THE INFLUENCE OF MONOVALENT CATIONS ON MYOFIBRILLAR FUNCTION

by

Jonathan Charles Kentish

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University of London

Cardiothoracic Institute,
2 Beaumont Street,
LONDON
W1N 2DX

ABSTRACT

An intracellular acidosis has a pronounced negative inotropic effect on cardiac muscle but potentiates the twitch of skeletal muscle. Conversely, mild extracellular hypertonicity, which causes an increase in intracellular [salt], increases the cardiac beat and decreases the skeletal twitch, although severe extracellular hypertonicity inhibits force development in both types of muscle. To determine whether these responses resulted from the direct influences of $[H^+]$ and [salt] on cardiac and skeletal myofibrils, I measured the Ca^{2+}-regulated ATPase activity of purified cardiac and skeletal myofibrils and the Ca^{2+}-regulated force production of detergent-skinned cardiac muscle in solutions of different pH and [salt]. Reducing the pH from 7.20 to 6.40 markedly decreased the Ca^{2+}-sensitivities of rabbit cardiac and rabbit skeletal myofibrillar ATPases. The opposite effects of acidosis on cardiac and skeletal muscle are not therefore due to contrasting pH-sensitivities of cardiac and skeletal myofibrils. Decreases in pH over the same range produced a similar decrease in the Ca^{2+}-sensitivity of force in skinned cardiac muscle and also depressed the maximum force. The combined actions of a raised $[H^+]$ on the Ca^{2+}-sensitivity and on maximum force were sufficient to entirely account for the reduction in the cardiac contraction during an intracellular acidosis.

Increases in [salt] over the ionic strength range 100 - 400 mM produced by the addition of KCl or other monovalent salts decreased the maximum ATPase activities of cardiac and skeletal myofibrils and the maximum force production of skinned cardiac muscle. This action was primarily due to the increase in ionic strength per se. In contrast, an additional effect of a raised [KCl] to decrease the Ca^{2+}-sensitivity of force resulted from a specific inhibitory action by K^+ ions. The combined deleterious effects of a raised ionic strength and a raised $[K^+]$ quantitatively account for the negative inotropic effect of severe extracellular hypertonicity but cannot explain the positive inotropic effect of slight extracellular hypertonicity.

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ABBREVIATIONS AND SYMBOLS:

ATP	adenosine 5'-triphosphate
ADP	adenosine 5'-diphosphate
cAMP	3':5'-cyclic adenosine monophosphate
Pi	inorganic phosphate
EGTA	ethyleneglycol-bis-(β-amino-ethyl ether) N, N, N', N'-tetra-acetic acid
HDTA	2,6-diaminohexane - N,N,N',N'-tetra-acetic acid
CP	creatine phosphate
Triton X-100	octyl phenoxy polyethoxyethanol
CK	creatine kinase (E.C. 2.7.3.2)
TMACl	tetramethylammonium chloride
ChCl	choline chloride

[X]	concentration of X
$\Gamma/2$	ionic strength
pH_i	intracellular pH
pH_e	extracellular pH
pCa	$-\log_{10} [Ca^{2+}]$
pCa_{50}	pCa required for 50% Ca^{2+}-activation
$\Delta pCa_{50}/\Delta pH$	change in pCa_{50} for unit change in pH

SUPPLIERS

(a) Animals. Immature adult male guinea-pigs (Dunkin Hartley strain, 200 - 250 g) were obtained from Redfern Limited (Brenchley, Kent) and male adult rabbits (New Zealand White strain, 2.0 - 2.5 kg) from Hyline (Marston, Cheshire). All animals were allowed free access to chow and water.

(b) Chemicals. Reagent grade chemicals were used whenever possible. Chemicals were supplied by B.D.H. Limited (Poole, Dorset) except for the following:

Chemicals	Suppliers
Imidazole, Triton X-100, H_4EGTA, crystalline bovine serum albumin, quinidine Cl, ouabain, Na pyruvate, Na_2CP, creatine kinase, caffeine, cAMP, cAMP-dependent protein kinase.	Sigma Limited (Poole, Dorset).
Dithiothreitol	Calbiochem (La Jolla, Calif., U.S.A.).
Na_2H_2ATP	Boehringer Limited (Lewes, East Sussex).
H_4HDTA	Fluorochem Limited (Glossop, Derbyshire).
All materials for electron microscopy.	EMscope Limited (Ashford, Kent).

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OBJECTIVES

This work was undertaken initially to investigate the direct influence of H^+ ions on the Ca^{2+}-regulated activity of cardiac and skeletal myofibrils. It was known that an intracellular acidosis in cardiac muscle produced an abrupt decline in contractile performance, which contrasted with the small, potentiating effect of a similar acidosis on the twitch tension of white skeletal muscle. To understand the reason for these different responses to an acidosis it was essential to know the relative pH-sensitivities of cardiac and skeltal myofibrils. However such a comparison was difficult to make because most previous studies on the myofibrillar ATPase activities had used either cardiac or skeletal myofibrils, but not both. In addition, the studies on the effect of pH on the cardiac myofibrillar ATPase had used myofibrillar preparations that without doubt contained contaminant membrane ATPases, each of which would have had its own pH-dependence. My studies on cardiac and skeletal myofibrillar ATPases therefore had the following objectives:

(i) To determine whether the Ca^{2+}-activation of cardiac myofibrillar ATPase, in a myofibrillar preparation freed of contaminant membrane ATPases, was truly sensitive to $[H^+]$.

(ii) To compare the $[H^+]$-sensitivities of purified cardiac myofibrils and white skeletal myofibrils, when assayed under identical conditions, in order to determine whether there were differences that would account for the contrasting effects of an acidosis on the two types of muscle.

In a complementary study I hoped to determine the pH-sensitivity of Ca^{2+}-regulated force in 'skinned' cardiac and skeletal muscle. However this objective was fulfilled by two independent reports, which were published while I was in the early stages of my study on skinned muscle. I therefore extended the scope of my research to investigate the influence of other monovalent cations, besides H^+, and of ionic strength on skinned cardiac muscle. This was relevant to the effects of extracellular hypertonicity on intact muscle, because hypertonicity causes an increase in the concentrations of ions in the cytosol and an increase in the cytosolic ionic strength. Gordon et al. (1973) had found that the inhibitory action of

extracellular hypertonicity on intact skeletal fibres could be accounted for entirely by the direct, deleterious effect of a raised ionic strength on the maximum force production in skinned skeletal fibres. However Ashley & Moisescu (1977) obtained some evidence that some of the effects of a raised [KCl] that had previously been attributed to the increased ionic strength per se may have been at least partially due to other factors. In my work on skinned cardiac muscle, I set out with the following objectives:

(i) To investigate the action of an increased [KCl] on the Ca^{2+}-regulated force production in skinned cardiac muscle in order to see whether the effects were sufficient to explain the effects of extracellular hypertonicity on intact cardiac muscle, in which the effects of hypertonicity are more complex than in skeletal muscle.

(ii) To establish whether the observed effects of a raised [KCl] were due to the increased ionic strength itself, or to other factors, such as the concomitant increase in tonicity or a specific inhibitory effect of K^{+} ions.

PART I

INTRODUCTION

Chapter 1.

GENERAL INTRODUCTION: THE REGULATION OF MYOFIBRILLAR CONTRACTILE ACTIVITY, WITH PARTICULAR REFERENCE TO CARDIAC MUSCLE.

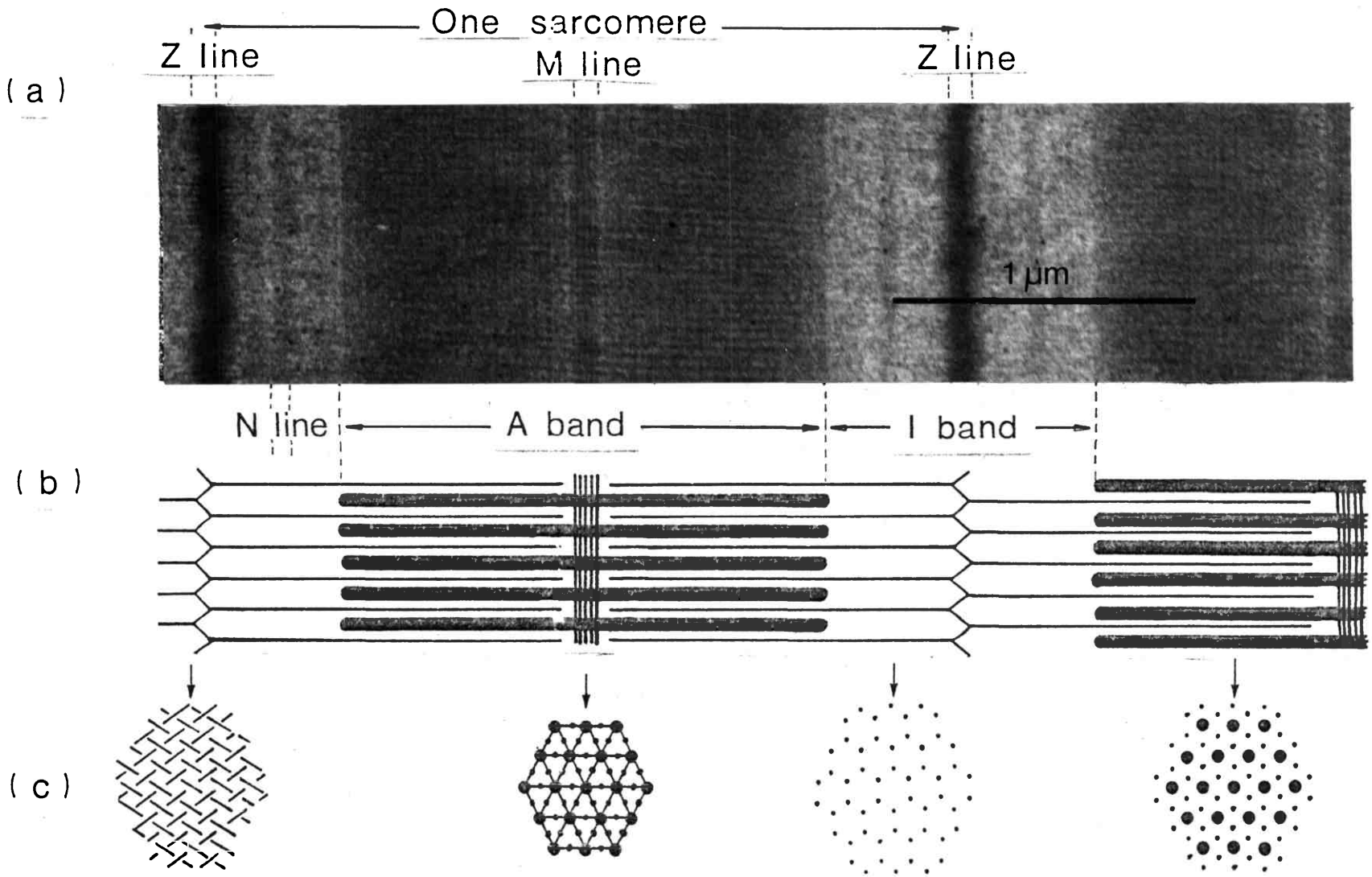
1.1 Structure and Organisation of the Myofibrillar Proteins

The contractile elements of vertebrate striated muscle - the myofibrils - are rod-like structures running the length of each muscle cell and connecting with bone via tendons in the case of skeletal muscle or with other muscle cells via intercalated discs in the case of cardiac muscle. Each myofibril is composed of a long succession of partially overlapping, interdigitating arrays of longitudinal protein filaments (Figure 1). The arrays of the 1.6 μm long 'thick' filaments and the 1.0 μm long 'thin' filaments give rise to the characteristic banding pattern of striated muscle. It is now universally accepted that muscle shortening results from a relative sliding of the two sets of filaments, as proposed independently by A.F. Huxley & Niedergerke (1954) and H.E. Huxley & Hanson (1954) after both groups of workers had observed alterations in I band width but not A band width. The central role played by Ca^{2+} in the regulation of muscle contraction was recognised a few years later by Weber (1959). However it is only more recently that an increasing knowledge of the detailed structure and localisation of the protein components of the myofibril has allowed insight into the molecular mechanisms of contraction and of the regulation of contraction by Ca^{2+} ions. Most of the pioneering ultrastructural, mechanical and biochemical studies were undertaken with skeletal muscle (usually from frog or rabbit). Subsequent work has shown that the mechanism of contraction in cardiac muscle is essentially the same as in skeletal muscle but that differences exist in the regulatory processes in the two types of muscle. We now understand the molecular bases for several of these differences.

(a) Proteins of the Thick Filament

(i) Myosin

More than 95% of each thick filament is composed of matchstick-shaped myosin molecules arranged side by side with their 'tails' forming the rigid backbone of the filament (Figure 2a). Negative staining of isolated thick filaments has highlighted numerous projections located on either side of a central bare zone (Huxley,


One sarcomere
Z line
M line
Z line
(a)
1 μm
N line
A band
I band
(b)
(c)

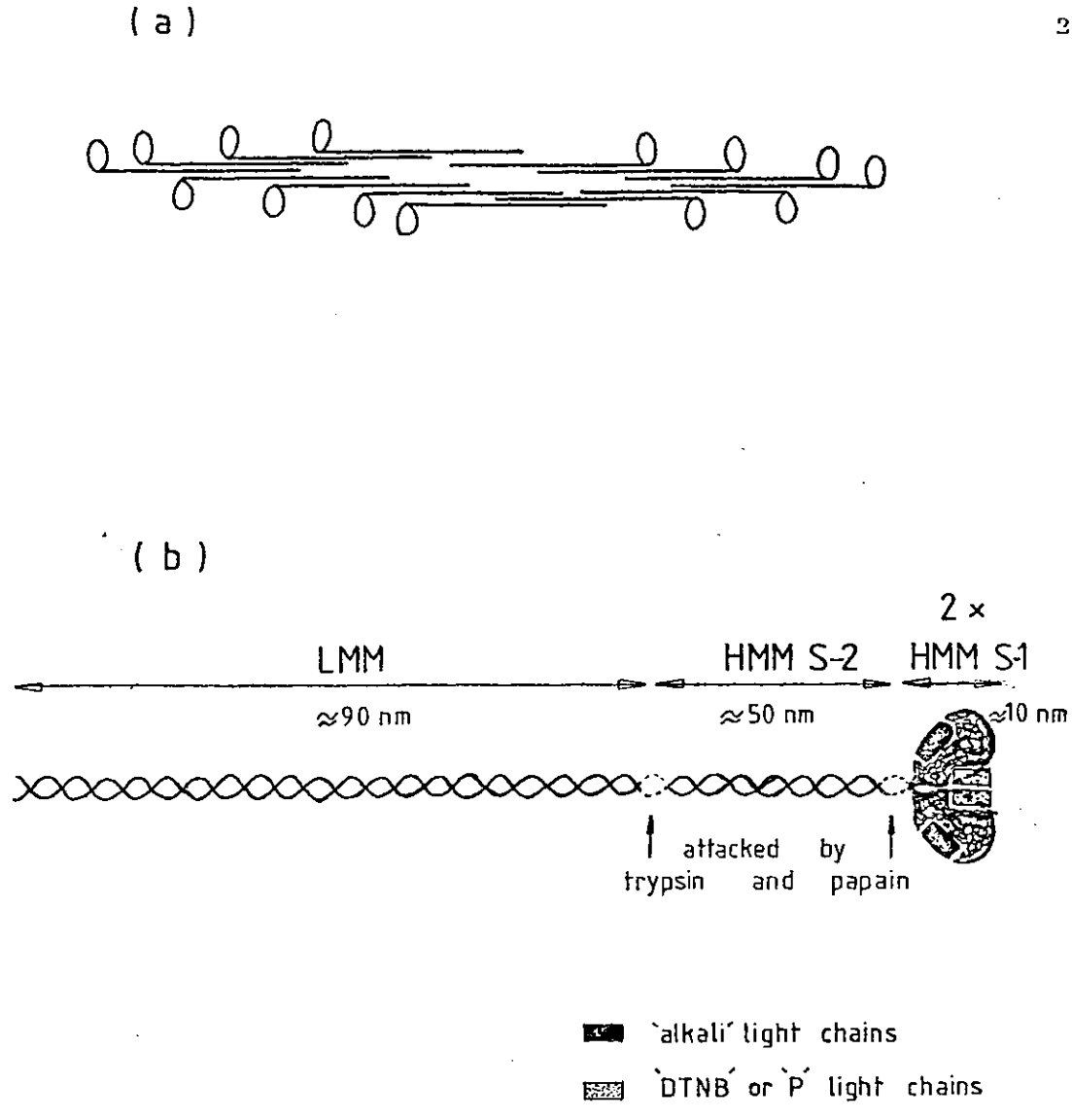


Figure 2. Aggregation and structure of myosin molecules

(a) H.E. Huxley's proposal as to how the myosin molecules aggregate to form a thick filament with a projection-free shaft in the middle and a reversed polarity of the molecules on either side of the centre region. (Modified from Huxley, 1963).
(b) Schematic diagram of a myosin molecule. Cleavage by proteolytic enzymes yields two sections of α-helical rod - light meromyosin (LMM) and heavy meromyosin subfragment 2 (HMM S-2) - plus two globular heads - heavy meromyosin subfragment 1 (HMM S-1). Most of the myosin molecule is composed of two 'heavy chains'; the remainder consists of four 'light chains'. Each HMM S-1 head contains one alkali light chain and one DTNB light chain (also called the P light chain).

1963). These projections are thought to be myosin 'heads' which probably correspond to the 'cross-bridges' spanning most of the 11 nm gap from the thick to the thin filaments in muscle in rigor. Huxley (1957) suggested such cross-bridges mediate the physical interaction between myosin and actin that results in the development of force in living muscle. Selective proteolytic digestion of myosin molecules (Szent-Gyorgi, 1953; Mueller & Perry, 1962; see Figure 2 b) has shown that much of the myosin tail consists of an α-helical rod, light meromyosin (LMM), which is insoluble at low ionic strengths (less than 300 mM). The insolubility of LMM causes myosin molecules to aggregate spontaneously if the ionic strength is low enough. Heavy meromyosin (HMM) (see Figure 2b) is composed of a short α-helical rod (HMM S-2) linked to two globular heads (2 x HMM S-1). The two biological activities of myosin - ATPase activity (Engelhardt & Ljubimova, 1939) and the ability to combine with actin (Straub, 1942) - reside in the HMM S-1 heads. Each HMM S-1 has one ATP-binding site and one actin-binding site. HMM S-1, unlike whole myosin, is soluble at low ionic strengths and has therefore been of great value in biochemical studies of ATPase kinetics. The structure of the thick filament, then, is determined largely by the self-aggregation properties of LMM, whilst its enzymic activity is conferred entirely by the HMM S-1 moieties.

In natural thick filaments or aggregates of pure myosin molecules the myosin heads point away from the midpoint of the filament (Figure 2a). This, together with a corresponding reversal of polarity in the thin filaments ensures that the forces generated by actin-myosin interaction in each half of the sarcomere are oppositely directed, so that the sarcomere tends to shorten. It is not clear exactly how the several hundred myosin molecules in each thick filament are packed, though most of the recent evidence favours a helical model in which three pairs of S-1 heads project every 14.3 nm (discussed fully in Squire, 1981).

Each molecule of skeletal myosin has six polypeptide chains (Figure 2b). Two structurally-similar 'heavy chains' comprise much of the mass of the myosin molecule (Dreizen & Gerschmann, 1970; Lowey & Risby, 1971; Weeds & Pope, 1971). The four homologous 'light chains' fall into two classes (Dreizen & Gerschmann, 1970; Weeds & Pope, 1971; Sarker et al., 1971): (i) the DTNB light chains (or P light chains), 2 moles/mole myosin, which are

selectively removed by the thiol reagent 5,5' dithiobis-(2-nitrobenzoic acid) and (ii) the alkali 1 and 2 light chains (ALC_1 and ALC_2), total 2 moles/mole myosin, which are released only under denaturing conditions such as high pH and which are therefore probably an integral part of the myosin head. In agreement with this idea, removal of the alkali light chains abolishes myosin ATPase activity although loss of the DTNB light chains has little effect (Dreizen & Gerschmann, 1970). ALC_1 and ALC_2 are not present in equimolar amounts in skeletal fibres, and so myosin isoenzymes must exist (Lowey & Risby, 1971).

Cardiac myosin has a similar overall structure to fast-twitch skeletal myosin except that it contains only two species of light chain (Sarker et al., 1971; Lowey & Risby, 1971): an alkali light chain that is structurally homologous with the alkali light chains of skeletal muscle and a lighter chain, of molecular weight 19000 Daltons. The latter chain is structurally and functionally similar to the DTNB light chain of fast skeletal muscle except that it cannot be removed by DTNB. However, a characteristic common to the 19000 Dalton light chains from skeletal, cardiac and smooth muscle is that they can be specifically phosphorylated by a myosin light chain kinase that exists in these muscles (Perry, 1975). Perry has suggested these light chains be termed 'P' (for phosphorylatable) light chains. This terminology will be followed in this thesis.

Each P light chain can bind one mole of Ca^{2+} or Mg^{2+} per mole with high affinity and this binding entirely accounts for the high-affinity divalent cation-binding capacity of native myosin which amounts to two moles/mole myosin (Morimoto & Harrington, 1974). Possible regulatory roles for this cation binding or for the phosphorylation of the P light chain are discussed later (Sections 1.3(c) and 1.5(b) respectively).

(ii) C-Protein

C-protein molecules bind strongly to LMM and so are probably associated with the thick filament backbone (Offer, 1972). Electron micrographs of muscle treated with antibody to C-protein have shown the C-protein molecules to be arranged in seven stripes about 43 nm apart in each half of the A-band (Offer, 1972). The function of C-protein is not known for certain but it could act to stabilise the myosin filament (Koretz et al., 1982) or control its length (Squire, 1981).

(iii) M-Protein(s)

Probably at least two proteins, apart from myosin, comprise the M band since the band appears to consist of (i) 'M bridges' connecting each thick filament to six surrounding thick filaments and (ii) 'M filaments', lying parallel to the thick filaments and connecting the 'M bridge' networks (Knappeis & Carlsen, 1968; also see Figure 1). A 43000 Dalton protein present in the M-line of skeletal myofibrils has been identified as the MM isoenzyme of creatine kinase by Eppenberger and colleagues (Turner et al., 1973). This creatine kinase, in combination with cytosolic creatine phosphate, forms a powerful ATP-regenerating system located only a short distance (<0.8 μm) from the site of ATP hydrolysis by actomyosin:

$$MgADP^{-} + HCP^{2-} + H^{+} \underset{\text{kinase}}{\overset{\text{creatine}}{\rightleftharpoons}} MgATP^{2-} + \text{creatine}$$

There is some indirect evidence that the primary M-bridges are composed predominantly of creatine kinase (Strehler et al., 1980). A 165000 Dalton protein, now termed 'M-protein', also appears to be a true constituent of the M-line (Trinick & Lowey, 1977), though its precise location remains to be determined.

(b) Proteins of the Thin Filament

(i) Actin

The backbone of the filament consists of globular actin monomers (G-actin) polymerised together to form filaments (F-actin) which are coiled into a double helix of pitch 2 x 37 nm or, less probably, 2 x 36 nm (Hanson and Lowey, 1963; Huxley, 1963; see Figure 3). The subunits repeat every 5.46 nm, which results in 13 - 14 monomers, i.e. a non-integral number, per turn (Huxley and Brown, 1967). Each thin filament is about 1.0 μm long and terminates at the Z-disc, where it is joined to four other thin filaments by interconnecting 'Z-filaments' in a tetragonal lattice of 'basket-weave' structure (Knappeis and Carlson, 1962 ; see Figure 1).

Actin participates in many different contractile processes in muscle and non-muscle cells. Despite actin's almost universal role in biological movement, the primary and tertiary structures of actin are relatively invariant. Not surprisingly, then, mammalian cardiac actin appears to be very similar to mammalian skeletal actin in its amino acid composition, physicochemical properties and biological activity (see Katz, 1970).

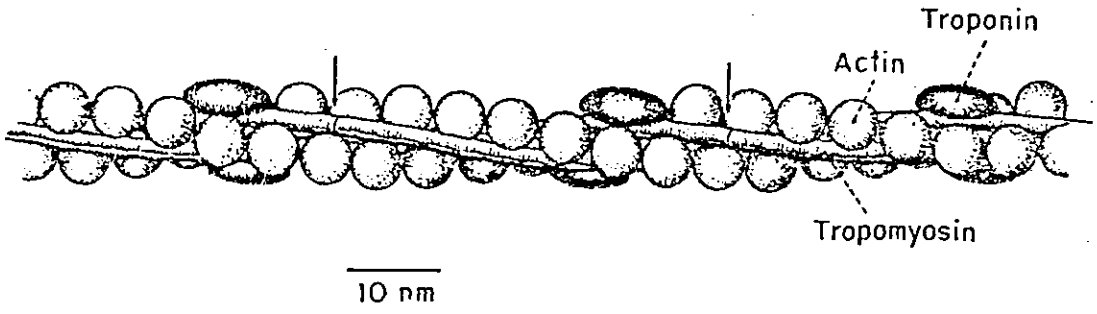


Figure 3. Structure of the thin filament

Tropomyosin molecules lie end-to-end in the grooves of the actin helix, with each tropomyosin extending over seven actin monomers (Ebashi & Endo, 1968). One troponin molecule binds to each tropomyosin at a site about 13 nm from one end of the tropomyosin molecule (Ohtsuki, 1974).

(ii) Tropomyosin

Together, the so-called 'regulatory proteins' tropomyosin and troponin confer Ca^{2+}-sensitivity on the interaction between actin and myosin. Tropomyosin is a rod-shaped molecule of length about 41 nm and consists of two α-helical subunits wound round one another to form a coiled coil. The tropomyosin molecules run the length of the thin filaments and lie end-to-end and slightly off centre in each groove between the strands of actin monomers. Each tropomyosin molecule binds to seven successive actin molecule (see Figure 3).

Rabbit skeletal tropomyosin is heterogeneous in that it is comprised of two types of subunit, termed α- and β-tropomyosin, which differ slightly in molecular weight and cysteine content (Cummins and Perry, 1972; Hodges and Smillie, 1972). The ratio α:β is 3.8:1 in rabbit psoas muscle. Cardiac muscle apparently contains only one type of tropomyosin subunit, which is indistinguishable from skeletal β-tropomyosin (Cummins and Perry, 1974).

(iii) Troponin (TN)

Troponin, the only Ca^{2+}-sensitive protein in thin filaments, is in fact a complex of three subunits: troponin-I (TN-I), which inhibits actin-myosin interaction; troponin-C (TN-C), which binds Ca^{2+} over the Ca^{2+} concentration range 10^{-7} M to 10^{-5} M; troponin-T (TN-T) which interacts with tropomyosin and TN-C and which may act to anchor the entire TN complex to the tropomyosin filaments. Although it is widely believed that the holo complex contains these three subunits in equimolar amounts, Heilmeyer and colleagues have recently claimed that skeletal TN in fact contains 2 moles TN-I/mole complex i.e. that whole TN has the composition TI_2C (Sperling et al., 1979). By using ferritin-labelled antibody to TN, Ohtsuki et al.(1967) demonstrated that TN is located along the entire length of the thin filaments at intervals of about 41 nm. This periodicity, which is the same as that of tropomyosin, corresponds to one TN for every seven actin monomers (Figure 3). In accord with this distribution, the molar ratio of TN-C:actin in 1:7 in both skeletal and cardiac muscle (Head and Perry, 1974). More recent work by Ohtsuki (1974) has shown TN to be positioned about 13 nm from the end of each tropomyosin molecule.

The amino acid sequences of most of the troponin subunits from skeletal and cardiac muscle have now been determined. Although

corresponding subunits from the two types of muscle show considerable homology, there are differences which have functional consequences for the regulation of myofibrillar activity.

Troponin-C (TN-C). Rabbit skeletal TN-C was the first myofibrillar polypeptide to be sequenced (Collins et al., 1973). Its amino acid sequence contains four regions, coded I - IV, which are homologous with each of the two Ca^{2+}-binding sites of parvalbumins, a group of Ca^{2+}-binding muscle proteins of which the three dimensional structure has been determined by X-ray diffraction. Collins et al. (1973) therefore postulated that skeletal TN-C should bind 4 moles Ca^{2+}/mole, a prediction that was in accord with Ca^{2+}-binding studies (Section 1.3(b)). In bovine cardiac TN-C too the polypeptide chain contains four sections which show homology with parvalbumin Ca^{2+}-binding sites (van Eerd & Takahashi, 1975). The primary structures of three of these are similar to the corresponding sites of skeletal TN-C but the fourth (region I, residues 28 - 40) contains far fewer acidic residues than its skeletal counterpart. Van Eerd & Takahashi (1975) suggested this site may have lost the ability to bind Ca^{2+}, so that cardiac TN-C might bind only three moles Ca^{2+}/mole. This prediction has been confirmed (Section 1.3(c)).

Troponin-I (TN-I). Skeletal and cardiac TN-I are on the whole similar but not identical in terms of primary structure and physical and chemical properties (Ebashi & Endo, 1968; Grand & Wilkinson, 1976). One striking difference between them is the presence of an extra 26 residues at the N-terminus of the cardiac protein. This additional sequence contains a residue (Serine 20) which is readily phosphorylated by protein kinase and which may therefore play a central role in the regulation of cardiac myofibrillar function by phophorylation of troponin (Section 1.5(b)). This regulatory mechanism appears to be denied to skeletal muscle because of the absence of the necessary N-terminal sequence.

Troponin-T (TN-T). Of the three TN components, TN-T is the least well characterised. To date, the primary structure of only rabbit skeletal TN-T has been determined (Pearlstone et al., 1977) but physicochemical properties indicated some differences between TN-T of fast skeletal, slow skeletal and cardiac muscles (Perry, unpublished, in Perry, 1975).

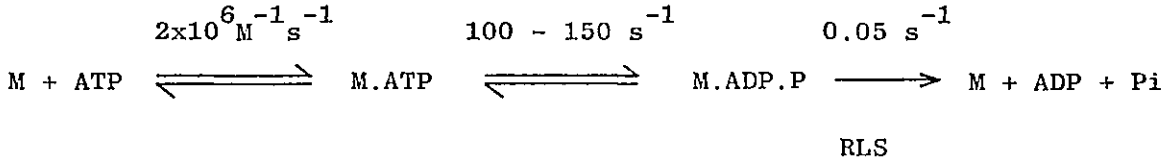
(iv) Other myofibrillar proteins

Several other minor proteins associated with the thin filaments have been discovered though their functions are in general poorly understood. α-actinin appears to be located at the Z-disc, where it may have a structural role (Ebashi & Ebashi, 1965). β-actinin seems to act as an 'ending factor' to inhibit the polymerisation of actin monomers at the free ends of the I filaments (Maruyama et al., 1977a). Maruyama and colleagues have also discovered an elastic protein, connectin, which is particularly abundant in cardiac myofibrils and which may contribute toward the 'parallel elastic element' in muscle (Maruyama et al., 1977b). A high molecular weight protein, yet to be named, appears to be a component of the N lines seen on either side of the Z line in striated muscle (Wang & Williamson, 1980). It is not yet known whether any of these proteins form the 'Z-filaments' linking actin filaments at the Z-disc.

1.2 Actomyosin ATPase Activity and the Mechanism of Force Generation

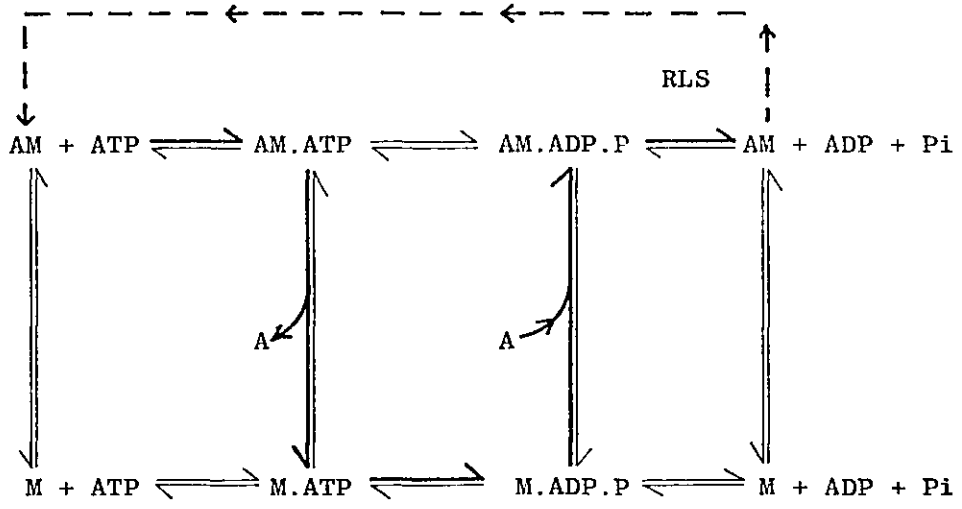
The sliding filament theory, as originally proposed, did not attempt to explain how the system of filaments could generate force and produce shortening of muscle. It is now widely, although not universally, believed that force is generated by the cyclical attachment and detachment of cross-bridges extending from myosin to actin, with each cycle involving a conformational change in the cross-bridge while attached to actin so that sliding force is produced. Current understanding of the coupling of ATP hydrolysis to force development has drawn on a wide range of biochemical, physiological and ultrastructural studies and has advanced to the stage where we can tentatively link stages in the kinetic scheme for the ATPase reaction to distinct physical steps in the cross-bridge cycle.

In the last decade or so detailed mechanisms for the myosin and actomyosin ATPase reactions have been deduced from steady-state and pre-steady-state kinetic data. These reactions exhibit unusual kinetics, in that there is a faster hydrolysis of ATP (as measured by the phosphate liberated by an acid quench) at the beginning of the reaction than during the steady-state (Tokiwa & Tonomura, 1965; Lymn & Taylor, 1970). Lymn & Taylor (1970) proposed that this 'initial Pi Burst' resulted from a rapid formation of an acid-labile myosin.ADP.P complex, the slow breakdown of which accounted for the slow steady-state rate of hydrolysis, i.e.

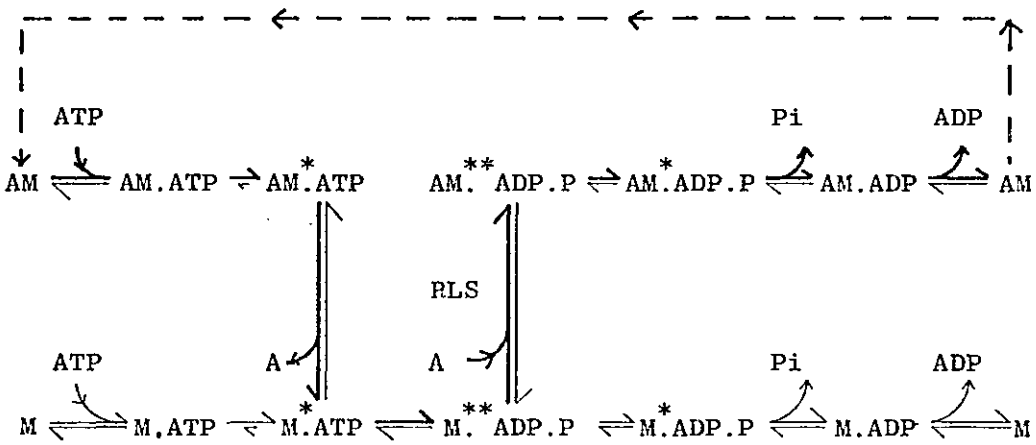


RLS = rate-limiting step

Actin activated the HMM ATPase activity by apparently increasing the rate of dissociation of the myosin-products complex from $0.05\ s^{-1}$ to $10\ s^{-1}$ (Lymn and Taylor, 1970, 1971). The action of ATP to dissociate actin and myosin (a process recognised by Szent-Györgyi in 1943) was found to be an essential step in the actomyosin ATPase reaction: the actual splitting of ATP occurred on the dissociated HMM, for the rate of dissociation of acto-HMM by ATP was at least ten times greater than the rate of ATP hydrolysis. Lymn and Taylor (1971) amalgamated these findings into the following reaction sequence for actomyosin ATPase activity (preferred pathway in bold arrows):



One source of contention between kineticists has been the location of the rate-limiting step, because there is now some evidence that, in disagreement with the original Lymn-Taylor model, the rate-limiting step occurs after the formation of M.ADP.P but before the release of products from actomyosin. From the Lymn-Taylor scheme it could be predicted that when actomyosin was maximally activated, almost all the HMM should be bound to actin (as AM.ADP.P, the state preceding the rate-limiting step). However, X-ray diffraction of skeletal muscle (Miller & Tregear, 1970), biochemical studies on actoHMM ATPase activity (Eisenberg & Kielley, 1972) and measurements of instantaneous stiffness of skeletal muscle (Yamamoto & Herzig, 1978) indicated that conditions of maximal activation are associated with only about 20% - 40% of cross-bridges being attached to actin at any moment. To explain this low degree of attachment most biochemists now believe the rate-limiting step in the presence of actin is the reassociation of actin to myosin and that the dissociation of products from actomyosin is relatively rapid. This, together with fluorimetric evidence for more intermediates than in the original Lymn-Taylor scheme, has lead to the following probable reaction sequence (Bagshaw et al., 1974; Koretz & Taylor, 1975; Huxley, 1980):



where the asterisks indicate the degree of fluorescence enhancement of a tryptophan residue in the myosin molecule.

There is at present no evidence for the appropriateness of the asterisks in the actin-myosin intermediates.

Eisenberg and colleagues suggest that the M.ADP.Pi complex has an additional 'refractory' state unable to bind to actin (Eisenberg & Kielley, 1972) and that the 'non-refractory' M.ADP.Pi complex can associate weakly with actin without release of products occurring (Stein et al., 1979). Furthermore, they also believe that some ATP hydrolysis can occur without detachment of myosin from actin. These proposals remain to be confirmed .

In Figure 4 the above reaction sequence is linked to possible mechanical events in the cross-bridge cycle. This scheme demonstrates one way in which the chemical steps of actomyosin association, product release and actomyosin dissociation can be related to the physical steps of cross-bridge formation, force development and cross-bridge detachment (though the temporal relationship between product release and the force-generating step is poorly understood). To date, there are more known biochemical steps than mechanical steps and only those biochemical steps which can be tentatively linked to distinct stages in the cross-bridge cycle are shown in Figure 4. Note that energy provided by ATP hydrolysis is not used directly for the development of force but rather for returning the myosin projection to its strained conformation. If muscle shortening is allowed, each cross-bridge cycle produces a relative sliding of thick and thin filaments by 5 - 10 nm. A steady average force during muscle contraction results from the asynchronous cycling of cross-bridges (about 5×10^{12} per half sarcomere).

Although the scheme in Figure 4 allows some agreement between the biochemical and physiological aspects of muscle contraction, exactly how well the reaction sequence parallels the mechanism occurring in living, contracting muscle is uncertain, for two main reasons. Firstly, the kinetic studies used actomyosin devoid of the regulatory proteins and used chemical conditions that were often unphysiological (such as very low ionic strengths or temperatures). Secondly, these studies necessarily used soluble fragments of myosin (HMM or S-1) together with actin in solution. As T. Hill & Simmons (1976) have pointed out, the reaction sequence and rate-limiting step in the intact myofibril may differ from that given above because of constraints arising from the highly-ordered filament lattice and from the coupling of the reaction to the production of force. This caveat has very recently been justified by Kerrick et al. (1980), who demonstrated that the Ca^{2+}-dependent tension in 'skinned' muscle

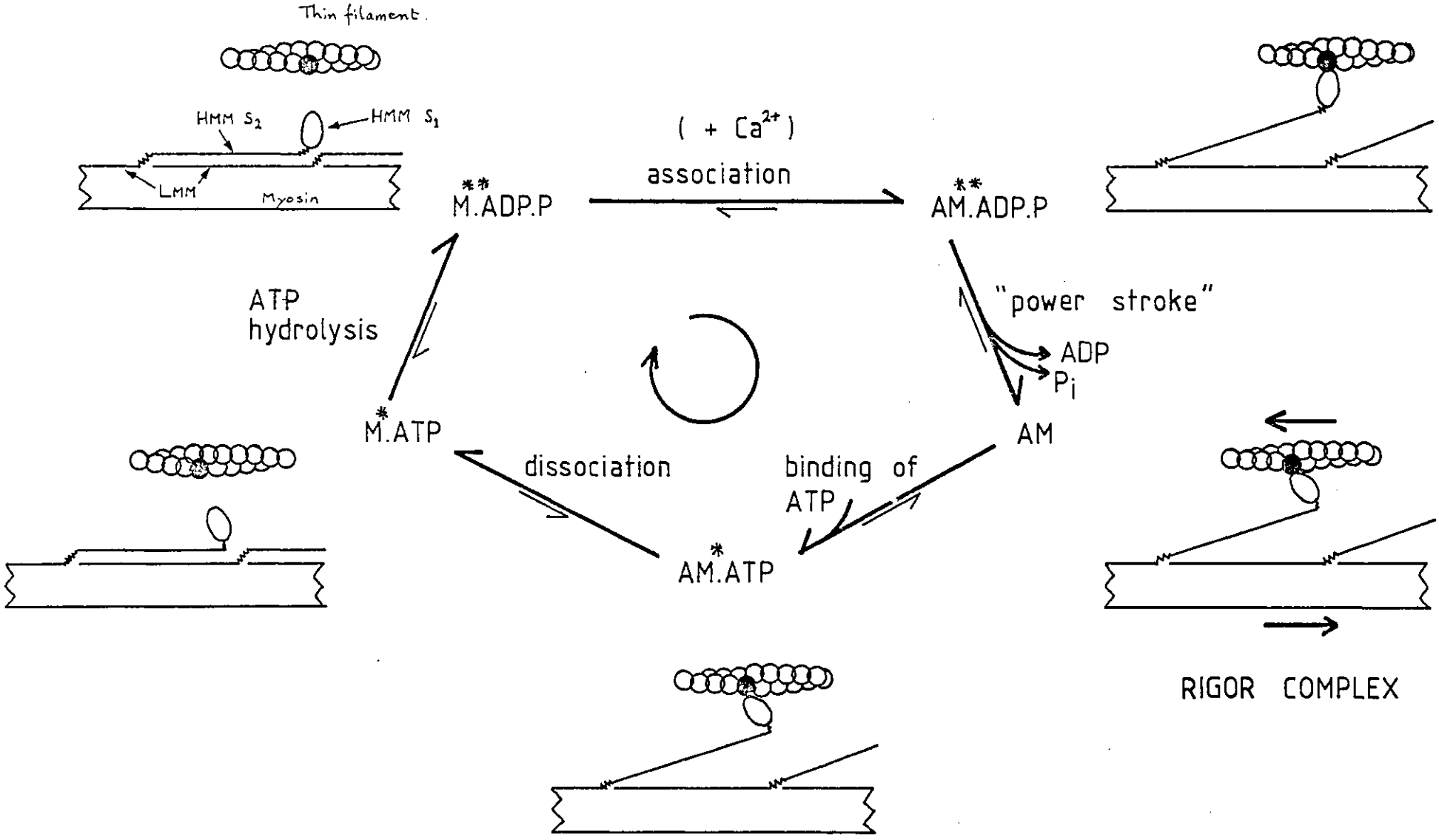


Figure 4. An outline of the possible sequence of biochemical and mechanical events during force production in myofibrils: the 'cross-bridge cycle'.

correlated well with the Ca^{2+}-regulated ATPase activity of isolated myofibrils but poorly with the Ca^{2+}-regulated ATPase activity of actomyosin.

The fact that myosin ATPase activity is very low unless it is activated by actin implies that HMM heads and actin monomers must come into close proximity during the contractile process, when the ATPase activity is high. The existence of stretch-resisting links between thick and thin filaments was initially inferred from the pronounced stiffness of glycerinated muscles in rigor (i.e. in the absence of ATP) compared with that of muscles bathed in MgATP, but the first substantial evidence was provided by direct observation of actin-myosin 'cross-bridges' in the electron microscope (Huxley, 1960). A combination of electron microscopy and X-ray diffraction (Reedy et al., 1965) showed that the myosin projections apparently stuck out at right angles from the thick filament in relaxed muscle, but in rigor muscle were elongated and were attached to actin at 45°, pointing towards the M-line. A widely-accepted interpretation of these data is due to Huxley (1969), who considered that it was just the S-1 head of the myosin projection which produced the angled appearance in rigor and that rotation of the head to the 45° angle pulled the thin filament along relative to the thick filament (see Figure 4). Huxley's 1969 model also incorporated the idea that each projection possesses two flexible hinges so that the correct stereospecific contact between actin and myosin molecules could be maintained despite large variations in interfilament distance (Figure 4). Such variations occur because the myofilament lattice in an intact fibre behaves as a constant-volume system, with the thick and thin filaments moving closer together as the muscle is stretched (Elliott, 1967). For example, the surface-to-surface separation between thick and thin filaments decreases from about 11 nm to about 5 nm as the sarcomere length increases from 1.8 μm to 3.5 μm. In spite of this, the force generated by each cross-bridge is independent of sarcomere length (above 2.2 μm) and is thus independent of filament separation (Gordon et al., 1966). Huxley's model for the cross-bridge provides a neat explanation for this. An attractive, though unproven, possibility is that the two flexible hinges correspond to the sites which are most vulnerable to proteolytic digestion: the links between LMM and HMM, and between HMM S-1 and HMM S-2.

Direct evidence for movement of myosin heads in living skeletal muscle has been obtained by X-ray diffraction, though this technique can only detect relatively large changes in orientation of the projections. When the muscle contracts the helical array of myosin heads becomes less ordered; there is also evidence for longitudinal movement or tilting of myosin heads and substantial radial circumferential or radial movement (Huxley & Brown, 1967). If the muscle is made to go into rigor there is an apparent movement of mass from the thick to the thin filaments. Huxley (1968) supposed this to represent a radial movement of myosin heads, though a possible alternative (Lymn, 1975) is a circumferential swinging of projections which are longer than in the Huxley model. In actively-contracting muscle the mass transfer is much less than in rigor (Miller & Tregear, 1970) and indicates that perhaps as few as 20% - 30% of cross-bridges are attached at any one moment.

In mammalian striated muscle Ca^{2+} ions regulate contractile activity by overcoming the inhibitory effect of troponin on the association of actin and myosin (Section 1.3). However even in the absence of Ca^{2+} some actin-myosin interaction may occur, for if the ATP is removed from a relaxed skinned fibre a 'rigor contracture' develops within a minute (e.g. see Section 10.5). This contracture is almost certainly due to an accumulation of rigor linkages (consisting of nucleotide-free HMM bound to actin) and this implies a basal rate of cross-bridge formation occurs even if no Ca^{2+} is present.

The cross-bridge cycle illustrated in Figure 4 shows only one of the two myosin S-1 heads binding to actin. Whether both S-1's can bind to actin simultaneously, and whether there is interaction between the two S-1's, is not established at present. Offer & Elliott (1978) have calculated that the S-1's of each myosin molecule could theoretically bind to two separate but adjacent actin filaments in insect flight muscle. This phenomenon would add considerably to the stability of the two arrays of filaments but it has yet to be observed experimentally in mammalian muscle.

The cross-bridge theory can account for many biochemical and mechanical properties of striated muscle. However there are several phenomena that it cannot explain, notably the longitudinal stability of the sarcomeres during muscle contraction at long sarcomere lengths (Noble & Pollack, 1977). Other theories, such as those assuming that electrostatic interaction between thick and thin filaments produces

the motive force (e.g. Iwazumi, 1970), have their own drawbacks and, unlike the cross-bridge theory, have not yet advanced to a stage where they can be tested.

Compared with skeletal muscle, cardiac muscle suffers from a dearth of information about the exact mechanism of force generation. With rather little experimental justification it is widely assumed that the mechanism is similar to that proposed for skeletal muscle. Electron microscopy has demonstrated that cardiac myofibrils are similar in appearance to skeletal myofibrils (Stenger and Spiro, 1961; see also Figure 1). Cardiac actomyosin is generally like skeletal actomyosin in its physicochemical and enzymatic properties (Katz, 1970), except that the maximum ATPase activity of cardiac actomyosin is approximately half that of the skeletal ATPase enzyme. X-ray diffraction of cardiac muscle has indicated a general similarity between the contractile machinery of cardiac muscle and skeletal muscle; however the cross-bridges are less well ordered than in skeletal muscle (Matsubara & Millman, 1974). Despite this, the cardiac cross-bridge cycle may be more complex than in skeletal muscle: analysis of the tension responses to sudden length changes in cardiac muscle reveals a delayed, transient increase in tension ('stretch activation') which is not seen in skeletal muscle (Steiger, 1977; Herzig and Ruegg, 1980).

Although the actomyosin ATPase reaction and the cross-bridges cycle can be related in a comprehensible way the direct extrapolation of ATPase activity to muscle function is difficult. The maximum activity of actin-activated myosin ATPase should theoretically correspond to the maximum rate of cross-bridge cycling, which in turn should determine the maximum speed of shortening in unloaded muscle (V_{max}). In fact there is an excellent correlation between V_{max} and actin-activated myosin ATPase activity in different types of striated muscle (Bárány, 1967).

Muscle force will depend among other things upon the number of attached cross-bridges, for the cross-bridges in each half sarcomere act as independent force generators effectively in parallel. Changes in cross-bridge number can be estimated from the 'instantaneous stiffness' of a muscle subjected to a rapid change in length. (This stiffness appears to reside mainly in the cross bridges; the magnitude of stiffness is therefore proportional to the number of cross-bridges (Huxley & Simmons, 1971)). Stiffness measurements have shown that, at least in glycerinated pig cardiac muscle, isometric

tension increases in parallel with the number of cross-bridges as the Ca^{2+} concentration is raised (Herzig & Ruegg, 1980). An indirect measure of the number of cross-bridges binding to actin in unit time is given by the Mg^{2+}-dependent ATPase activity, which has also been found to increase in proportion to isometric tension in Ca^{2+}-activated glycerinated muscle (Schädler, 1967; Levy et al., 1976).

1.3 The Regulation of Myofibrillar Contractile Activity by Ca^{2+} Ions

(a) The Ca^{2+}-dependence of 'models' of muscle contraction

Although it had been known for many years that extracellular Ca^{2+} was essential for the contraction of cardiac muscle (Blake, 1841; Ringer, 1883), it was in fact studies on skeletal muscle which suggested that Ca^{2+} might play a role in excitation-contraction coupling. The significance of the demonstration by Heilbrum & Wiercinski (1947) that the intracellular injection of Ca^{2+} induced skeletal muscle contraction was not fully appreciated until the flurry of research activity in the 1950's to identify the 'Marsh relaxing factor' culminated in two studies which established the role of Ca^{2+} in skeletal excitation-contraction coupling: Weber (1959) pointed out that myofibrillar ATPase activity depended upon the presence of a minute quality of Ca^{2+}, and Ebashi (1960) showed that the 'relaxing factor', and thus the sarcoplasmic reticulum from which it was derived, relaxed muscle models by sequestering Ca^{2+} and thereby removing it from the contractile proteins. Ebashi (1961) suggested that in intact muscle the SR was also the source of the Ca^{2+} which activated the myofibrils; this explained why the skeletal twitch was insensitive to the external Ca^{2+} concentration. Further work (Ebashi, 1961; Weber & Herz, 1963; Seidel & Gergely, 1963) showed the rate of ATP hydrolysis by isolated myofibrils or 'natural actomyosin' (now known to consist of actomyosin + regulatory proteins) to be activated by an increase in $[Ca^{2+}]$ over the range 10^{-7} M to 10^{-5} M Ca^{2+}. If the ATPase activity was plotted against log $[Ca^{2+}]$ a characteristic sigmoidal relationship was observed. This Ca^{2+}-dependency was subsequently confirmed with the tension development of glycerinated skeletal muscle (Filo et al., 1965) and mechanically-skinned single skeletal fibres (Hellam & Podolsky, 1969). The view that skeletal activation involved an increase in the cytosolic $[Ca^{2+}]$ over the range 10^{-7} M to 10^{-5} M was further strengthened by the work of Portzehl et al. (1964), who injected Ca-EGTA buffers of known $[Ca^{2+}]$ into living

muscle fibres and showed the tension-Ca^{2+} relationship to be similar to that obtained with the less physiological preparations.

The same Ca^{2+}-dependency was also exhibited by cardiac muscle: the relationship between log [Ca^{2+}] and the ATPase activity of cardiac actomyosin (Fanburg, 1964) or the tension development of glycerinated papillary muscle (Schädler, 1967) was sigmoidal, with the [Ca^{2+}] for half-maximal activation being, like that for skeletal muscle, about 10^{-6} M. These results, together with the demonstrations that both skeletal and cardiac sarcoplasmic reticulum could take up and release Ca^{2+} and that the cytosolic [Ca^{2+}] in intact cells rose quickly but transiently after muscle stimulation (Section 1.4), firmly established the intermediary role of Ca^{2+} in excitation-contraction coupling in both cardiac and skeletal muscle.

Although the sigmoidal relationship between log [Ca^{2+}] and contractile activity of striated muscle myofibrils has been subsequently confirmed in numerous studies using a variety of preparations, different authors, even if they use the same type of preparation, disagree as to (i) the steepness of the relationship and (ii) the exact Ca^{2+}-sensitivity, that is, the range of Ca^{2+} concentrations over which activation occurs. There are probably several reasons for such discrepancies. Firstly, some variability must arise from the wide range of ionic conditions (such as pH, ionic strength, [Mg^{2+}], [$MgATP^{2-}$]) used in the different studies. Another factor is the diversity of values used for the association constant for Ca^{2+} binding to the Ca^{2+}-buffer, EGTA: the apparent constants (at pH 7.0) have ranged from 1.1×10^{6} M^{-1} (determined by Ogawa, 1968) to 4.9×10^{6} M^{-1} (calculated from the value determined by Schwarzenbach et al., 1957) though in the last few years there has been a growing concensus that the proper value lies in between these two extremes, at about 2.5×10^{6} M^{-1} (Allen et al., 1977; Harifugi & Ogawa, 1980; Appendix A). Furthermore, it does not seem to be widely appreciated that commercially-available EGTA may be up to 5% impure (personal observation). A failure to correct for this impurity results in Ca^{2+}-activation curves that are artificially steep (Section 10.2). The Ca^{2+}-activation of myofibrillar contractile activity will also depend upon the sarcomere length (Section 1.5 (a)) and degree of phosphorylation (Section 1.5 (b)) of the myofibrils; both these factors are likely to vary between different types of preparation. Finally, there

may be true differences between the Ca^{2+}-sensitivities of myofibrils from different muscle types or from different species.

Figure 5 illustrates the relationships between log [Ca^{2+}] and Ca^{2+}-binding, ATPase activity and isometric force development of cardiac myofibrils. The steepness of the [Ca^{2+}]-ATPase and [Ca^{2+}]-force relationships, corresponding to a second-order relationship between activity and [Ca^{2+}], has been taken to indicate positive co-operativity, with at least two Ca^{2+} ions interacting to produce the elementary 'contractile event'. It is unlikely that this apparent co-operativity reflects co-operative interaction between the Ca^{2+}-binding sites on cardiac troponin because the myofibrils do not seem to bind Ca^{2+} in a co-operative fashion (Figure 5). In addition, there is evidence that only one Ca^{2+}-binding site is involved in the regulation of cardiac myofibrils (Section 1.3 (c)). Similarly in skeletal myofibrils, the relationship between force and [Ca^{2+}] may be as steep as fifth or sixth order (Brandt et al., 1980) although there are only four Ca^{2+}-binding sites on skeletal troponin and probably only two of these sites are of regulatory significance (Section 1.3 (c)). Thus the apparent positive co-operativity is probably the result, not of co-operativity between Ca^{2+}-binding sites, but of a more complex mechanism, such as a dependence of the inherent Ca^{2+}-sensitivity of the myofibrils upon force development (Brandt et al., 1980).

The results in Figure 5 were obtained from steady-state measurements of Ca^{2+} binding, ATPase activity or force. The experimental conditions were, to some extent, unphysiological since the cytosolic Ca^{2+} concentration varies continuously throughout a normal contraction of cardiac or skeletal muscle. Force will therefore also depend on the rate at which the activation and deactivation of the myofibrils follow the changes in Ca^{2+} concentration. Moisescu (1976) has developed a 'rapid activation' procedure by which the Ca^{2+} concentration within a skinned skeletal fibre can be increased within about 200 ms. From the Ca^{2+}-dependence of the rate of force development after such a 'Ca^{2+} jump', he concluded that 4 or more Ca^{2+} ions may interact to produce the unit contractile event. Thus the Ca^{2+}-activation of myofibrils may be more complex than suggested by the curves in Figure 5.

To date, it appears that Ca^{2+} ions control the contraction of all muscle systems, but there are various mechanisms by which an

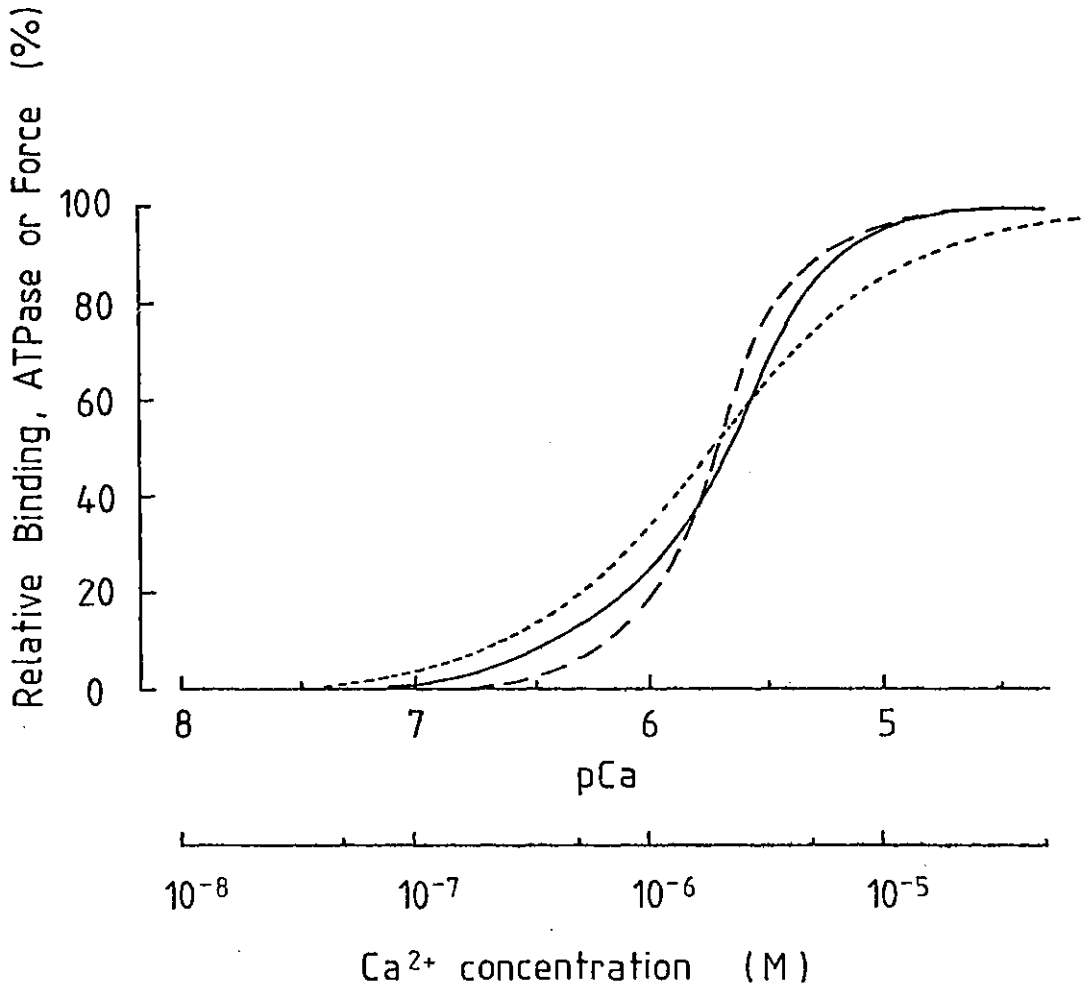


Figure 5. The Ca^{2+}-regulation of cardiac myofibrils

Relationships between log [Ca^{2+}] and Ca^{2+}-binding (dotted line), ATPase activity (solid line) and isometric force development (dashed line) in cardiac myofibrils. Ca^{2+}-binding and ATPase activity measured in isolated cardiac myofibrils; force production measured in 'skinned' cardiac muscle. Modified from Solaro & Briggs (1974).

increase in cytosolic Ca^{2+} concentration leads to muscular contraction. In vertebrate striated muscle, regulation involves the troponin-tropomyosin protein system on the thin filament, although some other types of muscle possess, in addition or instead, myosin-linked control.

(b) The molecular mechanism of Ca^{2+} regulation via tropomyosin and troponin

In 1963, Ebashi demonstrated that a difference in the Ca^{2+}-sensitivity of two crude actomyosin preparations was due to the presence of a third protein component, 'native tropomyosin', which was located in the thin filaments. 'Native tropomyosin' was subsequently found to be a complex of tropomyosin and a previously unknown Ca^{2+} binding protein, 'troponin' (Ebashi & Kodama, 1965). Because pure actomyosin had a fully active ATPase activity it was clear that the tropomyosin-troponin system served to inhibit this activity in the absence of Ca^{2+} and that Ca^{2+} therefore activated actomyosin by relieving an existing inhibition. Later, kinetic studies showed that, in the absence of Ca^{2+}, the tropomyosin-troponin complex inhibited actomyosin ATPase activity by decreasing the affinity of actin for myosin heads (Eisenberg & Kielley, 1970; Koretz et al., 1972). This inhibition was maximal when the mole ratio of troponin:tropomyosin:actin was 1:1:7 (Spudich & Watt, 1971), the ratio found in vivo. Together with the finding that, if Ca^{2+} binds to troponin, all the actin monomers are available to react with myosin (Moore et al., 1970) these results indicated that the binding of Ca^{2+} to a single troponin molecule could 'switch on' the seven actin monomers with which that troponin was associated.

Ebashi and Endo (1968) had suggested that tropomyosin could communicate changes in the conformation of troponin to the associated actin monomers, which would thus function in a co-operative manner. This proposal was further supported by the results of Bremel & Weber (1972). These workers found that, under conditions of low [MgATP] and absence of Ca^{2+}, the formation of a critical number of actin-myosin rigor linkages resulted in a Ca^{2+}-independent 'switching on' of the remaining, unoccupied actin monomers, which could then bind myosin and release ADP plus P_i. This co-operativity was conferred by tropomyosin, since pure actin filaments showed no such behaviour.

The observations of Bremel & Weber explain why, at micromolar concentrations of MgATP, myofibrils can hydrolyse significant amounts of ATP and skinned muscle can develop a substantial force ('rigor

contracture') in the absence of Ca^{2+}. At physiological concentrations of MgATP (about 5 mM) the number of rigor linkages is below the critical level for the co-operative activation of adjacent monomers - this can then only be effected by Ca^{2+} binding to troponin.

Bremel & Weber (1972) also found that the formation of rigor links increased the Ca^{2+}-affinity of the 'low affinity' (K_{Ca} - 5 x 10^6 M^{-1}) binding sites on troponin. Thus there is a very tight and bidirectional functional linkage between the conformation of troponin with Ca^{2+} bound and the conformation of actin-tropomyosin which allows the binding of myosin heads to actin.

In the early 1970's several lines of evidence suggested a possible structural basis for the Ca^{2+}-regulation of actin-myosin interaction and for the co-operative nature of actin-tropomyosin-troponin interaction. Three-dimensional reconstruction from electron micrographs of negatively-stained thin filaments (Moore et al., 1970) showed that each tropomyosin molecule was situated to one side of the groove in the actin helix, in a position that, it was suggested, could sterically interfere with the binding of myosin heads to actin (Figure 6). Changes in the thin filament X-ray reflections from living skeletal muscle indicated that during activation, the tropomyosin filaments move by about 1.5 nm further into the actin groove (Vibert et al., 1972; Huxley, 1972; Haselgrove, 1972). It seemed reasonable to suppose that this movement exposed previously-covered sites on actin which could then interact with myosin (Figure 6). Furthermore this movement could occur along the length of the tropomyosin molecule, so that all 7 actin monomers would be free of the steric blocking of myosin-binding sites by tropomyosin. It is readily apparent how binding of Ca^{2+} to troponin could activate 7 actin monomers by causing the inward movement of tropomyosin. The model in Figure 6 can also explain how the formation of several rigor linkages in low ATP could push the tropomyosin into its activated position, so exposing the myosin-binding sites of the remaining actin monomers controlled by the tropomyosin filament.

Unfortunately this comprehensible and attractive model requires considerable modification in the light of recent data obtained by Seymour & O'Brien (1980), who have determined the 'sidedness' of tropomyosin in the thin filament. If the site of myosin attachment is actually as indicated in previous work (Huxley, 1963; Moore et al., 1970) then the results of Seymour & O'Brien indicate that tropomyosin

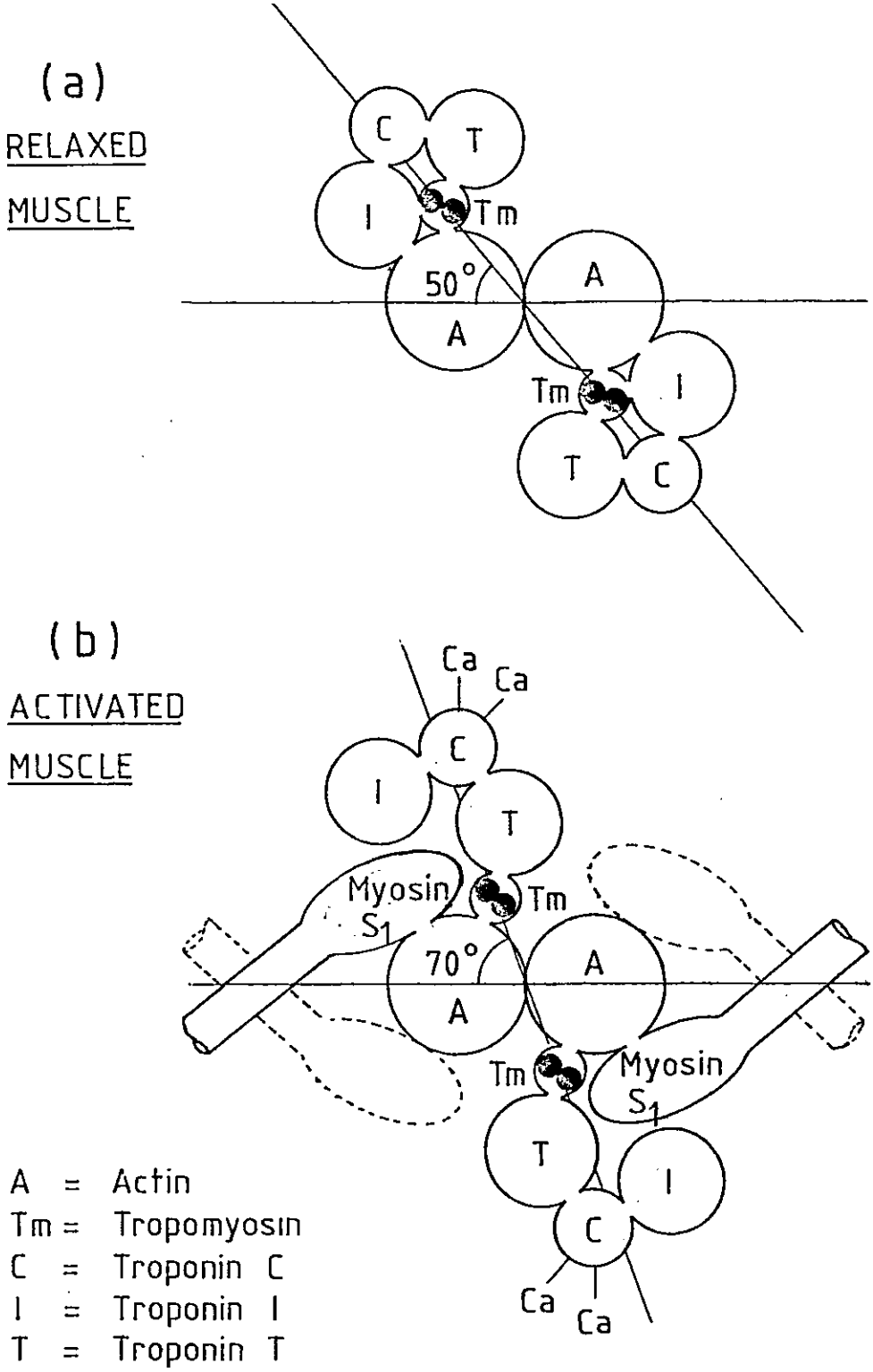


Figure 6. A model for the regulation of actin-myosin association by Ca^{2+}. Modified from Parry & Squire (1973). End-on views of the thin filament at the level of two troponin molecules. (a) In relaxed muscle tropomyosin is bound to actin in a position where it can prevent attachment of myosin S-1 heads to actin. (b) In activated muscle Ca^{2+} binds to troponin. Tropomyosin swings further into the grooves of the actin helix, myosin heads bind to actin and force is generated.

Parry & Squire (1973) considered tropomyosin could sterically block the attachment of the S-1 heads (solid lines) but the recent determination of the position of the bound S-1 (dashed lines) indicates that the inhibition must be via a co-operative affect on actin (Seymour & O'Brien, 1980).

and myosin do not lie on the same side of the actin groove. This makes steric hindrance between the two much less likely. We must therefore consider the possibility that the movement of tropomyosin controls actin-myosin association not by a steric effect but by a co-operative effect on the myosin-binding site of actin. This postulate is also consistent with the results of Stein et al. (1979), who reported that dissociated actin and myosin are in rapid equilibrium with a weakly associate species, whether or not Ca^{2+} is present, and that Ca^{2+} binding to troponin regulates a non-reversible reaction subsequent to cross-bridge attachment rather than the attachment reaction itself.

(c) Binding of Ca^{2+} by troponin

Although the interactions between the regulatory proteins and actin remain to be clarified, the initial event in the activation of myofibrils - the binding of Ca^{2+} to troponin - is reasonably well characterised. Rabbit skeletal TN-C contains four Ca^{2+}-binding sites (Ebashi and Endo, 1968), though the sites are not all equivalent (Potter and Gergely, 1975): isolated whole troponin has two Ca-specific 'low affinity' sites ($K_{Ca} \approx 5 \times 10^{-6}\ M^{-1}$) and two 'high-affinity' Ca^{2+}-binding sites ($K_{Ca} \approx 5 \times 10^{8}\ M^{-1}$) which can also bind Mg^{2+} ($K_{Mg} \approx 5 \times 10^{3}\ M^{-1}$). There are also two Mg-specific sites ($K_{Mg} \approx 2\text{-}4 \times 10^{3}\ M^{-1}$). At physiological concentrations of Mg^{2+}, i.e. about 3 mM, the apparent Ca^{2+}-affinity of Ca-Mg sites is reduced to about $5 \times 10^{6}\ M^{-1}$, so that the four Ca^{2+} sites have effectively the same affinity. The apparent binding constants for all four sites are thus consistent with the range of Ca^{2+} concentrations required for activation (Figure 5). Despite this, it seems that only the Ca-specific sites are directly involved in the physiological regulation of skeletal myofibrillar ATPase. This was originally suggested by Potter & Gergely (1975) on the basis that Mg^{2+} did not inhibit the Ca^{2+}-activation of skeletal myofibrillar ATPase, though it was suggested by Ashley & Moisescu (1977) that this apparent insensitivity to Mg^{2+} may have been due to a compensatory inhibition by the K^{+} used to replace the Mg^{2+}. More recently, Potter's group have studied the Ca^{2+}-induced changes in fluorescence of dansylaziridine-labelled skeletal TN-C and have found that Ca^{2+} dissociation from the Ca-specific sites occurs with a half-time of 2-3 ms, while Ca removal from the Ca-Mg sites has a half-time of $\approx$ 100 ms (Johnson et al., 1979).

Thus only in the case of Ca-specific sites is the Ca exchange fast enough to account for the speed of skeletal muscle relaxation, and this strongly suggests that the Ca-specific sites are the functionally important sites for the Ca^{2+}-activation of the myofibrils.

Studies on the Ca^{2+}- and Mg^{2+}-binding properties of proteolytic fragments of skeletal TN-C (Leavis et al., 1978) and on the [^{3}H]-ethanolanine reactivity of carboxyl Ca^{2+}-binding groups of troponin at various Ca^{2+} concentrations (Sin et al., 1978) have established sites I and II as the Ca-specific sites and sites III and IV as the Ca-Mg sites. From a comparison of the amino acid sequence of cardiac and skeletal TN-C, van Eerd and Takahashi (1975) predicted that the region of cardiac TN-C corresponding to site I in skeletal TN-C may have lost the ability to bind Ca^{2+}. By analogy with skeletal TN-C, this lost site should have been a 'low-affinity' Ca-specific site. Although several initial reports produced conflicting results, there is now general agreement that cardiac troponin does in fact contain two 'high-affinity' Ca-Mg sites ($K_{Ca} \approx 3 \times 10^8\ M^{-1}$, $K_{Mg} \approx 3 \times 10^3\ M^{-1}$) and, as predicted, only one 'low affinity' Ca-specific site ($K_{Ca} \approx 2 \times 10^6\ M^{-1}$) (Leavis & Kraft, 1978; Johnson et al., 1980; Holroyde et al., 1980). There are also several Mg-specific sites (Holroyde et al., 1980). The presence of Mg^{2+} decreases the K_{Ca} of the Ca-Mg sites, but only to $\approx 2 \times 10^7\ M^{-1}$ at a physiological [Mg^{2+}] of 4 mM (Holroyde et al., 1980). Thus in 4 mM Mg^{2+} only the Ca-specific site of cardiac troponin has a K_{Ca} ($2 \times 10^6\ M^{-1}$) that is consistent with the range of Ca^{2+} concentrations required for myofibrillar activation - the K_{Ca} for the Ca-Mg sites is too high. Accordingly it would appear that the single Ca-specific site is the only Ca^{2+}-binding site involved directly in the Ca^{2+}-regulation of cardiac myofibrils (Holroyde et al., 1980).

Nevertheless there are numerous instances in which Mg^{2+} ions have been found to inhibit the Ca^{2+}-sensitivity of ATPase activity and force development in both skeletal and cardiac myofibrils (Ebashi & Endo, 1968; Kerrick & Donaldson, 1975; Solaro & Shiner, 1976; Ashley & Moisescu, 1977). It may be that the binding of Mg^{2+} to the Ca-Mg sites or the Mg-specific sites modulates the effect of Ca^{2+} binding to the Ca-specific sites.

(d) Binding of Ca^{2+} by Myosin

Troponin is not the only myofibrillar Ca^{2+}-binding protein: the P light chains of skeletal and cardiac myosin bind Ca^{2+} and Mg^{2+} with

high affinity (Morimoto & Harrington, 1974; Holroyde et al., 1979). In 1 mM Mg^{2+} the apparent K_{Ca} is about 10^5 M^{-1}, which is probably too low for the P light chains to be directly involved in the Ca^{2+}-activation of myofibrillar ATPase. On the other hand, the P light chains could conceivably mediate an action of Ca^{2+} which was not reflected in stimulation of the ATPase activity. For instance, Ca^{2+} binding to P light chains might increase the freedom of movement of myosin heads - this could explain why in muscles, stretched to the point of no A-I overlap, stimulation nevertheless leads to outward movement of myosin heads (Haselgrove, 1975). Ca^{2+} binding to the P light chains could alter cross-bridge kinetics (see below). However, the effective rate of Ca^{2+} binding to these light chains is probably very slow, since it would be limited by the slow dissociation of Mg^{2+} from the light chain (time constant $\approx$ 18 s, Bagshaw & Reed, 1977).

(e) Influence of Ca^{2+} concentration on cross-bridge properties

Measurements of the 'instantaneous stiffness' (Section 1.2) of Ca^{2+}-activated skinned fibres have proved that the Ca^{2+}-regulated increase in force is largely due to an increase in the number of cross-bridges (Herzig & Ruegg, 1977, 1980). This fits neatly with the above scheme (Figure 6) which shows how a Ca^{2+}-dependent movement of tropomyosin could act as a simple on/off switch to control the number of available myosin-binding sites on actin. Rather surprisingly, there is now considerable evidence that Ca^{2+} not only regulates cross-bridge number but also affects cross-bridge kinetics, as judged by the maximum velocity of isotonic shortening of muscle (V_{max}). V_{max} has been found to increase with Ca^{2+} concentration in both skinned skeletal (Julian, 1971) and cardiac muscle (de Clerck et al., 1977; Fabiato and Fabiato, 1978a). If the rate-limiting step in the cross-bridge cycle is indeed associated with myosin which is not attached to actin (Section 1.2), the Ca^{2+}-sensitivity of V_{max} implies that Ca^{2+} acts directly on the myosin heads to alter cross-bridge kinetics. It seems likely that the Ca^{2+}-binding 'P' light chains could mediate this effect. In this regard, it of particular interest that the Ca^{2+}-sensitivity of V_{max} occurs over a range of Ca^{2+} concentrations that is not consistent with the Ca^{2+}-affinity of troponin but is consistent with the lower apparent Ca^{2+}-affinity of the P light chain (Herzig & Ruegg, 1980). Alternatively, a Ca^{2+}-dependent phosphorylation of the P light chain could be involved (Section 1.5 (b)). The influence of Ca^{2+} on cross-bridge

kinetics probably contributes little, if at all, to the effect of Ca^{2+} on isometric force, because the increase in isometric force can be entirely explained by the increase in the number of cross-bridges (Herzig & Ruegg, 1977, 1980).

1.4 The Regulation of the Intracellular Ca^{2+} Concentration during Cardiac Contraction and Relaxation

Although this thesis is concerned predominantly with the later stages in cardiac excitation-contraction coupling - the steps between the rise in cytosolic Ca^{2+} concentration and the subsequent development of force - an introduction to the regulation of myofibrillar activity would not be complete without a brief summary of the earlier stages in excitation-contraction coupling. The chain of events involved in the activation of fast-twitch skeletal muscle is now well-established and has been extensively reviewed (see, for example, Ebashi and Endo, 1968; Fuchs, 1974; Costantin, 1975). The fast time course of the skeletal twitch and the insensitivity of the twitch to external Ca^{2+} are consequences of the fact that almost all of the activator Ca^{2+} for contraction is released from internal stores (the sarcoplasmic reticulum or SR). In the case of cardiac muscle, the situation is more complex (Figure 7). The dependence of the heart beat upon external Ca^{2+}, the small size of cardiac cells (about 10 μm in diameter) and the slow onset of contraction all allowed for the possibility that activator Ca^{2+} entered the cell from the extracellular space during each beat. In mammalian heart the activator Ca^{2+} probably comes from both extracellular and intracellular locations. Ca^{2+} enters the cell during each beat and comprises most of the 'slow inward current' of the cardiac action potential (Beeler and Reuter, 1970 a, b). This current exhibits a dependence on external Ca^{2+} which parallels that of the force of contraction (New & Trautwein, 1972). In addition, Ca^{2+} could enter via a Na/Ca exchange during depolarisation of the cell (Mullins, 1979). However, the amount of Ca^{2+} entering the cell during a single beat (about 1-5 μmoles/kg cell water: Niedergerke, 1963; New & Trautwein, 1972) is much too small for the force developed at normal heart rates, for cardiac myofibrils require at least 100 μmole Ca^{2+}/kg cell water for full activation (Solaro et al., 1974; Kitazawa, 1976). Even if allowance is made for the likelihood that cardiac myofibrils are only 25% - 50% activated with Ca^{2+} during a normal beat, there remains a considerable discrepancy, which implies

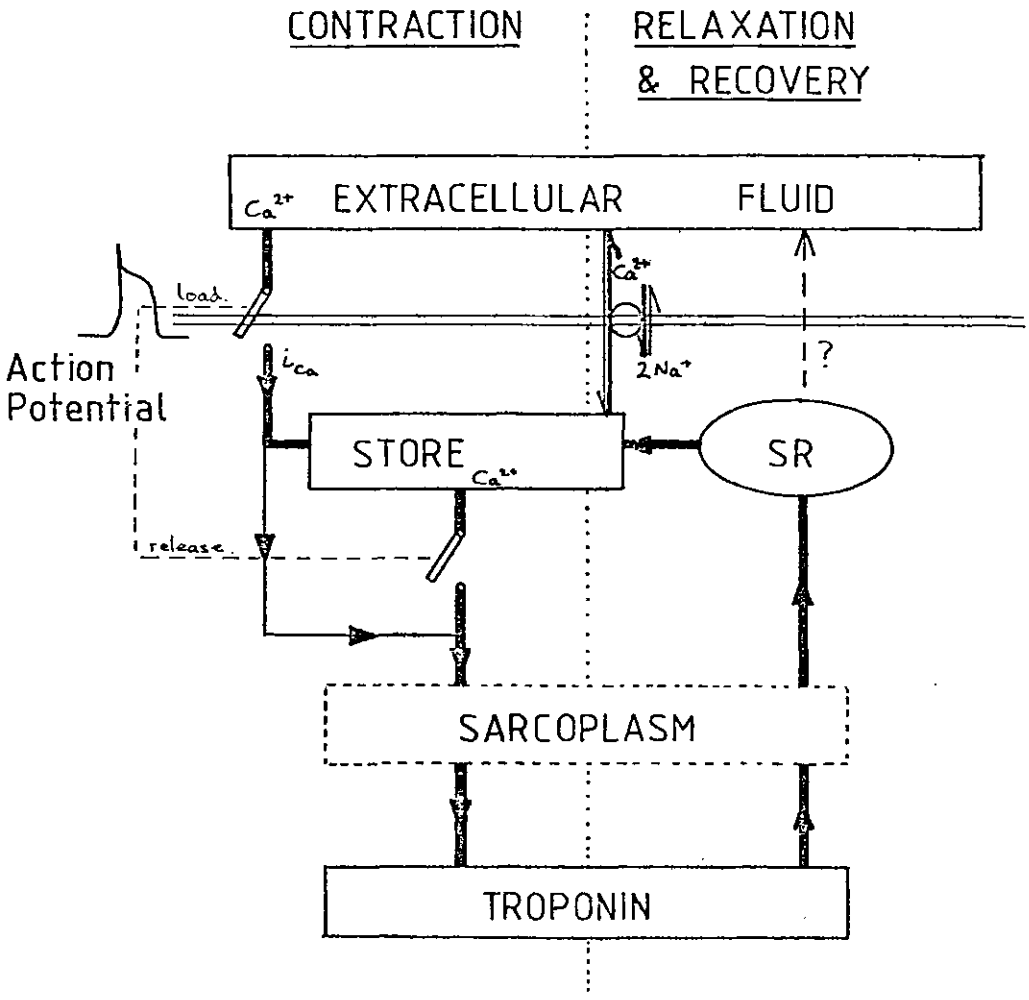


Figure 7. Movements of Ca^{2+} during contraction (left hand side) or relaxation and recovery (right hand side) in cardiac muscle cells. Depolarisation of the sarcolemma during the action potential triggers Ca^{2+} release from an intracellular store, probably the sarcoplasmic reticulum (SR). Ca^{2+} diffuses through the sarcoplasm and binds to troponin. Actin-myosin interaction is thereby allowed and contraction ensues. The store is loaded during the plateau phase by the slow inward current, i_{Ca}. Some of this Ca^{2+} may enter the sarcoplasm directly to add to that released from the store.

Relaxation results from the active uptake of Ca^{2+} by the SR. Most of this Ca^{2+} is returned to the releasable store. The loading of the store by i_{Ca} is opposed by net extrusion of Ca^{2+} from the cell in the interval between beats, mainly via a $Ca^{2+}/2Na^{+}$ exchange. The content of the store, which is a major factor determining the amount of Ca^{2+} released from the store by an action potential, therefore depends on the rate and pattern of stimulation. (Modified from Jewell, 1977).

that intracellular stores must provide much of the activator Ca^{2+}.

The morphological correlate of the Ca^{2+} store, which was inferred from the above physiological experiments, is still disputed. By analogy with skeletal muscle, a likely candidate for the site of storage is the cardiac SR. SR-rich microsomal fractions from cardiac muscle rapidly sequester Ca^{2+} by an ATP-dependent process (Katz & Repke, 1967; Fuchs et al., 1968). Furthermore, agents such as caffeine that inhibit this Ca^{2+} uptake also slow relaxation in intact muscle (Blinks et al., 1972) whilst phosphorylation of the SR stimulates Ca^{2+} uptake and accelerates relaxation (Meinertz et al., 1974). On the other hand, the abundance of mitochondria in heart muscle, coupled with the fact that mitochondria can take up large quantities of Ca^{2+} by an energy-dependent process, has prompted the suggestion that mitochondria may be intimately involved in the movements of Ca^{2+} during activation and relaxation (Carafoli, 1972). This is on the whole unlikely because the uptake and release of Ca^{2+} from isolated cardiac mitochondria seems to be too slow and the K_m for uptake too high (10^{-5} M - 10^{-4} M) to enable mitochondria to contribute significantly to excitation-contraction coupling or to relaxation, though they may act as intracellular Ca buffers (Scarpa and Williamson, 1974). A third likely site for Ca^{2+} storage is the glycocalyx of the basement coat, which covers the surface sarcolemma and extends deep into T-tubules. This has a large number of high-affinity Ca^{2+}-binding sites which may be in equilibrium with the extracellular fluid but its location on the external surface of the sarcolemma would seem to preclude a role as the prime source of activator Ca^{2+}. However, as Langer (1980) has pointed out, it may well be a source of 'trigger' Ca^{2+} (see below).

If the activator Ca^{2+} derives mainly from intracellular stores why then is the force of contraction dependent upon the quantity of Ca^{2+} entering the cell as the 'slow inward current'? An attractive possibility is that this small amount of Ca^{2+} 'triggers' the release of a much larger quantity of Ca^{2+} from the SR. Such a Ca^{2+}-induced release of Ca^{2+} from the SR was first observed in mechanically-skinned skeletal fibres (Endo et al., 1970; Ford and Podolsky, 1970) but it now seems that a physiological role for a Ca^{2+}-induced release of Ca^{2+} is much more likely in cardiac muscle than in skeletal muscle (Fabiato & Fabiato, 1977). In cardiac muscle this phenomenon occurs at a much lower [Ca^{2+}] than is required

to initiate Ca^{2+} release in skinned skeletal fibres and no preloading of SR with Ca^{2+} is necessary. Unfortunately the experiments of Fabiato & Fabiato give no indication as to the amount of 'trigger' Ca^{2+} needed to induce Ca^{2+} release. The Ca^{2+}-induced release of Ca^{2+} cannot be a simple positive feedback system, since this would result in an all-or-none release of Ca^{2+}; on the contrary, the in vivo Ca^{2+} release, as judged by contractile force, is graded according to the external Ca^{2+} concentration (Lüttgau and Niedergerke, 1958) and to the degree of membrane depolarisation (Niedergerke, 1956; Beeler and Reuter, 1970b).

Although the SR removes Ca^{2+} from the cytoplasm in the short term, Ca^{2+} must eventually be pumped out of the cell to balance the Ca^{2+} that enters passively during the action potential. Much of this Ca^{2+} extrusion is likely via a coupled $Ca^{2+}/2\ Na^{+}$ exchange, a system which was first suggested by Lüttgau & Niedergerke (1958) to explain the dependence of force on the $[Ca^{2+}]/[Na^{+}]^2$ ratio of the extracellular fluid. The driving force for this Ca^{2+} extrusion is derived from the large transsarcolemmal Na^{+} gradient maintained by the Na^{+} pump. In addition, there may also be a MgATP-dependent Ca^{2+} pump in the cardiac sarcolemma (Sulakhe & Dhalla, 1971).

