# CALCIUM TRANSPORT BY SARCOPLASMIC RETICULUM

by

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ABSTRACT

Microsomal fractions rich in sarcoplasmic reticulum (SR) were isolated from guinea pig ventricular muscle and used to measure rates of unidirectional calcium efflux. Calcium efflux rate increased with increasing free calcium concentration in the medium. Calcium efflux rate was inhibited by magnesium, but was not affected by verapamil, ouabain, or diltiazem. Increasing the pH from 6.4 to 7.2 increased the amount of calcium initially taken up by cardiac SR in the presence of oxalate, but at pH 7.2 uptake was followed by the early onset of a relatively rapid phase of net calcium release. Increasing the pH from 6.4 to 7.2 also increased the rate of unidirectional calcium efflux.

In both cardiac and skeletal SR, the amount of calcium bound in the absence of precipitating ions was greater at pH 6.5 than at pH 7.6. In skeletal SR, increasing the pH to 7.6 following calcium binding at pH 6.5 led to an immediate rapid release of accumulated calcium. In cardiac SR, a sudden increase in pH before calcium binding was complete, caused the slow release of a small amount of calcium. A sudden increase in pH after the completion of calcium binding had no effect on the calcium content of cardiac SR.

These results are discussed in relation to the possible in vivo trigger for calcium release from the SR.

Attempts were made to purify the calcium activated ATPase enzyme from rabbit or guinea pig cardiac and skeletal SR. The ATPase was solubilised by treatment with deoxycholate, Triton X100, or digitonin, and recovered by dilution, or by passage through a Sepharose 4B column. Using skeletal SR, a 3- to 4-fold increase in the specific activity of the enzyme was obtained, and the purified protein appeared as one band after polyacrylamide gel electrophoresis. The cardiac enzyme was more susceptible to inactivation by the detergents, and although some increase in specific activity was achieved, the high basal ATPase activity observed in intact cardiac SR persisted after purification.

## ABBREVIATIONS

SR - sarcoplasmic reticulum

ATP - adenosine triphosphate

ITP - inosine triphosphate

GTP - guanosine triphosphate

ADP - adenosine diphosphate

T.C.A.- trichloracetic acid

EGTA - ethyleneglycol-bis(β-amino ethyl ether)NNN'N'-tetra acetic acid

EDTA - diaminoethanetetra-acetic acid

TRIS - tris-(hydroxymethyl)aminomethane

SDS - sodium dodecyl sulphate

ATPase- adenosine triphosphatase

EPR - electron paramagnetic resonance

ESR - electron spin resonance

S.E.M.- standard error of the mean

$P_i$ - inorganic phosphate

$Ca_o$ - free calcium concentration outside the sarcoplasmic reticulum

$Ca_i$ - free calcium concentration inside the sarcoplasmic reticulum

μ - micron

Å - Angstrom

μm - micrometer

nm - nanometer

cm - centimeter

μg - microgram

mg - milligram

gm - gram

Kg - kilogram

nM - nanomolar

μM - micromolar

mM - millimolar

M - molar

Abbreviations (continued).....

nmole - nanomole

µmole - micromole

µℓ - microlitre

ml - millilitre

C - centigrade

w/w - weight by weight

v/v - volume by volume

Hepes - 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid

Tes - N-tris(hydroxymethyl)methyl-2-aminomethane sulfonic acid

EP - phosphoprotein intermediate

$K_m$ - Michaelis constant

# CHAPTER I

# GENERAL INTRODUCTION

SECTION I

Structure of the Sarcoplasmic Reticulum

(a) Structure of sarcoplasmic reticulum in intact muscle

The sarcoplasmic reticulum of skeletal muscle consists of a plexus of smooth-faced tubules applied to the surface of the myofibrils. The tubules overlying the A band of each sarcomere are longitudinal in orientation, communicating laterally with each other in the region of the H band. At the end of each sarcomere longitudinal meshes are confluent with dilated transverse branches, the terminal cisternae. The terminal cisternae of successive segmental units of the reticulum are situated on either side of the Z band and are separated by a row of small vesicles. The complex of the terminal cisternae and the intermediate vesicles is called a triad (Fawcett, 1961; Porter and Palade, 1957). In cardiac muscle the triads contain a single, relatively large, intermediary vesicle with a less constant relationship to one or both of the associated terminal cisternae (Porter and Palade, 1957). These triads are thought to be the structures responsible for the inward spread of excitation (Huxley and Taylor, 1958).

The sacroplasmic reticulum of cardiac muscle is less extensive than that of skeletal muscle (Fawcett and McNutt, 1969). In skeletal muscle the sarcoplasmic reticulum accounts for 11-12% of the total cell volume, whereas in cardiac muscle it only represents 6-7% of the total cell volume (Nayler, 1975). In cardiac muscle there is no lateral anastomosis of the sarcotubules in the middle of the A band, but the sarcoplasmic reticulum ramifies over the surface of the fibrils and extends into the narrow clefts which subdivide the mass of myofibrils. In the region of the I band, flattened saccular expansions of the sarcotubules are applied locally to the sarcoplasmic surface of the cell

membrane. These specialised dilations of the sarcoplasmic reticulum are known as subsarcolemmal cisternae. They lie parallel to the sarcolemma for a distance of 0.1-1 μ and are separated from it by a space 150-200 Å in diameter (Fawcett and McNutt, 1969). These areas where the sarcoplasmic reticulum membrane approaches that of the sarcolemma are highly specialised and exhibit small extensions known as 'feet'. These specialised areas also occur along the T tubules. The facing membrane of the sarcolemma remains flat, and it is not clear whether the extensions of the sarcoplasmic reticulum actually link with the membrane of the T system or sarcolemma. The space between the two membrane systems is fairly uniform in width and is known as the junctional gap (Nayler, 1975).

(b) Structure of isolated sarcoplasmic reticulum

Electron micrographs obtained by sectioning the pellet of isolated preparations of skeletal SR reveal spherical or oval vesicles (Hasselbach and Elfvin, 1967; Deamer and Baskin, 1969; Yu et al., 1968). The diameter of the vesicles varies between 300-2,200 Å. The vesicular membrane is triple layered with an overall thickness of about 60 Å. It consists of two opaque components separated by a light layer. The membrane appears to be asymmetric, the peripheral opaque layer being denser and thicker than the innermost layer (Hasselbach and Elfvin, 1967). However, negative staining of SR preparations reveals tadpole-shaped particles consisting of a spherical head (800-1,300 Å) and a tail of variable length. The diameter of the tail is 130-310 Å (Ikemoto et al., 1968; Martinosi, 1968; Deamer and Baskin, 1969). The surface of the vesicles is densely covered with spherical particules of approximately 35-40 Å diameter (Martinosi, 1968a;Deamer and Baskin, 1969; Inesi and Asai, 1968; Ikemoto et al., 1968). Freeze etch microscopy reveals the presence of a second type of particule 80-90 Å in diameter on the fractured concave

surfaces of the outer half of the membrane bilayer (Inesi and Scales, 1974). The density of the 90 Å particles is 5700 particles per $\mu m^2$ of membrane surface (Inesi and Scales, 1974; Scales and Inesi, 1976b). The density of the particles as observed by freeze fracture electron microscopy is greater on the outer than on the inner surface of the SR (Jilka et al., 1975; Inesi and Scales, 1974).