Although Ca^{2+} is the primary regulator of cardiac myofibrillar activity, it must be recognised that in vivo the Ca^{2+}-mediated link between excitation of the heart and contraction is itself subject to modulation by various factors. Unlike that of skeletal muscle, the contractile performance of the heart cannot be controlled by alterations in the number of muscle cells activated because the whole myocardium contracts in all-or-none fashion in response to a stimulus. Instead, the heart beat is regulated by alterations in the strength of contraction of each myocardial cell. As in skeletal muscle, cardiac muscle contraction is influenced by changes in resting length (Frank-Starling relationship). Even at constant resting length, however, nervous and hormonal influences can affect the force developed by myocardial cells. Much indirect evidence has accumulated to support the view that most inotropic agents act predominantly by altering the supply of Ca^{2+} to the myofibrils during systole (for reviews see Langer, 1968; Nayler & Merrillees, 1971). This implies that during a normal beat the myofibrils are not maximally activated by Ca^{2+} ions. These premises have been recently verified directly by injection of cardiac cells with the

Ca^{2+}-sensitive bioluminescent protein, aequorin (Allen & Blinks, 1978; Allen & Kurihara, 1980; Fabiato, 1982). The luminescence signals during contraction - 'Ca^{2+}-transients' - exhibited a qualitatively similar, although longer, time course to the transients obtained in barnacle muscle (Ashley & Ridgway, 1970) and frog skeletal muscle (Blinks et al., 1978). The slowness of Ca^{2+} release is probably largely responsible for the slower time course of cardiac contraction. All interventions that increased force also increased the magnitude of the Ca^{2+} transient. In addition, Fabiato (1982) has demonstrated that, even in intense positively-inotropic stimulation, the $[Ca^{2+}]$ reached during systole is sufficient to produce only about half-maximal Ca^{2+}-activation of the myofibrils. Thus the Ca^{2+} supply to the myofibrils is a major determinant of myocardial force production.

The long-standing and considerable evidence in favour of this view has until recently rather overshadowed the possibility that some physiological mechanisms that regulate the heart beat may be at least partly mediated by alterations in the inherent properties of the myofibrils. We now know however that cardiac myofibrillar activity can be modulated in both the short term and long term by changes in the properties of the myofibrils themselves.

1.5 Physiological Factors which change the Inherent Properties of Cardiac Myofibrils

(a) Sarcomere length

One of the triumphs of the sliding filament theory was the demonstration (Gordon et al., 1966) that filament geometry provided a ready explanation for the decrease in the active tension of skeletal muscle as muscle length is altered in either direction from an optimum value, designated L_{max}. Gordon et al. attributed the linear descending limb of the length-tension relationship (at sarcomere lengths above 2.2 μm) to decreased overlap of thin filaments with the cross-bridge bearing regions of thick filaments. They further suggested that the ascending limb (at sarcomere lengths below 2.0 μm) was due to physical factors either interfering with tension generation or producing forces opposing shortening. It was generally assumed that similar factors were responsible for the length-tension curve ('Frank-Starling relationship') in cardiac muscle. However it is now clear that, in both skeletal and cardiac muscle, the ascending limb of the length-tension relationship results

mainly from a length-dependence of excitation-contraction coupling rather than from detrimental interactions between filaments (for review see Jewell, 1977). This is of particular physiological significance for cardiac muscle, which normally works at lengths below the optimum (L_{max}).

It is now established that sarcomere length influences both the Ca^{2+} supply to the myofibrils and the sensitivity of the contractile proteins to Ca^{2+}. The latter phenomenon was first observed by Endo (1972), who found that the Ca^{2+}-sensitivity of skinned skeletal fibres increased as the sarcomere length was raised from 2.0 - 2.3 μm to 3.0 - 3.3 μm. The phenomenon caused L_{max} in partially-activated skinned fibres to occur at about 2.8 μm, i.e. the greater force produced by the increase in Ca^{2+}-sensitivity at suboptimal Ca^{2+} concentrations more than compensated for the tendency of the diminished filament overlap to reduce developed force. Subsequent work has shown that increases in sarcomere length over the range 2.2 μm to 3.0 μm shift the relative force-pCa relationships of skinned skeletal fibres (Moisescu & Thieleczek, 1979) and skinned cardiac cells (Fabiato & Fabiato, 1978a) to higher pCa values. A similar increase in Ca^{2+}-sensitivity also occurs when the sarcomere length is increased over the ascending (i.e. physiological) limb of the length-tension curve for skinned cardiac muscle (Hibberd & Jewell, 1979). The mechanism for this length-dependence of Ca^{2+}-sensitivity is not known at present.

(b) Phosphorylation state

To date, the phosphorylation of two protein components of the myofibril - troponin-I and myosin P light chains - has been implicated in the modulation of myofibrillar contractile properties.

Troponin-I. Cardiac muscle contains a cAMP-dependent protein kinase which can rapidly phosphorylate TN-I and an active phosphatase which can remove the phosphate group (Cole & Perry, 1975). A possible role for phosphorylation/dephosphorylation was suggested by the demonstration that the phosphorylation of TN-I increased in parallel with contractile force after administration of adrenaline to perfused rat hearts (England, 1975). Was the positive inotropic effect therefore caused by the increase in TN-I phosphorylation? The answer appears to be no: phosphorylation of TN-I *in vitro* shifts the Ca^{2+}-activation curves for the ATPase activity of cardiac

myofibrils (Ray & England , 1976) and for the force production in 'hyperpermeable' cardiac muscle (Mope et al., 1980) to higher Ca^{2+} concentrations, i.e. phosphorylation of TN-I decreases the Ca^{2+}-sensitivity, though maximum ATPase activity or force is not affected. Thus phosphorylation of myofibrils would, in itself, have a negative inotropic effect. Possibly however the phosphorylation of cardiac TN-I could partly explain the action of catecholamines to abbreviate systole (England, Personal communication, 1982).

Myosin P light chains. Perry and colleagues have established that the 18000-20000 Dalton 'P' light chains of myosins from skeletal, cardiac or smooth muscle can be specifically phosphorylated by a sarcoplasmic enzyme, myosin light chain kinase (Perrie et al., 1973; Frearson & Perry, 1975). This enzyme requires calmodulin plus micromolar concentrations of Ca^{2+} for activity. Another specific cytosolic enzyme - myosin light chain phosphatase - can dephosphorylate the 'P' light chains. In skeletal muscle, conditions which will tend to increase the cytosolic Ca^{2+} concentration are associated with increased phosphorylation of the P light chains, presumably because myosin light chain kinase is activated (Bárány & Bárány, 1977). In contrast the degree of phosphorylation of P light chains in cardiac muscle is relatively invariant (England, Personal communication, 1981).

(c) Pattern of myosin subunits

Not only are the maximum myosin and actomyosin ATPase activities correlated with the maximum velocity of unloaded shortening (V_{max}) in different muscles (Bárány, 1967), but they also change in parallel with long-term changes in contractile performance in the same muscle. Such changes occur during development and can be induced by physical training or by changes in the innervation of the muscle or in the hormonal status of the body (reviewed by Scheuer & Bahn, 1978). These changes in ATPase activity and V_{max} occur in parallel with alterations in the patterns of the heavy and light chains of myosin, though it is not yet clear which type of chain is responsible for determining the ATPase activity.

Whereas the above factors may directly alter the intrinsic properties of cardiac myofibrils as part of physiological regulatory mechanisms, other factors may become important in pathological conditions. In this thesis I am concerned with two of these: the direct influence of intracellular pH and the direct influence of

intracellular salt concentration on cardiac myofibrillar function. The significance of each of these factors is reviewed in the next two chapters.

Chapter 2

INFLUENCE OF pH ON THE MECHANICAL ACTIVITY OF MUSCLE

Since the first report by Klug (1879), many studies have confirmed that an acidosis produced by an elevated P_{CO_2} ('respiratory acidosis') results in an immediate and precipitous fall in the contractile performance of heart muscle both *in vivo* and *in vitro* (see for example: Wallace and Sowton, 1896; Smith, 1926; Pannier and Leusen, 1968; Williamson et al., 1975a, b. Also see Figure 8). Conversely, a respiratory alkalosis has a positive inotropic action. In a detailed study, Smith (1926) pointed out that the developed tension of cardiac muscle fell more slowly, and to a lesser extent, if the decrease in extracellular pH (pH_e) was produced by a reduction in the $[HCO_3^-]/[Cl^-]$ ratio ('metabolic acidosis') than if it was produced by a respiratory acidosis. From this he concluded that the negative inotropic action of a high P_{CO_2} was not due to the decrease in pH_e *per se*, but rather to an intracellular action of CO_2, probably its ability to produce an intracellular acidosis by the reaction:

$$CO_2 + H_2O \underset{\text{carbonic anhydrase}}{\rightleftharpoons} H_2CO_3 \rightleftharpoons H^+ + HCO_3^-.$$

Smith and others (Pannier and Leusen, 1968; Cingolani et al., 1970) proposed that the faster action of a respiratory acidosis was because CO_2 could readily diffuse across the sarcolemma and lower the intracellular pH (pH_i) whereas H^+ or HCO_3^- ions would be relatively impermeant. By the use of intracellular pH electrodes, the different time courses for a decrease in pH_i in respiratory and metabolic acidoses have been shown directly, first in crab skeletal muscle (Caldwell, 1958) and more recently in mammalian cardiac muscle (Ellis and Thomas, 1976).

The demonstration that pH_i is a major determinant of myocardial performance is of clinical relevance, for much of the initial decline in cardiac work seen when the myocardium becomes ischaemic (such as after coronary occlusion) may be attributable to the effects of an intracellular acidosis (Williamson et al., 1975a; Poole-Wilson, 1975). This acidosis is caused by a combination of two factors: (i) stimulation of lactic acid production via glycolysis in the hypoxic regions below the occlusion and (ii) accumulation of this lactic acid plus

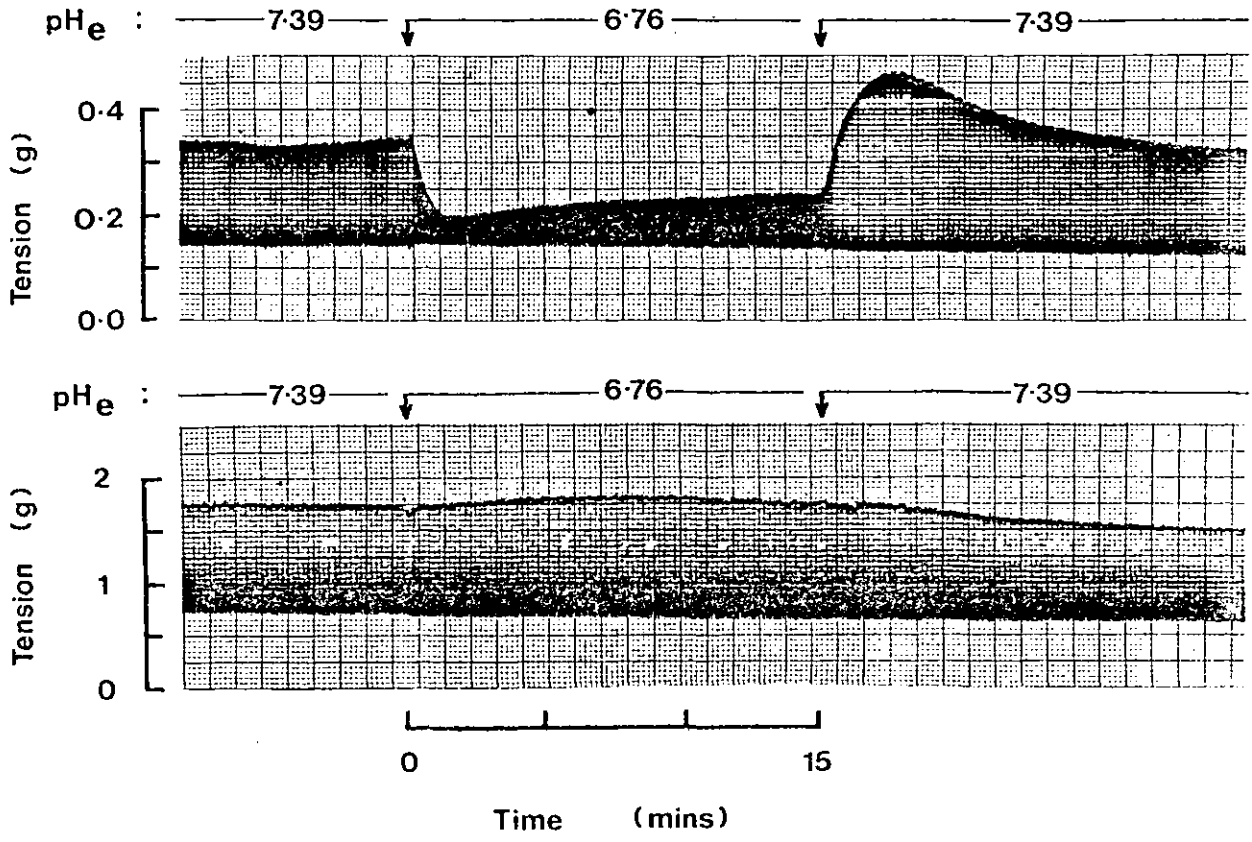


Figure 8. The effects of a respiratory acidosis on the force development in cardiac and white skeletal muscle.

Isolated preparations of a guinea-pig ventricular trabecula (upper panel) and a guinea-pig toe muscle (lower panel). Respiratory acidosis produced by switching from a physiological saline (Section 9.2(a)) equilibrated with 95% O_2 + 5% CO_2 to one equilibrated with 70% O_2 + 30% CO_2. 10^{-5} M tubocurare was added to the solutions bathing the skeletal muscle. 30°C. Both muscles stimulated at 1 s^{-1}.

other acidic metabolites and retention of CO_2 as a result of the reduced blood flow. However, the relative contributions of acidosis and depletion of high-energy phosphates to the initial decline in myocardial performance in ischaemia are still disputed.

Although a knowledge of the pH_i in normal conditions and during acidosis or ischaemia is obviously vital for the discussion of how acidosis reduces myocardial performance, attempts to unequivocally evaluate the pH_i have encountered numerous technical difficulties. The estimation of pH_i from the transsarcolemmal distribution of weak electrolytes such as DMO (dimethyl-2 ,4-oxazeolidinedione) is complex, requires an accurate knowledge of interstitial pH and can follow only very slow changes in pH. A new and promising non-invasive technique uses the phosphate N.M.R. signals from intracellular HPO_4^{2-} and $H_2PO_4^-$ to calculate the pH_i (Jacobus et al., 1977). Both methods however, suffer from the disadvantage that they measure the 'overall' intracellular pH_i, which may differ from the cytosolic pH owing to the likely compartmentalisation of protons. The true cytosolic pH can be measured with pH-sensitive microelectrodes but it is only with the development of recessed-tip microelectrodes (Thomas, 1974; Ellis & Thomas, 1976), that the pH-sensitive part of the glass has been made small enough to ensure that only the intracellular pH is being measured.

Nevertheless most studies indicate that the pH_i in normal cardiac muscle, like that in skeletal muscle, lies between pH 6.8 and 7.1,and is most frequently about pH 7.0 (Poole-Wilson, 1978; Roos & Boron, 1981). The pH_i in ischaemic cardiac muscle depends on the duration and severity of the ischaemia, but values after 30 minutes ischaemia are generally in the range pH 6.4 - pH 6.8, although the estimation of pH_i by phosphate N.M.R. has given a value of pH 5.7 for the pH_i after 25 minutes of ischaemia (Jacobus et al., 1977). To date only pH-sensitive microelectrodes are fast enough to continuously measure the changes of pH_i in ischaemia or acidosis (though their stiffness limits their use to quiescent preparations). Using such electrodes, Ellis and Thomas (1976) found that an increase or decrease in P_{CO_2} produced a biphasic response in pH_i in mammalian cardiac muscle: an initial rapid shift in pH_i, which reached a peak in about one minute, was followed by a slower, incomplete recovery. On return to the previous P_{CO_2}, pH_i overshot its initial value and then slowly

returned. It is interesting that the force developed by cardiac muscle responds in a similar biphasic manner to changes in P_{CO_2} (Serur et al., 1976; see also Figure 8). This can be construed as further evidence that myocardial contractile performance depends on pH_i rather than on pH_e.

The mechanism responsible for the partial restoration of myocardial pH_i after changes in P_{CO_2} is not known. Under normal conditions protons must be actively extruded from the muscle cells (or HCO_3^- or OH^- ions injected) because the resting pH_i is greater than the pH of about 6.0 predicted from the Nernst equation on the basis of a passive distribution of protons across the sarcolemma (Caldwell, 1958). In snail neurones (Thomas, 1976), squid axons (Russell & Boron, 1976) and barnacle muscle (Boron, 1977), all of which exhibit biphasic alterations in pH_i in response to a change in P_{CO_2} , the compensatory recovery of pH_i may be due to the activity of an OH^- or HCO_3^- pump, for the recovery is inhibited by SITS (4-acetamido-4'-isothiocyanatostilbene-2,2'-disulphonic acid) a well-established inhibitor of anion exchange in red cells. However in ventricular muscle SITS does not block the recovery of pH_i (Ellis & Thomas, 1976), so some other process may regulate pH_i in this tissue.

In contrast to that of cardiac muscle, the contractile performance of fast twitch skeletal muscle is potentiated, albeit slightly, by a respiratory acidosis (Wallace & Sowton, 1896; Pannier et al., 1970; see also Figure 8). The divergent actions of a respiratory acidosis on these two types of muscle cannot be explained in terms of differences in the resulting pH_i changes, for Thomas and co-workers have shown by the use of microelectrodes that the changes in pH_i resulting from alterations in P_{CO_2} are similar in direction and magnitude in both types of muscle (Ellis & Thomas, 1976; Aicken & Thomas, 1977). As in mammalian cardiac muscle, the pH_i response in mammalian skeletal muscle is biphasic (Aitken & Thomas, 1977). Despite this, the acidosis-induced potentiation of the skeletal twitch follows a monophasic time course (Pannier et al., 1970; see also Figure 8). The fact that the skeletal twitch is rather insensitive to pH_i is important for the function of skeletal muscle, for during sustained muscular exercise an intracellular acidosis will develop as a result of lactic acid production by anaerobic glycolysis. Thus in skeletal muscle, in contrast to cardiac muscle, an acidosis is a likely consequence of physiological, rather than pathological, events. If the skeletal twitch were as sensitive as the cardiac twitch to pH_i, muscular exercise could last only a few seconds.

Thus there are two problems: what is the primary mechanism for the negative inotropic effect of an intracellular acidosis in cardiac muscle and why does this mechanism apparently not operate in fast twitch skeletal muscle? It would seem likely that the difference in pH-sensitivities resides in a feature of cardiac E-C coupling that is present in cardiac muscle but lacking in skeletal muscle, or vice versa. That different mechanisms are involved in the two tissues is indicated by the fact that acidosis in cardiac muscle decreases the peak developed tension and rate of rise of tension, with no change in time to peak tension (Pannier & Leusen, 1968; Cingolani et al., 1970), whereas the potentiation of the skeletal twitch is associated with a prolonged time to peak tension but little alteration in the rate of rise of tension (Pannier et al., 1970).

As yet we do not know the primary mode of action of acidity on either type of muscle. It might be expected a priori that any negatively-charged Ca^{2+}-binding sites would be able to bind protons. Certainly, in cardiac muscle, acidosis influences Ca^{2+} movements in numerous ways. For example, it reduces the slow inward current, which is carried predominantly by Ca^{2+} ions, in frog atrial fibres (Chesnais et al., 1975) and mammalian ventricular muscle (Kohlhardt et al., 1976). A decrease in pH also reduces the influx of ^{45}Ca into rabbit septa (Poole-Wilson & Langer, 1979) and decreases Ca^{2+} binding to both 'high-affinity' sites ($K_m \approx 16$ μM) and 'low-affinity' sites ($K_m \approx 800$ μM) on isolated sarcolemmal vesicles from guinea-pig ventricles (Williamson et al., 1975b), though whether any of these actions are connected with the effects on the slow inward current is not clear.

The effects of pH on the Ca^{2+}-accumulation and ATPase activity of SR-rich microsomal fractions have been studied in some detail, but the results are complex. In general, the steady-state quantity of Ca^{2+} accumulated in the absence of a precipitating anion is little affected or is slightly decreased by acidity. This is true for both skeletal SR (Sreter, 1969; Sorenson & De Meis, 1977) and cardiac SR (Dunnett & Nayler, 1979). Before the steady-state is reached however, there is a transient accumulation of Ca^{2+}, which attains a peak at 1 - 2 min after the addition of Ca^{2+} and which is followed by a spontaneous release of Ca^{2+} from the SR until the steady-state level of accumulated Ca^{2+} is reached. This transient accumulation of Ca^{2+} only occurs at pH<7.0 and is greatly increased as the pH is reduced

(Sorenson & De Meis, 1977; Dunnett & Naylor, 1979).

These results are however difficult to interpret because the SR vesicles are usually suspended in a medium which contains a low concentration of unbuffered Ca^{2+}; the accumulation of Ca^{2+} by the vesicles in fact depletes the bathing medium of much of its Ca^{2+}. The 'spontaneous' release of Ca^{2+} may therefore be due to the changing Ca^{2+} gradients, since it is known that the SR Ca^{2+} pump can be reversed under conditions of low Ca^{2+} outside the vesicles and high Ca^{2+} inside (Barlogie et al., 1971).

Although the steady-state quantity of Ca^{2+} accumulated is little influenced by the pH existing during the incubation period, Nakamaru & Schwarz (1970) have reported that the amount of Ca^{2+} bound to skeletal or cardiac SR can be altered by rapidly changing the pH: increasing the pH from 6.5 to 7.8 induced a rapid release of Ca^{2+} from pre-loaded SR vesicles, whilst a change of pH in the opposite direction stimulated an uptake of Ca^{2+}.

The SR ATPase activity is also very sensitive to pH, but its pH-dependence does not parallel that of Ca^{2+}-accumulation. The general finding is that a decrease in pH over the range pH 7.5 - pH 6.0 reduces the ATPase activity by about 70% (Hasselbach, 1964; Huxtable & Bressler, 1974; Tomlinson et al., 1976).

Recently the pH-sensitivity of SR function has also been studied with intact SR in mechanically-skinned cardiac and skeletal muscle cells (Fabiato & Fabiato, 1978b). These workers found that the pH optimum for Ca^{2+}-loading of skeletal SR was between pH 6.6 and pH 6.2 at pCa 6.0 whereas the optimum was about pH 7.4 for cardiac SR. If release of Ca^{2+} from the SR is directly related to the Ca^{2+} loading of the SR, these results suggest that acidosis would reduce Ca^{2+} release from cardiac SR but might increase the release from skeletal SR. This is one possible explanation for the divergent effects of acidosis on cardiac and skeletal muscle and it will be discussed in more detail later (Section 12.1).

Changes in $[H^+]$ also alter the activity of the other Ca^{2+}-sequestering organelles in cardiac muscle - the mitochondria. Cardiac mitochondria possess an electroneutral H^+/Ca^{2+} antiport system in which two protons exchange for one Ca^{2+} ion (Williams & Fry, 1979). It is presumably this exchange which is responsible for the efflux of Ca^{2+} from mitochondria that can be induced by acidification of the mitochondria suspension (Fry & Poole-Wilson, 1981).

Such H^+-induced changes in Ca^{2+} sequestration or release by

intracellular organelles may explain why an intracellular acidosis increases the resting cytosolic [Ca^{2+}] in barnacle muscle (Lea & Ashley, 1978) and in cardiac Purkinje cells (Coray et al., 1980). In the case of barnacle muscle this rise in cytosolic [Ca^{2+}] is almost certainly the result of a release of Ca^{2+} by the SR (Lea & Ashley, 1981).

The pH_i also affects the intracellular concentrations of ions other than Ca^{2+}. For instance, a respiratory acidosis in cardiac muscle causes a net gain of K^+ (Poole-Wilson & Langer, 1975). In contrast, acidosis in skeletal muscle results in a net loss of K^+ (Adler et al., 1965), which probably accounts for the observed depolarisation in skeletal fibres subjected to a high P_{CO_2} (Aicken & Thomas, 1975). It is however unlikely that these ionic disturbances cause the divergent actions of pH on the contractions of cardiac and skeletal muscle because, at least in cardiac muscle, changes in developed tension are not related to the net uptake of K^+ (Poole-Wilson & Langer, 1975).

It should also be mentioned that pronounced falls in pH_i may have serious metabolic consequences in both cardiac and skeletal muscle because acidity inhibits glycolysis at the level of phosphofructokinase (Neely & Morgan, 1974) and reduces citric acid cycle activity by an action on citrate synthase (Schaffer et al., 1978).

When I started my studies (in 1976) there was considerable confusion in the literature as to whether the contractile machinery of cardiac or skeletal muscle was sensitive to pH. Studies on the Ca^{2+}-regulated ATPase of isolated skeletal myofibrils had produced conflicting reports that acidity increased (Mühlrad & Hegyi, 1965) or decreased (Schädler, 1967; Portzehl et al., 1969) the maximum ATPase activity and Ca^{2+}-sensitivity. In apparent contradiction to either type of result, the Ca^{2+}-dependent force production of mechanically-skinned skeletal fibres did not seem to be sensitive to pH changes over the range pH 6.4 to pH 7.0 (Ebashi & Endo, 1968; Hellam & Podolsky, 1969; Chen-lui and Endo, 1973). Only in one preliminary report was acidity shown to decrease the Ca^{2+}-sensitivity of skinned barnacle fibres but maximum tension was unaffected (Ashley & Moisescu, 1974).

With cardiac muscle too the results were inconclusive. Schädler (1967) and Williams et al. (1975) reported that the Ca^{2+}-sensitivity

of the ATPase activity in glycerinated papillary muscle and isolated cardiac myofibrils, respectively, was decreased by an increase in $[H^+]$. In addition, Schädler (1967) found the maximum Ca^{2+}-regulated ATPase activity was severely reduced by acidity whereas Williams et al. (1975) found this ATPase activity was enhanced by acidity. However both studies can be criticised on the grounds that the myofibrillar preparations used almost certainly contained contaminant membrane ATPases (discussed in Chapter 4). In such studies it is imperative to exclude all membrane-bound ATPases, especially the SR ATPase, since this is activated by Ca^{2+} over the same range as is the myofibrillar ATPase (pCa 7 - pCa 5) and its activity is known to be highly pH-sensitive (see above). Furthermore, the report by Schädler (1967) that the Ca^{2+}-dependent force production in glycerinated papillary muscle was, like the ATPase activity, reduced by acidity was not confirmed by Winegrad (1971), who reported that in cardiac muscle which had been made 'hyperpermeable' by soaking in EDTA, the Ca^{2+}-dependent tension was unaffected by a change in pH from 7.0 to 6.5.

In 1969 Katz & Hecht, citing Schädler's report that acidity decreased the Ca^{2+}-sensitivity of cardiac myofibrils, suggested that such a H^+/Ca^{2+} antagonism could explain the negative inotropic effect of an acidosis: if protons reduced the Ca^{2+}-sensitivity of the troponin-tropomyosin regulatory protein system there would be less contractile activation for the same systolic level of cytosolic Ca^{2+}. An obvious locus for the competition between H^+ and Ca^{2+} was the Ca^{2+}-binding sites of troponin. This hypothesis was originally supported by the studies of Fuchs and colleagues (1970), who reported that Ca^{2+} binding by isolated skeletal troponin was decreased by acidity. However this finding was later retracted after an improvement in technique had shown the Ca^{2+} binding to be pH-insensitive over the range pH 5 - pH 8 (Fuchs, 1974).

Thus by the mid-1970s there was a considerable need for clarification of the influence of H^+ ions on cardiac and skeletal myofibrils. In the experiments described in Part II of this thesis I have investigated the pH-sensitivities of the Ca^{2+}-regulated ATPase of cardiac and white skeletal myofibrils. Considerable care was taken to ensure that both myofibrillar preparations contained no active contaminant ATPases. Furthermore I assayed the ATPase activities of both cardiac and skeletal myofibrils under carefully-controlled and identical conditions, so that their pH-sensitivities could be directly

compared. Using this approach, I hoped to accomplish two objectives:
(i) to determine whether cardiac myofibrils, in a myofibrillar preparation freed of contaminating membrane ATPase, truly displayed a pH-sensitivity in their Ca^{2+}-activation.
(ii) To compare the pH-sensitivities of cardiac and skeletal myofibrils to discover whether there were any differences that could explain the different mechanical responses of cardiac and skeletal muscle to an intracellular acidosis.

The next stage was to investigate the influence of H^{+}concentration on the Ca^{2+}-regulated force generated by myofibrils, by the use of 'skinned' preparations of cardiac and skeletal muscle. For various reasons, discussed in Chapter 8, I regarded the available techniques for skinning cardiac muscle (glycerination, soaking in EDTA or mechanical removal of the sarcolemma) as unsuitable for my studies. I therefore developed an alternative procedure which used a detergent (Triton X-100) as the primary skinning agent. However, during this period two separate studies were published in which the pH-sensitivities of skinned cardiac and skeletal were compared (Fabiato & Fabiato, 1978; Donaldson &Hermansen, 1978). It seemed advisable to extend the scope of my research and so I investigated the influence of salts of other monovalent cations, besides H^{+}, on the Ca^{2+}-dependent force generated by skinned cardiac muscle. Although a considerable amount of research on this topic had been done using skeletal muscle, very few comparable data were available for cardiac muscle. My investigations on detergent-treated cardiac muscle from guinea-pig ventricle are described in Part III of this thesis. Because the effects of salt concentration on the contractile machinery are of particular relevance to the actions of extracellular hyper- and hypo-tonicity on intact muscle, in the next chapter I shall briefly review the effects of tonicity changes on intact fibres and shall outline possible mechanisms for the observed effects.

Chapter 3

INFLUENCE OF INTRACELLULAR IONIC STRENGTH* ON THE MECHANICAL ACTIVITY OF MUSCLE

3.1 The link between extracellular tonicity* and intracellular ionic strength

As a result of osmosis between intra- and extracellular compartments, muscle cells swell in hypotonic solutions and shrink in hypertonic solutions (within certain mechanical limits at the extremes of hypo- or hypertonicity). In fact cells of both skeletal muscle (Blinks, 1965; Dydynska & Wilkie, 1963) and cardiac muscle (Page & Storm, 1966) behave as perfect osmometers provided that allowance is made for a non-solvent fraction of the cell volume. One of the consequences of the partial cellular dehydration that results from extracellular hypertonicity is an increase in the intracellular concentrations of ions and thus an increase in intracellular ionic strength; conversely hypotonicity of the external medium leads to a decrease in intracellular ionic strength. These changes are of importance because there is now good evidence (discussed below) that changes in intracellular ionic strength mediate at least some of the actions of changes in extracellular tonicity on muscle contractile performance. This is of more than academic interest because such effects may underlie the alterations in cardiac contractility that may arise when the blood tonicity differs from its normal value (300 mOsm l^{-1}), such as in hyperosmolar diabetic coma or clinical treatment with hyperosmolar solutions (Giammona et al., 1963; Pogatsa & Dubecz, 1978).

* Terms used in this chapter:
Osmolarity indicates the molarity an ideal solution of a non-dissociating substance must possess in order to exert the same osmotic pressure as the solution under consideration. Osmolarity is related to the total number of particles in the solution, and is given by:

$$\text{Osmolarity} = \Sigma\, g_i\, m_i\, v_i$$

where g_i = osmotic coefficient of solute 'i'
m_i = concentration of the undissociated solute 'i'
v_i = no. of particles into which solute 'i' dissociates.

Tonicity is that portion of the osmolarity that is effective across a biological membrane that is selectively permeable to certain solutes. In this chapter hypertonicity and hypotonicity indicate values above and below, respectively, the normal blood tonicity of ≈ 300 mOsmol l^{-1} (330 mOsmol kg water^{-1}).
Ionic strength ($\Gamma/2$) is a factor determining the degree of ionic interaction in a solution of electrolytes. It is given by:

$$\text{Ionic strength} = 0.5\, \Sigma\, c_i z_i^2$$

where c_i = concentration of ion 'i'
z_i = charge on ion 'i'.

One of the major problems in this field is that we do not know with any accuracy the value of the intracellular ionic strength in muscles bathed in isotonic media, let alone muscles bathed in anisotonic media. Estimates of intracellular ionic strength, calculated from the total intracellular concentrations of ions, have given values between 100 and 200 mM (see Gordon et al., 1973), but the value depends critically on the estimated magnitudes of the corrections for binding or compartmentalisation of ions.

Fortunately, in the last few years the measurement of the cytosolic activities of certain ions with ion-selective microelectrodes have made estimates of cytosolic concentrations possible; from such estimates I calculate the intracellular ionic strength in cardiac muscle to be in the region of 160 mM (Section 12.2). However it is still not clear to what extent cytosolic proteins and amino acids (which may account for a considerable fraction of the intracellular solutes) contribute to the ionic strength.

However, it would be wrong to give the impression that only the intracellular ionic strength changes if the extracellular tonicity is altered, for there would also be changes in the cytosolic viscosity and probably in the surface areas of cellular membranes such as the sarcolemma and S.R.. In addition, the volume of the myofilament lattice reflects the osmotic behaviour of the whole muscle cell and so the distances between the myofilaments decrease if the muscle cell is bathed in a hypertonic medium (Rome, 1968; April et al., 1972). Thus there are known to be at least four variables - intracellular ionic concentrations (and thus intracellular ionic strength), cytosolic viscosity, membrane surface area and myofilament lattice spacing - that could mediate the effects of hypo- or hypertonicity on muscle performance. Indeed, the actions of tonicity changes are so numerous and varied (see below) that it would seem unlikely that any single factor was responsible for them all. Nevertheless it is ionic strength that has received by far the most attention.

In spite of the need to understand more about the intracellular mechanisms that are responsible for hypertonicity-induced changes in cardiac contractility, the vast majority of the work on the effects of tonicity on intact muscle and of ionic strength on muscle models has been carried out using skeletal muscle. It is therefore apposite to review some of the results from skeletal muscle before any discussion is made of the few studies on cardiac muscle.

3.2 The influence of ionic strength on models of skeletal muscle

Soon after the actin-activation of myosin became a recognised phenomenon, Hasselbach (1952) found that increases in ionic strength over the range 0.06 - 0.6 M progressively decreased the Mg^{2+}-dependent ATPase activity of skeletal actomyosin to zero. This effect was subsequent confirmed with the ATPase activities of: (i) heavy meromyosin (HMM) and acto-HMM (Leadbeater & Perry, 1963; Eisenberg & Moos, 1968); (ii) glycerinated muscle (Bowen & Kerwin, 1955); (iii) isolated myofibrils (Weber & Herz, 1963; Portzehl et al., 1969; Goodno et al., 1978). The work on isolated myofibrils has shown that the total ATPase activity in the presence of Ca^{2+} is largely unaffected by ionic strength up to 0.1 - 0.12 M, but above this level the total activity falls because of a reduction in Ca^{2+}-regulated ATPase activity. Decreases in ionic strength below about 0.1 M also lower the Ca^{2+}-regulated ATPase activity but this fall is compensated by a rise in the Ca^{2+}-insensitive ('basal') ATPase activity and so the total ATPase activity remains constant. This increase in basal activity at low ionic strengths probably corresponds to the increase in acto-HMM ATPase activity observed by Eisenberg & Moos (1968), who showed that the latter increase resulted from an increased interaction between actin and myosin rather than from a rise in V_{max} for the enzyme. Thus it is generally believed that the elevation in basal ATPase activity at low ionic strengths is caused by an increase in the steady-state number of cross-bridges between thick and thin filaments. In other words, low ionic strength overcomes the inhibitory effect that troponin-I imposes on actin-myosin interaction in the absence of Ca^{2+}.

This idea is supported by studies by Portzehl et al. (1969), who found that the $[Ca^{2+}]$ for half-maximal activation of skeletal myofibrillar ATPase decreased five-fold if the ionic strength was dropped from 0.1 M to 0.05 M, although changes in ionic strength over the higher range of 0.1 to 0.2 M did not shift the $[Ca^{2+}]$-ATPase relationship at all. This increase in Ca^{2+}-sensitivity below 0.1 M ionic strength is reflected in an increased binding of Ca^{2+} to the myofibrils (Solaro et al., 1976), presumably because the affinity of troponin for Ca^{2+} has increased. An increase in Ca^{2+}-sensitivity of the myofibrils is exactly what one would predict from Bremel & Weber's hypothesis (Section 1.3(b)) if there was an increase in the time-

averaged number of cross-bridges at low ionic strengths. Alternatively, it could be argued that a low ionic strength was acting directly on troponin to increase its affinity for Ca^{2+}, but this seems unlikely given that the binding of Ca^{2+} to isolated skeletal troponin is unaffected by changes in [KCl] from 0.05 to 0.6 M (Fuchs, 1974).

The effects of ionic strength on the force development of skeletal fibres in general parallel the effects on the ATPase activities of isolated myofibrils. The first evidence for this was provided by April et al. (1968), who demonstrated that the tension response of intact fibres to intracellular iontophoretic injection of Ca^{2+} was reduced when the intracellular ionic strength was raised by adding K^+ and Cl^- (both permeant ions) to the bathing solution. Subsequently many workers have shown that increasing the ionic strength over the range 0.1 - 0.5 M results in an approximately linear fall of maximum Ca^{2+}-regulated force development in glycerinated fibres (Jacobs & Guthe, 1970) and in mechanically-skinned fibres (Gordon et al., 1973; Homsher et al, 1974; Thames et al., 1974; Ashley & Moisescu, 1977; Julian & Moss, 1981). In all these studies ionic strength was raised by addition of KCl, but addition of NaCl or tetramethylammonium chloride had similar inhibitory effects on maximum force (Gordon et al., 1973). However ionic strength is not the sole determinant of maximum force in different salt solutions. For example, there are specific ion effects, since anions differ in their inhibitory effectiveness (Jacobs & Guthe, 1970; Gordon et al., 1973; Ashley & Moisescu, 1977) e.g. Br^- > Cl^- > propionate$^-$ (order of decreasing inhibition). In addition, Ashley & Moisescu (1977) found that the inhibitory effect of a raised [KCl] on maximum Ca^{2+}-regulated force was not solely the result of the increase in ionic strength: about one-third of the inhibition disappeared if the tonicity was kept constant with sucrose and so this fraction of the inhibitory action of KCl was in fact due to the concomitant increase in tonicity.

A reduction in ionic strength below about 0.12 M leads to a decrease in Ca^{2+}-regulated force in skinned fibres; however, as with the total ATPase activities of isolated myofibrils, the total force in the presence of Ca^{2+} is little affected because there is a compensatory rise in resting force (Gordon et al., 1973; Thames et al., 1974). This increase in resting force supports the view that the interaction between actin and myosin cannot be completely inhibited by troponin at low ionic strengths and so Ca^{2+}-insensitive cross-bridges are formed.

Skinned muscles have an advantage over isolated myofibrils in that they can be used to study the kinetics of muscle contraction. Several groups have investigated the influence of ionic strength on contraction kinetics, but the results are as yet equivocal. Thames et al. (1974) reported that at 5°C - 7°C the maximum velocity of isotonic shortening (V_{max}) was independent of ionic strength above 190 mM but fell considerably at lower ionic strengths. However more recent studies by the same group using lower temperatures (0°C - 1°C, Gulati & Podolsky, 1978) or by a different group using the same temperatures (1°C - 10°C, Julian & Moss, 1981) have failed to show any effect on V_{max} of ionic strength changes over the range 0.09 M - 0.2 M. This is perhaps a little surprising, for if there are indeed more Ca^{2+}-independent cross-bridges at low ionic strengths, these cross-bridges, if they were like rigor linkages, would constitute an internal load which would act to reduce V_{max}. Thus the recent evidence from contraction kinetics suggests that these cross-bridges are not rigor-like but are of normal activity, except that their formation does not require Ca^{2+}.

An increase in ionic strength by addition of KCl also influences the pCa-relative force relationship in skinned muscle. Ashley & Moisescu (1977) observed that increasing the ionic strength from 195 mM to 245 mM with KCl reduced the pCa_{50} in skinned barnacle by about 0.6 pCa units. However Ashley & Moisescu (1977) also found that changing the ionic strength by replacing 5 mM ATP + 10 mM CP by 15 mM ATP did not alter the pCa-relative force relationship. They concluded that the effects of [KCl] on this relationship were due to a specific antagonism of Ca^{2+} ions by K^{+} ions, and were not related to the accompanying changes in ionic strength.

From the above discussion it is clear that raising the ionic strength with KCl affects several of the contractile properties of myofibrils. In almost all studies the effects of the raised [KCl] have been ascribed to the consequent increase in ionic strength, but the work of Ashley & Moisescu (1977) has shown that the observed effects may be at least partly due to the concomitant increase in osmolarity or to the specific inhibitory actions of certain ions, e.g. K^{+}. Many of the observed effects of 'ionic strength' detailed above may need therefore to be reinterpreted in the light of these findings. For the moment it is probably better to describe the effects of [KCl] as effects of [salt], and not be more specific than

this unless the evidence merits otherwise. Given then that [salt] directly influences the activity of skeletal myofibrils, how well can this account for the effects of extracellular tonicity changes on intact skeletal muscle?

3.3 Relevance of the [salt]-dependence of model systems to the effects of changes in tonicity on the contractile properties of intact skeletal fibres.

One of the most dramatic actions of a tonicity change is on the magnitude of twitch force or tetanus force. Since the original report by Overton (1902) many studies have confirmed that hypertonic solutions decrease, and in sufficient concentrations abolish, the twitch or tetanus in skeletal muscle, whereas hypotonic solutions potentiate force development (e.g.see Ernst, 1926; Hodgkin & Horowicz, 1957; Howarth, 1958; Gordon & Godt, 1970; see also Figure 9). The inhibitory effect of hypertonicity is not due to a failure of muscle excitability, since normal action potentials are still conducted (Hodgkin & Horowicz, 1957). The functional lesion must therefore be either a derangement of E-C coupling or a direct inhibition of the activity of the contractile machinery. There seems little doubt that the direct inhibitory action of a raised [salt] on myofibrillar function (discussed above) contributes at least partly to the diminution of the twitch in hypertonic media, as was first suggested by Howarth (1958). One of the best pieces of evidence for this derives from work by April et al. (1968). They showed that when the intracellular [KCl] was raised by adding KCl to the solution bathing an intact skeletal fibre, the response to intracellular iontophoresis of Ca^{2+} was depressed, even though fibre volume (and thus myoplasmic viscosity and myofilament lattice spacing) remained constant; conversely, isosmotic replacement of NaCl by KCl led to an increase in fibre volume at constant intracellular ionic strength but the tension response was unaffected or was diminished only slightly. (Note that these experiments did not indicate the relative contribution of intracellular tonicity as compared with intracellular ionic strength). However in electrically-stimulated skeletal muscle the inhibitory actions of hypertonicity may not be mediated solely by a direct inhibition of actin-myosin interaction; there could also be a failure of E-C coupling. The relative contributions of the two mechanisms have remained controversial. In general it seems that in the range of one to two times normal

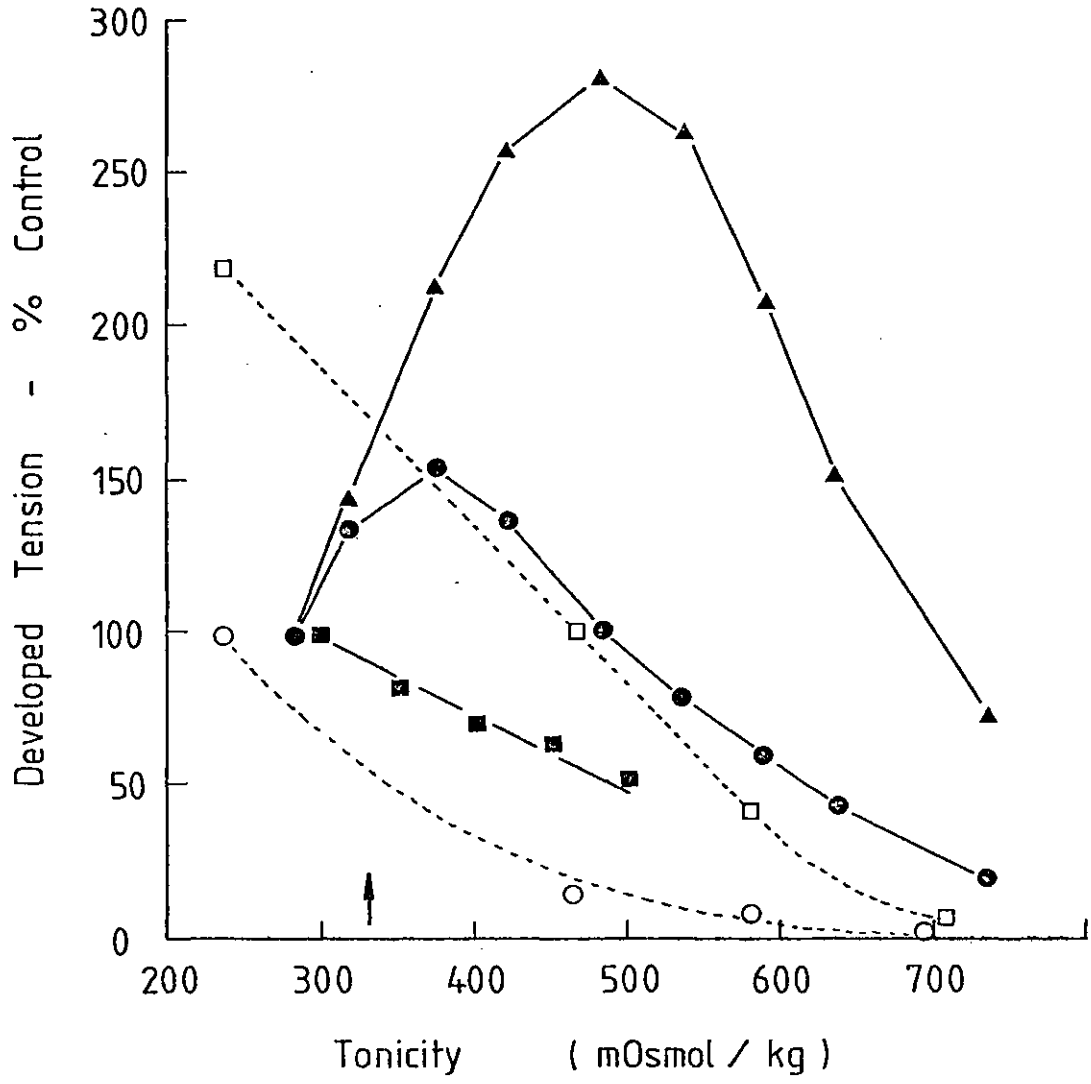


Figure 9. The influence of extracellular tonicity on the force development in cardiac muscle (filled symbols) and white skeletal muscle (open symbols). Twitch force (○) and tetanic force (□) in frog skeletal muscle (Gordon & Godt, 1970); developed force in cat papillary muscle (●) and atrial strip (▲) at 1 s^{-1} and 37°C (Koch-Weser, 1963); developed force in cat papillary muscles (■) with paired stimulation in 4 mM Ca^{2+} at 29°C (Wildenthal et al., 1975). All forces expressed relative to the force obtained in single contractions in the control solution used in the same study. Arrow indicates normal tonicity of mammalian plasma.

tonicity, reduction of the skeletal twitch by hypertonic solutions is due to a direct action of an increased intracellular salt concentration on the contractile apparatus. At higher tonicities, E-C coupling starts to be impaired too and this effect probably contributes increasingly as tonicity is raised further. Since a raised $\Gamma/2$ in fact has a small stimulatory effect on the Ca^{2+}-sequestering ability of isolated SR vesicles (Muir et al., 1971), it would seem likely that the loss of E-C coupling in strongly hypertonic solutions results from the osmotically-induced changes in size of the SR and/or sarcolemma.

Rather surprisingly perhaps, intact fibres in hypotonic media do not appear to display the elevated resting force that might be expected from the observed rise in the Ca^{2+}-insensitive activity of isolated myofibrils or skinned fibres at low [salt]. The reason for this discrepancy is not clear.

By contrast, a change from an isotonic to a hypertonic solution may induce in intact skeletal fibres a large transient contracture, and/or a small maintained increase in resting force (Hill, D.K., 1968; Isaacson, 1969; Lännergren & Noth, 1973). However there are no stimulatory effects of a raised ionic strength on the basal ATPase activity of skeletal myofibrils or resting force of skinned skeletal fibres that could account for these phenomena. The hypertonicity-induced transient contractures have properties similar to those of caffeine-induced contractures and thus probably arise from Ca^{2+} release from the SR (Lännergren & Noth, 1973). The cause of the maintained contractures is unknown.

Another phenomenon that the results from skinned fibres fail to explain is the influence of tonicity on the maximum speed of muscle shortening (V_{max}) in intact fibres. Howarth demonstrated in 1958 that increases in extracellular tonicity slowed the development of isometric twitch tension and reduced the velocity of isotonic shortening. In hypotonic solutions the muscles shortened at approximately the normal speed. With skinned fibres, however, V_{max} is independent of [salt] (Section 3.2), i.e. the effects of hypertonicity on intact fibres do not parallel the effects of a raised [salt] on skinned fibres. Neither can the effects be due to the change in myofilament lattice spacing, which has no influence on V_{max} in intact fibres (Edman & Hwang, 1977). Thus, by exclusion, the actions of tonicity changes on intact fibres are probably the result of alterations in E-C coupling or in cytosolic viscosity.

3.4 The influence of salt concentration on models of cardiac muscle

In contrast to that of skeletal muscle, the sensitivity of cardiac muscle to 'ionic strength' has received little attention from biochemists and physiologists. Katz et al. (1966) showed that the Mg^{2+}-dependent ATPase activity of reconstituted cardiac actomyosin (i.e. pure actin and pure myosin) was inhibited by about 90% as the [KCl] was increased from 60 mM to 120 mM. The ATPase activity remained at a low level (equal to that of myosin alone) as the ionic strength was raised further to 0.4 M. The Ca^{2+}-independent interaction between actin and myosin at low [salt] is thus similar to that observed with skeletal actomyosin and myofibrils (Section 3.2). Muir et al. (1971) found the total ATPase activity of cardiac myofibrils was depressed by an increased [salt], although the peak activity was found at ionic strengths of < 100 mM rather than the 100 - 120 mM for skeletal myofibrils.

The relationship between relative ATPase activity of cardiac myofibrils and pCa is, as with skeletal myofibrils, sensitive to the [KCl]: an increase in ionic strength from 25 mM to 123 mM made by the addition of KCl decreased the pCa_{50} for the ATPase activity of rabbit cardiac myofibrils by about 0.5 (Solaro & Briggs, 1974). It is not known how changes of ionic strength in the 'physiological' range (100 - 200 mM) affect the myofibrillar Ca^{2+}-sensitivity, although this is particularly important because cardiac myofibrils are probably never fully activated by Ca^{2+} *in vivo* (Section 1.4), and so the influence of [salt] on *submaximal* Ca^{2+}- regulated force is in fact more relevant than its influence on the maximum Ca^{2+}-regulated force.

To the best of my knowledge these are the only studies on the ionic strength-dependence of the biochemistry of the cardiac myofibrils. There have been no studies on the contractile properties of skinned cardiac muscle. This neglect of cardiac muscle is surprising because the effects of extracellular tonicity on the mechanical properties of intact cardiac muscle are more complex than in skeletal muscle (see below), and so it is by no means the case that the mechanisms proposed to explain the tonicity-dependence of intact skeletal muscle can also account for the tonicity-dependence of intact cardiac muscle.

3.5 Relevance of these observations to the effects of changes in tonicity on the contractile properties of cardiac muscle

The dependence of cardiac contractile performance on the extracellular tonicity was first described by Koch-Weser (1963) and subsequently confirmed by several others (Little & Sleater, 1969; Wildenthal et al., 1969, 1975). Koch-Weser showed that moderate increases in tonicity (i.e. up to 1.5 times the normal value of 300 $mOsmol.l^{-1}$) potentiated the contractions of isolated papillary muscles from cat hearts, but further increases in tonicity had a pronounced negative inotropic effect (Figure 9). The stimulating effect of mild hypertonicity on cardiac muscle is of course in marked contrast to its action on skeletal muscle, in which even small degrees of hypertonicity produce substantial decreases in contractile force. In mildly hypertonic solutions the potentiation of cardiac contraction was due to an increased rate of rise of force (positive dF/dt) with no change in time to peak force; the reduction in peak force produced by severe hypertonicity was associated with an increased latent period, a decreased positive dF/dt and a slowing of relaxation, the net result of which was a prolongation of the contraction (Koch-Weser, 1963). The plateau component of the cardiac action potential is reduced in synchrony with the decrease in developed force (Hermsmeyer et al., 1972). An analysis of cardiac muscle mechanics (Wildenthal et al., 1969) showed that, as tonicity was altered, the maximum velocity of shortening changed in parallel with the changes in positive dF/dt. In fact, all the alterations in cardiac muscle mechanics at high levels of tonicity resembled those in skeletal muscle under similar conditions. All this lent support to the suggestion by Koch-Weser (1963) that the negative inotropic effect of severe hypertonicity was due, as in skeletal muscle, to a direct inhibitory action of a raised intracellular ionic strength on myofibrillar function, whilst the positive inotropic effect of mild hypertonicity resulted from some other mechanism such as a stimulatory action on E-C coupling (in fact he suggested that a store of 'trigger Ca^{2+}' became more concentrated). Additional indirect evidence for this view was provided by Wildenthal et al. (1975) who showed that under conditions designed to maximise the intracellular $[Ca^{2+}]$ in papillary muscles (4 mM external Ca^{2+} plus paired stimulation) the usual positive inotropic effect of mildly hypertonic solutions was reversed, i.e. contractile force decreased (Figure 9). These results are consistent with the hypothesis that

hypertonicity normally depresses myofibrillar contractile performance but that this effect is obscured at low degrees of hypertonicity by an overriding stimulatory action on E-C coupling. Exactly how the latter effect could occur is not clear, for though an increase in ionic strength does slightly stimulate the ATPase activity of cardiac microsomes (Muir et al., 1971), the ATPase activity increases linearly over the ionic strength range 60 - 300 mM.

Hypertonicity was found to induce a sustained contracture in cardiac muscle (Chapman & Leoty, 1974; Kawata et al., 1974), as in skeletal muscle (Section 3.3). In frog atrial trabeculae, hypertonicity did not elicit contractures directly but potentiated Na^+-free or high-K^+ contractures (Chapman, 1978); there was also an 'off response' (i.e. transient contracture) on return from hypertonic to isotonic solutions.

Because of the dearth of studies on the influence of [salt] on the contractile properties of cardiac myofibrils, it is impossible to decide to what extent the above influences of tonicity on intact cardiac muscle can be explained by direct actions of ionic strength on the contractile machinery. Some of Part III of this thesis is directed towards the question. Using chemically-skinned cardiac muscle I investigated the influence of [KCl] on: (i) Ca^{2+}-regulated force; (ii) Ca^{2+}-insensitive force (i.e. resting force); and (iii) Ca^{2+}-sensitivity. In addition, an attempt was made to determine whether the observed effects of an altered [KCl] were due to changes in ionic strength per se, or to other factors such as changes in tonicity or specific inhibitory actions of K^+ or Cl^-.

PART II

THE ATPase ACTIVITIES OF ISOLATED CARDIAC AND SKELETAL MYOFIBRILS.

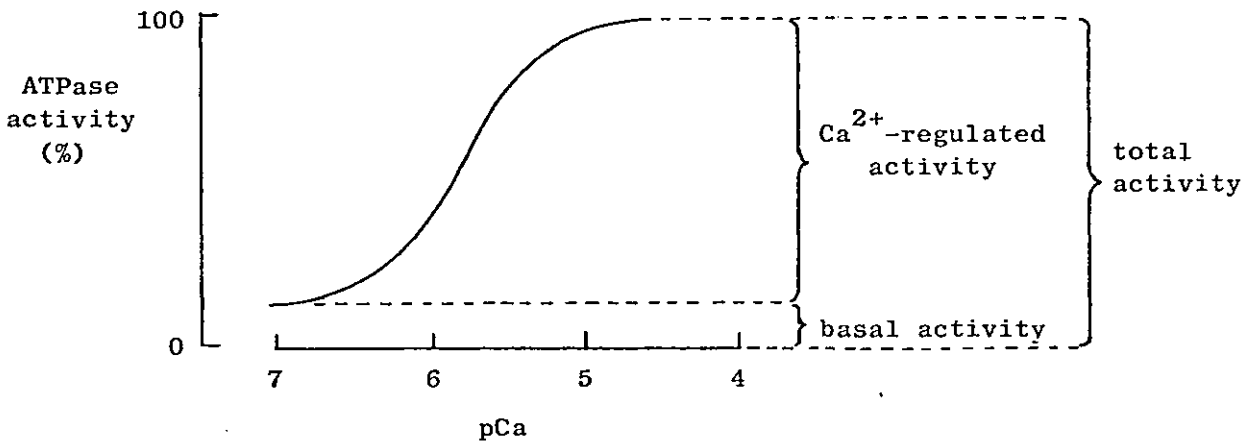
Chapter 4.

INTRODUCTION TO THE PREPARATION

Preparations of isolated myofibrils, due to Perry & Grey (1956), have proved to be attractive models for the study of muscle biochemistry because the myofibrils retain their *in vivo* structure and yet are relatively free of other intracellular organelles. Other structurally-intact preparations such as glycerinated muscles have also been used to study the ATPase activity of intact myofibrils, but glycerinated muscles present practical difficulties because: (i) they contain contaminant membrane ATPases; and (ii) there are almost certainly diffusional restrictions to the movement of molecules into and out of the muscle, with the result that during activity of the myofibrillar ATPase the composition of the bathing solution will not adequately control the chemical environment of the myofibrils in the muscle core. (As discussed in Chapter 8 other types of 'skinned' muscle also suffer from this problem). Provided that care is taken to ensure any bundles of myofibrils consist of only a few myofibrils, similar diffusional restriction should not be encountered in preparations of isolated myofibrils.

Actomyosin, either 'natural' or reconstituted from its purified protein components, has also been used extensively in enzymic studies but the lack of structural integrity in this preparation may lead to different enzymic properties from those of the *in vivo* myofibrillar ATPase (Section 1.2). Isolated myofibrils have considerable advantages over actomyosin gels because the actin and myosin are highly organised in their natural arrays and proportions.

Each of these preparations - myofibrils, glycerinated muscle, natural or reconstituted actomyosin - exhibits an ATPase activity that is activated by Ca^{2+} over the range 10^{-7} M - 10^{-5} M Ca^{2+}. This Ca^{2+}-regulated ATPase activity corresponds to the myofibrillar ATPase activity that is coupled to force production *in vivo* (discussed in Chapter 1). However it is a characteristic of these preparations that typically only 80% - 90% of the total ATPase activity is sensitive to Ca^{2+}; the residual 10% - 20% persists even at very high pCa values and is termed the 'Ca^{2+}-insensitive' or 'basal' ATPase activity. A typical pCa-ATPase activity relationship would thus be as follows:



The basal ATPase activity appears to be unphysiological because its activity greatly exceeds the ATPase activity observed in resting muscle (Weber et al., 1964; Bendall, 1969). It is not at all certain where this basal activity originates, or why the basal activity of cardiac myofibrils or actomyosin is usually greater than that of the corresponding skeletal preparation (e.g. Katz et al., 1966; Muir et al., 1971). The presence of contaminant ATPases can be ruled out because the basal ATPase activity is still observed in actomyosins reconstituted from pure actin + myosin + regulatory proteins (Katz et al., 1966). The basal activity could be due to the ATPase activity of myosin alone. Alternative or additional factors could be the loss or partial denaturation of troponin or tropomyosin, or the loss of a soluble factor which inhibits basal ATPase activity in the intact muscle.

Preparations of isolated myofibrils have been extensively used in studies on the Ca^{2+}-regulated ATPase activity, but they do have some drawbacks. Unlike actomyosin, which can be made completely pure if reconstituted from its purified constituent proteins, myofibrils suffer from the problem of contamination by remnants of cellular organelles, some of which will contain their own ATPase activities. These contaminant ATPase activities may well have properties different from those of the myofibrillar ATPase under study and their activities will probably add to the basal ATPase activity discussed above. This difficulty, which is perhaps the main disadvantage of isolated myofibrillar preparations, arises because, during homogenisation of the muscle, myofibrils tend to trap the organelles and prevent their mutual separation by subsequent differential centrifugation. Myofibrils prepared by the classical method of Perry & Grey (1956) are usually

contracted, which exacerbates this problem. A modification of this method to include chelation of Ca^{2+} ions by EDTA or EGTA during the homogenisation procedure (Perry & Corsi, 1959; Briggs & Fuchs, 1963) resulted in relaxed myofibrils and thus a cleaner preparation. An extension of this was to relax the muscle prior to homogenisation by soaking it in an EGTA-containing 'relaxing buffer' (Zak et al., 1971), a procedure which enabled a less disruptive homogenisation to be used, so that structural alteration of the myofibrils was minimised.

Skeletal myofibrils prepared by these improved methods are usually free of contaminant membrane ATPases, as judged by (i) the similar EGTA- and ionic strength-sensitivities of skeletal myofibrils to those of skeletal 'natural' actomyosin, and (ii) by the lack of effects of detergents on the ATPase activity (Seidel & Gergely, 1963; Zak et al., 1971; Solaro & Shiner, 1976). However this is not true for myofibrils prepared from cardiac muscle. Cardiac cells contain a large proportion of mitochondria and SR, which are intimately associated with the myofibrils. Consequently, cardiac myofibrils prepared by the methods originally developed for skeletal myofibrils are heavily contaminated with such membranes, the ATPase activities of which contribute significantly to the enzymic characteristics of the whole preparation (Fanburg, 1964; Solaro et al., 1971; Reddy, 1976). Indeed it at one time seemed that the ATPase activity of cardiac myofibrils was, unlike that of skeletal myofibrils, largely insensitive to Ca^{2+}-chelators or to ionic strength (Finkel & Gergely, 1961) until Fanburg (1964) demonstrated these Ca^{2+}- and ionic strength-insensitivities were due to a contaminant enzyme which masked the true myofibrillar ATPase activity. This non-myofibrillar ATPase was at least partly mitochondrial in origin, since it was substantially inhibited by 5 mM sodium azide (NaN_3), a known inhibitor of the mitochondrial ATPase (Fanburg, 1964). For this reason, azide is often included in the incubation medium for the assay of cardiac myofibrillar ATPase. However, azide does not inhibit the SR ATPase, which may be active in cardiac myofibrillar preparations because such preparations exhibit significant 'relaxing factor' activity (Briggs & Fuchs, 1963). When studying myofibrillar ATPase activity, it is particularly important to remove any SR ATPase activity, since this too is activated by Ca^{2+} over the range 10^{-7} - 10^{-5} M Ca^{2+}. In addition, the sarcolemmal (Na^+ + K^+)-ATPase is a likely contaminant of cardiac myofibrils (Solaro et al,, 1971). Consideration of the gross contamination of cardiac myofibrillar preparations with membrane-

bound ATPases led Katz to write in 1971: 'at the present time, therefore, this type of preparation cannot be recommended for study'.

Fortunately, in the next year two groups of workers demonstrated that cardiac myofibrils could be essentially purged of contaminant membrane ATPases by washing the myofibrillar preparation with solutions containing the non-ionic detergent Triton X-100 (Solaro et al., 1971; Zak et al., 1971). These Triton-treated cardiac myofibrils had a purity which approached that of skeletal preparations because their ATPase activity was unaffected by azide but was inhibited by more than 80% by EGTA (Solaro et al., 1971), and by about 90% by high ionic strength (0.2 M KCl: Zak et al., 1971).

In the remainder of Part II I shall describe experiments in which I re-examined the influence of pH on the Ca^{2+}-regulation of myofibrillar ATPase, using Triton-treated cardiac and white skeletal myofibrils. Because of the above considerations, considerable care was taken to ensure the homogeneity of the myofibrillar preparations. ATPase activity was assayed by colorimetric measurement of the Pi liberated from ATP during a 5-minute incubation period at 37°C. The assay was performed at four pH values (6.40, 6.80, 7.00 and 7.20) chosen to cover the range of intracellular pH values encountered in normal and acidotic muscle (Chapter 2).

For some of the preliminary experiments I used cardiac myofibrils from guinea-pigs. However in this species it proved difficult to find a skeletal muscle of sufficient size that was unequivocally white; all the muscles seemed to contain some red fibres. It was desirable to ensure the homogeneity of fibres, since red and white fibres have different myofibrillar ATPase activities (Bárány, 1967). Therefore most of the experiments were carried out using cardiac and skeletal tissue from rabbit, in which the sartorius muscle appears completely white.

Chapter 5

METHODS

5.1 Isolation of Cardiac Myofibrils

(a) General procedure

The extraction procedure was based on the methods of Chandler et al. (1967), Zak et al. (1971) and Solaro et al. (1971). A summary of the procedure is given in Figure 10 together with the rationale for the various stages; the detailed procedure is given below. All stages were carried out at 2 - 4°C. Throughout, 'relaxing buffer' refers to a solution containing 70 mM KCl, 20 mM imidazole, 0.1 mM dithiothreitol, 2 mM $MgCl_2$, 2 mM K_2H_2EGTA, pH 7.0 at 4°C and calculated pCa > 8. Unless specified otherwise, pellets were resuspended in 20 pellet volumes of the appropriate solution.

(b) Dissection

Rabbits or guinea-pigs were killed by cervical dislocation. Their hearts were rapidly excised and immersed in relaxing buffer containing 2 mM sodium pyrophosphate. Atria and connective tissue were removed. The ventricles were blotted, weighed and then minced with scissors on an ice-cold Petri dish.

(c) Homogenisation

Five volumes of relaxing buffer containing 2 mM sodium pyrophosphate were added to the ventricular mince in an ice-cold boiling tube. The resulting mixture was homogenised with an Ultra-Turrax (Janke & Kunkel, W. Germany) for three periods, each of 20 seconds duration, at speed setting '5' with the tube kept in the ice. The mixture was allowed to cool for 20 sec between homogenisations.

(d) First high-speed centrifugation

After it had been diluted 1:1 with relaxing buffer + 2 mM sodium pyrophosphate, the homogenate was spun at 500 x g (1200 r.p.m., Mistral 6L, M.S.E. Ltd., Crawley, Sussex) for 20 minutes.

(e) Filtration

The pellet was resuspended (with a 'Whirlimixer') in relaxing buffer containing 0.3 M sucrose and was filtered through 2 layers of cotton gauze.

(f) Low-speed centrifugation

The suspension was spun at 50 x g (400 r.p.m., Mistral 6L) for 2 minutes. The resulting supernatant was kept on ice. Meanwhile, the pellet was resuspended in relaxing buffer + 0.3 M sucrose, was homogenised twice for 20 seconds at speed setting '5' (Ultra-Turrax)

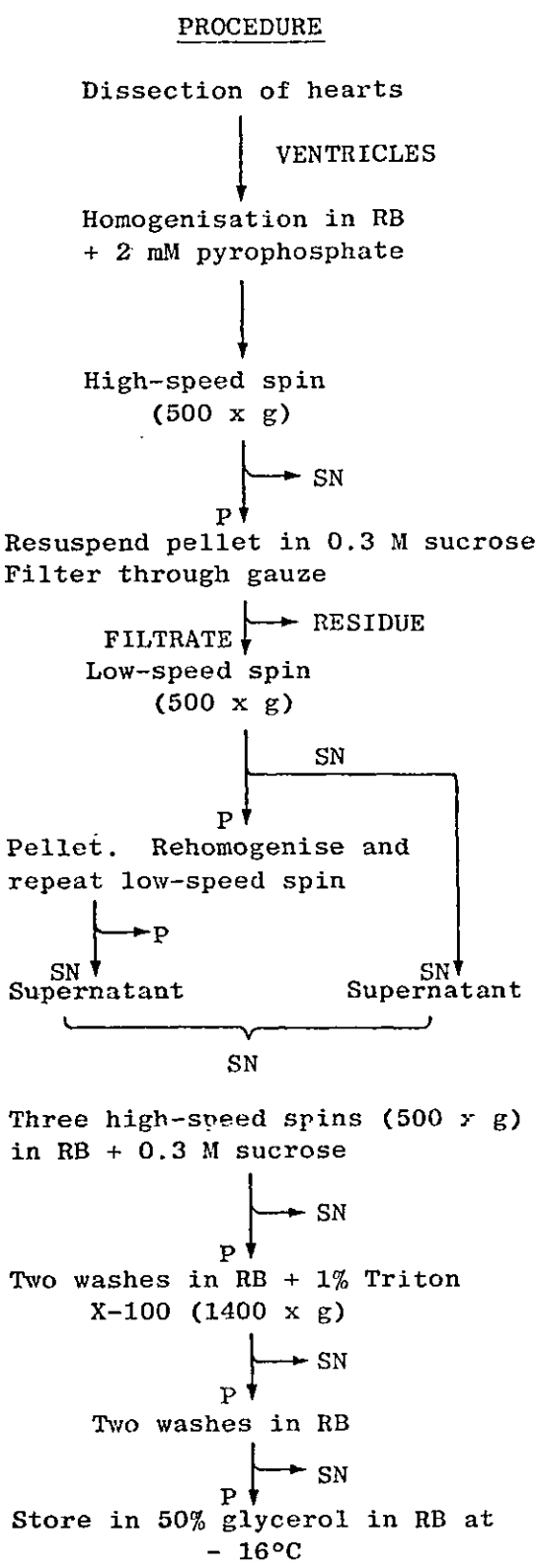


Figure 10. Stages in the preparation of purified isolated myofibrils from mammalian ventricles.

RB, 'relaxing buffer'; SN, supernatant; P, pellet.

and was spun at 50 x g for 2 minutes. The supernatants from both low speed spins were then combined.

(g) High-speed centrifugations

The crude myofibrils were then spun three times for 20 minutes at 500 x g, with resuspension of the pellet in relaxing buffer + 0.3 M sucrose following the first and second spins. At this stage the pellet was mid-brown.

(h) Washes in Triton X-100

After the third 500 x g spin the pellet was suspended in relaxing buffer containing 1% (v/v) Triton X-100. To minimise foaming, I used a thin glass rod for this and all subsequent resuspensions. The myofibrils were sedimented by a 10 minite spin at 1400 x g (2000 r.p.m., Mistral 6 L). This Triton treatment was repeated once.

(i) Removal of Triton X-100

The myofibrillar pellet, which was now white, was washed twice in relaxing buffer (two spins of 10 minutes each at 1400 x g, with resuspension of the pellet after each).

(j) Storage

The final pellet was resuspended in 50% (v/v) glycerol in relaxing buffer and was stored at -16°C. Myofibrils stored thus lost less than 5% of their maximum ATPase activity per 24 hours.

(k) Use of myofibrils

The myofibrils were assayed for ATPase activity on the day after they had been isolated. Before use, myofibrils were freed of glycerol and dithiothreitol by three washes (three 10 minute spins at 1400 x g) in 20 volumes of a solution containing 70 mM KCl, 20 mM imidazole, 2 mM $MgCl_2$ and 2 mM K_2H_2EGTA (pH 7.0 at 4°C). Washed myofibrils were suspended in a solution of 70 mM KCl and 20 mM imidazole (pH 7.0 at 4°C) and kept on ice until required. Aliquots of this suspension were taken routinely and were processed for electron microscopy by the method described in Section 5.8.

One gram of wet but blotted ventricle yielded about 30 mg of myofibrils. A single rabbit heart provided enough myofibrils for a complete day's experiment but the smaller size of guinea-pig ventricles necessitated the pooling of ventricles from 3 - 5 guinea-pig hearts.

5.2 Isolation of white skeletal myofibrils

Once the heart had been removed from the rabbit, the muscles of one leg were exposed and pieces of visually 'white' skeletal muscle were cut from the superficial layers of the sartorius. (To the eye, the rabbit sartorius muscle contained predominantly white fibres near the surface;however it seemed to merge with red fibres towards the femur). Myofibrils were prepared from these pieces by the same procedure as for cardiac myofibrils except that the homogenisation times were shorter: each step was for 10 seconds (speed '5', Ultra-Turrax) instead of 20 seconds. Yields were about 40 mg g^{-1} blotted fresh tissue.

The successful preparation of purified cardiac and skeletal myofibrils depended critically on the duration and severity of homogenisation: too much homogenisation resulted in a very low yield because the myofibrils were broken into such small fragments that they were lost in the supernatants after the 500 x g spins; insufficient homogenisation also produced a low yield, in this case because the myofibrils remained as large fragments of cells after homogenisation and therefore sedimented in the 50 x g spins.

5.3 Incubation for ATPase assay

(a) Assay procedure

To determine myofibrillar ATPase activity I measured the inorganic phosphate (Pi) liberated from MgATP by a 5 minute incubation at 37.0 ± 0.1°C. The 'standard' compositions of the incubation media, which were based on those used by Hönig & Stam (1967), were as follows (final concentration in a total volume of 3 cm^3): 70 mM KCl; 20 mM imidazole; 5 mM NaN_3; 5 mM K_2Na_2ATP; 6mM $MgCl_2$; 1 mM (nominal) K_2EGTA; 0.0 - 1.1 mM added Ca to provide a range of pCa values from > 8 to 4.4 (Section 5.6.d); 100 - 200 µg cm^{-3} myofibrils; pH 7.20, 7.00, 6.80 or 6.40 (± 0.01 pH units) at 37.0°C. 'Blank' solutions containing no myofibrils were also incubated to allow correction for non-enzymic production of Pi. Centrifuge tubes containing the incubation media minus K_2Na_2ATP and $MgCl_2$ were pre-incubated in a water bath for 3 minutes at 37°C before the reaction was started by the addition of 0.1 cm^3 of a stock solution containing 150 mM K_2Na_2ATP + 180 mM $MgCl_2$ (pH 7.0). After the 5 minute incubation period, during which the tubes were shaken in the water bath, the reaction was stopped by the addition of 2.5 cm^3 of ice-cold 22% (w/v) trichloroacetic acid and the tubes were plunged into ice. Myofibrils were then added to the 'blank' tubes. The tubes were centrifuged at 2 - 4°C for 15 minutes at 1400 x g to sediment the precipitated protein. Aliquots of the supernatants were removed for the colorimetric determination of Pi by

the method described below (Section 5.4). The remaining supernatant was decanted off and 1 cm^3 of 0.1 M NaOH was added to the drained precipitates. The tubes were covered with 'Parafilm' and incubated overnight at 37°C. Next day the protein concentration was assayed by the method described in Section 5.5.

All tubes were duplicated. The mean ATPase activity of each pair was used as a single datum for the results and for any statistical analysis. ATPase activities are usually given in terms of nanomoles Pi hydrolysed min^{-1} mg $protein^{-1}$, although in some cases the activities have been normalised by expressing all the ATPase activites in each experiment as a percentage of the maximum total ATPase activity in the same experiment.

A different preparation was used for each experiment.

(b) MgATP concentration

To check that the standard concentration of $MgATP^{2-}$ (5 mM) was a saturating concentration for the cardiac myofibrillar ATPase, the rate of Pi production was determined in incubation media of 6 different $MgATP^{2-}$ concentrations, ranging from 5 μM to 5 mM. Ionic strength was kept constant at 116 mM by varying the KCl concentration. To maintain the starting $MgATP^{2-}$ concentration an ATP-regenerating system comprising 6 mM phosphoenolpyruvate + 60 units cm^{-3} pyruvate kinase was added to the incubation solutions. As Figure 11 a shows, the rate of Pi liberation in the presence 0.01 mM added $CaCl_2$ increased with increasing $MgATP^{2-}$ concentration up to 1 mM and showed no further rise above this. The Ca^{2+}-sensitive portion of this Pi liberation (i.e. total liberation in 0.01 mM added $CaCl_2$ minus Pi liberation in 0.4 mM EGTA) also rose over the range of $MgATP^{2-}$ concentrations from 5 μM to 1 mM, but decreased slightly above this because of a continuing rise in the Ca^{2+}-insensitive liberation of Pi. Thus 5 mM $MgATP^{2-}$ is certainly a saturating concentration for the Ca^{2+}-regulated cardiac myofibrillar ATPase. Lineweaver-Burk plots of these data produced a straight line only in the case of the Ca^{2+}-sensitive portion of the total ATPase activity (Figure 11 b). From the intercepts of this line K_m for Ca^{2+}-regulated ATPase was estimated to be about 80 μM $MgATP^{2-}$ and V_{max} to be about 66 nmoles Pi min^{-1}.

(c) Time course of Pi liberation

In preliminary experiments in which the incubation period was varied, the rate of Pi liberation, both in the absence and in the presence of Ca^{2+}, was found to remain constant over the first eight

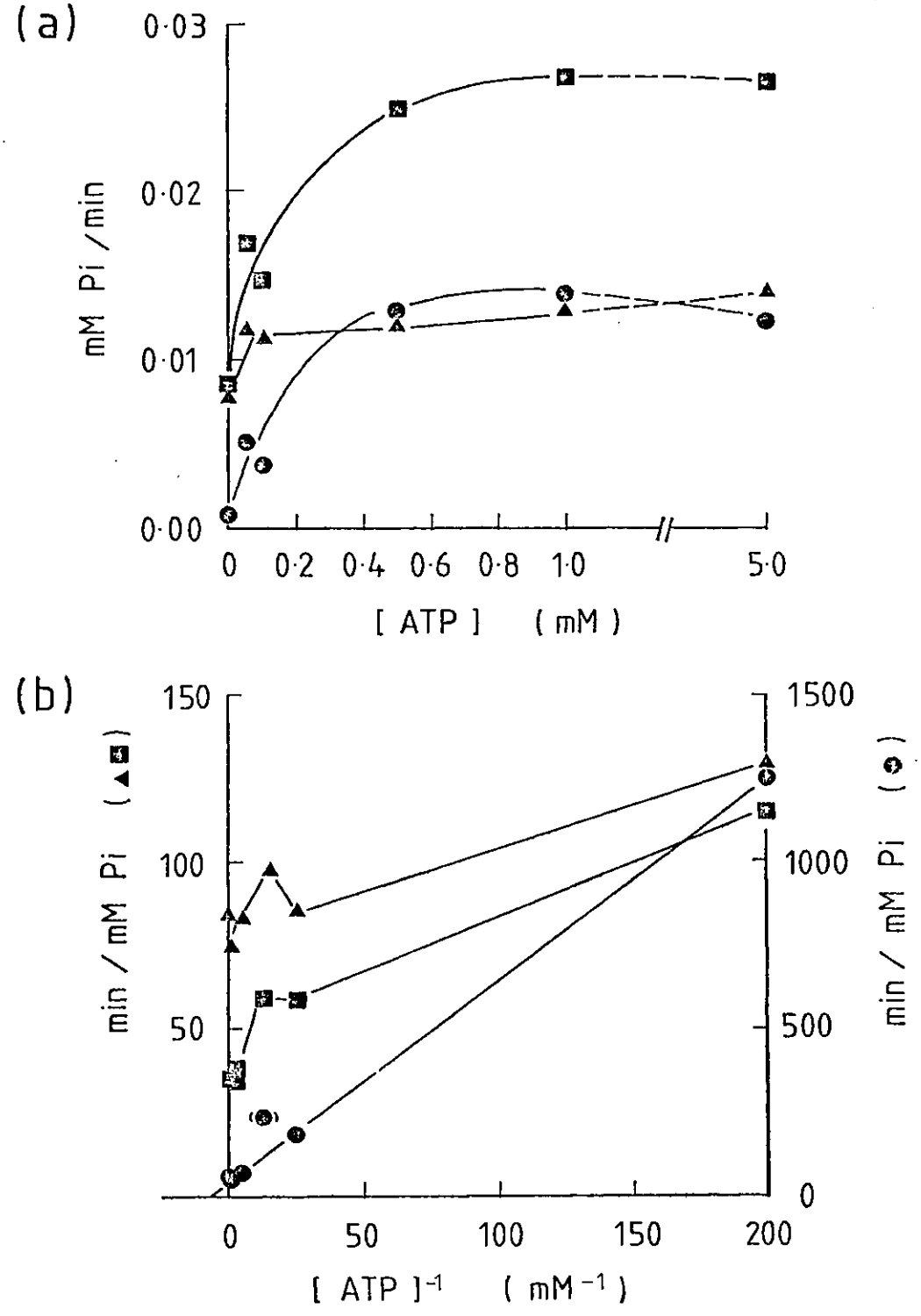


Fig. 11. The influence of [ATP] on the rate of liberation of Pi from ATP by cardiac myofibrils. Guinea-pig cardiac myofibrils (not washed in Triton X-100). (a) (▲) Basal ATPase activity measured in presence of 0.4 mM K_2EGTA (pCa>8); (■) total ATPase activity measured in presence of 0.1 mM $CaCl_2$ (pCa 4); (●) Ca^{2+}-regulated ATPase activity i.e. total activity - basal activity, pH 7.00, 37°C. [Pi] measured at the end of a 5 min incubation period by the method described in Section 5.4(a).
ATPase activities are not given in terms of per mg protein because the measured [protein] included a substantial contribution from the added pyruvate kinase.
(b) Lineweaver-Burk plot of the data in (a).

minutes or so of incubation (Figure 12). Thereafter the rate decreased progressively with time. Because Pi liberation was linear over the standard 5 minute incubation period, the amount of Pi liberated enzymatically over the 5 minutes could justifiably be taken as a measure of the initial reaction rate of the steady-state ATPase activity. (Any Pi liberated in the 'initial burst' was too small to be observed - it would have contributed only about 10^{-7} M Pi to the total Pi concentration of about 0.2 mM).

(d) Concentration of myofibrils

If cardiac myofibrils were being assayed, I added enough to produce a concentration of about 200 μg cm^{-3} in the incubation solution. White skeletal myofibrils were found to have approximately double the ATPase activity of cardiac myofibrils (Section 6.1), so I halved the concentration of white skeletal myofibrils in the incubation solutions to about 100 μg cm^{-3} in order that the amount of ATP hydrolysed would be similar in the two cases. Less than 0.3 mM (i.e. 6%) of the ATP was hydrolysed during the 5 minute incubation. Thus an ATP-regenerating system was unnecessary.

Other preliminary experiments, in which the incubation was carried out in a 100 cm^3 beaker in the water bath, verified that (i) on addition of the $K_2Na_2ATP + MgCl_2$ stock solution any changes in temperature and pH lasted only a few seconds and (ii) the fall in pH which resulted from the proton-generating hydrolysis of ATP was less than 0.02 pH units over the 5 minutes incubation.

5.4 Measurement of the concentration of inorganic phosphate

(a) Procedure

The inorganic phosphate liberated from ATP was determined spectrophotometrically by measuring the formation of a blue molybdate-phosphate complex in the presence of acid. The procedure was basically that of Fiske & Subba Row (1925) except for two modifications: (i) the molybdate stock solution contained no acid, since sufficiently acid conditions for the reaction were produced by the presence of trichloroacetic acid; (ii) the molybdate stock usually contained 1% Cirrasol ALN-WF to prevent precipitation in the presence of alkaloids (see below). Stock solutions of 'Fiske - Subba Row reagent' (15% $Na_2S_2O_5$ + 0.25% 1-amino-2-naphthol-4-sulphonic acid + 0.5% Na_2SO_3) and 2.5% ammonium molybdate + 1% (w/v) Cirrasol were mixed in the ratio 2:1 on the day of each ATPase assay. 0.5 cm^3 of this reagent mixture was added to 2.5 cm^3 of the test supernatants,

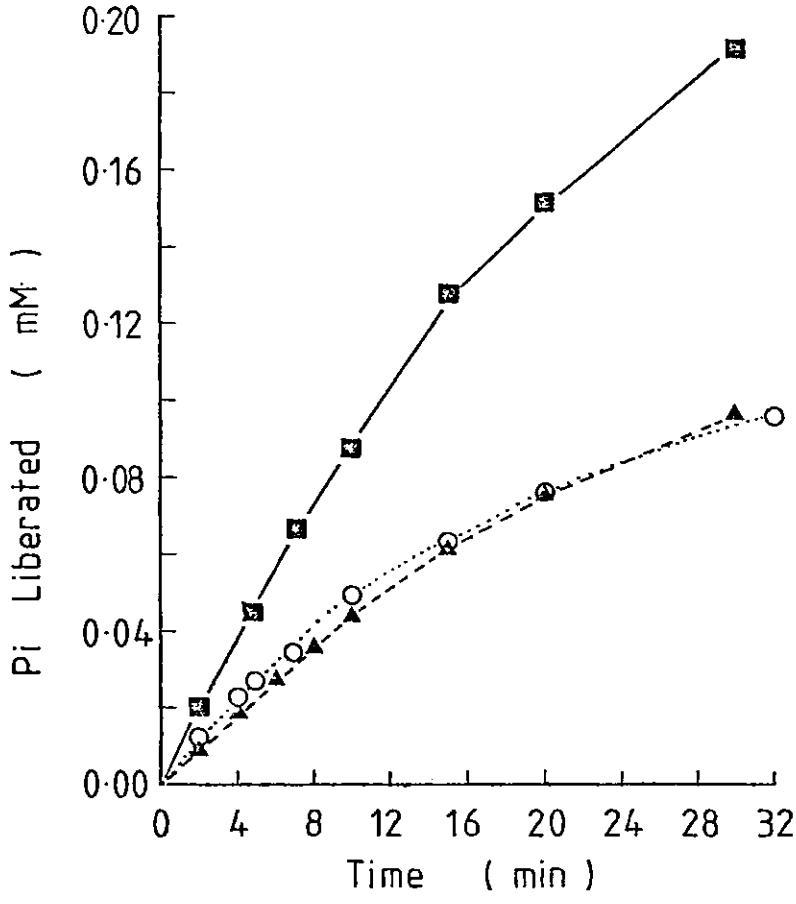


Figure 12. Time course of liberation of Pi by cardiac myofibrils.

Guinea-pig cardiac myofibrils (not washed in Triton X-100) were incubated in the presence of (▲) 0.4 mM EGTA or (◙) 0.1 mm $CaCl_2$. (O) Ca^{2+}-regulated liberation of Pi. Standard incubation media (Section 5.3(a)), pH 7.00, 37°C. At the various times shown the reactions in selected test-tubes were stopped with ice-cold trichloroacetic acid. [Pi] measured as in Section 5.4(a).

standards or blanks, which had been made up as follows:

(test supernatants = 3 cm^3 incubation medium + 2.5 cm^3 TCA)
standards = 0.05 - 0.2 mM Pi in 3 cm^3 H_2O + 2.5 cm^3 TCA
blank = 3 cm^3 H_2O + 2.5 cm^3 TCA
where TCA = trichloroacetic acid.

The solutions were immediately mixed. After precisely 10 minutes the optical densities at λ = 700 nm were read in a spectrophotometer (Gilford Inc., Oberline, Ohio, U.S.A.) against the blank. Accurate timing was essential because the optical density never reached a constant value; instead the reaction was characterised by an initial rapid increase in optical density followed by a slow but steady development of colour (Figure 13a).

In most of the preliminary experiments, the detergent Cirrasol ALN-WF was not included in the molybdate solution. However quinidine, which was used in the contamination studies (Section 5.9), was found to interfere with the optical density readings since it formed a pale yellow precipitate with molybdate. Accordingly, the molybdate solution was modified by the addition of 1% Cirrasol, which prevents the formation of a precipitate in the presence of alkaloids such as quinidine (Price and Williams, 1977). An additional benefit was an increase in the sensitivity of the Pi determination if Cirrasol was present (Figure 13b); for this reason Cirrasol was thereafter routinely included in the molybdate stock solution.