The 30-40 Å particles occur at a density of 21,000 per $\mu m^2$. This corresponds closely to the calculated density of ATPase polypeptide chains (Scales and Inesi, 1976 a and b). Since both types of projection are associated with ATPase protein, it was suggested that the ATPase polypeptide chains are amphiphilic molecules with polar ends protruding individually, corresponding to the 35 Å outer projections, and the hydrophobic ends appearing as 90 Å particles on the concave fracture faces. The discrepancy between the density of the outer projections and the 90 Å particles, was suggested to be due to the formation of oligomers containing 3-4 hydrophobic ends (Scales and Inesi, 1976 a and b).

That the intramembranous particles are associated with the ATPase protein is suggested by the parallel development of the particles with that of calcium transport activity and ATPase activity in the SR (Tillack et al., 1974), and by the loss of both calcium transport activity and surface particles during trypsin digestion (Martinosi, 1968; Deamer and Baskin, 1969), although ATPase activity is not impaired, suggesting that the ATPase particles are deeply buried in the membrane. Extraction of the minor protein components of the SR with EDTA leaves the surface particles intact, suggesting that they are an intrinsic part of the ATPase molecule (Inesi and Scales, 1974).

SECTION II

## Historical Review of the Function of the Sarcoplasmic Reticulum

### (a) Skeletal muscle sarcoplasmic reticulum

The presence of a factor in skeletal muscle homogenates necessary for the relaxation of skeletal muscle fibres was first observed by Marsh (1951; 1952) and Bendall (1952 a and b), who termed it the 'relaxing factor'. The activity of the relaxing factor was shown to be dependent on magnesium, and was inhibited by calcium (Bendall, 1953). The effect of the factor could be completely imitated by EDTA (Bozler, 1954; Watanabe, 1955). The nature of the relaxing factor was the subject of controversy for several years, and was variously identified as an unspecified enzymic factor (Goodall and Szent-Gyorgi, 1953), myokinase (Bendall, 1954), pyruvate phosphokinase (Lorand and Moos, 1956) and ATP-creatine transphosphorylase (Lorand, 1953). However, it was later shown that the relaxing factor activity was associated with a particulate fraction isolated by high speed centrifugation (Kumagai et al., 1955; Bendall, 1958; Ebashi, 1958; Molnar and Lorand, 1959). Although it was established that the relaxing activity of muscle homogenates occurred in a relatively small granular fraction, the way in which the factor exerted its activity remained unclear. It was postulated by several authors that the microsomal fraction effected relaxation by producing a soluble relaxing substance (Briggs and Fuchs, 1960; Fuchs and Briggs, 1961; 1962/3; Nagai et al., 1960; Parker and Gergely, 1960; 1961). Relaxing activity was later conclusively demonstrated to be associated with the light granular fraction of the muscle homogenate and its activity was enhanced by 5 mM oxalate (Baird and Perry, 1960; Berne, 1962). It was postulated that the factor had a strong affinity for a trace metal, possibly calcium, which was essential

for the magnesium activated ATPase activity of the myofibrils. Relaxation would be caused by the removal of calcium by the relaxing factor, and stimulation of the muscle would lead to release of calcium from the granules, causing contraction (Baird and Perry, 1960; Ebashi, 1961 a and b). It was also suggested that relaxing factor activity could be associated with the reticular structures, i.e. the sarcoplasmic reticulum, observed under the electron microscope by Porter and Palade (1957), Baird and Perry (1960) and Muscatello et al. (1961). Ebashi and Lippman (1962) showed that preparations of granular relaxing factor consisted mainly of closed fragments of sarcoplasmic reticulum. It was demonstrated that the sarcoplasmic reticulum could accumulate calcium in the presence of ATP, and that calcium accumulation was magnesium dependent (Hasselbach and Makinose, 1961; Ebashi and Lippman, 1962). Calcium accumulation was also stimulated by the presence of oxalate or phosphate in the incubation medium (Hasselbach and Makinose, 1961; 1963). The stimulatory effect of oxalate on calcium accumulation was shown to be due to a reduction of calcium outflux rate, rather than to an increase in calcium influx rate (Martinosi and Feretos, 1964a). During calcium accumulation from oxalate-containing solutions, the product of the active concentration of calcium and oxalate inside the vesicles was assumed to be equal to the solubility product of calcium oxalate (Hasselbach and Makinose, 1963; Hasselbach, 1964a). Granules of calcium oxalate are stored inside the SR (Hasselbach and Makinose, 1961; Martinosi and Feretos, 1964a). Sarcoplasmic reticulum fragments exhibit an ATP, magnesium and calcium dependent transport of oxalate against a concentration gradient. Using $^{14}C$-labelled oxalate, the uptake of 1 mole of calcium was shown to be accompanied by the uptake of 1 mole of oxalate. However, since no oxalate uptake occurs in the absence of

calcium, it was considered unlikely that a specific oxalate transport system exists in the SR (Martinosi and Feretos, 1964a). The influence of oxalate on calcium transport was thought to be due to the reduction of the free calcium concentration inside the SR vesicles (Makinose and Hasselbach, 1965). These authors investigated the effect of the concentration of free calcium inside the SR on calcium transport, and showed that the maximal capacity of the vesicles for calcium decreases with increasing free calcium concentration inside the SR, i.e. at lower concentrations of oxalate and phosphate.

Other nucleotide triphosphates besides ATP also support calcium transport by the SR, but at a lower rate than ATP (Makinose and The, 1965).

Calcium uptake is accompanied by a 7- to 8-fold increase in the rate of calcium-dependent ATPase activity and also by phosphate exchange between ATP and ADP (Hasselbach and Makinose, 1961; 1962; Hasselbach, 1964a). Calcium accumulation, but not ATPase activity, was shown to be inhibited by deoxycholate (Ebashi and Lippman, 1962). Calcium transport and ATPase activity exhibit a similar dependence on the calcium concentration in the medium (Weber et al., 1964; 1966). It was therefore suggested that calcium transport, calcium activated ATPase activity and phosphate exchange are closely linked (Hasselbach and Makinose, 1962).

At medium calcium concentrations above $10^{-7}$ M, the ratio of calcium ions transported to ATP molecules split was found to be two. At calcium concentrations of $10^{-8}$ M this ratio was at first reported to be reduced to one (Hasselbach and Makinose, 1963; Makinose and The, 1965). However, ratios of calcium ions transported to ATP molecules hydrolysed above two have been reported even at low ATP concentrations (Ebashi and Yamanouchi, 1964), and Weber et al. (1966) have found that the ratio remains constant at two under all conditions.