Figure 13b also shows that, whether Cirrasol was present or not, the optical density at 700 nm was proportional to Pi concentration up to 0.5 mM. Thus Beer's Law was obeyed to well above the maximum concentration of Pi introduced by the ATPase reaction ($\approx$ 0.3 mM after 5 minutes incubation at 37°C).

(b) Source of Pi in 'blank' centrifuge tubes.

The blank centrifuge tubes had considerable quantities of Pi, ranging from 0.05 mM to 0.12 M. This blank Pi represented a considerable fraction of the total Pi in the test incubation solutions. Several experiments were undertaken to find the source of this Pi and to investigate whether the amount of blank Pi was influenced by the conditions of incubation.

A high blank Pi was observed only if ATP had been present in the incubation solution. Pi originating from the myofibrils was negligible. The concentration of Pi in the blanks was unaffected by the changes in the pH of the incubation solution over the range pH 7.20 to pH 6.40 or in the pCa over the range pCa > 8 to pCa 3. Keeping the blank test-tube on ice for 5 minutes did not alter the Pi concentration.

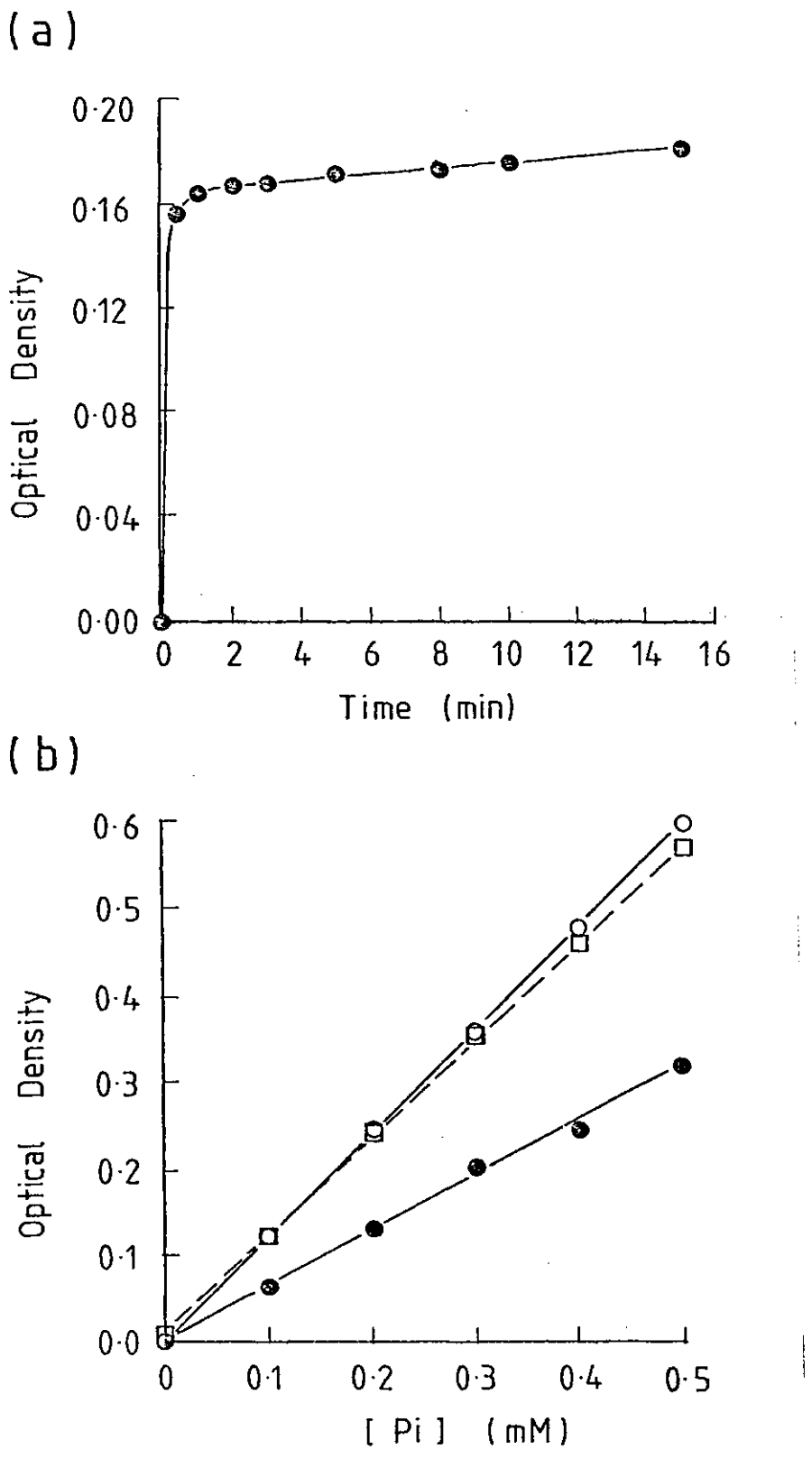


Figure 13. Characteristics of the assay for Pi. (a) Time course of colour development after the addition of 'Fiske-SubbaRow reagent', ammonium molybdate and Cirrasol ALN-WF to a standard solution containing 0.1 mM Pi + trichloroacetic acid. Optical density read at λ = 700 nm. 18°C.
(b) Standard curves for the modified Fiske-SubbaRow procedure using ammonium molybdate with 1% Cirrasol ALN-WF (open symbols) or without (filled symbols). Assay carried out in the presence (□) or absence (○●) of 1 mM quinidine. If Cirrasol ALN-WF was omitted a precipitate formed in the presence of quinidine and no O.D. readings could be taken.

It was concluded that the Pi in the blank test-tubes was not formed from ATP during the incubation period. Some Pi may have been present in the $MgCl_2$ + Na_2K_2ATP stock solution, though the magnitude of blank Pi varied from experiment to experiment and was not correlated with the age of this stock. Probably most Pi was formed by acid hydrolysis of ATP after trichloroacetic acid had been added to the centrifuge tubes. It seemed that in either case, there was little one could do to reduce the magnitude of the Pi in the blank tubes except to carry out the assay for Pi as soon as possible after the termination of the incubation of myofibrils.

5.5 Measurement of protein concentration

Protein concentrations of the myofibrillar digests were determined by the method of Lowry et al. (1951) using crystalline bovine serum albumin as a standard.

A reagent mixture containing 50 cm^3 2% Na_2CO_3 in 0.1 M NaOH + 0.5 cm^3 2% NaK tartrate + 0.5 cm^3 1% $CuSO_4$ was mixed freshly each day. This solution was added in 5 cm^3 aliquots to 1 cm^3 samples of the myofibrillar digest, standard and blank solutions. These had been prepared as follows:

Test = 0.5 cm^3 myofibrillar digest (in 0.1 M NaOH) + 0.5 cm^3 H_2O

Standards = 0.5 cm^3 0.1 M NaOH + 0.05 - 0.5 cm^3 bovine serum albumin stock solution (1 mg cm^{-3}) + H_2O to give a final volume of 1 cm^3.

Blank = 0.5 cm^3 0.1 M NaOH + 0.5 cm^3 H_2O.

The solutions were mixed and were allowed to stand for 10 minutes at room temperature. Then 0.5 cm^3 of Folin-Ciocalteau phenol reagent (diluted 1 volume : 2 volumes H_2O before use) was added and the solutions were mixed. After a further period of 30 minutes, during which the solutions needed to be mixed twice more to ensure homogeneity, the optical densities of the solutions were read at λ = 546 nm (Gilford) against the reagent blank. All optical densities had reached constant values by 30 minutes.

A typical standard curve (Figure 14) illustrates the curvilinear relationship between protein concentration and optical density which is a characteristic of the Lowry protein determination.

5.6 Solutions

All solutions were prepared with glass-distilled water which had been deionised to a resistance of >4 MΩ by passage through a mixed-bed ion exchange deioniser (type B102, Elga Ltd., Lane End, Bucks.).

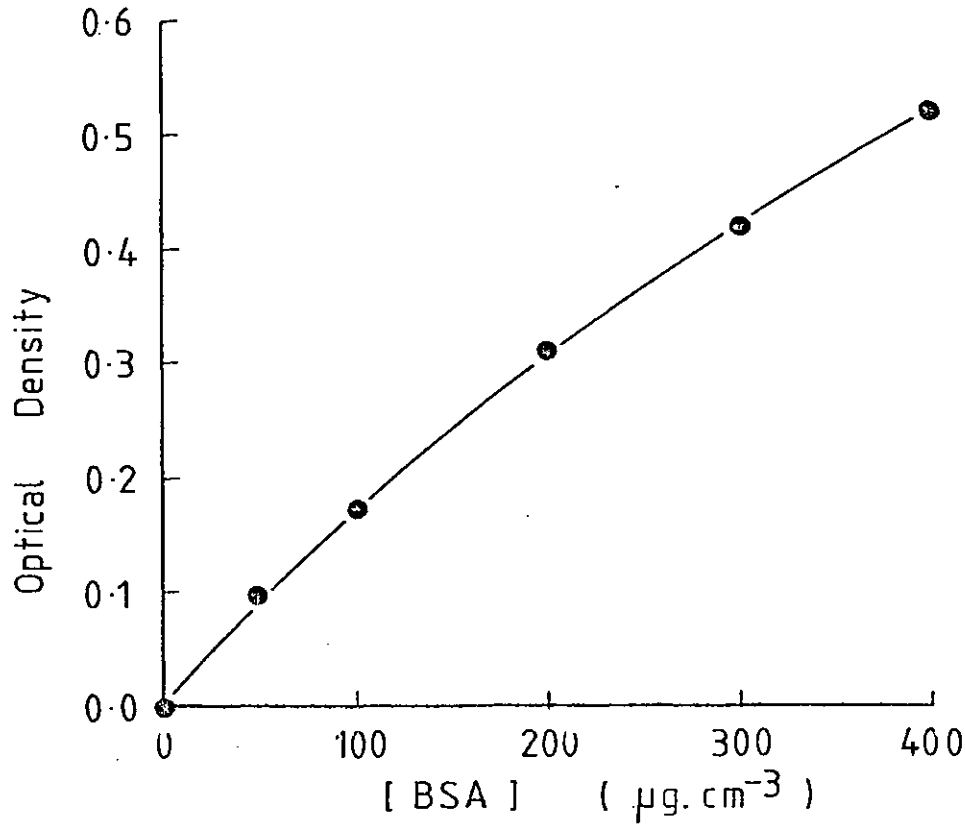


Figure 14. Typical standard curve for the determination of [protein] by the Lowry method.

Optical density read at λ = 546 nm. BSA = bovine serum albumin.

(a) Incubation media

The incubation media were made freshly each day from stock solutions of: 1 M KCl; 200 mM imidazole, pH ≈ 10; 150 mM K_2Na_2ATP + 180 mM $MgCl_2$, pH 7.0; Ca/EGTA solutions containing 10 mM total EGTA and 0 - 11 mM total Ca, pH 7.0. NaN_3 was added in solid form and the incubation solutions were brought to the required pH at 37°C with HCl using a Pye 290 pH meter (Pye Unicam Ltd., Cambridge).

(b) Stock solutions

The 150 mM Na_2K_2ATP + 180 mM $MgCl_2$ stock solution was prepared by neutralising 150 mM Na_2H_2ATP + 180 mM $MgCl_2$ with KOH (final concentration of KOH was about 300 mM). To make a stock solution of 10 mM EGTA + 10 mM Ca, accurately weighed dried samples of $CaCO_3$ and H_4EGTA were mixed in equimolar amounts and a little water was added. The mixture was warmed to 70°C - 80°C and stirred to aid liberation of CO_2 (Ashley & Moisescu, 1977):

$$CaCO_3 + H_4EGTA \rightleftharpoons CaEGTA^{2-} + H_2CO_3 \rightleftharpoons H_2O + CO_2\ (g)$$

The solution was allowed to cool and was neutralised with 1 M KOH (final concentration about 20 mM). A solution of 10 mM EGTA was also accurately prepared and was neutralised with KOH. The two solutions, 10 mM $K_2CaEGTA$ and 10 mM K_2H_2EGTA, were mixed in various proportions to produce stock solutions containing 10 mM total EGTA and 0, 1, 2,5, 4, 6, 8, 9 and 10 mM total Ca^{2+}. If necessary, the pH was readjusted to 7.0 with KOH. $CaCl_2$ was added to an aliquot of the 10 mM CaEGTA stock to make one solution with 11 mM total Ca^{2+}. The exact ratio of [Ca]:[EGTA] in these stock solutions was determined by a pH-metric method (see below).

All stock solutions and the relaxing buffer for the extraction procedure were prepared weekly and were stored at 4°C, except for the 150 mM K_2Na_2ATP + 180 mM $MgCl_2$ solution, which was stored frozen in 5 cm^3 aliquots. Contaminant Ca in the stock solutions and in nitric acid digests of the final incubation solutions was measured by atomic absorption spectroscopy (model 290, Perkin-Elmer, Norwalk, Conn., U.S.A.) in 1% $LaCL_3$.

(c) Measurement of the ratio [total Ca]:[total EGTA]

At the time that I did these experiments I assumed that EGTA was 100% pure. However, in my later studies on skinned muscle I

discovered that this was not the case: the purity of EGTA from Sigma Ltd. is only ≈ 96% (Section 9.2). It is vital to know exactly the ratio between [total Ca] and [total EGTA] in the solutions, for the calculated [free Ca^{2+}] depends critically upon this ratio. A failure to take into account an impurity in EGTA would result in a large underestimate in the calculated [free Ca^{2+}], especially at values of pCa (pCa 4 - 5) where the EGTA is almost saturated with Ca. In turn this underestimate would produce an artificially steep relationship between muscle force and pCa. Accordingly, during my studies on skinned muscle I determined the effective [EGTA] in the myofibrillar ATPase incubation solutions. This involved pH titration of 1 M $CaCl_2$ (B.D.H.) against a solution containing 1 mM EGTA (nominal concentration based on the weight of dried EGTA solid), 100 mM KCl and 2 mM imidazole, pH 9.4 (modification of the method of Moisescu & Pusch 1975; see Section 9.1 for details). The effective [EGTA] was found to be 0.947 ± 0.006 mM (mean ± S.E.M., n = 8). This value is close to that expected (0.950 mM) for 1 mM (nominal) EGTA of 96% purity with 10 μM contaminant Ca bound to it. In the computation of the free Ca^{2+} described in Section 5.6(d) I used the value of 0.947 mM for the effective total concentration of EGTA.

(d) Computation of the free Ca^{2+} concentrations in the incubation media.

Calculation of the free concentrations of ions in media containing interacting ionic species requires solving a set of simultaneous linear equations which describe the multiple equilibria in the solutions. The complexity of my incubation solutions, which involved at least 20 equilibria, necessitated the use of a computer to solve the simultaneous equations. An ALGOL version of a computer program, based on the iterative procedure of Perrin & Sayce (1967), was kindly given to me by Dr. R. Abbott, University of Oxford. I translated the program into FORTRAN and modified it to run on the Imperial College Computer Centre system via a dial-up linkage. Input to the program consisted of the total concentrations of metals and ligands, the pH of the solution, the compositions of the likely complex species, and the absolute constants for each complex. The free concentrations of metal ions, ligands and complex ions were then computed and printed, together with the ionic strength, which was calculated taking into account all the ionic species. Throughout this thesis the free Ca^{2+} concentration will be given in terms of the pCa value which, by

analogy with pH, is equal to $-\log_{10}$ [free Ca^{2+}].

A problem with the computation of free ion concentrations is that the calculated concentrations depend not only upon the [Ca]:[EGTA] ratio but also upon the values chosen for the association constants, some of which may have been determined under conditions that were far from physiological. I chose the association constants from Sillén & Martell (1971) so that the temperature and ionic conditions in which they had been determined were as close as possible to the conditions of my incubation solutions. The values chosen are listed, in logarithmic form, in Tables 1 and 2. The proton binding constants have been converted from 'concentration constants' to 'mixed constants' by the addition of 0.11 log units (Martell & Smith, 1974). Unfortunately the constants which largely determine the pCa - i.e. those for $EGTA^{4-}/H^{+}$ and $EGTA^{4-}/Ca^{2+}$ have not been measured at 37°C. The values given for these constants in Table 1 were determined in 0.1 M KCl by Schwarzenbach et al. (1957). I have recently confirmed these values at 20°C (Appendix A). Little error should however result from my use of these values for solutions at 37°C because Murphy & Hasselbach (1968) reported that the total binding of Ca to EGTA was constant over the range 0°C - 40°C at pH 7.0. The values in Table 1 give an apparent log K_{CaEGTA} at pH 7.0 = 6.43, which is close to the values that have recently been determined by myself and other workers by direct means (Appendix A).

The total Ca concentration or EGTA concentration entered in the program took into account the contaminant Ca in the incubation media (about 10 μM). I estimated the maximum binding of Ca^{2+} by myofibrils at pCa 4.5 to be about 3 μmoles/g myofibrillar protein (from Figure 5 in Solaro & Shiner, 1976); this would have reduced the total Ca concentration in the final incubation media by less than 0.2 μM. Such binding was therefore neglected in the calculation of pCa. Total Ca (added + trace Ca) was varied between 10 μM and 1.1 mM to generate a range of pCa values from >8 to 4.3. The total Ca concentrations and the corresponding calculated pCa values at pH 7.20, 7.00, 6.80, and 6.40 are listed in Table 3. Computed concentrations of free Mg^{2+} (about 1.4 mM) and $MgATP^{2-}$ (about 4.6 mM) varied only slightly between solutions of different pCa or pH. Ionic strength increased from 115 mM at pH 7.20 to 119 mM at pH 6.40.

By using the computer program, I was also able to model the progress of the ATPase reaction and to predict the pH and pCa

Reactants	log K
imidazole; H^+	6.80
$EGTA^{4-}$; H^+	9.58
$HEGTA^{3-}$; H^+	8.96
H_2EGTA^{2-}; H^+	2.77
H_3EGTA^-; H^+	2.11
$EGTA^{4-}$; K^+	0.96
$EGTA^{4-}$; Na^+	1.38
$EGTA^{4-}$; Ca^{2+}	10.97
$HEGTA^{3-}$; Ca^{2+}	5.30
$EGTA^{4-}$; Mg^{2+}	5.21
$HEGTA^{3-}$; Mg^{2+}	3.36
ATP^{4-}; H^+	6.63
$HATP^{3-}$; H^+	3.98
ATP^{4-}; K^+	0.90
ATP^{4-}; Na^+	1.20
ATP^{4-}; Ca^{2+}	3.94
$HATP^{3-}$; Ca^{2+}	2.13
ATP^{4-}; Mg^{2+}	4.28
$HATP^{3-}$; Mg^{2+}	2.29
N_3^-; Na^+	4.58

Table 1. Association constants, in logarithmic form, used in the computation of the pCa values in the incubation solutions.

Reactants	log K
ADP^{3-}; H^{+}	6.55
$HADP^{2-}$; H^{+}	4.04
ADP^{3-}; K^{+}	0.67
ADP^{3-}; Na^{+}	0.65
ADP^{2-}; Ca^{2+}	2.80
$HADP^{2-}$; Ca^{2+}	1.54
ADP^{3-}; Mg^{2+}	3.30
$HADP^{2-}$; Mg^{2+}	1.78
HPO_4^{2-}; H^{+}	6.84
HPO_4^{2-}; Ca^{2+}	2.60
HPO_4^{2-}; Mg^{2+}	2.91

Table 2. Additional association constants used for the estimation of the effects of ATP hydrolysis on pH and pCa.

[total Ca] (mM)	pH			
	7.20	7.00	6.80	6.40
0.010*	8.77	8.38	7.99	7.20
0.025	-	-	-	6.80
0.1	7.72	7.34	6.95	6.16
0.25	7.24	6.85	6.46	5.68
0.4	6.93	6.55	6.16	5.38
0.6	6.56	6.17	5.79	5.03
0.8	6.07	5.69	5.32	4.65
0.9	5.57	5.25	4.95	4.43
1.0	4.62	4.51	4.48	4.22
1.1	4.20	4.18	4.16	-

* Usual concentration of contaminant Ca.

Table 3. Computed values of pCa in the incubation media at different pH values and at different total concentrations of Ca.

Incubation media contained: 70 mM KCl; 20 mM imidazole; 5 mM NaN_3; 5 mM K_2Na_2ATP; 6 mM $MgCl_2$; 1 mM (nominal) K_2EGTA (= 0.947 mM effective EGTA); 0.010 - 1.1 mM Ca. Appropriate association constants are listed in Tables 1 and 2.

changes resulting from the hydrolysis of the ATP to known amounts of ADP, Pi and H^+ ions. For this study I included the logarithmic association constants given in Table 2. My computations showed that hydrolysis of 0.5 mM (i.e. 10%) of the ATP would reduce the pH by less than 0.02 pH units and increase the pCa by less than 0.01 pCa units. Pilot experiments confirmed that the observed fall in pH was never greater than 0.02 pH units. I concluded that the effects of ATP hydrolysis on pH and pCa over the 5 minute incubation period could be neglected.

5.7. Curve-fitting

The relationship between relative ATPase activity and the pCa of the incubation solutions was found to be approximately sigmoidal (Chapter 6). To assist comparison of the relative ATPase activity - pCa curves, non-linear least squares regression was used to fit a modified form of the Hill equation (Hill, 1910) to the data:

$$\text{ATPase activity} = \text{basal activity} + \left(\begin{matrix} \text{maximum} \\ Ca^{2+}\text{-regulated} \\ \text{activity} \end{matrix} \times \frac{[Ca^{2+}]^{n_H}}{K^* + [Ca^{2+}]^{n_H}} \right)$$

where all ATPase activities are relative ATPase activities (i.e. are expressed as a percentage of the total activity) and where

n_H = the Hill coefficient

and K^* = a compound association constant.

The pCa at 50% (i.e. half-maximal) Ca^{2+}-activation is then

$= -\frac{1}{n_H} \log K^*$. This pCa value is termed pCa_{50} in this thesis.

The Hill coefficient 'n_H' determines the steepness of the curve. Its value gives some information on the degree of cooperativity in the system; if $n_H > 1$ this is consistent with positive cooperativity. The value of the association constant K* is related to, but is not identical with, the association constant for Ca^{2+} binding to the myofibrillar site which regulates ATP hydrolysis and force generation. The value of K* also depends upon the cooperativity in the system and upon other unknown factors. It should be emphasised that fitting of the above equation to the data is empirical and assumes no knowledge of the steps between the binding of Ca^{2+} to troponin and the subsequent hydrolysis of ATP.

5.8 Electron microscopy

Aliquots of the myofibrillar suspension in 70 mM KCl + 20 mM imidazole (pH 7.0 at 4°C) were centrifuged at 1400 x g for 10 minutes. The supernatant was drained off. Samples of this pellet and of pellets obtained during the preparation of the myofibrils were fixed overnight at room temperature by immersion in 0.1 M Na cacodylate buffer (pH 7.3) containing 4% glutaraldehyde. The myofibrils were rinsed three times in buffer (0.1 M Na cacodylate) and were processed for electron microscopy according to the following schedule:

- postfixation in 1% OsO_4 in buffer for 1 - 2 hours
- staining in a saturated aqueous solution of uranyl acetate for 1 hour
- dehydration in 70%, 90%, and then 100% ethanol
- clearing in propylene oxide
- infiltration and embedding in Araldite
- examination of ultrathin sections in the electron microscope (A.E.I. model 801).

5.9 Tests for contamination of the myofibril preparations

In addition to examining electron micrographs of the preparations, I investigated the nature of any contaminant membrane ATPases in Triton X-100 treated or untreated preparations of rabbit cardiac and white skeletal myofibrils, as follows: during the isolation of these myofibrils, I washed half of the myofibrils from each type of muscle with 1% Triton X-100 in relaxing buffer (as in Section 5.1); the other half I treated with relaxing buffer containing no Triton X-100. Aliquots of the final myofibrillar suspensions (in 70 mM KCl + 20 mM imidazole, pH 7.0 at 4°C) were processed for electron microscopy as described above (Section 5.8). The remainder of each suspension was assayed for ATPase activity at pCa = 4.5 and at pCa>8. Contaminant membrane ATPases in the preparation were quantified by assaying the ATPase activities under the following conditions: (i) no inhibitor added; (ii) 1 μM ouabain to inhibit sarcolemmal ($Na^+ + K^+$)-ATPase activity (Schatzmann, 1953); (iii) 5 mM NaN_3 to inhibit mitochondrial ATPase activity (Fanburg, 1964); (iv) 1 mM quinidine to inhibit the SR Ca^{2+}-regulated ATPase activity (Fuchs et al., 1968); (v) 5 mM NaN_3 + 1 mM quinidine.

5.10 Statistical analysis

Where applicable, differences between means have been analysed by Student's t-test or the Paired t-test. The difference was considered significant if the probability (p) that the Null Hypothesis was correct (i.e. that the means were derived from the same population) was less than 0.05.

Chapter 6

RESULTS

6.1 The Ca^{2+}-activated and Ca^{2+}-insensitive ('basal') ATPase activities of the myofibrils

Table 4 lists the mean ATPase activities of the various myofibrillar preparations measured in the presence of an optimal Ca^{2+} concentration (pCa = 4.5) or in the virtual absence of Ca^{2+} (pCa>8). These are termed respectively the 'maximum total ATPase activity' and the 'basal ATPase activity', with the difference between them being 'maximum Ca^{2+}-activated ATPase activity'. Table 4 includes results not only from my standard Triton-washed preparations (guinea-pig cardiac, rabbit cardiac and rabbit white skeletal myofibrils) but also from classically-prepared (i.e. not Triton-treated) guinea-pig cardiac myofibrils, which I used before I routinely included Triton washes in the extraction procedure.

The results illustrate the well-known fact that the ATPase activity of skeletal myofibrils exceeds that of cardiac myofibrils taken from the same species (in this case, rabbit): the skeletal ATPase activities in the absence and presence of Ca^{2+} were approximately double the corresponding activities for the rabbit cardiac myofibrils. On the other hand, the ATPase activities of the rabbit Triton-treated cardiac myofibrils were not significantly different from those of the guinea-pig Triton-treated cardiac myofibrils.

As previous investigators have noted(Weber et al., 1964), the absolute values for myofibrillar ATPase activity vary considerably between experiments and produce a substantial scatter about the mean. This was particularly severe with the Triton-washed skeletal myofibrils, where the standard error (117.5 nmol Pi.min^{-1}.mg $protein^{-1}$) was more than one quarter of the mean total activity (413.7 nmol Pi. min^{-1}. mg $protein^{-1}$). To overcome this variability, I normalised the data by expressing the absolute ATPase activities in each experiment as a percentage of the maximum total ATPase activity obtained with the same type of myofibril in the same experiment. The mean percentages are shown in Table 4 below the absolute activities. The normalisation procedure reduces the scatter because, although the absolute values for total, basal and Ca^{2+}-activated ATPase activities vary from preparation to preparation, the relative proportions of these three are approximately constant. (Possible reasons for the variability in

Myofibrillar preparation		Basal ATPase activity	Max. Ca^{2+}-activated ATPase activity	Max. Total ATPase activity
Guinea-pig cardiac untreated (n = 10)	ABS	107.8 ± 12.6	164.5 ± 13.4	272 ± 17.2
	REL	39.3 ± 3.2	60.7 ± 3.2	100
Guinea-pig cardiac Triton-treated (n = 7)	ABS	39.7 ± 2.8	178.3 ± 10.6	218.0 ± 10.8
	REL	18.5 ± 1.5	81.5 ± 1.5	100
Rabbit cardiac Triton-treated (n = 14)	ABS	38.9 ± 8.0	149.7 ± 29.0	188.2 ± 32.4
	REL	20.9 ± 4.2	79.3 ± 4.4	100
Rabbit skeletal Triton-treated (n = 9)	ABS	70.8 ± 12.6	342.9 ± 113.0	413.7 ± 117.5
	REL	17.8 ± 1.4	82.2 ± 1.4	100

Table 4. Mean ATPase activities from different types of myofibrillar preparation. pH 7.00, 37°C.
Other conditions of incubation as in Section 5.3. 'Basal ATPase activity' measured at pCa>8.
'Max. total ATPase activity' measured at pCa 4.5. 'Max. Ca^{2+}-activated ATPase activity' =
Max. total ATPase activity mirus basal ATPase activity.
ABS = absolute ATPase activities in nmol $Pi.min^{-1}.mg\ protein^{-1}$.
REL = relative ATPase activities, i.e. percentage of the max. total ATPase activity.
All values are means ± S.E.M.

the absolute activities are discussed in Section 7.4). Normalisation also facilitated comparison between the Ca^{2+}-sensitivities of cardiac and skeletal myofibrils.

As will be shown later (Section 6.4), the maximum total ATPase activity at 37°C and pH 7.00 was obtained at a pCa of about 4.5 for the cardiac myofibrils and about pCa 5.3 for the skeletal myofibrils; at pCa 4.5, the skeletal activity was 98% of that at pCa 5.3.

The normalised values indicate that the relative basal activities of all the Triton-washed myofibrillar preparations were not significantly different from one another and comprised about 20% of the total activities. Thus 80% of the maximum activity of cardiac and skeletal ATPases was dependent upon Ca^{2+}. However, the 'untreated' myofibrils from guinea-pig hearts seemed to be less sensitive to Ca^{2+}, with a basal ATPase activity accounting for about 40% of the total. The absolute values of ATPase activity for this preparation show that in fact these myofibrils exhibited as much Ca^{2+}-activated ATPase activity as did the Triton-treated cardiac myofibrils, but that the basal ATPase activity (108 nmol Pi. min^{-1}. mg protein $^{-1}$) was 2 - 3 times greater than that of the treated myofibrils. This extra Ca^{2+}-insensitive ATPase activity in the untreated myofibrils suggests the presence of a contaminant ATPase which was not present in the Triton-treated preparations. Note that the assays were performed in the presence of 5 mM azide, so this contaminant ATPase is unlikely to be mitochondrial in origin. Thus these pilot studies with guinea-pig cardiac myofibrils revealed that washing with Triton X-100 was an essential step if the purity of the myofibrils was to be maximised.

6.2 Purity of the rabbit myofibrils prepared with and without washes in Triton X-100

I carried out further experiments to investigate more thoroughly the nature and extent of any contaminant ATPase activities in Triton X-100-treated or untreated preparations of the myofibrils chosen for eventual study - rabbit cardiac and rabbit white skeletal myofibrils (see Section 5.9 for details of method).

(a) Ultrastructure

Electron microscopy showed that much residual membrane, cellular debris and nuclei contaminated the rabbit cardiac myofibrils that had been prepared without washes in 1 % Triton X-100 (Figure 15). In places it was possible to discern mitochondria trapped between the myofibrils. More frequently, the contaminant membrane presented an amorphous appearance and so its source(s) could not be identified.

The untreated skeletal myofibrils (Figure 16) also contained some extraneous membrane, though much less than in the cardiac preparations. I was surprised to find some of the membrane in the skeletal preparations consisted of tubules adhering to the myofibrils at the level of the Z-line (arrows, Figure 17). These could not have been transverse tubules (T-tubules), which occur at the junction between the A and I bands in mammalian skeletal muscle. Similar tubules were not visible in the untreated cardiac preparations.

Two washes with 1% Triton X-100 removed almost all the contaminating membrane from the cardiac myofibrils (Figure 18), though occasionally nuclei were still present. Skeletal myofibrils were completely purified by Triton X-100 (Figure 19). The appearance of the myofibrils themselves was not noticeably altered by detergent treatment.

One general difference between cardiac and skeletal myofibrils prepared under identical conditions was that the sarcomeres of the former were shorter, so that often no I-band was visible: compare Figures 18 and 19 .

(b) Enzyme studies

To quantify the activity of non-myofibrillar ATPases in the preparations under my standard ionic conditions, I measured the basal and total ATPase activities in the presence of one or two of the following inhibitors of membrane ATPases: 10^{-6} M ouabain, 5 mM azide (as in my standard incubation solutions), 1 mM quinidine. Incubation medium containing no inhibitor served as control in this case. The observed ATPase activities of the Triton-treated and untreated rabbit myofibrils are plotted in the form of histograms (Figures 20 and 21).

(i) Cardiac myofibrils. The most striking difference between the Triton-treated and untreated cardiac preparations was in the sensitivity of their measured ATPase activities to certain inhibitors, notably azide and quinidine. None of the inhibitors used significantly decreased the basal or total ATPase activities of the treated myofibrils below the control values i.e. in the absence of any inhibitor (Figure 20). In contrast, the ATPase activities of myofibrils prepared without washes in 1% Triton X-100 were very sensitive to azide and quinidine, though 10^{-6} M ouabain was without effect. The basal activity in the absence of any inhibitor - 332 nmoles $P.min^{-1}.mg\ protein^{-1}$ - exhibited by the Triton-treated cardiac myofibrils was reduced to about a third of its activity by 5 mM azide

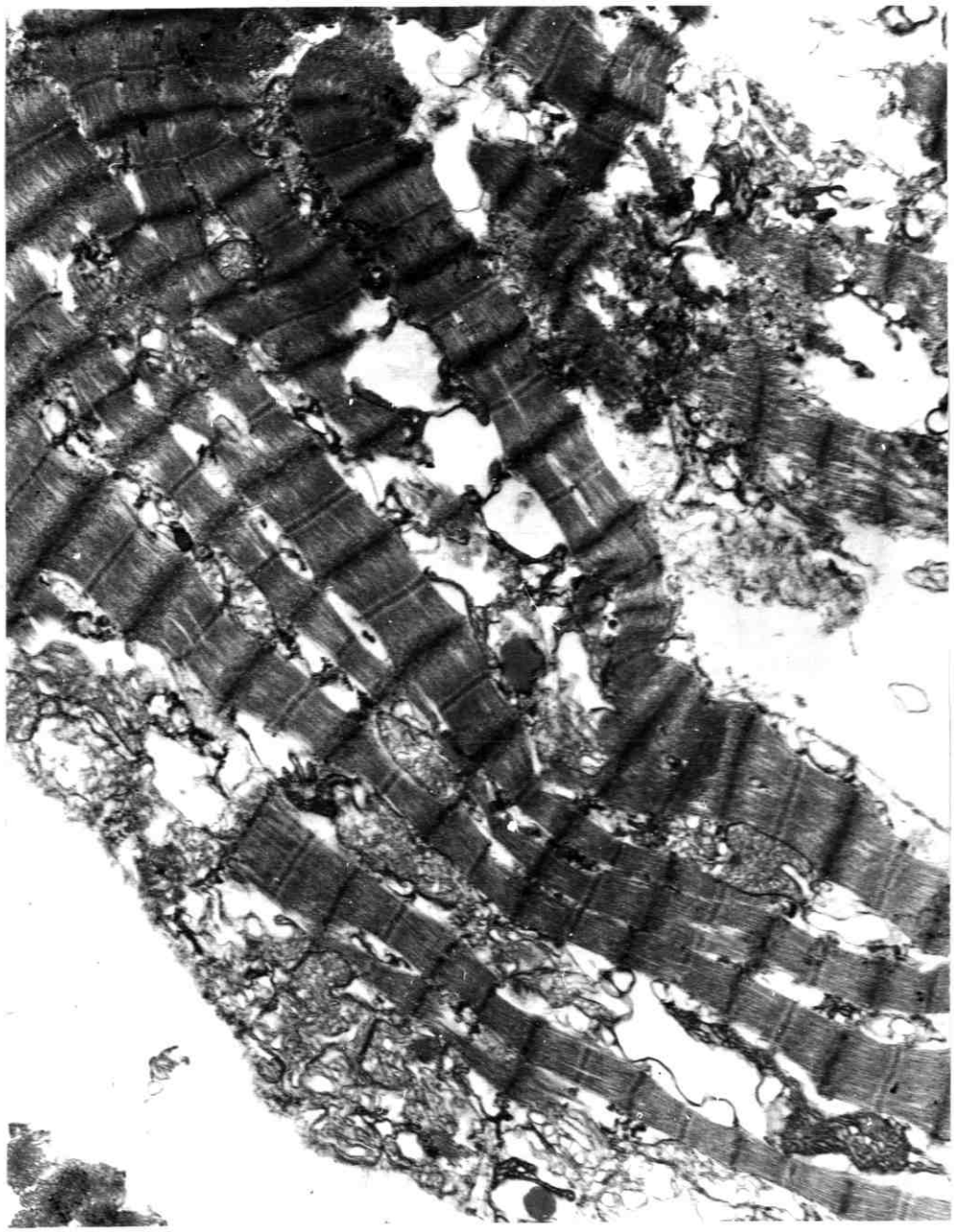


Figure 15. Rabbit cardiac myofibrils prepared without treatment with Triton X-100. x 9000.



Figure 16. Rabbit white skeletal myofibrils prepared without treatment with Triton X-100. x 9000

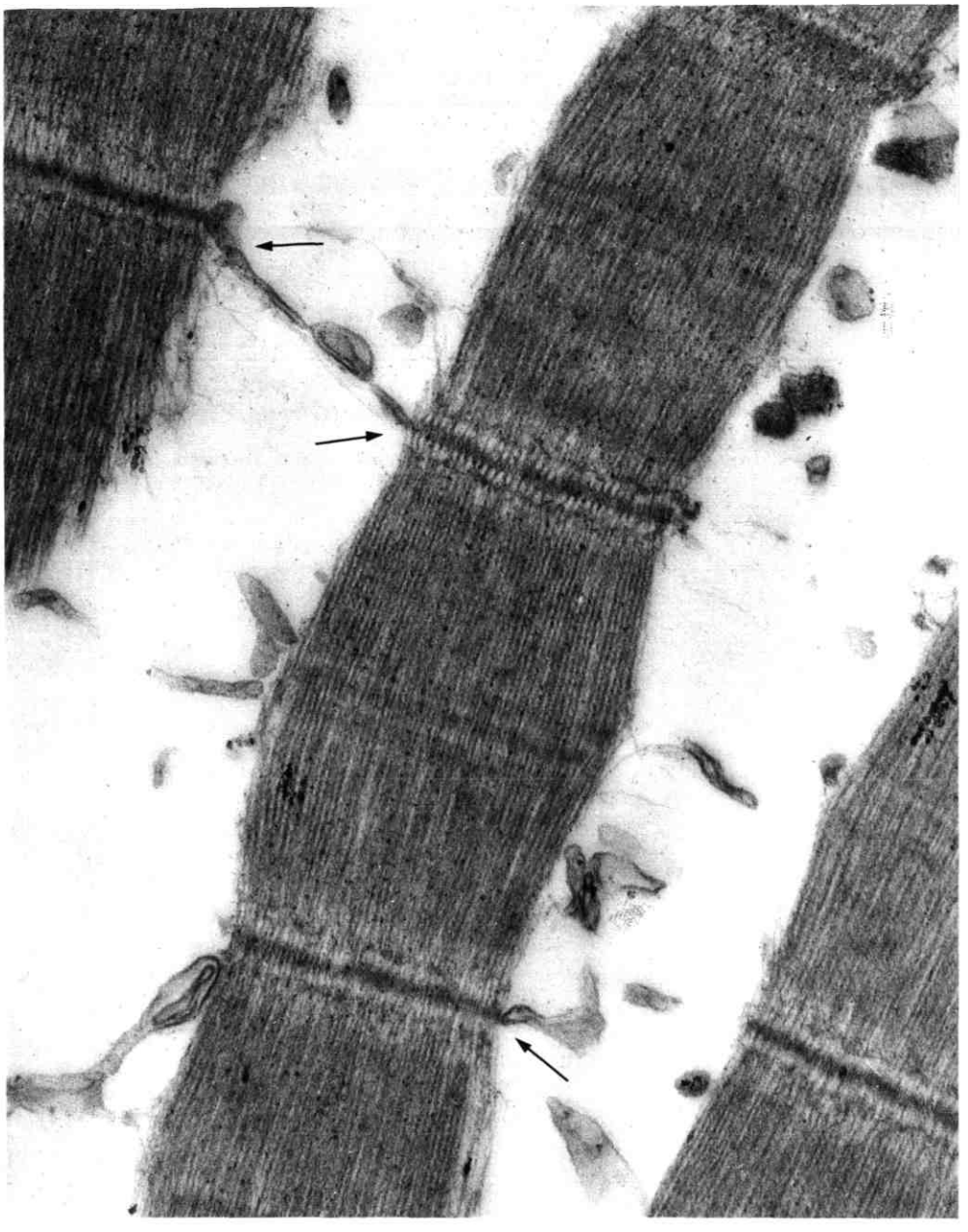


Figure 17. Rabbit white skeletal myofibrils prepared without treatment with Triton X-100. Same preparation as in Figure 16 but at higher magnification (x 42000). Arrows show the attachment of tubules at the level of the Z-lines.

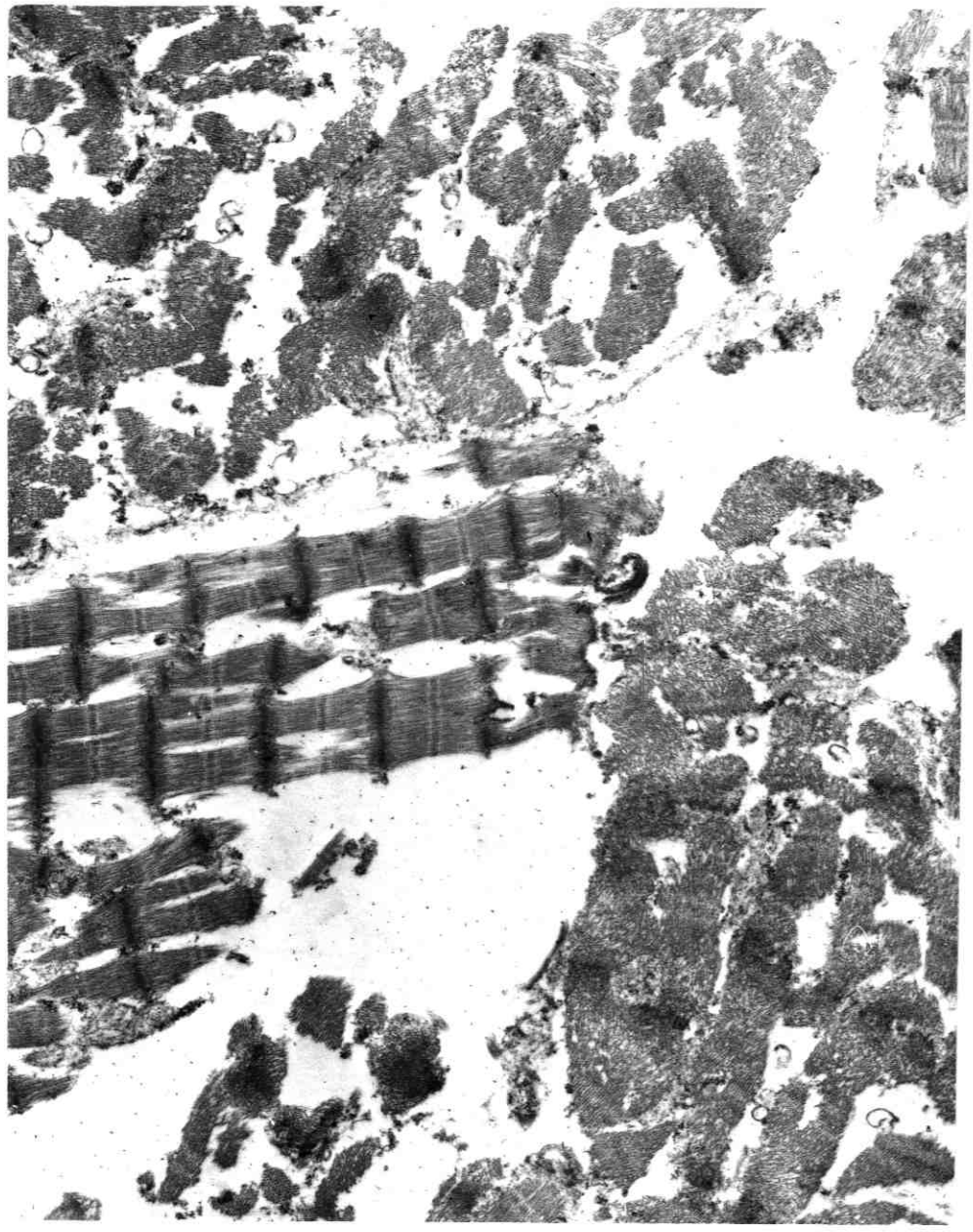
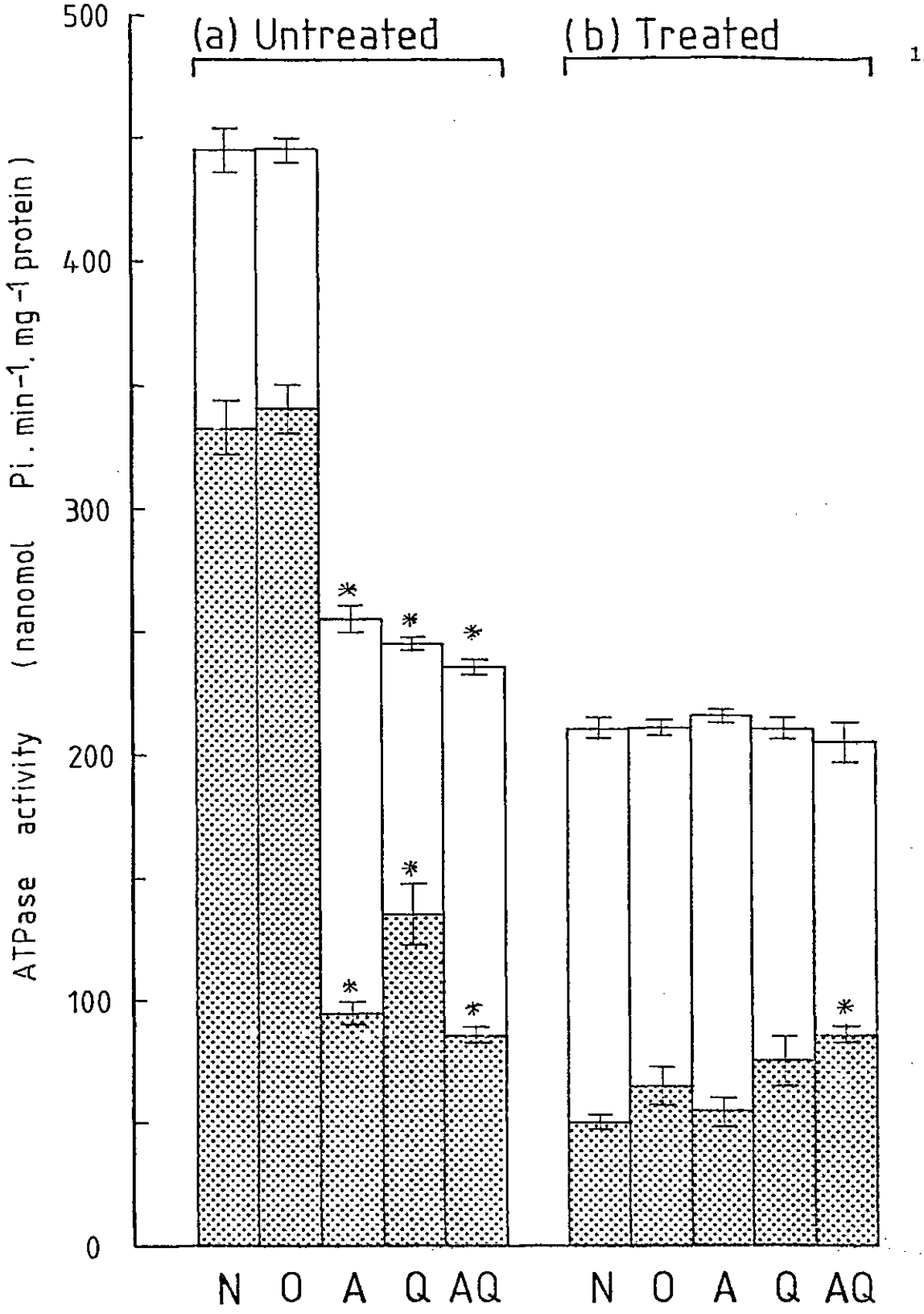


Figure 18. Rabbit cardiac myofibrils washed twice with a solution of 1% Triton X-100 in 'relaxing buffer'.
Compare with Figure 15. x 9000.

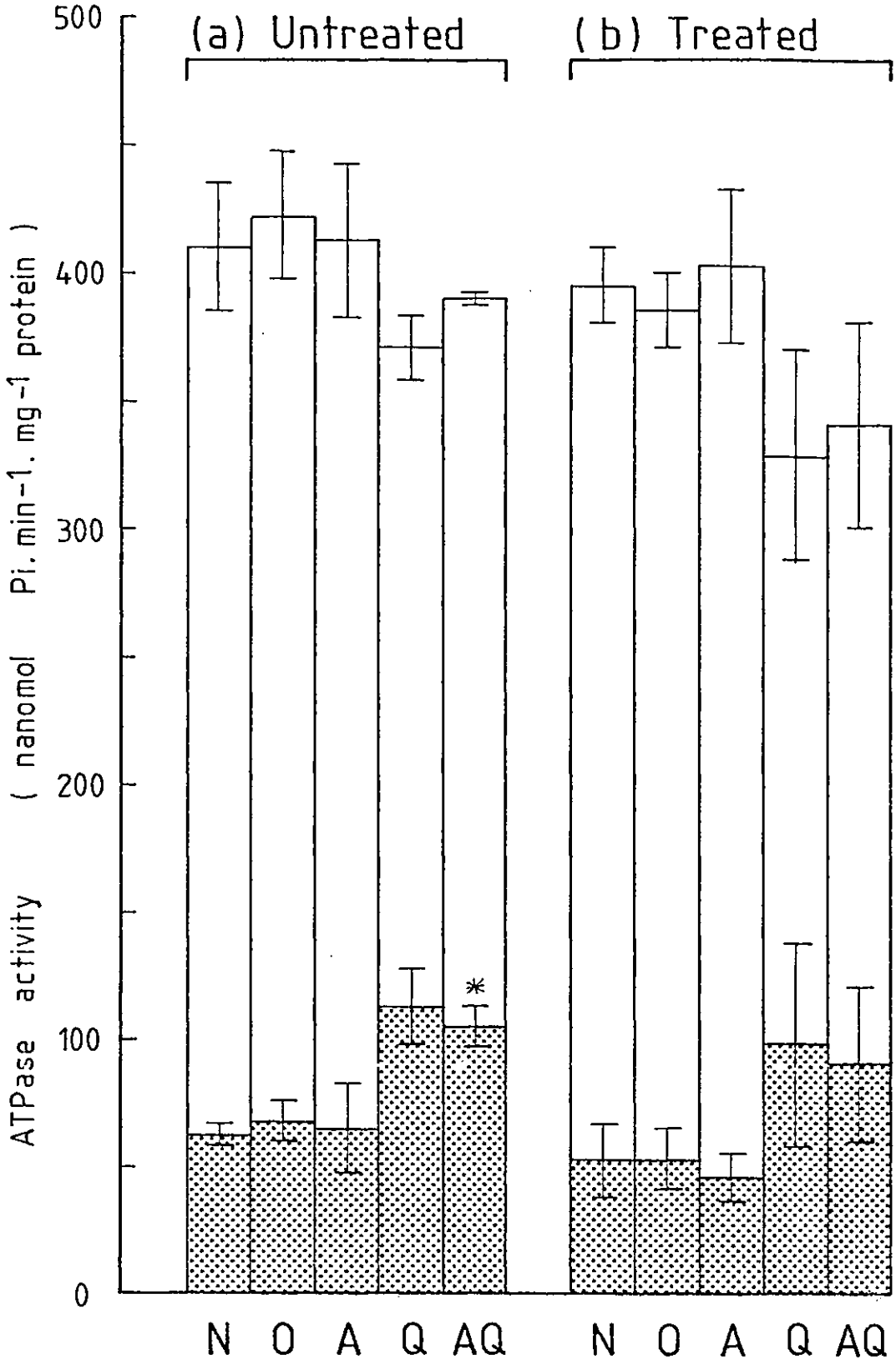


Figure 19. Rabbit white skeletal myofibrils washed twice with 1% Triton X-100 in 'relaxing buffer'.
Compare with Figure 16. x 9000.



* Significantly different ($p<0.05$, paired t-test) from the activity exhibited by the same type of myofibrillar preparation when assayed at the same pCa but in the absence of inhibitor (N).

Figure 20. ATPase activities of cardiac myofibrillar preparations which (a) had not been treated in detergent or (b) had been washed twice in 1% Triton X-100 in 'relaxing buffer'. Shaded area = basal ATPase activity (pCa>8), clear area = maximum Ca^{2+}-regulated ATPase activity (pCa 4.5). ATPase assayed in the presence of inhibitors of various membrane ATPases: (N), no inhibitor; (O), 1μM ouabain; (A), 5 mM azide; (Q), 1 mM quinidine; (AQ), 5 mM azide + 1 mM quinidine. See Section 5.3 for other incubation conditions. Means ± S.E.M. from 4 preparations.



* Significantly different ($p<0.05$, paired t-test) from the activity exhibited by the same type of myofibrillar preparation when assayed at the same pCa but in the absence of inhibitor (N).

Figure 21. ATPase activities of preparations of white skeletal myofibrils which (a) had not been treated in detergent or (b) had been washed twice in 1% Triton X-100 in 'relaxing buffer'. Other details as for Figure 20. Means ± S.E.M. from 4 preparations.

or 1 mM quinidine. Azide and quinidine together produced no further inhibition. The results also demonstrate that, even when assayed under the widely-used conditions of 5 mM azide, the untreated cardiac myofibrils still had a basal ATPase activity that, at 95 nmol Pi. min^{-1}.mg $protein^{-1}$, was almost double that of the Triton-washed myofibrils (55 nmol Pi.min^{-1}.mg $protein^{-1}$). Thus these results supported the conclusion from the pilot experiments on guinea-pig myofibrils (Table 4) that not all of the contaminant ATPase activity in untreated cardiac preparations can be inhibited by 5 mM azide.

The azide- or quinidine-induced reduction in the basal ATPase activity of the untreated myofibrils was largely reflected by a decrease in the total ATPase activity, for none of the inhibitors greatly altered the Ca^{2+}-activated ATPase activity (control value = 112 nmol Pi. min^{-1}. mg $protein^{-1}$), with the exception of 1 mM quinidine, which did seem to increase basal activity at the expense of Ca^{2+}-activated activity.

(ii) Skeletal myofibrils. There were no significant differences between the total ATPase activities of Triton-treated skeletal myofibrils and the corresponding activities of the untreated myofibrils (Figure 21). Furthermore, no inhibitor significantly decreased the basal ATPase activities of either type of preparation below control values. Any ATPases present in the tubules visible in Figure 17 must therefore have had minimal activity under the chosen assay conditions. Nevertheless, I retained Triton washes in the preparation of skeletal myofibrils to maximise purity.

The basal ATPase activities of both Triton-treated and untreated skeletal myofibrils were significantly greater in the presence of 1 mM quinidine than in its absence. Some evidence for a quinidine-induced stimulation of the basal activity is also apparent from the data on the cardiac preparation (Figure 20).

(c) Other signs of purity or impurity

Some indication of purity could be gained merely from the colour of the preparations: skeletal myofibrils prepared with or without washes in Triton X-100 were always white, as were cardiac myofibrils that had been washed twice in the detergent. In contrast, cardiac myofibrils prepared without washes in Triton X-100 were light brown, probably owing to contamination by mitochondria cytochromes. The change in colour of cardiac preparations from brown to white during the washes with detergent provided a ready way of checking

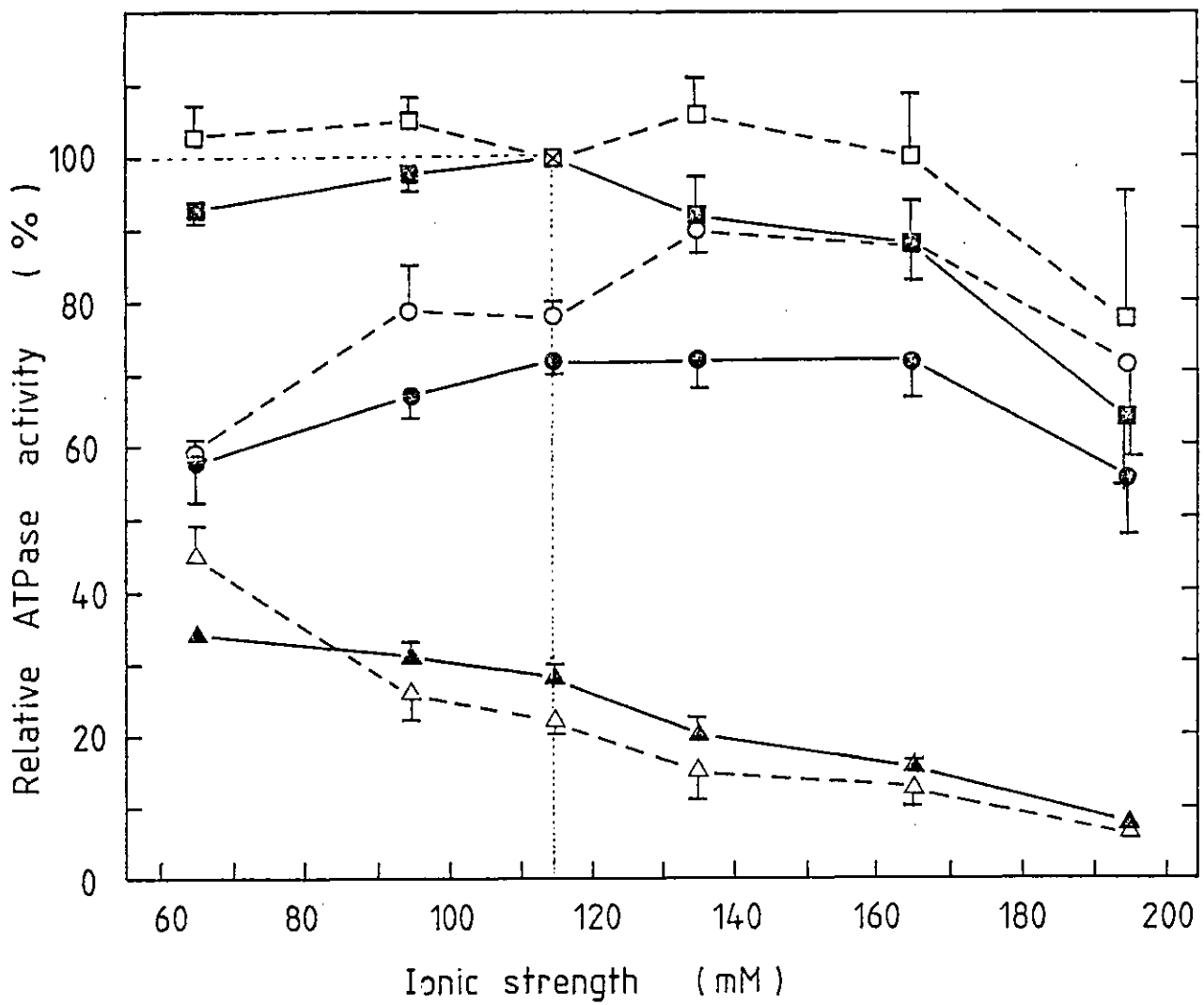
that at least some of the contamination had been removed.

6.3 The influence of ionic strength on the ATPase activities of the purified myofibrils

I examined the influence of ionic strength ($\Gamma/2$) on ATPase activity, (i) to see whether the differences in ionic strength between the solution at pH 7.2 ($\Gamma/2$ = 115 mM) and the solution at pH 6.4 ($\Gamma/2$ = 119 mM) could cause significant effects and (ii) to find out whether these ionic strengths, although in the physiological range, produced anything like maximal ATPase activity. Although these experiments were originally carried out to characterise the assay conditions, the results are reported in detail here because they bear on results obtained with skinned muscle preparations (Part III). Ionic strength was varied over the range 65 - 205 mM by alteration of the KCl concentration. The ATPase activities of the Triton-washed rabbit cardiac and rabbit white skeletal myofibrils, assayed at pCa>8 and at pCa 4.5, are displayed in Figure 22. To reduce the scatter in the data, all activities have been expressed relative to the total ATPase activity at pCa 4.5 and ionic strength 116 mM (my standard ionic strength at pH 7.0). This 100% point corresponded to 174 ± 19 nmol Pi. min^{-1}.mg $protein^{-1}$ for the rabbit cardiac myofibrils and to 367 ± 46 nmol Pi.min^{-1}.mg $protein^{-1}$ for the rabbit skeletal myofibrils (means ± S.E., n = 3).

As the ionic strength was increased from 65 mM, the total ATPase activity of rabbit cardiac myofibrils rose to a maximum (100%) at $\Gamma/2$ = 115 mM and then fell again to reach 64% of maximum at $\Gamma/2$ = 205 mM. The basal ATPase activity decreased almost linearly over this range of ionic strengths, from 34% at $\Gamma/2$ = 65 mM to 8% at $\Gamma/2$ = 205 mM. This resulted in the Ca^{2+}-activated ATPase activity exhibiting a broad plateau over the ionic strength range 115 - 165 mM.

Skeletal myofibrils displayed an ionic strength-sensitivity that was generally similar to that of cardiac myofibrils except that the maxima were shifted to slightly higher ionic strengths. In fact, the skeletal Ca^{2+}-activated ATPase had a peak at about $\Gamma/2$ = 135 mM. This was because the basal activity, like that of cardiac myofibrils, decreased monotonically with increasing ionic strength whereas the total skeletal ATPase activity was little affected by ionic strength changes over the range 65 - 165 mM. Above 165 mM, the total ATPase activity and thus the Ca^{2+}-activated activity, were depressed.


Relative ATPase activity (%)
100
80
60
40
20
0
60
80
100
120
140
160
180
200
Ionic strength (mM)

These studies indicated that the standard ionic strength (115 mM at pH 7.00) was in the range of ionic strengths which produced maximal ATPase activity, but was at the lower end of this range. From the broad plateau it can be seen that the difference in ionic strengths (115 mM - 119 mM) in the solutions of different pH would have by themselves a negligible effect on the ATPase activities.

6.4 The influence of pH on cardiac and skeletal myofibrillar ATPase activity

I determined the pCa-ATPase activity relationships for the Triton-treated myofibrils from rabbit ventricles, guinea-pig ventricles and rabbit white skeletal muscle at four values of pH: 7.20, 7.00, 6.80 and 6.40 (all ± 0.01 pH unit at 37°C). These relationships are shown in Figure 23 to 25.

(a) Basal ATPase activity

Changes in pH over the range pH 7.20 - pH 6.40 did not significantly alter the relative basal ATPase activities of rabbit and guinea-pig cardiac myofibrils from the values at pH 7.00 (21.9 ± 1.0% and 20.2 ± 2.4% respectively). However the basal ATPase activity of the skeletal myofibrils was slightly influenced by pH: although the relative ATPase activity was constant at about 14 - 15 % from pH 6.40 to 7.00, at pH 7.20 the activity (22.6 ± 2.7%) was significantly greater than that at pH 7.00 (15.3 ± 1.0%). (Paired t-test, $p<0.05$).

(b) Maximum Ca^{2+}-regulated and total ATPase activities

The absolute values of ATPase activities at pH 7.00 that correspond to the 100% points in Figures 23 to 25 were (in nmol $Pi.min^{-1}.mg^{-1}$ protein): rabbit cardiac myofibrils, 199 ± 15; guinea-pig cardiac myofibrils, 205 ± 16; rabbit white skeletal myofibrils, 480 ± 71 (means ± S.E.M., n = 4).

As the pH was reduced from 7.20 to 6.40 the maximum relative value for the total ATPase activity of the cardiac myofibrils fell, from 103.9 ± 1.6% to 90.1 ± 6.1% in the case of rabbit cardiac myofibrils and from 101.9 ± 3.9% to 92.5 ± 5.9% with the guinea-pig cardiac myofibrils. Neither of these reductions were however significant ($p>0.05$, Paired t-test). The maximum total ATPase activities of the skeletal myofibrils ranged from 105.1 ± 3.5% at pH 7.20 to 98.4 ± 1.6% at pH 6.40, though there was no consistent trend over the pH range studied and no differences were significant.

As a consequence of the pH-insensitivity of the cardiac basal ATPase activity, the reduction in total ATPase activity of the cardiac

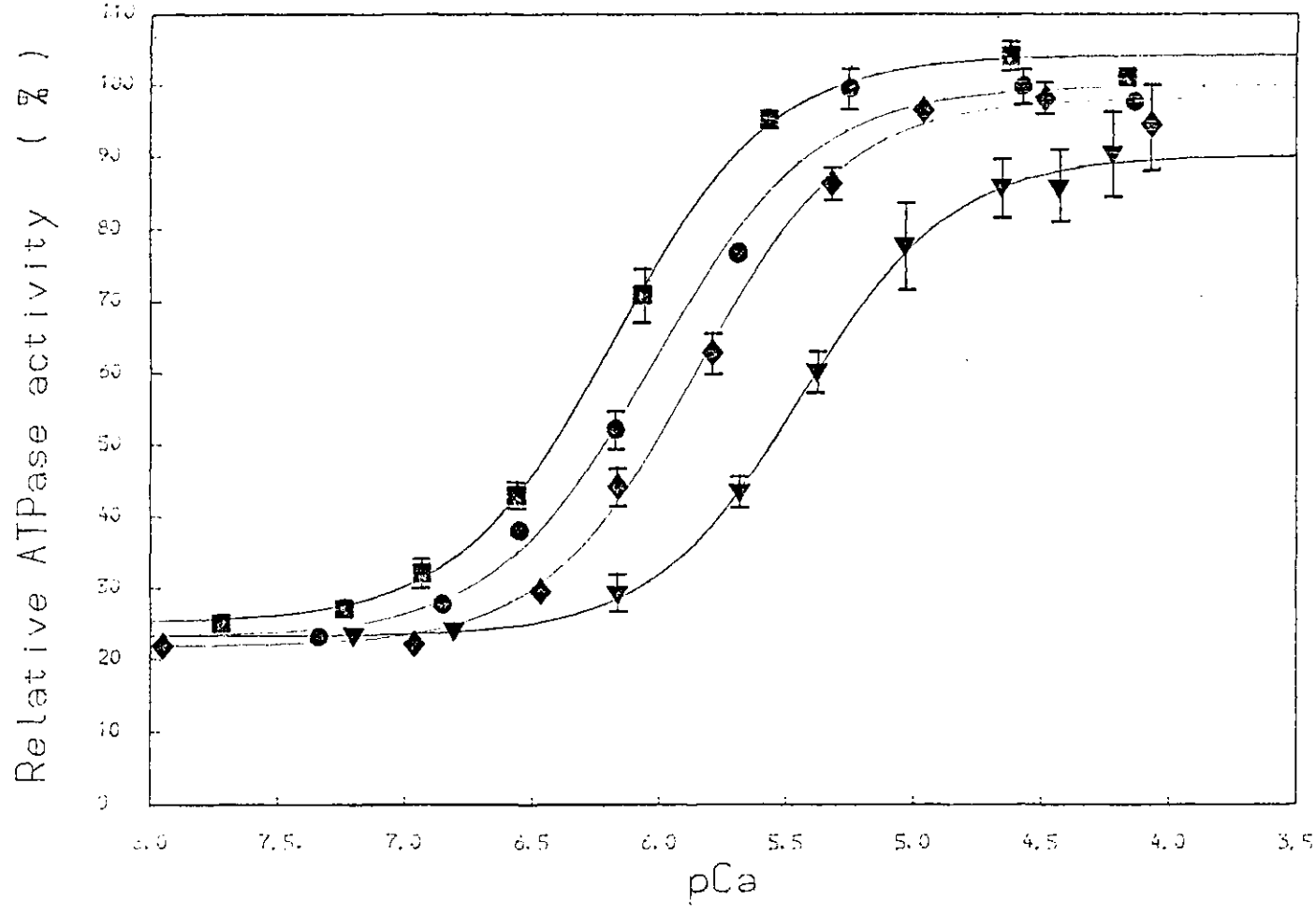


Figure 23. Influence of pH on the pCa-ATPase relationship of Triton-washed myofibrils from rabbit cardiac muscle. Assays performed at: (■) pH 7.20; (●) pH 7.00; (◆) pH 6.80; (▼) pH 6.40. Other incubation conditions as in Section 5.3(a). All ATPase activities expressed relative to the total ATPase activity at pCa 4.5 and pH 7.00. Each point shows the mean ± S.E.M. of four experiments. Absence of error bars indicates S.E.M. ≤ size of symbol. Solid lines show the fittings of the modified Hill equation. The fitted coefficients are listed in Table 5.

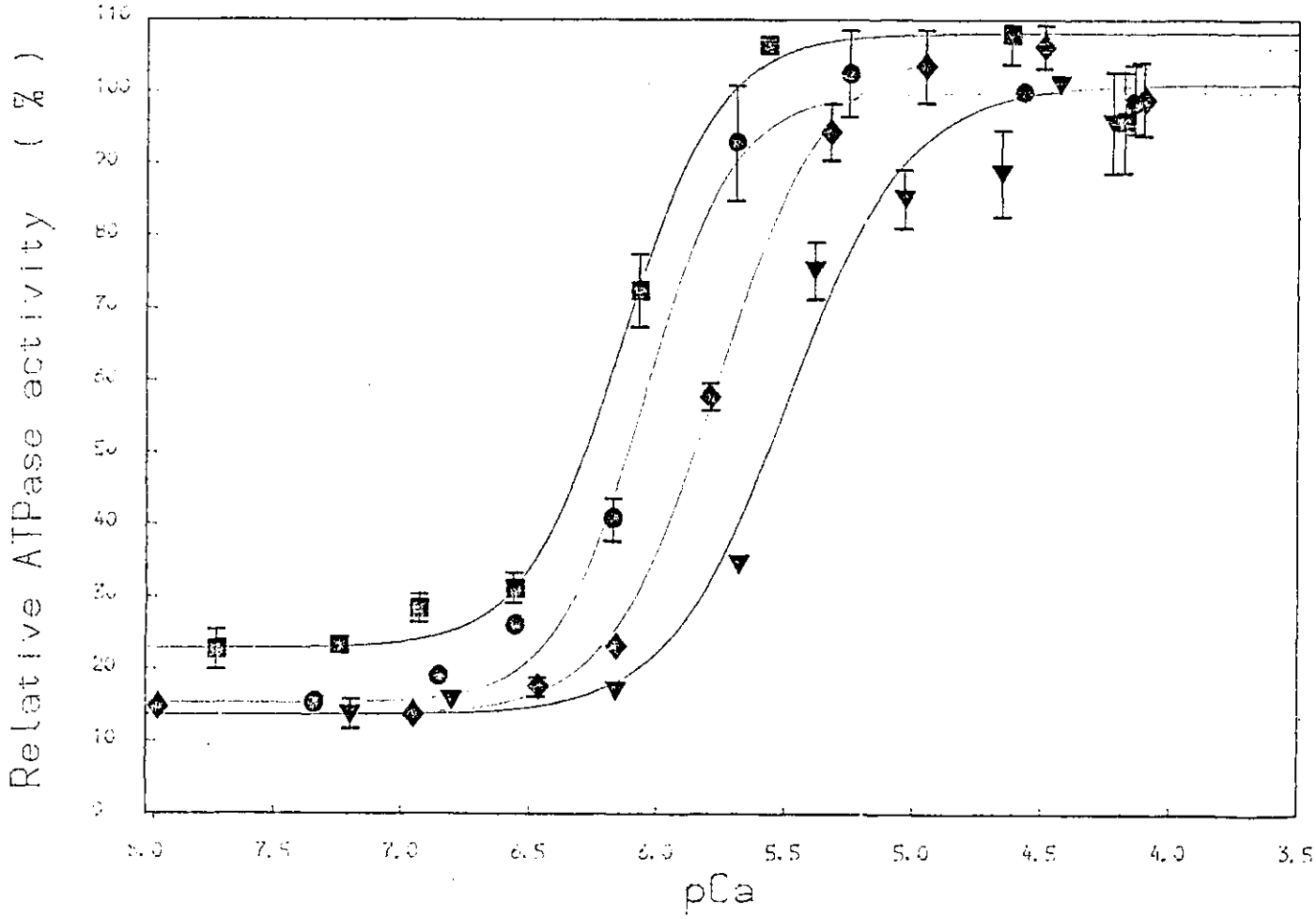


Figure 24. Influence of pH on the pCa-ATPase relationship of Triton-washed myofibrils from rabbit white skeletal muscle. Assays performed at: (■) pH 7.20; (●) pH 7.00; (◆) pH 6.80; (▼) 6.40. ATPase activities expressed relative to the total ATPase activity at pH 4.5 and pH 7.00. Each point shows the mean ± S.E.M. of four experiments. Other details as in Figure 23.

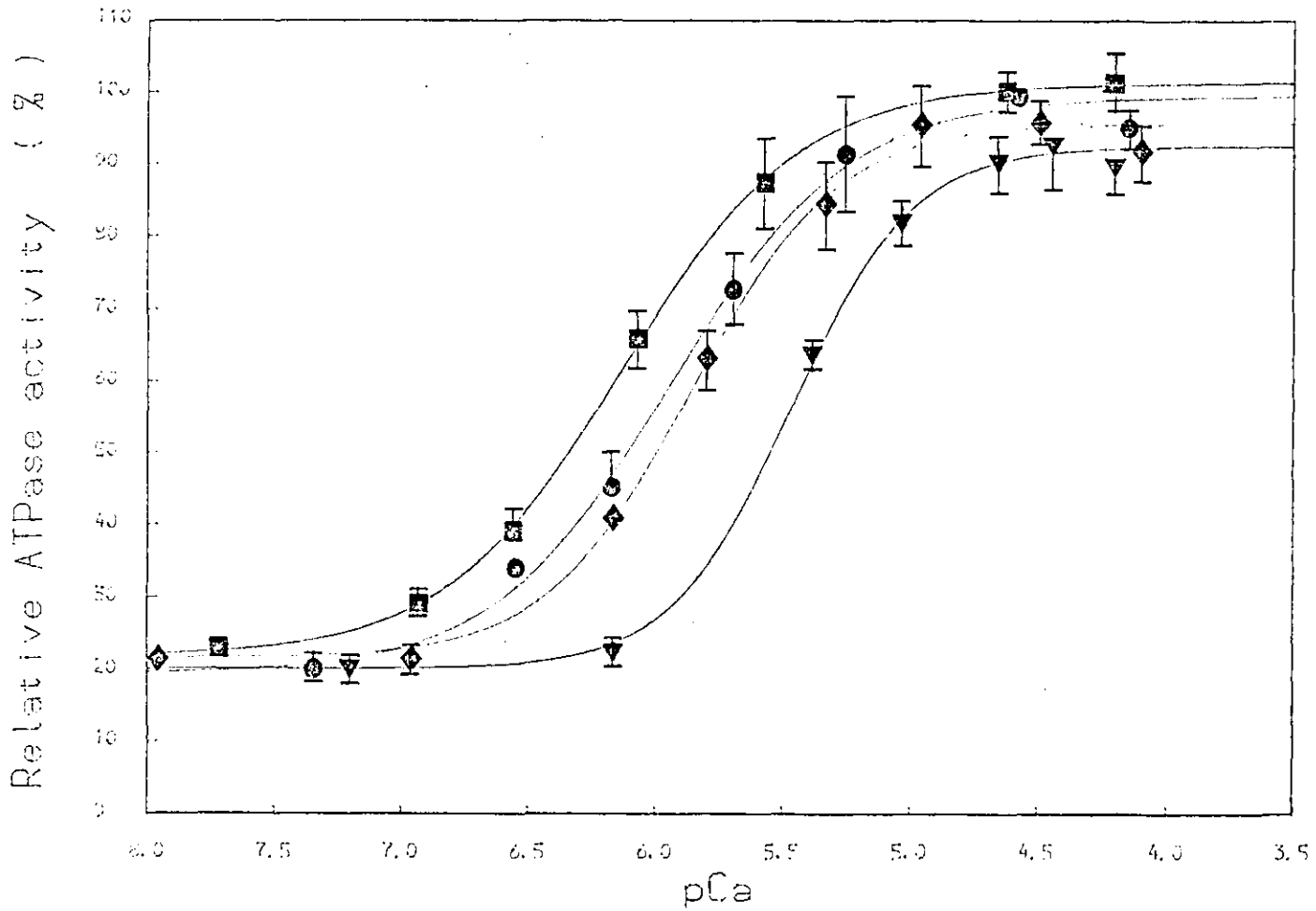


Figure 25. Influence of pH on the pCa-ATPase relationship of Triton-washed myofibrils from guinea-pig cardiac muscle. Assays performed at: (■) pH 7.20; (●) pH 7.00; (◆) pH 6.80; (▼) pH 6.40. ATPase activities expressed relative to the total ATPase activity at pCa 4.5 and pH 7.00. Each point shows the mean ± S.E.M. of three experiments. Other details as in Figure 23.

myofibrils that was seen as the pH was reduced was attributable entirely to a fall of the Ca^{2+}-regulated ATPase activity. Over the range pH 7.20 to 6.40 the Ca^{2+}-regulated ATPase activity of rabbit cardiac myofibrils fell from 79.0 to 66.9% and that of guinea-pig myofibrils from 79.6 to 72.5%. Neither of these reductions was statistically significant however. Although the total ATPase activity of the skeletal myofibrils was also reduced by acidity, in fact the pH-sensitivity of the skeletal basal activity resulted in a slight (but statistically not significant) increase in the Ca^{2+}-regulated ATPase activity as the pH was lowered from 7.20 to 6.40.

(c) Relationship between pCa and relative ATPase activity

The ATPase activities of all three types of myofibrils increased in sigmoidal fashion as the pCa was reduced from 7.0 to 4.5 i.e. as the $[Ca^{2+}]$ was raised from 0.1 μM to 30 μM (Figures 23 to 25). Using non-linear least squares analysis, I was able to fit a sigmoid curve, defined by the modified Hill equation (Section 5.7), to each pCa-ATPase activity relationship (solid lines in Figures 23 to 25). The cardiac data were fitted very well by these sigmoid curves but the fit was not quite so good for some of the points in the skeletal Ca^{2+}-activation curves. From the computed values for the parameters n_H and K* in the modified Hill equations, I was able to obtain an objective estimate of the pCa_{50} for each Ca^{2+}-activation curve: $pCa_{50} = -\frac{1}{n} \log K^*$. (It should be recalled that the pCa_{50} is the pCa value required for half-maximal Ca^{2+}-activation of the myofibrils; it is not the pCa value at which the total ATPase activity is 50% of maximum). The values for n_H, K* and pCa_{50} for all the Ca^{2+}-activation curves of Figures 23 to 25 are given in Table 5 . To facilitate the comparison between the pH-sensitivities of Ca^{2+}-regulation in the different myofibrils, I have plotted the pCa_{50} values against pH in Figure 26 . It is important to emphasise that these values of n_H, K* and pCa_{50} were derived from the curves fitted to the mean data in Figures 23 to 25 . Unfortunately the individual Ca^{2+}-activation curves in each experiment were not smooth enough to enable the computer to fit each with a sigmoid curve - the fitting procedure was successful only if the mean curves were used. Thus there are no standard errors for the mean values of n_H, K* or pCa_{50} that are listed in Table 5 .

At pH 7.00, the pCa_{50} values for rabbit cardiac myofibrils (6.02) and for rabbit skeletal myofibrils (6.05) were almost identical. Despite this, the relationsips between pCa and relative ATPase activity

Type of myofibril		pH (± 0.01)			
		7.20	7.00	6.80	6.40
Rabbit cardiac (n = 4)	n_H	1.41	1.38	1.45	1.47
	K^*	1.9×10^{-9}	4.8×10^{-9}	3.1×10^{-9}	1.0×10^{-8}
	pCa_{50}	6.18	6.02	5.85	5.44
Rabbit white skeletal (n = 4)	n_H	2.29	2.50	2.08	1.87
	K^*	8.4×10^{-15}	7.4×10^{-16}	1.1×10^{-12}	5.9×10^{-11}
	pCa_{50}	6.14	6.03	5.76	5.48
Guinea-pig cardiac (n = 3)	n_H	1.24	1.25	1.52	1.83
	K^*	2.3×10^{-8}	3.7×10^{-8}	1.2×10^{-9}	1.0×10^{-10}
	pCa_{50}	6.13	5.94	5.85	5.46

Table 5. Hill coefficients for the sigmoid curves fitted to the data in Figures 23 to 25.

See Section 5.7 for details of fitting procedure.

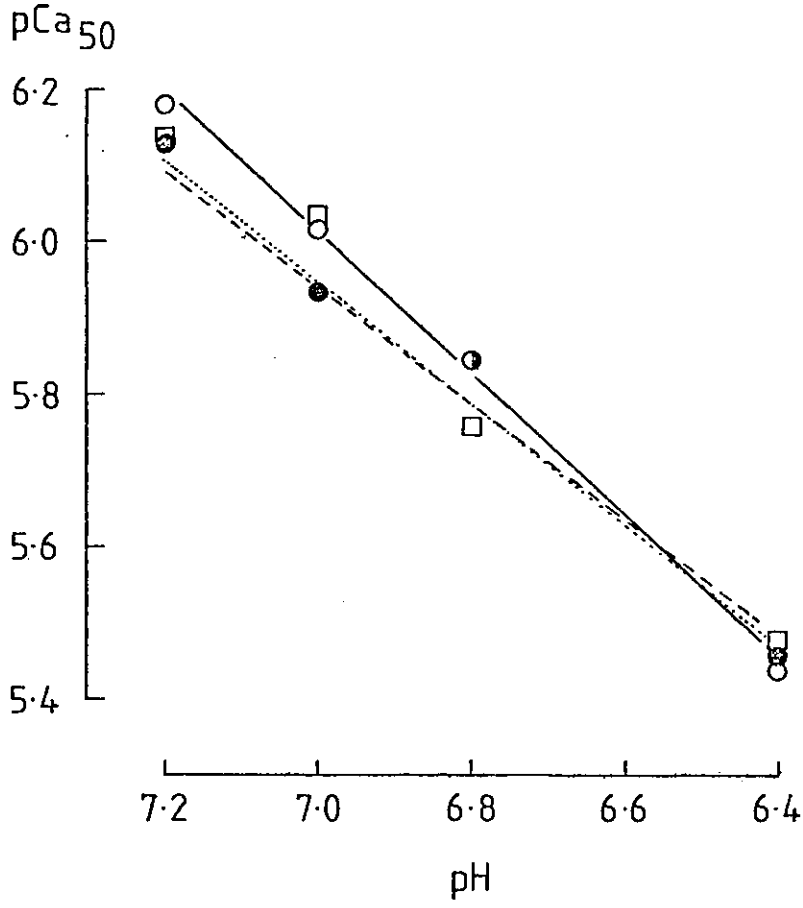


Figure 26. Influence of pH on the pCa_{50} values for the three types of myofibril. (○) Rabbit cardiac myofibrils; (□) rabbit white skeletal myofibrils; (◑) guinea-pig cardiac myofibrils. pCa_{50} values were derived from the Hill coefficients n_H and K^*.

The linear regression lines were as follows:

rabbit cardiac myofibrils (solid line): $pCa_{50} = 0.930 \times pH - 0.498$

rabbit white skeletal myofibrils (dotted line) : $pCa_{50} = 0.856 \times pH - 0.009$

guinea-pig cardiac myofibrils (dashed line) : $pCa_{50} = 0.820 \times pH + 0.228$

for the two types of rabbit myofibril were obviously different, for the skeletal curve was considerably steeper than the cardiac curve (Figures 23 and 24). A small part of this difference in slope was due to the fact that the relative basal activity in these experiments was slightly smaller for the skeletal myofibrils (14% - 22%) than for the cardiac myofibrils (22% - 24%). However, the chief cause of the difference in slope was that the skeletal myofibrillar ATPase activity was activated over a smaller pCa range (pCa 7.0 - 5.3 at pH 7.00) than was the cardiac myofibrillar ATPase activity (pCa 7.4 - 5.0 at pH 7.00). The steepness of each pCa-ATPase relationship was quantified by the value for n_H, which was 2.5 for the skeletal ATPase activity at pH 7.00, compared with only 1.4 for the rabbit cardiac ATPase activity at the same pH (Table 5).

The pCa-relative ATPase activity relationship for the guinea-pig cardiac myofibrils at pH 7.00 was very similar to that for the rabbit cardiac myofibrils except that (i) the pCa_{50} (5.94) was slightly lower than that for the rabbit myofibrils (6.02) and (ii) the relationship was not quite so steep as that for rabbit cardiac myofibrils (n_H = 1.25 for guinea-pig cardiac myofibrils and 1.38 for rabbit cardiac myofibrils).

All three types of myofibril showed a small inhibition of the Ca^{2+}-regulated ATPase activity at Ca^{2+} concentrations above the optimum i.e. at pCa < 4.5 for the cardiac myofibrils at pH 7.00 and pCa < 5.0 for the skeletal myofibrils.

In contrast to the rather small effects of pH on the magnitude of the basal and maximum ATPase activities (Sections 6.4(a) and 6.4(b)), changes in pH produced substantial alterations in the relationship between pCa and relative ATPase activity. With each step decrease in pH, the pCa-relative ATPase relationships for both cardiac and skeletal myofibrils were shifted to lower pCa values i.e. to higher Ca^{2+} concentrations (Figures 23 to 25). In other words, a decrease in pH lowered the Ca^{2+}-sensitivities of the myofibrils. The pCa_{50} for each type of myof ibril fell almost linearly with pH, with each 0.2 unit reduction in pH decreasing the pCa_{50} by almost 0.2 pCa units (Table 5 and Figure 26). The mean change in pCa_{50} for unit change in pH ($\Delta pCa_{50}/\Delta pH$) was 0.93 for rabbit cardiac myofibrils, 0.82 for guinea-pig cardiac myofibrils and 0.86 for rabbit skeletal myofibrils (values taken form the slopes of the linear regression lines in Figure 26). In other words, for each ten-fold increase in $[H^+]$

the [Ca^{2+}] required for half-maximal Ca^{2+}-activation increased by a factor of 8.5 for rabbit cardiac myofibrils, 6.6 for guinea-pig cardiac myofibrils and 7.2 for rabbit skeletal myofibrils. Thus the pH-sensitivities of Ca^{2+}-regulation were slightly different for the three types of myofibril studied, with pH-sensitivity decreasing in the order: rabbit cardiac myofibrils > rabbit skeletal myofibrils > guinea-pig cardiac myofibrils.

If no allowance had been made for the impurity in EGTA (i.e. if it had been assumed EGTA was 100% pure), all the Ca^{2+}-activation curves would have been shifted by 0.20 - 0.22 pCa units to higher pCa values (see curves in Kentish & Nayler, 1979) and the n_H values would have been greater than those in Table 5 by 0.15 - 0.30 units. Nevertheless, the changes in pH would have had the same effect on pCa_{50} as described above, and the mean values for $\Delta pCa_{50}/\Delta pH$ would have been 0.88, 0.73 and 0.75 for rabbit cardiac myofibrils, guinea-pig cardiac myofibrils and rabbit skeletal myofibrils, respectively.

In the case of the rabbit cardiac myofibrils, each alteration in pH shifted the pCa values required for threshold activation, for half-maximal activation and for maximum activation by approximately equal amounts - about 0.2 pCa units for each 0.2 change in pH. Consequently, the Ca^{2+}-activation curves at the four values of pH were 'parallel'. Thus the steepness of the pCa-ATPase relationship for rabbit cardiac myofibrils was pH-insensitive. This was reflected by the n_H values, which remained constant at about 1.4 throughout the pH range 7.20 - 6.40 (Table 5). In contrast, n_H for the rabbit skeletal myofibrils decreased from 2.3 at pH 7.20 to 1.9 at pH 6.4 i.e. the Ca^{2+}-activation curve for the skeletal myofibrils became shallower as the pH was reduced. Despite this, at pH 6.40 the curve for the skeletal myofibrillar ATPase (n_H = 1.9) was still steeper than that for the rabbit cardiac myofibrillar ATPase at the same pH (n_H = 1.4). The pCa-ATPase activity for guinea-pig cardiac myofibrils seemed to become steeper as the pH was reduced from 7.20 to 6.40. Unfortunately, the lack of any estimates for the S.E.M. of the n_H values made it impossible to calculate whether any of these differences in n_H was statistically significant.

In summary, a change in pH of the incubation solutions over the range pH 7.20 - pH 6.40 had statistically insignificant effects on the basal, maximum total and maximum Ca^{2+}-regulated ATPase activities of both cardiac and skeletal myofibrils (except for the increase in basal activity from pH 7.00 to pH 7.20 with skeletal myofibrils). The main influence of pH was on the pCa_{50}, which was lowered by almost

0.2 pCa units for each decrease of 0.2 pH units, though the rabbit cardiac myofibrils were slightly more sensitive to pH in this respect than were the rabbit skeletal myofibrils, and the guinea-pig cardiac myofibrils were the least sensitive of all.

Chapter 7

DISCUSSION

7.1 Compositions of the incubation media

The primary aim of this study was to establish whether the Ca^{2+}-activation of the ATPase activity of cardiac and skeletal myofibrils was sensitive to pH, and so to judge whether a direct influence of pH on myofibrils could account for the observed actions of an intracellular acidosis on cardiac and skeletal muscle. Thus it was desirable that the chosen assay conditions should simulate as closely as possible the *in vivo* myofibrillar environment. To achieve this, I chose the compositions of the incubation media to reproduce the pH, pCa, temperature and ionic conditions existing in intact muscle cells. The 'standard' pH for the incubation media was selected to be pH 7.0, which is approximately the normal intracellular pH in both cardiac and skeletal muscle (Chapter 2). I assayed the myofibrillar ATPase activity over a pH range (pH 7.2 - 6.4) that includes values found in cardiac muscle during an intracellular acidosis (pH $\approx$ 6.4 - 6.6, Chapter 2). It was not advisable to use pH values lower than this because the Ca-EGTA system is a very poor buffer of $[Ca^{2+}]$ at pH values below about 6.2.

Again to simulate *in vivo* conditions I used an incubation temperature of 37°C. Most of the previous studies on myofibrillar ATPase activity, including those on its pH-sensitivity (Mühlrad & Hegyi, 1965; Schädler, 1967; Portzehl et al., 1969; Williams et al., 1975), have been at 20°C or 25°C. My use of higher temperatures had the disadvantage that many association constants have been determined only at 20°C or 25°C. If these association constants had been very sensitive to temperature, my calculation of the pCa at 37°C would have been in error. Fortunately, Murphy & Hasselbach (1968) have found that the apparent binding constant of EGTA for Ca^{2+} is constant over the temperature range 0°C - 40°C, and so it is unlikely that there was an appreciable error in my estimation of the pCa in the incubation solutions. In any case, a small error in the calculated pCa would not have invalidated any conclusions made from comparison of the cardiac and skeletal myofibrillar ATPases, since I assayed these under identical conditions. Another potential disadvantage of a high incubation temperature is that it has been reported that the relative basal ATPase rises with increasing temperature, so that at 37°C, for example, 50% of the total ATPase activity of skeletal

myofibrils is Ca^{2+}-insensitive (Murphy & Hasselbach, 1968). However, the basal ATPase activities in my experiments at 37°C were about 20% of the total-ATPase activities - no greater than in previous experiments performed at 20°C or 25°C. Although an incubation temperature on 37°C may have had its disadvantages, these are minor and were, I believe, a small price to pay for the use of 'physiological' rather than 'biochemical' conditions.

Other conditions, such as the precise ionic composition of the incubation media (20 mM imidazole, 1 mM K_2EGTA, 0 - 1 mM added $CaCl_2$, 5 mM Na_2ATP, 6 mM $MgCl_2$, 5 mM NaN_3, 70 mM KCl) were based on those used by Hönig & Stam (1967). These were deemed to be suitable for the reasons given below.

20 mM imidazole (pK_a 6.8 at 37°C) provided a powerful H^+ buffer over the pH range studied. It was essential to keep the pH constant, in spite of the liberation of up to about 0.3 mM H^+ from ATP hydrolysis, because the $[Ca^{2+}]$ in a Ca-EGTA buffer system is highly sensitive to pH. (In fact, $[Ca^{2+}]$ varies with $[H^+]^2$, because two H^+ ions compete with one Ca^{2+} for binding to $EGTA^{4-}$). Both calculation and pilot experiments demonstrated that over the 5-minute incubation period the pH never dropped more than 0.02 pH units below the initial pH.

A Ca-EGTA buffer system was used to control $[Ca^{2+}]$. It does not buffer $[Ca^{2+}]$ well at pCa<5 or pH<6.2 but its use is necessary to provide controlled concentrations below the concentration of contaminant Ca^{2+} (approximately 10 μM i.e. pCa 5).

5 mM Na_2ATP plus 6 mM $MgCl_2$ formed the MgATP substrate for the myofibrillar ATPase. This combination resulted in a $[Mg^{2+}]$ of 1.4 mM and $[MgATP^{2+}]$ of 4.6 mM in the incubation solutions. At the time when I performed these experiments the true intracellular $[Mg^{2+}]$ was not accurately known, but was believed to be in the range 0.1 - 1 mM Polimeni & Page, 1973). In fact recent work with Mg^{2+}-selective microelectrodes has produced values for intracellular $[Mg^{2+}]$ of 3.1 mM and 3.3 mM in cardiac and skeletal cells, respectively (Hess & Weingart, 1981), so 1.4 mM Mg^{2+} is a little below the physiological concentration. The concentration of $MgATP^{2-}$ (4.6 mM) is probably close to the in vivo value, however: in isolated perfused rat hearts the total [ATP] is about 5 mmol /kg fresh weight (England & Randle, 1967; Opie et al., 1971), though some of this ATP may be compartmentalised or not bound to Mg. An $[MgATP^{2-}]$ of 4.6 mM is sufficient to saturate the myofibrillar ATPase (Figure 11) but is below the

concentration (about 7 mM) at which formation of ATP-molybdate complexes begins to interfere with the Fiske-SubbaRow assay for P_i (Blum & Chambers, 1955).

I found that Triton-treated myofibrils in three different preparations had no azide-sensitive ATPase activity (Figure 20). Nevertheless I retained 5 mM NaN_3 in the standard incubation media to ensure that mitochondrial ATPase was not active in any preparation and to keep the conditions similar to those in the pilot experiments.

Of all the intracellular conditions, the ionic strength is perhaps the least certain, though it has been estimated to lie within the range 100 mM - 200 mM (Sections 3.1 and 12.2). I chose the standard ionic strength at pH 7.00 to be 115 mM, which gave the greatest total and Ca^{2+}-regulated ATPase activities for the rabbit cardiac myofibrils, although 115 mM was a little below the optimum for the Ca^{2+}-regulated ATPase activity of the white skeletal myofibrils (Figure 22). Nevertheless an ionic strength of 115 mM is within the probable physiological range; this is not true for the ionic strengths used in most previous studies on myofibrillar ATPase activity which, with only a few exceptions (Hönig & Stam, 1967; Portzehl et al., 1969; Muir et al., 1971; Solaro et al., 1971), have used ionic strengths in the range 20 mM - 70 mM.

In summary, the experimental conditions, especially temperature and ionic strength, were much closer to in vivo intracellular conditions than was the case in previous studies on the influence of pH on myofibrillar ATPase activity (Mühlrad & Hegyi, 1965; Schädler, 1967; Portzehl et al., 1969; Williams et al., 1975).

7.2 Purity of myofibrils

(a) Cardiac myofibrils

In the pilot experiments for my work, I prepared cardiac myofibrils by the procedure of Chandler et al. (1967). This procedure was based on methods classically used to isolate skeletal myofibrils and so it did not include washes in a detergent. My comparative studies on the purity of the Triton-treated and untreated myofibrils confirm previous observations that cardiac myofibrils prepared without detergent treatment contain much non-myofibrillar ATPase activity (Fanburg, 1964; Solaro et al., 1971; Reddy, 1976). This contaminant ATPase was probably associated with the abundant residual membrane, some of it obviously from mitochondria, seen in the electron micrographs

(Figure 15). A mitochondrial origin for much of this contaminant ATPase activity is confirmed by the large inhibitory effect of 5 mM azide (Figure 20), a known inhibitor of mitochondrial ATPase activity. In previous contamination studies (Solaro et al., 1971; Reddy, 1976) it was shown that azide inhibits the total ATPase activity but it has not previously been demonstrated that this is due to an action of azide on the basal ATPase activity and not on the Ca^{2+}-sensitive fraction of the ATPase activity. Neither has a comparison been made previously between the basal activities of Triton-washed and unwashed myofibrils assayed in the presence of azide. My results (Table 4 and Figure 20) show that the basal activity of the untreated cardiac myofibrils in the presence of 5 mM azide was almost double that of the treated myofibrils. The source of this additional basal activity cannot be established conclusively from my experiments, but since it can be destroyed by treatment of the myofibrils with Triton X-100, it would seem to be associated with a membranous component of the untreated myofibrils. Probably the contaminant ATPase is due to a non-mitochondrial membrane ATPase. Alternatively there exists the possibility that 5 mM azide inhibits only 80% - 90% of mitochondrial ATPase activity, leaving the rest active in the cardiac preparations. Whatever the origin of this additional basal ATPase activity, my results demonstrate that the assumption implicit in all previous work on cardiac myofibrils prepared without detergent treatment - that 5 mM azide completely inhibits the contaminant ATPase activity - is not justified.

There appeared to be no ouabain-sensitive ($Na^+ + K^+$)-ATPase activity in the untreated cardiac myofibrils under my standard incubation conditions (Figure 20). At first sight this result conflicts with the demonstration of an ouabain-sensitive ATPase activity in similar myofibrils prepared by other workers (9% of total activity - Solaro et al., 1971; 14% of total activity - Reddy, 1976). However this discrepancy can be explained by the conditions of incubation in the different studies: Solaro et al. (1971) and Reddy (1976) used ionic conditions (100 mM NaCl, 30 mM KCl) that were chosen to maximise the ($Na^+ + K^+$)-ATPase activity, whereas I was concerned only with the activity of this enzyme in my standard incubation solutions, which contained 82 mM K^+ and 15 mM Na^+. These ionic conditions are probably in themselves inhibitory to the sarcolemmal ($Na^+ + K^+$)-ATPase.

Azide or quinidine alone substantially inhibited the basal and total ATPase activities of the untreated cardiac myofibrils, but together they produced an inhibition that was no greater than that exerted by azide alone. Quinidine is a potent inhibitor of SR Ca^{2+}-regulated ATPase activity (Fuchs et al., 1968), but most of the large reduction in basal ATPase activity with quinidine present was probably due to the known action of quinidine to inhibit mitochondrial ATPase activity (Solaro et al., 1971). The absence of an extra inhibition in the presence of azide plus quinidine, compared to azide alone, could be taken as evidence for a lack of SR ATPase activity in the preparation; this cannot be stated categorically, however, for there exists the possibility that any inhibition of an SR ATPase by quinidine could have been obscured by the slight stimulation which quinidine appeared to exert on the basal activity of the myofibrils themselves. More work would be needed to establish whether this apparently direct effect of quinidine on myofibrillar function was real or was merely an artifact. However, it would seem likely that the SR ATPase activity was removed from myofibrillar preparations by Triton X-100, since this detergent abolishes all ATPase activity in SR-rich microsomal fractions (Weber, 1971).

After the cardiac myofibrils had been washed in Triton X-100, neither the basal nor the total ATPase activity was inhibited by azide, quinidine or ouabain, even though electron microscopy showed that not all the extraneous membrane had been removed from the myofibrils. It therefore seems unlikely that any mitochondrial ATPase, SR Ca^{2+}-ATPase or sarcolemmal (Na^{+} + K^{+})-ATPase were active in these treated myofibrils under the standard incubation conditions I employed. This conclusion is supported by the fact that Triton treatment of the cardiac myofibrils reduced the basal ATPase activity to levels associated with the skeletal myofibrils ($\approx$ 20% of total ATPase activity, Table 4 and Figure 20) or with pure cardiac actomyosin (22% of total activity, Fanburg, 1964). In previous reports the basal activity in Triton-treated cardiac myofibrils has ranged from 11% of total activity (Ray & England, 1976) to 24% (Reddy, 1976). The agreement between my results and those from these studies is to some extent fortuitous, since my use of a higher ionic strength than in these studies would act to decrease the relative activity of the basal ATPase (Figure 22) but the higher temperature – 37°C as opposed to 20°C or 25°C - would tend to increase the relative

basal ATPase activity (Murphy & Hasselbach, 1968).

(b) Skeletal myofibrils. The basal and total ATPase activities of the skeletal myofibrils were not inhibited by ouabain, azide or quinidine whether or not the myofibrils had been washed in Triton X-100 (Figure 21). The contribution of membrane ATPases to the ATPase activities of these preparations was therefore judged to be insignificant. Nevertheless, electron microscopy of the untreated skeletal myofibrils showed them to possess residual membrane, as tubules adhering to the Z-line (Figure 17). These were not T-tubules, which occur at the level of the junction between A and I bands in mammalian skeletal muscle. Probably the tubules were remnants of SR. The connections between these tubules and the myofibrils must have been strong to survive the homogenisation procedure. A close association of such tubules, 'Z tubules', with the Z-line has been reported in cardiac muscle (Simpson & Rayns, 1968) but no similar linkages have been observed previously in white skeletal muscle. It is possible that the 'Z-cables' attached to the Z-lines in rat diaphragm muscle (Pierobon-Bormioli, 1981) are another manifestation of the same structure.

These biochemical and ultrastructural studies established the purity of the Triton-treated cardiac and skeletal myofibril preparations, an essential step if one is to ascribe the Ca^{2+}-sensitivity and pH-sensitivity of the ATPase activity to properties of the myofibrillar enzyme alone.

7.3 The influence of [salt] on the myofibrillar ATPase activities

The ATPase activities of both cardiac and skeletal myofibrils were found to change as the ionic strength was varied with KCl, but in general these influences of [salt] were rather small over the ionic strength range studied ($\Gamma/2$ = 65 mM - 205 mM). The maximum total ATPase activity of the skeletal myofibrils (at pCa 4.5) exhibited a broad plateau over most of this range, and only fell appreciably at $\Gamma/2$>160 mM (Figure 22). In other studies with skeletal myofibrils at 20°C or 25°C the [salt]-dependence has been more noticeable, with a peak of total ATPase activity at about 100 mM - 120 mM (Portzehl et al., 1969; Muir et al., 1971; Goodno et al., 1978). In the case of the rabbit cardiac myofibrils, the total ATPase activity at pCa 4.5 attained a peak at $\Gamma/2$ = 115 mM (Figure 22). In apparently the only other

study using cardiac myofibrils, Muir et al. (1971) reported that the total ATPase activity decreased linearly over the ionic strength range 60 mM - 100 mM; over the same range I observed an increase in ATPase activity. It is possible that some of the ATPase activity observed by Muir et al. (1971) was non-myofibrillar in origin, because their myofibrils were not washed in detergent.

My results are in some respects similar to those obtained previously with preparations of actomyosin. An increase in [KCl] has been found to reduce the ATPase activity of skeletal actomyosins (Hasselbach, 1952; Leadbeater & Perry, 1963; Muir et al., 1971) and cardiac actomyosins (Fanburg, 1964; Katz et al., 1966). However the influences of [salt] on myofibrils and actomyosin are not exactly the same, for at an ionic strength of 200 mM, for example, the ATPase activity of actomyosin is little more than that of myosin alone, whereas the isolated myofibrils at the same ionic strength exhibit considerable acti n-activation of myosin in the presence of Ca^{2+} (as do skinned muscle fibres - Chapter 10). This again illustrates that the contractile proteins in less organised preparations, such as natural or reconstituted actomyosin, may not have the same properties as when the contractile proteins are in their natural arays in the myofibrils.

In most of the previous studies mentioned above, only the total ATPase activity was measured i.e. there was no attempt to separate this into Ca^{2+}-regulated and Ca^{2+}-insensitive (basal) ATPase activities. In the two studies in which this separation was carried out it was reported that the basal ATPase activity of skeletal myofibrils fell monotonically as the [KCl] was raised (Portzehl et al., 1969; Goodno et al., 1978). I confirm this, and have found in addition that the same effect occurs with cardiac myofibrils (Figure 22). As discussed in Section 3.2, there is good evidence that, at least with skeletal myofibrils, the raised Ca^{2+}-insensitive ATPase activity at low ionic strengths is due to an increase in actin-myosin interaction that is not subject to the usual inhibition by Tn-I in the absence of Ca^{2+}.

As a result of my measurement of the basal ATPase activity, I was able to determine the influence of [salt] on the Ca^{2+}-regulated ATPase activity alone. This part of the ATPase activity reached a peak at a higher ionic strength than that for the total ATPase activity because the Ca^{2+}-insensitive activity fell with increasing $\Gamma/2$ (Figure 22). The peak Ca^{2+}-regulated ATPase activity for the

skeletal myofibrils occurred at $\Gamma/2$ = 135 mM - 165 mM, which was a little higher than that observed by Portzehl et al. (1969) and Goodno et al. (1978), who found the peak at $\Gamma/2 \approx$ 120 mM. In my experiments the Ca^{2+}-regulated ATPase of the rabbit cardiac myofibrils displayed a broad optimum over the ionic strength range 115 - 165 mM. Thus in the case of the Ca^{2+}-regulated ATPase activity, the cardiac myofibrils were slightly less sensitive than the skeletal myofibrils to a change in [KCl].

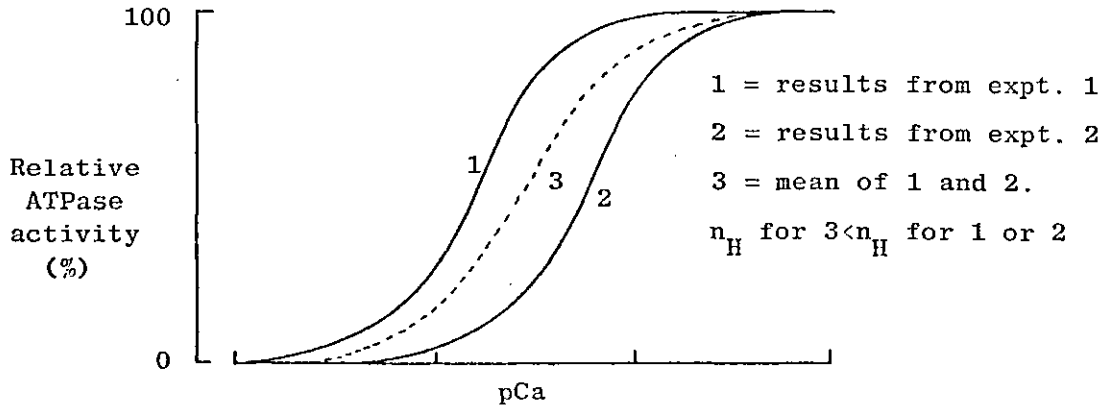
My finding that, as the ionic strength was increased from 65 mM to 205 mM, the Ca^{2+}-regulated ATPase activity of cardiac myofibrils rose to a maximum and then decreased again can provide a ready explanation for the positive inotropic effect of mild hypertonicity and the negative inotropic effect of severe hypertonicity on intact myocardial cells (Section 3.4), provided that the normal intracellular ionic strength in cardiac muscle is considerably less than the optimum (115 mM) for the Ca^{2+}-regulated myofibrillar ATPase. However, it is unlikely that the intracellular ionic strength is much less than 115 mM. It is probably more (discussed more fully in Section 12.2), in which case my results can explain only the negative inotropic effect of severe hypertonicity: they do not account for the positive inotropic effect of mild hypertonicity. The detrimental effects of a high ionic strength on the Ca^{2+}-regulated ATPase activity of the skeletal myofibrils provides an obvious explanation for the effects of hypertonicity on skeletal muscle, provided that the intracellular ionic strength is normally greater than about 165 mM. There is however the problem that the [salt]-dependence of the Ca^{2+}-regulated ATPase activity of the skeletal myofibrils exhibits a peak in the ionic strength range 120 - 165 mM (Figure 22 ; Portzehl et al.,(1969); Goodno et al., 1978), whereas the Ca^{2+}-regulated force production in skinned skeletal fibres decreases almost linearly as the ionic strength is raised over the same range (Section 3.2). Thus there seem to be differential effects of [salt] on ATPase activity and force production. These considerations mean that, before the cardiac actions of hypertonicity can be ascribed unequivocally to the direct effects of a raised [salt] on the activity of cardiac myofibrils, it is necessary to establish the influence of [salt] on the force production of the myofibrils. My studies on the [salt]-dependence of force production in skinned cardiac muscle are described in Part III (Section 10.11).

Possible mechanisms by which [salt] influences the ATPase activity and force production of myofibrils are also discussed later (Section 11.10).

7.4 The relationships between pCa and the ATPase activities of cardiac and skeletal myofibrils at pH 7.00

As has been demonstrated many times previously (see Section 1.3(a)), the myofibrillar ATPase activity increases sigmoidally as the pCa is reduced from pCa 7.0 to about pCa 4.5 at pH 7.00 (Figures 23, 24 and 25). The curve was steeper for rabbit skeletal myofibrils than for rabbit cardiac myofibrils and this was reflected in the values for n_H, which for skeletal myofibrils was 2.50 compared with only 1.38 for the cardiac preparation. This difference has also been found by Kerrick et al. (1980), who reported n_H values of 2.35 and 1.72 for rabbit skeletal myofibrils and rabbit cardiac myofibrils, respectively. Values for n_H are usually taken to indicate the minimum number of interacting sites involved in regulation; this would imply there are two functionally-important sites for cardiac myofibrils and three for skeletal myofibrils. It is tempting to speculate that this difference is related to the fact that cardiac troponin has one less Ca^{2+}-binding site than skeletal troponin (Section 1.3(c)). It has been suggested that cardiac myofibrils are regulated by the single Ca-specific site on cardiac troponin, whilst myofibrils are regulated by the two Ca-specific sites on skeletal troponin (Johnson et al., 1979; Holroyde et al., 1980). If this is so, the steepness of the pCa-ATPase relationships, which suggests a minimum of two interacting sites in cardiac myofibrils and three in skeletal myofibrils, rule out the possibility that the co-operativity observed in the Ca^{2+}-activation curves is due solely to an interaction between the functionally-important Ca^{2+}-binding sites on troponin. We must postulate additional or alternative mechanisms. For example, Brandt et al. (1980) have suggested that the positive cooperativity in the force-pCa relationship of skinned skeletal muscle could be explained if the inherent Ca^{2+}-sensitivity increased as isometric force production increased. However, this explanation probably cannot account for the co-operativity in the Ca^{2+}-activation curves of isolated myofibrils, which may shorten almost isotonically during activity. Another possible way in which co-operativity could arise is if the 'switching on' of the tropomyosin molecule in one groove of the actin helix increased the ease with which the tropomyosin molecule in the other groove 'switched on'.

It should be noted that each computed n_H value in Table 5 may well be an underestimate of the true steepness of the ATPase-pCa relationships since any between-experiment variability in the Ca^{2+}-sensitivity of the preparations will cause the mean Ca^{2+}-activation curve to be flatter than any of the individual Ca^{2+}-activation curves (Moisescu & Thieleczek, 1979). This point is illustrated below.



Thus it is preferable to determine the values of n_H and K* for each experiment and then to calculate the mean n_H and K*, rather than to estimate n_H and K* from the curves fitted to the mean data. Unfortunately I could not do this because the individual Ca^{2+}-activation curves in each experiment were not smooth enough to enable the computer to provide a close fit according to the Hill equation.

Although co-operativity appears to be greater in the activation of skeletal myofibrils than in the activation of cardiac myofibrils, the finding that the pCa for half-maximal activation is the same in both cases (pCa 6.0) could be taken as evidence that Ca^{2+}-regulation involves the same type of Ca^{2+}-binding site in the two systems. This is likely to be the Ca^{2+}-specific site or sites on troponin (Section 1.3(c)).

The optimal pCa was 4.5 - 5.0 for the cardiac myofibrils and about 5.3 for the skeletal myofibrils. At lower pCa values the myofibrillar ATPase activities were slightly inhibited, as has been observed previously with skeletal myofibrils (Portzehl et al., 1969). Although it is conceivable that this was due to a direct action of a supra-optimal [Ca^{2+}] on the myofibrils, for example arising from Ca^{2+}-binding to the myosin P light chains, it is more likely to have been

due to disturbances in the free concentrations of ions in the incubation media. At pCa<5, EGTA is saturated with Ca^{2+} and some of the excess Ca^{2+} ion bind to ATP. Probably the depression of ATPase activity results from an increased concentration of CaATP which, unlike MgATP, is a poor substrate for the actomyosin ATPase (Bendall, 1969).

It is difficult to compare values for the maximum absolute ATPase activities with earlier work because most previous studies have been performed at lower ionic strengths, or at lower temperatures, or both. One exception is that by Chandler et al. (1967), who reported the maximum total ATPase activity of their cat cardiac myofibrils to be 180 nmol $Pi.min^{-1}.mg\ protein^{-1}$ at 37°C and $\Gamma/2$ = 150 mM. In general the other investigations at 25°C and at an ionic strength of about 100 mM, such as those by Fanburg et al. (1964) and Solaro & Shiner (1976), have given cardiac myofibrillar ATPase activities of 30 - 100 nmol $Pi.min^{-1}.mg\ protein^{-1}$ and skeletal myofibrillar ATPase activities of 200 - 350 nmol $Pi.min^{-1}.mg\ protein^{-1}$. (The species was usually dog or rabbit). Given that myofibrillar ATPase activity has a Q_{10} of 2.9 - 3.0 (Bárány, 1967), these figures correspond to cardiac ATPase activities at 37°C of 90 - 300 nmol $Pi.min^{-1}.mg\ protein^{-1}$ and skeletal activities of 600 - 1050 nmol $Pi.min^{-1}.mg\ protein^{-1}$. The ATPase activity of my cardiac myofibrils - about 200 nmol $Pi.min^{-1}.mg\ protein^{-1}$ - was within this range but the skeletal activity - about 400 nmol $Pi.min^{-1}.mg\ protein^{-1}$ - was lower than in these other studies for a reason that I do not know.

The absolute myofibrillar ATPase activity varied from experiment to experiment, particularly with the skeletal myofibrils. This was probably due to a variable degree of inactivation or washout of regulatory proteins during myofibrillar extraction or to the unavoidable inclusion of variable numbers of slow skeletal fibres with the desired fast skeletal fibres.

7.5 Influence of pH on the relationship between pCa and ATPase activity

One of the objectives of these studies was to determine whether the Ca^{2+}-activation of cardiac myofibrillar ATPase activity was genuinely influenced by pH. This question had been addressed previously by Schädler (1967) and Williams et al. (1975), but in neither study were steps taken to check for, or remove, contaminant membrane ATPases from the myofibrillar preparations. As discussed in Section 7.2, cardiac myofibrils extracted without washes in detergent

contain considerable quantities of non-myofibrillar ATPase activity. It is particularly important to remove any SR ATPase activity, because this is activated over the same range of Ca^{2+}-concentrations as the myofibrillar ATPase, and is sensitive to changes in pH (Chapter 2). Although the myofibrillar preparations used by Schädler (1967) were glycerinated cardiac muscles, glycerination does not inactivate the SR ATPase, for the SR in glycerinated papillary myscles can sequester and release Ca^{2+} (Brenner, 1979). It is clear that the preparations of Schädler (1967) and Williams et al. (1975) contained considerable contaminant ATPase activity because, even when assayed in the presence of 5 mM azide, the preparations used in these studies had basal activities that comprised 30 - 50% of the total activity, as I too found with the cardiac myofibrils that had not been washed in Triton X-100 (Table 4 and Figure 20).

Used purified cardiac myofibrils, I have shown that the basal ATPase activity is unaffected by changes in pH over the range pH 7.2 to 6.4 (Figures 23 and 25). This contradicts the report by Williams et al. (1975) that the basal activity of canine cardiac myofibrils shows a bell-shaped pH-activity profile, with the maximum at pH 8.0 being 180% of the activity at pH 7.0. In their experiments, basal activity fell by 66% from pH 7.2 to 6.4. One possibility is that this marked pH-sensitivity was a property of the azide-insensitive contaminant ATPase(s) making up part of the basal ATPase activity in their preparation. In the other previous study Schädler (1967) did not state whether or not the basal activities in his preparations were pH-sensitive. The basal ATPase activity of my skeletal myofibrils was only slightly increased by a rise in pH from 6.4 to 7.2. This confirms a previous report (Portzehl et al., 1969). Although the source of the basal ATPase activity is obscure, if indeed it is largely due to the activity of the myosin ATPase (Chapter 4) then the general pH-insensitivity of the basal ATPase activity is consistent with the observed broad minimum of myosin ATPase activity in the range pH 6 - pH 8 (Bárány et al., 1963).

I found that a reduction in pH from 7.2 to 6.4 lowered the maximum Ca^{2+}-regulated ATPase of cardiac myofibrils by a small amount. This result is similar in direction to that obtained by Schädler, although he observed a larger effect, and is thus opposite to that found by Williams et al. (1975). The latter workers reported the

total ATPase activity to be unaffected by pH while the basal activity decreased as pH fell; the net result was that Ca^{2+}-activated ATPase activity doubled from pH 7.6 to 6.5. In my experiments the slight inhibition of cardiac ATPase activity by an increase in $[H^+]$ at optimal Ca^{2+} concentrations could not be overcome by further increases in $[Ca^{2+}]$, and it is therefore unlikely that it was due to competitive interaction between H^+ and Ca^{2+} for a functionally-important binding site. It might have been caused by a direct antagonism by H^+ at the active site of the enzyme i.e. an inhibition of the ATPase activity itself, rather than of its regulation by Ca^{2+}. Since the ATPase reaction liberates protons, one might expect the overall reaction to be slowed in acidic conditions.

The maximum skeletal ATPase activity was not significantly influenced by pH, though the variability in the data was sufficient to have obscured any small genuine changes.

I confirm the reports by Schädler (1967) and Williams et al. (1975) that the Ca^{2+}-sensitivity of cardiac myofibrillar ATPase is reduced dramatically by a decrease in pH. My results indicate that the Ca^{2+}-sensitivity of skeletal myofibrils is also decreased. This is in disagreement with the early study by Mühlrad & Hegyi (1965) but confirms the later one by Portzehl et al. (1969). Not only was the Ca^{2+}-activation of cardiac and skeletal myofibrillar ATPase influenced in the same direction by pH but also both cardiac and white skeletal myofibrils had similar pH-sensitivities (Figures 23 to 25). The pCa for half-maximal activation (pCa_{50}) was reduced by almost 0.2 pCa units for each 0.2 unit drop in pH; for each unit fall in pH, the $[Ca^{2+}]$ required for half-maximal activation increased by a factor of 8.5 in the case of the rabbit cardiac myofibrils and 7.2 for the rabbit skeletal myofibrils, i.e. the skeletal ATPase was slightly less sensitive to pH. These results therefore differ in some respects from those obtained in the only previous study comparing the pH-sensitivities of cardiac and skeletal myofibrils (Schädler, 1967) in which it was reported that the skeletal ATPase was seven times more sensitive to pH than was the cardiac enzyme. My study has been subsequently confirmed by investigations of the pH-sensitivity of force generation in skinned cardiac and skeletal muscle; these reports will be discussed in detail in Part III of this thesis (Section 11.9).

Where then is the site of H^+/Ca^{2+} competition? As suggested by Katz & Hecht (1969), an obvious locus is the Ca^{2+}-binding sites

on troponin, since these are the only Ca^{2+}-binding sites known with certainty to be involved in the regulation of myofibrillar ATPase activity. The fact that a 0.2 pH unit change caused a parallel shift in the pCa-ATPase relationship along the pCa axis by about 0.2 pCa units is consistent with a simple competition between H^+ and Ca^{2+} on a 1:1 basis for a Ca^{2+}-binding site involved in the regulation of myofibrillar ATPase activity. This attractive idea is however not supported by binding studies, which have shown that neither the Ca^{2+}-affinity nor the capacity of isolated skeletal troponin (Fuchs, 1974) or cardiac troponin (Stull & Buss, 1978) varies with pH. Possibly when troponin is dissociated from the thin filament it undergoes a conformational change such that it loses a pH-sensitivity of Ca^{2+}-binding that it possessed in situ. Alternatively, the Ca^{2+}-affinity of troponin in the myofibril may indeed be unaffected by pH, the pH-sensitivity of the Ca^{2+}-regulated myofibrillar ATPase being due instead to a later step in the coupling between Ca^{2+}-binding and ATP hydrolysis. The second alternative is favoured by Fuchs' demonstration (1979) that Ca^{2+}-binding to glycerinated skeletal muscle fibres, and by implication Ca^{2+}-binding to troponin in situ, was not affected by pH changes over the range pH 6.2 - 7.4. However, contrary to these previous results, a preliminary report by Robertson et al. (1978) has claimed that Ca^{2+}-binding to the Ca-specific sites of skeletal troponin-C did show a pH-dependence that fits with the pH-dependence of myofibrillar activation, although Ca^{2+}-binding to the Ca-Mg sites was insensitive to pH. However the results of Fuchs (1974, 1979) and Robertson et al. (1978) are not necessarily contradictory, for Fuchs reported that isolated skeletal troponin bound only two moles Ca^{2+}/mole troponin over the range pH 4 - pH 8. Thus it seems likely that he was measuring the binding of Ca^{2+} to only one class of Ca^{2+}-binding site, possibly the Ca-Mg sites, which Robertson et al. (1978) found were not affected by pH.

Godt (1981) has suggested a rather different explanation for the pH-sensitivity of the Ca^{2+}-regulation of myofibrils. He pointed out that changes in $[H^+]$ would alter the degree of dissociation of ionisable groups on the surface of the thin filaments and thereby change the electrostatic potential surrounding the negatively-charged thin filaments. This would in turn change the concentration of Ca^{2+} in the immediate vicinity of the troponin molecules. For example, an increase in $[H^+]$ would decrease the negative surface charge and thus

the local [Ca^{2+}] would fall, with the result that a higher [Ca^{2+}] would be needed in the bulk solution to produce the same level of Ca^{2+}-activation of the myofibrils. By this means, an increase in [H^+] would decrease the Ca^{2+}-sensitivity. This is an attractive theory because it can explain how there can be a pH-dependence of Ca^{2+}-activation without a pH-dependence of the binding of Ca^{2+} to isolated troponin : isolated troponin would not be under the electrostatic influence of the thin filaments. This theory is plausible, but it does predict that an increase in ionic strength should reduce the Ca^{2+}-sensitivity because of an increased shielding of surface charges and the consequent decrease in surface potential. Evidence will be presented in Part III that an increase in ionic strength does not in fact alter the Ca^{2+}-sensitivity in skinned cardiac muscle (Section 10.11).

We cannot rule out the possibility that the P light chains on myosin are the sites of H^+/Ca^{2+} antagonism, though it is hard to see how these light chains could confer pH-sensitivity on the Ca^{2+}-regulation of myofibrils, since they are probably not involved directly in the Ca^{2+}-activation process (Section 1.3(d)).

Whatever the mechanism by which a decrease in pH reduces the pCa_{50}, my demonstration that the pH-sensitivities of pCa_{50} were similar, both in direction and magnitude, in rabbit cardiac and rabbit-skeletal myofibrils (Figure 26) suggests that the mechanism is probably the same in the two types of myofibril. However, the finding that the steepness of the skeletal Ca^{2+}-activation curve fell with pH, whereas the steepness of the curve for rabbit cardiac myofibrils was insensitive to pH (Table 5), may mean that protons have additional effects on skeletal myofibrils.

The similarity in the pH-sensitivities of cardiac and skeletal myofibrillar ATPase activity would appear to rule out the possibility that the opposite responses of cardiac and skeletal muscle to an intracellular acidosis resulted from divergent actions of H^+ on the two types of myofibril (this is discussed in more detail in Section 12.1). However it could be argued that ATPase activity is a rather indirect measure of myofibrillar contractility. These experiments on myofibrillar ATPase activity could not rule out the possibility that the mechanism coupling ATP hydrolysis to tension development had a pH-sensitivity that differed in the two types of muscle. To answer

this point I decided to investigate the influence of pH on the force development in 'skinned' cardiac and skeletal muscle. Satisfactory preparations of skinned skeletal muscle were available at this time but to study cardiac muscle I needed to develop a simple procedure to adequately 'skin' cardiac muscle. In Part III of this thesis I shall describe the preparation and characterisation of a detergent-skinned cardiac muscle preparation, and show how its force development is influenced by pH. The results are discussed in relation to my work on isolated myofibrils and to the results from two studies (Fabiato & Fabiato, 1978b; Donaldson & Hermansen, 1978) that compared the pH-sensitivity of force production in mechanically-skinned cardiac and skeletal muscle. In addition, I shall describe the influence of ionic strength and of other monovalent cations, besides H^+, on the force development in the detergent-skinned cardiac muscles.

PART III

THE FORCE DEVELOPMENT OF DETERGENT-SKINNED CARDIAC MUSCLE.

Chapter 8

INTRODUCTION TO THE SKINNED MUSCLE PREPARATION

Our understanding of the later stages in excitation-contraction coupling has been aided by the development and use of 'skinned' muscle preparations. These are muscles or muscle fibres in which the sarcolemma has been removed by dissection ('mechanical skinning') or has been disrupted by chemical means ('chemical skinning'). Once the selective permeability barrier that is the sarcolemma has been removed, the ionic composition of the myofibrillar environment should approximate to that of the solution bathing the muscle. The ionic environment of the myofibrils is thus under direct experimental control, unlike the situation in living muscle cells, where the sarcolemma is the primary controller of the composition of the cytoplasm. Skinned muscles and muscle fibres are much larger than isolated myofibrils (10 - 200 μm in diameter compared with 1 - 1.5 μm) and it is possible to attach a force transducer to them and thus study their force production in relation to the concentration of Ca^{2+} and other ions in the bathing solution.

Several features of skinned muscle preparations make them very useful models for studying muscle contraction. The contractile proteins are in their natural arrays in the myofibrils and it is possible to record the myofibrillar force production or ATPase activity, or both. Force production is probably the more meaningful of the two measurements because (i) it relates directly to the force production in living, unskinned muscle, and (ii) any observed Ca^{2+}-regulated force must have been developed solely by the myofibrils, whereas the measurement of ATPase activity may suffer from the problem of contamination by other cellular ATPases (as in preparations of isolated myofibrils). One great advantage of skinned muscle preparations is that, because both ends of the muscle can be held, muscle length or better still, sarcomere length, can be held constant or set to required values. This is desirable because maximum force production, maximum ATPase activity, and their Ca^{2+}-sensitivities are dependent upon the sarcomere length (Section 1.5). Isolated myofibrils and less organised preparations of myofibrillar proteins can shorten to an unknown extent during activation, with unpredictable consequences.

Unfortunately, skinned muscle preparations are not without their drawbacks. By far the greatest disadvantage is concerned with diffusional restrictions to the movement of molecules between the

muscle and the bathing solution. Even if the sarcolemma is freely permeable to all the constituents of the solution bathing the muscle (and this may not always be so), a diffusion barrier to movement will effectively exist for molecules that are bound or metabolised by the muscle. For example, Ca^{2+} supplied by the bathing solution could be bound by the SR or by the myofibrils themselves, and MgATP from the bathing solution may be hydrolysed by the myofibrillar ATPase. In this case, Ca^{2+} and MgATP molecules diffusing into the muscle may never reach the core of the muscle. Conversely, molecules formed or released inside the muscle (e.g. MgADP, Pi and H^{+} produced by MgATP hydrolysis) will be present in higher concentrations in the muscle core than in the outer regions of the muscle or in the bathing solution. Thus the ionic milieu of the myofibrils may vary throughout the cross-section of the muscle and could differ in some respects from the ionic composition of the bathing solution. This problem is negligible in isolated myofibrils because of the small diffusion distances involved (myofibrils are only 1 - 1.5 μm in diameter) but it takes on great significance in skinned muscles, which may be as large as 200 μm in diameter. The magnitude of the ionic inhomogeneity in skinned muscles can be minimised by (i) using the thinnest possible preparations and (ii) using powerful buffer systems to maintain the concentrations of H^{+}, Ca^{2+}, $MgATP^{2-}$, etc. inside the muscle.

Another problem is that the disruption of the sarcolemma results in the loss of most of the normal constituents of cytoplasm (soluble enzymes, metabolic intermediates, binding proteins, etc.). Such substances are not usually included in the experimental solutions bathing the skinned muscle, partly because we do not know the true composition of the cytosol and partly because even if the composition were known, it would be too difficult for technical reasons to reproduce it exactly. Thus the environment of the myofibrils in skinned muscles represents only a very simplified version of that in intact muscles. In addition, the loss of those cytosolic proteins that are normally excluded from the myofilament lattice causes the lattice, and thus the whole muscle fibre, to swell by 25% - 100% and to lose constant-volume behaviour (Matsubara & Elliott, 1972; April et al., 1972; Godt & Maughan, 1977). For these reasons one must take care when extrapolating results from skinned muscle experiments to explain the properties of intact muscles.

There are various methods of skinning muscles or muscle fibres. The technique of mechanical skinning, introduced by Natori (1954), has been widely applied to single skeletal fibres. In this technique the sarcolemma is dissected away from a length of a single fibre using fine needles or forceps. The sarcolemma-free portion is then attached to a force transducer. Although some superficial myofibrils come away with the sarcolemma during dissection, the internal structure of the fibre appears unaffected (Costantin et al., 1965). Thus mechanically-skinned fibres can be used to study the properties of both the myofibrils and the sarcoplasmic reticulum. (The Ca^{2+}-binding and release properties of the sarcoplasmic reticulum are determined indirectly, by recording the transient increase in muscle force after the sarcoplasmic reticulum has been made to release its Ca^{2+}). It should however be borne in mind that the T-tubules probably remain in these preparations and may contribute in an unknown way to the Ca^{2+} uptake and release phenomena which some investigators have attributed entirely to the activity of the sarcoplasmic reticulum.

Mechanical skinning can be satisfactorily performed only with single muscle cells. The small size of cardiac muscle cells ($\approx$10 μm diameter) compared with skeletal cells ($\approx$100 μm diameter) had seemed to preclude the extension of this technique to cardiac cells. However Fabiato & Fabiato (1975, 1977, 1978a, b) have pioneered a micro-dissection procedure to mechanically remove the sarcolemma from single cardiac cells, which they obtain by brief homogenisation of ventricular tissue.

Unfortunately the apparatus required for preparing and studying single mechanically-skinned cardiac fibres is complex and expensive. The technique of chemical skinning offers a simpler and cheaper approach because whole natural bundles of cells can be skinned by this procedure. Bundles of cells are larger, are more robust and develop a greater total force than do single cells. Accordingly the recording equipment can be less sensitive and the dissection techniques less skilful than if single cells were used. During chemical skinning the sarcolemma of the cells is rarely removed completely, but it is disrupted to an extent which depends on the chemical agent used. A disadvantage of chemical skinning is that agents that disrupt the sarcolemma will probably alter the characteristics of the other cellular membranes. Thus the normal function of, for example, the sarcoplasmic reticulum cannot be satisfactorily studied in chemically-

skinned muscle.

At the time when I started these studies (1977) there were two main classes of substance that had been used to disrupt the sarcolemma of muscle cells:

(i) glycerol. Glycerol-extraction of muscle is an example of chemical skinning which has been in use for many years since it was developed by Szent-Györgyi (1949). However the contractile properties of the myofibrils may alter during the period of storage in glycerol (Abbott, 1973). This undesirable change is probably avoided in a modified technique suitable for single skeletal fibres, which are given brief glycerination (30 minutes) followed by a 30 - 60 minute soak in a non-ionic detergent, Lubrol-WX (Julian, 1971).

(ii) Ca^{2+}-chelators. In 1971 Winegrad demonstrated that the sarcolemma of frog ventricular strips could apparently be made 'hyperpermeable' to Ca^{2+} ions by soaking the muscle pieces for 30 minutes in a 'disruption solution' containing the Ca^{2+}-chelators EDTA or EGTA. The rapidity of this process allowed this investigator to record, in the same experiment, the electrically-stimulated contractions of the intact cardiac muscle and the Ca^{2+}-regulated contractures of the subsequent 'hyperpermeable' muscle (Winegrad, 1971). This illustrates another advantage of chemical skinning over mechanical skinning, namely that the unskinned preparation can be attached to a force transducer and the forces in the muscle can be recorded before, during and after the skinning procedure, provided that the process of skinning is sufficiently rapid. In this way a check can be made on the viability of the muscle before skinning is started and the force responses of intact and 'skinned' muscle can be compared. Unfortunately Winegrad's original procedure did not work well with bundles of mammalian cardiac muscle; to make such tissue sufficiently hyperpermeable it was necessary to bathe the muscle overnight in an EGTA-containing 'disruption solution' at 4°C (McClellan & Winegrad, 1977). Although the EDTA-treated frog cardiac muscle and EGTA-treated mammalian (rat) cardiac muscle contract in micromolar concentrations of Ca^{2+} and have very small resting membrane potentials, the interpretation that these muscles are 'functionally skinned' (McClellan & Winegrad, 1977) has recently been questioned by Miller (1979). He pointed out that a high Ca^{2+}-sensitivity and a low resting membrane potential are characteristics also displayed by unskinned frog cardiac muscle in high K^+/low Na^+

physiological salines. Other evidence that EGTA-treated rat trabeculae are not truly 'functionally skinned' comes from the low rate of diffusion of Ca^{2+} and/or CaEGTA into these muscles (Jewell & Kentish, 1982). These considerations demonstrate that Ca^{2+}-dependence of force is not an adequate criterion by which to assess the permeability of the sarcolemma in chemically skinned muscle preparations.

During the preliminary stages of my investigations I attempted to skin cardiac trabeculae from guinea-pigs by superfusing the muscles with a 'disruption solution' containing KCl, ATP, a pH buffer (imidazole) and 10 mM EDTA or 10 mM EGTA, as in Winegrad's procedure (1971). However after 30 minutes' exposure to either of these solutions the trabeculae did not generate force when exposed to Ca^{2+} in the micromolar range. Because Triton X-100 had proved effective in removing the membrane from isolated cardiac myofibrils (Section 6.2) I thought it likely that this detergent would disrupt the sarcolemma in strips of cardiac muscle. This proved to be so: the inclusion of 1% Triton X-100 in my 'disruption solution' produced a solution which rapidly made cardiac trabeculae sensitive, in terms of force production, to micromolar concentrations of Ca^{2+}. Since these preliminary experiments I have modified the composition of the 'disruption solution' (now termed 'skinning solution') to improve the viability of the muscles but the solution still contains 1% Triton X-100. My skinning procedure therefore differs fundamentally from previous methods to chemically skin cardiac muscle in that it employs a detergent to render the cardiac sarcolemma permeable to externally-applied molecules. It was therefore necessary to adequately characterise the Triton-treated preparation.

Chapter 9

METHODS

9.1 An outline of the procedure

The basic protocol was similar to that of Winegrad (1971). A strip of cardiac muscle was mounted in a muscle bath, was superfused with a modified Krebs-Henseleit solution and was stimulated electrically. The muscle was allowed to equilibrate until resting and developed forces were stable. The sarcolemma of the cells was then chemically disrupted by a brief superfusion of the muscle with a 'skinning solution'. The muscle could then be made to contract and relax repeatedly by alternately exposing it to ATP-containing solutions of pCa 7.0 - 3.8 ('activating solutions') and pCa>8 ('relaxing solutions'). I investigated the influence of bathing solution composition, and in particular, the influences of pH, pCa and [salt] on the force developed in these ATP-dependent Ca^{2+}-regulated contractures.

The skinning procedure was first reported in a communication to the Physiological Society (Kentish & Nayler, 1978).

9.2 Solutions

(a) Experimental solutions

'Modified Krebs-Henseleit solution' contained (in mM): NaCl, 118; KCl, 5.0; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 25; glucose, 5.6; Na pyruvate, 3.0; $CaCl_2$, 1.8. This solution was of standard composition (Krebs & Henseleit, 1932) except for the inclusion of Na pyruvate, which provided an additional source of substrate for the tricarboxylic acid cycle. Before and during use modified Krebs-Henseleit solution was bubbled with a gas mixture of 95% O_2 + 5% CO_2, giving a pH of 7.3 - 7.4 at room temperature (20°C - 25°C).

Table 6 lists the standard compositions of the other bathing solutions: 'skinning solution', 'high-EGTA relaxing solution', 'low-EGTA relaxing solution' and 'activating solutions'. Their compositions were kept as close as possible to the compositions of the incubation media in the myofibrillar ATPase experiments, provided that this did not jeopardise the viability of the skinned muscles. (The compositions of the solutions are discussed in Chapter 11). The ionic compositions of all three types of solution were (in mM): $[K^+]$, 130; $[Na^+]$, 30; $[Cl^-]$, 124; $[Mg^{2+}]$, 1.3 - 1.5; $[MgATP^{2-}]$, 4.1 - 4.2.

	'Skinning solution'	'High EGTA relaxing solution'	'Low EGTA relaxing solution'	'Activating solutions'
KCl	100 mM	100 mM	100 mM	100 mM
imidazole	20 mM	20 mM	20 mM	20 mM
K_2Na_2ATP	5 mM	5 mM	5 mM	5 mM
$MgCl_2$	6 mM	6 mM	6 mM	6 mM
Na_2CP	10 mM	10 mM	10 mM	10 mM
Creatine kinase*	25 $\mu g.cm^{-3}$	25 $\mu g.cm^{-3}$	25 $\mu g.cm^{-3}$	25 $\mu g.cm^{-3}$
K_2H_2EGTA†	10 mM	10 mM	0.15 mM	0-7.5 mM ǂ
K_2H_2HDTA	-	-	9.85 mM	-
$K_2CaEGTA$	-	-	-	2.5-10 mM ǂ
Glucose	5.6 mM	-	-	-
Na pyruvate	3 mM	-	-	-
Triton X-100	1% (v/v)	-	-	-
pH	7.00	7.00	7.00	7.00
Calculated pCa	>8	>8	>7	6.79- 3.75 ǂ
Calculated ionic strength	≈205 mM	200 mM	200 mM	200 mM

* Omitted in the later experiments.
† Nominal concentrations based on the weight of EGTA. Effective [EGTA] = 0.96 x nominal [EGTA].
ǂ 'Control' values = 0 mM K_2H_2EGTA, 10 mM $K_2CaEGTA$ (calculated pCa = 3.75).

Table 6. Standard compositions of the bathing solutions.

All bathing solutions were prepared freshly each day by mixing the appropriate stock solutions (see below), adding any other chemicals such as Na_2HCP and creatine kinase in the solid form and then diluting the solutions with deionised distilled water. The pH was checked with a Pye 290 pH meter and adjusted if necessary with HCl or KOH to be within 0.01 pH units of the required value at the appropriate temperature. A separate pH electrode and calomel reference electrode were used rather than a combination pH/reference electrode because the calomel electrode was found to be much less susceptible than Ag/AgCl reference electrodes (such as used in combination electrodes) to drift caused by EGTA in the experimental solutions.

Except for the modified Krebs-Hensleit solution, all bathing solutions were held in plastic beakers or test-tubes before use.

(b) Stock solutions

The stock solutions for the modified Krebs-Henseleit solution contained (in mM): NaCl, 1180; KCl, 50; KH_2PO_4, 12; $MgSO_4$, 12; $NaHCO_3$, 250. This was prepared weekly and stored at 4°C.

A range of CaEGTA stock solutions containing 50 mM (nominal) EGTA and 0 - 50 mM Ca at pH 7.0 were prepared by the general procedure described in Section 5.6. The concentration of excess EGTA in these solutions was checked by titrating aliquots against 1 M $CaCl_2$, using a modification of the pH-metric method of Moisescu & Pusch (1975). Details of the procedure are given below.

A solution of 49.25 mM hexamethylene diamine tetra-acetic acid (HDTA) + 0.75 mM EGTA, pH 7.0, was prepared in the same manner as the 50 mM EGTA solution.

Tetramethylammonium chloride (TMACl) and choline chloride (ChCl) were available only as technical grade, so I purified these substances further by dissolving them in deionised water, filtering, recrystallising at 70°C under reduced pressure and drying. Stock solutions of 1 M KCl, 1 M TMACl, 1 M ChCl, 1 M NaCl and 200 mM imidazole were prepared and were passed down a column of the ion-exchange resin Chelex-100 (in the potassium form) to remove contaminant Ca^{2+}.

The preparation and storage of a stock solution containing 100 mM K_2Na_2ATP + 120 mM $MgCl_2$, pH 7.0, was as described in Section 5.6.

All the above stock solutions were stored in plastic bottles at 4°C except for the ATP + $MgCl_2$ stock, which was kept at -16°C.

Atomic absorption spectroscopy in 0.5% $LaCl_3$ established that the

concentration of contaminant Ca^{2+} in all stock solutions was 20 μM or less and that of contaminant K^{+} in the TMACl, ChCl, NaCl and imidazole stock solutions was less than 0.5 mM.

(c) Determination of the ratio [total Ca]:[total EGTA] in the Ca/EGTA stock solutions

As mentioned previously (Section 5.6), the calibrated pCa depends critically on the ratio between [total Ca] and [total EGTA] in the solutions. I determined this ratio in the Ca/EGTA stock solutions by a titration procedure modified from that of Moisescu & Pusch (1975).

Exactly 20 cm^3 of the Ca/EGTA stock solution, containing nominally 50 mM total EGTA, was pipetted into a 100 cm^3 plastic beaker. Ca^{2+}-free 1 M KCl, 200 mM imidazole, 1 M KOH and water were added to give 100 cm^3 of a solution containing: 10 mM (nominal) EGTA + 0 - 10 mM Ca; 100 mM KCl; 20 mM imidazole; pH ≈ 9.4. Then 1 M $CaCl_2$ was added in 0.1 cm^3 or 0.01 cm^3 aliquots using calibrated automatic pipettes. (In some titrations 0.1 cm^3 100 mM $CaCl_2$ was used instead of 0.01 cm^3 1 M $CaCl_2$). The 1 M $CaCl_2$ used was either bought from B.D.H. Limited or was prepared in the laboratory from HCl + dried $CaCO_3$ (the same $CaCO_3$ as was used to prepared the Ca/EGTA stock solutions). After each addition of $CaCl_2$ the titrant was briefly stirred and the steady pH value was read after stirring had ceased. Each addition of $CaCl_2$ produced a drop in pH, as protons were displaced from EGTA by Ca^{2+} ions, until the [total Ca] was sufficient to saturate all the EGTA; from that point, further addition of $CaCl_2$ produced little change in pH. The titration curve consisted of two straight lines meeting at an obtuse angle. Back extrapolation of these lines enabled the end-point to be estimated with a precision of ≈ 0.1%.

This end-point corresponded to a total concentration of added Ca of 9.579 ± 0.005 mM (mean ± S.E.M., n = 12). Thus 9.58 mM added $CaCl_2$ was needed to just saturate the nominally 10 mM EGTA. The same results were obtained whether the 1 M $CaCl_2$ used was that from B.D.H. or that produced in the laboratory; this suggested that the concentration of $CaCl_2$ was accurate. Because it is known that one mole of EGTA binds exactly one mole of Ca^{2+}, I concluded that the effective [total EGTA] in my solutions was only 9.58 mM i.e. the EGTA was only 95.8% pure. A little of the apparent impurity would have resulted from the 15 - 20 μM of contaminant Ca^{2+} in the titrant binding to the EGTA, so the true effective concentration of the EGTA was in fact 9.60 mM, corresponding to a purity of 96.0%.

(d) Calculation of the pCa in the activating solutions

The pCa in the activating solutions was calculated by the iterative computer program described in Section 5.6(d). However, the bathing solutions used in the skinned muscle experiments generally had higher ionic strengths and were at lower temperatures than the myofibrillar ATPase incubation solutions. Thus the chosen values for the association constants (Table 7) were in some cases slightly different from those listed in Part II (Table 1).

The association constants for the binding of one and two protons and of Ca^{2+} to $EGTA^{4-}$ (K_{HEGTA} , K_{H_2EGTA}, K_{CaEGTA} respectively) largely determine the calculated pCa for a given total concentrations of Ca and EGTA. Unfortunately the values of these constants had not previously been measured at an ionic strength of 200 mM, which I chose as the standard ionic strength for the relaxing and activating solutions. Furthermore, in the study on the effect of salt concentration on the relative force - pCa relationship of skinned muscle it was essential to know whether changes in salt concentration altered the values of K_{HEGTA}, K_{H_2EGTA} and K_{CaEGTA}. Failure to correct for any changes in these association constants would have resulted in artifactual shifts in the relative force - pCa relationships as the salt concentration was varied. To see whether the constants did indeed vary with solution composition, I determined the apparent K_{CaEGTA} in solutions of salt compositions as similar as possible to those of the activating solutions. The method used was the pH-metric method of Moisescu & Thieleczek (1979), with modifications to correct for errors I found to be inherent in the equations they formulated. The details of the methods and results in this study are described in Appendix A. From these experiments I was able to estimate how the apparent K_{CaEGTA} varied with salt concentration. Although I could not tell whether any changes in the apparent K_{CaEGTA} were due to changes in K_{HEGTA}, in K_{H_2EGTA} or in K_{CaEGTA}, this did not matter, because the effect on the calculated pCa would have been the same. For the purposes of the computer program, I assumed that K_{HEGTA} and K_{H_2EGTA} had constant values in the different solutions and that any changes in the apparent K_{CaEGTA} in these solutions must have been due to alterations in the K_{CaEGTA}. Estimates of the values of these constants in solutions of different [salt] are given in Table 8. With these values, plus those chosen from Martell & Smith (1974), I was able to calculate the pCa values in the activating solutions of different pH (Table 9) and of different [salt] (Table 10).

Reactants	$\log_{10}K$	Reactants	$\log_{10}K$
imidazole; H^+	7.17	$HDTA^{4-}$; Ca^{2+}	4.60
$EGTA^{4-}$; H^+	9.58*	$HHDTA^{3-}$; Ca^{2+}	3.79
$HEGTA^{3-}$; H^+	8.96*	$HDTA^{4-}$; Mg^{2+}	4.80
H_2EGTA^{2-}; H^+	2.77*	$HHDTA^{3-}$; Mg^{2+}	3.59
H_3EGTA^-; H^+	2.11*	ATP^{4-}; H^+	6.62*
$EGTA^{4-}$; K^+	0.96	$HATP^{3-}$; H^+	4.17*
$EGTA^{4-}$; Na^+	1.38	ATP^{4-}; K^+	0.90
$EGTA^{4-}$; Ca^{2+}	ǂ	ATP^{4-}; Na^+	1.20
$HEGTA^{3-}$; Ca^{2+}	ǂ	ATP^{4-}; Ca^{2+}	3.77
$EGTA^{4-}$; Mg^{2+}	5.21	$HATP^{3-}$; Ca^{2+}	1.95
$HEGTA^{3-}$; Mg^{2+}	3.36	ATP^{4-}; Mg^{2+}	4.06
$HDTA^{4-}$; H^+	10.92*	$HATP^{3-}$; Mg^{2+}	2.10
$HHDTA^{3-}$; H^+	9.90*	HCP^{2-}; Ca^{2+}	1.15
H_2HDTA^{2-}; H^+	2.81*	HCP^{2-}; Mg^{2+}	1.30
H_3HDTA^-; H^+	2.31*		

* Proton constants converted from concentration constants to mixed constants by addition of 0.11 (Martell& Smith, 1974).

ǂ Value depends on [salt] - see Table 8.

Table 7. Association constants (in log. form) used in the computation of pCa values in the relaxing and activating solutions.

All values derived from Martell & Smith (1974) except those for formation of $CaEGTA^{2-}$ and $CaHEGTA^-$.

Reactants	$\log_{10}K$ in [salt] of:				
	90 mM KCl	190 mM KCl	90 mM KCl + 100 mM TMACl	90 mM KCl + 100 mM ChCl	90 mM MCl + 100 mM NaCl
$EGTA^{4-}$; Ca^{2+}	10.952	10.895	10.910	10.930	10.949
$HEGTA^{3-}$; Ca^{2+}	5.416	5.333	5.500	5.440	5.344

Table 8. Association constants (log form) for formation of $CaEGTA^{2-}$ and $CaHEGTA^{-}$ (K_{CaEGTA} and $K_{CaHEGTA}$, respectively) in solutions of different salt compositions.

The 'true' association constants were calculated from the experimentally-determined apparent constants (Appendix A) assuming

$\log K_{HEGTA} = 9.58$, $\log K_{H_2EGTA} = 8.96$, $\log K_{H_3EGTA} = 2.77$ and

$\log K_{H_4EGTA} = 2.11$ as in Table 7.

[total Ca] (mM)	pH			
	7.20	7.00	6.80	6.40
0.020*	9.40	9.02	8.62	7.83
2.5	7.18	6.79	6.40	5.61
4.0	6.87	6.48	6.09	5.30
5.0	6.68	6.30	5.91	5.12
6.0	6.50	6.11	5.72	4.94
7.0	6.29	5.90	5.51	4.73
8.0	6.02	5.64	5.25	4.48
8.5	5.83	5.45	5.06	4.31
9.0	5.55	5.17	4.80	4.10
9.5	4.87	4.58	4.32	3.84
10.0	3.76	3.75	3.72	3.55

* Usual concentration of contaminant Ca.

Table 9. Computed values of pCa in the activating solutions at different total concentrations of Ca and at different values of pH.

Solutions contained nominally 10 mM EGTA (= 9.60 mM effective EGTA) and 100 mM added KCl. See Table 6 for detailed compositions of of the solutions.

[total Ca] (mM)	Added salt				
	None	100 mM KCl	100 mM TMACl	100 mM ChCl	100 mM NaCl
0.020*	9.07	9.02	9.03	9.05	9.07
2.5	6.84	6.79	6.80	6.82	6.84
4.0	6.54	6.48	6.50	6.52	6.53
5.0	6.36	6.30	6.31	6.33	6.35
6.0	6.17	6.11	6.13	6.15	6.17
7.0	5.96	5.90	5.92	5.94	5.96
8.0	5.69	5.64	5.65	5.67	5.69
8.5	5.51	5.45	5.46	5.48	5.50
9.0	5.23	5.17	5.19	5.20	5.22
9.5	4.62	4.58	4.59	4.61	4.62
10.0	3.77	3.75	3.75	3.76	3.76

* Usual concentration of contaminant Ca.

Table 10. Computed values of pCa in the activating solutions of different [salt], pH 7.00.

Other solutions details as in Table 6. With no added salt the total ionic strength of the activating solution was 100 mM.

9.3 Apparatus

The muscle bath and some of the ancillary apparatus are shown in Figure 27. The muscle bath, which was of the flow-through type, consisted of a 3 mm x 3 mm channel milled in a Perspex block. Total bath volume was 0.3 cm^3. Platinum electrodes, through which the muscle could be field stimulated, were set into the channel walls and were connected to an isolated stimulator (type 6032, S.R.I. Limited, Croydon, Surrey). The bath was lit from below by a 2.5 V torch bulb. A non-pulsatile roller pump (Varioperpex, LKB A.B., Bromma, Sweden) propelled the solutions from beakers or test-tubes into the muscle bath via plastic tubing. The effluent in the collecting well was sucked away by an air pump and was not recirculated. The temperature of the solution flowing over the muscle was maintained at the required value (usually 25°C or 30°C) ± 0.1°C by a proportional control system. A thermistor (GM222, ITT Limited, Harlow, Essex) was held just distal to the muscle in the channel. The resistance of this thermistor was monitored by the temperature control box, which passed current through a heating wire coiled around the perfusion line immediately proximal to the bath.

Two fine hooks were made from stainless steel wire and were glued with Araldite to pieces of stainless steel syringe needle. One needle piece was glued with Collodion to the beam of an isometric force transducer and the other was attached to a rigid plastic tube (Figure 28). The force transducer (AE875, Aksjeselskapet Mikro-Elektronikk, Horten, Norway), when coupled to the hook and needle, had a overall sensitivity of 12 $\mu V/\mu N$ at 5 V excitation, a compliance of 21 $nm/\mu N$ and a natural frequency of 260 Hz. A microdrive allowed fine horizontal movement of the transducer relative to the fixed hook. Both the microdrive and fixed plastic tube were mounted on a micropositioner (type 23, Prior Limited, Bishop's Stortford, Herts.) so that the muscle could be moved in all planes without a change in muscle length. The transducer was connected to a bridge preamplifier (Lectromed 3559, Ormed Limited, Welwyn Garden City, Herts.). Electrical output from the preamplifier was filtered (-3 dB at 5 or 0.5 Hz) to remove spurious oscillations in the force signal which were due to vibration of the building. The filtered force signal was differentiated with respect to time (dF/dt). Force and its differential were displayed on a storage oscilloscope (5103N, Tektronix Limited, Harpenden, Herts.) and recorded

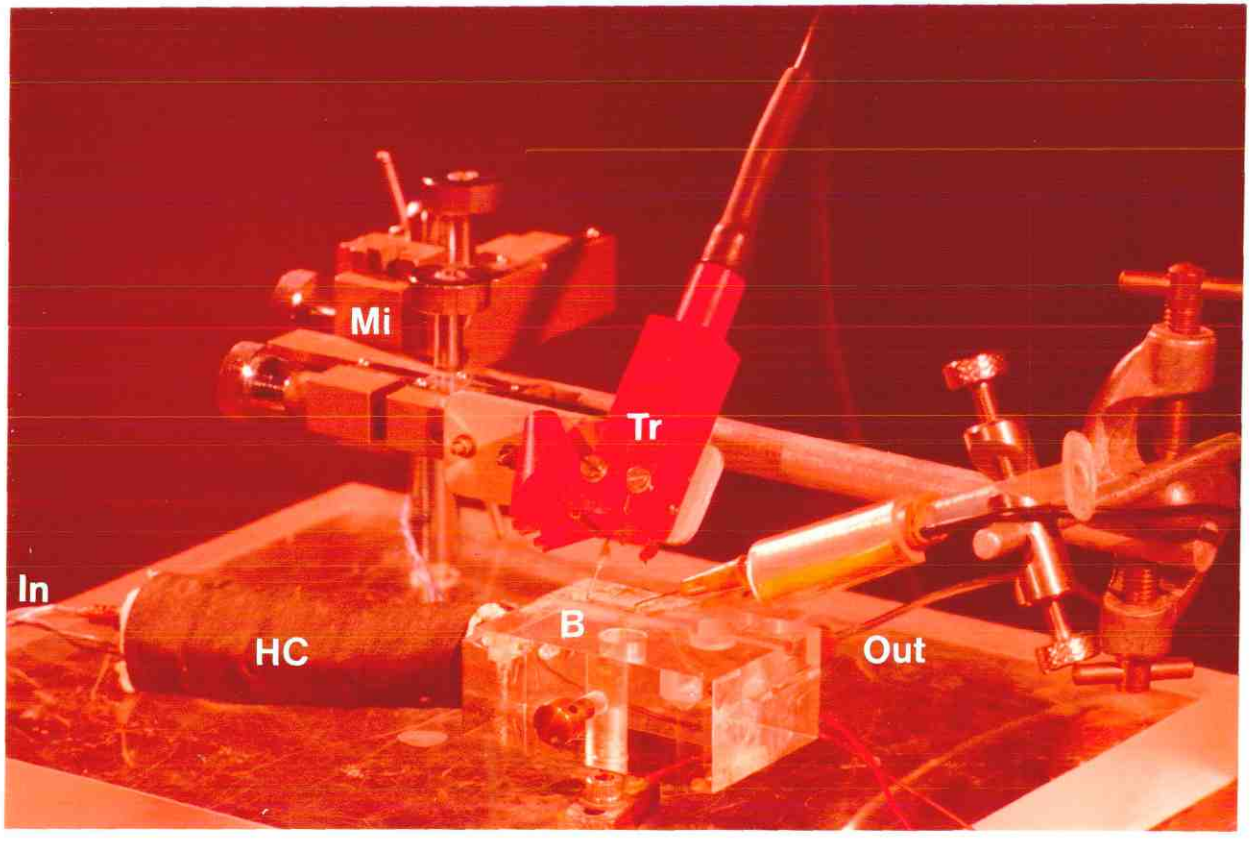


Figure 27. General view of the apparatus.

(Mi) micropositioner; (tr), force transducer; (B), muscle bath; (HC) heating coil; (In) inlet tube; (Out), outlet tube.

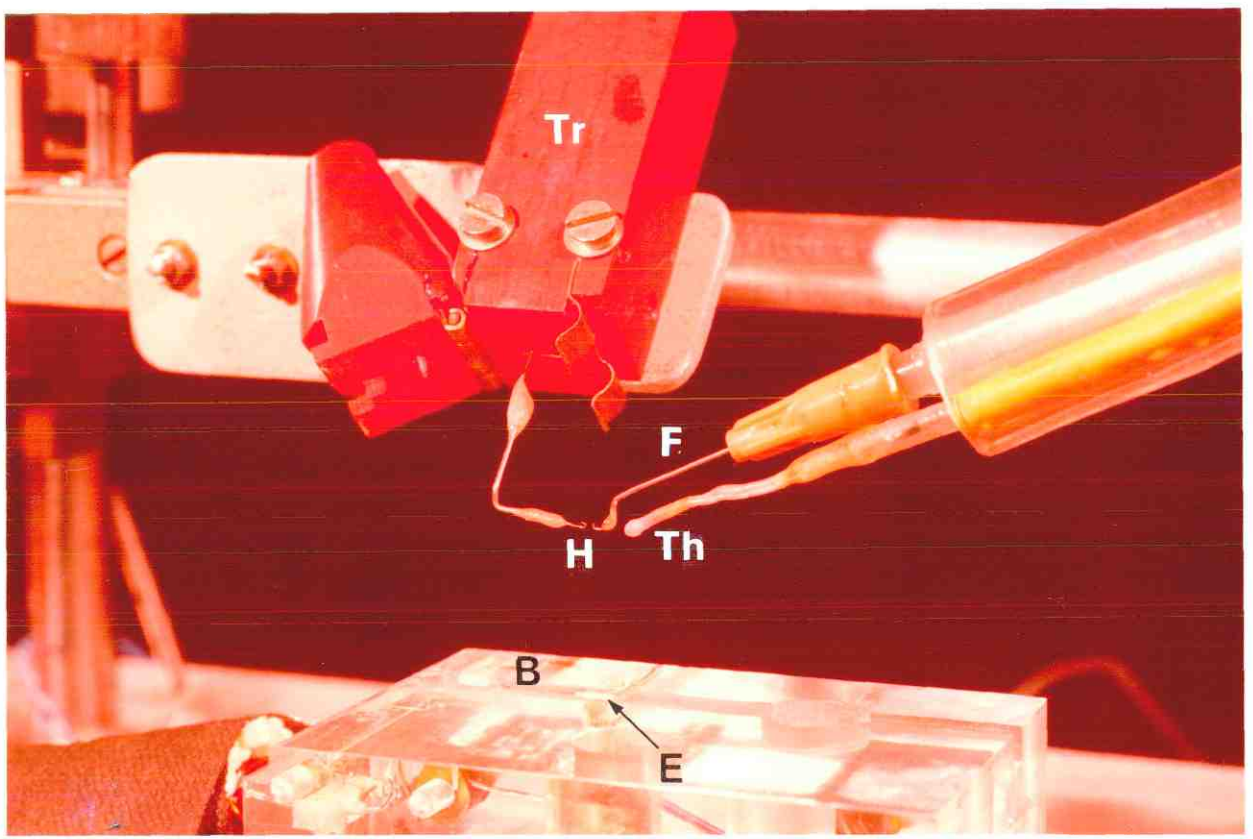


Figure 28. Detailed view of the transducer assembly. Assembly raised out of bath for clarity. (Tr), transducer; (B), muscle bath; (H), hooks; (F), fixed rod; (Th), thermistor; (E), electrodes.

on a chart recorder (Lectromed MX412, Ormed Limited).

An earthed aluminium box with a Perspex front shielded the muscle bath and transducer from air currents and electrical noise. The entire apparatus stood on an anti-vibration table.

9.4 Measurement of muscle forces

The forces developed in the muscle were measured from the 5 cm - wide chart record to the nearest 0.2 mm (i.e. 0.4% of total chart width).

I defined 'resting force' in the skinned muscle as the force in a relaxing solution containing EGTA and MgATP but no added Ca^{2+} (pCa>8) - see Figure 29a. An increase in Ca^{2+} concentration resulted in a contracture - a tonic increase in muscle force - which reached a steady plateau if the superfusion of the muscle with the Ca^{2+}-containing solution ('activating solution') was of sufficient duration. The difference between the steady total force in the contracture and the steady resting force established after reapplication of the relaxing solution was termed 'Ca^{2+}-regulated force'. Usually the resting force before and after a contracture were identical. Under some conditions however the resting force after a contracture was greater than that before, that is, relaxation was incomplete (Figure 29b). The increment in resting force was called 'residual force', after the terminology of Thames et al. (1974).

9.5 Preparation of the 'skinned' muscle

(a) Preparation of the intact muscle bundle

All the following experiments were performed using cardiac muscle from male guinea-pigs (200 - 250 g). The guinea-pigs were killed by cervical dislocation. Their hearts were rapidly excised and immersed in the oxygenated modified Krebs-Henseleit solution at room temperature (20°C - 25°C). Each heart was dissected on a bed of silicone rubber (Sylgard 184, Dow Corning Corporation, Michegan, U.S.A.) in a dish filled with Krebs-Henseleit solution. The right ventricular wall was cut along the ventral midline and was pinned back. A suitable trabecula (length > 500 μm, diameter < 200 μm) was selected. Strands teased from opthalmic silk were tied into small loops around each end of the muscle. The muscle was then cut out and the loops were placed over the fine hooks in the muscle bath. Modified Krebs-Henseleit solution at room temperature was pumped through the bath at 3 - 5 cm^3 min^{-1} (10 - 15 bath volumes min^{-1}). The trabecula was stimulated

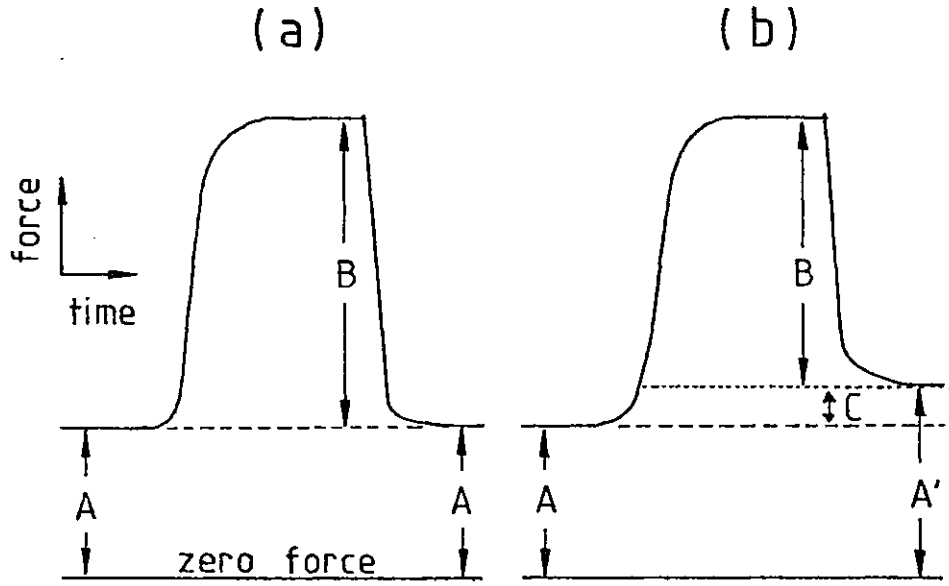


Figure 29. Diagrammatic representation of Ca^{2+}-regulated contractures in a skinned muscle to illustrate the definitions of the forces measured.

(a) Contracture followed by complete relaxation
(b) Contracture followed by incomplete relaxation
A = 'resting force'
B = 'Ca^{2+}-regulated force'
C = 'residual force'

electrically with supramaximal rectangular pulses of amplitude 5 - 10 V, duration 2 ms and frequency 1 s^{-1}. If the muscle failed to develop force after 15 minutes of stimulation it was discarded. During an equilibratory period of several hours the muscle was progressively stretched until developed force was maximal.

(b) Skinning procedure

Once the trabecula had equilibrated and resting and developed forces were stable the modified Krebs-Henseleit solution was replaced by the 'skinning solution' (Table 6). Flow of perfusate was thereafter maintained at 1 cm^3 min^{-1}. Skinning solution was perfused for 12 - 30 minutes at room temperature (20°C - 25°C) unless stated otherwise. During this period, the extent of sacrolemmal disruption was periodically assessed by one or both of the methods described in the next section.

(c) Assessment of the time course of sarcolemmal disruption

(i) Functional assessment

Superfusion of the muscle with 'skinning solution' of standard composition (Table 6) was interrupted at intervals by 1 minute's superfusion with a 'high-Ca skinning solution'. This solution was identical to the standard 'skinning solution' except that it contained 10 mM CaEGTA instead of 10 mM H_2EGTA, and thus had a pCa of 3.75 instead of pCa>8.0. Each application of 'high-Ca skinning solution' resulted in a contracture of the muscle. The maximum force attained in each contracture was taken as a functional measure of the degree to which the muscle was skinned at that time. (This maximum force was not always equivalent to the steady 'Ca^{2+}-regulated force' defined in Section 9.4 because some of the earlier contractures did not exhibit a plateau of force).

An example of the series of contractures produced by several applications of high-Ca skinning solution during the skinning period is shown in Figure 30. In Figure 31 the maximum forces in this muscle and in several other typical muscles are plotted against the total duration of perfusion with the skinning solutions. The maximum force rapidly increased with increasing time in skinning solution, reached a peak and declined slowly thereafter. All the muscles tested exhibited qualitatively the same behaviour, though the perfusion time necessary to reach the peak varied considerably from muscle to muscle and depended to a large extent on the muscle diameter.

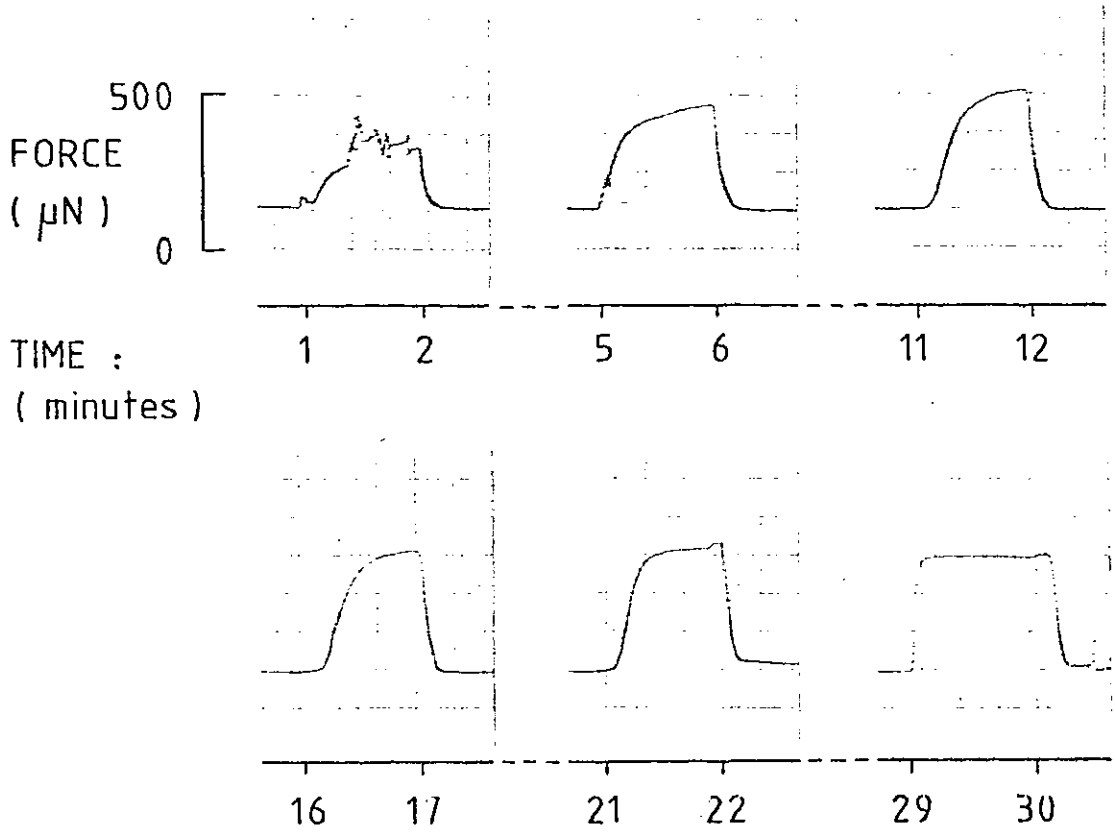


Figure 30. An example of the 'functional assessment' of skinning.

Contractures were produced, at the times shown after the start of the skinning procedure, by superfusing the muscle with 'high-Ca skinning solution' (pCa 3.75) in place of the standard skinning solution (Table 6). The record for 1 - 2 min contains artefacts due to bubbles in the muscle bath. Muscle diameter 160 μm.

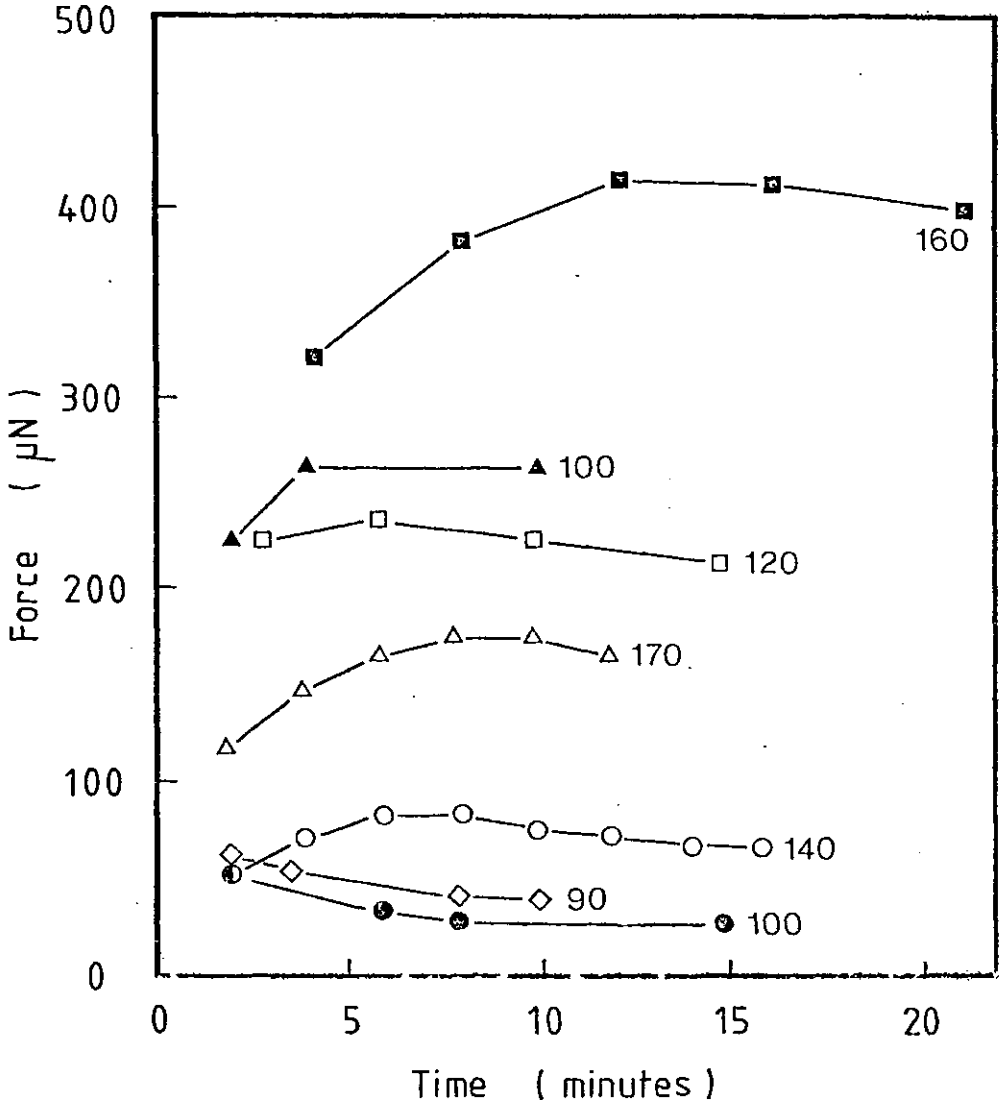


Figure 31. Maximum forces in contractures produced by application of 'high-Ca skinning solution' at various times throughout the skinning procedure.

Force was measured at the end of each 1 minute superfusion with 'high-Ca skinning solution'. In between, standard 'skinning solution' was perfused. Abscissa gives time since start of skinning procedure. Results from 7 muscles. Muscle diameters given in μm.

In the thickest trabeculae used (diameter 150 - 200 μm) this perfusion time was about 15 - 20 minutes. In the thinnest muscles (diameter ≈80 μm) the very first application of the high-Ca skinning solution, starting 1 minute after the beginning of the skinning period, produced the maximum force. The faster time course of skinning in the thinner muscles probably reflected the smaller distance over which Triton X-100 had to diffuse to reach all the cells in the bundle.

The rate of rise of force in each contracture also became greater as the length of perfusion with 'skinning solution' increased (Figure 30). I could not however routinely use the rate of rise of force as a functional measure of the degree of sarcolemmal disruption because the development of force was often slowed to a variable extent by the presence of detergent-stabilised bubbles in the muscle bath. In the few experiments in which this problem was minimal the time to half-maximal force in each contracture was measured and was found to fall to a minimum with a time course which lagged 5 - 10 minutes behind that of the maximum force.. For this reason the perfusion with skinning solution was routinely continued for 10 minutes after the peak value of force in the high Ca skinning solution had been reached.

(ii) Visual assessment

The progress of sarcolemmal disruption was also monitored by visual inspection of the trabeculae under the dissecting microscope. Soon after the modified Krebs-Henseleit solution had been replaced by skinning solution the outer regions of each muscle lost their reddish-brown colour and became translucent. The translucent layer slowly spread inward until the entire muscle was colourless. This point was usually reached at about the time that the application of high-Ca skinning solution produced the maximum force in the muscle. I assumed that the loss of colour was due to sarcolemmal disruption that was sufficient to allow the loss of myoglobin molecules from the muscle cells. Thus when the entire cross-section of the muscle had become translucent I concluded that the great majority of muscle cells were adequately 'skinned'.

As judged by these two criteria, the muscles became adequately 'skinned' after 2 - 20 minutes in skinning solution , the optimal period varying from muscle to muscle. Superfusion with skinning solution was continued for 10 minutes after the peak force in high-Ca skinning solution had been attained. The total perfusion period therefore ranged from 12 to 30 minutes. At the end of this period skinning solution was replaced by 'high-EGTA relaxing solution' (Table 6).

The question of to what extent the cells in these detergent-treated muscles are truly skinned, that is, to what extent the sarcolemma is non-selectively permeable to externally-applied molecules, is addressed in Sections 10.7 and 11.3.