All the above results assume that calcium is transported across the SR membrane, and is stored inside the SR vesicles. An alternative hypothesis suggests the formation of a complex with calcium at the surface of the SR membrane (Ebashi and Endo, 1964). Using the calcium sensitive dye, murexide, and a dual beam spectrophotometer, Ohnishi and Ebashi (1963) showed a rapid ATP and magnesium dependent binding of calcium to SR membranes in the absence of precipitating ions. Haragaya and Schwartz (1969), using the murexide technique, showed that calcium binding in the absence of precipitating ions was followed by spontaneous calcium release (see Chapter 2). In the presence of oxalate a three-phase pattern of calcium uptake is observed using the murexide technique. They consist of an initial rapid phase, thought to correspond to a calcium binding; a lag phase; and a steady phase of calcium accumulation, thought to correspond to transport of calcium across the membrane, and its deposition as calcium oxalate inside the vesicles (Harigaya and Schwartz, 1969; Ogawa, 1970). Using a stopped-flow attachment to the dual beam spectrophotometer, it was possible to measure the initial rate of calcium binding (Ohnishi and Ebashi, 1964; McCollum et al., 1972) and it has been calculated that the initial rate of calcium binding is fast enought to account for relaxation in vivo (Ohnishi and Ebashi, 1964). It is possible that the calcium binding described above represents the first stage of the reaction in which calcium is transported across the SR (see Section III).

(b) Cardiac muscle sarcoplasmic reticulum

Relaxing granules, later shown to be sarcoplasmic reticulum, were also isolated from cardiac muscle (Parker and Berger, 1963; Fanburg et al., 1964; Weber et al., 1963; 1966; Inesi et al., 1964; Carsten, 1964), and they also inhibited synaeresis of cardiac myofibrils, but were less effective than skeletal muscle grana (Inesi et al., 1964;

Fanburg et al., 1964). Cardiac SR was also shown to accumulate calcium in an ATP-dependent fashion, both in the absence (Inesi et al., 1964; Weber et al., 1966) and in the presence (Fanburg et al., 1964; Inesi et al., 1964; Carsten, 1964; Weber et al., 1966) of precipitating ions. In all cases the rate of calcium accumulation by cardiac SR was found to be slower than that of skeletal SR. The calcium binding capacity of cardiac SR was found to be half to a quarter of calcium binding capacity of skeletal SR.

Calcium accumulation by cardiac SR has also been measured using the murexide technique. The pattern of calcium accumulation is similar to that found in skeletal SR, both in the absence and in the presence of oxalate, but the capacity of the cardiac SR preparations was lower than that of skeletal SR (Nayler et al., 1975; Dunnett, 1976; Harigaya and Schwartz, 1969; Entman et al., 1972).

## SECTION III

## Reaction Mechanism of the Calcium Activated ATPase in Skeletal Muscle Sarcoplasmic Reticulum

The reaction mechanism of this enzyme has been most extensively studied in skeletal SR. The main features of the reaction mechanism will therefore first be discussed in relation to skeletal SR, and a comparison will than be made with the mechanism in cardiac SR.

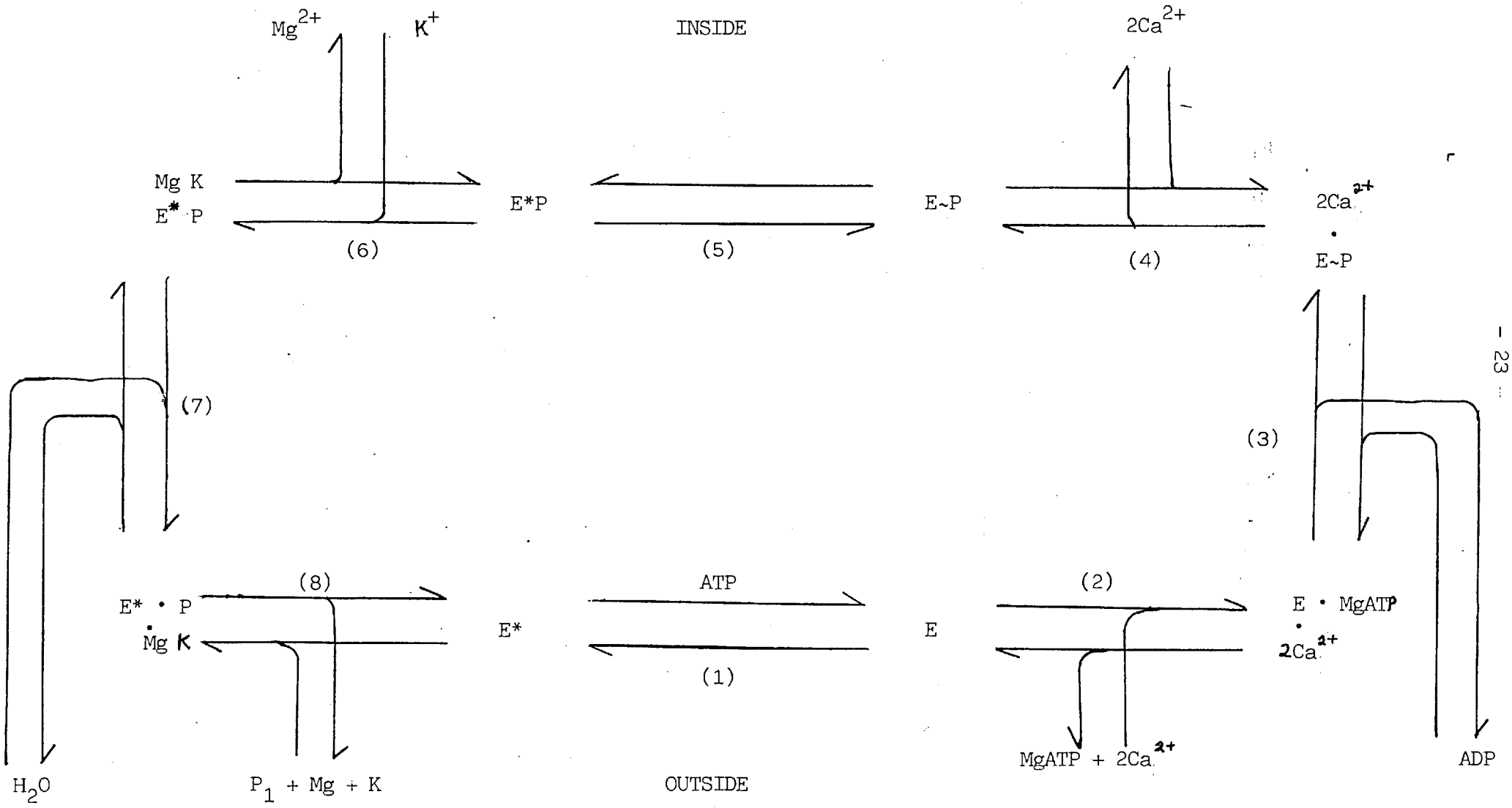
Figure 1 is a schematic diagram representing the main features of the cation transport mediated by the calcium activated ATPase enzyme.