9.6 Activation of the 'skinned' muscles with Ca^{2+}

After the skinning period, the muscle was perfused with high-EGTA relaxing solution for 15 minutes at room temperature to wash out all the detergent-containing skinning solution. All subsequent perfusates were usually maintained at 25°C or 30°C.

In my original procedure the muscles were then made to contract and relax by superfusions with activating solutions of pCa 3.75 - 7.0 and high-EGTA relaxing solutions of pCa>8. After each change to an activating solution, force usually reached a new steady value within 1 - 5 minutes. However it was found that muscle viability was improved if contractures were kept as short as possible, since protracted contractures sometimes produced an irreversible increase in resting force (Section 10.3). During the course of these studies a combination of two factors resulted in a considerable reduction in the time to maximum force in the contractures, so that contracture duration could be minimised. Firstly, my dexterity in handling the trabeculae improved over a period of months so that I was able to use muscles of smaller diameter than before. This allowed a more rapid equilibration of Ca^{2+}, $CaEGTA^{2-}$ and other ions between the bathing solution and the solution inside the skinned muscle. Secondly, a more immediate acceleration of the development of force in each contracture was accomplished by the use of a 'rapid activation' procedure devised by Moisescu (1976). Basically, this technique involved interposing a short perfusion with a solution of low EGTA concentration ('low-EGTA relaxing solution', Table 6) between the superfusions with high-EGTA relaxing solution and activating solution. The low-EGTA relaxing solution washed out most of the EGTA from the skinned muscle and thereby minimised the Ca^{2+}-buffering capacity of the solution within the muscle. When the perfusate Ca^{2+} concentration was then raised by replacing the low-EGTA relaxing solution by an activating solution, the increase in Ca^{2+} concentration within the muscle was much faster than it would have been if considerable amounts of EGTA had remained in the preparation. The acceleration of the rise in Ca^{2+} concentration by the rapid activation technique was reflected by an increased rate of development force in a contracture and

an ealier plateau (illustrated in Figure 32). Thus the duration of each contracture could be reduced, with a consequent improvement in the viability of the preparation.

Total EGTA concentrations of 0.15 mM in low-EGTA relaxing solution and of 10 mM in high-EGTA relaxing solution and activating solutions were found to provide a substantial increase in the rate of development of force without an excessive contribution of EGTA-containing complexes to the total ionic strength of the solutions. The ionic strength in low-EGTA relaxing solution was brought up to that in the high-EGTA relaxing solution and activating solutions by the inclusion of 9.85 mM HDTA, an EGTA analogue which does not bind Ca^{2+} or Mg^{2+} to any significant extent (Moisescu, 1976).

Whether or not it was preceded by low-EGTA relaxing solution, each activating solution was pumped through the bath for a sufficient time to allow the contracture to reach a plateau. The following perfusion regimes were used most frequently:

	perfusion time
Original activation procedure	
High-EGTA relaxing solution	1 - 5 minutes
Activating solution	1 - 5 minutes
Rapid activation procedure	
High-EGTA relaxing solution	30 seconds
Low-EGTA relaxing solution	30 seconds
Activating solution	30 seconds

9.7 Protocols for data collection

Two problems introduced complications into the analysis and interpretation of muscle forces. Firstly, absolute force varied considerably from muscle to muscle, even if it was normalised by conversion to force/unit area. This made comparisons of absolute force between muscles unreliable. The second problem was that the Ca^{2+}-regulated force in any given activating solution was not constant, but decreased as the number of contractures increased. This deterioration in the contractile performance hampered a comparison of the absolute forces produced in the same muscle by different activating solutions. In this thesis this phenomenon is termed the decline in Ca^{2+}-regulated force to differentiate it from the decrease in force recorded when an activating solution is replaced by a relaxing solution.

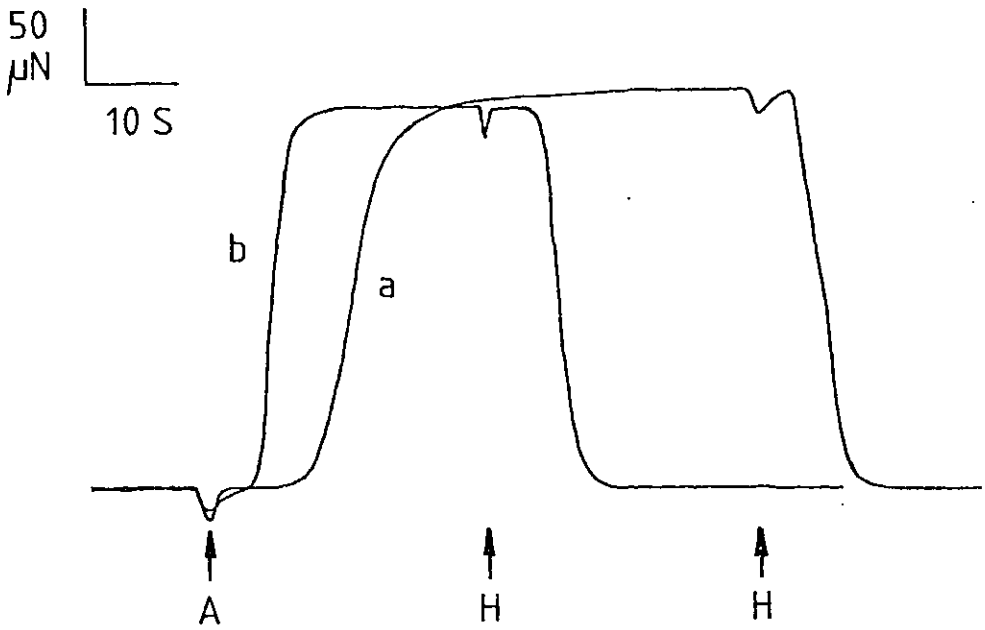


Figure 32. Comparison of contractures produced by (a) the original activation procedure and (b) the 'rapid activation' procedure.

Force traces from oscilloscope screen superimposed to synchronise moment when activating solution (A) reached muscle. Perfusion regimes were:

(a) 30 s H - 30 s A - 30 s H

(b) 30 s H - 30 s L - 30 s A - 30 s H

where H = high-EGTA relaxing solution

L = low-EGTA relaxing solution

A = activating solution (pCa 3.75)

Dips in force were due to bubbles hitting the transducer tip. 25°C.

Like many other investigators who use skinned muscle, I circumvented both problems by using each muscle as its own control: all Ca^{2+}-regulated forces in each experiment were expressed relative to the Ca^{2+}-regulated force in a 'control' activating solution, which was chosen to be the activating solution of standard composition (Table 6) containing 10 mM $K_2CaEGTA$ (calculated pCa = 3.75). This solution produced maximum Ca^{2+}-regulated force in the skinned muscles at pH 7.00 and ionic strength 200 mM. Most of the Ca^{2+}-regulated forces are thus given in relative, rather than absolute, terms. The skinned muscles were frequently superfused with the 'control' activating solution throughout each experiment. From the Ca^{2+}-regulated forces elicited by this solution I was able to quantitate the decline in the ability of the muscles to generate force and so could make corrections for this decline.

The corrections were based upon the following assumptions (see Appendix B for experimental support for these assumptions): (i) in a series of regularly-spaced contractures produced by the same activating solution, the steady Ca^{2+}-regulated force in any contracture is a constant fraction of the steady force in the preceding contracture. In other words, Ca^{2+}-regulated force declines exponentially with time or with the number of contractures; (ii) the decline in the absolute magnitude of Ca^{2+}-regulated force does not affect the position of the Ca^{2+}-activation curve on the pCa axis. The Ca^{2+}-regulated force in a given activating solution is expressed relative to the Ca^{2+}-regulated force I estimated would have been recorded if, instead of this activating solution, control activating solution had been applied to the muscle. This estimated Ca^{2+}-regulated force was derived by interpolation from the forces of contractures in control activating solution applied before and after the 'test' contracture. An exponential decline of Ca^{2+}-regulated force with increasing contracture number was assumed for the interpolation.

Two protocols were used for data collection. In both protocols a change in the basic composition of the solution bathing the muscle (that is, a change involving more than just an alteration in Ca^{2+} or EGTA concentration) was made with the muscle in a high-EGTA relaxing solution, so that any alterations in resting force would be apparent. After such a change, the perfusion with the new relaxing solution was prolonged as necessary to allow time for resting force to reach a new steady level.

(a) 'Staircase' protocol (Figure 33a)

In this protocol the muscles were superfused with solutions of identical composition but containing successively greater concentrations of free Ca^{2+}. The series of solutions began with zero added Ca (high-EGTA relaxing solution) and ended with 10 mM added Ca (control activating solution). The muscle was made to relax by superfusion with high-EGTA relaxing solution only when the series had been completed; a 'staircase' of fused contractures was thus produced. The usual perfusion time for each activating solution was 1 - 5 minutes. To enable a correction to be made for the decline in force, contractures were elicited by the superfusion of the muscle with control activating solution (pCa 3.8) before and after each staircase of contractures.

Let F_{C1} and F_{C2} be the Ca^{2+}-regulated forces in the leading and trailing contractures in control activating solution of pCa 3.75 and let F_x be the Ca^{2+}-regulated force in the xth contracture in a staircase of i contractures. Then F_x, corrected for the force decline and expressed relative to the force in control activating solution is given by:

$$\text{relative force} = F_x/\text{interpolated force in control activating solution}$$

$$= F_x/(F_{C1}.(F_{C2}/F_{C1})^{x/(i+1)}).$$

Usually i had a value of 6 or 7.

Sometimes however the muscles developed a residual force after having been bathed in the series of activating solutions for many minutes (Section 10.3) The data from any muscles exhibiting this behaviour were discarded. Another disadvantage of the 'staircase' protocol was that the validity of the correction procedure was equivocal: although in a series of discrete contractures produced by regular application of the same activating solution the decline in force is indeed an exponential function of contracture number (Section B.3(b)), the same may not have held for the force in a staircase of contractures. Because of these considerations the 'staircase' protocol was eventually abandoned in favour of the more time-consuming but inherently more accurate 'discrete' protocol.

(b) 'Discrete' protocol (Figure 33b)

Here activating and relaxing solutions were applied alternately, with the result that discrete contractures were generated by the skinned muscles. As in the 'staircase' protocol, application of the activating

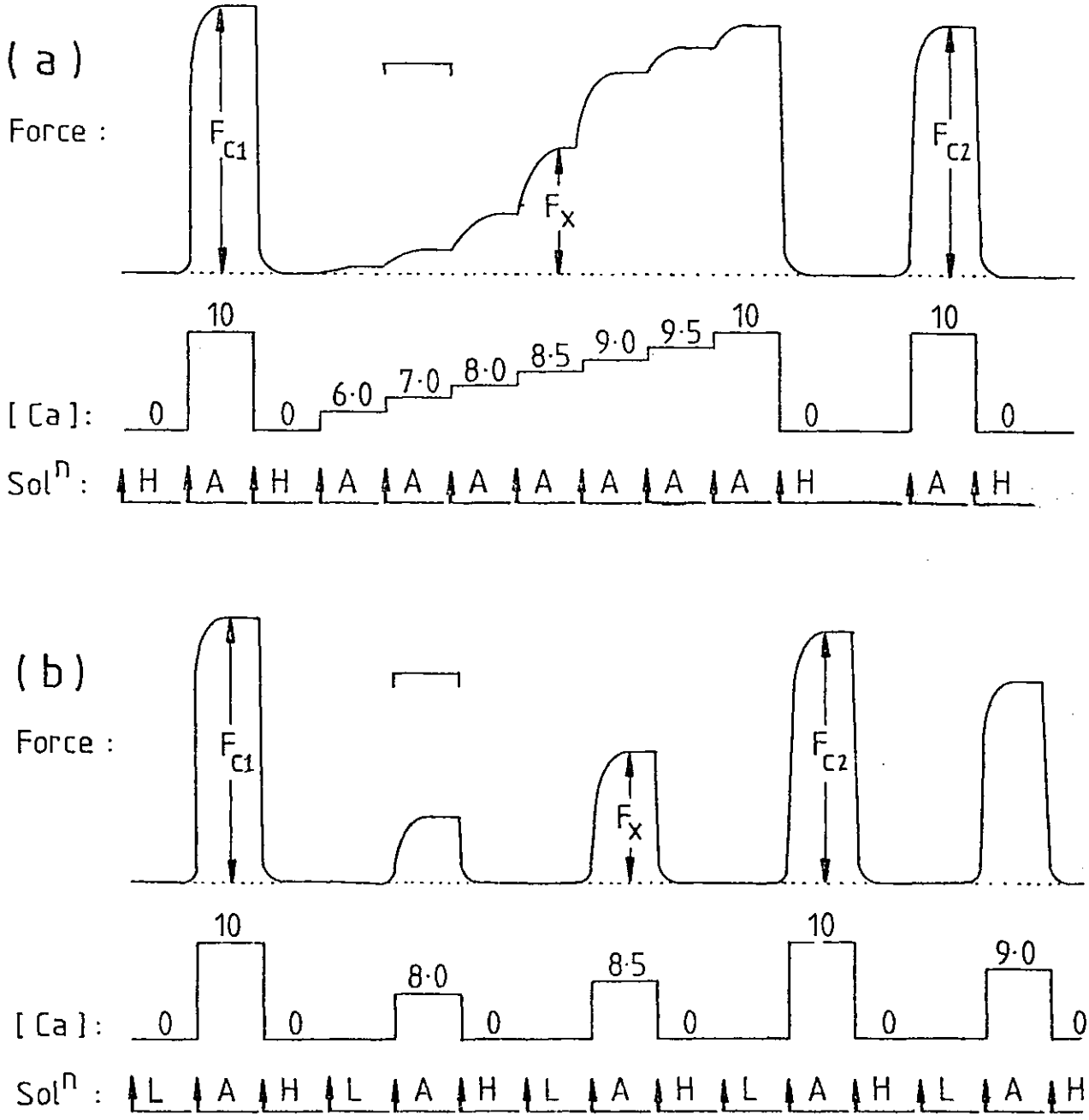


Figure 33. Diagrammatic representation of the two types of experimental protocol used to correct for the decline in Ca^{2+}-regulated force.

(a) 'Staircase protocol'.
(b) 'Discrete protocol'.

For each protocol, the bottom trace shows the types of solution (H = high-EGTA relaxing solution, L = low-EGTA relaxing solution, A = activating solution), the middle trace represents the total Ca concentrations of the solutions (in mM) and the top trace shows typical force responses. Note that section b shows only part of the procedure necessary to produce the six or seven points for a sufficiently detailed relative force-pCa relationship. Time calibration = 1 - 5 min for a, 30 s (usually) for b.

solutions under test was preceded by and followed by perfusions with the control activating solution. The force, F_x, in the xth contracture in a series of i discrete test contractures was expressed relative to the corrected force in control activating solution. If F_{C1} and F_{C2} are, respectively, the Ca^{2+}-regulated forces produced by the first and second perfusions of control activating solution, then the corrected relative force is given, as with the staircase protocol, by:

$$\text{relative force} = F_x/\text{interpolated force in control activating solution}$$

$$= F_x/(F_{C1}.(F_{C2}/F_{C1})^{x/(i+1)})$$

In most experiments the value of i was 1 or 2. It was never greater than 3. When only a single test contracture was bracketed by the control contractures so that i = x = 1, the above equation simplified to:

$$\text{relative force} = F_x/(F_{C1}\sqrt{F_{C2}/F_{C1}})$$

The 'discrete' protocol was more time-consuming than the 'staircase' protocol because each contracture in a particular activating solution was followed by relaxation. However because the contractures were discrete, more reliance could be placed upon the estimate of the force that would have been observed if, instead of the test activating solution, control activating solution had been applied at a given point. A further advantage of the 'discrete' protocol was that, because each activating solution was immediately preceded by relaxing solution, the 'rapid activation' technique could be used.

The relative force-pCa relationships produced by the two protocols were not significantly different (Section 10.2).

9.8 Experiments to determine the effects of pH on the force production of skinned cardiac muscle

I investigated the dependence of Ca^{2+}-regulated force on the pCa of the activating solution at the four values of pH(7.20, 7.00, 6.80 and 6.40) used in my study on the myofibrillar ATPase activity. Unfortunately the skinned muscles were not viable at 37°C (Appendix B), which was the temperatures used in the ATPase experiments, and so I used a perfusion temperature of 30.0 ± 0.1°C. The 'staircase' protocol was employed to determine the pCa - relative force

relationship at each pH. (These experiments were performed before I started to use the 'discrete'protocol and the rapid-activation procedure). The four sets of relaxing and activating solutions of different pH were applied in the order:

pH 7.00 (i) - pH x - pH y - pH z - pH 7.00 (ii)

where pH 7.00 (i) and pH 7.00 (ii) refer to the first and second applications, respectively, of the same solutions at pH 7.00 and where (x,y,z) represents any permutation of (6.40, 6.80, 7.20).

The permutation was different in each of the six experiments.

9.9 Experiments to determine the effect of salt concentration on the force production of skinned cardiac muscle

All the following experiments were performed at 25°C and at pH 7.00. The rapid-activation procedure and the 'discrete' protocol were used throughout. There were two types of experiment in this study.

(a) The influence of [salt] on resting force and maximum Ca^{2+}-regulated force

I investigated the effect of increases in ionic strength over the range 100 - 400 mM on the resting force and maximum Ca^{2+}-regulated force (at pCa $\approx$ 3.8) in the skinned cardiac trabeculae. The increases in ionic strength over this range were accomplished by the addition of four chloride salts (KCl, NaCl, TMACl, and ChCl) to try to distinguish between effects due to ionic strength per se and effects due to specific inhibitory of certain cations, particularly K^+ ions. With no added salt, the solutions had a calculated ionic strength of 100 mM, due mainly to the 30 mM K^+, 30 mM Na^+ and 22 mM Cl^- and to polyvalent complexes such as $MgATP^{2-}$, HCP^{2-} and H_2EGTA^{2-} or $CaEGTA^{2-}$. All changes in salt concentration of the solution bathing the muscle were made while the muscle was in high-EGTA relaxing solution. Each activating solution was bracketed by the control activating solution (pCa 3.75, pH 7.00, 100 mM added KCl, total ionic strength 200 mM). The 'test' activating solutions were applied throughout the experiments in order of increasing ionic strength, with all the solutions of a particular ionic strength being applied to the muscle before a change was made to the next ionic strength. When all the solutions had been applied once it was possible in some experiments to then reverse the sequence i.e

to reapply the activating solutions in order of decreasing ionic strength.

To check whether the activating solutions of pCa $\approx$ 3.8 did indeed produce maximal activation at each salt concentration studied, the Ca^{2+}-regulated forces in these solutions were compared with the forces in solutions of pCa $\approx$ 3.6, which were made by adding 0.1 mM $CaCl_2$ to the activating solutions of pCa $\approx$ 3.8.

To assess the contribution of changes in osmolarity to the observed effects of changes in salt concentration, in some experiments I increased osmolarity at constant ionic strength by adding 0 - 600 mM sucrose to the solutions.

(b) The influence of [salt] on the relationship between relative force and pCa.

This relationship was determined at two ionic strengths: 100 mM (no added salt) and 200 mM. The 100 mM increase in ionic strength was produced by the addition of one of four chloride salts: KCl, NaCl, TMACl or ChCl. The relative force - pCa relationship at each [salt] was determined using the 'discrete' protocol. As with the experiments on pH, the relationship between relative force and the pCa in the activating solutions of 'standard' composition (pH 7.00, 100 mM added KCl, total $\Gamma/2$ = 200 mM) was determined both at the start and at the end of each experiment.

9.10 Curve-fitting

In a similar way to that in Part II (Section 5.7), sigmoid curves were fitted to the relative force - pCa relationships by non-linear least squares analysis. The curves were of a modified form of the Hill equation:

$$\text{relative force} = \frac{[Ca^{2+}]^{n_H}}{K^* + [Ca^{2+}]^{n_H}}$$

where n_H = the Hill coefficient

and K^* = a compound association constant.

The pCa for half-maximal force (pCa_{50}) is then = $\frac{-1}{n_H} \log_{10} K^*$.

9.11 Statistics

Where applicable, differences were tested for significance using Student's t-test or the Paired t-test.

9.12 Microscopy

(a) Light microscopy

In the more recent experiments muscle length and mid-length diameter were measured at the end of each experiment by means of an eyepiece graticule fitted in the dissecting microscope (magnification = x40). Distances were estimated to the nearest 10 μm. A cover slip was placed over the muscle to eliminate the diminishing effect of the concave solution meniscus. Whenever the muscle cross-sectional area was to be calculated it was assumed that the trabecula was perfectly cylindrical. Cross-sectional areas were not estimated from the weight, density and length of the muscles because the weight (<10μg) could not be accurately determined.

(b) Electron microscopy

Some unskinned and some skinned muscles were fixed, while still tied between the hooks in the muscle bath, by two hour's immersion at room temperature in a solution containing: 4% glutaraldehyde; 0.1 M Na cacodylate buffer, pH 7.3; 1 mM K_2H_2EGTA. Each muscle was then given two ten-minute rinses in buffer (0.1 M Na cacodylate, pH 7.3). The muscle was unhooked from the transducer and was processed for electron microscopy by the method described in Part II (Section 5.8). The ultrathin sections were viewed and photographed with a Philips 301 electron microscope.

Chapter 10

RESULTS

10.1 Muscle forces before, during and after the period of perfusion with skinning solution

A typical chart record of the forces in the intact and skinned muscle is shown in Figure 34. The intact muscle was bathed in modified Krebs-Henseleit solution (KH) and was given single or paired shocks (pulse interval = 350 ms in the experiment shown in Figure 34). In the experiment shown, the Krebs-Henseleit solution was replaced by the standard skinning solution (Table 6) for the full 30 minutes - there was no superfusion of the muscle with 'high-Ca skinning solution'. The muscle became unresponsive to electrical stimulation within a few seconds of the change to skinning solution. This rapid loss of muscle excitability may have been due to the virtual absence of Ca^{2+} in the skinning solution (pCa>8) or to the high concentration (130 mM) of K^{+}, which would have depolarised the cells. However, after the solution change there was no transient contracture corresponding to the K^{+}-induced phasic responses in skeletal muscle (e.g. Reuben et al., 1971).

The length at which force production was maximal in the unskinned trabeculae (L_{max}) was associated with a resting force that varied considerably from muscle to muscle; usually resting force was 0.2 - 0.5 times developed force. Resting force sometimes fell slightly (by up to 20%) during the first few minutes of the skinning period but thereafter remained constant.

Once the muscle had been skinned, Ca^{2+}-regulated contractures could be produced by alternately perfusing the muscle with relaxing solutions and activating solutions. The magnitude of the first contracture produced by bathing the skinned muscle in 'control' activating solution (pCa 3.75, pH 7.00, $\Gamma/2$ = 200 mM) was generally 150% - 250% of the force that had been developed by the intact muscle stimulated electrically at 1 s^{-1} (e.g. Figure 34). In 14 experiments performed at 25°C throughout, the Ca^{2+}-regulated force in the skinned muscles at pCa 3.75 was 209% ± 76% (mean ± S.E.M.) of the force developed by the unskinned muscle stimulated at 1 s^{-1} in modified Krebs-Henseleit solution.

In six experiments I attempted to produce the maximum possible activation of the myofibrils in the unskinned trabeculae so that

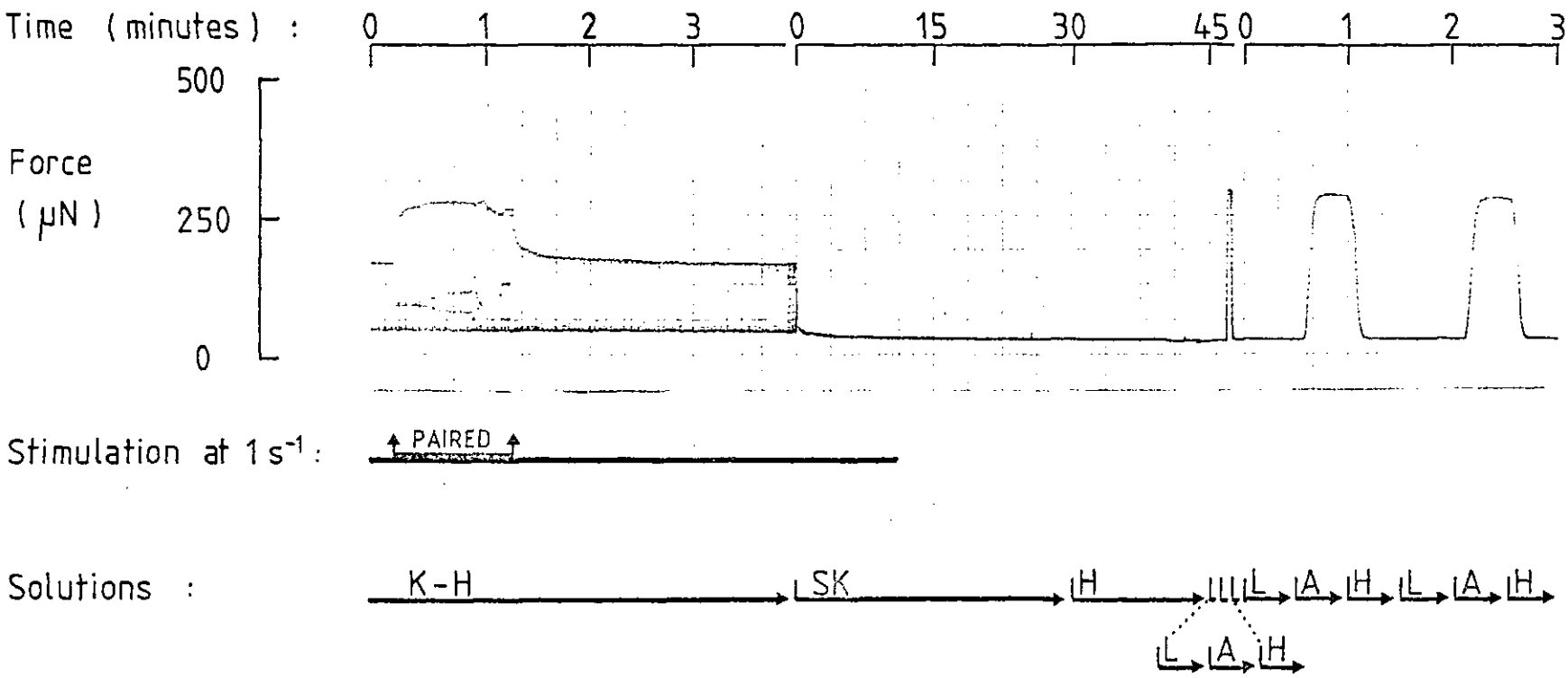


Figure 34. Forces in a guinea-pig trabecula before, during and after the skinning procedure. Solutions were: (K-H) modified Krebs-Henseleit solution; (SK) skinning solution; (H) high-EGTA relaxing solution; (L) Low-EGTA relaxing solution; (A) activating solution. Chart speed was reduced during the skinning period and for 5 minutes after. Temperature 25°C throughout. Muscle diameter 110 μm.

their force development could be compared with the maximum Ca^{2+}-regulated force in the skinned muscles (attained at pCa 3.75). Since cardiac muscles cannot ordinarily be tetanised it is almost impossible to unequivocally produce maximal activation of myofibrils in the intact cardiac cell. However it has been reported that stimulation of the muscles with paired electrical pulses in 4.0 mM Ca^{2+} produces what is probably the greatest degree of activation that can be obtained in mammalian cardiac muscles at 29°C (Wildenthal et al., 1975). Accordingly, in the six experiments the unskinned trabeculae were stimulated with paired pulses (pulse interval = 300 - 450 ms) in modified Krebs-Hensleit solution. The Ca^{2+} concentration of this solution was kept at 1.8 mM because at room temperature an increase in $[Ca^{2+}]$ to 4.0 mM produced no further increase in developed force (personal observation). The change in stimulation pattern from single pulses at 1 s^{-1} to paired pulses doubled the developed force (Figure 34), which was then similar in magnitude to the force of the first Ca^{2+}-regulated contracture in the skinned muscle. In fact in the six experiments the maximum Ca^{2+}-regulated force in the skinned muscle was 116% ± 29% (mean ± S.E.M.), which was not significantly different from 100%.

The resting force was constant during the second half of the skinning period and during the subsequent perfusions with relaxing solutions, if these solutions were used at room temperature (Figure 34). However this was not so in the pilot experiments for these studies, in which a perfusion temperature of 30°C was used. Under these conditions the muscles often developed an apparently irreversible contracture during or soon after the skinning procedure. The same phenomenon was sometimes seen at lower perfusion temperatures (e.g. 25°C) if the muscle diameter exceeded about 300 μm. The muscles could not be relaxed by an increase in the concentration of MgATP or EGTA in the skinning and relaxing solutions or by bubbling the skinning solution with 100% O_2. I found that these difficulties could be avoided if the skinning procedure was performed at a temperature of 25°C or below with muscles of diameter less than about 200 μm.

Figure 35 shows the relationship between the nominal cross-sectional areas of 29 skinned trabeculae and the corresponding Ca^{2+}-regulated forces produced by the first application of control activating solution at room temperature (20°C - 25°C). The solid line is the line of best fit determined by linear least-squares regression. Over the range of muscle diameters used (70 μm - 180 μm) force was proportional to cross-sectional area, though the scatter of data about the regression line was considerable ($r = 0.56$). The mean value for force/unit area was 20.7 kN m^{-2} (20.7 mN mm^{-2})

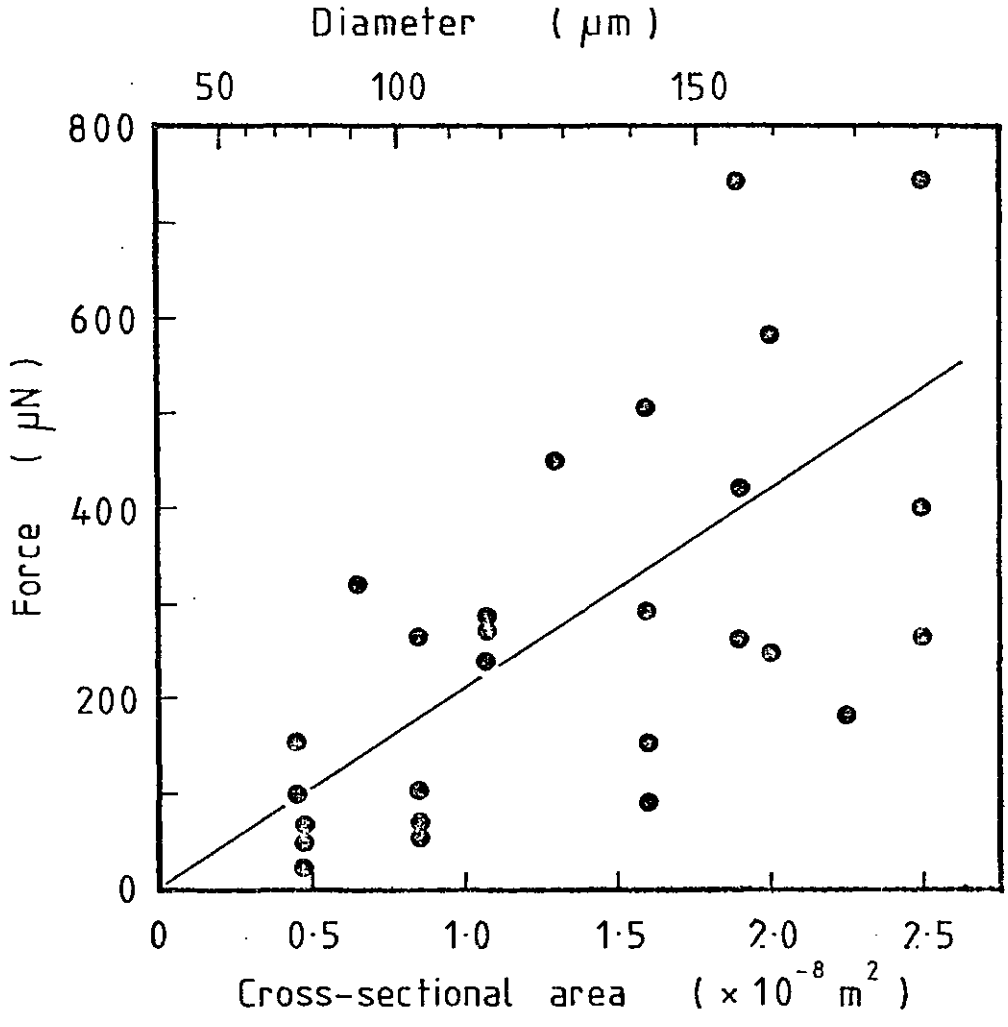


Figure 35. Relationship between the cross-sectional area of the skinned muscle and the Ca^{2+}-regulated force in the first contracture in control activating solution (pCa 3.75) at room temperature (20°C - 25°C).

Results from 29 muscles. Cross-sectional area calculated from the measured diameter assuming a circular cross-section (Section 9.12(a)). Details of least-squares regression line (solid line): slope = 20.7 kN m^{-2}, intercept on y-axis = 9.3 μN, r = 0.56.

and the maximum value was 49.2 kN m^{-2}. There was no evidence for a drop of force/unit area at the higher values of cross-sectional area, such as might have been expected if some of the cells in the muscle cross-section had not been adequately skinned.

Because both the absolute Ca^{2+}-regulated force and force/unit area varied widely from muscle to muscle, in this thesis the Ca^{2+}-regulated forces in each muscle are not generally given in terms of absolute force, but in relative terms, as a proportion of the Ca^{2+}-regulated force elicited in the control activating solution (pH 7.00, pCa 3.75, I/2 200 mM).

10.2 The relative force-pCa relationship determined by the 'staircase' and 'discrete' protocols

Figure 36 shows typical force records obtained while determining the relative force-pCa relationship by the 'staircase' protocol and the 'discrete' protocol. The staircase protocol was used in the earlier experiments but in the later experiments this was replaced by the discrete protocol, for reasons given in Section 9.7. During this change-over period I carried out experiments to determine whether the relative force-pCa relationships determined by the two protocols were the same. The mean results are given in Figure 37. The solid lines represent the least-squares fits to the modified Hill equation (Section 9.10); the fitted coefficients are given in the figure legend.

The Ca^{2+}-regulated force rose in sigmoidal fashion as the pCa of the activating solution was lowered from 6.5 to 3.75 (Figures 36 and 37), with half-maximum activation occurring at pCa $\approx$ 5.5, i.e. the pCa_{50} was $\approx$ 5.5. The relative force-pCa relationships obtained from both protocols were reasonably well fitted by the modified Hill equation. It is clear that the relative force-pCa relationships produced by the two protocols were very similar; in fact, the relative forces at each pCa were not significantly different ($p>0.05$, paired t-test). This similarity in the Ca^{2+}-activation curves was borne out by the values for the Hill parameter n_H, which reflects the steepness of the curve, and the pCa_{50}, which indicates the position of the curve on the pCa axis: the mean values for n_H and pCa_{50} were 1.70 and 5.51 for the staircase protocol and 1.75 and 5.50 for the discrete protocol. Thus it can be concluded that the results obtained by the two protocols were the same.

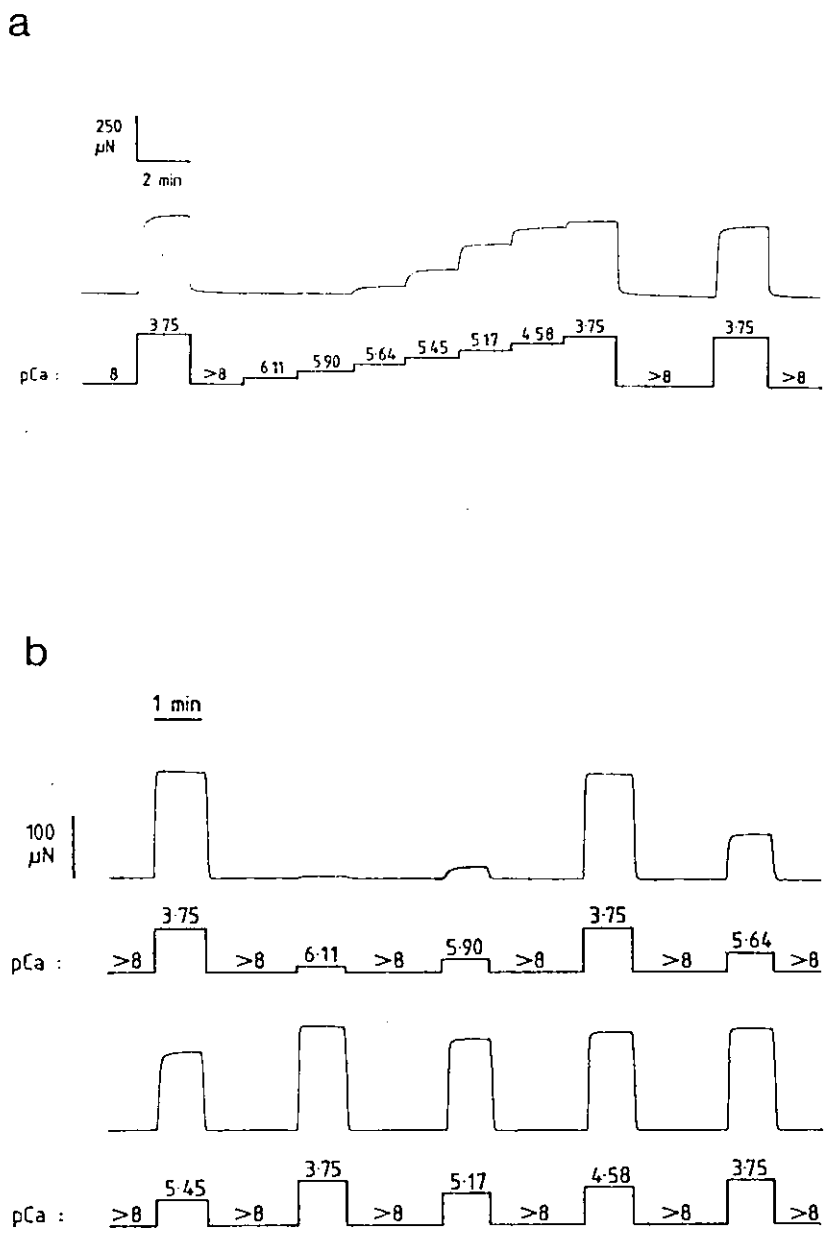
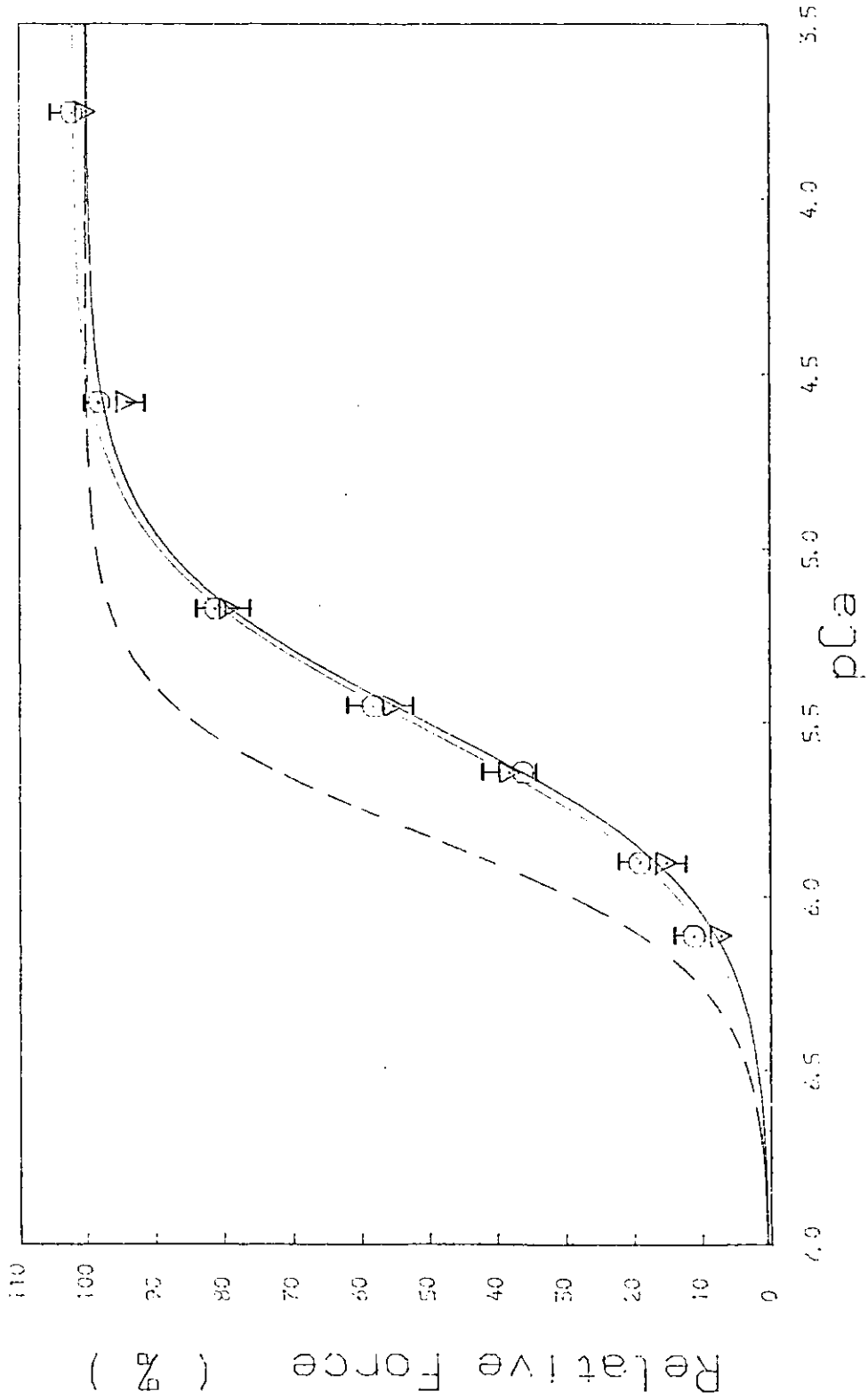


Figure 36. Chart records of forces in the two protocols that were used to determine the relative force-pCa relationship
(a) 'Staircase' protocol.
(b) 'Discrete' protocol.
Results from different muscles. 25°C.


Relative Force (%)
pCa
110
100
90
80
70
60
50
40
30
20
10
0
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5

In some experiments (not those shown in Figure 36) the staircase protocol gave relative forces of >1.00 at pCa 3.75. This occurred because the force produced by a given activating solution near or at the end of the 'staircase' was greater than the force produced by the same solution if it was used to produce a 'discrete' contracture; thus, for example, the force at pCa 3.75 at the end of the staircase exceeded that in the subsequent 'control' contracture at pCa 3.75 (see Section 9.7(a)). This resulted in a relative force of >1. This phenomenon was related to an upward 'creep' of force, which could be seen even in a contracture elicited by a single activating solution provided that the perfusion period was long enough. This behaviour is described in more detail in Section 10.3 below. The data from any muscles exhibiting this behaviour were discarded.

A rise in the Ca^{2+} concentration not only increased the steady Ca^{2+}-regulated force but also increased the rate of development of force in a contracture and thereby shortened the time taken to reach the plateau of force (Figure 36). This dependence of rate of force development upon pCa was not investigated further because it probably reflected merely the rate at which Ca^{2+} diffused through the skinned muscles from the bathing solution.

Figure 37 also shows (dashed line) the relative force-pCa relationship that would have been obtained from the discrete protocol if the purity of EGTA had been taken as 100%, rather than the 96.0% which was actually found by determination of the [Ca]:[EGTA] ratio (Section 9.2(c)). The fitted Hill equation gave n_H = 2.21 and pCa_{50} = 5.83 for 100% EGTA purity and n_H = 1.75 and pCa_{50} = 5.50 for 96.0% purity. This illustrates that a failure to take into account the impurity in EGTA results in relative force-pCa relationships that have artifactually-high values for n_H and pCa_{50}.

10.3 'Creep' of force during a prolonged contracture and appearance of 'residual force' after the contracture

One of the reasons for my eventual abandonment of the 'staircase' protocol was that the skinned muscles sometimes displayed an upward 'creep' of force during the 5 - 20 minutes that it took to apply all the activating solutions. Often, but not always, the contracture was then followed by incomplete relaxation i.e. a 'residual force' (Section 9.4) appeared. These phenomena could also be observed if

the skinned muscle was perfused with a single activating solution of low pCa for several minutes. An extreme example is shown in Figure 38, which is a force record from a muscle that exhibited this behaviour with only one minute's perfusion with control activating solution (second contracture, Figure 38). The creep of force or residual force was not seen in this muscle if the perfusion with activating solution was limited to only 15 s (first contracture, Figure 38).

It was as though the Ca^{2+}-dependent force remained constant throughout the contracture but that the true resting force increased and was manifest as an increase in the total force measure by the force transducer. A similar creep of force has been observed during prolonged tetanic contractions in frog skeletal muscle and has been attributed to a slowly-developing heterogeneity of sarcomere length along the muscle (discussed fully in Huxley, 1980). Whatever the cause, the creep in force during prolonged contractures made it difficult to estimate the true Ca^{2+}-regulated force. To avoid this problem, contractures were kept as short as possible. Contracture duration was minimised by (i) replacing the 'staircase' protocol by the 'discrete' protocol and (ii) using the 'rapid activation' procedure.

10.4 The relative force-pCa relationships at 25°C and at 30°C

Although the initial experiments on the detergent-treated muscles were carried out at 30°C, in later experiments the temperature was lowered to 25°C to improve muscle viability (Appendix B, Section B.3(e)). Figure 39 shows the mean relative force-pCa relationships from 14 experiments carried out at 25°C using the discrete protocol only and from 20 other experiments carried out at 30°C using either the staircase or the discrete protocol. There was no significant difference between the relative forces obtained at a given pCa at 25°C and at 30°C. Because the relative force-pCa curves were insensitive to temperature over this range, meaningful comparisons could be made between the results from experiments performed at 25°C and the results from experiments at 30°C.

A rigorous study was not carried out on the influence of temperature on the absolute magnitude of Ca^{2+}-regulated force; however I did note that raising the perfusion temperature usually resulted in a slight increase in Ca^{2+}-regulated force at pCa 3.75 (e.g. Figure 64, Appendix B). This apparently beneficial effect

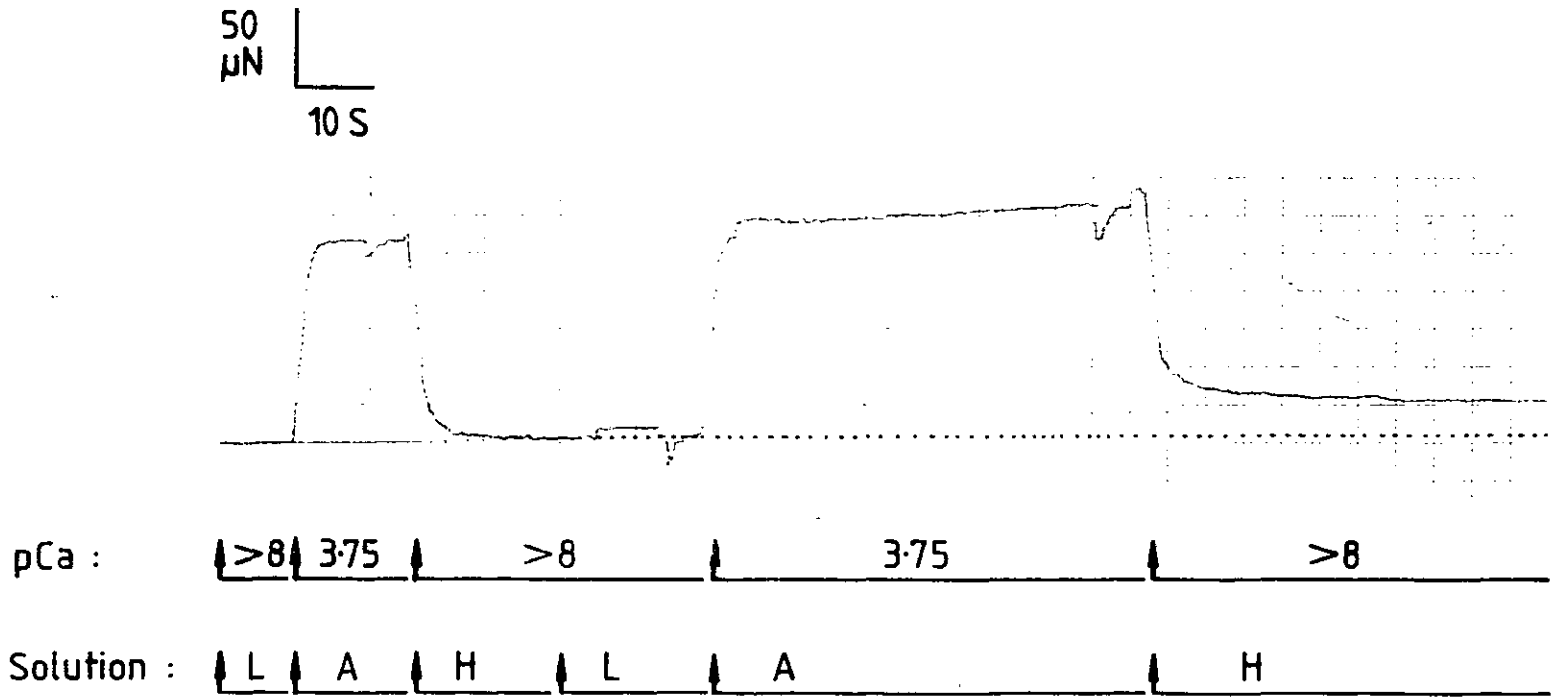


Figure 38. Creep of Ca^{2+}-regulated force during a 60 s contracture at low pCa and appearance of residual force after the contracture. Solutions were:
(H) high-EGTA relaxing solution, (L) low-EGTA relaxing solution, (A) control activating solution, pCa 3.75. Magnitude of resting force before the 60 s contracture is shown by the dotted line.

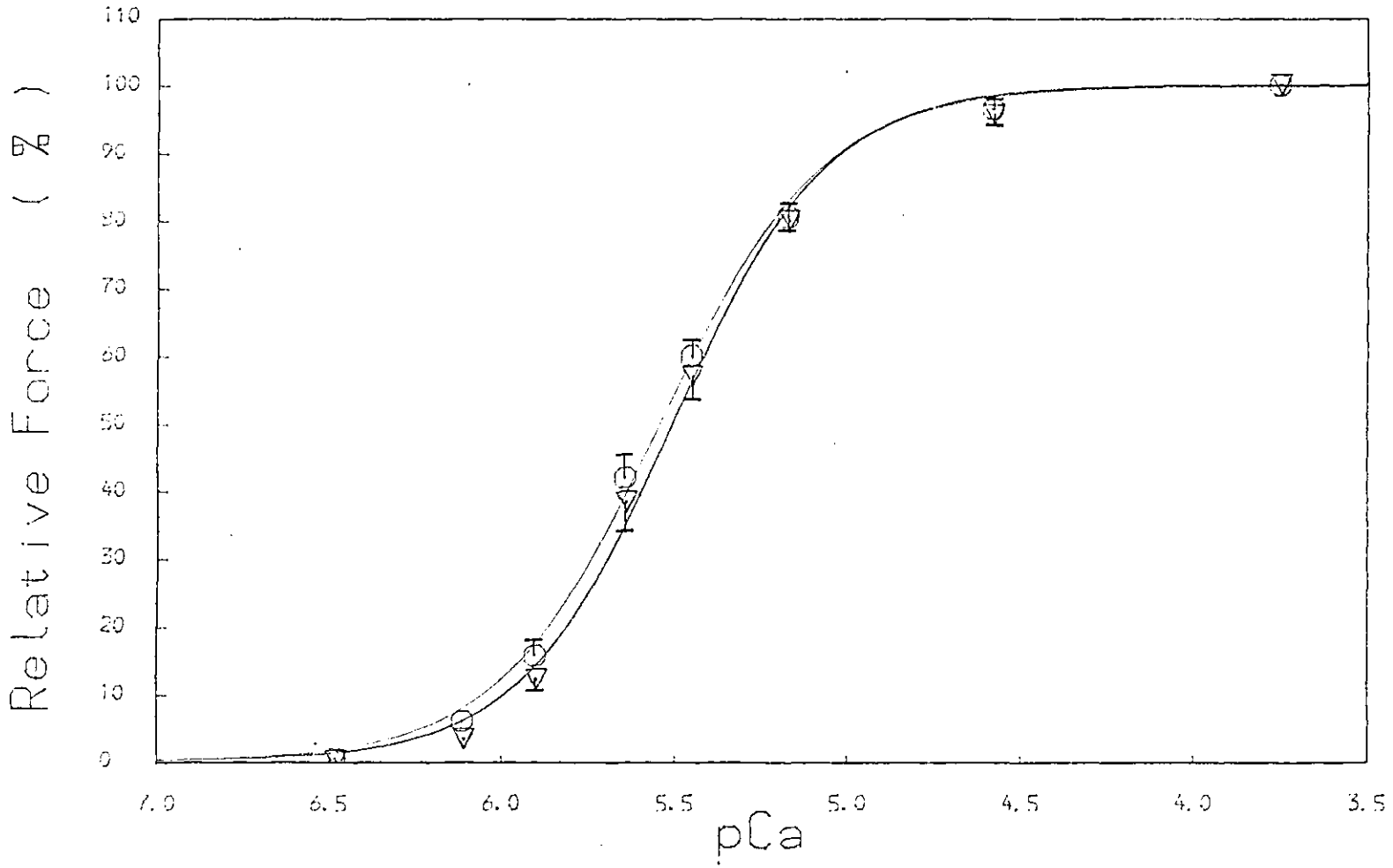


Figure 39. Relative force-pCa relationship at 25°C (⊙) and at 30°C (▽). Mean values ± S.E.M. of 14 experiments at 25°C and of 20 experiments at 30°C.
Coefficients of the modified Hill equation (solid lines) were:
25°C: n_H, 1.96; K*, 1.6×10^{-11}; pCa_{50}, 5.51
30°C: n_H, 1.86; K*, 5.1×10^{-11}; pCa_{50}, 5.54.

of a higher temperature was however overwhelmed by the detrimental effects of a higher temperature on muscle viability (Appendix B, Section B.3(e)).

10.5 Relaxation of rigor contractures by CP or phosphoenolpyruvate: a test for the presence of ATP-regenerating enzymes

If the MgATP is removed from the solution bathing a skinned muscle, actin and myosin remain attached at the end of the cross-bridge stroke with the cross-bridges held in a constrained configuration (Figure 4). The number of such attachments slowly increases, with the result that the muscle becomes stiff and develops a maintained force - a 'rigor contracture' - even if there is no Ca^{2+} present (Reuben et al., 1971; Fabiato & Fabiato, 1975). When the MgATP is resupplied the actin and myosin dissociate and the muscle relaxes. I employed this relaxation of rigor contractures as an indirect means of testing for the presence and activity of ATP-regenerating enzymes in my skinned muscles (as in McClelland & Winegrad, 1978). The reactions of interest were:

$$MgADP^{-} + HCP^{2-} + H^{+} \xrightleftharpoons[\text{creatine kinase}]{} MgATP^{2-} + \text{creatine}$$

$$MgADP^{-} + \underset{(PEP)}{\text{phosphoenolpyruvate}} \xrightleftharpoons[\text{pyruvate kinase}]{} MgATP^{2-} + \text{pyruvate}^{-}$$

The rationale of the test was that if, for example, creatine kinase activity was present, the addition of MgADP plus CP to a skinned muscle in rigor contracture should result in regeneration of MgATP and thus lead to relaxation of the rigor contracture. A similar procedure using MgADP + PEP was employed to test for pyruvate kinase activity. In these experiments no creatine kinase (or pyruvate kinase) was added to the bathing solutions, and no CP was present unless otherwise stated. All solutions contained 10 mM K_2H_2EGTA and thus had a pCa>8.

The muscles were first put into rigor by replacing the 5 mM K_2Na_2ATP + 6 mM $MgCl_2$ in the relaxing solution with 10 mM NaCl + 1 mM $MgCl_2$ + 15 mM KCl. After the solution changeover the muscles slowly developed a rigor contracture after a lag period of about 20 - 30 seconds (Figure 40). The peak force attained in a rigor contracture was generally about half of that observed in a Ca^{2+}-regulated contracture in the control activating solution (pCa 3.75, pH 7.00, $[MgATP^{2-}]$ = 4.2 mM), in accord with previous investigations using cardiac muscle

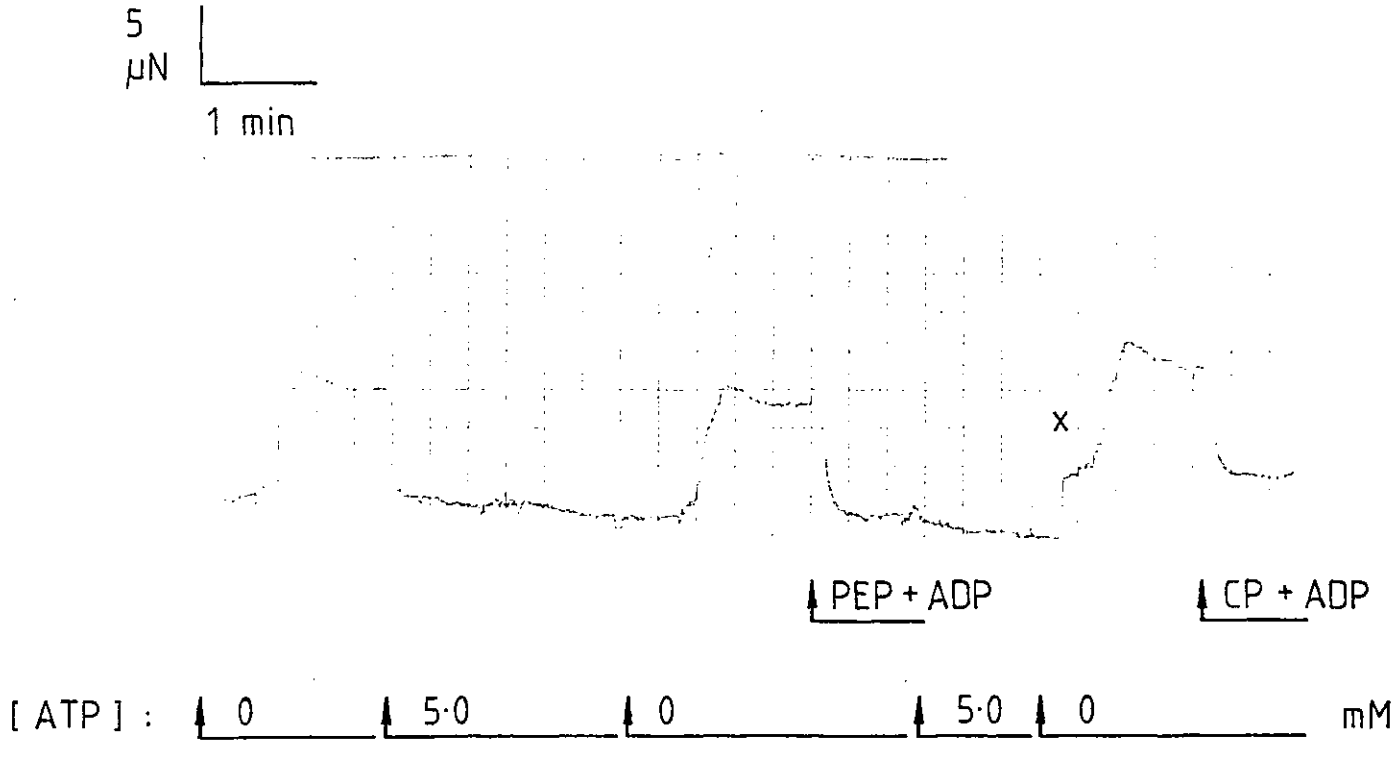


Figure 40. Relaxation of rigor contractures by high-energy phosphate compounds.

PEP = phosphoenolpyruvate. pCa>8 throughout. Offset introduced at point 'X' to prevent the pen going off scale.

(Fabiato & Fabiato, 1975). All rigor contractures displayed a peak in force, followed by a small and incomplete relaxation. The skinned muscle relaxed rapidly and completely if it was resupplied with MgATP (Figure 40). Similar relaxation of a rigor contracture was seen if the muscle was perfused with a solution containing 10 mM CP + 5 mM MgADP or 10 mM PEP + 5 mM MgADP in place of the 5 mM MgATP (Figure 40). Neither CP nor PEP had this effect if the MgADP was omitted from the solution. These results provided good evidence for the retention of considerable creatine kinase and pyruvate kinase activity in the skinned trabeculae.

10.6 Structure of the detergent-treated muscles

(a) Light microscopy

A photomicrograph of a skinned trabecula is shown in Figure 41 . The diameter of this muscle (110 μm) was typical but the length (3 mm) was 2 - 3 times that usually found. Except for the loss of colour, which was used as a visual assessment of skinning (Section 9.5(c)), no changes in the structure of the trabeculae could be detected by observation through the dissecting microscope (at x40) during and after the skinning procedure. Although muscle diameters could be measured to the nearest 10 μm with the eyepiece graticule, there was no evidence for any change in the diameter of each muscle during the superfusion with skinning solution.

(b) Electron microscopy

Trabeculae that had been fixed and processed for electron microscopy immediately after the period of equilibration in modified Krebs-Henseleit solution exhibited the ultrastructure typical of healthy myocardium (Figure 42). There were apparently-relaxed myofibrils (but see below), mitochondria packed with cristae, abundant granules (probably glycogen granules), and an intact sarcolemma with an amorphous basement coat. In most of the muscles there was a considerable amount of collagen running parallel with the myofibrils, though this is not visible in Figure 42 .

In muscles treated for 30 minutes with the skinning solution there were obvious changes in morphology (Figure 43). The most striking alteration was the reorganisation of the cellular membranes into multi-laminar whorls, resembling myelin sheaths seen in cross-section. Membranes throughout the entire cross-section of the muscle were affected in this way. The sarcolemmal membrane appeared to be absent in places but the basement coat remained. Glycogen granules were

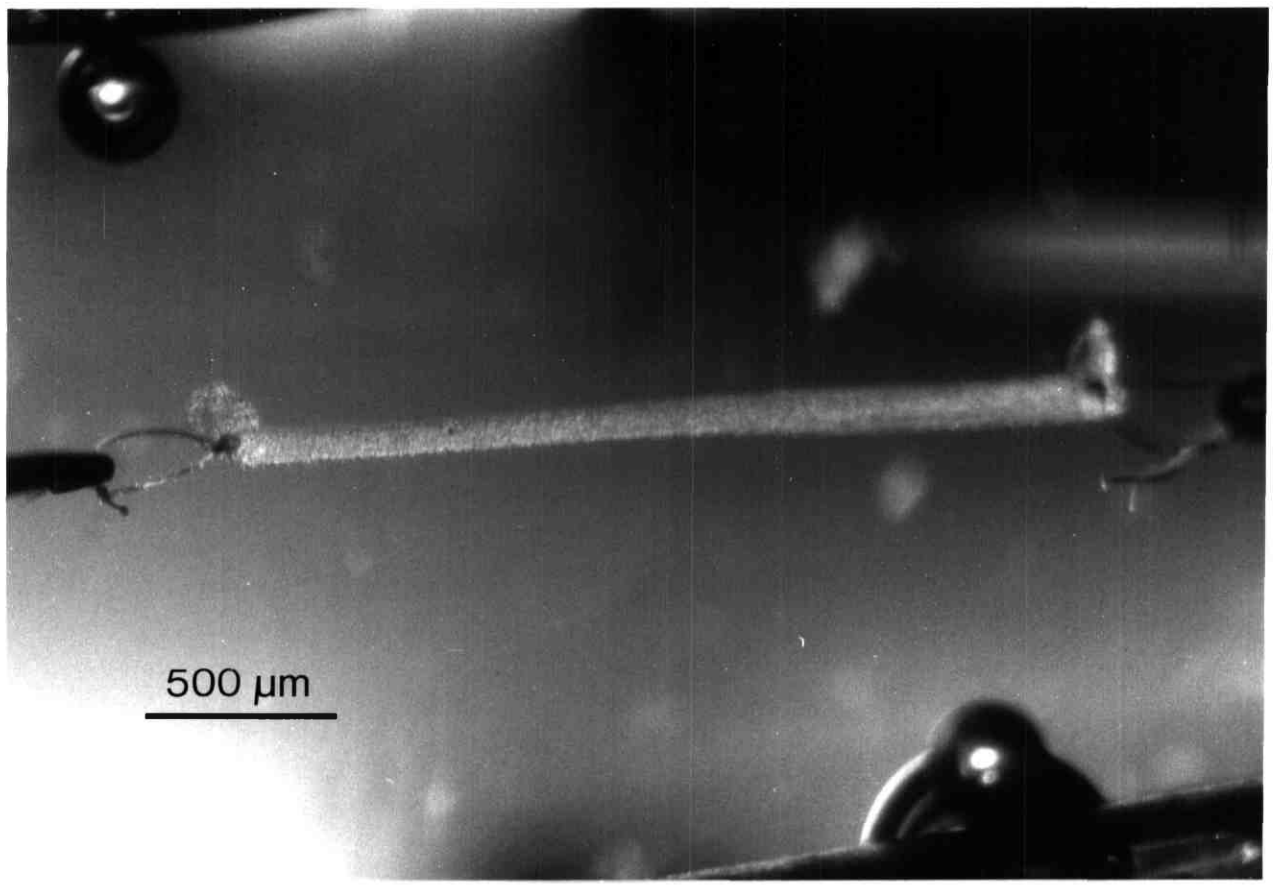


Figure 41. Photomicrograph of a skinned trabecula. Muscle tied between the two hooks in the muscle bath and bathed in high-EGTA relaxing solution.

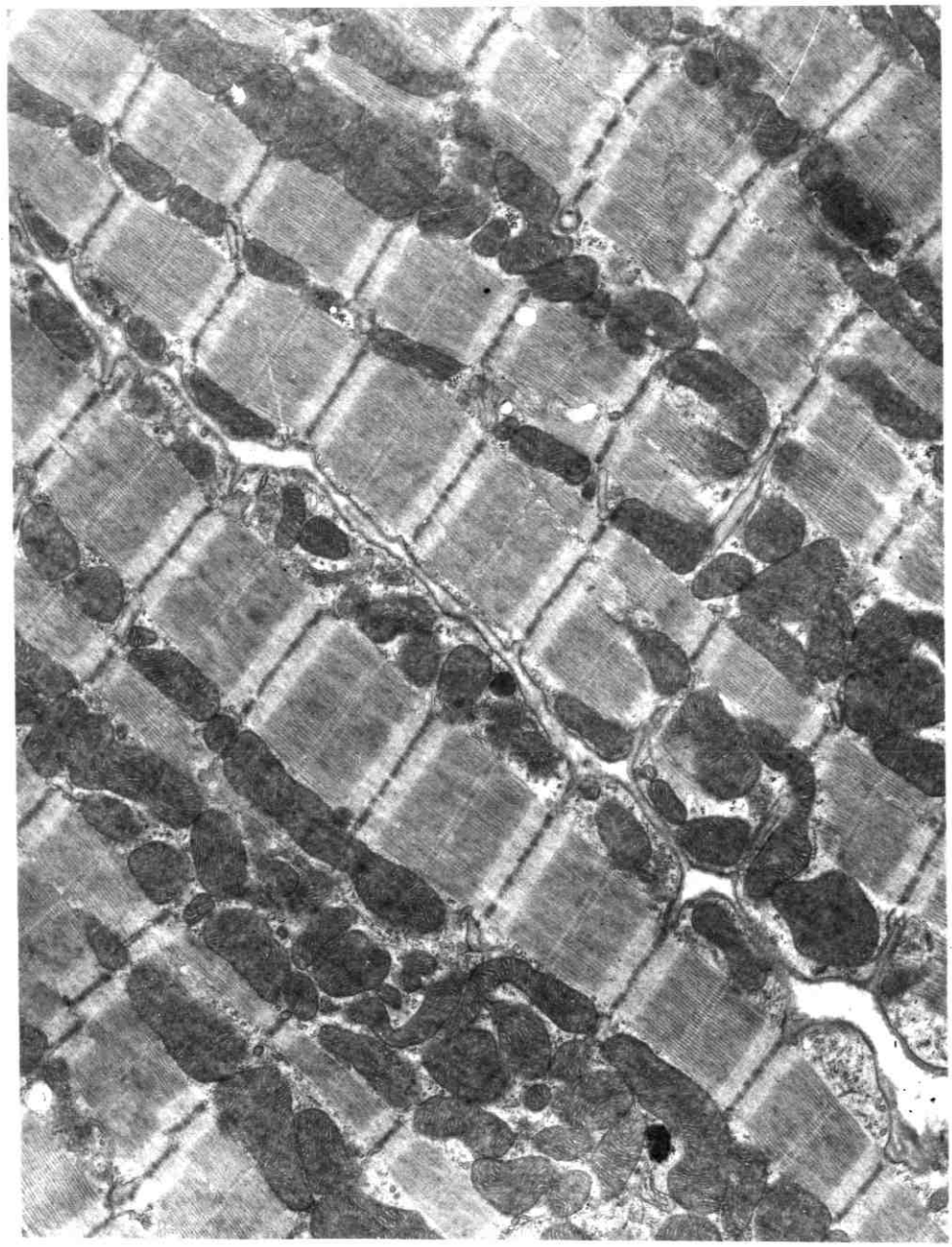


Figure 42. Electron micrograph of an unskinned trabecula. Muscle fixed in 4% glutaraldehyde after the equilibration period in modified Krebs-Henseleit solution at 22°C. Sarcomere length in this photo ≈ 2.4 μm. x 9000

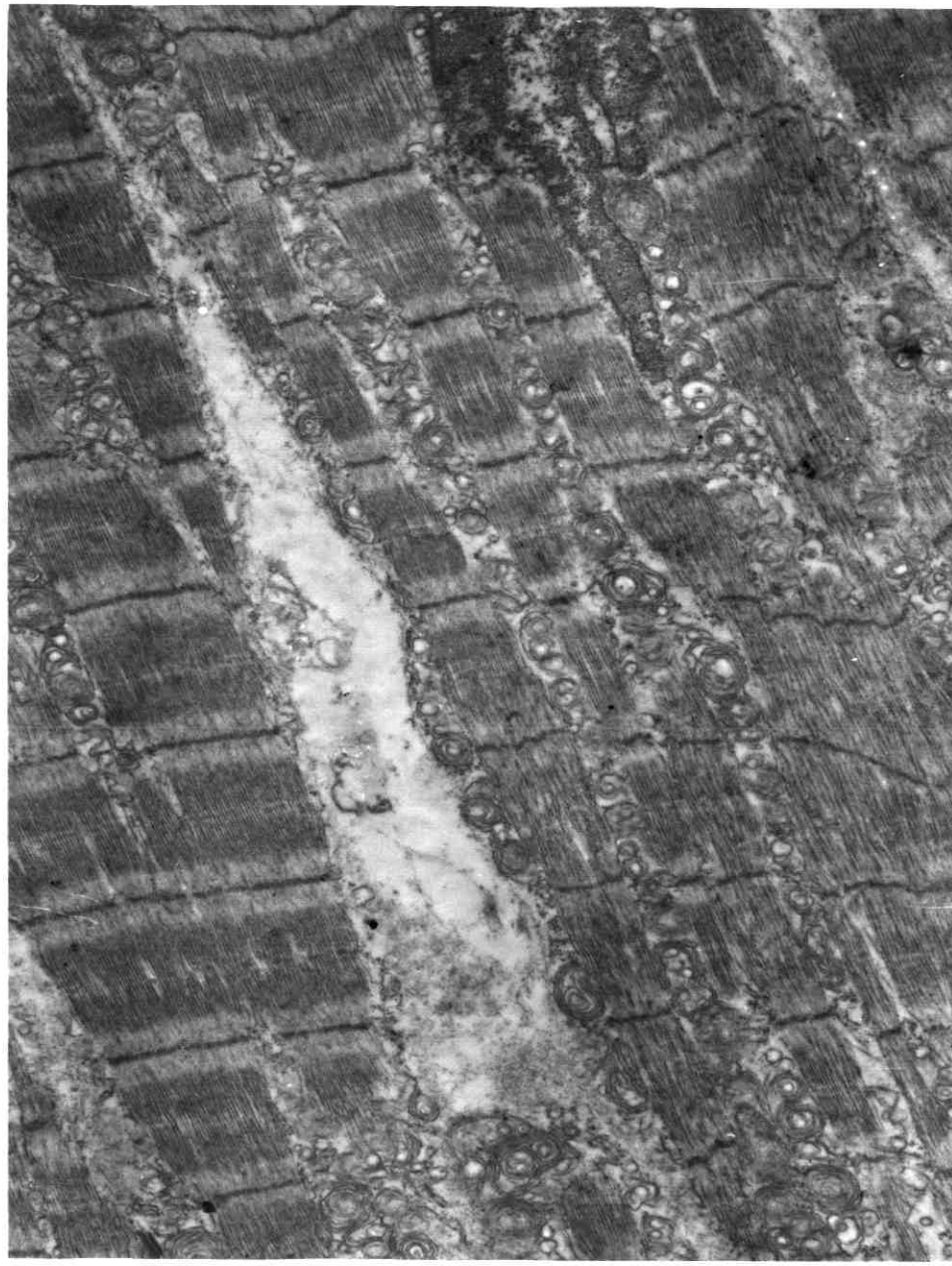


Figure 43. Electron micrograph of a skinned trabecula. Muscle fixed after 30 min in skinning solution plus 15 min in high-EGTA relaxing solution at 23°C. Sarcomere length in this photo ≈ 2.4 μm. x 8000.

usually lost. However, the skinning solution did not apparently alter the morphology of the myofibrils. The Z-line and M-line were still present and the arrays of thick and thin filaments generally remained in register, although some distortion of sarcomere structure was apparent in one or two places.

The continued recording of force during the fixation period indicated that both unskinned and skinned muscles developed moderate contractures during this period, probably because of lack of MgATP i.e. the muscles developed rigor contractures.

10.7 Permeability of the detergent-treated muscles to ferritin: a test for skinning

If, as I assumed (Section 9.5(c)), myoglobin molecules were lost from the cardiac muscle cells during treatment with skinning solution, then the sarcolemma must have become permeable to large molecules. To examine this further I tested whether ferritin molecules could readily enter the Triton-treated muscles.

Each ferritin molecule (molecular weight $\approx$ 750000 Daltons) consists of an 11 nm-wide protein shell enclosing a dense core of ferric hydroxide. This core, which contains about 3000 iron atoms, is electron-dense and so is visible in the electron microscope. This allows the location of individual ferritin molecules to be determined.

At the end of several experiments I transferred the muscles, which were still tied to the transducer, to a beaker containing 50 mg of ferritin in 5 cm^3 of low-EGTA relaxing solution. Ten minutes later the muscles were rinsed in low-EGTA relaxing solution without ferritin and were fixed and processed for electron microscopy.

Electron micrographs from such muscles revealed that ferritin molecules were dispersed throughout each muscle (Figure 44). Ferritin molecules were visible in every intermyofibrillar space but were usually excluded from the spaces between the myofilaments. The distribution of ferritin molecules throughout these muscles would appear to be good evidence that the selective permeability barrier of the sarcolemma had been destroyed by the detergent.

It is interesting that some of the ferritin attached specifically to the N-lines, which lie between the Z-lines and the ends of the thick filaments (see Figure 1). This is precisely how Franzini-Armstrong (1970) first came to notice the N-lines in skinned skeletal muscle.

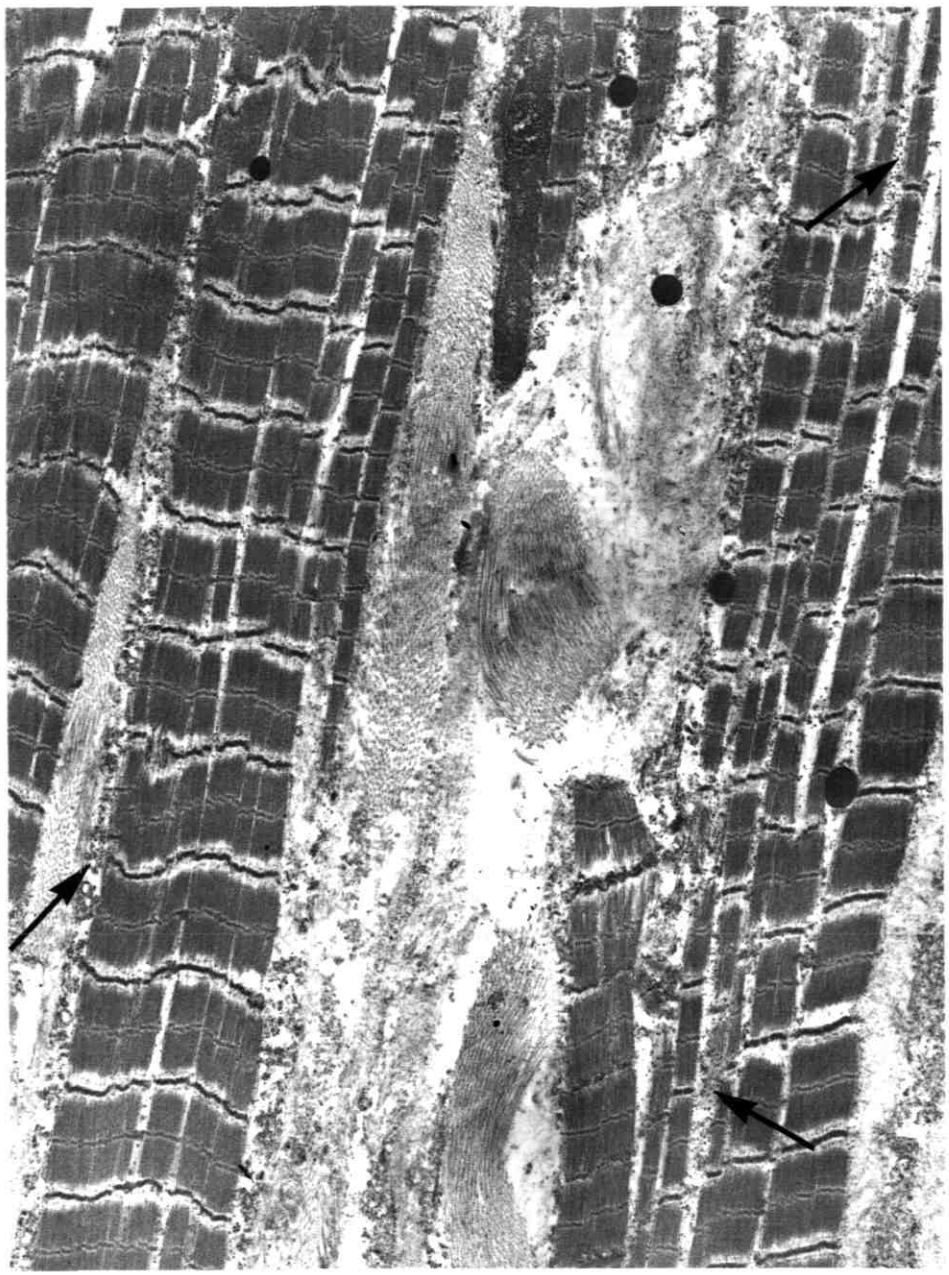


Figure 44. Electron micrograph of a detergent-treated trabecula that was fixed after exposure to ferritin.
Muscle fixed after a 15 min soak in 10 mg cm^{-3} ferritin in low-EGTA relaxing solution. Prior to this, muscle had undergone many contractures. Ferritin is shown by the arrows. x 7000.

10.8 The effect of the residual membrane vesicles in the skinned trabeculae on Ca^{2+}-activation

Electron micrographs of the skinned muscles showed that Triton X-100 treatment of the muscles did not remove all the cellular membranes - some membranes were merely reorganised into whorls. The possibility existed that these vesicular structures could have taken up Ca^{2+} after the change from a relaxing to an activating solution and released this Ca^{2+} when relaxing solution was reapplied. To test for Ca^{2+}-sequestering activity, I added 10 mM caffeine or 5 mM NaN_3 to the relaxing and activating solutions. These substances inhibit Ca^{2+} uptake by the SR and mitochondria, respectively (Fanburg, 1964; Weber, 1968). If there had been any Ca^{2+}-sequestering activity that could be inhibited by caffeine or NaN_3, the addition of the substances would have been expected to have accelerated the rate of rise of the $[Ca^{2+}]$, and thus of force, in a Ca^{2+}-activated contracture. However neither 10 mM caffeine nor 5 mM NaN_3 altered the rate of change of force in muscles activated by the rapid activation procedure (Figure 45). This suggested that the membrane whorls had no appreciable Ca^{2+}-sequestering activity.

10.9 Deterioration of the contractile performance of the skinned muscles: the 'decline' in Ca^{2+}-regulated force

If the skinned muscles were repeatedly perfused with the same relaxing solution and activating solution, the Ca^{2+}-regulated force was not reproduced but fell slightly in successive contractures. Examples of this progressive deterioration in contractile performance can be seen in Figures 34 and 36 . This phenomenon, which is termed the 'decline' in Ca^{2+}-regulated force in this thesis, is in fact a characteristic property of almost all types of skinned muscle preparation. This decline in force is a nuisance because it limits the viability of the skinned muscle and makes corrections necessary. More seriously it implies that the properties of myofibrils in unskinned and skinned muscle may be different. Therefore in a series of experiments I varied the experimental conditions and solution composition so as to characterise the decline of force, with a view to reducing its magnitude. Because these experiments are not central to the main theme of this thesis, they are described in detail in an appendix (Appendix B). However some of the conclusions from these experiments have a bearing on the next two sections. These conclusions are summarised below:

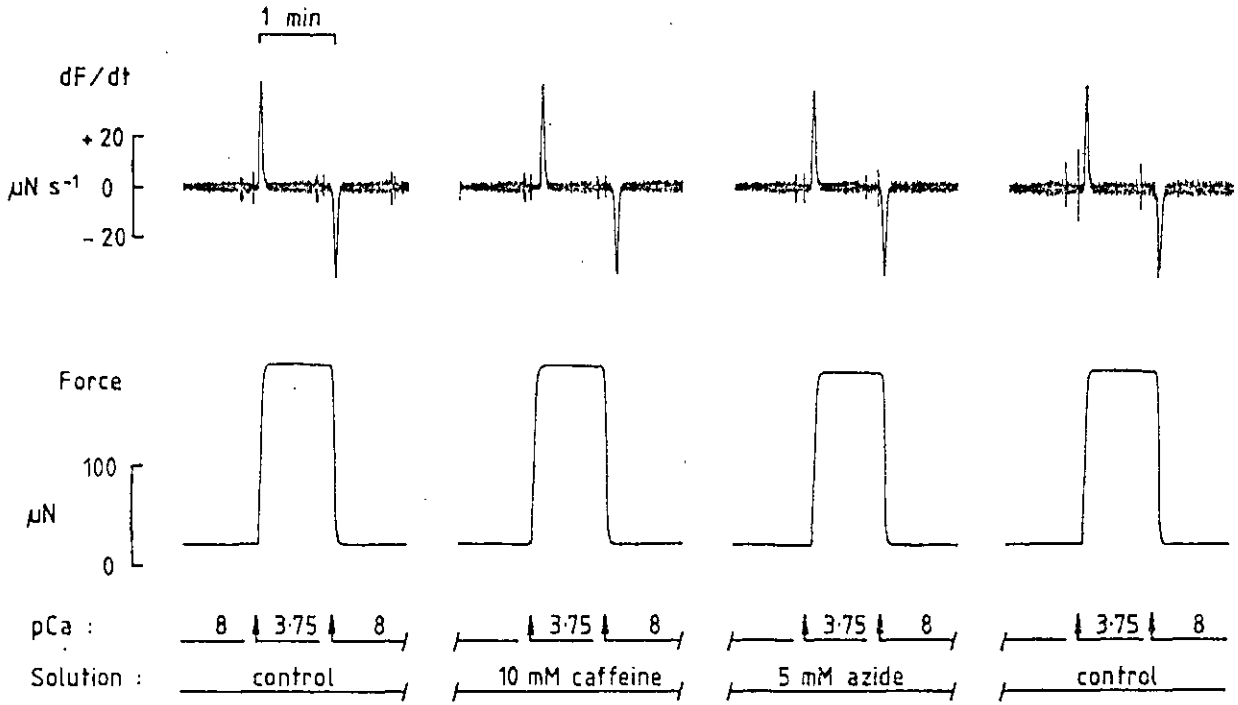


Figure 45. Lack of effect of 10 mM caffeine or 5 mM NaN_3 on the rate of rise of Ca^{2+}-regulated force in a skinned trabecula.

Rapid activation procedure. Muscle equilibrated in high-EGTA relaxing solution plus caffeine or azide before the contracture was produced.

(i) The decline in Ca^{2+}-regulated force was due to deterioration of the muscle rather than of the solutions.

(ii) In a series of regularly-spaced contractures produced by the repeated application of the same activating solution, the Ca^{2+}-regulated force declined as an exponential function of time (or of the number of contractures). Thus plots of log force against time were linear.

(iii) The relationship between relative force and pCa was not altered by the decline in the absolute magnitude of force.

10.10 The influence of pH on the force production of the skinned cardiac muscle

The 'staircase protocol' (Section 9.7(a)) was used to determine the relative force-pCa relationship at each pH studied (7.20, 7.00, 6.80 and 6.40) at 30°C. All solutions were of standard composition and contained 25 μg cm^{-3} creatine kinase. Typical results from this protocol are illustrated in Figure 46, which shows a chart record of resting and Ca^{2+}-regulated forces at pH 7.00 and at pH 7.20.

(a) Resting force

It is apparent from Figure 46 that the resting force was not altered when the pH of the relaxing solution was changed from 7.00 to 7.20 or when the pH was returned to 7.00. In fact I found the resting force to be unaffected by any changes in pH within the pH range 7.20 - 6.40.

(b) Maximum Ca^{2+}-regulated force

In contrast to the resting force, the maximum Ca^{2+}-regulated force (at pCa<4.0) was highly sensitive to the pH of the bathing solution. In an activating solution of optimal pCa, the muscle developed more force at pH 7.20 than at 7.00 (Figure 46). Comparisons between the effects of all four values of pH on the maximum Ca^{2+}-regulated force can be made with reference to Figure 47, which shows the mean relative force-pCa relationship for each pH value studied. In this figure all forces are expressed relative to the steady Ca^{2+}-regulated force in the 'control' activating solution (pH 7.00, pCa 3.75, I/2 = 200 mM). The solid lines represent the fittings of the modified Hill equation to the data. The coefficients for the fitted equations are listed in Table 11 (page 205). Note that the relative force-pCa relationship at pH 7.00 was determined twice in each experiment, once soon after the muscle had been skinned (filled circles, Figure 47) and again at the end of the experiment (open circles). Corresponding points in these two curves were not significantly

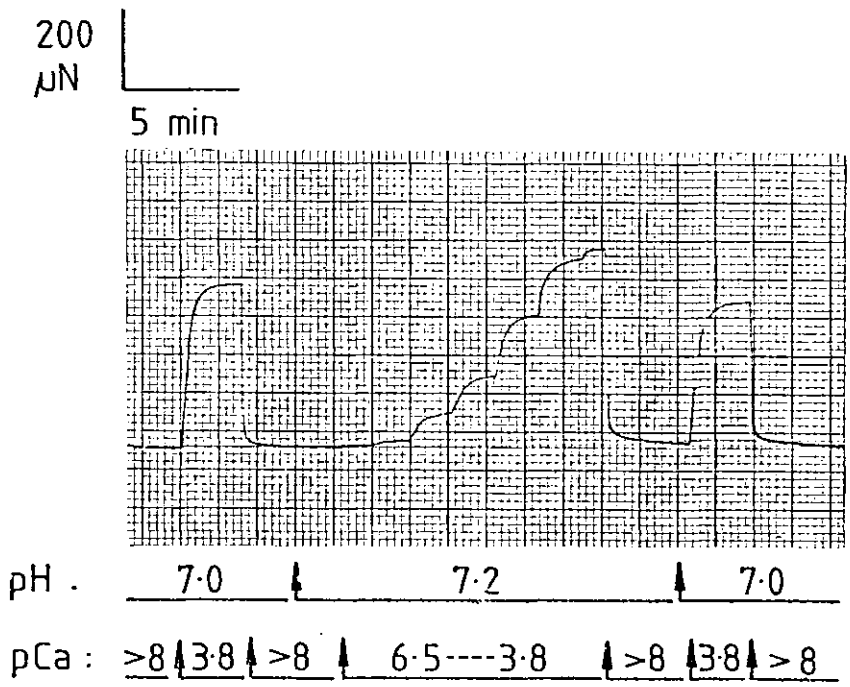


Figure 46. Typical record of forces in the detergent-skinned cardiac muscle at pH 7.00 and at pH 7.20.