Step 1 in the reaction scheme represents the isomerisation of the enzyme from a form E*, which can be phosphorylated by phosphate, to a form E, which binds calcium with high affinity and can be phosphorylated

by ATP. The isomerisation reaction may be catalysed by relatively high concentrations of ATP or other nucleotide triphosphates. This step is followed by the binding of 1 mole of magnesium ATP and 2 calcium ions to 1 mole of the enzyme E (step 2). Step 3 involves the transfer of the terminal phosphate of ATP to the enzyme to form a phosphoprotein intermediate $E_1 \sim P$, still with 2 calcium ions bound to it. Phosphate is probably bound to the enzyme via a high energy phosphate bond, and ADP is liberated to the outside of the membrane. The rate of the phosphorylation reaction is very rapid and, since it occurs faster than the overall rate of ATP hydrolysis, it cannot be a rate limiting step. A change in the affinity of the phosphoprotein for calcium results in the liberation of two calcium ions to the inside of the SR, leaving the phosphoprotein in a form with a low affinity for calcium, designated $E_2 \sim P$. An acid labile form of phosphoprotein may also be formed during the reaction sequence, designated $E_3 \sim P$ by Ikemoto (1976) or E•P by Froehlich and Taylor (1975; 1976) and this could be identical with the E*P form of phosphoenzyme shown in Fig.1, which has a low affinity for calcium, and a high affinity for magnesium and probably potassium. Binding of magnesium and potassium to E*P (step 6) is followed by phosphoenzyme hydrolysis (step 7) and the liberation of magnesium, potassium and phosphate to the outside of the membrane (step 8). The rate of enzyme dephosphorylation measured in isolation, is similar to the overall rate of ATP hydrolysis, and may thus be the rate limiting step of the reaction (Martinosi et al., 1974).

Under certain conditions, the reaction mechanism of the enzyme can be reversed. The enzyme E* can be phosphorylated by phosphate in the presence of magnesium, with the sequential formation of two or more phosphoproteins, varying in their sensitivity to ADP and potassium.

Figure I Reaction mechanism of the calcium-dependent ATPase in skeletal muscle sarcoplasmic reticulum



In the presence of high internal calcium concentration, ATP synthesis can occur, coupled with the transport of two calcium ions from inside to outside.

(a) Binding of calcium and ATP to the membrane

Using a purified ATPase preparation, Meissner (1973) measured binding of labelled ATP to SR vesicles, and passive binding of labelled calcium in the absence of ATP. Analysis of the results obtained by Scatchard plots gave straight lines for both calcium and ATP, indicating the presence of only one type of binding site for calcium, and one type for ATP. The purified ATPase exhibited a high affinity for both calcium and ATP, but binding of ATP required the presence of magnesium. The number of calcium binding sites was twice the number of ATP binding sites, and it was calculated, from the levels of phosphoenzyme formed, that each active site contained one high affinity ATP binding site and two high affinity calcium binding sites. The existence of two high affinity calcium binding sites on the ATPase molecule is confirmed by electron manganese paramagnetic resonance studies carried out by Kalbitzer et al. (1978), and by studies using the fluorescent calcium indicator, calcein (Chui and Haynes, 1977). The association constant of the high affinity calcium binding sites corresponds to the Km value for calcium of the ATPase and phosphorylation reactions of the isolated SR, suggesting the involvement of these sites in the activation of the ATPase enzyme and calcium transport across the SR (Ikemoto, 1974).

On lowering the temperature to $0^{\circ}C$ the number of high affinity calcium binding sites per ATPase molecule is reduced to one. This is consistent with an observed ratio of calcium transport to ATP hydrolysis of two at $22^{\circ}C$, dropping to one at $0^{\circ}C$ (Ikemoto, 1975).

In addition to the high affinity calcium binding sites located on the ATPase protein itself, the existence in SR vesicles of at least one other type of calcium binding site, with a low affinity, but high capacity for calcium, have been demonstrated (Chevalier and Butow, 1971; Chui and Haynes, 1977). Using stopped-flow kinetic studies, and the calcium sensitive dyes, calcein or arsenazo III, Chui and Haynes (1971) have shown that calcium binding to the high affinity sites is very fast, while the binding reaction to the low affinity sites is comparatively slow. These authors suggest that calcium is bound with high affinity to the ATPase protein at the outside surface of the SR membrane, and following calcium transport across the membrane, is bound to low affinity sites at the inside surface of the SR. These would probably be located on the acidic proteins, calsequestrin and the 54,000 Dalton protein, which are thought to be located on the inside surface of the SR (see Chapter 5). The location of the high affinity binding sites on the outside surface of the SR, and of the low affinity sites on the inside surface is also suggested by the experiments of Myamoto and Kasai (1979), in which calcium bound to the internal surface of the SR was measured after removing calcium bound to the outside surface by washing the vesicles with EGTA. Calcium bound externally was calculated by subtracting the calcium bound at internal sites from the total calcium bound before washing the vesicles.

Binding of calcium to the enzyme may occur by a two step sequence:

$$E^* + Ca^{++} \rightleftharpoons E^*Ca^{++} \rightleftharpoons E{\cdot}Ca$$

The evidence for this is derived from the observation that calcium bound to SR vesicles in the absence of ATP (estimated by phosphoenzyme formation on addition of ATP) was removed only slowly when EGTA was

added to the membrane (Rauch et al., 1978), and that calcium-dependent phosphorylation of the SR membrane is faster when the reaction is started by the addition of ATP to a calcium-containing medium, than when calcium is added to an ATP-containing medium (Rauch et al., 1978; Vieyra et al., 1979). These results have been interpreted to indicate that an initial rapid binding of calcium to the enzyme occurs, to form the first intermediate $E^*Ca^{++}$, followed by a slow isomerisation step in which $E^*Ca^{++}$ is converted to the second intermediate $E \cdot Ca^{++}$, which can react with ATP. The rate of the slow isomerisation reaction was shown to be equal to the overall rate of calcium turnover during calcium uptake and release, and could therefore be the rate limiting step in the overall ATPase reaction (Rauch et al., 1978). An alternative explanation, put forward by Vieyra et al. (1979), suggests that E* and E represent the form of the enzyme reactive with phosphate, E*, and the form of the enzyme reactive with $ATP_1E_1$ respectively. The slow isomerisation step in which E* is converted to E (step 1, Fig.1) would be followed by the rapid binding of calcium to E. Addition of ATP at high concentration accelerates the conversion of E* to E (Vieyra et al., 1979).

(b) Enzyme phosphorylation by ATP

Binding of ATP and calcium to the enzyme is followed by the incorporation of the terminal phosphate of ATP into the enzyme to form a phosphoprotein intermediate, E ~ P. Reversible phosphorylation of an unidentified substance in the membrane was first postulated as a step in the ATPase reaction on the basis that isolated skeletal muscle SR catalyses a rapid exchange of phosphate between ATP and ADP (Ebashi and Lipmann, 1962; Hasselbach and Makinose, 1963; Hasselbach, 1964b; Inesi, 1967; Inesi and Almendares, 1968). This would represent steps 2 and 3 in Figure 1, proceeding in the forward and backward direction.

The maximum rate of ATP-ADP phosphate exchange was 4-5 μmoles of phosphate exchanged/minute/mg protein. Further evidence for the existence of the intermediate came from the observation that $^{32}P$-labelled ATP interacts with the membrane (Inesi and Almendares, 1968), and its existence was confirmed by the observation that $^{32}P$ from $^{32}P$-labelled ATP is incorporated into the SR membrane (Yamamoto and Tonomura, 1967; Makinose, 1969), and by the isolation of a T.C.A. stable phosphoprotein intermediate (Yamamoto and Tonomura, 1967; Martinosi, 1969; Tonomura, 1973). The maximum amount of $^{32}P$ incorporated varied according to the conditions used. In the presence of saturating concentrations of calcium, 6-8 moles of phosphate are incorporated/$10^6$ gram protein (Martinosi, 1967). The rate of formation of phosphoprotein is extremely rapid, steady state being reached within 10 seconds. Calcium is essential for phosphoprotein formation (Makinose, 1969; Martinosi, 1967; Kanazawa et al., 1971; Yamamoto and Tonomura, 1967; Tonomura, 1973). The calcium sensitivity of phosphoprotein formation is similar to that of the ATPase reaction and reaches saturation levels at concentrations of calcium that are inhibitory to the ATPase reaction (Yamamoto and Tonomura, 1967; Makinose, 1969; Inesi and Almendares, 1968; de Meis and de Mello, 1973). The Km for calcium is 2-4 μm (Kanazawa et al., 1971). Double reciprocal plots of the rate of phosphoprotein formation versus the square of the free calcium concentration are linear, indicating that the enzyme binds to two calcium ions (Kanazawa et al., 1971; Tonomura, 1973). Kurzmack et al. (1977) and Verjovski Almeida et al. (1978), using a rapid mixing device and an EGTA quenching medium, demonstrated a burst of calcium uptake within 20 milliseconds of ATP addition. The size of the burst was dependent on the calcium concentration and reached a maximum level of 6-8 nmoles/mg protein. Under identical experimental conditions, 3.5-4 nmoles of phosphoenzyme are

formed/mg protein within 20 milliseconds, indicating a ratio of calcium transport to phosphoenzyme formation of 2 : 1. This is in agreement with the observation that 2 calcium ions are transported across the SR membrane per mole of ATP hydrolysed (Weber et al., 1966). There is no lag phase in phosphoprotein formation, and addition of EGTA to the external medium results in an immediate cessation of phosphoprotein formation. Since the SR membrane is impermeable to EGTA (Weber et al., 1966) it is concluded that the formation of phosphoprotein requires calcium on the outside of the membrane (Makinose, 1969; Kanazawa et al., 1971; Tonomura, 1973). Similarly, addition of large amounts of unlabelled ATP to the reaction medium immediately reduces the rate of incorporation of radioactivity from $^{32}P$, indicating that ATP must also be present at the outside of the membrane in order for phosphoprotein formation to occur (Tonomura, 1973).

(c) Dependence on substrate concentration

Phosphoprotein formation is accelerated by magnesium in the external medium (Yamamoto and Tonomura, 1967; Makinose, 1969; Kanazawa et al., 1971; Tonomura, 1973). Although some phosphoprotein formation can occur in the absence of added magnesium (Kanazawa et al., 1971; Tonomura, 1973), this is probably due to contaminating magnesium in the medium, which could also account for some reports which claim that phosphoprotein formation does not require magnesium (Panet et al., 1971). The requirement for magnesium for phosphoprotein formation led to the suggestion that magnesium ATP is the true substrate for the ATPase reaction, since at a concentration of ATP of 25 µM the calcium dependence of the ATPase is unaffected by increasing the free magnesium concentration from 0.5 to 5 µM (Yamamoto and Tonomura, 1971; Kanazawa et al., 1971), and since magnesium is required for the binding of ATP to the SR

(Meissner, 1973). This is confirmed by kinetic evidence which has shown that a double reciprocal plot of ATPase activity against magnesium ATP concentration at concentrations ranging from 0.2-3 μM gave a straight line, whereas double reciprocal plots of activity versus ATP concentration, when ATP and magnesium were varied in equimolar fashion, were non-linear, due to the decrease in the proportion of nucleotide present as a complex when the total concentration of the components decreases (Vianna, 1975). Inhibition of ATPase activity at high calcium concentrations has been attributed to the formation of the inactive calcium ATP complex (Vianna, 1975). This is supported by the observation that the inhibition of ATPase activity at high calcium concentrations can be overcome by increasing the concentration of ATP (Tonomura, 1973; Kanazawa et al., 1971).

Adenosine triphosphatase activity exhibits a biphasic dependence on magnesium ATP concentration (Yamamoto and Tonomura, 1967; Inesi et al., 1967; Vianna, 1975; Neet and Green, 1977; Moller et al., 1980; Dean and Tanford, 1978; Dupont, 1977; Horgen, 1974). At low magnesium ATP concentrations ATPase activity exhibits a linear dependence on magnesium ATP concentration (as described above), until saturating concentrations of magnesium ATP are reached. However, at high magnesium ATP concentrations a second phase of stimulation of ATPase activity occurs. Although there is variation in the precise concentrations of magnesium ATP at which the two phases of stimulation are reported to occur, it appears from most reports that the first phase of stimulation occurs at magnesium ATP concentrations of up to approximately 25 μM, and that the second phase of stimulation occurs at magnesium ATP concentrations between 100 and 1,000 μM (Yamamoto and Tonomura, 1967).

The Kms for magnesium ATP for the first phase of stimulation of 0.3-10 μM have been reported (Vianna, 1975; Yamamoto and Tonomura, 1967;

Inesi et al., 1967). For the second phase of stimulation the Km appears to be between 18.5 μM (Vianna, 1975) and above 1 mM (Inesi et al., 1967). Both regions of the ATP rate curve respond similarly to variations in pH, temperature, metal ion transported, and the effect of partial inactivation and product inhibition (Taylor and Hattan, 1979).