'Staircase' protocol. 30°C.

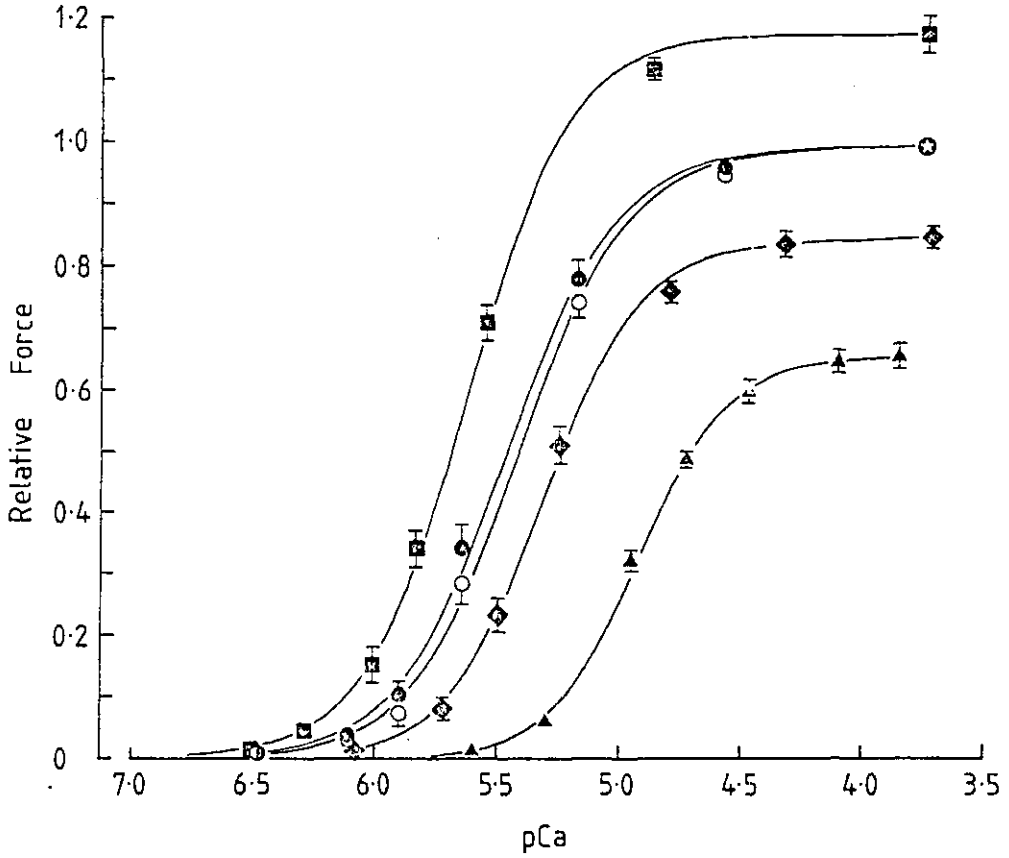


Figure 47. Influence of pH on the relative force-pCa relationship in detergent-skinned cardiac muscle. (▣) pH 7.20; (○●) pH 7.00; (◈) pH 6.80; (▲) pH 6.40. All forces expressed relative to the maximum Ca^{2+}-regulated force at pH 7.00. Relative force-pCa relationships at pH 7.00 were determined at the beginning, (●) and at the end (○) of each experiment. Each point shows the mean ± S.E.M. of 6 experiments. Where error bars are not visible S.E.M. < size of symbol. The coefficients of the fitted Hill equations are given in Table 11.

different ($p>0.05$, paired t-test).

At each pH the maximum force was produced by activating solutions of pCa<4.0, but it is clear that the magnitude of this maximum force fell markedly with each step reduction in pH over the range pH 7.20 - pH 6.40. Relative to the maximum Ca^{2+}-regulated force at pH 7.00, force at optimal pCa was 1.18 ± 0.03 at pH 7.20, 0.85 ± 0.02 at pH 6.80 and 0.66 ± 0.02 at pH 6.40 (mean ± S.E.M., n = 6). All these values were significantly different from 1.00 ($p<0.002$, paired t-test). The decrease in force was not linearly related to the reduction in pH; rather the pH-sensitivity of maximum Ca^{2+}-regulated force became less as the solution was made more acid. However the decrease in the log of force was almost linearly related to the decrease in pH of the solution (Figure 48).

(c) Relationship between pCa and relative force

Figure 47 also reveals that the relative force-pCa relationship was shifted to lower pCa values, that is to higher Ca^{2+} concentrations, as the pH of the activating solutions was reduced. This decrease in Ca^{2+}-sensitivity of force with decrease in pH is shown more clearly if the data in Figure 47 are normalised so that the forces at a given pH are expressed relative to the maximum force at the same pH (Figure 49). Each step alteration in pH produced an almost symmetrical shift in the Ca^{2+}-activation curve along the pCa axis. To obtain an objective estimate of the pCa_{50} at each pH, its value was calculated from the Hill coefficients n_H and K^*, which were obtained from the least-squares fits of the modified Hill equation to the mean data shown in Figures 47 and 49 . The values for n_H, K^* and pCa_{50} at each pH are listed in Table 11 . Also, because the relative force-pCa relationships in each experiment were well fitted by the modified Hill equation, I was able to calculate the n_H, K^* and pCa_{50} at each pH in each experiment and then find their mean values. These values are also listed in Table 11 . Corresponding values for pCa_{50} obtained by the two procedures should be, and were identical. However, as discussed in Section 7.4, the mean value for n_H is likely to be greater than the corresponding n_H relating to the mean data. This was what was indeed observed (Table 11). In other words, the true slope of the relative force-pCa relationship at a given pH was slightly greater than slope of the curve fitted to the mean data in Figures 47 and 49 . Below I shall refer only to the mean values for n_H, K^* and pCa_{50}.

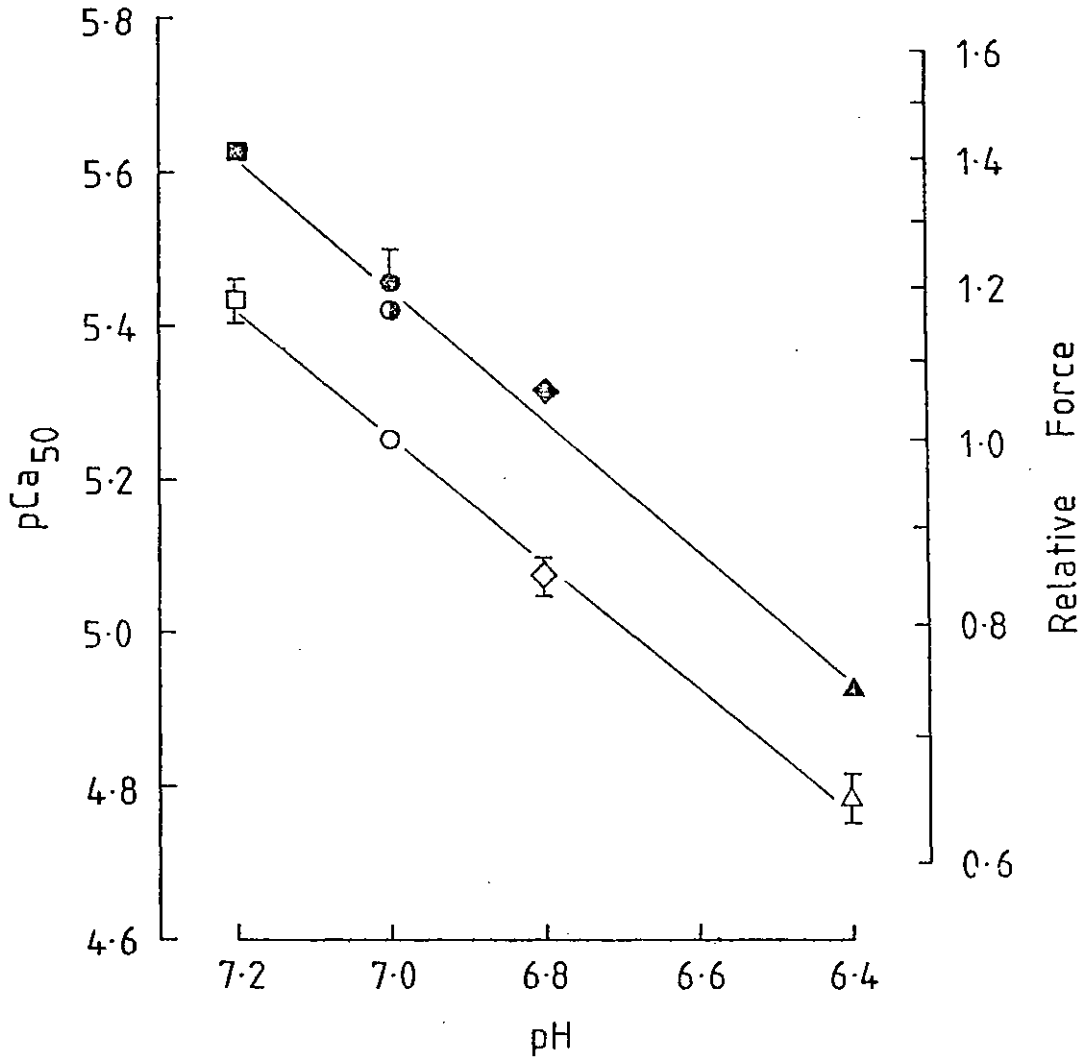


Figure 48. Influence of pH on the maximum Ca^{2+}-regulated force (open symbols) and on the pCa_{50} (closed symbols).

Force expressed relative to the maximum Ca^{2+}-regulated force at pH 7.00. Note log scale for force. pCa_{50} calculated from the Hill coefficients n_H and K* (see Table 11). 30°C.

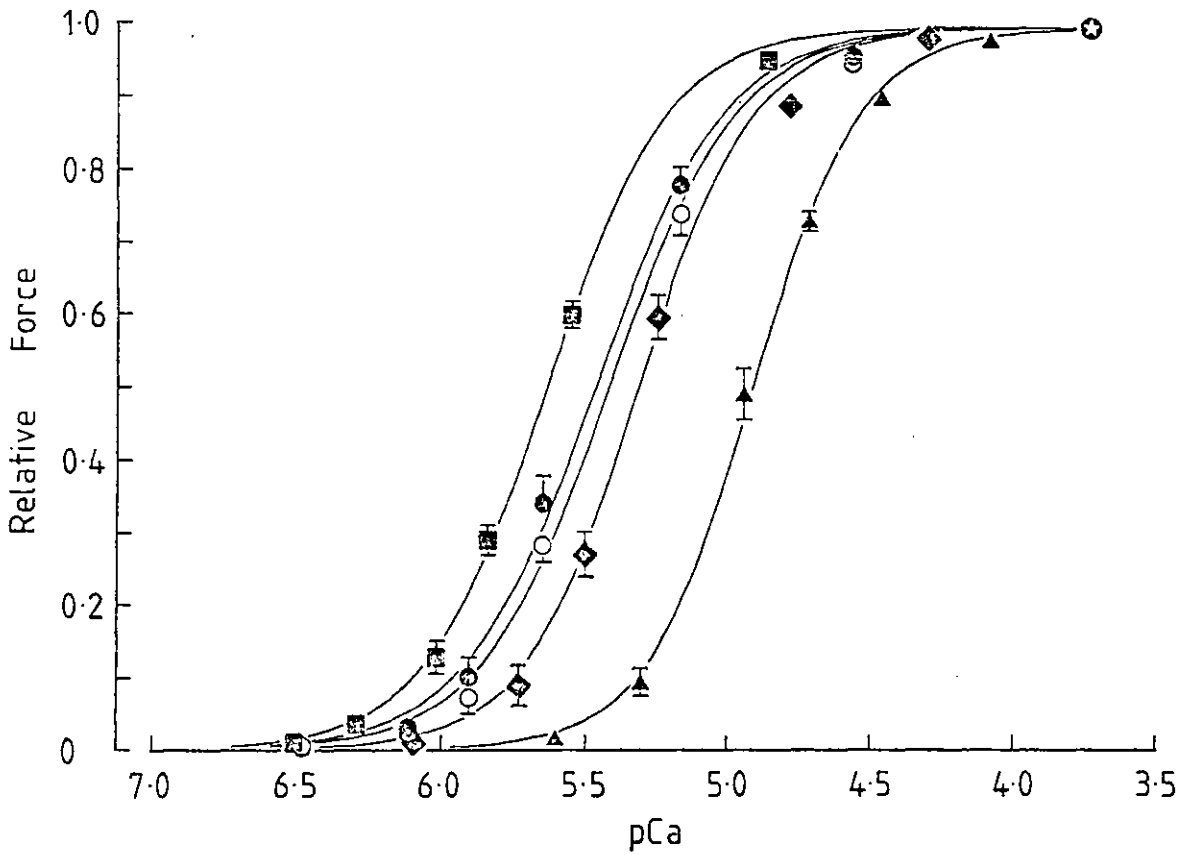


Figure 49. Influence of pH on the relative force-pCa relationship in detergent-skinned cardiac muscle. (▣) pH 7.20; (⊙○) pH 7.00; (◈) pH 6.80; (▲) pH 6.40. Data of Figure 46 normalised so that each force at a particular pH is expressed relative to the maximum Ca^{2+}-regulated force at the same pH. Other details as in Figure 46.

		pH				
		7.20	7.00(i)	7.00(ii)	6.80	6.40
(a)	n_H	2.06	1.94	1.98	2.18	2.35
	K* (x 10^{-12}M)	2.39	25.3	19.4	2.46	2.77
	pCa_{50}	5.63	5.46	5.42	5.31	4.92
(b)	n_H	2.12 ± 0.13	2.01± 0.06	2.01± 0.09	2.20*± 0.02	2.43*± 0.11
	K* (x 10^{-12}M)	42.11 ±35.14	27.50±16.68	82.48±66.33	2.38± 0.56	7.64± 3.50
	pCa_{50}	5.64* ± 0.02	5.46± 0.04	5.42± 0.02	5.31*± 5.03	4.92*± 0.02

* significantly different from the corresponding value for pH 7.00(i) (paired *t*-test, $p<0.05$).

Table 11. Details of the sigmoid curves fitted to the data in Figures 47 and 49.
pH 7.00 (i) and pH 7.00 (ii) refer to the relative force-pCa relationships at pH 7.00 that were determined at the beginning and end, respectively, of each experiment.

(a) Hill coefficients n_H and K*, and the pCa_{50} calculated from them (Section 9.10), for the curves fitted to the mean data of Figures 47 and 49.

(b) Mean values of n_H, K* and pCa_{50} for the curves fitted to the data in each experiment. Mean ± S.E.M. of six experiments.

The mean pCa_{50} at pH 7.00 was 5.44 (average of the mean values for pH 7.00 (i) and pH 7.00 (ii)). The pH-sensitivity of pCa_{50} was almost linear over the pH range 7.20 - 6.40. This is seen most clearly if pCa_{50} is plotted against pH (Figure 48). For each 0.2 unit reduction in pH the pCa_{50} was also reduced by almost 0.2 units (Figures 48 and 49 and Table 11). The mean $\Delta pCa_{50}/\Delta pH$ was 0.89 (calculated from the slope of the line in Figure 48). Over the entire pH range studied the pCa_{50} fell from 5.64 at pH 7.20 to 4.92 at pH 6.40, corresponding to a five-fold increase in the $[Ca^{2+}]$ required to produce half-maximal activation.

The values of n_H for all the relative force-pCa relationship were about 2.0 (Table 11). Although the curves in Figure 49 appear almost 'parallel', the mean n_H values for pH 6.80 (2.20) and pH 6.40 (2.43) were in fact significantly greater than the value for pH 7.00 (2.01). Thus a decrease in pH from 7.00 to 6.80 or 6.40 produced a slight but statistically significant steepening of the relative force-pCa relationship. However the slope was not significantly altered by the change in pH from 7.00 to 7.20.

That these pH-induced changes in the Ca^{2+}- activation curves were fully reversible is demonstrated by the insignificant differences between the values for pCa_{50} or n_H of the pH 7.00 (i) and pH 7.00 (ii) curves (and see also Figures 47 and 49). Furthermore, from the small scatter about the mean of any of the parameters (relative force, pCa_{50} or n_H) it would appear that these effects of pH are independent of time and of the order in which the solutions of different pH were applied.

It should be pointed out that the combination of the reduction in maximum force and the decrease in Ca^{2+}-sensitivity caused by a fall in pH meant that at suboptimal values of pCa the Ca^{2+}-regulated force was extremely sensitive to pH. This can be observed in Figure 47 but is better seen in Figure 50 , in which I have estimated the effects of pH on the forces obtained at certain pCa values, by substituting appropriate values for $[Ca^{2+}]$ into the modified Hill equation with the experimentally-determined values for n_H and K^*. Figure 50 demonstrates that the Ca^{2+}-regulated force was most sensitive to pH at pCa 5.0 - 6.0. For example, whereas the reduction in pH from 7.00 to 6.80 depressed the <u>maximum</u> Ca^{2+}-regulated force (at pCa< 4.0) by less than 20%, a similar change in pH halved the Ca^{2+}-regulated force at pCa 5.5.

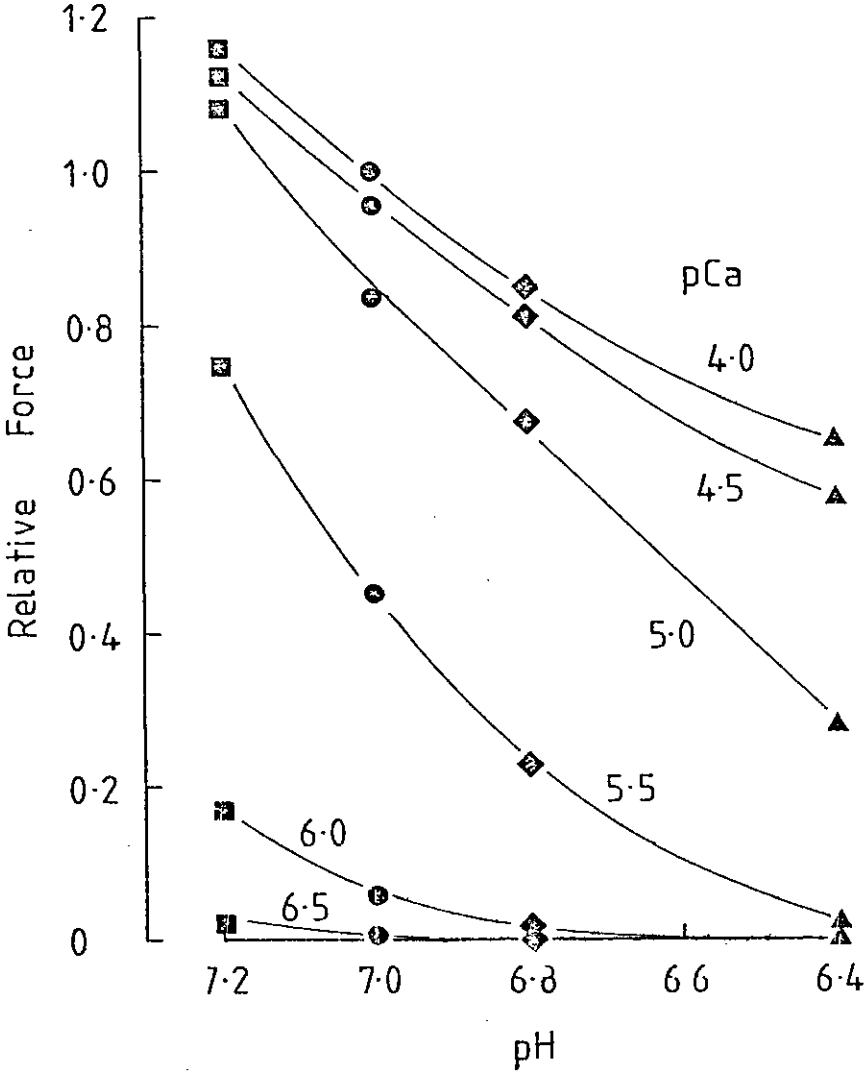


Figure 50. Influence of pH on the Ca^{2+}-regulated force at certain values of pCa. All forces expressed relative to the maximum Ca^{2+}-regulated force (at pCa 3.75) at pH 7.00. The data were obtained by substituting values for $[Ca^{2+}]$ into the modified Hill equation (Section 9.10) with the appropriate values for coefficients n_H and K*.

(d) Rate of decline of Ca^{2+}-regulated force

Some indication of the influence of pH on the rate of decline of force was obtained from the forces of the 'control' contractures preceding and following each staircase of test contractures. The ratio of force in the second control contracture (F_{C2}) to the force in the first control contracture (F_{C1}) was in fact a term in the correction formula for the 'staircase protocol' (Section 9.7(a)). In Figure 51 I have plotted the mean values of this ratio (F_{C2}/F_{C1}) against the pH of the solutions that were interposed between the two control contractures. A decrease in pH from 7.00 to 6.80 or 6.40 caused a slight increase in F_{C2}/F_{C1}. Conversely, an increase from pH 7.00 to 7.20 resulted in a considerable fall in this ratio. Thus, decreasing the pH over the range pH 7.20 - 6.40 improved the viability of the skinned muscle preparations.

It could be argued however that this 'protective' effect of acidity was perhaps merely a consequence of the inhibition of Ca^{2+}-regulated force at lower pH values. This suggestion is not supported by the data in Figure 52 , which shows results from two experiments in which discrete, regularly-spaced contractures were elicited by activating solutions of pH 7.20 or 6.40 (at 30°C). For both pH values, semilogarithmic plots of Ca^{2+}-regulated force against time were linear, as had been observed in other experiments (Appendix B). The pCa values of the activating solutions were chosen so that the Ca^{2+}-regulated forces were of similar magnitude at the two values of pH. Despite this, the decline of force was slower at pH 6.40 than at pH 7.20. At least some of the 'protective' effect of a reduction in pH that was suggested by the data in Figure 51 may thus have been due to a genuine influence of pH on the viability of these muscles.

(e) Muscle diameter

Within the limits of measurement with the eyepiece graticule (± 5μm), no changes were observed in the diameter of the skinned muscles as the pH was varied over the range pH 7.20 - pH 6.40.

10.11 The influence of salt concentration on the force production of the skinned cardiac muscle

A chart record of the resting and maximum Ca^{2+}-regulated forces at two ionic strengths - 100 mM (no added salt) and 200 mM (100 mM added KCl) - is shown in Figure 53. Figure 54 gives the mean results

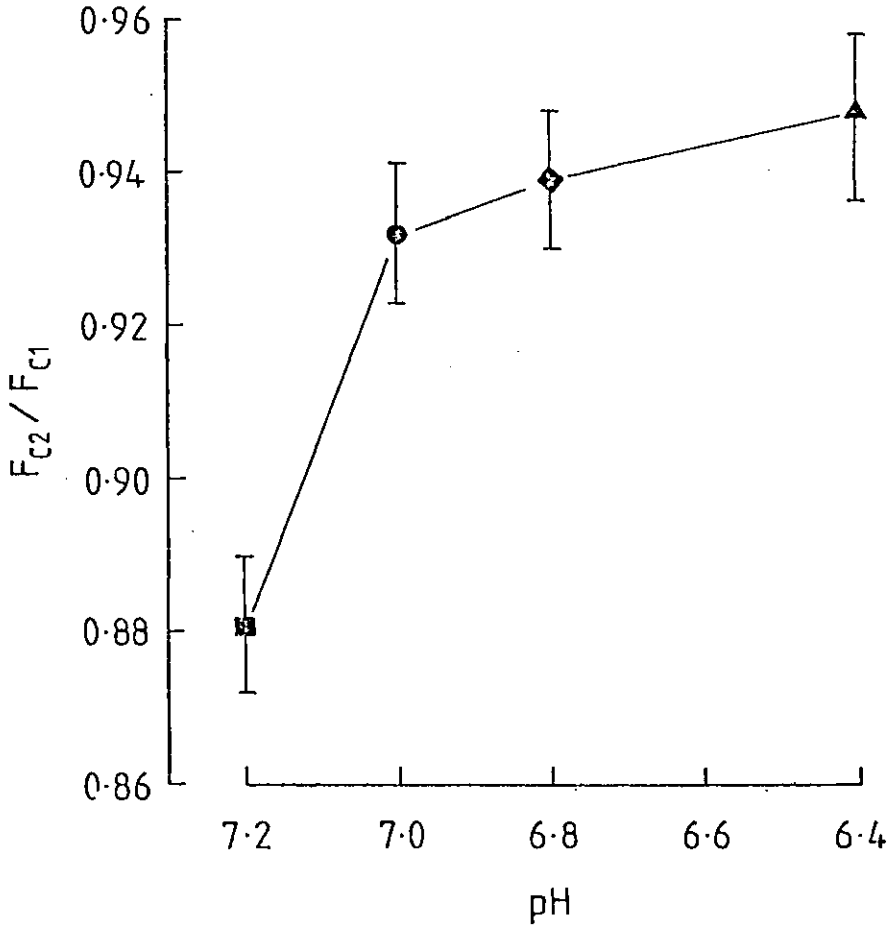


Figure 51. Influence of pH on the rate of decline of Ca^{2+}-regulated force. Ordinate shows the ratio between the force in the leading control contracture (F_{C1}) and the force in the trailing control contracture (F_{C2}) in the 'staircase' protocol. Abscissa shows the pH of the intervening test solutions.

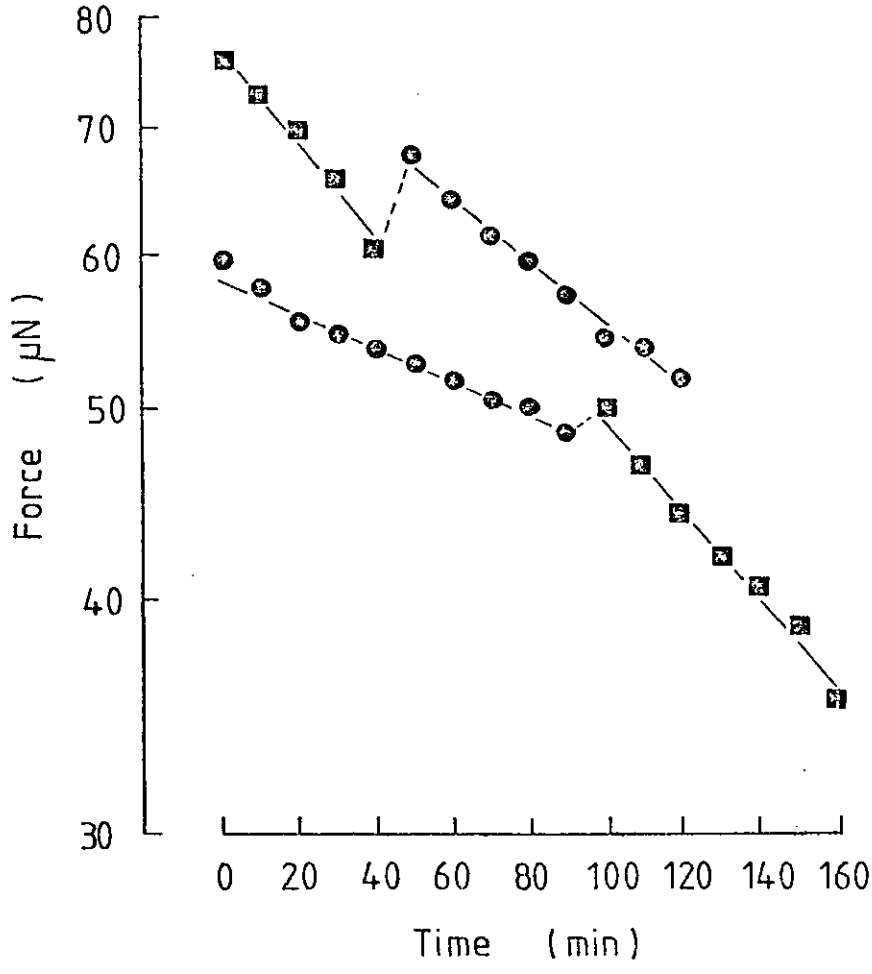


Figure 52. Decline of Ca^{2+}-regulated force in a series of regularly-spaced contractures at pH 7.20 (■) pH 6.40 (●). Results from two experiments at 30°C. Note the log scale for force. pCa at pH 7.20 = 5.55 and at pH 6.40 = 4.10.

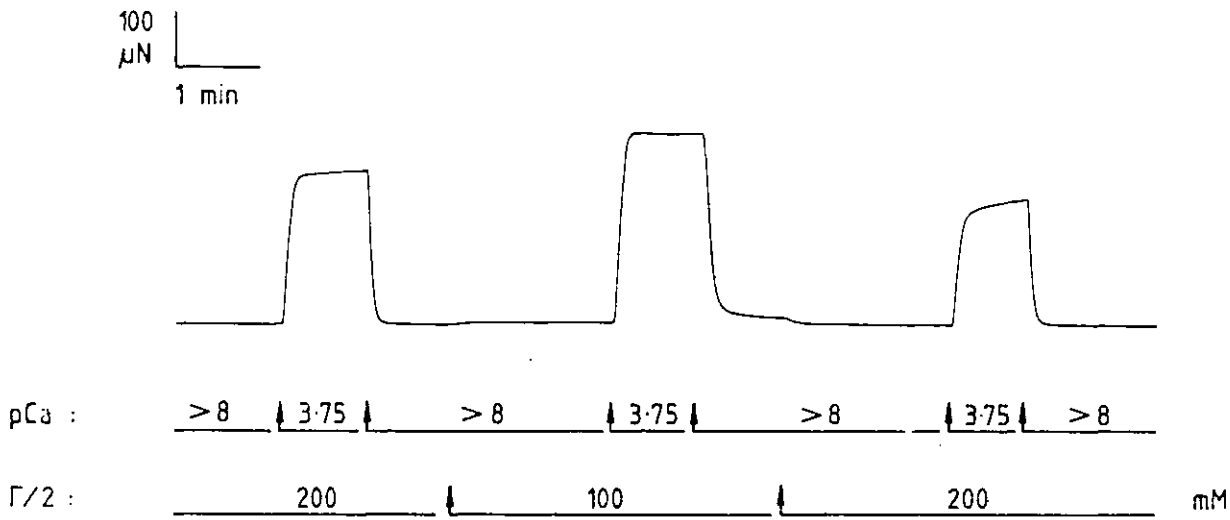


Figure 53. Typical force record from a detergent-skinned trabecula in solutions of ionic strength (Γ/2) 100 mM and 200 mM. 25°C.

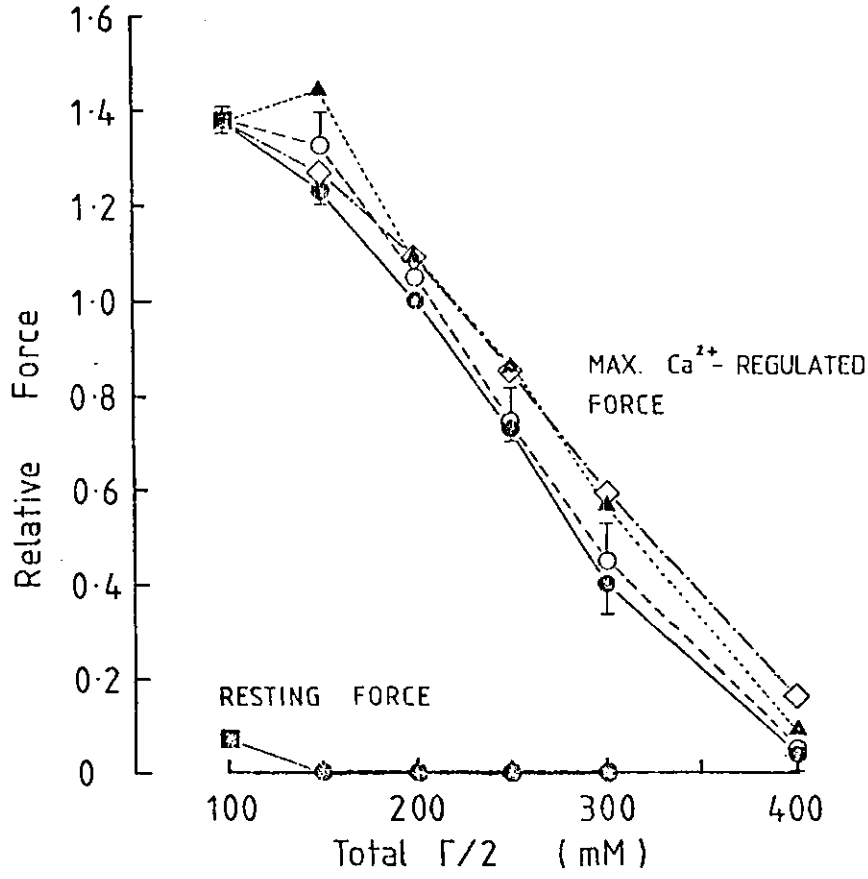


Figure 54. Influence of ionic strength (Γ/2) on the resting force and maximum Ca^{2+}-regulated force of detergent-skinned cardiac muscle. With no added chloride salt Γ/2 = 100 mM (■). Increases in ionic strength were produced by addition of KCl (●, n = 8), NaCl (○, n = 4), TMACl (◇, n = 3) or ChCl (▲, n = 2). 'Maximum-Ca^{2+}-regulated force' measured at pCa ≈ 3.8, 'resting force' measured at pCa>8. All forces expressed relative to the maximum Ca^{2+}-regulated force in 100 mM added KCl (total Γ/2 = 200 mM). Points represent mean ± S.E.M.

of 8 experiments in which ionic strength ($\Gamma/2$) was varied over the range 100 mM - 400 mM with KCl, NaCl, tetramethylammonium Cl (TMACL) or choline Cl (ChCl). In Figure 54 all forces are expressed relative to the maximum Ca^{2+}-regulated force in 100 mM KCl i.e. the Ca^{2+}-regulated force in the 'control' activating solution (pCa 3.75). The points for resting force show the increase in resting force above the value of the resting force in standard high-EGTA relaxing solution (100 mM added KCl, $\Gamma/2$ = 200 mM).

(a) Influence of [salt] on resting force

The resting force was not affected by ionic strength ($\Gamma/2$) changes in the range 200 - 400 mM, no matter which chloride salt was used to adjust ionic strength (Figure 54). However if the ionic strength was lowered to 100 mM (no added salt), resting force increased. As Figure 53 shows, this increase began soon after the drop in ionic strength and force reached a new steady value within one minute. The increment in force was equivalent to about 10% of the maximum Ca^{2+}-regulated force at $\Gamma/2$ = 200 mM (Figure 54). In some experiments the resting force at $\Gamma/2$ = 150 mM was also a little greater than at $\Gamma/2$ = 200 mM.

After contraction of the muscles in an activating solution of ionic strength 100 mM, incomplete relaxation was often seen (Figure 53). This 'residual force' persisted until the relaxing solution was changed for one of $\Gamma/2$>150 mM, when the original resting force at this ionic strength was re-established. The resting forces before and after the contracture were averaged to supply data for Figure 54 .

(b) Influence of [salt] on maximum Ca^{2+}-regulated force

Increasing the ionic strength over the range 100 - 400 mM with the chloride salts resulted in an almost linear decrease in the Ca^{2+}-regulated force measured at pCa of $\approx$ 3.75 (Figure 54). (The pCa was not known precisely at $\Gamma/2$ >200 mM because the association constant for the binding of Ca^{2+} to EGTA was not known with accuracy at these high ionic strengths). In activating solutions with 300 mM added NaCl or KCl (calculated $\Gamma/2$ = 400 mM) the force was not significantly different from zero ($p>0.05$). Although there is some suggestion from the data in Figure 54 that the effects of KCl and NaCl on maximum Ca^{2+}-regulated force were a little greater than those of TMACl and ChCl, in fact none of the four values of relative force at each ionic strength was significantly different from any other ($p>0.05$, Student's t-test). Thus the inhibitory effect on maximum force of an increase in ionic strength is the same whether this increase is brought

about by addition of KCl, NaCl, TMACl or ChCl.

These decreases were not due to a reduction in Ca^{2+}-sensitivity (i.e. a shift of the relative force-pCa relationship to higher Ca^{2+}-concentrations) because lowering the pCa further to 3.56 from 3.75 did not result in any greater force.

I obtained the data in Figure 54 using a perfusion regime in which all the solutions at one ionic strength were tested, relative to the control, before a change was made to solutions with the next highest ionic strength. When all the solutions had been perfused at least once, I was able in some experiments to reverse this perfusion sequence. The relative forces obtained in this reverse sequence were not significantly different from those in the initial sequence provided that ionic strengths were in the range 200 - 400 mM (Figure 55), but at lower ionic strengths the relative forces determined in the reverse sequence (i.e. towards the end of the experiment) were considerably greater than those obtained in the initial sequence at the start of the experiment. In other words, the dependence of maximum Ca^{2+}-regulated force upon ionic strength increased with time for ionic strengths below 200 mM. This was a consequence of the fact that, although the maximum Ca^{2+}-regulated forces at all ionic strengths declined throughout the experiment, the forces at $I/20$ >200 mM declined more quickly than the forces at $I/2$ <200 mM. The same phenomenon occurred in one experiment in which 1 mM of reduced dithiothreitol was included in all solutions.

(c) Influence of tonicity on maximum Ca^{2+}-regulated force

Ashley & Moisescu (1977) obtained evidence that part of the inhibitory action of an increase in salt concentration on maximum force in barnacle skinned fibres resulted in fact from the concomitant increase in tonicity of the solution. To test whether the same was true for skinned cardiac trabeculae, I added sucrose to the solutions of 100 mM ionic strength to increase the tonicity but not the ionic strength. Figure 56 compares the effect of increasing ionic strength and tonicity with KCl with that of increasing tonicity alone with sucrose. In none of the sucrose-containing solutions were the Ca^{2+}-regulated and resting forces significantly different from those in the solutions of $I/2$ = 100 mM ($p>0.05$, paired t-test, n = 3).

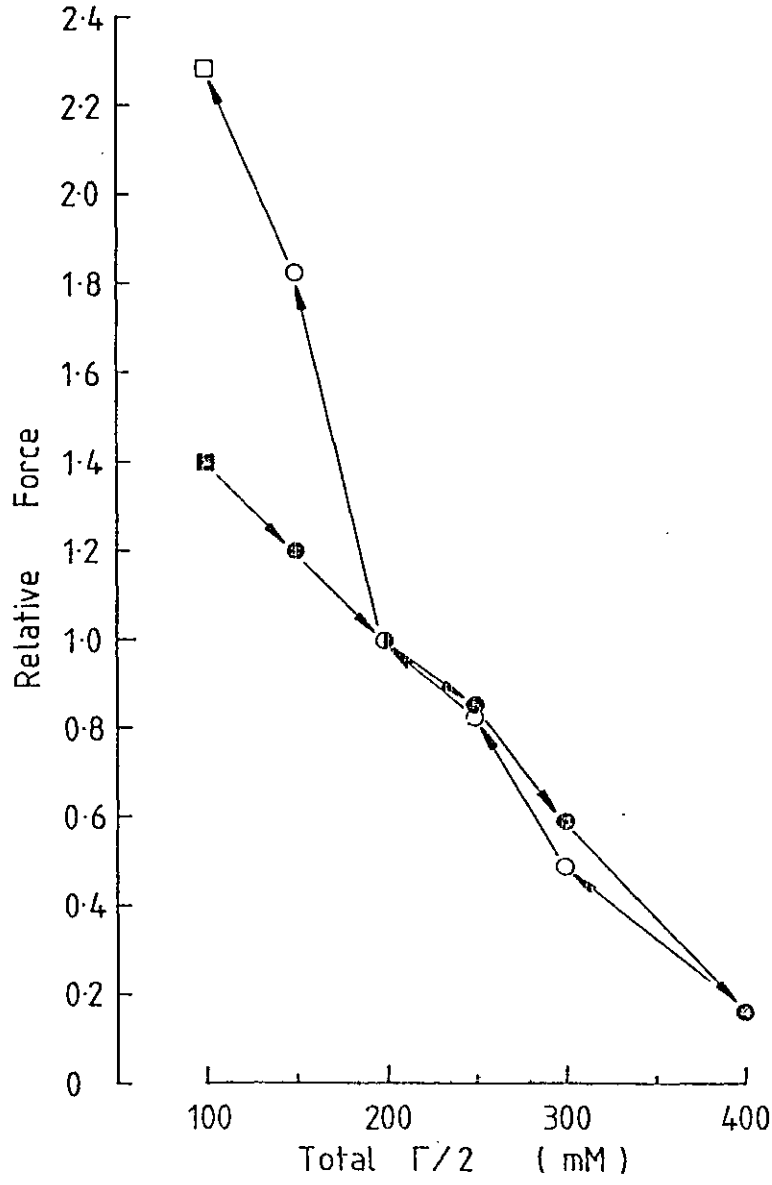


Figure 55. Change in the ionic strength-dependence of maximum Ca^{2+}-regulated force with time. Results from a typical experiment. Arrows indicate the order of application of the activating solutions of different Γ/2. Increases in Γ/2 were produced by the addition of KCl. 26°C. Other details as in Figure 54.

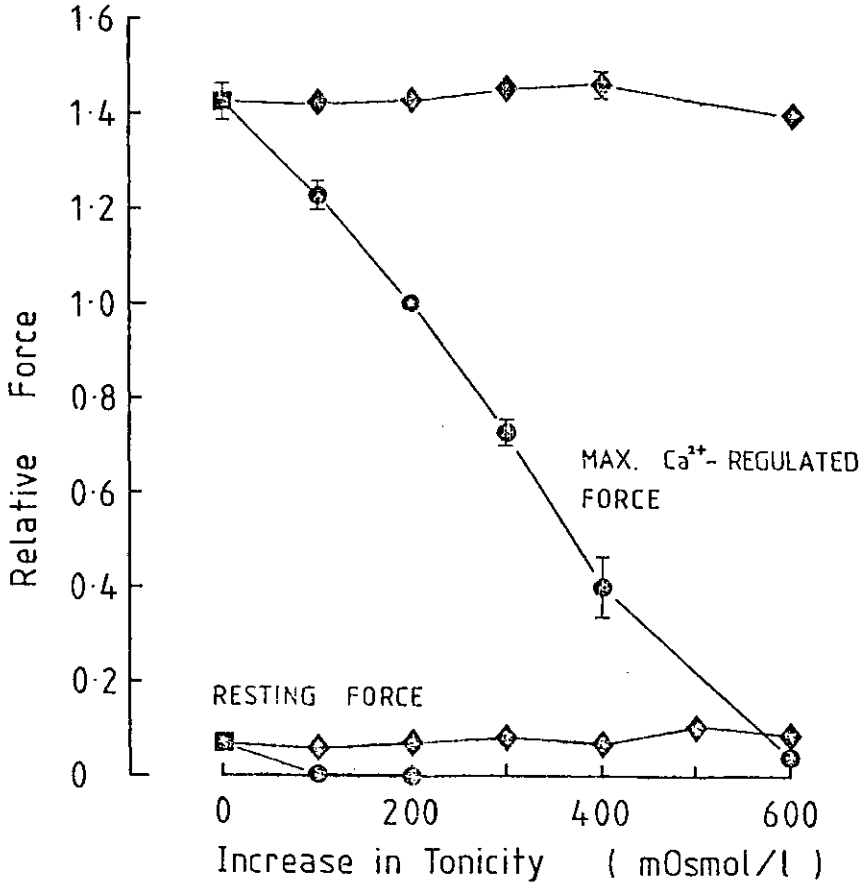


Figure 56. Influence of tonicity on the resting force and maximum Ca^{2+}-regulated force in detergent-skinned cardiac muscle. Increases in tonicity were produced by addition of KCl (○; data as in Figure 54) or by addition of sucrose (◇). Points represent mean ± S.E.M. of 3 experiments. Other details as in Figure 54.

(d) Influence of [salt] on the relative force-pCa relationship

I determined the relative force-pCa relationship at two ionic strengths (100 mM and 200 mM) that probably lie on either side of the physiological ionic strength in cardiac muscle. As with the above experiments, the higher ionic strength was produced by the addition of KCl, TMACl, ChCl or NaCl.

Figure 57 shows the relative force-pCa relationships in the solutions of different salt composition. Once again all forces are expressed relative to the maximum force in control activating solution (pCa 3.75, 100 mM added KCl, total ionic strength 200 mM). The pCa values were computed taking into account the effect of salt on K_{CaEGTA} and $K_{CaHEGTA}$ (Appendix A), but in fact this correction made at most a 0.06 unit difference between the pCa values calculated for the different salt compositions. Thus the relative force-pCa relationships with these pCa corrections (Figure 57) were little different from those that would have been obtained if it had been assumed that the calculated pCa was not affected by the [salt] (results not shown).

Figure 57 confirms that an increase in ionic strength depresses the maximum Ca^{2+}-regulated force, and gives some indication that the position of the relative force-pCa relationship on the pCa axis depends on the [salt] of the activating solution. The latter effect is seen much more clearly if the curves are normalised so that all forces in activating solutions of a given [salt] are expressed relative to the Ca^{2+}-regulated force in a maximally-activating solution of the same [salt] (Figure 58).

Each relative force-pCa relationship was fitted by a sigmoid curve of the form of the modified Hill equation, using non-linear least-squares analysis (lines in Figures 57 and 58). The fitted values for the Hill coefficients n_H and K^* are given in Table 12 , together with the values for pCa_{50} calculated from them (Section 9.10).

Table 12 also gives the mean values for n_H, K^* and pCa_{50} that were calculated from the individual sigmoid curves fitted to the relative force-pCa relationships in each experiment. Note that whenever the relative force-pCa relationship at a particular salt concentration was determined more than once in an experiment, the mean value of n_H, K^* or pCa_{50} in that experiment was used as a single datum in the statistical analysis.

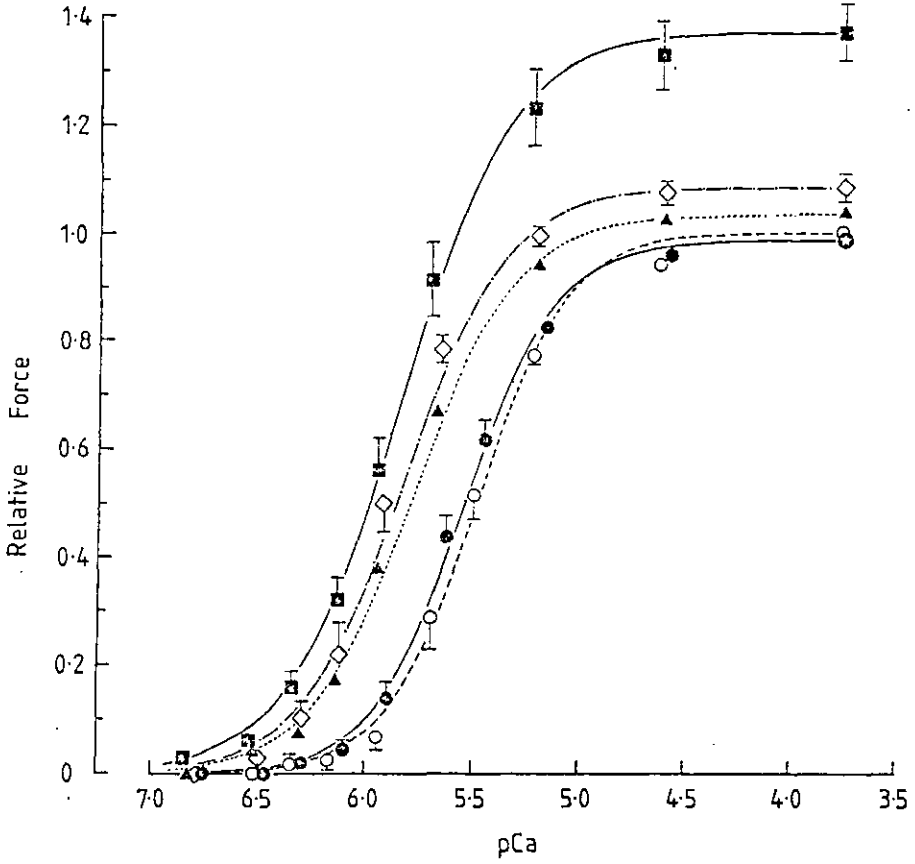


Figure 57. Relative force-pCa relationships for skinned cardiac muscle in solutions of different [salt]. With no salt added $\Gamma/2$ = 100 mM (■). $\Gamma/2$ was raised to 200 mM by addition of KCl (●), NaCl (○), TMACl (◇) or ChCl (▲). All forces expressed relative to the maximum Ca^{2+}-regulated force in 100 mM added KCl (⊙; total $\Gamma/2$ = 200 mM). Points represent the mean ± S.E.M. of n experiments. Values of n, and of the coefficients for the fitted modified Hill equations, are listed in Table 12.

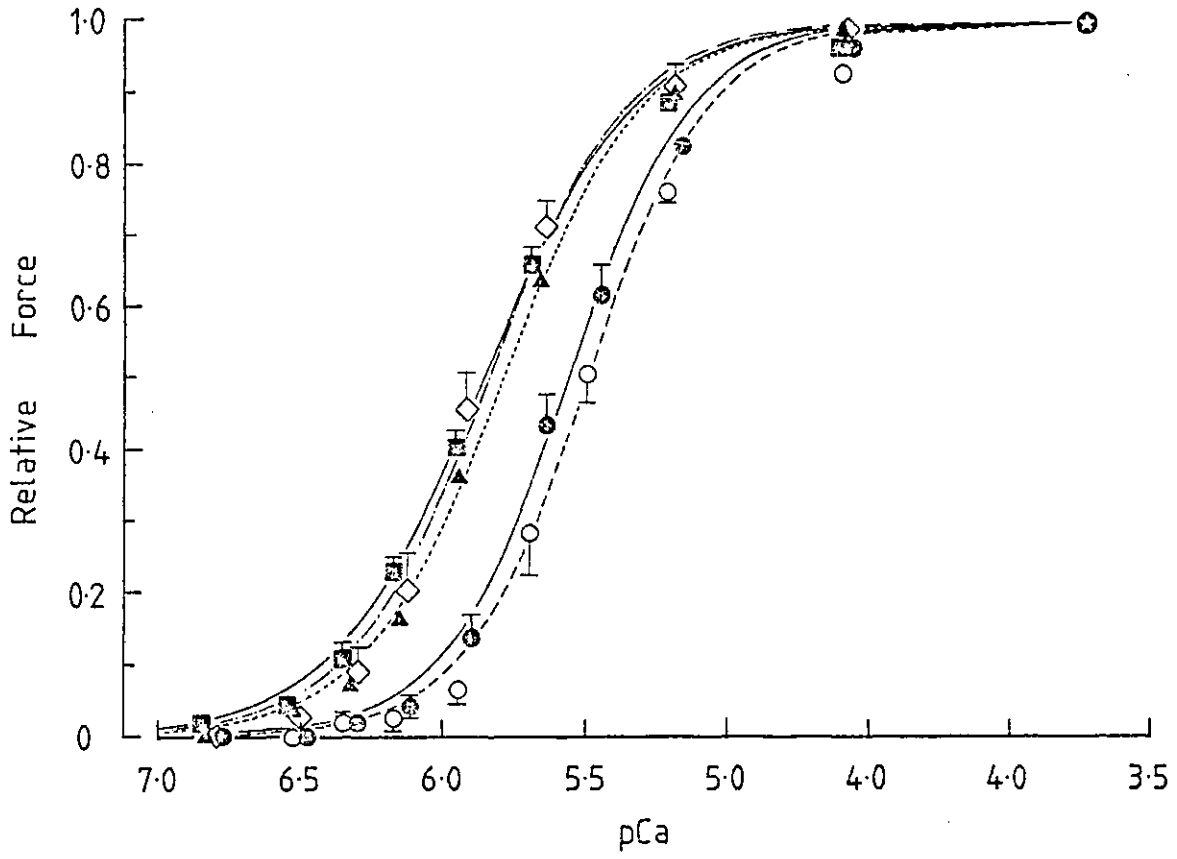


Figure 58. Relative force-pCa relationships for skinned cardiac muscle in solutions of different [salt]. Forces at each [salt] expressed relative to the maximum Ca^{2+}-regulated force at the same [salt]. Other details as in Figure 57.

	Added salt				
	None	100 mM KCl	100 mM TMACl	100 mM ChCl	100 mM NaCl
Total Γ/2 (mM)	100	200	200	200	200
n (no. of experiments)	4	10	4	2	4
(a) n_H	1.64	2.04	1.81	1.81	2.05
(a) K* (x 10^{-12}M)	255	4.48	26.7	31.4	5.72
(a) pCa_{50}	5.85	5.55	5.84	5.79	5.49
(b) n_H	$1.67^{*} \pm 0.02$	2.14 ± 0.08	2.09 ± 0.27	1.82	2.10 ± 0.13
(b) K* (x 10^{-12}M)	19.8 ± 51	54.4 ± 36.6	144 ± 96	62.3	12.5 ± 10.2
(b) pCa_{50}	$5.85^{*} \pm 0.03$	5.56 ± 0.03	$5.86^{*} \pm 0.04$	5.79	5.49 ± 0.04

* significantly different from the corresponding value in 100 mM added KCl (Students t-test, $p<0.05$)

Table 12. Details of the sigmoid curves fitted to the data in Figures 57 and 58.

(a) Hill coefficients n_H and K*, and the pCa_{50} calculated from them (Section 9.10) for the curves fitted to the mean data of Figure 57 or 58.

(b) Mean values of n_H, K* and pCa_{50} for the curves fitted to the data in each experiment. Mean ± S.E.M. of 'n' experiments.

In the standard activating solutions (100 mM added KCl, total $[K^+]$ = 130 mM, total $\Gamma/2$ = 200 mM) the relative force-pCa relationship had a pCa_{50} of 5.56 ± 0.03 and an n_H of 2.14 ± 0.08. This value for n_H was not significantly different from 2.0. If the 100 mM KCl was omitted from the solutions, so that the total ionic strength fell to 100 mM and the $[K^+]$ fell to 30 mM, the pCa_{50} was increased from 5.56 to 5.85 (Figure 58 and Table 12). This increase in Ca^{2+}-sensitivity was accompanied by a significant reduction in n_H from 2.14 ± 0.08 to 1.67 ± 0.02. The shift of the relative force-pCa relationship to the left was also observed if the 100 mM KCl in the standard activating solutions was replaced by 100 mM TMACl or 100 mM ChCl to keep the ionic strength constant at 200 mM. In fact the pCa_{50} value in 100 mM TMACl (5.86 ± 0.04)was significantly different from that in 100 mM added KCl but not from that (5.85) in zero added salt. Only two experiments were carried out with 100 mM ChCl and so the difference between the pCa_{50} in 100 mM ChCl (5.79) and that in zero or 100 mM KCl could not be tested for significance, but it is clear that the results in 100 mM ChCl were similar to those in 100 mM TMACl. Thus the pCa_{50} of force was increased by a reduction in $[K^+]$ from 130 mM to 30 mM at constant ionic strength but it was not affected by an increase in ionic strength at a constant $[K^+]$ of 30 mM.

On the other hand, if the 100 mM KCl was replaced by 100 mM TMACl or 100 ChCl the steepness of the force-pCa relationship was not significantly altered, as evinced by the values for n_H. The n_H in zero added salt (1.67) was significantly different from all the other n_H values. Thus the slope of the Ca^{2+}-activation curve was not altered by a reduction in $[K^+]$ at constant ionic strength but it was decreased if the ionic strength was reduced from 200 mM to 100 mM.

In 100 mM NaCl (total $[Na^+]$ = 130 mM, total $\Gamma/2$ = 200 mM) the relative force-pCa relationship appeared to be shifted to lower values of pCa compared with the relationship in 100 mM KCl (Figure 58). However the mean values for pCa_{50} in 100 mM NaCl and 100 mM KCl (5.49 ± 0.04 and 5.56 ± 0.03 respectively) were not significantly different; neither did the replacement of the KCl by NaCl significantly affect the value for n_H.

Because the pCa_{50} was influenced by the $[K^+]$ or $[Na^+]$, I could calculate from the data in Table 12 a value for the change in pCa_{50} for a unit change in the negative log of the ion concerned. By analogy with $\Delta pCa_{50}/\Delta pH$, these values were termed $\Delta pCa_{50}/\Delta pK$ and

$\Delta pCa_{50}/\Delta pNa$. The values for $\Delta pCa_{50}/\Delta pK$ and $\Delta pCa_{50}/\Delta pNa$ were 0.46 and 0.56 respectively.

(e) Rate of decline of Ca^{2+}-regulated force

I did not study the influence of [salt] on the rate of force decline in a regularly-timed series of contractures, but a good indication of this influence of [salt] could be obtained from a comparison of the Ca^{2+}-regulated forces in the contractures produced by application of the control activating solution before and after the test activating solution (as in Figure 53). In Figure 59 I have plotted the ratio between the forces in these leading and trailing control contractures (F_{C2}/F_{C1}) against the ionic strength of the intervening test solution. This ratio was in fact a term in the procedure that corrected for the decline in force (Section 9.7(b)). The smaller was this ratio, the faster was the decline of force. The magnitude of the decline of force did not appear to depend on the type of chloride salt that was used to vary the ionic strength, but it was very sensitive to the ionic strength per se. The decline was least in the ionic strength range 200 mM - 250 mM, with F_{C2}/F_{C1} being approximately 0.96 - 0.98. This ratio fell to about 0.9 at $\Gamma/2$ = 100 mM. At $\Gamma/2$ = 400 mM the decline of force was even more severe and the force in the trailing control contracture was only about 70% of that in the leading control contracture ($F_{C2}/F_{C1} \approx 0.7$).

(f) Muscle diameter

I could detect no changes in the muscle diameter as the $\Gamma/2$ was varied over the range 100 mM - 400 mM or as the tonicity was raised by the addition of up to 600 mM sucrose.

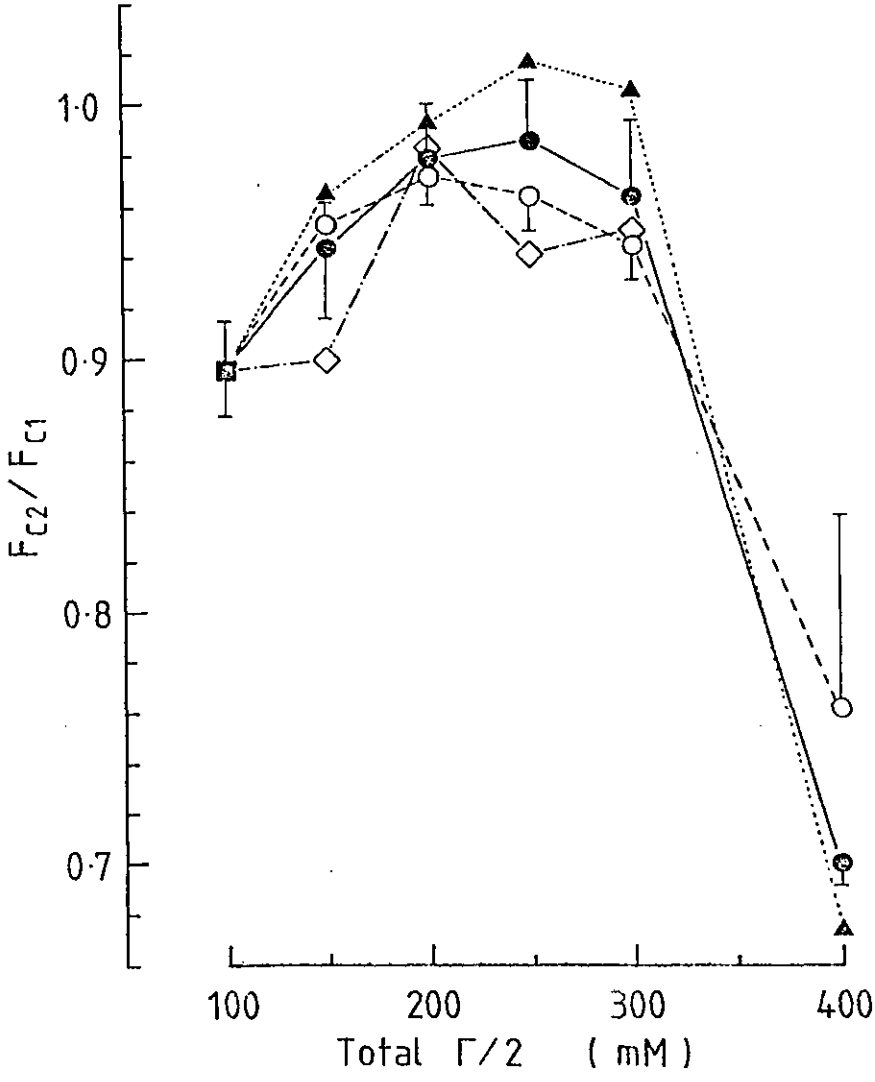


Figure 59. Influence of ionic strength on the rate of decline of force. The ordinate shows the ratio between the force in the leading control contracture (F_{C1}) and the force in the trailing control contracture (F_{C2}) in the 'discrete protocol' (e.g. Figure 53). The abscissa shows the ionic strength of the intervening test solutions. Each point shows the mean ± S.E.M.. pCa 3.8. Other details as in Figure 54.

Chapter 11

DISCUSSION

11.1 Experimental procedure

Some aspects of my experimental procedure differ from those in other studies on skinned muscle and are worthy of comment.

A flow-through muscle bath was used in preference to the movable series of wells that is commonly used in skinned muscle work. The flow-through system was needed to allow continuous superfusion of the muscle with an oxygenated physiological saline ('modified Krebs-Hensleit solution') before skinning was begun. One advantage of this apparatus over the series of wells was that the bathing solution could be changed without subjecting the muscle to any possibly harmful passages through an air/water interface. Furthermore, the continuous flow of solution past the muscle removed the need for stirring of the solution. The solutions were not reperfused, so that cross-contamination between solutions was negligible.

Continuous perfusion was not wasteful of solutions: usually each activating or relaxing solution was perfused for 30 s at a flow rate of 1 cm^3 min^{-1}, i.e. 0.5 cm^3 of solution was used each time. However, use of a flow-through muscle bath did suffer from the disadvantage that the solution change was neither instantaneous nor uniform along the length of the muscle. It would therefore have been inadvisable to use such a bath to study, for example, the kinetics of force development in Ca^{2+}-regulated contractures (cf. Moisescu, 1976).

Apart from the original report by Winegrad (1971) on cardiac muscle and the recent studies by Gordon (1978) on smooth muscle and Fabiato (1982) on cardiac muscle, this appears to be the only study in which a muscle with intact cells was mounted in a muscle bath and stimulated to contract before the skinning procedure was begun. This protocol was followed so that it was possible to stretch each muscle until twitch force development was maximal; in this way I could ensure that, prior to skinning, average sarcomere length was approximately constant from preparation to preparation. This precaution has been justified by reports that the relative force-pCa relationship of skinned cardiac muscle is dependent on sarcomere length (Section 1.5(a)). Although the setting up and stimulation of the unskinned muscle considerably prolonged the total duration of each experiment, it

brought several additional benefits. Ensuring that mechanical performance of the unskinned muscle was satisfactory proved to be a useful complement to visual inspection as a means of checking that the preparation was not damaged. The experimental design also made it possible to compare, in the same muscle, the twitch (the magnitude of which is a complex function of muscle excitation, myofibrillar activation and relaxation) with the Ca^{2+}-regulated contractile properties of the myofibrils *per se*.

11.2 Compositions of the bathing solutions

As far as possible the compositions of the perfusates were kept the same as the compositions of the incubation media for the ATPase experiments so as to facilitate comparisons between the two studies. This ideal could not be fully realised because constraints were placed on the bathing solution compositions by the need to (i) maximise the viability of the skinned muscles and (ii) minimise ionic concentration gradients within the muscles, while at the same time reproducing as closely as possible the conditions of pH, pCa, ionic strength, [high energy phosphates], etc. that obtain in intact cardiac cells. The ionic concentrations that were common to both isolated myofibril and skinned muscle studies are: imidazole, 20 mM; K_2Na_2ATP, 5 mM; $MgCl_2$, 6 mM; pH 7.20, 7.00, 6.80 or 6.40. The concentrations of the other constituents of the bathing solutions were chosen for the reasons given below.

As mentioned in Chapter 8, the inclusion of 1% Triton X-100 in the skinning solution was essential for the rapid skinning of the trabeculae; if Triton X-100 was omitted, the muscles did not become sensitive to micromolar levels of Ca^{2+} after 30 minutes of superfusion with the skinning solution. The concentration of Triton X-100 (1% v/v) was the same as that used to remove contaminating membrane from the preparations of isolated myofibrils. Skinning solution also contained 5 mM glucose and 3 mM Na pyruvate, which were present in the modified Krebs-Hensleit solution. These two substances were retained in the skinning solution to provide substrates for intracellular ATP synthesis before sarcolemmal disruption was sufficient to ensure an adequate supply of ATP from the bathing solution.

100 mM KCl was used to raise the calculated total ionic strength to 200 mM. Muscle force declined rapidly at ionic strengths below about 150 mM (Section 10.11(e)) and so the ionic strength of $\approx$ 115 mM used in the myofibrillar ATPase study could not be used. Force decline

was slowest in the ionic range 150 - 200 mM. I chose to add 100 mM KCl, giving a total ionic strength of 200 mM and bringing the $[K^+]$ to 130 mM, which is the cytosolic $[K^+]$ in cardiac muscle cells (Section 12.2).

10 mM Na_2CP plus creatine kinase formed a powerful ATP-regenerating system:

$$H^+ + MgADP^- + HCP^{2-} \underset{\text{kinase}}{\overset{\text{creatine}}{\rightleftharpoons}} MgATP^{2-} + \text{creatine} .$$

The practical reason for adding Na_2CP was that it greatly slowed the rate of force decline (Appendix B, Section B.3(g)). This may have been because the regenerating system would have helped to maintain the $[MgATP^{2-}]$ and to reduce the $[MgADP^-]$ in the face of continous myofibrillar ATPase activity during Ca^{2+}-regulated force development in the skinned muscles. Of course, this is the normal function of the CP/creatine kinase system in intact muscle cells. A secondary benefit is that the breakdown of HCP^{2-} to creatine is a reaction that absorbs protons ($\approx$ 0.7 H^+ per phosphate group transferred at pH 7.0). Thus most of the protons formed during $MgATP^{2-}$ hydrolysis ($\approx$ 1 H^+ per phosphate group hydrolysed at pH 7.0) would have been reabsorbed in the regeneration of $MgATP^{2-}$ from $MgADP^-$ via the creatine kinase reaction, and this would have limited the fall in pH caused by MgATP hydrolysis. The beneficial effects of raising $[MgATP^{2-}]$ or $[HCP^{2-}]$ on the viability of the skinned muscles appeared to approach a maximum at a total concentration of high-energy phosphate compounds of 10 - 15 mM (Section B.3(g)). It may be no coincidence that the physiological intracellular concentrations of ATP and CP ($\approx$ 5 mmol g^{-1} fresh tissue and 6 - 10 mmol g^{-1} fresh tissue, respectively - Opie et al., 1971) sum to give a total concentration in the same range. Thus, to reproduce conditions in the cytosol and to minimise the decline in force, the bathing solutions were given 'standard' concentrations of total CP = 10 mM and total ATP = 5 mM. Once I had found that the creatine kinase in the skinned muscles was of sufficient activity to prevent a fall in [MgATP] in the core of the muscles (Section B.3(h)), the addition of creatine kinase to the bathing solutions was discontinued.

The concentration of the Ca/EGTA buffer system was not 1 mM as in the myofibrillar ATPase studies but 10 mM, in order to reduce the likelihood of gradients of Ca^{2+} across the skinned muscles. Gradients of Ca^{2+} would have been present for a short time after the

change from a relaxing solution to an activating solution and then would have been reversed after reapplication of the relaxing solution. However it is unlikely that any gradients of Ca^{2+} existed when the muscle forces were steady. It was only when the forces were steady that measurements of force were made.

The use of HDTA in the 'rapid-activation' procedure to wash out excess EGTA has already been described (Section 9.6).

Finally, the temperature of the perfusates was kept as low as possible to prolong the viability of the skinned muscles (Section B.3(e)) and to prevent irreversible contractures during and immediately after the skinning period (Section 10.1). Solution temperature was maintained just above room temperature to ensure adequate temperature regulation by the proportional feedback system.

11.3 To what extent were the Triton-treated muscle cells 'skinned'?

A major question that arises in studies on skinned muscle is whether it can be assumed that externally-applied molecules have free access to all the myofibrils in the muscle. It is important to determine whether the sarcolemma in chemically-skinned muscle is truly non-selectively permeable to the entry and exit of molecules from the cells. Only if this is so can it be assumed that the ionic composition of the myofibrillar environment approximates to that of the bathing solution.

There are several lines of evidence to suggest that the trabeculae which had been superfused with the Triton-containing skinning solution were truly skinned. For instance, the electron micrographs of the Triton-treated muscles (e.g. Figure 43) showed that the reorganisation of the cellular membranes occurred throughout each and every cell. It therefore seems likely that, at least during the skinning procedure, the sarcolemma of each cell was disrupted sufficiently to allow entry of Triton X-100 molecules into the cells. Although the electron micrographs also showed the sarcolemma to be lost in places, this does not prove functional skinning because it could be suggested that the skinning procedure merely increased the lability of the membrane, which was then disrupted by the fixative.

The electron micrographs showed that all the cells in the muscle had been reached by the detergent. That all these cells were indeed skinned is suggested by the linear relation between maximum Ca^{2+}-regulated force and muscle cross-sectional area over the range of muscle diameters employed (Figure 35). Insufficient skinning in

the core of the thicker muscles would have resulted in a fall-off of the values for force/unit area in the muscles of largest diameter; there was no evidence for this however.

The high permeability of the Triton-treated muscle cells to medium-sized ions was convincingly demonstrated by the rapid relaxation of rigor contractures after addition of MgATP or CP + MgADP or phosphoenolpyruvate + MgADP to the muscles (Figure 40). These ions do not readily cross the normal, intact sarcolemma. The force production of the Triton-treated muscles was also very responsive to changes in the $[Ca^{2+}]$ in the micromolar range. However this Ca^{2+}-sensitivity is not good evidence for functional skinning because patently-unskinned frog cardiac muscle displays a similar Ca^{2+}-sensitivity in high K^+/low Na^+ media (Miller, 1979).

By far the most direct evidence that the trabeculae were genuinely skinned derives from their permeability to ferritin (Section 10.7). If ferritin molecules (molecular weight $\approx$ 750,000 Daltons) could enter the intermyofibrillar spaces it seems probable that the disrupted sarcolemma was freely permeable to all molecules that were much smaller than ferritin.

A consideration of all the above factors leads me to conclude that cardiac trabeculae that had been superfused for 12 - 30 minutes with Triton-containing skinning solution were freely permeable to all the ions in my standard bathing solutions, i.e. the muscles were adequately skinned.

11.4 Comparison of my skinning procedure with other methods of disrupting or removing the sarcolemma of cardiac muscle cells

I found that the Triton-containing skinning solution very rapidly disrupted the sarcolemma in guinea-pig ventricular trabeculae. If it can be assumed that when the Ca^{2+}-regulated force in 'high Ca skinning solution' had reached its peak value, all the muscle cells were skinned, it follows that complete skinning was attained after the muscles had been superfused with skinning solution for 2 - 20 minutes (Section 9.5). A similarly rapid time course has been observed with a skinning procedure based on saponin, a glycoside from plants (Endo & Kitazawa, 1978). In contrast, Ca^{2+}-chelators such as EDTA (Winegrad, 1971) and EGTA (McClellan & Winegrad, 1977) disrupt the sarcolemma of rabbit or rat cardiac muscles only very slowly - a soaking of many hours is usually needed, although Brandt & Hibberd (1976) reported

that rat ventricular muscle was apparently skinned by 30 minutes' exposure to a 'skinning solution' containing 10 mM EGTA. It seems likely that the ease with which Ca^{2+}-chelators can disrupt the cardiac sarcolemma reflects species-specific differences in the lability of the cardiac cell membrane. Guinea-pig trabeculae seem particularly refractory to skinning with Ca^{2+}-chelators (Chapter 8).

Chemical skinning has an advantage over mechanical skinning in that technically it is very much simpler. However a drawback of skinning with Triton X-100 is that the detergent altered the morphology of all the intracellular membranes; thus it would have been impossible to study the normal function of any of these membranes, such as the SR. (This is probably true for all other chemical skinning procedures, too). This disrupting action of Triton X-100 upon the SR has been observed previously with isolated SR-rich microsomal preparations from skeletal muscle (Weber, 1971). Provided that only the myofibrils are of interest, the lack of any SR Ca^{2+}-binding activity in the Triton-treated muscle is a positive benefit, for it would reduce the non-myofibrillar binding of Ca^{2+} and thus tend to enhance the rate of development of force in a Ca^{2+}-regulated contracture.

The myofibrils themselves appeared to be little altered by the skinning procedure. Electron micrographs of freshly-skinned trabeculae showed almost no discernible damage to sarcomere structure. Furthermore, the force in the first contracture was usually double that recorded in the electrically-stimulated muscle prior to skinning (Section 10.1). It is therefore unlikely that the skinning solution greatly reduced the maximum force-generating capability of the myofibrils. In addition, there was some evidence that Triton X-100 had little effect on the Ca^{2+}-sensitivity of the myofibrils, because the relative force-pCa relationships in different muscles were similar (e.g Figure 39) despite the variation in the duration of the skinning period (12 - 30 minutes).

11.5 Maximum force generation in the skinned trabeculae

Calculated values for force per unit area in the first contracture in 'control' activating solution (pCa 3.75, pH 7.00, $\Gamma/2$ = 200 mM) had a mean of 20.7 kN m^{-2} (20.7 mN mm^{-2}) and a maximum of 49.2 kN m^{-2}. These values were not only considerably lower than those reported for mechanically-skinned frog skeletal fibres (137 kN m^{-2} - Hellam & Podolsky, 1969; 108 kN m^{-2} - Gordon et al.,

1973) as might have been expected because of the lower proportion of myofibrils in cardiac muscle, but also were below those observed in mechanically-skinned rat ventricular cells (85 kN m^{-2} - Fabiato & Fabiato, 1976). The small values for force per unit area in my experiments did not seem to result from a detrimental action of the skinning solution because the force in the first contracture was usually double the force developed in the unskinned muscle stimulated at 1 s^{-1}. Rather the low values resulted from an inherent property of the guinea-pig trabeculae. Other workers have also observed low values of force per unit area with guinea-pig trabeculae (18 kN m^{-2}, J.S. McGuigan, personal communication, 1981). Although it is possible that the differences between mechanically-skinned rat muscle and detergent-skinned guinea-pig muscle resulted from genuine species-differences in the force-generating ability of the myofibrils, the low values for force per unit area in the guinea-pig trabeculae (and the considerable scatter of these data about the mean - see Figure 35) are probably attributable to the large and variable amounts of collagen in these muscles (see for example Figure 44). Collagen fibres and other connective tissue would have added to the muscle diameter without contributing to the Ca^{2+}-regulated force. In contrast, the mechanically-skinned cardiac cells used by Fabiato and Fabiato lack any extracellular tissue. If collagen fibres were effectively in parallel with the myofibrils in my skinned trabeculae, one would have expected the muscle length at which developed force was maximal to be associated with a large resting force. This was indeed observed in several muscles (Section 10.1).

Variability in the values for force per unit area could have been due to several factors. These include: variable amounts of collagen, as described above; the assumption, which is certainly an oversimplification, that the trabeculae are perfectly circular in cross-section at the point of measurement of their diameter; finally, there probably was some loss of structural integrity at the ends of the trabeculae because of the loops tied around them - this would probably have reduced the force-generating ability of the trabeculae in a fashion which varied from muscle to muscle.

The first contracture in control activating solution (pH 7.00, pCa 3.75) exhibited a steady force that was approximately equal to the force developed by the unskinned muscle during paired stimulation in 1.8 mM Ca^{2+} at room temperature. This does not necessarily indicate

that the myofibrils in the unskinned guinea-pig muscles were maximally activated under these conditions. The equivalence of force may have been fortuitous, because the true intracellular ionic strength may be lower than the ionic strength in my control activating solution (200 mM), in which case the maximum Ca^{2+}-regulated force would be greater (e.g. by 20 - 30% if the ionic strength was 160 mM and not 200 mM - see Figure 54). On the other hand the presence of a 'series elastic component' in the muscles would mean that the peak force developed during the phasic contraction of unskinned muscle would be expected to be less than the steady force the myofibrils could generate, if provided with the same intracellular conditions but with a constant Ca^{2+} concentration. Nevertheless the similarity of forces in the intact and Triton-skinned muscles is reassuring evidence that the skinning procedure was not damaging to the myofibrils.

11.6 Lack of swelling of the cardiac muscles during the skinning with Triton X-100

When single skeletal fibres are skinned mechanically they swell by 25% - 100% and lose constant-volume behaviour; these changes reflect similar alterations in the properties of the myofilament lattice and are at least partly due to a change in the Donnan equilibrium between cytosol and intermyofilament solution as a consequence of the loss of cytosolic proteins after skinning (Matsubara & Elliott, 1972; April et al., 1972; Godt & Maughan, 1977). One might therefore have expected the cardiac trabeculae to have swollen as the cytosolic proteins were washed out during the skinning procedure, but in fact there was no observable change in muscle diameter during this period. One possible explanation is that any myofibrillar swelling in the trabeculae took place entirely at the expense of other intracellular structures, resulting in no net increase in muscle diameter. In intact cardiac cells mitochondria account for about 40% of the cell volume, so a reduction in mitochondrial volume by Triton X-100 could compensate for any increase in myofibrillar volume.

Alternatively, it is conceivable that the cardiac sarcolemma, although disrupted, retained sufficient structural integrity to restrain the myofilaments from outward movement. Similar reasoning may explain the retention of constant-volume behaviour in glycerinated

psoas muscle (Rome, 1972) and the lack of swelling in skeletal muscles during skinning with detergent (Altringham, personal communication, 1981).

11.7 Creatine kinase and pyruvate kinase activity

The rapid relaxation of rigor contractures by CP + MgADP or phosphoenolpyruvate + MgADP argues for the retention of creatine kinase activity and pyruvate kinase activity, respectively, in the skinned trabeculae. I expected that creatine kinase activity would be present, since in myocardial cells 20% of the total activity of this enzyme is associated with the myofibrils (possibly as a component of the M-line) and 40% with the mitochondria (Sachs et al., 1974). On the other hand, pyruvate kinase is usually regarded as a predominantly cytosolic enzyme, so it was a surprise to find it still active in the skinned muscles. Possibly pyruvate kinase molecules had non-specifically bound to structures in the skinned muscles.

11.8 Comparison of the relative ATPase - pCa relationship of the isolated cardiac myofibrils with the relative force-pCa relationship of the skinned cardiac muscles.

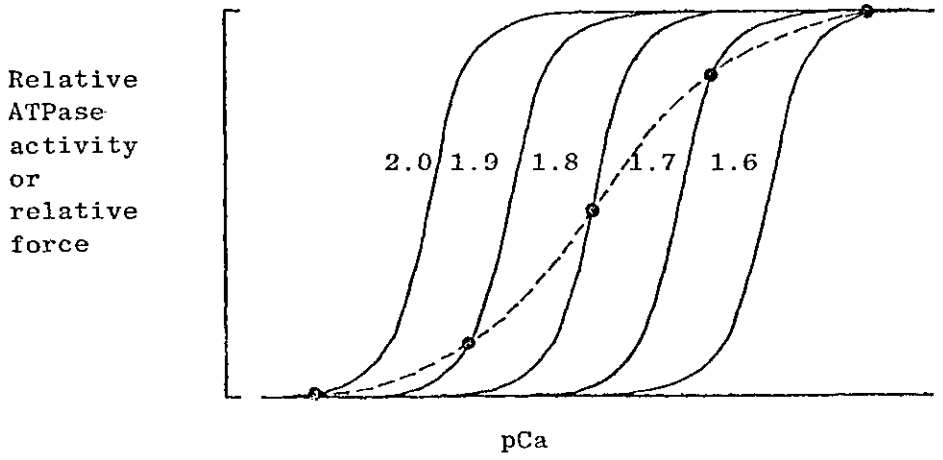
There were several differences between the Ca^{2+}-activation curves for the myofibrillar ATPase activity (Figure 25) and for the force production (Figure 47) in the two types of preparation from guinea-pig cardiac muscle. Firstly, the pCa_{50} for the myofibrillar ATPase activity was 5.94 at pH 7.00 (Table 5), whereas the pCa_{50} for force at pH 7.00 occurred at pCa 5.56 (experiments on [salt] - Table 11) or 5.44 (experiments on pH - Table 12). Much of this difference between the Ca^{2+}-sensitivities of ATPase and force would have been due to the fact that in the experiments on skinned muscle the total concentration of K^+ and Na^+ ions was 160 mM, compared with 95 mM in the experiments on the isolated myofibrils. Both K^+ and Na^+ ions reduce the Ca^{2+}-sensitivity of force (Section 10.11), and I estimate from Figure 58 that the 65 mM difference in [K^+ + Na^+] between the two studies would have caused the Ca^{2+}-activation curve for the skinned muscles to lie about 0.2 pCa units to the right of that for isolated myofibrils. This factor accounts for about half of the discrepancy in the observed pCa_{50} values for ATPase activity and force. The remainder was probably due to the fact that the isolated myofibrils were free to shorten during activity whereas the skinned muscles were held isometric (although some internal shortening would have occurred

in the skinned muscles). Thus the sarcomeres in actively-contracting isolated myofibrils would have been much shorter than in the skinned muscles and this would have caused a lower Ca^{2+}-sensitivity in the isolated myofibrils (Section 1.5 (a)). I cannot rule out the additional possibility that differences in experimental conditions (e.g. temperature) contributed to the differences in pCa_{50} between the ATPase activity of isolated myofibrils and the force production of skinned muscle.

Another difference between the two studies was that the slope of the Ca^{2+}-activation curve was steeper for force production than for ATPase activity. For example, n_H at pH 7.00 was 2.00 - 2.14 in the skinned muscles (Tables 11 and 12) as opposed to only 1.25 for the isolated myofibrils from guinea-pig (Table 5). This has also been observed by Kerrick et al. (1980), who found that n_H at pH 7.0 was 1.90 for the force production of rabbit cardiac 'mechanically-skinned' muscle (probably chemically-skinned by EGTA in fact) and 1.72 for the ATPase activity of rabbit cardiac myofibrils. Certainly some of this difference would have been due to the lower ionic strength in the myofibrillar ATPase experiments because I found that a reduction in ionic strength from 200 mM to 100 mM decreased n_H in the skinned muscles from 2.14 to 1.67 (Table 12). There are several other factors that could explain the rest of the discrepancy in n_H values. One possibility arises from the work of Brandt et al. (1980), who proposed that the extreme steepness of the relative force-pCa relationship in skinned skeletal fibres was due to a mechanism whereby the inherent Ca^{2+}-sensitivity of each myofibril was directly related to the force being generated in the myofibril at that moment. If this suggestion is correct, the Ca^{2+}- activation curve for the isolated myofibrils, which develop little force because they contract isotonically rather than isometrically, would be expected to be shallower than that for the myofibrils in an isometrically-contracting skinned muscle.

The difference in steepness between the ATPase-pCa and force-pCa relationships could also have been another consequence of the freedom of the isolated myofibrils to shorten. As discussed above, the decrease in sarcomere length would have decreased the Ca^{2+}-sensitivity. If shortening is greater the lower the pCa, then it follows that the lower the pCa, the lower the Ca^{2+}-sensitivity. The measured ATPase activity-pCa relationship would then have been

equivalent to a line joining a series of ATPase-pCa curves for the isometric condition. For example, the Ca^{2+}-activation curve for a myofibril starting with a resting sarcomere length of 2.0 μm and shortening to 1.6 μm upon full Ca^{2+}-activation would be comprised of the following isometric Ca^{2+}-activation curves:



—— individual Ca^{2+}-activation curves for sarcomeres held isometric at the length shown (values in μm)

--- observed Ca^{2+}-activation curve for a myofibril which shortens during activity to an extent dependent upon the pCa

11.9 Influence of pH on the force production of the skinned cardiac muscles

The objective of this series of experiments was to determine whether the pH-sensitivity of the Ca^{2+}-regulated force in skinned muscles was similar to the observed pH-sensitivity of the Ca^{2+}-regulated ATPase activity of isolated myofibrils. Originally my intention had been to study both cardiac and skeletal muscle. However during the course of my experiments on skinned cardiac trabeculae two papers were published in which the pH-sensitivities of skinned cardiac and skeletal muscles were compared (Fabiato & Fabiato, 1978b; Donaldson & Hermansen, 1978). Therefore it was decided that it would not be worthwhile for me to extend my experiments to include skeletal muscle.

I investigated the relationship between the pCa of the bathing solution and the force at the four values of pH (7.20, 7.00, 6.80 and

and 6.40) that had been used in the myofibrillar ATPase experiments. It can be reasonably assumed that the pH of the solution immediately surrounding the myofibrils in the skinned muscle was equal to the pH of the external bathing solution because (i) all solutions contained a high concentration (20 mM) of the pH buffer, imidazole, and (ii) any tendency for the solution inside the skinned muscle to acidify owing to the production of protons during MgATP hydrolysis would have been largely counteracted by the simultaneous activity of the proton-consuming regeneration of ATP catalysed by creatine kinase.

(a) Maximum Ca^{2+}-regulated force

Decreases in pH over the range pH 7.20 - pH 6.40 had two main effects on the Ca^{2+}-activation of the detergent-skinned cardiac muscles: the maximum Ca^{2+}-regulated force was reduced and the relative force-pCa relationship was shifted to lower pCa values (Section 10.10). The former effect - the depressant action of acidity on maximum force - confirms the data obtained from glycerol-extracted papillary muscle (Schädler, 1967; Steiger, 1977) and therefore refutes the results of Winegrad (1971), who reported that the Ca^{2+}-activation of EDTA-treated frog cardiac muscle was independent of pH over the pH range 6.5 - 7.0. Further confirmation that a decrease in pH depresses force development came from the studies on the pH-sensitivity of mechanically-skinned muscle from rat hearts (Fabiato & Fabiato, 1978b) and rabbit hearts (Donaldson & Hermansen, 1978). However the detergent-skinned trabeculae in my experiments exhibited a pH-dependence of maximum force that was approximately double the pH-dependence observed in these other studies. The most likely cause of this discrepancy is that my experiments were carried out at a higher temperature (30°C) than in these other investigations (20°C - 24°C); Donaldson & Hermansen (1978) mention preliminary data of theirs which indicate that raising the temperature from 21°C to 30°C increased the acidotic depression of maximum Ca^{2+}-regulation force. It may be that at true *in vivo* temperatures ($\approx$37°C) the pH-sensitivity of maximum force development is greater still.