The complex dependence of enzyme activity on magnesium ATP concentration has led to the suggestion that in addition to acting as the substrate for the ATPase enzyme, magnesium ATP also binds to an effector site on the enzyme molecule, distinct from the active site. This is supported by the identification of two different binding sites for ATP, one being a high affinity site ($K_d$ 2-3 μM) with a high specificity for ATP (Dupont, 1977; Meissner, 1973) and the other a low affinity site ($K_d$ approximately 500 μM), which is relatively non-specific for ATP, being easily displaced by ATP analogues (Dupont, 1977).

Since it is thought that the functional unit of the ATPase enzyme consists of at least two polypeptide chains of molecular weight 100,000 (see Chapter 5) the effector site could be present on the same polypeptide chain as the phosphorylation site, or it could be on a separate polypeptide chain and exert its effect through the dimeric interaction of the two polypeptide chains. Experiments in which these two types of effect can be resolved are those in which vesicular SR preparations are compared with active monomeric ATPase preparations made by delipidation of the SR with non-ionic detergents such as dodecyl octaethylene glycol monoether ($C_{12}E_8$) or Triton X100, at high detergent to protein ratios (Moller et al., 1980; Neet and Green, 1979). Two reports show that monomeric preparations of the

ATPase exhibit a similar biphasic dependence of ATP concentration as the intact SR (Neet and Green, 1977; Taylor and Hattan, 1979). On the other hand, another report shows that, although in detergent-treated preparations double reciprocal plots of activity versus ATP concentration are linear, linear plots were obtained whether the protein is in an aggregated state (low $C_{12}E_8$) concentration, or in a monomeric form (high $C_{12}E_8$ concentration) (Dean and Tanford, 1978). Although these reports are contradictory, they all agree in ruling out protein-protein interaction to explain the biphasic dependence on ATP concentration.

However, evidence that the activating effect of a high ATP concentration may be related to the interaction between two monomers comes from the experiments of Verjovski-Almeida and Inesi (1979). These authors calculated that at saturating ATP concentrations only half the available ATPase polypeptide chains were phosphorylated, and that the saturating concentration of ATP for phosphoenzyme formation is 100 μM. However, the turnover of the enzyme, measured by the velocity of calcium uptake, was doubled when the ATP concentration was raised to the millimolar range, with no increase in the level of phosphoprotein formed. On the basis of these results it was suggested that while the phosphorylated site occurs on one chain of the postulated enzyme dimer, ATP occupancy of an effector site on the other polypeptide chain accelerates the turnover of the phosphorylated site. Additional evidence for the dimeric interaction of ATPase polypeptide chains comes from electron microscopic evidence, and is discussed in Section Ib.

An explanation for these conflicting interpretations may come from the incomplete disaggregation of supposedly monomeric ATPase

preparations (Moller et al., 1980). An alternative explanation is that there are two effector sites, which are themselves activated by different ATP concentrations, although both sites would require an ATP concentration higher than that required by the phosphorylated site. It has been shown that in monomeric ATPase preparations, magnesium ATP exerts an activating effect only at concentrations above 100 μM, suggesting that an effector site exists on the same polypeptide chain as the phosphorylated site. In vesicular preparations, magnesium ATP also exerts an activating effect at concentrations of 2-50 μM, suggesting that another effector site is present on a different polypeptide chain from the phosphorylated site, and exerts its effect by protein-protein interaction between polypeptide chains (Moller et al., 1980).

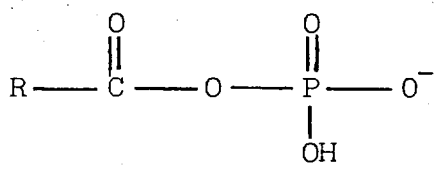
(d) Effect of pH and monovalent cations

The pH optimum for phosphoprotein formation was found to be at pH 8.3 and above (Yamamoto and Tonomura, 1967; Martinosi, 1969). A second pH optimum at pH 4.5 has also been reported (Martinosi, 1969). This contrasts with the pH optimum for the calcium uptake and calcium activated ATPase reactions which exhibit a bell-shaped curve for pH dependence, with an optimum between pH 7 and 8 (Tonomura, 1973). Potassium and other monovalent cations have no effect on the level of phosphoprotein formed (Shigekawa et al., 1976).

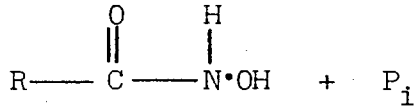
(e) Chemical nature of the phosphoprotein intermediate

Since ATP formation from EP and ADP has been demonstrated (see below), it was postulated that EP must contain a high energy phosphate bond (Tonomura, 1973). The protein component of the sarcoplasmic reticulum which is phosphorylated by ATP has been identified as the

ATPase by polyacrylamide gel electrophoresis (Martinosi, 1969a. The phosphoprotein has been isolated and its properties studied. It has been shown to be stable in acidic media, but unstable at alkaline pH, and in 0.5 M hydroxylamine (Yamamoto and Tonomura, 1968; Tonomura, 1973; Inesi et al., 1970). Since all the above properties are exhibited by acyl phosphate compounds it was suggested that the phosphoprotein is an acyl phosphate, with the following structure:



in which R–C(=O)– represents an amino acid residue in the enzyme. Treatment with hydroxylamine ($NH_2OH$) should result in the formation of hydroxamate, which would be stable:



Although formation of hydroxamate has been demonstrated on treatment of the phosphoprotein with hydroxylamine (Yamamoto et al., 1971), the expected irreversible inhibition of ATPase activity (Inesi et al., 1970) and phosphoprotein formation (Tonomura, 1973) on treatment by hydroxylamine, has not been shown. Similarly, if an acyl phosphate was an intermediate in the reaction chain, hydrazine ($NH_2NH_2$) should inhibit the calcium pump, since, in phosphorylating media at neutral or weakly alkaline pH, hydrazine should split the acyl phosphate bone forming a stable hydrazide. However, hydrazine has no effect on the calcium activated ATPase enzyme (Makinose, 1969). In addition, acyl phosphate

compounds are rapidly decomposed by molybdate (Lipmann and Tuttle, 1944), whereas molybdate does not accelerate phosphate liberation from phosphoenzyme (Makinose, 1969). It therefore remains to be established conclusively that the phosphoprotein is an acyl phosphate compound.

(f) Coupling of calcium transport to the phosphorylation reaction

Formation of the phosphorylated intermediate is accompanied by the transport of the two calcium ions bound to the enzyme from the outside to the inside of the membrane, and by the liberation of ADP. Since the reverse reaction, i.e. the formation of ATP from ADP and magnesium, occurs with no lag phase when ADP is added to the external medium, it is concluded that ADP must be liberated to the outside of the membrane (Kanazawa et al., 1971).

Use of EGTA to quench the phosphorylation reaction in experiments such as those described by Kurznack and Inesi (1977), suggests that calcium must be transported across the membrane during phosphoprotein formation. Direct evidence of a relationship between calcium transport and phosphoprotein formation is provided by the experiments of Sumida and Tonomura (1974), who showed that when calcium uptake was stopped by adding 1 mM EGTA and 1 mM magnesium chloride, the rapid hydrolysis of phosphoprotein to phosphate and enzyme was not accompanied by a concomitant release of calcium ions.