I found the pH-sensitivity of maximum Ca^{2+}-regulated force in the cardiac trabeculae to be slightly greater in the alkaline pH range (pH 7.20 - 7.00) than in the acidic range (7.00 - 6.40). Fabiato & Fabiato (1978b) reported the converse to be true with mechanically-skinned rat cardiac muscle, but in fact the large scatter in their data precludes such a definite conclusion because none of the

differences in maximum force at the different pH values were statistically significant, except for the difference between the forces at pH 6.60 and pH 6.20 (compare this with the small scatter in Figure 47). Schädler's data, like mine, appeared to indicate a reduction of pH-sensitivity as the pH was lowered. However over the wide pH range studied by Schädler (pH 7.0 - pH 5.5) it is probable that the free concentrations of several ionic species such as Mg^{2+} and $MgATP^{2-}$ varied so much that they affected the Ca^{2+}-regulated force directly.

(b) Relative force-pCa relationship

In addition to reducing maximum Ca^{2+}-regulated force, a fall in pH also shifted the relative force-pCa relationship to lower pCa values. The pCa required for half-maximal Ca^{2+}-regulated force (pCa_{50}) fell linearly with the decrease in pH. Over the pH range 7.20 - 6.40 the pCa_{50} dropped by 0.71 pCa units, giving a mean value for $\Delta pCa_{50}/\Delta pH$ of 0.89 (Figure 48). A pH-dependent shift in the Ca^{2+}-activation curve has been observed in some of the other studies but the degree of pH-sensitivity varies considerably: values for $\Delta pCa_{50}/\Delta pH$ range from 0 (Winegrad, 1971) to 1.2 (Schädler, 1967). Fabiato & Fabiato (1978b) found a mean $\Delta pCa_{50}/\Delta pH$ of 0.58 with rat cardiac cells, while Donaldson & Hermansen (1978) obtained 0.68 rabbit cardiac muscle. In the two other studies, apart from mine, in which the Ca^{2+}-activation curve has been determined at more than two values of pH (Schädler, 1967; Fabiato & Fabiato, 1978b), it has been reported that the pH-sensitivity of pCa_{50} is non-linear, but while Schädler (1967) observed the pH-sensitivity to be more pronounced as acidity increased, Fabiato & Fabiato (1978b) reported that pH-sensitivity was lower in the acidic than in the near-neutral range. As mentioned above, the results from both these studies must be interpreted with care. My data, which indicate a linear fall in pCa_{50} with pH, lie between these two results and confirm neither.

The steepness of the Ca^{2+}-activation curves was also dependent upon the pH. The curves were shallowest at pH 7.00, where n_H was 2.0. In my experiments the maximum slope was found at pH 6.40, where n_H was 2.4. This pH-sensitivity of n_H does not agree with the results of Donaldson & Hermansen (1978), who reported n_H was constant at 2.14 over the pH range 7.0 - 6.5.

My results, and those of Fabiato & Fabiato (1978b) and Donaldson & Hermansen (1978), in general confirm those of Schädler (1967) and refute those of Winegrad (1971). However the pH-sensitivities of

maximum Ca^{2+}-regulated force and the relative force-pCa relationship varied considerably between the different studies. One factor causing this variability could have been the different skinning techniques used. Glycerol-extracted muscle, which was used by Schädler (1967) and Steiger (1977), may have an altered response to Ca^{2+} (Abbott, 1973), while EDTA-treated cardiac muscle, which displayed no pH-sensitivity (Winegrad, 1971), may not be functionally skinned (Miller, 1979). It is unlikely that either mechanically-skinned muscles, as used by Fabiato & Fabiato (1978b), or Triton-treated muscles suffer from these problems. The cardiac muscle used in the skinned muscle experiments described above has been taken from various species: guinea-pig (my experiments), rat (Fabiato & Fabiato, 1978b), rabbit (Winegrad, 1971; Donaldson & Hermansen, 1978; Steiger, 1977), and dog (Schädler, 1967). It is not unreasonable to postulate species-specifc differences in the pH-sensitivity of force development. However species differences probably cannot account for any differences between my results obtained with guinea-pig muscle and those obtained with rabbit muscle because, from the similar pH-sensitivities of the ATPase activities of rabbit and guinea-pig myofibrils (Section 6.4(c)), it might be expected that the pH-sensitivities of force development should also be similar in the two species.

The most likely explanation for the differences in pH-sensitivity discussed above is a difference in the experimental conditions: variations in the composition of the solution would modulated the effects of pH on force development. For instance, a rise in the Mg^{2+} concentration has been found to decrease the pH-dependence of Ca-regulated force in both cardiac and skeletal mechanically-skinned muscle (Donaldson & Hermansen, 1978). The shape and position of the relative force-pCa curves would also depend upon whether allowance was made for the impurity in EGTA (e.g. Figure 37) but this factor would not affect the pH-sensitivity.

(c) Possible mechanisms for the effects of H^+ ions on Ca^{2+}-regulated force

What are the mechanisms then for the observed actions of $[H^+]$ on Ca^{2+}-regulated force? In considering this question, it is relevant to compare the pH-sensitivity of Ca^{2+}-regulated force with that of the ATPase activity of isolated myofibrils. Originally I had hoped that the skinned muscles could be investigated under the same experimental

conditions as in the myofibrillar ATPase experiments but unfortunately the different characteristics of the two preparations made this impossible. For example, whereas the isolated myofibrils had been incubated at 37°C in solutions of ionic strength 120 mM, the skinned muscles were found to need lower temperatures (30°C or below - Section B.3(e)) and higher ionic strengths (150 mM or above - Section 10.11(e)) for adequate viability. There were several other differences between the incubation solutions for the isolated myofibrils and the activating solutions for the skinned muscles, though wherever possible the ionic conditions in the skinned muscle experiments were chosen to be identical to those in the experiments on isolated myofibrils. It should also be borne in mind that the isolated myofibrils would have shortened in the Ca^{2+}-containing incubation solutions whereas the skinned muscles were held virtually isometric in the Ca^{2+}-containing activating solutions; the decrease in sarcomere length in the isolated myofibrils may have reduced their Ca^{2+}-sensitivity (discussed in Section 11.8). Despite these limitations, a comparison of the influences of $[H^+]$ on isolated cardiac myofibrils and on skinned cardiac muscle can yield some pertinent information. In both preparations a reduction of 0.2 pH units shifted the Ca^{2+}-activation curves by almost 0.2 pCa units to lower pCa values, and the decrease in the pCa_{50} for a drop of 0.2 pH units was linear over the pH range 7.20 - 6.40 (Figures 26 and 48). These data are consistent with a simple 1:1 competition between H^+ and Ca^{2+} ions for a Ca^{2+}-binding site that is involved in the regulation of contraction, although it is necessary to postulate an additional action of H^+ ions to account for the pH-sensitivity of the steepness of the Ca^{2+}-activation curves. Because there was such a striking similarity between the influence of pH on the pCa_{50} of the cardiac myofibrillar ATPase activity and the influence of pH on the pCa_{50} of force production in the skinned muscles, this strongly suggests that the pH-dependence of the Ca^{2+}-sensitivity of force was due entirely to the pH-dependence of the Ca^{2+}-sensitivity of the ATPase activity. Possible causes for this pH-dependence of the Ca^{2+}-regulated myofibrillar ATPase activity have already been discussed (Section 7.5).

There is an alternative explanation for the observed effects of $[H^+]$ on the Ca^{2+}-sensitivity of the skinned trabeculae: the increase in Ca^{2+}-sensitivity at higher pH values could conceivably have been due to a deficiency in the supply of $MgATP^{2-}$ to the myofibrils. It is known that the rate of regeneration of MgATP in the creatine kinase

reaction decreases from 100% to zero as the pH is raised from pH 5 to 8 (Nikei et al., 1961). Any severe dysfunction of the CP/creatine kinase ATP-regenerating system at higher levels of pH could have resulted in a significant drop in [$MgATP^{2-}$] in the core of the muscle during activity of the myofibrillar ATPase. If the internal [$MgATP^{2-}$] fell to a sufficiently low level, rigor links would form and this would increase the apparent Ca^{2+}-sensitivity of the preparation (Bremel & Weber, 1972; Godt, 1974). However it is unlikely that the observed effects of pH on the Ca^{2+}-regulated force are even partly attributable to this mechanism, for several reasons. Firstly, in these experiments on pH 25 $\mu g\ cm^{-3}$ of creatine kinase was present in all solutions, in addition to the creatine kinase which remained in the muscle and which was shown to be enzymatically active at pH 7.00 (Section 10.5). Secondly, if the [$MgATP^{2-}$] had indeed been limiting in the muscle at the highest values of pH, one would have expected an increase in the resting force as a consequence of the formation of rigor links. In fact, resting force did not alter at all over the pH range studied.

In contrast to the effect of pH on submaximal force, the effect of pH on maximum Ca^{2+}-regulated force could not be overcome by increasing the free Ca^{2+} concentration, and it was therefore probably not due to Ca^{2+}/H^{+} antagonism at a Ca^{2+}-binding site. Some of the depression of the maximum force in acidic solutions almost certainly resulted from the inhibition of Ca^{2+}-regulated ATPase activity that was observed in the experiments on isolated myofibrils (Section 6.4(b)). However, the detrimental action of H^{+} ions on the maximum Ca^{2+}-regulated force was much greater than on the maximum Ca^{2+}-regulated ATPase activity of the myofibrils. For instance, decreasing the pH from 7.20 to 6.40 reduced the maximum force in the guinea-pig skinned trabeculae by 56% (Figure 47) but the maximum ATPase activity of the guinea-pig myofibrils fell by only 9% over this range (Figure 25). This discrepancy could have arisen from the differences in the preparations or in the experimental conditions, as discussed in Section 11.8 above. On the other hand it could represent a real difference that resulted from a deleterious action of H^{+} ions on the force generated in each cross-bridge for each ATP molecule split. In other words H^{+} ions may have decreased the efficiency of the mechano-chemical transduction. To test this possibility it would be necessary to simultaneously measure ATPase activity and force development in the same skinned muscle. However, it would appear that the effects of pH on maximum force are not mediated by changes in cross-bridge kinetics, since Steiger (1977) reported that a decrease in pH from 7.0 to 6.5 did not alter the contractile kinetics of glycerol-extracted muscle subjected to quick releases or stretches.

Steiger's results do not however exclude the possibility that the mechano-chemical transduction is sensitive to pH.

Although I do not know the mechanism responsible for the pH-dependence of maximum Ca^{2+}-regulated force, there is one possible explanation which is based upon an alteration in myofilament lattice spacing. Incrases in the $[H^+]$ shrink the myofilament lattice, perhaps because of a partial neutralisation of the net negative charges on the myofibrillar proteins (Rome, 1968; April et al., 1972; Godt & Maughan, 1977). Similar reductions in lattice volume can be accomplished by addition of long-chain polymers, such as polyvinylpyrrolidone (PVP), to the bathing solutions and shrinkage of skinned muscles by PVP is accompanied by a decrease in maximum Ca^{2+}-regulated force(Godt & Maughan, 1977; Fabiato & Fabiato, 1978a). Thus it is conceivable that acidity depresses maximum force by a similar shrinkage of the myofilament lattice, though it is not clear whether this shrinkage would also reduce the myofibrillar ATPase activity.

Because cardiac myofibrils in vivo are probably never maximally activated by Ca^{2+}, it is the influence of pH on the submaximal Ca^{2+}-regulated force that is of chief physiological significance for the function of intact cardiac muscle. My results showed that the actions of H^+ ions on the maximum force and on the relative force-pCa relationship combined to result in an extreme pH-sensitivity of force at any suboptimal Ca^{2+} concentration. For example, at pCa 5.5, which was the pCa that produced about half maximal force at pH 7.00, a drop in pH from 7.00 to 6.40 decreased force by more than 90% (Figure 50); for comparison, the maximum Ca^{2+}-regulated force (at pCa <4.0) was reduced by only 34% by the same fall in pH. Whether this very marked pH-dependence of submaximal force can account for the magnitude of the negative inotropic effect of an intracellular acidosis in intact cardiac muscle is discussed in detail in the next chapter (Section 12.1).

(d) Decline of Ca^{2+}-regulated force

In spite of the detrimental actions of acidity on the force production of the skinned cardiac trabeculae, a reduction in pH increased the viability of the preparations. Much of this 'protective' effect may have been due to the smaller forces at the lower values of pH, but there was also some evidence for a more direct beneficial effect of acidity on the longevity of the muscle: if the Ca^{2+}-regulated forces were matched in the solutions of different pH, the

decline in force was still slower in the more acidic solutions (Figure 52). This finding has been supported by the results of Robertson & Kerrick (1979), who found that a decrease in pH over the pH range pH 8 - pH 5 increased the viability of mechanically-skinned skeletal fibres.

In whole cardiac muscle, too, acidosis has a protective effect on force development during and after a period of low-flow or hypoxic perfusion (Nayler et al., 1979). Although these authors argued that the protection resulted from a preservation of high-energy compounds because of the reduction in developed force, the results from my skinned muscle experiments indicate there may also be a direct protective effect of acidity on myofibrillar function in intact cardiac muscle.

11.10 Influence of [salt] on the force production of the skinned cardiac muscles

(a) Maximum Ca^{2+}-regulated force

Changes in the salt composition of the solutions had several actions on force development in the skinned trabeculae. Perhaps the most dramatic effect was the linear decrease in maximum Ca^{2+}-regulated force (at pCa $\approx$ 3.75) seen when the ionic strength was increased over the range 100 mM - 400 mM (Figure 54). In this respect, the detergent-skinned cardiac muscle displayed the same characteristics as skinned skeletal muscle (Gordon et al., 1973; Homsher et al., 1974; Thames et al., 1974; Gulati & Podolsky, 1981) and skinned barnacle muscle (Ashley & Moisescu, 1977). There was no evidence for a plateau of Ca^{2+}-regulated force at $\Gamma/2 \approx 130$ mM like that seen in one study on skeletal muscle (Gordon et al., 1973) but not in the others. At an ionic strength of 400 mM the Ca^{2+}-regulated force of the cardiac muscle was not significantly different from zero. Thus skinned cardiac muscle is more sensitive to [salt] than are skinned skeletal muscle and barnacle muscle, which are both able to develop some Ca^{2+}-regulated force at $\Gamma/2$ = 400 mM (Gordon et al., 1973; Homsher et al., 1974; Ashley & Moisescu, 1977).

Equivalent increases in ionic strength by the addition of KCl, NaCl, TMACl or ChCl produced similar decreases in maximum Ca^{2+}-regulated force (Figure 54). In skeletal muscle too, corresponding K^+, Na^+ and TMA^+ salts are equally effective in their inhibition of maximum Ca^{2+}-regulated force (Gordon et al., 1973). These findings suggest that the reduction in force in the cardiac muscles was not due to specific

inhibitory actions of the cations used but rather to the increase in ionic strength per se.

My results do not concur therefore with those of Katz et al. (1966), who reported that replacement of half of the KCl by NaCl at $\Gamma/2 \approx 80$ mM led to a 20% enhancement of the ATPase activity of reconstituted cardiac actomyosin (i.e. the inhibition by K^+ ions was greater than that by Na^+ ions). I found that increases in KCl or NaCl over the range 100 mM - 400 mM had almost identical inhibitory actions of Ca^{2+}-regulated force (Figure 54). The data from the actomyosin experiments of Katz et al. (1966) must be viewed with caution, however, for Katz et al. (1966) found virtually no actin-activation of cardiac myosin at ionic strengths in the physiological range.

The similar inhibitory effects of the different chloride salts in my studies do not prove conclusively that the inhibition of force was due to the increase in ionic strength per se. There are two other possible explanations. One is that the reduction in force was specifically the result of the increase in $[Cl^-]$. This could be tested by comparing the effects of KCl with those of other K^+ salts, such as K propionate. The second possibility is that at least part of the inhibitory effect of a raised [salt] on maximum Ca^{2+}-regulated force resulted from the accompanying increase in tonicity of the solution. This seemed likely since Ashley & Moisescu (1977) have reported that the maximum force in skinned barnacle fibres fell by 20% when the osmolarity of the solutions was increased by 320 mosmol l^{-1} with sucrose. In contrast to their findings, I observed that the Ca^{2+}-regulated force in the skinned cardiac trabeculae was insensitive to increases in osmolarity produced by the addition of 0 - 600 mM sucrose at a constant $\Gamma/2$ of 100 mM (Figure 56). (Addition of 600 mM sucrose would have been osmotically equivalent to the addition of 300 mM KCl). This insensitivity of force to the osmolarity of the solution indicates that not even a small part of the depressant action of a raised [salt] on maximum force can be ascribed to the concomitant increase in tonicity. I do not know why there is this discrepancy between my data and those of Asley & Moisescu (1977). One major difference between the two preparations is that the mechanically-skinned barnacle fibres, unlike the detergent-skinned cardiac muscle, would possess an intact SR. The SR in mechanically-skinned fibres shrinks in a sucrose-containing medium (Godt, unpublished observations,

in Godt & Maughan, 1977), but it is difficult to see how such a change in volume of the SR could alter the steady force development in the skinned barnacle fibres. Perhaps cardiac muscle and barnacle muscle do truly possess a species-difference in their sensitivities to solution osmolarity.

A distinction should be made here between changes in solution osmolarity produced by molecules such as sucrose that probably can readily enter the myofilament lattice and changes produced by larger molecules such as proteins or PVP that may be excluded from the lattice. An increase in solution osmolarity produced by addition of impermeant molecules would cause shrinkage of the myofilament lattice, and this would directly inhibit Ca^{2+}-regulated force (Godt & Maughan, 1977; Fabiato & Fabiato, 1978a).

In summary, the decrease in force in skinned cardiac muscles seen when the ionic strength was raised with KCl was not due to a specific inhibition by K^{+} ions or to the increase in tonicity; almost certainly it resulted from the increase in ionic strength per se.

The inhibitory action of a raised [salt] on maximum Ca^{2+}- regulated force was similar to its action on the maximum Ca^{2+}-regulated ATPase activity of isolated cardiac myofibrils, which suffered a 20% decrease in ATPase activity as the ionic strength was raised from 100 mM to 200 mM (Figure 22). This would indicate that much of the influence of [salt] on force was a consequence of its influence on the ATPase activity. However there is not a perfect parallelism between the ATPase activity of isolated myofibrils and the force development of skinned muscles because the ATPase activities of both cardiac and skeletal myofibrils reach a peak at ionic strengths in the range 100 - 140 mM (Figure 22; Portzehl et al., 1969; Muir et al., 1971) whereas, with one exception (Gordon et al., 1973), in studies on skinned cardiac and skeletal muscle the peak in Ca^{2+}-regulated force occurs at ionic strengths below 100 - 120 mM (Figure 54; Homsher et al., 1974; Thames et al., 1974). Why there is such a discrepancy between the two types of preparation is not clear.

Nevertheless, the fact remains that there is an approximate parallel between the influence of [salt] on the Ca^{2+}-regulated force in skinned muscles and the influence of [salt] on the Ca^{2+}-regulated ATPase activity of isolated myofibrils: both are decreased as the $\Gamma/2$ is raised from 100 mM to 200 mM. At least part of the influence of [salt] on force is therefore probably a direct result of its depression of the ATPase activity, though (as in the study on pH) I

cannot exclude the possibility that there is an ionic strength-dependence in the force generated in each cross-bridge per molecule of ATP split.

These effects on the biochemical and contractile properties of myofibrils could arise from (i) a change in the number of sites at which cross-bridges were formed, or from (ii) a change in the kinetic properties of each cross-bridge. Studies on the contractile kinetics of skinned skeletal muscle have attempted to resolve these possibilities, but as yet the results are equivocal. Most workers have reported that the maximum steady speed of shortening, which reflects the maximum turnover rate of cross-bridges, is independent of $\Gamma/2$ over the range 100 mM - 300 mM (Gulati & Podolsky, 1978, 1981; Julian & Moss, 1981). However, if the muscles develop 'residual force' (see below), shortening is slowed (Thames et al., 1974; Gulati & Podolsky, 1978). This ionic-strength dependence of steady shortening in normally-contracting muscles would appear to preclude an action of $\Gamma/2$ on cross-bridge kinetics, but Gulati & Podolsky (1978) have observed that the amplitude of the presteady motion during shortening of skinned skeletal muscle is increased as $\Gamma/2$ is increased with 50 - 210 mM KCl. This indicates a subtle change in the properties of cross-bridge kinetics with change in ionic strength. Whether this change in kinetics can account for all, or even a part of, the $\Gamma/2$-dependence of isometric force in skeletal muscle is not clear at present. As yet, there have been no equivalent experiments with skinned cardiac muscle.

This still leaves the question, what is the physico-chemical mechanism by which an increase in [salt] decreases the ATPase activity or force production of myofibrils? The depression of force at high ionic strengths was not due to the decrease in Ca^{2+}-sensitivity that was observed in some solutions because a further decrease in pCa from 3.75 to 3.56 by the addition of $CaCl_2$ did not potentiate force at the high ionic strengths. One possible explanation (Gordon et al., 1973) is that neutral salts affect protein structure, and thus myofibrillar activity, by the effect on water structure. However it might be supposed that sucrose would also alter water structure, since the sucrose molecule readily forms hydrogen bonds with water molecules, and yet sucrose, in concentrations as large as 600 mM, did not depress the Ca^{2+}-regulated force (Figure 56). Thus a change in water structure may not be an important factor in the mechanisms of the ionic strength-dependence of ATPase activity and force.

One of the most attractive hypotheses is that an elevated ionic strength leads to an increased screening of charged regions on actin and myosin. Thus an increase in Γ/2, like a decrease in pH (Rome, 1968; April et al., 1972; Godt & Maughan, 1977), would reduce the mutual repulsion on the negatively-charged myofilaments, which would then draw closer together. As discussed in Section 11.9(c), a shrinkage of the myofilament lattice with PVP does reduce Ca^{2+}-regulated force*. This hypothesis appears to be refuted by the observations that changes in Γ/2 over the range 100 - 300 mM did not significantly alter the lattice spacing in glycerinated or mechanically-skinned skeletal fibres (Rome, 1968; Matsubara & Elliott, 1972) or reduce the diameter of detergent-skinned cardiac muscle (personal observation) or mechanically-skinned skeletal fibres (Godt & Maughan, 1977).

Eisenberg & Moos (1968) found that an increase in Γ/2 up to 100 mM decreased actin-myosin interaction by reducing the affinity of the two proteins; whether a similar phenomenon underlies the decrease in Ca^{2+}-regulated force at higher ionic strengths remains to be determined.

(b) Resting force

The salt composition of the solution also influenced the resting force, which increased as the Γ/2 fell below about 150 mM (Figure 54). An increase in resting force at low ionic strengths was observed even if the osmolarity had been raised with sucrose, so the effect of [salt] was not related to changes in osmolarity of the solution. The resting forces in relaxing solutions containing 100 mM KCl, NaCl, TMACl or ChCl were not significantly different, indicating that resting force was determined mainly by the ionic strength rather than by the salt composition or, more specifically, the cation composition of the relaxing solution. This find runs contrary to that of Gordon et al. (1973), who reported that the resting force was sensitive to the neutral salt used, especially the cation: for instance, they observed that the resting force was lower in K^+ salts than in TMA^+ salts, indicating some specific ion effects.

* On the other hand, it could be that the shrinkage of myofibrils in PVP decreases force because it produces an effective increase in ionic strength in immediate microenvironment of the myofilaments.

An increase in Ca^{2+}-insensitive interaction between actin and myosin at low ionic strengths is well documented and has been observed with the force in skinned skeletal fibres (Gordon et al., 1973; Julian & Moss, 1981) and with the ATPase activities of skeletal myofibrils (Figure [22]; Portzehl et al., 1969; Goodno et al., 1978) and cardiac myofibrils (Figure 22). The increase in actomyosin ATPase activity at low ionic strengths (Hasselbach, 1952; Leadbeater & Perry, 1963; Katz et al., 1966; Eisenberg & Moos, 1968) may be a reflection of the same phenomenon - an increase in the steady-state number of cross-bridges (Section 3.2). Although the Ca^{2+}-sensitivity of the myofibrils increased as the [salt] was lowered (Figure 57), the pCa in the relaxing solutions (>9) was nevertheless far too high to induce any significant Ca^{2+}-activation. Thus the cross-bridges responsible for the resting force must have been either normal cross-bridges that were no longer inhibited by troponin-I at high pCa values or abnormal cross-bridges. The existence of the latter was favoured by Thames et al. (1974), who proposed that long-lived abnormal cross-bridges, perhaps similar to rigor cross-bridges, could account for the rise in resting force and the decrease in shortening velocity observed at low ionic strengths in skinned skeletal muscle. However the more recent demonstrations that steady shortening velocity is unchanged at ionic strengths low enough to produce a rise in resting force (see Section 11.10(a) above) indicate that the elevated resting force cannot be due to long-lived cross-bridges, for these would tend to retard motion and reduce the speed of shortening. This is supported by the observed rise in Ca^{2+}-insensitive ATPase activity at low ionic strengths (e.g. Figure 22), since this is what one would have expected to see if the steady-state number of normal cross-bridges had increased, whereas an increase in the number of rigor links would not have been expected to directly raise the ATPase activity. In addition, Gordon et al. (1973) observed that total force remained constant as resting force increased and Ca^{2+}-regulated force decreased with a drop in $\Gamma/2$ - again this is consistent with the idea that the properties and the total possible number of normal cross-bridges were not altered by low ionic strength but that the formation of some of these cross-bridges was no longer inhibited in the absence of Ca^{2+}. However I cannot exclude the possibility that the primary event was a small increase in the number of rigor links and that this induced a larger increase in the number

of 'normal' cross-bridges by the co-operative mechanism described by Bremel & Weber (1972 - Section 1.3(b)). One way to test for the presence of rigor links might be to measure the instantaneous stiffness (Section 1.3(e)) of the skinned muscles in relaxing solutions: if the stiffness at low ionic strengths was found to be greater than the stiffness in an activating solution of $\Gamma/2$ = 200 mM that produced the same isometric force as the low-$\Gamma/2$ relaxing solution, this would indicate the presence of rigor links at the lower ionic strength.

Gordon et al. (1973) in fact found that if the skinned skeletal fibres were slackened manually at low ionic strength the increased resting force redeveloped at the new muscle length, just as normal Ca^{2+}-regulated force, but not rigor force, would. Again, this would imply the raised resting force is not due to long-lived abnormal cross-bridges.

However, such cross-bridges could account for another phenomenon seen at ionic strengths below 150 mM: the incomplete relaxation after each contracture (e.g. Figure 53). The increase in resting force was termed 'residual force' by Thames et al. (1974) and has been observed in other studies on skinned skeletal fibres (Gordon et al., 1973; Gulati & Podolsky, 1981; Julian & Moss, 1981). Podolsky's group have found that whenever the muscle exhibits residual force, the maximum shortening velocity is reduced and they have therefore suggested that both phenomena reflect an increase in the number of long-lived 'abnormal' cross-bridges (Thames et al., 1974; Gulati & Podolsky, 1981). Thames et al. (1974) stated that residual force could not be reversed by a return to a relaxing solution of $\Gamma/2 \geqslant 150$ mM, but I find that this solution change did abolish residual force in detergent-skinned cardiac muscle (Figure 53).

In the preliminary studies for these experiments I often saw an upward 'creep' of Ca^{2+}-regulated force, followed by a residual force upon relaxation, in solutions of $\Gamma/2$ = 200 mM if the skinned muscles were thick and were perfused for many minutes with an activating solution of low pCa (Section 10.3). This residual force may be comparable to that seen by Thames et al (1974) in skeletal fibres at $\Gamma/2 > 150$ mM. It would appear that there are several ways in which residual force can be induced.

(c) Decline in Ca^{2+}-regulated force

The 'decline' in Ca^{2+}-regulated force - the deterioration of contractility in the muscle in successive contractures - was found

to vary considerably with ionic strength (Figure 59). The rate of decline was least in the ionic strength range 150 - 250 mM. A deleterious action of high ionic strength on muscle viability has also been noted with skinned skeletal muscle (Gordon et al., 1973). This decrease in contractility was almost certainly due to the solvation and subsequent wash-out of myofibrillar proteins: washing in solutions of high $\Gamma/2$ has long been used to extract myofibrillar proteins, particularly myosin.

At ionic strengths below about 150 mM, the decline was faster than at $\Gamma/2$ = 200 mM; this phenomenon has not been reported before. Thus a low ionic strength and a high pH had similar actions: both increased Ca^{2+}-regulated force (Figures 47 and 54) but both were in fact detrimental because they accelerated the loss of contractility (Figures 51 and 59). The mechanism by which the decline of force was accelerated may however have been different. It is possible that the pH-dependence of the rate of force decline resulted from shrinkage or swelling of the myofilament lattice; in contrast, the lattice spacing is relatively insensitive to ionic strength. One explanation for the increased rate of force decline at $\Gamma/2 < 150$ mM could be that the low $\Gamma/2$ causes a washout of the regulatory proteins. It is known that washes at low $\Gamma/2$ desensitize myofibrils by removal of troponin (Perry et al., 1966).

Possibly related to this decline of force was my observation that the $\Gamma/2$-dependence of maximum Ca^{2+}-regulated force at $\Gamma/2 < 200$ mM increased with time during the experiment (Figure 55). This increase in $\Gamma/2$-sensitivity arose because the absolute Ca^{2+}-regulated force at $\Gamma/2$ = 100 mM or 150 mM declined more slowly during an experiment than did the absolute Ca^{2+}-regulated force at higher ionic strengths ($\Gamma/2 \geq 200$ mM). Dancker & Hasselbach (1971) have previously demonstrated that the ionic strength-dependence of skeletal actomyosin ATPase activity is influenced by the state of oxidation of the actomyosin SH groups. In my experiments, however, the addition of dithiothreitol failed to abolish the increase in ionic strength-dependence of force, so this increase is not a consequence of an oxidation of myofibrillar SH groups.

It may be relevant that the alteration in myofibrillar sensitivity to $\Gamma/2$ occurred only in the range of ionic strengths that influenced resting force. As discussed above, the increase in resting force at low $\Gamma/2$ almost certainly reflected an increase in Ca^{2+}-independent interaction between actin and myosin. It may be that, during the course of the

experiment, the inherent ability of actin and myosin to interact declined more slowly than did the ability of the troponin-tropomyosin system to allow this interaction in the presence of Ca^{2+}. In other words, the activity of the actin-myosin system itself may have deteriorated more slowly than the activity of the proteins that confer Ca^{2+}- sensitivity. The result of this would have been that the force in normal Ca^{2+}-regulated contractures (e.g. at I/2 = 200 mM) would have declined more rapidly than the force in contractures in which at least part of the Ca^{2+}-activation process was by-passed (e.g. at I/2 = 100 mM). Consequently there would have been an apparent time-dependent increase in ionic strength-sensitivity at low ionic strengths.

(d) Relative force - pCa relationship

Although ionic strength had marked effects on maximum Ca^{2+}-regulated force and on resting force, by itself it exerted little influence on the relationship between relative force and pCa (Section 10.11(d)). A decrease of I/2 from 200 mM to 100 mM by the omission of the 100 mM KCl that was present in the 'control' activating solutions increased the pCa_{50} from 5.56 to 5.85. However, this shift of the relative force-pCa relationship was also seen if the ionic strength was maintained at 200 mM by the substitution of 100 mM TMACl or ChCl for the KCl. From this it can be deduced that the shift seen if the KCl was omitted was not due to the decrease in ionic strength per se or to the decrease in $[Cl^-]$ or to the decrease in osmolarity, since all three factors would have been constant in solutions in which the KCl was replaced on an equimolar basis by TMACl or ChCl. It must be concluded that the difference between the solutions of zero added KCl (total $[K^+]$ = 30 mM) and 100 mM KCl (total $[K^+]$ = 130 mM) was due entirely to a specific inhibition by K^+ ions upon the Ca^{2+}-sensitivity of the myofibrils. A similar argument leads me to conclude that Na^+ ions also specifically inhibit the Ca^{2+}-sensitivity; indeed, because the relative force-pCa relationship seemed to be shifted slightly to lower values of pCa when the 100 mM KCl was replaced by 100 mM NaCl it appeared that the specific antagonism of Ca^{2+} by Na^+ ions was even greater than that by K^+ ions. However the Ca^{2+}-activation curves in 100 mM NaCl and 100 mM KCl were not in fact significantly different.

Whereas the pCa_{50} was influenced by the $[K^+]$ or $[Na^+]$ and was not affected by the ionic strength, the converse was true for the slope of the force-pCa relationship. If the $[K^+]$ was reduced at constant ionic strength by replacing the 100 mM KCl in control activation solutions with 100 mM TMACl or 100 mM ChCl, the value for n_H was not significantly altered from that (2.14) in 100 mM KCl (Table 12).

In contrast, n_H in zero added salt (1.67) was significantly below that in 100 mM KCl. Thus the n_H was not altered by a change in $[K^+]$ but it was decreased by a fall in ionic strength per se from 200 mM to 100 mM, although I cannot exclude the possibility that the concomitant decreases in $[Cl^-]$ and tonicity were contributory factors.

None of the effects of [salt] on the relative force-pCa relationship were due to differences in the apparent association constant of EGTA for Ca^{2+} at pH 7.0 ($K^{7.0}_{CaHEGTA}$) in the solutions of different [salt], because the value of the $K^{7.0}_{CaEGTA}$ was determined in these different salt concentrations (Appendix A) and this was allowed for in the computation of pCa. (In fact, however, the $K^{7.0}_{CaEGTA}$ was found to be altered only slightly by the different salt compositions).

My observation that K^+ and Na^+ ions exerted specific inhibitory actions on the Ca^{2+}-regulation of cardiac myofibrils agrees with and extends a similar finding by Ashley & Moisescu (1977). These workers noted that an increase in $\Gamma/2$ over the range $\approx$ 175 mM to $\approx$ 245 mM with KCl shifted the relative force-pCa curve for skinned barnacle muscle to lower pCa values, whereas a further increase in $\Gamma/2$ to $\approx$ 270 mM by substitution of 5 mM ATP + 10 mM CP with 15 mM ATP had no effect on the position of this curve on the pCa axis. Ashley & Moisescu (1977) concluded that the decrease in Ca^{2+}-sensitivity as the [KCl] was raised was not due to ionic strength but was a specific effect of K^+ ions. Similar reductions in Ca^{2+}-sensitivity as [KCl] was increased had previously been seen in studies on the ATPase activity of isolated myofibrils (Portzehl et al., 1969; Solaro & Briggs, 1974) but in these studies it had been assumed that ionic strength was the important variable.

In these studies on the Ca^{2+}-activation of isolated myofibrils, the KCl-induced shifts in the relative ATPase activity - pCa curve were observed only at ionic strengths below physiological levels. Using rabbit cardiac myofibrils, Solaro & Briggs (1974) observed that a decrease in $\Gamma/2$ from 123 mM to 25 mM raised the pCa for half-maximal activation. In the only study to date that encompassed 'physiological' ionic strengths, Portzehl et al. (1969) found the Ca^{2+}-activation curve of rabbit skeletal myofibrils was not shifted by a drop in $\Gamma/2$ from 200 mM to 100 mM, but was shifted to higher values of pCa if the $\Gamma/2$ was reduced further to 50 mM. Similarly the amount of Ca^{2+} bound to skeletal myofibrils decreased when the $\Gamma/2$ was lowered from 140 mM to 50 mM (Solaro et al., 1976). It is relevant that all these shifts

in the Ca^{2+}-sensitivity of isolated myofibrils were observed in the $\Gamma/2$ range in which there is considerable Ca^{2+}-insensitive ('basal') ATPase activity. This elevated basal activity is widely thought to reflect an increase in the steady-state concentration of cross-bridges at low ionic strengths. The presence of these cross-bridges would enhance thin filament binding of Ca^{2+} and thus ATPase activity (Bremel & Weber, 1972), thereby explaining why the Ca^{2+}-sensitivity is increased at ionic strengths below about 100 mM.

However it is unlikely that this mechanism could also account for the shifts in the relative force-pCa relationship seen in skinned muscles as the $\Gamma/2$ was varied (with KCl) in the $\Gamma/2$ range above 100 mM (Figure 57 ; Ashley & Moisescu, 1977). In the experiments of Ashley & Moisescu, the ionic strength was altered over a range (175 mM - 270 mM) that was well above the maximum $\Gamma/2$ at which an elevated resting force is observed (about 150 mM; Gordon et al., 1973; Thames et al., 1974). In my studies there was an increase in resting force at the lower ionic strength (100 mM) at which the relative force-pCa relationship was determined. However, the cross-bridges causing this increase in resting force cannot have been responsible for the greater Ca^{2+}-sensitivity seen in zero added KCl (total $\Gamma/2$ = 100 mM) compared with 100 mM added KCl ($\Gamma/2$ = 200 mM), because the higher Ca^{2+}-sensitivity was also obtained in 100 mM added TMACl or ChCl ($\Gamma/2$ = 200 mM), in which no increase in resting force was seen. In other words, the resting force was sensitive to the ionic strength but the Ca^{2+}-sensitivity of force development was not. On the contrary, the Ca^{2+}-sensitivity was affected only by the $[K^+]$ or $[Na^+]$. It is likely that at least part of the shifts in Ca^{2+}-sensitivity seen by Portzehl et al. (1969), Solaro & Briggs (1974) and Solaro et al. (1976) were also due to a specific inhibitory effect of K^+ ions. It is not clear why Portzehl et al. (1969) saw no shift in Ca^{2+}-sensitivity over the $\Gamma/2$ range 100 mM - 200 mM whereas I find the pCa_{50} for force is altered by 0.3 pCa units over the same range.

The exact locus of the antagonism of Ca^{2+} by K^+ or Na^+ ions must remain conjectural at present. Obviously the Ca^{2+}-binding sites on troponin-C are likely candidates, though the Ca^{2+}-binding site on each myosin 'P' light chain cannot be excluded. Since the $\Delta pCa_{50}/\Delta pK$ and $\Delta pCa_{50}/\Delta pNa$ were less than unity (0.46 and 0.56, respectively), a simple mechanism involving 1:1 competition between

Ca^{2+} ions and K^+ or Na^+ ions seems unlikely. It is interesting that monovalent cations also specifically antagonise the association of Ca^{2+} with aequorin and that, as with my skinned cardiac muscles, this antagonism is somewhat greater with Na^+ ions than with K^+ ions (Moisescu & Ashley, 1977). It seems likely that K^+ or Na^+ ions could, in the same way, compete with Ca^{2+} for a Ca^{2+}-binding site on troponin. One piece of evidence against this idea is that the binding of $^{45}Ca^{2+}$ to isolated troponin from skeletal muscle is not altered by an increase in $\Gamma/2$ from 100 mM to 500 mM by the addition of KCl or NaCl (Fuchs, 1974). As Fuchs (1974) pointed out, an ionic strength-insensitivity of Ca^{2+} binding to troponin would be predicted if in troponin, as in parvalbumins, the binding sites are in 'buried' locations shielded from the bulk of the solution.

Thus it would appear that K^+ and Na^+ ions can, like H^+ ions depress the Ca^{2+}-sensitivity of skinned cardiac muscle even though they do not alter the binding of Ca^{2+} to isolated troponin. The similarity between H^+, K^+ and Na ions can be carried further, because all three reduce the maximum Ca^{2+}-regulated force in the skinned muscle. However K^+ and Na^+ ions exerted this effect via their contribution to the increase in ionic strength, whereas H^+ ions have a specific inhibitory action on maximum force.

The effects of a raised $[K^+]$(or $[Na^+]$) to depress both maximum Ca^{2+}-regulated force and the Ca^{2+}-sensitivity add together, with the result that the submaximal Ca^{2+}-regulated force is very sensitive to $[K^+]$(or $[Na^+]$). This has an important bearing on the relevance of these effects to the influence of extracellular tonicity on the contraction of intact cardiac muscle (Section 12.2).

PART IV

GENERAL DISCUSSION

Chapter 12

CONCLUSIONS AND GENERAL DISCUSSION: THE RELEVANCE OF MY RESULTS TO THE INFLUENCES OF pH AND [SALT] ON MYOFIBRILS IN INTACT MUSCLES

12.1 The direct influence of pH on myofibrils

(a) Summary of my findings

Several main conclusions can be drawn from my studies on the influence of pH on isolated cardiac and skeletal myofibrils and on detergent-skinned cardiac muscle:

(i) Decreases in pH over the range pH 7.20 - 6.40 reduced the Ca^{2+}-sensitivities of both cardiac and skeletal myofibrillar ATPase activities. The $\Delta pCa_{50}/\Delta pH$ was 0.93 for rabbit cardiac myofibrils, 0.86 for rabbit skeletal myofibrils and 0.82 for guinea-pig cardiac myofibrils. Thus the pH-sensitivity of skeletal myofibrils from the rabbit was only slightly less than that of the cardiac myofibrils from the same animal.

(ii) A decrease in pH from 7.20 to 6.40 had small or negligible effects on the maximum Ca^{2+}-regulated ATPase activity of the myofibrils.

(iii) Decreases in pH over the range pH 7.20 - 6.40 reduced the Ca^{2+}-sensitivity of force production in detergent-skinned trabeculae from the guinea-pig. The $\Delta pCa_{50}/\Delta pH$ was 0.89. Almost all of this pH-sensitivity was accounted for by the pH-sensitivity of pCa_{50} for the myofibrillar ATPase (see (i)).

(iv) A decrease in pH also markedly depressed the maximum Ca^{2+}-regulated force in the skinned trabeculae. This effect was probably not due to an effect on the myofibrillar ATPase, the maximum value of which was rather insensitive to pH (see (ii)). The observed pH-sensitivity of maximum force may therefore have resulted from an action of H^+ ions to decrease the efficiency of the process coupling ATP hydrolysis to force development.

(v) In the skinned muscles the influences of pH on the Ca^{2+}-sensitivity and on the maximum force combined to produce a very marked pH-sensitivity of force at suboptimal concentrations of Ca^{2+}.

(b) The effect of an acidosis in cardiac muscle

With these results and conclusions in mind, can we account for the action of intracellular acidosis in intact muscle *in vivo*? In cardiac muscle the well-established negative inotropic effect of an intracellular acidosis *is* in the direction that would be predicted

from the deleterious effects of acidity on the Ca^{2+}-regulated force in the skinned trabeculae, but are the two effects quantitatively compatible? This is a difficult question to answer categorically because as yet no-one has simultaneously measured cytosolic pH and developed force in an intact cardiac muscle. Thus for this discussion I am forced to assimilate results from several studies that used different conditions and different types of cardiac muscle.

It is known that a decrease in extracellular pH (pH_e) from 7.4 to 6.8 produced solely by raising the P_{CO_2} reduces the developed force in mammalian ventricular muscle to 30% - 50% of its initial value (e.g. Figure 8; Serur et al., 1976; Fry & Poole-Wilson, 1981). The magnitude of the fall in intracellular pH (pH_i) in the cardiac muscle under these conditions is not certain, for while studies using DMO or other indirect methods has yielded values for $\Delta pH_i/\Delta pH_e$ of about 0.6 (reviewed by Poole-Wilson, 1978), the direct measurement of pH_i with pH-sensitive microelectrodes, albeit in Purkinje fibres, gave $\Delta pH_i/\Delta pH_e$ as 0.14 (Ellis & Thomas, 1976). Taking the larger value for the moment, we can calculate that a reduction in pH_e from 7.4 to 6.8 would cause pH_i to drop by about 0.4 pH units - let us say from pH 7.0 to pH 6.6. We must in addition assume a value for the cytosolic pCa attained during systole in living cardiac muscle. My observation that the developed force in the cardiac trabeculae before skinning was approximately half the maximum Ca^{2+}-regulated force obtained after skinning suggests that in the living trabeculae the systolic pCa was in the region of 5.5 (since this was a typical value for the pCa_{50} at pH 7.0 in the skinned muscles). Recent results with aequorin-injected cardiac cells have indicated that a systolic pCa of 5.5 is probably the lowest pCa (i.e. highest $[Ca^{2+}]$) that is ever reached (Allen & Kurihara, 1980; Fabiato, 1982). It can be seen from Figures 47 and 50 that it is at a pCa of about 5.5 that Ca^{2+}-regulated force is most sensitive to a change in pH, for it is about this pCa that the Ca^{2+}-activation curve is at its steepest. For a peak systolic pCa of 5.5, a fall in pH of 0.4 units would depress the force to about 20% of its value at pH 7.0 (Figure 50). Thus, assuming the systolic pCa is 5.5 and that $\Delta pH_i/\Delta pH_e$ is 0.6, the direct action of H^+ ions on the myofibrils can account for <u>all</u> of the 50% - 70% decrease in force seen in intact muscle when the external pH is lowered from 7.4 to 6.8. However this is not so if the true value for $\Delta pH_i/\Delta pH_e$ is as low as 0.14;

in this case, the fall in pH_e from 7.4 to 6.8 would cause the pH_e to drop from 7.0, say, to 6.92. At pCa 5.5, force would then decrease by only about 22% (Figure 50) - considerably less than the percentage reduction in developed force observed in the intact muscle. A definitive conclusion on the importance of the direct actions of pH on cardiac myofibrils must therefore await a more systematic investigation of force and cytosolic pH and pCa before and during an acidosis. In the meantime it can be stated that the direct inhibitory actions of protons on myofibrillar force production could account for a substantial fraction, if not all, of the negative inotropic effect of an acidosis in intact cardiac muscle.

However, the manifold effects of pH on intracellular systems involved in Ca^{2+} metabolism makes it unlikely that only the myofibrils are affected by an intracellular acidosis. In fact most of these systems have activities that are altered by acidity in a direction that would tend to decrease the amount of Ca^{2+} reaching the myofibrils. Ca^{2+} influx into cardiac cells decreases (Poole-Wilson & Langer, 1979), possibly because of a reduction in the slow inward current (Kohlhardt et al., 1975); the Ca^{2+}-induced release of Ca^{2+} from the cardiac SR is probably less in acidic conditions (Fabiato & Fabiato, 1978b), although whether this type of Ca^{2+} release is the predominant mechanism in intact cells is not yet clear. Countering these two effects, though, a decrease in intracellular pH would probably induce a release of Ca^{2+} and an uptake of H^+ by the mitochondrial $2H^+/Ca^{2+}$ exchange system. The time course of this buffering of Ca^{2+} and H^+ by the mitochondria is such that it could well be responsible for the partial recovery of pH_i and force after the first minute or two of the acidosis (Fry & Poole-Wilson, 1981). If indeed an intracellular acidosis in cardiac muscle does decrease the Ca^{2+} supply to the myofibrils, then this indirect inhibition of myofibrillar activity would act in concert with the direct, deleterious action of acidity on the myofibrils and as a consequence cardiac performance would be very severely reduced.

There is however one report which is at variance with the idea that a decrease in the Ca^{2+} supply may be an important factor during acidosis, at least in its initial stages. Allen et al. (1981) have found that the early, dramatic increase in force after the onset of an intracellular alkalosis was associated with no change in the magnitude of the light signal from aequorin-injected ventricular cells i.e. the Ca^{2+} transient was unaltered, which in turn suggests

that the Ca^{2+} supply to the myofibrils was unchanged. (There is however the remote possibility that alkalosis increased the Ca^{2+} supply to the myofibrils and increased the binding of Ca^{2+} to the myofibrils by the same amount, thus leaving the systolic concentration of cytosolic Ca^{2+} unchanged). If the Ca^{2+} supply is indeed constant, then the initial part of the positive inotropic effect of an alkalosis, and by inference the initial part of the negative inotropic effect of an acidosis, must be entirely due to the direct actions of H^+ ions on the myofibrils. One other possibility - that acidity could alter the Ca^{2+}-sensitivity by an indirect action that involved an increased phosphorylation of the troponin in the myofibrils - seems to be ruled out by the demonstration that the activity of the cAMP-dependent protein kinase is not affected by an intracellular acidosis (Chiappe de Mon et al., 1978).

After a few minutes of intracellular alkalosis, the Ca^{2+} transient seen by Allen et al. (1981) began to diminish. This apparent reduction in Ca^{2+} release was associated with the decrease in force after the initial potentiation by alkalosis. This decrease in systolic Ca^{2+} is not what we would predict from the pH-dependence of SR Ca^{2+} loading, which is increased by an increase in pH (Fabiato & Fabiato, 1978b) (see 12.1(c) below), but it is consistent with an uptake of Ca^{2+} by the mitochondrial Ca^{2+}/H^+ exchange system as the result of a decrease in the cytosolic $[H^+]$. These considerations suggest that over a time period of minutes, the pH-dependence of mitochondrial Ca^{2+} exchange may predominate over the pH-dependence of SR Ca^{2+} release. This idea is supported by the fact that white skeletal muscle, which possesses far fewer mitochondria than cardiac muscle, shows no biphasic response to a change in intracellular pH (Figure 8).

I therefore conclude that the direct inhibitory of H^+ ions on the contractile function of cardiac myofibrils at suboptimal values of pCa must play a large part in the negative inotropic effect of an intracellular acidosis, especially in the early stages of the acidosis.

(c) The effect of an acidosis in white skeletal muscle

The pH-sensitivity of the Ca^{2+}-activation of isolated cardiac myofibrils or skinned cardiac muscles can account for most, if not all, of the negative inotropic effect of an acidosis on cardiac muscle. Can the actions of an acidosis on the contractile performance of skeletal muscle be explained in a similar way? My results showed that the Ca^{2+}-regulation of the skeletal myofibrillar ATPase was

slightly less sensitive to pH than was the Ca^{2+}-activation of the cardiac myofibrillar ATPase. These findings have been supported by studies with skinned skeletal and cardiac muscles: for a drop in pH from 7.4 to 6.2, Fabiato & Fabiato (1978b) found that the $[Ca^{2+}]$ for half-maximal force was increased by a factor of 3 for frog skeletal skinned muscle and 5 for rat cardiac skinned muscle; Donaldson & Hermansen (1978) reported that a decrease in pH from 7.0 to 6.5 increased the $[Ca^{2+}]$ for half-maximal force by a factor of 1.4 in rabbit skeletal skinned muscle and 2.2 in rabbit cardiac skinned muscle. Taken together, all these results indicate that for a given drop in pH_i at a given pCa one would expect slightly smaller decreases in ATPase activity and force in skeletal muscle than in cardiac muscle. Nevertheless both ATPase activity and force in skeletal muscle would be expected to decrease if the pH_i fell. In fact what is actually observed is that an intracellular acidosis potentiates the skeletal muscle twitch (Figure 8: Pannier et al., 1970). Thus the slightly different pH-sensitivities of cardiac and skeletal myofibrils can explain why the response to an acidosis should be smaller in skeletal muscle than in cardiac muscle (e.g. Figure 8) but give no clue as to why acidosis has the opposite effects in the two types of muscle.

Other explanations are therefore required. As discussed above, the marked negative inotropic effect of an acidosis in cardiac muscle is not surprising because the systolic concentration of cytosolic Ca^{2+} is insufficient to fully activate the myofibrils, and it is at such suboptimal Ca^{2+} concentrations that the direct inhibitory action of acidity on myofibrillar activity is greatest. Either the same situation does not exist in skeletal muscle or it is obscured by some potentiating effect of an intracellular acidosis. The explanation cannot be due to differential changes in pH_i, since the responses of pH_i to changes in extracellular pH or P_{CO_2} appear to be similar in the two types of muscle (Ellis & Thomas, 1976; Aicken & Thomas, 1977). One possible explanation for the contrasting responses of cardiac and skeletal muscle to an acidosis (Tsien, 1976) was based on the belief that the magnitude of the Ca^{2+} supply to the myofibrils differed in the two types of muscle: Tsien (1976) considered that the systolic $[Ca^{2+}]$ in cardiac muscle was insufficient to produce full activation but that in skeletal muscle the cytosolic $[Ca^{2+}]$ was probably sufficient to completely activate the myofibrils. If

this were so, we could predict from Figure 24 (as Tsien predicted from the results of Schädler, 1967) that maximum ATPase activity and force in skeletal muscle would be little altered by an acidosis, provided that the acidosis did not simultaneously decrease the Ca^{2+} supply below saturating levels. However there are two objections to this hypothesis. First, it cannot explain why skeletal muscle performance is usually slightly potentiated by an intracellular acidosis. Secondly, and more critically, it is probable that the $[Ca^{2+}]$ attained in single twitches in skeletal muscle at temperatures above room temperature is, as in cardiac muscle, insufficient for full myofibrillar activation (Close, 1972), although the $[Ca^{2+}]$ is probably higher than in cardiac muscle (D.G. Allen, personal communication).

An alternative explanation for the effects of acidosis on skeletal muscle has been provided by Fabiato & Fabiato (1978b), who discovered a possible compensatory mechanism that could overcome the direct inhibitory effect of acidity on the skeletal myofibrils. These workers found that at pCa values below 7.75 the loading of SR in skinned skeletal fibres had a pH optimum which was more acidic (e.g. $\approx$ pH 6.6 at pCa 6.00) than the pH optimum for loading of cardiac SR (e.g. $\approx$ pH 7.4 at pCa 6.50). They therefore suggested that acidity could lead to an increase in Ca^{2+} loading into, and thus Ca^{2+} release from skeletal SR, whereas it would decrease Ca^{2+} loading into, and Ca^{2+} release from, cardiac SR. Provided that the peak cytosolic $[Ca^{2+}]$ in a skeletal muscle twitch is submaximal, this lower pH optimum of skeletal SR can explain how the skeletal twitch may increase under conditions of an intracellular acidosis despite the direct depressant action of acidity on the contractile machinery. An obvious way to test this supposition would be to observe the effect of an intracellular acidosis in the pH range 7.0 - 6.4 on the calcium transient from aequorin-injected skeletal fibres. Ashley et al. (1979) have demonstrated that an intracellular acidosis of $pH_i \approx 6.0$ in aequorin-injected barnacle fibres reduced the Ca transient and isometric force, prolonged relaxation, and increased resting light and resting force. All these changes are consistent with an impairment of SR function, as would be expected since the pH_i of ≈ 6.0 would probably be lower than the pH optimum of the SR for loading of the SR ($pH \approx 6.6$, Fabiato & Fabiato, 1978b). It would be interesting to know what happens with a milder acidosis, which, from the

results of Fabiato & Fabiato (1978b), might be expected to potentiate Ca^{2+} release and thereby increase the magnitude of the Ca transient in skeletal muscle.

The effect of pH on the maximum ATPase activity of skeletal myofibrils (Figure 24) and on the maximum force of skinned skeletal muscle (Fabiato & Fabiato, 1978b; Donaldson & Hermansen, 1978) leads me to predict that in a skeletal muscle maximally activated with Ca^{2+}, a mild acidosis should in fact decrease force slightly. However, Pannier et al. (1970) have reported that the tetanic force in skeletal muscle was increased by about 20% by a mild respiratory acidosis. However, their experiments were performed at 25°C and so it is possible that the muscle was not fully activated by Ca^{2+} even though it had achieved a fused tetanus. The experiments of Pannier et al.(1970) need to be repeated at lower temperatures to ensure the maximum possible release of Ca^{2+}.

The results from both the myofibrillar ATPase and skinned muscle studies show that the threshold $[Ca^{2+}]$ for activation of the cardiac and skeletal myofibrils increased as the pH was decreased. This observation leads me to predict that, if the Ca^{2+} supply to the myofibrils in the intact muscle remained constant, the latency of the skeletal and cardiac twitch should be increased by an acidosis, since it would take longer for the cytosolic $[Ca^{2+}]$ to reach the higher threshold for activation. However this increase in the latency might be too small to be observed, or it might be obscured by the actions of an acidosis on other Ca^{2+} transport processes in the cell.

12.2 The direct influence of [salt] on myofibrils

The main aim of the experiments with skinned cardiac muscle was to establish to what extent, and by what mechanism, the [salt] of the bathing solution directly influenced the Ca^{2+}-regulated force generated by the myofibrils. The ultimate objective was to establish whether in cardiac muscle, as in skeletal muscle (Gordon et al., 1973), the effects of extracellular tonicity on force production in intact muscle could be explained entirely by direct effects of the cytosolic [salt] on the myofibrils.

(a) Summary of my findings

From my experiments on isolated myofibrils and on skinned muscle I conclude the following:

(i) An increase in ionic strength from 100 mM to $\approx$ 130 mM produced a slight stimulation of Ca^{2+}-regulated ATPase activity in

both skeletal and cardiac myofibrils but further increases up to 200 mM reduced these ATPase activities considerably.

(ii) As the ionic strength was increased over the range 100 - 400 mM with KCl the Ca^{2+}-regulated force production in skinned cardiac trabeculae fell linearly to zero. The similarity in the inhibitory effects of different chloride salts suggested that the reduction in Ca^{2+}-regulated force was chiefly due to the rise in ionic strength per se.

(iii) In contrast, the additional action of a raised [KCl] or [NaCl] to depress the Ca^{2+}-sensitivity of force development in the skinned muscles was due to a specific antagonism by K^+ or Na^+ ions and was not related to the increase in ionic strength.

(iv) As the ionic strength was reduced below about 150 mM the Ca^{2+}-insensitive ('basal') ATPase activity and resting force increased.

(v) Most of these effects of [salt] on cardiac muscle models were qualitatively, and in some cases quantitatively, similar to those observed by other investigators with models of skeletal muscle.

(b) The positive inotropic effect of mild extracellular hypertonicity

Gordon et al. (1973) have argued that the direct inhibitory effects of [salt] on maximally-activated skinned skeletal myofibrils can account entirely for the influence of extracellular tonicity on skeletal contraction. Can a similar conclusion be reached for cardiac muscle? It is obvious from my results that the answer must be no: the direct actions of a raised salt concentration on cardiac myofibrils cannot account for all of the effects of extracellular tonicity on intact cardiac muscle, for whereas mild hypertonicity has a positive inotropic action on intact cardiac muscle (Section 3.5), the effects of a raised [salt] on the force development of skinned cardiac muscle were without exception inhibitory. One consequence of extracellular hypertonicity would be an increase in the cytosolic ionic strength, and this would act to depress the maximum Ca^{2+}-regulated force; in addition the increase in concentration of the major intracellular cation, K^+, would decrease the Ca^{2+}-sensitivity of the myofibrils and this would decrease the Ca^{2+}-regulated force generated at suboptimal concentrations of Ca^{2+}, such as are found in intact cardiac muscle during systole. It could not be argued that the differences between the results from intact and and skinned cardiac muscle were due to the unphysiological ionic

strengths or K^+ concentrations in my experiments because (i) the $\Gamma/2$ range I studied (100 mM - 400 mM) must encompass the true intracellular $\Gamma/2$ in cardiac muscle and (ii) the $[K^+]$ of 130 mM in my standard solutions is almost identical to the cytosolic concentrations of $[K^+]$ estimated from measurements with K^+-selective microelectrodes (Section 12.2(c) below).

If the positive inotropic effect of a mild hypertonicity is not related to a direct effect of ionic strength on the cardiac myofibrils, to what is it due? As discussed in Chapter 3, changes in extracellular tonicity would cause, besides an alteration in intracellular ion concentrations and ionic strength, changes in intracellular viscosity and tonicity; the latter would in turn affect myofilament lattice spacing. Of these factors, the only one that we might expect could cause an increase in force would be the increase in an intracellular ion concentration - more specifically, an increase in the intracellular concentration of Ca^{2+}. However the proportional increase in the $[Ca^{2+}]$ would be small at the levels of hypertonicity that stimulate the contraction of heart muscle. For instance, Koch-Weser (1963) found that the maximum stimulation in papillary muscles was obtained with an extracellular tonicity 15% above the normal tonicity of 300 mosmol l^{-1} (Figure 9); if we assume that this degree of hypertonicity also increased the systolic $[Ca^{2+}]$ by 15% - a drop in pCa by 0.06 units - force would increase by a maximum of 18% (estimated from Figure 57). By itself this could explain the observed 14% increase in the force developed by the intact papillary muscles (Koch-Weser, 1963), but it must be remembered that the intracellular ionic strength would also have increased by about 15%. The latter change would have caused force production in the myofibrils to fall by about 15% (Figure 54). Thus the effects of a rise in the systolic $[Ca^{2+}]$ and a rise in intracellular ionic strength would have cancelled each other out. A 15% rise in the systolic $[Ca^{2+}]$ is therefore not sufficient to account entirely for the positive inotropism of mild hypertonicity, although it could be a contributory factor.

Another intracellular condition that would be affected by a change in extracellular tonicity is the intracellular tonicity. My results suggest that the fraction of the intracellular tonicity that is due to molecules which can permeate the myofibrils would not be expected to influence myofibrillar force production (Figure 56). However if the increase

in intracellular tonicity was partly due an increased concentration of those cytosolic proteins that were excluded from the myofilament lattice, the myofilament lattice spacing would decrease and this in turn would inhibit Ca^{2+}-regulated force production in the myofibrils (Godt & Maughan, 1977; Fabiato & Fabiato, 1978a).

It is not known what effect an increase in cytosolic viscosity would have on force production, but it seems possible that contraction might be slowed - an action which could depress the peak force developed in each contraction.

Summarising the above, it would seem that the increase in cytosolic $[Ca^{2+}]$ which is one likely consequence of hypertonicity would act to increase force development in intact cardiac muscle but that this stimulatory effect would almost certainly be obscured by the inhibitory effects of increases in cytosolic ionic strength and viscosity, and of a decrease in myofilament lattice spacing. It must be concluded that, as originally suggested by Koch-Weser (1963), mild hypertonicity enhances the release of Ca^{2+} from the cardiac SR, and to such an extent that it overcomes the underlying tendency of hypertonicity to depress contractility by direct actions on the cardiac myofibrils. Koch-Weser (1963) suggested that the positive inotropism could be the result of an increase in the $[Ca^{2+}]$ in a functionally-important store of Ca^{2+}. In this regard it may be relevant that Page & Upshaw-Earley (1977) have found that mild and severe hypertonicity decrease the volume of the longitudinally-oriented SR in cardiac muscle but dilate the terminal cisternae. Taking a simplistic view, we might therefore expect hypertonicity to increase the $[Ca^{2+}]$ in the longitudinally-oriented SR and decrease the $[Ca^{2+}]$ in the terminal cisternae. On this basis, the functionally-important Ca^{2+} store that was suggested by Koch-Weser(1963)could be the Ca^{2+} in the longitudinally-oriented SR in cardiac muscle.

To understand more fully the effects of mild hypertonicity on E-C coupling, it would be interesting to know whether hypertonicity enhances the release of Ca^{2+} from the SR in mechanically-skinned cardiac cells.

(c) The negative inotropic effect of severe extracellular hypertonicity

The detrimental effects of high $\Gamma/2$ and $[K^+]$ on Ca^{2+}-regulated force are however in the right direction to account for the negative inotropic action of severe hypertonicity. Whether these factors can explain all of the cardiodepressant effect of severe hypertonicity can only be answered if it is known by how much the intracellular $\Gamma/2$ and $[K^+]$ rise during extracellular hypertonicity. Although these intracellular changes have not yet been measured directly, we can make reasonable guesses if we bear in mind that cardiac muscle cells behave as perfect osmometers with all their cell water osmotically active (Page & Storm, 1966). Thus, to a first approximation, the intracellular $\Gamma/2$ and $[K^+]$ will change in direct proportion to the change in extracellular tonicity. Estimates for the cytosolic $\Gamma/2$ and $[K^+]$ that obtain when the cardiac muscle is bathed in solutions of normal tonicity (300 mosmol l^{-1}) can be obtained from the results from recent work with ion-selective microelectrodes. The activity of K^+ ions in the cytosol of cardiac muscle is 90 - 100 mM (Lee & Fozzard, 1975; Wier, 1978), which corresponds to a $[K^+]$ of 125 - 139 mM if we assume an activity coefficient of 0.72 (the same as in the extracellular fluid). In a similar way, the work with micro-electrodes of other ion-selectivities leads to the following estimates for other cytosolic ion concentrations: $[Na^+]$, 8 - 10 mM (Lee & Fozzard, 1975; Deitmer & Ellis, 1978); $[Cl^-]$, 20 mM (Macchia et al., 1978; Baumgarten & Fozzard, 1978); $[Mg^{2+}]$, 3.1 mM (Hess & Weingart, 1981). From the Henderson-Hasselbalch equation the $[HCO^-_3]$ can be calculated to be 18 mM, assuming $pH_i = 7.0$ and $P_{CO_2} = 38$ mm Hg. These ionic concentrations give a minimum value for the cytosolic $\Gamma/2$ of 87 mM, and if we take into account the $[MgATP^{2-}]$ of ≈ 5 mM and the $[HCP^{2-}]$ of ≈ 10 mM (Section 11.2), this rises to 118 mM. The concentrations of hexose phosphates and of residual phosphates in cardiac muscle are not known with certainty, but if it is assumed that their concentrations are the same as in skeletal muscle (≈ 15 mM and ≈ 11 mM, respectively, Conway , 1957), the calculated total ionic strength of the cytosol is 164 mM. This is a minimum value, for no account has been taken of the concentrations of any other metabolites or ions. To what extent the amino acids and proteins contribute to the cytosolic $\Gamma/2$ is not known at all. For the following discussion I shall assume the normal cytosolic $\Gamma/2$ is

160 mM. (The true $\Gamma/2$ may be a little higher than this but would not affect the argument, which depends not so much on the absolute value of the cytosolic $\Gamma/2$ as on the proportional change in its value as the extracellular tonicity is varied).

Using conditions of stimulation designed to activate papillary muscles as much as possible, Wildenthal et al. (1975) observed that an increase in extracellular tonicity from the control value of 300 mosmol l^{-1} to 400 mosmol l^{-1} decreased the developed force by 30% (shown in Figure 9). If we assume that the intracellular $\Gamma/2$ and $[K^+]$ in the control situation were 160 mM and 130 mM respectively, after the tonicity of the extracellular solution had been raised to 400 mosmol l^{-1} these values would have increased to about 213 mM and 173 mM respectively, assuming there was no redistribution of ions between intra- and extracellular compartments. Of course, the intracellular $[Na^+]$ would also have risen by a similar proportion, but since the cytosolic $[Na^+]$ is small in comparison with the cytosolic $[K^+]$, I shall neglect the contribution of Na^+ ions in the following discussion. From Figure 54 I would predict that the increase in ionic strength from 160 mM to 213 mM would decrease the maximum Ca^{2+}-regulated force to 81% of the control value - not enough in fact to account for the 30% decrease in force observed by Wildenthal et al. (1975). Thus, in contrast to the conclusion reached by Gordon et al.(1973) for skeletal muscle, I conclude that the inhibitory action of a raised ionic strength on the maximum force production of cardiac myofibrils cannot account fully for the negative inotropic effect of severe hypertonicity. The difference between these conclusions arises chiefly from my finding that the maximum Ca^{2+}-regulated force in skinned cardiac muscle is less sensitive than that in skinned skeletal muscle to changes in ionic strength.

The increase in the cytosolic $[K^+]$ during extracellular hypertonicity would, by reducing the Ca^{2+}-sensitivity of the cardiac myofibrils, act in concert with the increase in ionic strength to reduce force, provided that the systolic $[Ca^{2+}]$ was not sufficient to fully activate the myofibrils. Although Wildenthal et al. (1975) used conditions designed to produce the maximum possible activation of the myofibrils in the papillary muscles, it is unlikely that the systolic $[Ca^{2+}]$ would have been saturating, for recent data by Fabiato (1982) indicate that in rat and rabbit ventricular muscle the systolic pCa does not fall below 5.5 even under conditions

designed to maximise the physiological release of Ca^{2+} from the SR. Assuming that the minimum cytosolic pCa attained in the experiments of Wildenthal et al. (1975) was 5.5, I estimate from Figure 57 that an increase in $[K^+]$ from 130 mM to 173 mM would by itself reduce the relative force by 11%. The combined inhibitory effects of the raised ionic strength and the raised $[K^+]$ would therefore decrease the force developed at pCa 5.5 to 72% (i.e. 81% x 89%) of the force in the control solution. The magnitude of this effect is sufficient to explain all of the negative inotropism observed by Wildenthal et al. (1975).

Of course, this does not exclude other mechanisms as factors contributing to the reduction in cardiac contractility seen in strongly hypertonic solutions. As discussed in Section 12.2(b), the increased concentration of any cytosolic proteins that are excluded from the myofibrillar lattice would tend to decrease the lattice spacing because of Donnan osmotic effects. This lattice shrinkage would lead to a reduction in force development by the myofibrils - an effect that would augment the decrease in force brought about by the increases in $\Gamma/2$ and $[K^+]$.

Changes in extracellular tonicity would also probably alter the efficiency of E-C coupling. It seems likely that slight hypertonicity potentiates E-C coupling (Section 12.2(b)) and there is no reason to suppose that E-C coupling is not affected by severe hypertonicity, though whether it is potentiated or attenuated cannot be stated categorically. By analogy with skeletal muscle, it could be suggested that marked degrees of hypertonicity (greater than twice normal tonicity) would depress E-C coupling, but if there is one general conclusion that arises from this thesis it is that it is often inadvisable to extrapolate from skeletal to cardiac muscle. As Morad & Goldman (1973) emphasised, cardiac muscle should not be regarded as 'a stepchild of skeletal muscle'. As with the effects of pH, it would seem that a definite conclusion about whether changes in extracellular tonicity alter cardiac E-C coupling must await studies with aequorin-injected cardiac cells or, in the more distant future, studies with intracellular Ca^{2+}-selective micro-electrodes (which at present are too slow to record the transient rise in $[Ca^{2+}]$ during activation of the muscle).

(d) The effects of extracellular tonicity on resting force

There were no stimulatory effects of a high [salt] on the resting force in skinned cardiac muscle (Figure 54) that could explain the hypertonicity-induced contractures seen in intact ventricular muscle from frog (Kawata et al., 1974) and rat (Chapman & Léoty, 1974). In skeletal muscle such contractures seem to be due to a release of Ca^{2+} from the SR (Lännergren & Noth, 1973) and it is possible that this could also apply to cardiac muscle.

My results (Figures 22 and 54) suggest that if the intracellular ionic strength were to drop below about 150 mM as the extracellular tonicity was reduced, there would be an increase in the Ca^{2+}-insensitive interaction between actin and myosin and thus the resting force in the living muscle would increase. Solutions made hypotonic by the removal of some of the NaCl do indeed produce a contracture in the living cardiac muscle (e.g. Kawata et al., 1974) but this is probably chiefly due to the accumulation of Ca^{2+} inside the cells as a result of the reversal of the sarcolemmal Na^{+}/Ca^{2+} exchange. To study the effects of hypotonicity alone, the cardiac muscle must first be equilibrated in a low-Na^{+} physiological saline that contains sucrose to make it isotonic to normal plasma, and then the hypotonicity can be produced by removal of the sucrose. In experiments of this sort (Koch-Weser, 1963; Chapman, 1978), hypotonicity has been found to have little or no influence on the resting force in cardiac muscle, as in skeletal muscle (Gordon & Godt, 1970). I do not know why Ca^{2+}-insensitive attachment of myosin to actin occurs at low ionic strengths in skinned muscle but not in living muscle.

To summarise, the direct inhibitory influences of a raised ionic strength and $[K^{+}]$ on cardiac myofibrils can, if combined, account for the direction and magnitude of the negative inotropic effect of severe hypertonicity in living cardiac muscle, but the stimulatory action of mild hypertonicity on developed force must be due to other factors, which act to facilitate E-C coupling. In addition, the influence of [salt] on the resting force in the skinned cardiac muscle does not parallel the influence of extracellular tonicity on the resting force in the living muscle.

APPENDICES

Appendix A

THE DETERMINATION OF THE APPARENT AFFINITY CONSTANTS OF EGTA FOR Ca^{2+} IN SOLUTIONS OF DIFFERENT IONIC STRENGTHS AND SALT CONCENTRATIONS

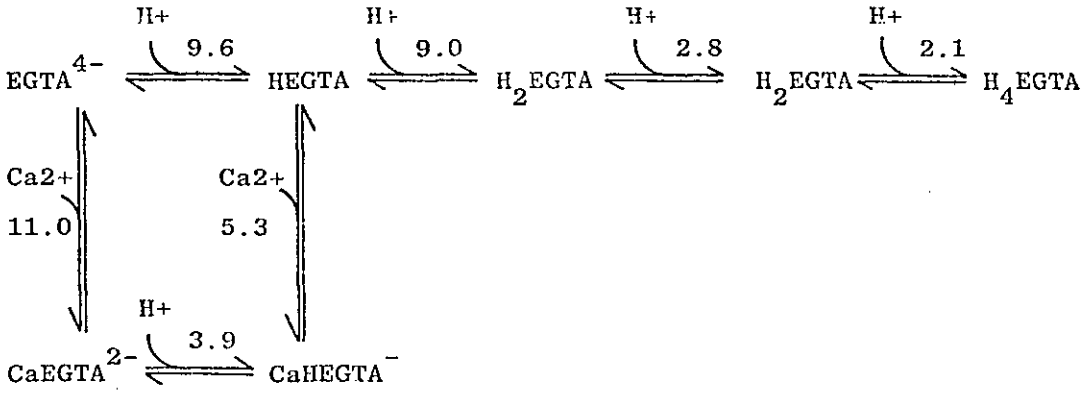
A.1 Objectives

The aim of this study was to measure the apparent affinity constants of EGTA for Ca^{2+} under the different conditions of ionic strength and salt concentration that were used in the skinned muscle experiments. In particular I needed to determine whether the changes in [salt] in the skinned muscle experiments altered the values of these association constants, for a failure to correct for any changes in the values of these constants would have resulted in artifactual shifts in the relative force - pCa relationship as the [salt] was varied.

The apparent affinity constants for the formation of CaEGTA and CaHEGTA at pH x (K^{x}_{CaEGTA} and $K^{x}_{CaHEGTA}$, respectively) were determined by a pH-metric method modified slightly from that of Moisescu & Thieleczek (1979).

A.2 Background

It is thought that EGTA can bind up to four protons and that Ca^{2+} binds only to $EGTA^{4-}$ or $HEGTA^{3-}$ (Sillèn & Martell, 1971; Martell & Smith, 1974). Thus the following equilibria obtain:



where the numbers are the approximate values of the true association constants (in logarithmic form) at 20°C (Martell & Smith, 1974).

Thus in the pH range 5.0 - 7.0 and in the absence of Ca^{2+}, almost all the EGTA is present as H_2EGTA^{2-}. Addition of Ca^{2+} leads predominantly to the formation of $CaEGTA^{2-}$. Therefore to a first approximation the overall reaction at pH 5.0 - 7.0 is:

$$Ca^{2+} + H_2EGTA^{2-} \rightleftharpoons CaEGTA^{2-} + 2H^{+}.$$

In fact the number of moles of H^+ released per mole of EGTA bound to Ca^{2+} is a little less than two because (i) some $CaHEGTA^-$ is formed and (ii) not all the EGTA binds to Ca^{2+}.

In the pH-metric method of Moisescu & Thieleczek (1979) the number of moles of H^+ released when Ca^{2+} is added to EGTA at a given pH, pH x, in the pH range 5.8 - 6.0 is determined by measuring the amount of OH^- needed to return the pH to its initial value. If this is done at two Ca concentrations the concentrations of $CaEGTA^{2-}$ and $CaHEGTA^{2-}$ can be calculated. Then the apparent association constants for the formation of $CaEGTA^{2-}$ and $CaHEGTA^-$ at pH x (K^x_{CaEGTA} and $K^x_{CaHEGTA}$ respectively) can be estimated from the following (Portzehl et al., 1964):

$$K^x_{CaEGTA} = \frac{[CaEGTA^{2-}]}{[Ca^{2+}][EGTA]_f} \qquad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

$$K^x_{CaHEGTA} = \frac{[CaHEGTA^-]}{[Ca^{2+}][EGTA]_f} \qquad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2)$$

where $[EGTA]_f$ is the total concentration of EGTA that is not bound to Ca^{2+}.

A combined apparent association constant can be defined by:

$$K^x_{CaEGTA\ total} = \frac{[CaEGTA^{2-}]+[CaHEGTA^-]}{[Ca^{2+}]\ [EGTA]_f}$$

$$= K^x_{CaEGTA} + K^x_{CaHEGTA} \qquad \ldots\ldots\ldots\ldots\ldots\ldots (3)$$

These pH-dependent apparent constants must be distinguished from the 'true' association constants K_{CaEGTA} and $K_{CaHEGTA}$ which are given by:

$$K_{CaEGTA} = \frac{[CaEGTA^{2-}]}{[Ca^{2+}][EGTA^{4-}]} \qquad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (4)$$

$$K_{CaHEGTA} = \frac{[CaHEGTA^-]}{[Ca^{2+}][HEGTA^{3-}]} \qquad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (5)$$

In practice it is usually easier to determine the apparent constants than the true constants because it is easier to estimate $[EGTA]_f$ rather than $[EGTA^{4-}]$ or $[HEGTA^{3-}]$. However the two classes of constants can be interconverted by the following equations (Portzehl et al., 1964):

$$K_{CaEGTA} = K^{x}_{CaEGTA} \cdot \alpha \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (6)$$

$$K_{Ca\overset{H}{\underset{1}{}}EGTA} = K^{x}_{Ca\overset{H}{\underset{1}{}}EGTA} \cdot \alpha/([H^{+}].K_1) \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (7)$$

where $\alpha = 1 + [H^{+}]\,K_1(1 + [H^{+}]K_2(1 + [H^{+}]K_3(1 + [H^{+}]\,K_4)))$

and K_1, K_2, K_3, K_4 are the true association constants for EGTA binding one, two, three and four protons, respectively.

Thus the apparent association constants determined at pH x can be used to estimate the true constants provided that values for K_1 - K_4 are assumed or measured. Alternatively, the apparent constants determined at pH x can be converted to apparent constants at another pH, pHy (provided that 5.8 < y < 7.5), by the following (Moisescu & Thieleczek, 1979):

$$K^{y}_{CaEGTA} = K^{x}_{CaEGTA} \times 10^{2(pHy - pHx)} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (8)$$

$$K^{y}_{CaHEGTA} = K^{x}_{CaHEGTA} \times 10^{(pHy - pHx)} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (9)$$

A.3 Methods

(a) Apparatus

For the titrations I used a three-place digital pH meter (model 120, Corning, Halstead, Essex) in combination with a miniature glass pH electrode (SJRM/45, Russell pH Ltd., Auchtermuchty, Fife) and calomel reference electrode (CRRM/45, Russell). Calomel reference electrodes are much less prone than Ag/AgCl electrodes (such as used in combination pH/electrodes) to drift caused by EGTA in the solutions (personal observation). To check for drift, the output of the pH meter was monitored with a chart recorder (model 2007, Gould,

Ballainvilliers, France). The titrant was contained in a 100 cm^3 or 250 cm^2 plastic beaker and was stirred with a magnetic follower. Solution temperature was maintained at 25°C with a water jacket.

(b) Solutions

For simplicity the titration solutions had to contain Ca^{2+} as the only divalent cation and EGTA as the only Ca^{2+} ligand (Moisescu & Thieleczek, 1979). It was therefore not possible to directly determine the apparent binding constants in the 'activating solutions' used in the skinned muscle experiments because these solutions also contained Mg^{2+}, which binds to EGTA, and ATP, which binds to Ca^{2+} and Mg^{2+}. Instead, the activating solutions of different [salt] were modelled by simplified solutions that initially contained: 2 mM (nominal) K_2H_2EGTA; 4 mM imidazole; 90 mM KCl, plus an added salt as follows:

added salt	final ionic strength (mM)
none	100
100 KCl	200
100 TMACl	200
100 ChCl	200
100 NaCl	200

The initial pH was adjusted to pH 5.9 with HCl. These solutions were made from the same stock solutions of K_2H_2EGTA, imidazole, KCl, TMACl, ChCl and NaCl that were used to prepare the solutions for the skinned muscle experiments. The preparation of these stock solutions has already been described (Section 9.2). During the titration procedure (see below) $CaCl_2$ was added as a 50 mM $CaCl_2$ solution, which was prepared by accurate dilution of 1 M $CaCl_2$ solution (B.D.H.). NaOH was added using 0.5 M and 0.1 M carbonate-free NaOH from B.D.H. The molarities of these solutions were frequently checked by titration under N_2 against dried potassium hydrogen phthalate with phenolphthalein as indicator.

The pH electrode was calibrated and its linearity checked with the following standard solutions, all of which I made by dissolving the dried solids in freshly double-distilled water (see Bates, 1973):

- 0.05 M potassium hydrogen phthalate, pH 4.001 at 20°C.
- 0.025 M KH_2PO_4 + 0.025 M Na_2HPO_4, pH 6.881 at 20°C.
- 0.00869 M KH_2PO_4 + 0.0304 M Na_2HPO_4, pH 7.429 at 20°C.

Many pH electrode/reference electrode combinations give erroneous pH readings when the ionic strength of the test solution differs from that of the pH standards (Illingworth, 1981). To check my electrodes, I calibrated them with the above pH standards (of total ionic strength $\approx$ 100 mM) and then measured the pH of a solution containing 0.025 M KH_2PO_4 + 0.025 M Na_2HPO_4 and 0.1 M NaCl (total ionic strength $\approx$ 200 mM). The pH reading was always within 0.003 pH units of the true pH value of this solution (pH 7.081 at 20°C - Table 5-7 in Bates, 1973).

Solution volumes of 1 cm^3 or greater were dispensed using class A glass pipettes. Adjustable automatic pipettes (P50 and P200, Gilson) were used for smaller volumes. I regularly calibrated all pipettes by weighing the amount of water they dispensed and assuming the density of water = 0.99823 g cm^{-3} at 20°C.

(c) Titration procedure

Figure 60 shows a typical chart record of the pH changes during the titration procedure. A 125 cm^2 aliquot of the titrant, which consisted of 2 mM (nominal) K_2H_2EGTA, 4 mM imidazole, 90 mM KCl, plus added salt as required, and which contained $\underline{a}$ moles EGTA, was adjusted to an initial pH (pHx) about pH 5.9 with HCl. The solution was stirred slowly and was left for 2 - 5 mins for the pH to stabilise. Then exactly $\underline{a}$ moles of $CaCl_2$ were added and the pH fell as protons were released from H_2EGTA^{2-}. The carbonate-free NaOH solutions were added in 0.1 or 0.01 cm^3 steps to titrate the solution back to pHx (moles of NaOH needed = n_1). Then 1 cm^3 of 1M $CaCl_2$ was added to give a final [total Ca] of 10 mM (i.e. in 8 mM excess of EGTA), causing another pH drop. pHx was again restored with NaOH (n_2 moles).

The recorded pH of the titrant, before addition of $CaCl_2$ was usually very stable, the drift being usually less than 0.001 pH units min^{-1} (Figure 60). This low rate of drift ensured that in the complete titration, which took 15 - 20 minutes, the amounts of NaOH (n_1, n_2) needed to return the pH to its initial value could be estimated to within 0.2% (assuming pipetting errors were negligible).

(d) Calculations

Moisescu has reasoned that values for the apparent association constants can then be calculated from the following algebraically-derived equations (Moisescu & Thieleczek, 1979):

$$K^x_{CaEGTA} = n_1(n_1 + n_2)\,(n_1 + n_2 - a).\,V.\,a^{-2}.\,n^{-2} \quad \ldots\ldots\ldots \quad (10)$$

$$K^x_{CaHEGTA} = n_1(n_1 + n_2)\,(2a - n_1 - n_2).\,V.\,a^{-2}.\,n_2^{-2} \quad \ldots\ldots\ldots \quad (11)$$

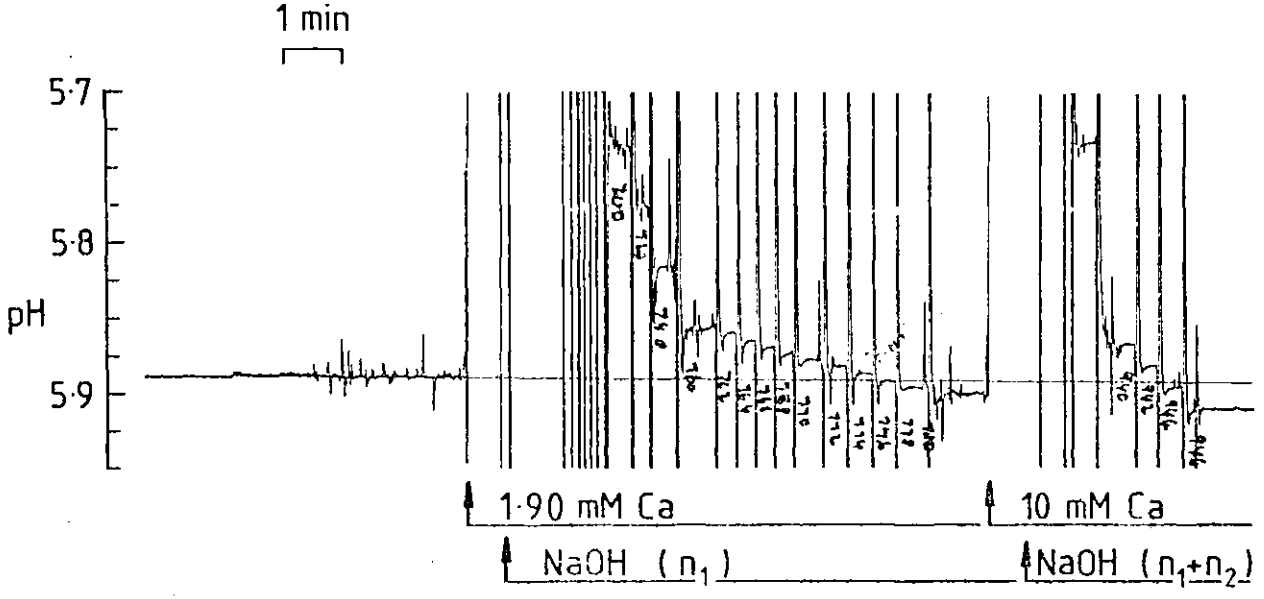


Figure 60. Typical record of the pH changes during the titration for the determination of K_{CaEGTA} and $K_{CaHEGTA}$.

In this case the solution contained 90 mM KCl + 100 mM TMACl. Numbers on chart indicate µl of 0.5 M NaOH added.

where V is the volume of the solution after the first addition of $CaCl_2$.