(g) Dephosphorylation of phosphoprotein

Dephosphorylation of the phosphoprotein has been studied after stopping the phosphorylation reaction by adding EGTA to remove external calcium, and measuring the rate of phosphate liberation. Under these conditions the level of phosphoprotein has been shown to decrease exponentially (Yamamoto and Tonomura, 1968; Kanazawa et al., 1971).

Magnesium is required for the dephosphorylation reaction (Inesi et al., 1970; Panet et al., 1971; Kanazawa et al., 1971), and magnesium dependent dephosphorylation is competitively inhibited by calcium. Chelation of external magnesium by the addition of an excess of EDTA led to the cessation of phosphoprotein decomposition only after a lag phase, which could be shortened by treatment of the SR with Triton X100. This result shows that phosphoprotein decomposition requires magnesium at the internal surface of the SR (Kanazawa et al., 1971).

When phosphate production is measured by starting the reaction by addition of calcium and ATP, and without stopping the phosphorylation reaction by adding EGTA, phosphate production exhibits an early time lag, showing that phosphate is liberated after phosphoprotein formation rather than in parallel with it (Froelich and Taylor, 1975). At high ATP concentrations an overshoot of phosphoprotein formation is observed, and a burst of phosphate production occurs over the same time range as the decay of the phosphoprotein overshoot. It was postulated that the burst of phosphate production represents the conversion of E ~ P, the acid stable form of phosphoenzyme to an acid labile form of the phosphoenzyme which would have a low affinity for calcium, and would decompose liberating phosphate and free enzyme (Froelich and Taylor, 1976). An initial burst of phosphate production has also been confirmed by Kanazawa et al. (1971) and by Kurzmack and Inesi (1977), but the interpretation of the result proposed by Froelich and Taylor has been questioned. It has been observed that the burst of phosphate production occurs both in the presence and in the absence of added calcium (Froelich and Taylor, 1976; Kurzmack and Inesi, 1977). This observation, together with the finding that the magnitude of the initial phosphate burst is three times greater than the number of available enzymatic sites, estimated by the

amount of phosphoenzyme formed, led to the suggestion that the proposed acid labile phosphoprotein is formed at sites other than, and independent of, the sites at which the calcium-dependent formation of acid stable phosphoprotein occurs (Kurzmack and Inesi, 1977). These authors did not, however, taken into account the initial burst of phosphoenzyme production when calculating the number of enzymatic sites. Froelich and Taylor (1976) considered that the burst of phosphate production in the absence of calcium and in the presence of a high concentration of ATP, was due to the direct formation of the acid labile phosphoenzyme mediated by the calcium independent ATPase activity present in SR. Since phosphoenzyme levels are usually measured following T.C.A. precipitation of protein, the acid labile phosphoprotein would be destroyed, and phosphoenzyme formation measured by this method would appear to be totally calcium dependent.

They also postulated the existence of a coupling mechanism by which the acid labile phosphoprotein could only be formed after the acid stable form. In the presence of calcium and low concentrations of ATP, at which the calcium independent ATPase activity is negligible, the acid stable intermediate E ~ P would be formed (Froelich and Taylor, 1976). Conversion of the acid stable intermediate to the acid labile form would be catalysed by high concentrations of ATP (Froelich and Taylor, 1975). This mechanism provides another site of action for the regulatory action of high concentrations of ATP described previously. Froelich and Taylor (1976) suggested that in the membrane the ATPase enzyme functions as a dimer, the subunits alternating between states of high calcium affinity and low calcium affinity, out of phase with each other (see Chapter 5).

A change in the affinity of the enzyme for calcium has been reported during decomposition of phosphoprotein (Yamada and Tonomura, 1972). It was calculated that the affinity of the purified enzyme for calcium during phosphoprotein formation is 3,000 times higher than the affinity for magnesium, whereas the affinity of the enzyme for calcium during phosphoenzyme decay is only 2-5 fold that for magnesium. These affinities were calculated by measuring the competitive inhibition of calcium-dependent phosphoprotein formation by excess magnesium, and of competitive inhibition by calcium of magnesium dependent phosphoenzyme decay. Using the purified ATPase, Ikemoto (1975) showed a reduction in the amount of calcium bound upon addition of ATP as the phosphoprotein is formed. This could be explained by a decrease in the affinity of the enzyme for calcium as the second phosphoprotein intermediate $E_2 \sim P$ is formed. Decay of phosphoenzyme was accompanied by the restoration of calcium binding, which would occur as the enzyme returns to the E form.

Using a stopped-flow apparatus fitted to a dual beam spectrophotometer, and the calcium sensitive dye, arzenazo III, Ikemoto (1976) demonstrated calcium release from the purified enzyme, and phosphoprotein formation over 500 milliseconds. While phosphoprotein formation occurred with no noticeable delay, calcium release from the enzyme occurred only after a 15 millisecond lag phase. Decomposition of the phosphoprotein was accompanied by a rebinding of calcium to the enzyme. These results all suggest that there are at least two acid stable phosphoprotein intermediates, differing in their calcium binding affinity, and providing a mechanism by which calcium bound to the first intermediate would be transported across the membrane where, on conversion to the second intermediate, calcium would be released to the inside of the SR (steps 3 and 4, Fig.I). The maximum amount of calcium release

from the purified enzyme exceeds the expected value of a 2 : 1 ratio with the amount of phosphoenzyme formed. It was found that 0.24 moles of calcium were released for 0.1 moles of phosphoenzyme formed (Ikemoto, 1976). This suggests the presence of an additional form of phosphoenzyme, which would have a low affinity for calcium, but would be acid labile, thus not contributing to the total measured amount of phosphoprotein, since this is isolated by T.C.A. precipitation. This acid labile form of phosphoprotein may be the same as that postulated by Froelich and Taylor (1976) to account for the initial burst of phosphate production (see page 36 ) and could represent the E*P form of the enzyme shown in Figure 1.

It has also been suggested that dephosphorylation of phosphoprotein and calcium release to the inside of the SR membrane is accompanied by an increase in the affinity of the enzyme for magnesium (Froelich and Taylor, 1976; Tonomura, 1973; Kanazawa et al., 1971). Since a linear relationship between the reciprocal of the ratio between phosphoprotein concentration and phosphate liberation and the reciprocal of magnesium concentration has been demonstrated, it was suggested that the activation of phosphoprotein decomposition is caused by the binding of one mole of magnesium to one mole of phosphoprotein (Kanazawa et al., 1971). Activation of phosphoprotein hydrolysis by potassium, and to a lesser extent by other monovalent cations has also been shown (Shigekawa and Pearl, 1976). It has been suggested that the two calcium ions exchange with internal magnesium and potassium which are transported from inside to outside the SR coupled with phosphoprotein decomposition (Kanazawa et al., 1971). This hypothesis is supported by the experiments of Carvalho and Leo (1967) who have shown that binding sites for calcium also bind magnesium, potassium and hydrogen ions, so that ATP-induced

calcium binding releases an equivalent amount of other cations. At pH values below 6.2, calcium is largely exchanged for hydrogen ions, whereas at pH values above 6.2, calcium is exchanged for magnesium and potassium. The total amount of bound magnesium, potassium, calcium and hydrogen remains constant at various calcium concentrations, indicating a stoichiometric exchange of calcium for these cations.