Using my computer program I was able to model the titration procedure, and so could estimate the free concentrations of ions at each stage of the titration by supplying the computer program with the pH, the total concentrations of solutes and assumed values for the true association constants. From the computed ionic concentrations at each stage of the titration I could estimate the amount of NaOH (n_1, n_2 moles) needed to restore pHx after each addition of Ca^{2+}. When those values for n_1 and n_2 were inserted into equations 10 and 11, I found that K^x_{CaEGTA}, and the K_{CaEGTA} calculated from it by equation (6), were overestimated by 6 - 9%, whilst $K^x_{CaHEGTA}$, and $K_{CaHEGTA}$ calculated from it by equation (7), were overestimated by about 100%. At first this discrepancy was blamed upon the computer program, so the computer results were checked using another program that used an algebraic method to find the $[Ca^{2+}]$ in a solution containing only Ca and EGTA (method suggested to me by D.J. Miller):

$$[Ca^{2+}] = (\sqrt{[\alpha + \beta\ (EGTA_t - Ca_t)]^2 + 4\alpha.\beta.Ca_t} - [\alpha + \beta\ (EGTA_t - Ca_t)])\ /2\beta \quad \ldots\ldots\ldots\ldots\ (12)$$

where $EGTA_t$ = total concentration of EGTA

and Ca_t = total concentration of Ca

and $\alpha = 1 + K_1[H^+](1 + K_2[H^+](+ K_3[H^+](1 + K_4[H^+])))$

and $\beta = K_{CaEGTA} + K_{CaHEGTA}.\ [H^+].\ K_1.$

From the $[Ca^{2+}]$ all the other ionic concentrations could be calculated. When the results of the two programs were compared they were found to agree to the tenth decimal place. Accordingly Moisescu's equations were suspected as introducing the discrepancy. To derive equations 10 and 11, Moisescu (personal communication) assumed that at the end of the titration, when Ca was in excess of EGTA, all the EGTA existed as either $CaEGTA^{2-}$ of $CaHEGTA^-$. However the computer results showed me that at this point a substantial fraction of EGTA still existed as H_2EGTA^{2-}; indeed the $[H_2EGTA^{2-}]$ was similar to the $[CaHEGTA^-]$. Reworking through all Moisescu's equations, with an extra term to allow for the EGTA that was not bound

to Ca^{2+} at the end of the titration, produced the following equations:

$$K^{x}_{CaEGTA} = \frac{n_1(n_1 + n_2)(n_1 + n_2 - a + b).V}{(a.n_2 + n_1.b)^2} \quad \ldots\ldots\ldots\ldots\ldots (13)$$

$$K^{x}_{CaHEGTA} = \frac{n_1(n_1 + n_2)(2a - n_1 - n_2 - 2b).V}{(a.n_2 + n_1.b)^2} \quad \ldots\ldots\ldots\ldots\ldots (14)$$

where b is the $[EGTA]_f$ (i.e. the total concentration of EGTA not bound to Ca^{2+}) at the end of the titration, when Ca is in excess over EGTA.

If the titration was modelled on the computer and the K^{x}_{CaEGTA} and $K^{x}_{CaHEGTA}$ calculated from n_1, n_2, a and b using equations 13 and 14, the values for the apparent and true K_{CaEGTA} were only 0.001% different from the values originally put into the program, and the values from the apparent and true $K_{CaHEGTA}$ were only 1% different from the original values. The calculated $K^{x}_{CaEGTA\ total}$ was 0.001% different from its original value.

A problem with using equations 13 and 14 to estimate the apparent association constants is that in the real titration the $[EGTA]_f$ is unknown and depends upon the apparent association constants. This difficulty was resolved by another computer program, which relied on an iterative technique to calculate both b and the association constants: first the actual experimentally-derived values for a, n_1 and n_2 were used to calculate K^{x}_{CaEGTA} and $K^{x}_{CaHEGTA}$ assuming that b was zero (as in equations 10 and 11). Assuming values for K_1 to K_4, the true association constants K_{CaEGTA} and $K_{CaHEGTA}$ were then calculated. From these true association constants and from the assumed values for K_1 - K_4, the $[EGTA]_f$ at the end of the titration - b - was estimated, and this concentration was inserted into equations 13 and 14 to obtain revised estimates for K^{x}_{CaEGTA} and $K^{x}_{CaHEGTA}$. The process of alternately calculating the association constants and b was repeated as necessary until values for K^{x}_{CaEGTA} and $K^{x}_{CaHEGTA}$ (and also the values for K_{CaEGTA} and $K_{CaHEGTA}$) changed by less than 0.1% with each iteration.

The values chosen for K_1 - K_4 ($\log K_1$ - $\log K_4$ = 9.58, 8.96, 2.77, 2.11) were obtained from Martell & Smith (1974) with suitable corrections to convert from concentration constants to mixed constants. I assumed that these values were constant in the solutions of different salt composition and that any changes in the apparent association

constants were due entirely to alterations in the K_{CaEGTA} and $K_{CaHEGTA}$.

For comparison with results from other laboratories I also calculated the values of the apparent constants at pH 7.0 using equations 8 and 9.

(e) [Ca]: [EGTA] ratio

The pH-metric method for determination of the apparent association constants required a very accurate knowledge of the ratio between the [total Ca] in the $CaCl_2$ stock solution and the [total EGTA] in the K_2H_2EGTA stock solution. Only if this ratio was known accurately could I be sure that in the first stage of the titration, Ca and EGTA were present in exactly equimolar amounts (a moles). The [Ca]:[EGTA] ratio was therefore determined by a modification of the pH-metric method developed by Moisescu & Pusch (1975). (A similar method had been used to determine the [Ca]:[EGTA] ratio in the CaEGTA stock solutions for the activating solutions). The initial solutions were exactly the same as the initial solutions for the association constant determinations (i.e. 4 mM imidazole, nominally 2 mM EGTA, 90 mM KCl plus added salt as required) except that the solutions were brought to pH $\approx$ 9.4 with 1 M NaOH rather than to pH $\approx$ 5.9 with HCl. These solutions were titrated with the 50 mM $CaCl_2$ used in the association constant determinations and the end point was taken as the point where the pH of the solution ceased to fall with further addition of $CaCl_2$.

The titration curve consisted of two straight lines meeting at an obtuse angle. Back extrapolation of these lines enabled the [Ca]:[EGTA] ratio to be measured to an accuracy of 0.1%.

In all titrations I assumed that the $CaCl_2$ was 100% pure. There is some justification for this view because the results were the same whether I used 1 M $CaCl_2$ from B.D.H. or used my own 1 M $CaCl_2$, made from dried $CaCO_3$ + HCl.

(f) [EGTA]:[OH] ratio

The association constant determination also required that the ratio between the total concentrations of EGTA and OH^- in the EGTA and NaOH stock solutions be accurately known. The $[OH^-]$ in the 0.5 M and 0.1 M NaOH solutions was measured by titration against potassium hydrogen phthalate (Section A.3(b)). The [EGTA] was determined by the above titration against 50 mM $CaCl_2$.

As a check that the [EGTA]:[OH] ratio was accurately known, I added excess $CaCl_2$ to a solution containing 2 mM EGTA (nominal) plus 90 mM KCl at pH $\approx$ 7.0. I then measured the amount of 0.5 M or 0.1 M NaOH needed to restore the pH to exactly its original value. Computer calculations showed that at pH $\approx$ 7.0, 1.999 moles of protons are released for each mole of EGTA that has complexed with Ca^{2+}. In other words, for each mole of EGTA saturated with Ca^{2+}, 1.999 moles of OH^- are needed to keep the pH constant. Knowing this figure, and knowing the volumes of 50 mM K_2H_2EGTA and 0.5 M or 0.1 M NaOH in the actual titration, I could calculate the [EGTA]:[OH] ratio.

A.4 Results

The ratio between the [total Ca] in the 50 mM $CaCl_2$ stock solution and the [total EGTA] in the 50 mM K_2H_2EGTA stock solution was found to be 1:0.9531 ± 0.0007 (mean ± S.E.M., n = 16) when 2 mM (nominal) EGTA was titrated against Ca^{2+} in the presence of 4 mM imidazole, 90 mM KCl plus other salts, added as required. This [Ca]:[EGTA] ratio was the same in all titrant solutions, irrespective of salt composition, and so the results from the solutions of different [salt] were combined to give the above statistics. If, as I assumed, the $CaCl_2$ was 100% pure, then these results indicate that the purity of EGTA was 95.3% and that the [EGTA] in the titrant was 1.906 mM rather than 2mM. In other [Ca]:[EGTA] determinations with the nominal [EGTA] = 10 mM the effective [EGTA] was estimated to be 9.60 ± 0.01 mM (mean ± S.E.M., n = 5), giving a purity of 96.0%.

These estimates of purity would have been lower than the true purity because Ca^{2+} contamination ($\approx$ 20 µM) from the other components in the titrant would have bound to the EGTA and reduced the effective [EGTA] by $\approx$ 20 µM. In fact the above ratios can be accounted for if the true purity of EGTA was 96.17% and the contaminant Ca^{2+} was 17 µM; then a solution of nominal [EGTA] = 10 mM would have an effective [EGTA] = (10 x 0.9617) - 0.017 = 9.600 mM and a solution of nominal [EGTA] = 2 mM would have an effective [EGTA] = (2 x 0.9617) - 0.017 = 1.906 mM, as observed.

The measured $[OH^-]$ of the 0.1 M NaOH was 0.1004 ± 0.0001 M (mean ± S.E.M., n = 5) and that of the 0.5 M NaOH was 0.5030 ± 0.0004 M (mean ± S.E.M., n = 9). The ratio of $[OH^-]$ in the 0.1 M NaOH to the [EGTA] in the 50 mM K_2H_2EGTA stock solution was found to be 1:0.961 ± 0.001 (mean ± S.E.M., n = 3) if the nominal [EGTA] in the titrant was 2 mM. On this basis, the effective [EGTA] in the titrant for the K_{CaEGTA} determination would have been 1.922 mM, which was somewhat greater than the [EGTA] (1.906 mM) estimated from the [Ca]:[EGTA] ratio.

This discrepancy probably arose because, in the solutions for the determination of the [OH]:[EGTA] ratio, the excess Ca^{2+} lead to the formation of some $Ca(OH)_2$ and so more OH^- was required to return the pH to its previous value than would otherwise have been necessary.

The values I determined for the 'true' constants K_{CaEGTA} and $K_{CaHEGTA}$ and for the apparent constants at pH 7.0, $K^{7.0}_{CaEGTA}$ and $K^{7.0}_{CaHEGTA}$, are listed in Table 13 in logarithmic form. The 'true' constants were computed using equations 13 and 14 and using the iterative procedure described in Section A.3(d); the apparent constants were calculated by equations 1, 2 and 3. Since at pH 7.0 the value for $K^{7.0}_{CaEGTA}$ exceeded that of $K^{7.0}_{CaHEGTA}$ by a factor of 10^4, the value for the total apparent association constant, $K^{7.0}_{CaEGTA\ total}$, was almost exactly the same as that for $K^{7.0}_{CaEGTA}$ alone.

None of the constants for Ca^{2+} binding to EGTA was greatly affected by the alterations in salt composition of the solution. The largest difference in values was observed between the solutions containing 90 mM KCl and 190 mM KCl. The extra 100 mM KCl caused all the constants to fall by about 15% (0.06 log units). For example, the $\log K^{7.0}_{CaEGTA\ total}$ dropped from 6.412 to 6.354. If however, the same 100 mM increase in ionic strength was produced with the other chloride salts, the reductions in the constants were less: compared with their values in 90 mM KCl, $\log K^{7.0}_{CaEGTA}$ and $\log K_{CaEGTA}$ were reduced by about 10% (0.04 log units) in 100 mM added TMACl, by about 5% (0.02 log units) in 100 mM added ChCl and by an insignificant amount in 100 mM added NaCl.

Neither the apparent constant nor the true constant for the binding of Ca^{2+} to HEGTA ($K^{7.0}_{CaHEGTA}$ and $K_{CaHEGTA}$) was significantly different from the appropriate value in 90 mM KCl. Any small changes in these two constants may have been obscured by the scatter in the measurements; the scatter was considerably greater than that for the estimates of $\log K^{7.0}_{CaEGTA}$ and $\log K_{CaEGTA}$

A.5 Discussion

The data in Table 13 are likely to be good estimates for the values of the association constants for the binding of Ca^{2+} to EGTA. Assuming no pipetting errors, the [Ca]:[EGTA] ratio, the $[OH^-]$:[EGTA] ratio and the magnitude of $\underline{a}$, n_1 and n_2 in equations 13 and 14 could be estimated with errors of 0.1%, 0.1% and 0.2% respectively. Taken

Salt composition (mM)	Final ionic strength (mM)	n	Apparent constants at pH 7.00			'True' constansts	
			$\log K^{7.0}_{CaEGTA}$	$\log^{7.0}_{CaHEGTA}$	$\log^{7.0}_{CaEGTA(total)}$	$\log K_{CaEGTA}$	$\log K_{CaHEGTA}$
90 KCl	100	5	6.412±0.014	2.328±0.199	6.412±0.015	10.952±0.014	5.416±0.177
190 KCl	200	7	6.354*±0.011	2.258±0.106	6.354±0.012	10.895*±0.011	5.333±0.101
90 KCl + 100 TMACl	200	3	6.370*±0.008	2.433±0.114	6.370±0.009	10.910*±0.008	5.500±0.110
90 KCl + 100 ChCl	200	3	6.389*±0.008	2.378±0.198	6.389±0.008	10.930*±0.008	5.440±0.190
90 KCl + 100 NaCl	200	3	6.409±0.016	2.275±0.212	6.409±0.017	10.949±0.016	5.344±0.197

* significantly different from corresponding value for 90 mM KCl.

Table 13. Apparent and 'true' association constants for Ca^{2+} binding to EGTA in solutions of different salt composition. Apparent constants at pH 7.0 calculated from the measured apparent constants at pH ≈ 5.9 using equations (3), & + (7). 'True' constants calculated from the measured apparent constants using equations (13) and (14) and assuming $\log K_1 = 9.58$, $\log K_2 = 8.96$, $\log K_3 = 2.77$, $\log K_4 = 2.11$. Each value represents the mean ± S.E.M. of n observations (Means and S.E.M. were calculated before logs were taken).

together with the errors in pipetting (estimated at ≈ 1%), the total experimental error for the determination of the apparent constants at pH ≈ 5.9 should be no greater than 2%, provided that systematic errors were negligible. This percentage would also apply to the apparent constants extrapolated to pH 7.0. However, the 'true' constants would be subject to an additional small error because I assumed that the values for K_1 - K_4 were constant and that any differences in the apparent constants in the solutions of different salt composition were due entirely to differences in the true constants. This assumption was necessary because the values for K_1 - K_4 have been determined only at an ionic strength of 100 mM (Schwarzenbach et al., 1957). While this assumption is certain to be an oversimplification, it would affect neither the apparent constants nor the calculated pCa of the activating solutions at any pH in the range pH 5.8 - 7.5.

Table 14 enables a comparison of my results for the apparent and true constants with those from other studies. Although earlier determinations (up to about 1976) gave rather divergent values for the association constants, the more recent studies have appeared to reach a consensus for the constants determined in 100 mM - 200 mM KCl. The data of Allen & Blinks (1977)† and Harifugi & Ogawa (1980) both give the log $K^{7.0}_{\text{CaEGTA total}}$ as 6.40 (my estimate from their data). My figure of 6.41 is not significantly different from this value. For many years the most commonly used constants were those determined by Schwarzenbach et al. (1957). These values were used by Schädler (1967), Portzehl et al. (1969), Williams et al. (1975), and Fabiato and Fabiato (1978b) in their studies on the effect of pH on myofibrillar ATPase or force development. As the Table shows, the apparent $K^{7.0}_{\text{CaEGTA total}}$ calculated from Schwarzenbach's constants (6.44) is not very different from the value of 6.40 in the most recent studies. However in all the above studies on pH in fact the $K^{7.0}_{\text{CaEGTA total}}$ was calculated as 6.65 because the investigators mistakenly put Schwarzenbach's constants for log K_1 - log K_4 (9.46, 8.85, 2.65, 2.00) into equations 6 and 7. Tsien, quoted in Fabiato (1982), realised that this was incorrect because the latter four values are concentration constants, which involve a term giving the concentration of hydrogen ions, e. g.:

$$K_1 = \frac{[HEGTA^{3-}]}{[H^+][EGTA^{4-}]}$$

However because pH electrodes measure hydrogen ion activity, for practical purpose (e.g. to calculate the apparent constant at a given

† unpublished, quoted in Fabiato & Fabiato (1978b).

	Method	I/2(mM)	log $K^{7.0}_{CaEGTA}$
Schwarzenbach et al. (1957)	potentiometric	100	6.43 ǂ
Ogawa (1968)	murexide	≈ 110	6.05*
Godt & Briggs (quoted by Godt, 1974)	chelex partition	≈ 130	5.88 ǂ
Dipolo et al. (1976)	arsenazo	100	6.68
Moisescu (1976)	pH-metric	-	6.58*
Allen & Blinks (1977)†	aequorin	155	6.40
Harifugi & Ogawa (1980)	murexide	106 200	6.40* 6.31*
Present results	pH-metric	100 200	6.41* 6.35*

* Apparent constant determined at pH ≠ 7.00; log $K^{7.00}_{CaEGTA}$ calculated from it by equation (8).

ǂ True constant determined; log $K^{7.00}_{CaEGTA}$ calculated from it by equation (6) using the proton constants listed in legend to Table 13.

† Unpublished, quoted by Fabiato & Fabiato (1978b).

Table 14. Reported values for the apparent association constant for Ca^{2+} binding to EGTA at pH 7.0 ($K^{7.0}_{CaEGTA}$). Values given in log form.

pH) a 'mixed constant' must be used that involves a term for hydrogen ion activity rather than concentration (Martell& Smith, 1974). Concentration constants can be converted to mixed constants by the addition of a correction factor (0.11 at $\Gamma/2$ = 100 mM, Martell & Smith, 1974). With this addition to the values of Schwarzenbach et al. (1957) for log K_1 - log K_4, the calculated $K^{7.0}_{\text{CaEGTA total}}$ is close to that of the more recent studies even though it was previously thought to be much higher (e.g. see Fabiato & Fabiato, 1978b). In fact my estimates of log K_{CaEGTA} and log K_{CaHEGTA} are not significantly different from those of Schwarzenbach et al. (1957) at $\Gamma/2$ = 100 mM.

Not all investigations have given a value for $K^{7.0}_{\text{CaEGTA total}}$ of $\approx$6.40 at $\Gamma/2$ = 100 mM. Ogawa (1968) reported a value of 6.04, but Harifugi & Ogawa (1980), revising this to 6.40, pointed out that the temperature control was probably inadequate in the earlier study. The value obtained by Godt & Briggs (quoted in Godt, 1974) was also lower than most others. On the other hand, Dipolo et al. (1976) and Moisescu (1976) obtained higher than average values. Moisescu's value would be reduced, but only by about 0.03 log units to 6.55 (my estimate), if allowance is made for the presence of free EGTA at the end of the titration. It is not clear why there is a discrepancy between these results and those of Allen & Blinks (1977),† Harifugi & Ogawa (1980) and myself.

With the exception of Godt & Briggs (reported in Godt, 1974) and Moisescu (1976), all studies of the log $K^{7.0}_{\text{CaEGTA total}}$ at different ionic strengths report a decrease in this constant with an increase in [KCl]. For an increase from 100 mM to 200 mM KCl, log $K^{7.0}_{\text{CaEGTA total}}$ fell by 0.16 (my estimate from Dipolo et al., 1976) and 0.09 (my estimate from Allen & Blinks, 1978; Harifugi & Ogawa, 1980). My data show a reduction of 0.06 - slightly less than in the above studies but in the same direction. It is possible that the different values obtained result from different ionic strength - sensitivities of the pH electrodes used in these studies. (Many electrodes give an incorrect reading of pH if the ionic strength is different from that in the pH standards - see Illingworth, 1981).

One of the main aims of my study was to see whether, and by how much, the apparent constants for Ca^{2+} - EGTA association varied between solutions of different salt composition, and thereby to determine whether the apparent shifts in the relative force - pCa relationship were real or were due to alterations in the binding constants. Only

† unpublished, quoted in Fabiato & Fabiato (1978b).

the K_{CaEGTA} and $K_{CaHEGTA}$ were studied because it is only these constants that, together with K_1 - K_4, determine the pCa at values of pCa greater than $\approx$ 4.0. Apparently no other investigations have studied the effects of chloride salts other than KCl on the association constants of EGTA for Ca^{2+}. My data show that increasing the ionic strength from 100 mM to 200 mM with TMACl or ChCl decreased $\log K^{7.0}_{CaEGTA\ total}$, though the fall was less than if the ionic strength was raised with KCl. However in 90 mM KCl + 100 mM NaCl the apparent constants for the binding of Ca to EGTA were not significantly different from their values in 90 mM KCl alone. I had expected the constants in 90 mM KCl + 100 mM NaCl to be even lower than in 190 mM KCl, since the ionic strength was the same in both cases and Na^+ reportedly binds to $EGTA^{4-}$ more strongly than K^+ does ($\log K_{NaEGTA}$ = 1.2, $\log K_{KEGTA}$ = 0.9, Sillèn & Martell, 1971).

The main conclusions to be drawn from my results are that (i) the $\log K^{7.0}_{CaEGTA\ total}$ was 6.41 at $\Gamma/2$ = 100 mM and (ii) this value was decreased if the ionic strength was raised by 100 mM with KCl, TMACl or ChCl but the changes were small: the maximum drop (with KCl) was 0.06 log units. Thus the effects on the position of the relative force - pCa relationship were small. If allowance was made for the [salt]-dependence of $K^{7.0}_{CaEGTA\ total}$, the pCa at half maximal Ca^{2+}-activation was raised at most by 0.06 pCa units - a shift that was barely noticeable in the relative force - pCa relationships such as those in Figures 57 and 58 .

Appendix B

STUDIES ON THE DECLINE OF Ca^{2+}-REGULATED FORCE IN A SERIES OF CONTRACTURES IN DETERGENT-SKINNED CARDIAC MUSCLE

B.1 Introduction

Unlike intact muscles, which can develop the same force over many hours of continual activity, skinned muscles exhibit a decrease in the Ca^{2+}-regulated force in successive contractures. This phenomenon, which I have termed the 'decline' in Ca^{2+}-regulated force in this thesis, has been observed in both mechanically-skinned and chemically-skinned preparations of skeletal and cardiac muscle (Hellam & Podolsky, 1969; Julian, 1971; Endo, 1972; Moisescu, 1976; Endo & Kitazawa, 1978) and was also a feature of Triton-skinned cardiac muscle (see Figures 34 and 36). This loss of contractility presents a technical inconvenience because it limits the viability of the skinned muscles and makes corrections necessary. More seriously, it implies that the properties of myofibrils in unskinned and skinned muscles are different. Although this deterioration had been linked with a progressive disruption of the striation pattern during contraction of skinned skeletal fibres (Hellam & Podolsky, 1969; Endo, 1972) there had been no investigation into its underlying biochemical basis in either skeletal or cardiac muscle. In this Appendix I shall describe the results from a series of experiments in which I changed the bathing solution composition or other experimental conditions in order to characterise the decline of Ca^{2+}-regulated force in the Triton-skinned trabeculae. The ultimate aim was to find the conditions that made the decline of force as slow as possible.

B.2 Methods

All the experimental procedures and solutions were as described in Chapter 9. Each experiment was performed at least twice unless otherwise stated.

B.3 Results

(a) Structure of the skinned trabeculae after many contractures

I used an electron microscope to examine the ultrastructure of skinned trabeculae that had undergone many contractures during the course of the experiments and that had consequently suffered a considerable decline in contractile performance. A typical electron

micrograph of such a muscle is shown in Figure 61 . There was obviously some derangement of sarcomere structure compared with its appearance in skinned muscles that had not undergone any contractures (Figure 43). The myofilament arrays were not as well ordered as those in Figure 43 and the A band did not always lie at the centre of the sarcomere. (The latter point is illustrated better in Figure 44 , which is an electron micrograph of another muscle that had undergone many contractures).

(b) Time- and contracture-dependence of the decline of force

If the skinned muscles were perfused with the same activating solution at regular intervals, the Ca^{2+}-regulated force attained in each contracture declined as an exponential function of time. This was shown clearly by the straight lines in semi-logarithmic plots of force versus time, such as those in Figure 62 . This fact made it simple for me to quantify the effects of changes in solutions or experimental protocol on the rate of decline, by measuring the slopes of the lines. The slopes could be expressed in terms of force lost per unit time or force lost per contracture. In the experiments described below the rate of decline is expressed as the percentage force lost per minute (% min^{-1}). In the figures, each value for the rate of decline is given next to the line from which it was derived.

To test whether the decline in Ca^{2+}-regulated force was related to the occurrence of contractures, i.e. was frequency-dependent, or was only time-dependent, I reduced the frequency of contractures for a certain period. Typical results are given in Figure 62 , together with an indication of the rate of decline that would have been expected if the decline had been purely time-dependent (dotted lines) or purely contracture-dependent (dashed lines). The results were not clear-cut. In some experiments the reduction in frequency had no effect on the rate of force decline, which was therefore solely time-dependent (circles, Figure 62); in other experiments the decline was slowed by the reduction in contracture frequency i.e. there was some contracture-dependence (squares, Figure 62). However, there was always some time-dependence, since the decline during the period of reduced contracture frequency was faster than would have been expected if the decline had been solely related to the occurrence of contractures.



Figure 61. Electron micrograph of a detergent-skinned trabecula that had undergone 40 contractures. x15000.

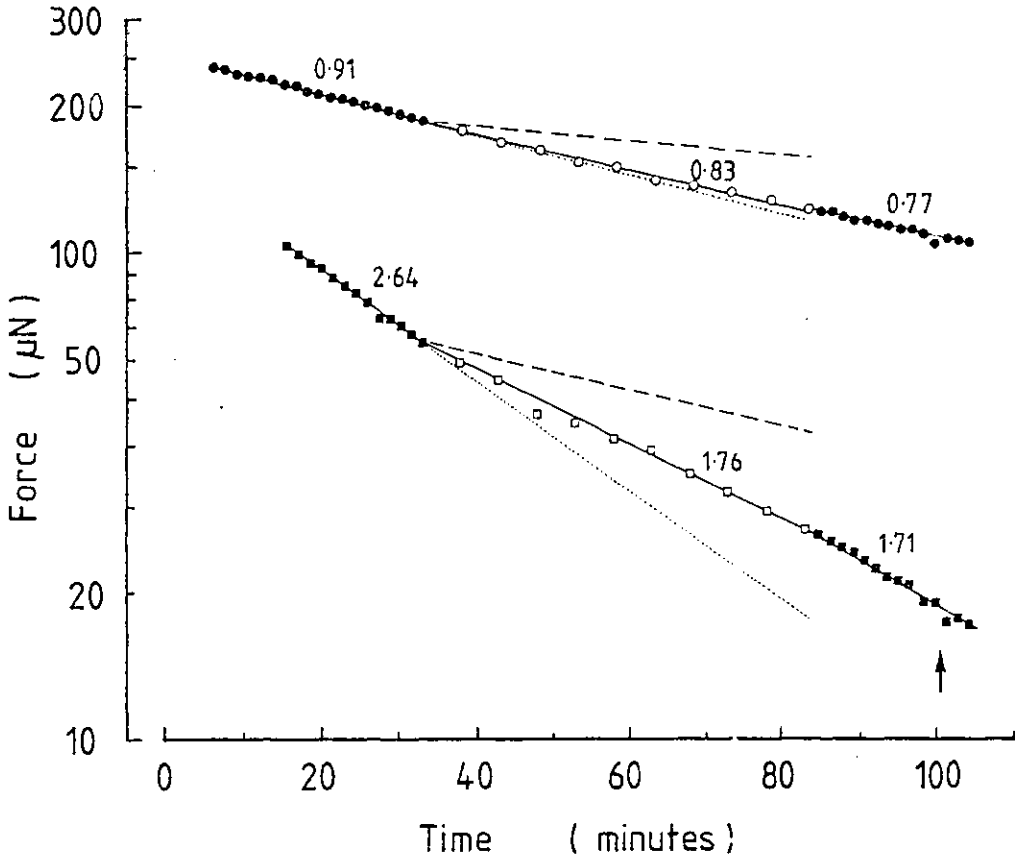


Figure 62. Effect of a change in contracture frequency on the rate of decline of Ca^{2+}-regulated force. Results from 2 muscles. Contracture frequency was one every 1.5 mm^{-1} (filled symbols) or one every 5 min (open symbols). The rates of force decline are given as % loss min^{-1}. See text for explanation of dashed and dotted lines. Temperature: 25°C for (●○), 29°C for (■□).

(c) Effect of freshly-made solutions

The decline in contractility could not be reversed or even arrested by substitution of the experimental solutions with identical solutions made freshly only minutes previously (arrow, Figure 62). Thus the deterioration was entirely a property of the skinned muscle itself rather than a property of the bathing solutions.

(d) Relationsip between relative force and pCa

One possible explanation for the apparent decline in Ca^{2+}-regulated force production was that the Ca^{2+}-sensitivity of the myofibrils progressively decreased, so that more Ca^{2+} was required to produce the same level of myofibrillar activation. Figure 63 shows the results from six experiments in which the relative force - pCa relationship at pH 7.00 was determined twice, once soon after the muscles had been skinned (closed circles) and again, one to two hours later, after the muscles had undergone an intervening series of 30 - 40 contractures (open circles). (These data are in fact from the pH experiments but they are reproduced here for convenience). Although the absolute magnitude of force in the control activating solution (pCa 3.75) had fallen by 50.3 ± 3.5 % (mean ± S.E.M. of 6 experiments), the sub-maximal forces when expressed relative to this, i.e. the relative forces, were not significantly altered (Paired t-test, $p>0.05$ for each pair of relative forces at a give pCa). The same was true for the values of n_H and pCa_{50}. Thus the decline in Ca^{2+}-regulated force occurred with no significant shift in the relative force - pCa relationship and therefore no change in the optimal pCa (3.75) for force production. In the experiments described below all the activating solutions had a pCa of 3.75.

(e) Influence of temperature on the rate of decline of force

Figure 64 shows the results from an experiment in which series of contractures were produced at different temperatures: room temperature (23°C), 30°C and 37°C. Different sets of solutions were used at the different temperatures so that each set could be brought to pH 7.00 at the appropriate temperature by adding KOH or HCl. The Ca^{2+}-regulated force at each temperature declined exponentially with contracture number but the rate of decline increased dramatically as the temperature was raised: at 23°C the decline was 0.8% min^{-1}, at 30°C it was 2.7% min^{-1} and at 37°C it was 9.5% min^{-1}. Thus each 7°C increase in temperature accelerated the decline by a factor of about 3.5; this corresponds to a Q_{10} of about 6. At 37°C force decline very quickly indeed and only a

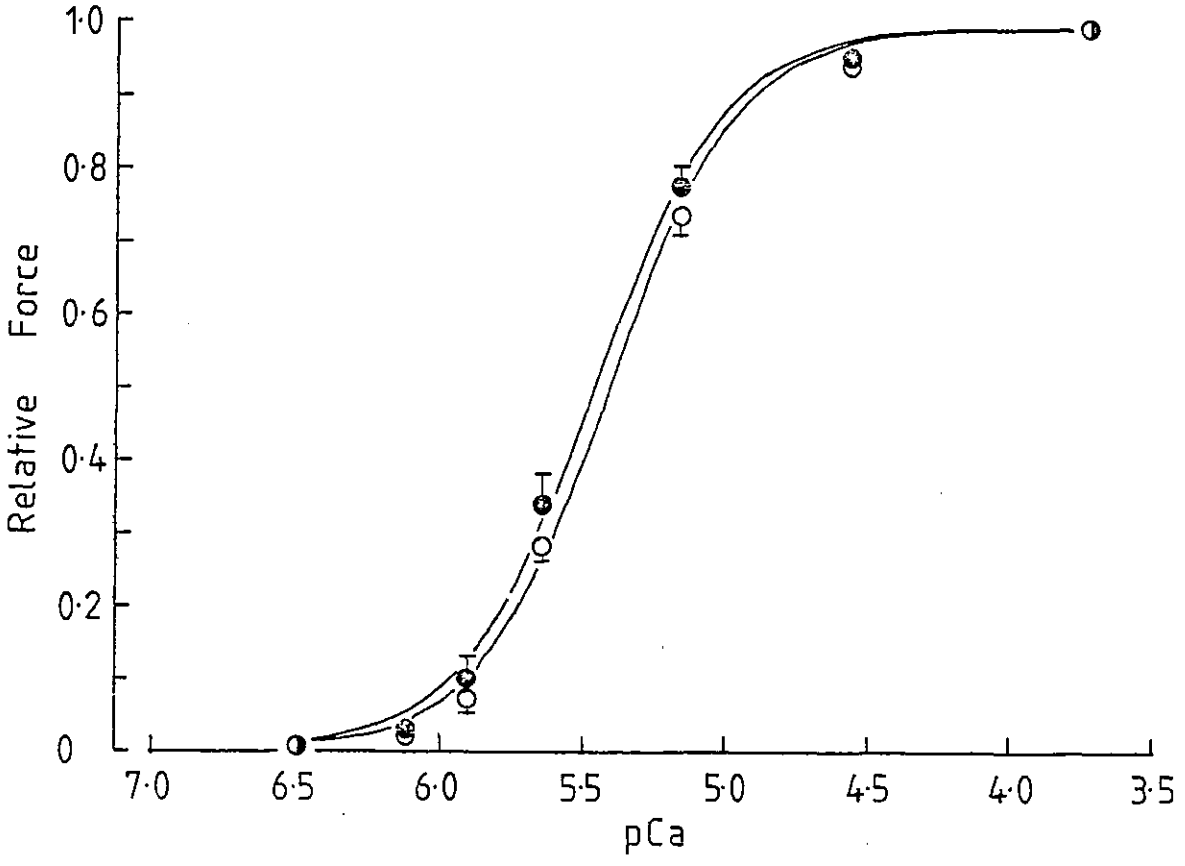


Figure 63. Lack of effect of the decline of force on the relative force-pCa relationship. Relationship determined soon after muscle was skinned (●) and much later, after 30 - 40 contractures (○). Standard solutions. 30°C.

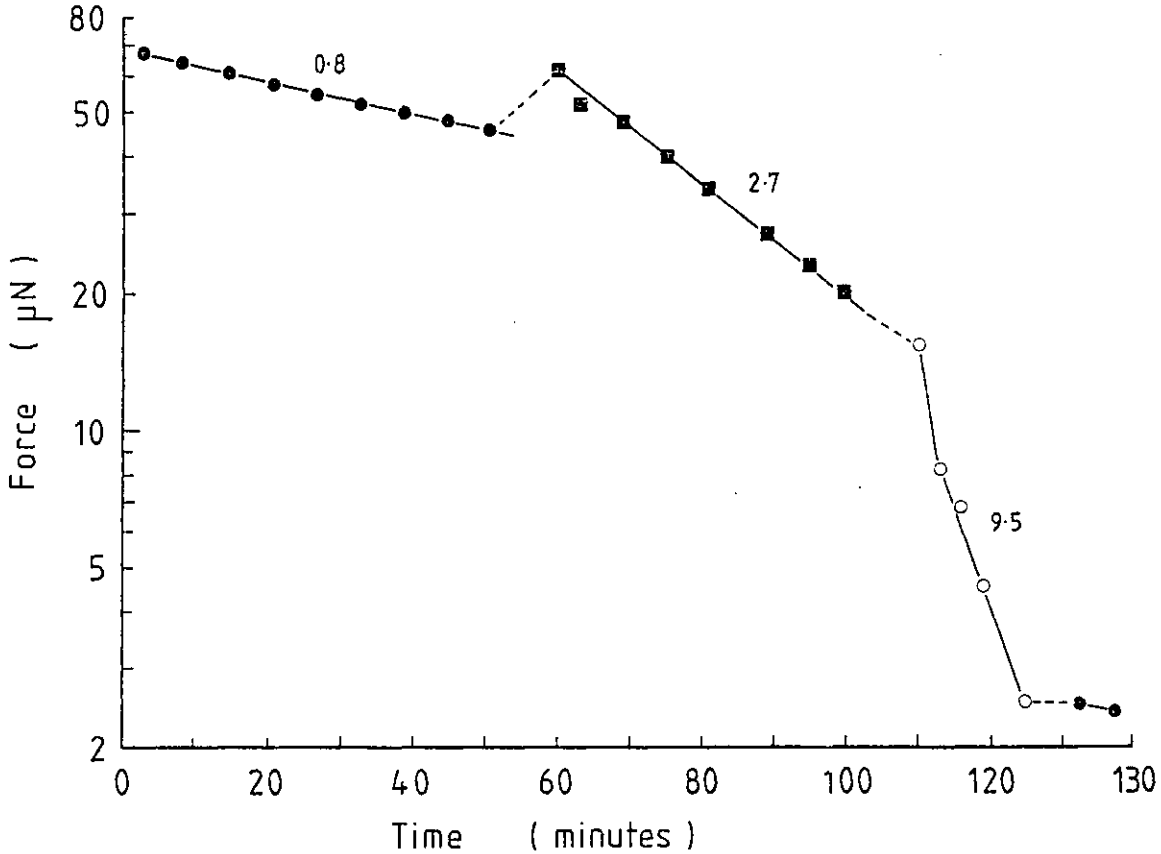


Figure 64. Influence of temperature on the rate of decline of force.
(O), 23°C; (■) 30°C; (O) 37°C. pH 7.00, pCa 3.75.

few usable contractures could be obtained at this temperatue. If the muscle was returned to room temperature the rate of decline decreased to its previous value at this temperature but the Ca^{2+}-regulated force itself was not restored.

(f) Influence of dithiothreitol

It seemed possible that some of the decline was due to oxidation of functionally-important sulphydryl groups in the myofibrils. To test this, in one experiment I added the sulphydryl-protecting agent dithiothreitol to the bathing solutions. However 1 mM dithiothreitol had no effect on the Ca^{2+}-regulated force or on the rate of force decline (Figure 65).

(g) Influence of 'high-energy' phosphate compounds

(i) Creatine phosphate. Replacement of the 10 mM Na_2CP in the control activating solution by 10 mM Na_2SO_4 had profound effects on the force production in the skinned muscles (Figure 66). Initially the Ca^{2+}-regulated force was potentiated but this effect was soon negated because the force decline was much faster in Na_2SO_4 than in Na_2CP e.g. in the experiment shown in Figure 66 the decline, increased from 0.5% min^{-1} in Na_2CP to 6.3% min^{-1} in Na_2SO_4. On return to Na_2CP the rate of decline fell to its previous value. (Possibly the increase in Ca^{2+}-regulated force after the substitution of Na_2CP by Na_2SO_4 was due to association of Na^+ or K^+ ions with SO_4^{2-} ions; this would have lowered the ionic strength, a change which would lead to an increase in force (see Section 10.11(b)).

(ii) MgATP. I varied the total concentration of MgATP in the relaxing and activating solutions from 0 mM to 15 mM. In all bathing solutions the 10 mM Na_2CP was replaced by 20 mM NaCl. The total [$MgCl_2$] was altered to maintain the [Mg^{2+}] at 1.4 mM and the [KCl] was adjusted to keep the ionic strength at 200 mM.

Increasing the [MgATP] over the range 1 mM to 15 mM slowed the deterioration from 6.0% min^{-1} to 0.9% min^{-1} (Figure 67), although this beneficial effect seemed to be near maximal at 10 mM ATP (1.1% min^{-1}). If MgATP was removed completely, the resulting rigor contractures (produced in the absence of Ca^{2+}) exhibited some decline of force but this was only 1.3% min^{-1}, much slower than that observed in 1 mM or 5 mM MgATP and only slightly faster than that in 10 mM ATP. Although each solution had a calculated ionic strength of 200 mM, the absolute Ca^{2+}-regulated force appeared to be decreased as the [MgATP] was raised.

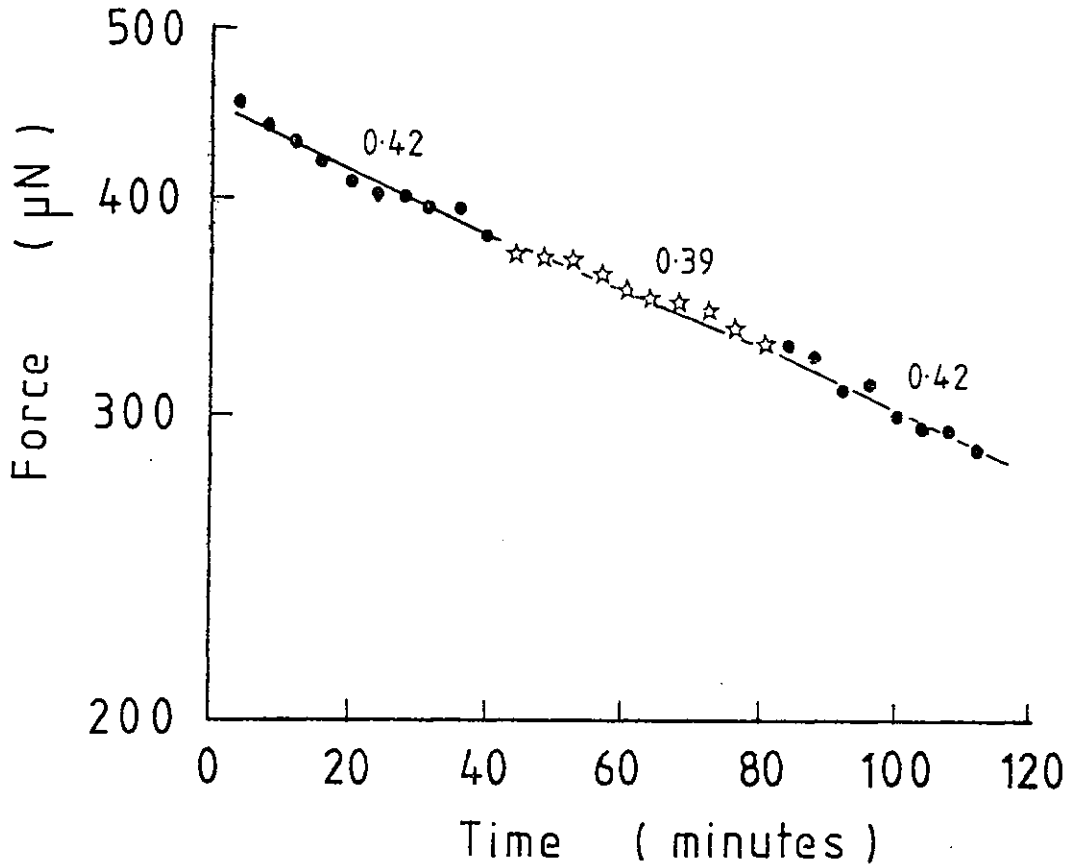


Figure 65. Rate of force decline in absence (●) and presence (☆) of 1 mM dithiothreitol. pH 7.00, pCa 3.75, 25°C.

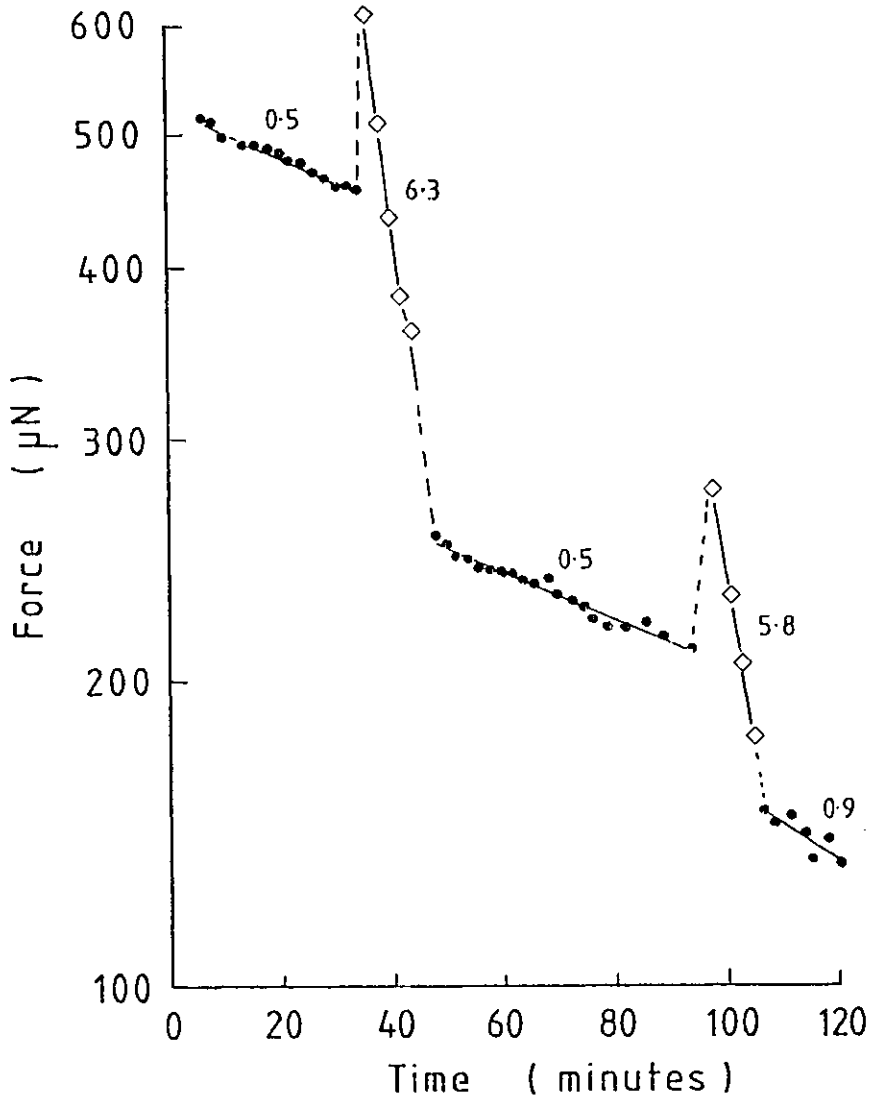


Figure 66. Effect of replacement of the 10 mM Na_2CP (●) by 10 mM Na_2SO_4 (◇) on the rate of force decline. pH 7.00, pCa 3.75, 30°C.

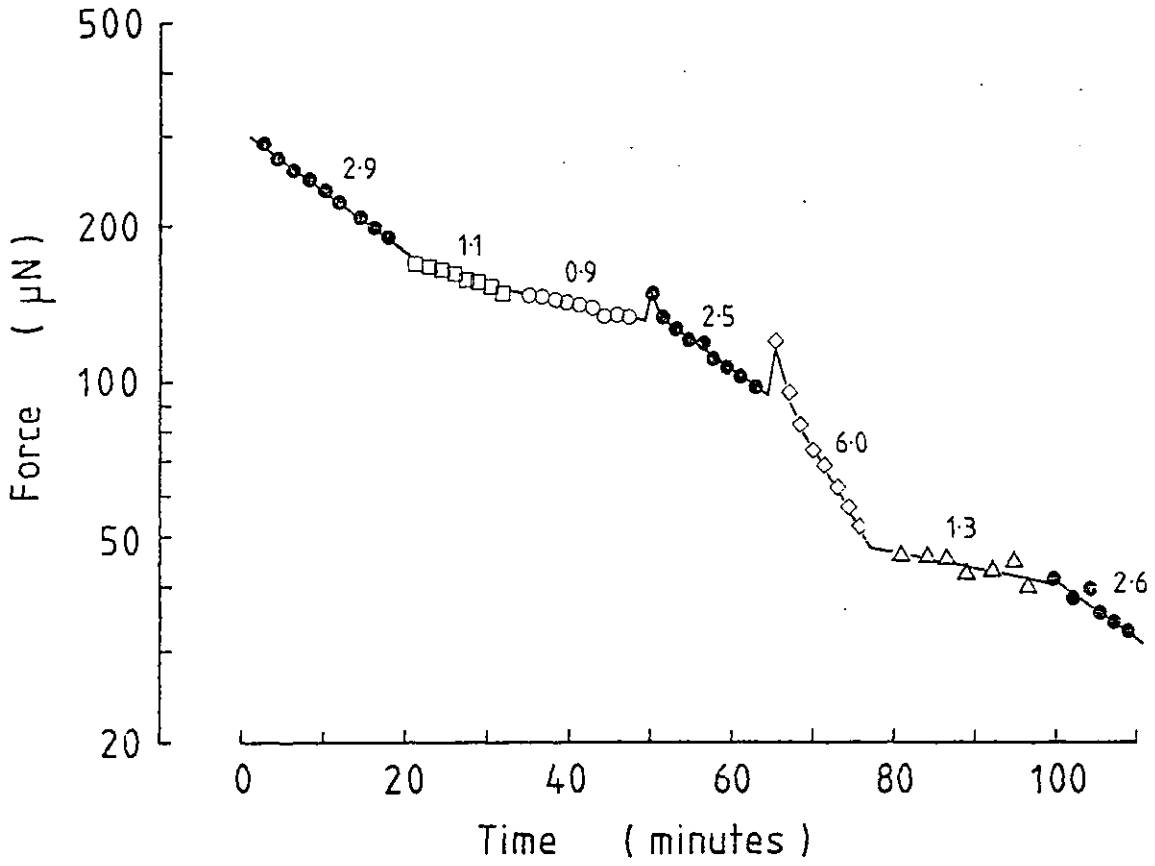


Figure 67. Effect of [MgATP] on the rate of force decline. [MgATP] as follows: (○) 15 mM; (□) 10 mM; (●) 5 mM; (◇) 1 mM; (△) 0 mM (rigor contracture). All contractures at pCa 3.75 except for rigor contractures, which were produced at pCa>8. CP was omitted from all solutions. 30°C.

When, in one experiment, a reduction of [MgATP] from 15 mM to 5 mM was compensated by the addition of 10 mM Na_2CP, there was no change in the rate of decline of force (Figure 68). In other words, applied MgATP and Na_2CP wereequipotent in their beneficial effect on muscle viability.

(h) Influence of creatine kinase

In the preliminary experiments with detergent-skinned cardiac muscles I added 25 μg cm^{-3} creatine kinase to the bathing solutions to form an ATP-regenerating system in combination with CP. However neither the Ca^{2+}-regulated force nor the rate of decline of force was altered if this creatine kinase was omitted from the skinning solution and other bathing solutions and then was added during the experiment (Figure 69).

(i) Influence of cAMP plus cAMP-dependent protein kinase

To investigate the possibility that some of the force decline was the result of phosphorylation or dephosphorylation of the myofibrils, I incubated the muscles in 10 μM cAMP + 20 μg cm^{-3} cAMP-dependent protein kinase for 30 minutes. There were no effects on Ca^{2+}-regulated force or on the rate of decline of force (Figure 70). However no biochemical studies were carried out to determine whether the phophorylation state of the myofibrils had increased, because such studies would have taken many months to set up.

B.4 Discussion

The data presented in this Appendix demonstrated that the Ca^{2+}-regulated forces obtained in a series of regularly-spaced contractures declined as a single exponential function of time or of contracture number. (The procedures I used to correct for the decline in force (Section 9.7) were based upon this finding; I usually measured contracture number rather than time because it was more quickly measured from the chart record). Many investigators correct for the deterioration in contractile performance of skinned muscles using the procedure of Julian (1971), who assumed force declinedlinearly in successive contractures of skinned skeletal muscle. While my results indicate that this assumption is not valid, at least for detergent-skinned cardiac muscle, in practice values for relative force that are calculated assuming a linear decline of force with time should be only a few percent smaller than values calculated on the basis of an exponential decline with time.

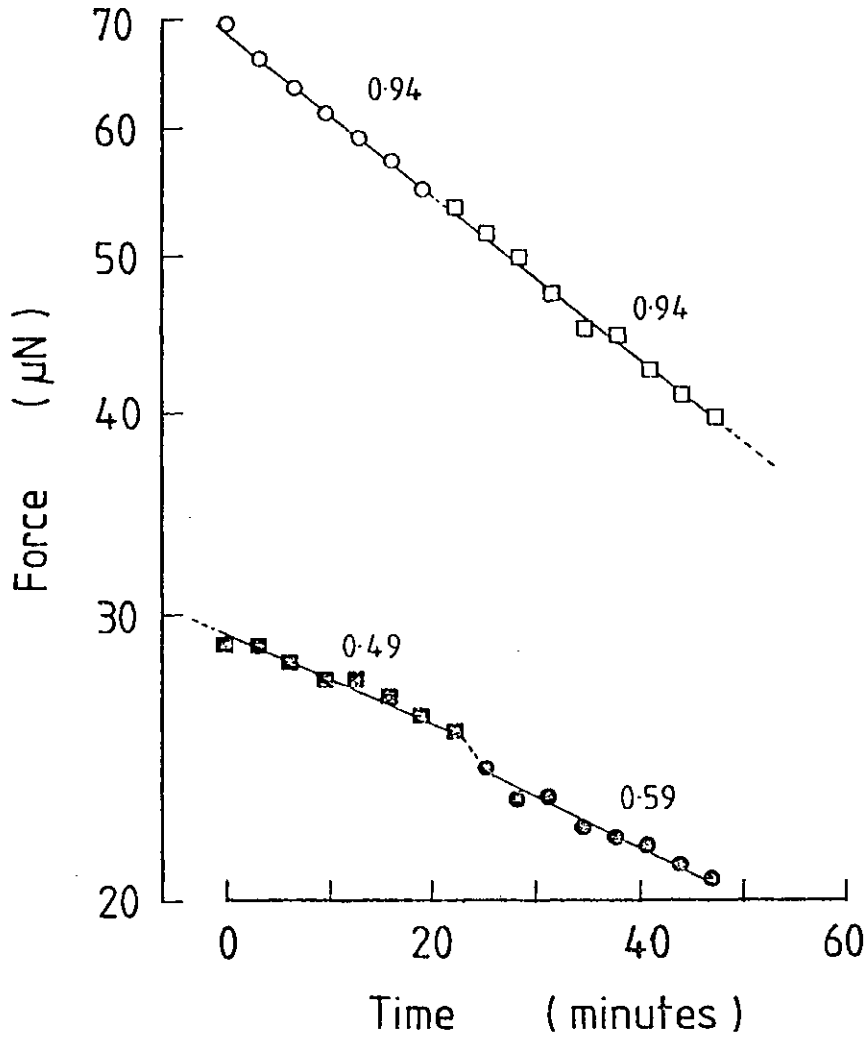


Figure 68. Rates of force decline in 15 mM MgATP (□■) and in 5 mM MgATP plus 10 mM Na_2CP (○●). Results from two muscles. pH 7.00, pCa 3.75, 25°C.

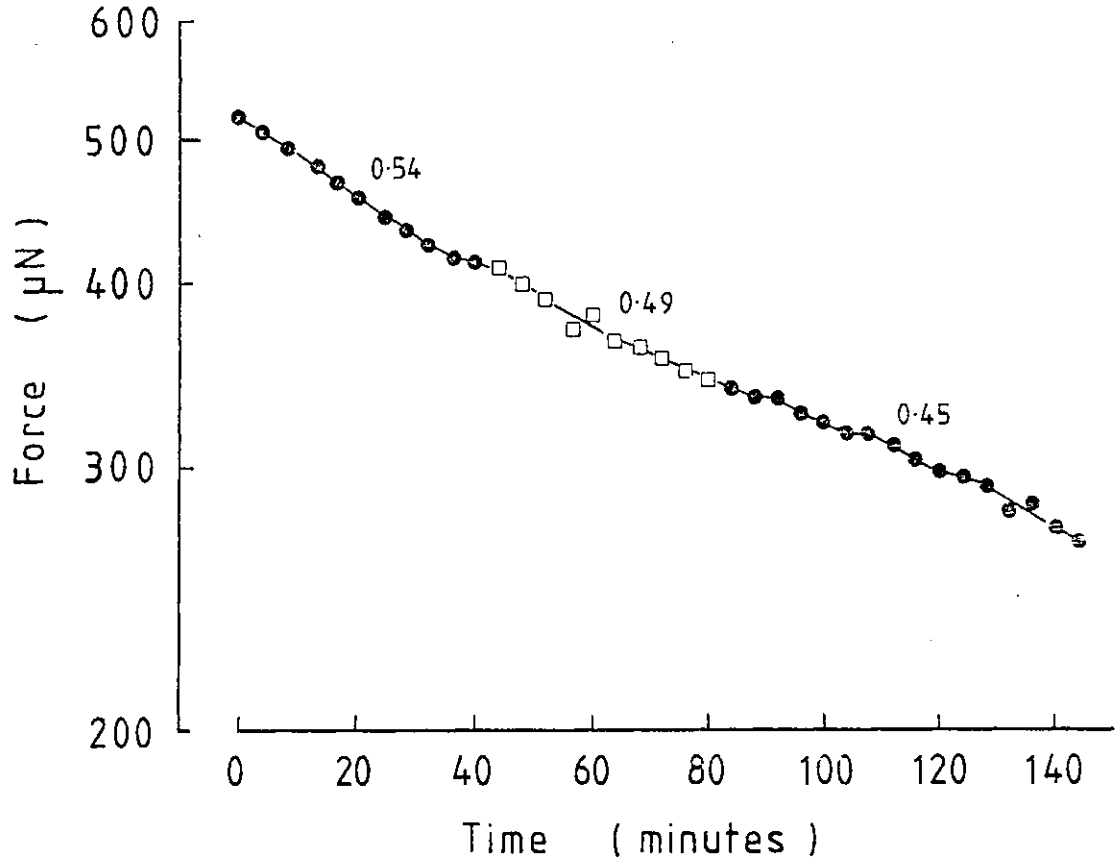


Figure 69. Force decline in the absence (●) and presence (□) of 25 μg cm^{-3} creatine kinase. pH 7.00, pCa 3.75, 25°C.

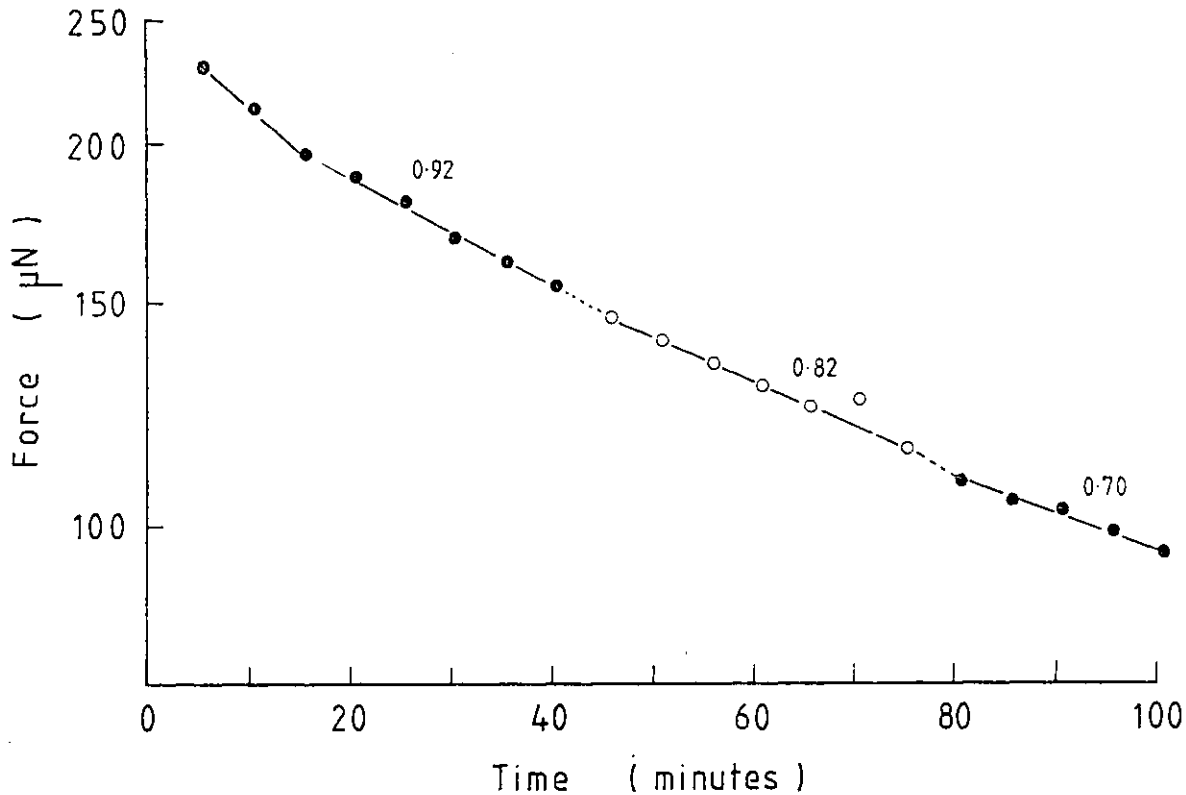


Figure 70. Force decline in the absence (●) and presence (○) of cAMP + cAMP-dependent protein kinase. pH 7.00, pCa 3.75, 25°C.

Fortunately the decline in force did not alter the relative force - pCa relationship. Thus justifiable comparisons could be made between relative force - pCa relationships that had been determined at different times during the same experiment, even if the absolute forces were different. This invariance of Ca^{2+}-sensitivity has also been noted in mechanically-skinned skeletal muscle from frog (Moisescu, 1976).

Can we say anything about the mechanism responsible for the decline in contractility? It is likely that disruption of sarcomere structure was involved at some stage in the process. There was some heterogeneity in the lengths of sarcomeres and half-sarcomeres in muscles that had undergone many contractures (Figures 44 and 61). Almost certainly, this heterogeneity would have been exacerbated during a Ca^{2+}-regulated contracture and may well have been responsible for some of the loss of contractility. Similarly, in mechanically-skinned skeletal muscle a progressive loss of the striation pattern is associated with the decline of Ca^{2+}-regulated force (Hellam & Podolsky, 1969; Endo 1972). However although a disruption of sarcomere structure may have been the final, direct cause of the decline of Ca^{2+}-regulated force, this does not answer the question of what was the primary biochemical lesion that was responsible for the decline.

In these experiments dithiothreitol had no effect on the rate of decline (Figure 65) and so it is unlikely that oxidation of functionally-important sulphydryl groups on the myofibrils was responsible.

However in later experiments done at Leeds University I found that dithiothreitol did slow the rate of force decline and even restored much of the lost contractility in muscles that had suffered a considerable decline in contractile performance (Jewell & Kentish, 1981). The reason for the different results in the two laboratories is currently under investigation.

It also seems unlikely that phosphorylation or dephosphorylation of the myofibrils caused the decline, since incubation in cAMP plus cAMP-dependent protein kinase was without effect, although I did not carry out biochemical tests to check that the myofibrils had indeed been phosphorylated by this procedure. However the fact that the relative force-pCa relationship did not alter as the muscle deteriorated is good indirect evidence against the decline being due to phosphorylation of dephosphorylation, since the Ca^{2+}-sensitivity varies with the phosphorylation state of the myofibrils (Section 1.5(b)).

Experiments in which the frequency of contractures was altered showed that the decline was time-dependent and was also sometimes contracture-dependent. This suggests that the loss of contractile performance resulted predominantly from a process that continued unabated whether the muscles were relaxed or contracted. For exampled there could have been inactivation of an enzyme (though not by sulphydryl oxidation - see above) or the washout of a protein involved in the Ca^{2+}-regulation of force development.

The decline of force was found to be very sensitive to temperature (Figure 64). At 37°C the decline was extremely fast and for this reason it was impossible to carry out the experiments on skinned muscle at the same temperature as in the experiments on isolated myofibrils (37°C). The mechanism of this extreme temperature-dependence was not clear. Because the values for Q_{10} were high (>3), an enzymic reaction would seem to be implicated. Whatever the reason for the temperature-dependence, my results indicated that lowering the temperature greatly improved the viability of the skinned muscles. Once this had become clear, all experiments were performed at room temperature rather than at 30°C.

An increase in the supply of CP or MgATP to the myofibrils slowed the decline of force (Figures 66 and 67). This beneficial effect seemed to be near-maximal at a [total ∿ P] of 10 mM - 15 mM. Since MgATP and not CP is the immediate source for the chemical energy used in the contraction of cardiac muscle (Katz, 1970), the beneficial effect of CP must have been due to its action to maintain the [MgATP] in the immediate vicinity of the myofibril and thus to counter any depletion of MgATP that could occur during ATP hydrolysis. This explains why MgATP and CP had equal effects on the rate of force decline (Figure 68). Because rigor contractures declined only slowly, it could be that a little ATP was needed for the decline to be maximal, although I cannot exclude the possibility that Ca^{2+} itself was the important factor, since Ca^{2+} was present during the Ca^{2+}-activated contractures but not during the rigor contractures.

One way to assimilate the effects of temperature and [∿ P] is by assuming that the decline of force resulted from a lack of MgATP in the core of the actively-contracting skinned muscles. This would be exacerbated (i) at lower concentrations of MgATP or CP and (ii) at higher temperatures, because the ATP hydrolysis is a reaction of high Q_{10} whereas the supply of ATP by diffusion from the external

solution is a process of low Q_{10}. However on this basis one would have expected the decline to be contracture - rather than time-dependent but in fact the converse was generally seen (Figure 62).

The fact that the rate of decline was sensitive to the supply of MgATP allowed me to test whether I needed to add creatine kinase to the bathing solutions or whether the creatine kinase present in the skinned muscles (Section 10.5) was sufficiently active to form an adequate ATP-regenerating system in combination with CP. Omission of the creatine kinase from the bathing solutions did not alter the rate of decline of force (Figure 69). If the supply of ATP to the myofibrils had been compromised by this procedure the decline of force would have accelerated (viz. Figure 67). Thus I concluded that the endogenous creatine kinase in the skinned muscles was active enough to provide an adequate ATP-buffering system. After these experiments, creatine kinase was not added to the bathing solutions.

In conclusion, the results from these experiments suggested two ways to maximise the viability of the detergent-skinned cardiac muscles: keep the temperature as low as possible and include at least 10 mM of a high-energy phosphate compound in the bathing solutions.

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Effect of pH on the Ca^{2+}-dependent ATPase of rabbit cardiac and white skeletal myofibrils

By J. Kentish and Winifred G. Nayler. *Cardiothoracic Institute, London University, 2 Beaumont Street, London, W1N 2DX*

When cardiac muscle becomes ischaemic, developed tension declines rapidly. The decline is accompanied by an intracellular acidosis which is known to depress myocardial function. Katz & Hecht (1969) suggested that the fall in developed tension results from a competition between H^+ and Ca^{2+} for binding sites on the troponin complex. Alternatively an acidosis may inhibit Ca^{2+} influx or directly affect myofibrillar ATPase. In contrast to cardiac muscle, the mechanical performance of skeletal muscle is relatively unaffected by an intracellular acidosis. To investigate the basis of this difference we have studied the effect of pH on the Ca^{2+}-activation curves of cardiac and skeletal muscle myofibrillar ATPases.

Myofibrils were isolated from rabbit ventricular and gastrocnemius muscle by homogenization and differential centrifugation. Washes with Triton X-100 were included to remove contaminant membranes, and the homogeneity of the preparations was checked by electron microscopy. Myofibrillar ATPase activity was determined at $37{\cdot}0 \pm 0{\cdot}1°$ C, in a solution containing 70 mM-KCl, 20 mM imidazole buffer (pH 7·20, 7·00, 6·80 or 6·40), 5 mM-NaN_3, 5 mM-Na_2ATP, 6 mM-$MgCl_2$, 1 mM EGTA and $CaCl_2$ to provide the required concentration of free Ca^{2+}.

Estimations of the free Ca^{2+} took account of the variation of the Ca^{2+}-affinity of EGTA with pH.

The cardiac and skeletal muscle myofibrillar ATPases were both activated over the pCa ranges 7·4 to 5·0. The pCa at 50 % activation was 6·2 at pH 7·0 for both types of myofibril. Decreasing the pH over the range 7·2 to 6·4 displaced the Ca-activation curves to the right (to higher Ca^{2+} concentrations), for skeletal and cardiac preparations. This apparent decline in the Ca^{2+}-sensitivity confirms the results of Schädler (1967), but unlike Schädler we find a reduction in pH only slightly lowers the maximal ATPase activity at saturating concentrations of Ca^{2+}.

These results show that acidosis depresses the Ca^{2+}-sensitivity of the myofibrillar ATPase in both cardiac and white skeletal muscle. The different mechanical response of the two tissues to acidosis could be explained if during acidosis skeletal muscle is maximally activated whilst cardiac muscle is on the steep slope of the Ca^{2+}-activation curve.

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[P.T.O.

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Ca^{2+}-dependent tension generation in chemically 'skinned' cardiac trabeculae: effect of pH

By J. C. Kentish and W. G. Nayler. *Department of Cardiac Medicine, Cardiothoracic Institute, University of London, 2 Beaumont Street, London, W1N 2DX*

We have previously reported (Kentish & Nayler, 1977) that the Ca^{2+}-activated ATPase of guinea-pig cardiac myofibrils is inhibited when the pH is reduced over the range 7·2–6·4. To investigate whether the tension developed by the myofibrils is similarly depressed when $[H^+]$ is increased, we have studied the effect of pH on the Ca^{2+}-dependent tension development of 'skinned' cardiac cells – cells in which the sarcolemma has been rendered permeable to externally applied ions. Disruption of the cell membranes was effected chemically by using a Triton-based procedure.

Guinea-pig right ventricular trabeculae (100–200 μm diam.) were mounted in a muscle bath and bathed in a modified Krebs–Henseleit solution containing: 118 mM-NaCl, 4·7 mM-KCl, 1·2 mM-KH_2PO_4, 1·2 mM-$MgSO_4$, 25 mM-$NaHCO_3$, 1·8 mM-$CaCl_2$, 5·0 mM-glucose, 3·0 mM-Na_2 pyruvate. The solution, equilibrated with a 95% O_2, 5% CO_2 gas mixture, had a pH of $\simeq$ 7·4 at room temperature (20 °C). The muscles were stimulated at 1 Hz and stretched until tension development was maximal. Once the muscles had equilibrated, the sarcolemmae and intracellular membranes (mitochondrial, sarcoplasmic reticular and nuclear) were disrupted by bathing the muscles for 30 min at 20 °C in a 'skinning solution', which contained: 100 mM-KCl; 20 mM-imidazole (pH 7·0); 10 mM-K_2EGTA; 5 mM-Na_2ATP; 10 mM-Na_2 creatine phosphate; 25 μg/ml. creatine kinase; 5 mM-glucose; 3 mM-Na_2 pyruvate; 1% (v/v) Triton X-100. The skinning solution was replaced by 'relaxation solution', which had a similar composition to the skinning solution except that it lacked glucose, Na_2 pyruvate and Triton X-100. This, and all subsequent solutions, were maintained at 30·0 $\pm$ 0·1 °C. Exposing the muscles to 'activating solutions' (composition as for relaxation solution, but with 2·5–10·0 mM total calcium) containing $10^{-7.0}$–$10^{-4.5}$ M-Ca^{2+} produced graded, maintained contractures. The tension developed during these Ca^{2+}-dependent contractures ('contracture tension') was recorded at four pCa's: 7·2, 7·0, 6·8 and 6·4.

Reducing the pH over this range produced a sevenfold decrease in the Ca^{2+} sensitivity of contracture tension. Maximum tension, relative to that developed at pCa 4·6, pH 7·0 (100%), was 118 $\pm$ 3% at pH 7·2, 85 $\pm$ 2% at pH 6·8, and 66 $\pm$ 2% at pH 6·4 (means $\pm$ S.E.M., n = 6). The depressant effect of increased $[H^+]$ on the Ca^{2+}-sensitivity of tension generation is similar in magnitude to the previously-reported effect of an identical increase in $[H^+]$ on the Ca^{2+} sensitivity of the myofibrillar ATPase activity; in contrast, the decrease in maximum contracture tension is approximately five times greater than the reduction in maximum ATPase activity.

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The Influence of pH on the Ca^{2+}-regulated ATPase of Cardiac and White Skeletal Myofibrils

J. C. KENTISH* AND W. G. NAYLER

Cardiothoracic Institute, 2 Beaumont Street, London W1N 2DX, England

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J. C. KENTISH AND W. G. NAYLER. The Influence of pH on the Ca^{2+}-regulated ATPase of Cardiac and White Skeletal Myofibrils. *Journal of Molecular and Cellular Cardiology* (1979) **11**, 611–617. An intracellular acidosis depresses the twitch tension of cardiac muscle but in general increases that of skeletal muscle. To determine whether these contrasting effects might be due to a difference in the pH-sensitivities of the Ca^{2+}-activation of the myofibrils, we assayed the ATPase activity of rabbit and guinea-pig cardiac, and rabbit white skeletal, myofibrils at 37°C in solutions of different pCa (8.0 to 4.3) and pH (7.20, 7.00, 6.80, 6.40). Care was taken to free the cardiac myofibrils of contaminant membrane ATPases. Reducing the pH from 7.20 to 6.40 decreased the maximum Ca^{2+}-regulated portion of the cardiac myofibrillar ATPase by 20% but had no significant effect on that of the skeletal myofibrillar ATPase. This reduction of pH also produced a five-fold increase in the $[Ca^{2+}]$ required for half-maximal cardiac ATPase activity at each pH; the corresponding $[Ca^{2+}]$ for the skeletal myofibrillar ATPase was increased four-fold. These slight differences in the pH-sensitivities of cardiac and skeletal myofibrillar ATPases probably do not account for more than a small part of the contrasting effects of acidosis on the two types of muscle.

KEY WORDS: pH; Myofibrillar ATPase; Ca^{2+}-activated; Acidosis; Cardiac; Skeletal.

1. Introduction

The abrupt decline in contractile performance seen when an intracellular acidosis is produced by hypercarbia in mamalian cardiac muscle [e.g. *10*, *15*] contrasts with the small, potentiating effect of a similar acidosis on the twitch tension of skeletal muscle [*7*]. Katz and Hecht [*4*] suggested that the cardiodepressant action of an acidosis could be explained if protons compete with cytosolic Ca^{2+} for the Ca^{2+}-binding sites on cardiac troponin. However, a similar effect might be expected to occur in skeletal muscle. To aid our understanding of the actions of an acidosis on cardiac and skeletal muscle, we need to compare the pH-sensitivities of the Ca^{2+}-activation of purified cardiac and skeletal myofibrils. Unfortunately, previous studies on the effect of pH on cardiac myofibrillar ATPase [*9*, *14*] have used myofibrillar preparations that almost certainly contained contaminant membrane ATPases, including the sarcoplasmic reticulum (SR) Ca^{2+}-regulated ATPase. In

* To whom reprint requests should be addressed.

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such studies it is imperative to exclude all SR ATPase activity, since this is Ca^{2+}-activated over the same pCa (negative $\log_{10}$ of the free Ca^{2+} concentration) range (7 to 5) as the myofibrillar ATPase and its activity, at least in skeletal muscle, is reduced by a decrease in pH [*13*]. Fortunately cardiac myofibrils can be freed of extraneous membranes by washes in the non-ionic detergent Triton X-100 [*12*]. We have therefore measured the ATPase activities of Triton X-100-treated cardiac and skeletal myofibrils from the same species (rabbit) under identical assay conditions, so as to directly compare their pH-sensitivities.

2. Materials and Methods

Myofibrils were extracted at 4°C from rabbit ventricular and white skeletal (gastrocnemius) muscle by homogenization in a "relaxing buffer" [70 mM KCl, 20 mM imidazole, 0.1 mM dithiothreitol, 2 mM $MgCl_2$, 2 mM K_2 ethyleneglycol-bis (β-aminoethylether) N, N′ tetraacetic acid (EGTA), pH 7.0 at 4°C] followed by differential centrifugation. The residual membranes that usually contaminate cardiac myofibrillar preparations were removed by two washes in relaxing buffer containing 1% (v/v) Triton X–100 [*12*]. Skeletal myofibrils were treated in the same way. Three further washes in relaxing buffer were given to remove the Triton X-100 from the myofibrillar preparations.

Myofibrillar ATPase activity was determined by measuring the liberation of inorganic phosphate (P_i) from ATP at 37.0±0.1°C in solutions containing: 80 mM KCl; 20 mM imidazole buffer; 5 mM NaN_3; 5 mM Na_2ATP; 6 mM $MgCl_2$ ($[Mg^{2+}]$= 1.4 mM); 1 mM K_2 EGTA; 0.1 to 1.1 mM $CaCl_2$ ($[Ca^{2+}] = 10^{-8.0}$ M to $10^{-4.3}$ M); 100 to 200 μg/ml myofibrils. The pH was adjusted to 7.20, 7.00, 6.80 or 6.40 with HCl. The reaction was started by adding the Na_2 ATP and was terminated 5 min later by adding ice-cold 22% trichloroacetic acid. Standard methods were used to determine the final concentrations of P_i [*3*] and protein [*6*].

An iterative computer program calculated the pCa values of the incubation media from the total concentrations of solutes and the association constants for the chemical equilibria.

Appropriate logarithmic association constants were [*11*]:

imidazole/H^+, 6.80; $EGTA^{4-}/H^+$, 9.54; $HEGTA^{3-}/H^+$, 8.93;
H_2EGTA^{2-}/H^+, 2.73; H_3EGTA^-/H^+, 2.08; $EGTA^{4-}/K^+$, 0.96;
$EGTA^{4-}/Na^+$, 1.38; $EGTA^{4-}/Ca^{2+}$, 11.00; $HEGTA^{3-}/Ca^{2+}$, 5.33;
$EGTA^{4-}/Mg^{2+}$, 5.20; $HEGTA^{3-}/Mg^{2+}$, 3.40; ATP^{4-}/H^+, 6.52;
$HATP^{3-}/H^+$, 3.87; ATP^{4-}/K^+, 0.90; ATP^{4-}/Na^+, 1.20;
ATP^{4-}/Ca^{2+}, 3.94; $HATP^{3-}/Ca^{2+}$, 2.13; ATP^{4-}/Mg^{2+}, 4.28;
$HATP^{3-}/Mg^{2+}$, 2.29; N_3^-/Na^+, 4.58.

The calculated ionic strength in all incubation solutions was 0.12 M.

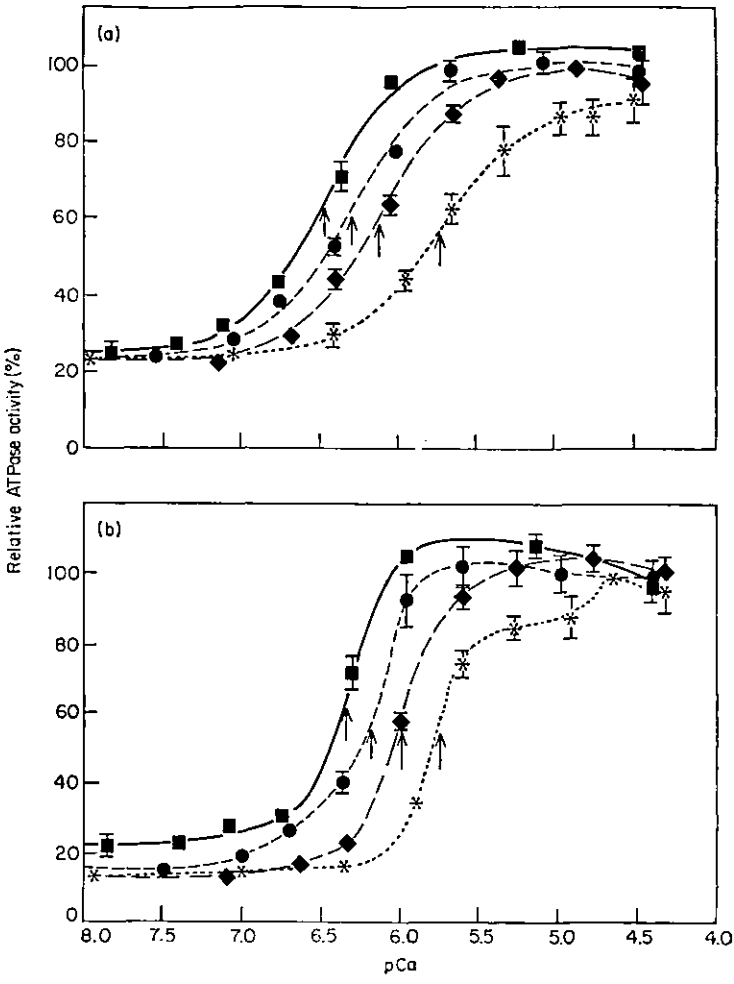


FIGURE 1. Effect of pH on the Ca^{2+} activation of myofibrillar ATPases from cardiac and skeletal muscle: (a) rabbit ventricle; (b) rabbit white skeletal (gastrocnemius) muscle. ATPase activities expressed as a percentage of the maximum ATPase activity at pH 7.0 of the appropriate type of myofibril in each experiment. Each point shows the mean percentage $\pm$ S.E.M. ($n = 4$). Absence of error bars indicates S.E.M. < size of symbol. ATPase assayed at 37°C in solutions of pH 7.20 (■), 7.00 (●), 6.80 (◆) or 6.40 (*) containing (mM): KCl, 80; imidazole, 20; NaN_3, 5; Na_2ATP, 5; $MgCl_2$, 6; K_2EGTA, 1; $CaCl_2$, 0 to 1.1. Arrows indicate the points of half-maximal Ca^{2+}-activated ATPase activity for each pH.

3. Results and Discussion

Figure 1 shows the pCa–ATPase relationships for rabbit cardiac and skeletal myofibrils at pH 7.20, 7.00, 6.80 and 6.40. To facilitate comparison between the cardiac and skeletal results we normalized the data by expressing the ATPase activities in each experiment as a percentage of the maximum total ATPase activity of the same myofibrils at pH 7.0. Each point in Figure 1 is the mean percentage, $\pm$ S.E.M., from four different preparations.

The "basal" cardiac ATPase activity (at pCa$>$7.5) at pH 7.00 was 22% of the total activity at that pH. This percentage is a little greater than the 12% reported by Solaro *et al.* [*12*] but is considerably less than the 50% found in the previous studies of pH on cardiac myofibrils [*9*, *14*]. The basal ATPase activity of our purified cardiac myofibrils was not inhibited by 5 mM NaN_3, 1 mM quinidine or 10^{-6} M ouabain—inhibitors of mitochondrial Mg^{2+}-ATPase, SR Ca^{2+}-ATPase and sarcolemmal ($Na^+ + K^+$)-ATPase, respectively (results not shown). If Triton X-100 treatment was omitted from the myofibril extraction procedure, basal ATPase activity constituted 50% of the total activity, and could be partially inhibited by 5 mM NaN_3 or 1 mM quinidine, although 10^{-6} M ouabain had no effect. We therefore believe that the contribution of membrane ATPases to the ATPase activity exhibited by our Triton-treated cardiac myofibril preparations was negligible under our assay conditions, although this may not have been so in the earlier cardiac studies of Williams *et al.* [*14*] and Schädler [*9*].

In our experiments the basal ATPase activity of cardiac myofibrils was unaffected by pH over the range pH 7.20 to 6.40. This contrasts with the observation of Williams *et al.* showing that the curve relating the ATPase activity of canine cardiac myofibrils in the absence of Ca^{2+} to pH is bell-shaped with a peak at pH 8.0 (Figure 1 in ref. [*14*]). It seemed possible that this discrepancy might arise from the use of different species. We therefore repeated our experiments using guinea-pig cardiac myofibrils, lest rabbit myofibrils be atypical in respect of their pH-sensitivity. However, the same results were obtained with guinea-pig cardiac myofibrils as with those from rabbit (results not shown). The pH-insensitivity of the basal ATPase is therefore not restricted to one species. The basal ATPase activity of the skeletal myofibrils did however appear to be slightly influenced by pH, an increased basal activity at pH 7.2 being a consistent finding.

The maximum absolute values of ATPase activity were (in nmol P_i liberated/min/mg protein): rabbit cardiac, 199 ± 15; guinea-pig cardiac, 205 ± 16; rabbit white skeletal, 480 ± 71 (means $\pm$ S.E., $n = 4$). Decreasing the pH from 7.20 to 6.40 lowered the maximum Ca^{2+}-activated portion of the rabbit cardiac ATPase activity by 20% (Figure 1). There was, however, no obvious alteration in the maximum Ca^{2+}-activated part of the skeletal activity over this pH range.

The most dramatic effect of the increase in $[H^+]$ was to shift the pCa–ATPase relationships for both cardiac and skeletal myofibrils to lower pCa values i.e. to

TABLE 1. pCa values at half-maximal activation* of the myofibrillar ATPase

Type of myofibril	pH (±0.01) 7.20	7.00	6.80	6.40
Rabbit cardiac	6.39	6.18	6.05	5.67
Guinea-pig cardiac	6.35	6.13	6.05	5.63
Rabbit white skeletal	6.33	6.17	5.97	5.73

* "Half-maximal activation" at each pH taken as the point where the ATPase activity = $\frac{V_{max} + V_{basal}}{2}$

where V_{max} = maximum ATPase activity *at that pH*
and V_{basal} = minimum ATPase activity *at that pH*.
The pCa values were read from the pCa–ATPase graphs (Figure 1).

higher Ca^{2+} concentrations. This effect has been observed in earlier studies on cardiac [*9*, *14*] and skeletal [*8*] myofibrillar ATPase. To quantify these shifts we measured from the graphs the pCa value at each pH at which the Ca^{2+}-activated portion of the ATPase had an activity half that of the maximum Ca^{2+}-activated activity *at that pH* (Table 1). Results from the guinea-pig experiments are included.

It can be seen that for both cardiac and white skeletal myofibrils a decrease in pH of 0.2 pH units lowered the pCa for half-maximal activation by about 0.2 pCa units. The pCa values for threshold and maximal activation of the ATPase were also shifted by about 0.2 pCa units per 0.2 pH unit, so that the Ca^{2+}-activation curves at the different pH values were "parallel" (Figure 1). Thus, reducing the pH from 7.20 to 6.40 resulted in a four-fold increase in the $[Ca^{2+}]$ required for 50% activation of the skeletal ATPase and a five-fold increase in that required for 50% activation of the cardiac ATPase. This similarity in the pH-sensitivities of rabbit cardiac and skeletal myofibrillar ATPases contrasts with Schädler's finding that the ATPase of skeletal myofibrils is much less sensitive than that of cardiac myofibrils to a change in pH.

During the preparation of this paper two studies were published in which the pH-sensitivities of tension development in mechanically-skinned cardiac and skeletal fibres were compared. Fabiato and Fabiato [*2*] reported that a decrease in pH from 7.40 to 6.20 led to a five-fold increase in the $[Ca^{2+}]$ required for 50% maximum tension development in rat cardiac myofibrils, but only a three-fold increase in the corresponding $[Ca^{2+}]$ for frog skeletal myofibrils. In a study that is directly comparable with ours, because both the cardiac and skeletal fibres were taken from the rabbit, Donaldson and Hermansen [*1*] found that a reduction in pH from 7.0 to 6.5 increased the $[Ca^{2+}]$ for 50% tension by a factor of 2.2 for cardiac fibres and 1.4 for skeletal fibres (at $[Mg^{2+}] = 1$ mM). The results from our study of the enzymic activity of myofibrils concur with the results from these investigations of the mechanical properties; thus there is general agreement that the effect of a raised $[H^+]$ on the $[Ca^{2+}]$ required for half-maximal activation is somewhat larger

in cardiac than in skeletal myofibrils. However the pH-sensitivities of both types of myofibril appear greater in our studies on ATPase than in the above studies on tension. The reason for this discrepancy is currently being investigated.

Our data and those in refs [*1*] and [*2*] also indicate that decreases in pH lower the maximum contractile activity of cardiac and skeletal myofibrils by small and comparable amounts.

The slightly different pH-sensitivities of cardiac and skeletal myofibrils discussed above cannot account for more than a small part of the contrasting mechanical responses of intact cardiac and skeletal muscle fibres to an intracellular acidosis. Other explanations are therefore required. The marked negative inotropic effect of an acidosis in cardiac muscle is not surprising because the systolic concentration of cytosolic Ca^{2+} is probably insufficient to fully activate the cardiac myofibrils, and it is at such Ca^{2+} concentrations that the direct, deleterious effect of acidity on the ATPase activity is the greatest (Figure 1). Although a similar situation may exist in single skeletal muscle twitches, a possible compensatory mechanism has been discovered: Fabiato and Fabiato [*2*] found that at pCa values below 7.75 the loading of SR in skinned skeletal fibres has a pH optimum which is more acidic (e.g. pH 6.6 to 6.2 at pCa 6.00) than is the pH optimum for loading of cardiac SR. They therefore suggest that acidity could lead to an increase in Ca^{2+} release from skeletal SR, whereas it would decrease Ca^{2+} release from cardiac SR. Provided that the peak cytosolic $[Ca^{2+}]$ in a skeletal muscle twitch is submaximal, this phenomenon can account for the finding that the skeletal twitch may increase under conditions of a hypercarbia-induced intracellular acidosis [*7*].

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