The phosphate formed during phosphoprotein decomposition is liberated to the external surface of the SR membrane. This is demonstrated by the observation that in SR incubated with $^{32}P$-labelled ATP, the total amount of $^{32}P$ in the filtrate does not change during calcium transport and ATP hydrolysis (Yamada et al., 1970).

(h) Regulatory effect of nucleotide triphosphates

De Meis and de Mello (1973) showed that the rate of phosphoprotein decomposition is increased by ATP, ITP and GTP, and Froelich and Taylor (1975) suggested that ATP at relatively high concentrations could catalyse the conversion of the acid stable phosphoenzyme to the postulated acid labile phosphoprotein, thus also accelerating the rate of phosphoenzyme decay. This would provide a possible mechanism of action for the regulatory effect of high ATP concentrations described previously. However, Martinosi et al. (1974) and Kanazawa et al. (1971) found no acceleration of phosphoprotein decomposition at high ATP concentrations.

It was suggested that high concentrations of ATP exert a regulatory effect on the formation of the enzyme-substrate-calcium complex, which would be formed in two sequential steps:

$$E^{*}\cdot Ca\cdot ATP \rightleftharpoons E\cdot Ca\cdot ATP$$

High concentrations of ATP would shift the equilibrium of this reaction to the right (Kanazawa et al., 1971).

This mechanism is similar to that proposed by Rauch et al. (1978) (page 26) and can be explained by the scheme of Vieyra et al. (1979), described on page 26 , by which the conversion of E*, the form of the enzyme reactive with phosphate, to E, the form of the enzyme reactive with ATP, is accelerated by high concentrations of ATP. As well as affecting the formation of the substrate complex in a similar way to that proposed by Kanazawa et al. (1971), this would also have the effect of accelerating the rate of phosphoenzyme decomposition, since the equilibrium of reactions 7 and 8 (Fig.1) would be shifted towards the right (Vieyra et al., 1979). This scheme could therefore explain the conflicting results reported for the effect of high concentrations of ATP on phosphoprotein. An alternative explanation for the lack of effect of ATP on phosphoprotein decomposition reported by Martinosi et al. (1974) and Kanazawa et al. (1971) is that in these experiments the phosphoenzyme was already present in a form which is insensitive to ATP (Froelich and Taylor, 1975).

(i) Reversal of the calcium pump

Adenosine diphosphate has been shown to decrease the concentration of phosphoprotein (Makinose, 1969; Yamada and Tonomura, 1972; de Meis and de Mello, 1973), and it was shown that the decrease in the amount of phosphoenzyme was due to an acceleration of the rate of phosphoprotein decomposition by ADP, but with no concomitant phosphate liberation (Kanazawa et al., 1970; 1971). By monitoring the change in $AT^{32}P$ concentration by thin layer chromatography, Kanazawa et al.

(1970) showed that the decrease in phosphoprotein concentration upon addition of ADP is accompanied by a parallel increase in ATP concentration. The formation of ATP from ADP and phosphoprotein did not require external calcium, but was dependent on calcium inside the vesicles (Kanazawa et al., 1970; 1971). It was therefore suggested that calcium is translocated from inside to outside the SR, coupled with ATP synthesis (Kanazawa et al., 1971; Yamada and Tonomura, 1972b).

Calcium release into a calcium-free medium from SR previously loaded with calcium oxalate was stimulated by addition of ADP and phosphate (Barlogie et al., 1971; Masuda and de Meis, 1973). Adenosine diphosphate-activated calcium release is dependent on magnesium in the external medium (Masuda and de Meis, 1973) and is inhibited by free calcium in the medium at the same concentrations as those which activate calcium uptake (Barlogie et al., 1971). Adenosine diphosphate activated calcium release was shown to be accompanied by the synthesis of one mole of ATP for every 2 calcium ions leaving the SR (Makinose, 1971; Makinose and Hasselbach, 1971). Adenosine triphosphate synthesis was dependent on the presence of a calcium concentration gradient across the membrane (Makinose and Hasselbach, 1971).

It was suggested that ATP synthesis and calcium release occur by a reversal of the ATPase reaction represented by the equation:

$$2Ca_o + ATP + E \xrightleftharpoons{Mg^{2+}} Ca^2E \sim P + ADP \qquad (1)$$

$$Ca^2E \sim P \rightleftharpoons 2Ca_i + P_i + E \qquad (2)$$

$$2Ca_o + ATP \xrightleftharpoons{Mg^{2+}} 2Ca_i + ADP + P_i$$

where E represents the enzyme, E ~ P the phosphoprotein, and o and i denote the outside and inside of the SR respectively, except in the case of $P_i$.

The reverse of this reaction could only occur in the presence of all the reaction products, and of the activator, magnesium, and would require phosphorylation of the enzyme by phosphate (Makinose, 1972).

(j) Calcium gradient dependent phosphorylation by phosphate

Incorporation of inorganic phosphate in the medium into SR protein was demonstrated using SR previously loaded with calcium (Makinose, 1972; Yamada and Tonomura, 1972b). The degree of phosphate incorporation was inhibited by the addition of ADP, which also led to increased ATP synthesis (Makinose, 1972; Yamada and Tonomura, 1972). A phosphoprotein intermediate was isolated, and was shown to have similar properties to the intermediate formed during phosphorylation of the enzyme by ATP, i.e. stable in acidic media, but decomposed in the presence of hydroxylamine and at alkaline pH (Makinose, 1972; Yamada et al., 1972). It was shown that $^{32}P$ was incorporated into the major protein band separated by polyacrylamide gel electrophoresis (Yamada et al., 1972).

The affinity of the SR membrane for phosphate varies according to the pH of the reaction medium. The $K_m$ for phosphate for calcium oxalate loaded vesicles at pH 6.1 is 3.1, whereas at pH 7.0 it is 8.7. At pH 6.0, a maximum of 3 moles of phosphate are incorporated/$10^6$ grams SR (de Meis, 1976), whereas at pH 7.0 only 1.5-2.0 moles phosphate are incorporated/$10^6$ grams SR (Yamada and Tonomura, 1972b; Makinose, 1972).

Membrane phosphorylation by phosphate is inhibited by calcium in the external medium (de Meis, 1976). The inhibition by calcium is

probably due to the observed dependence of phosphorylation by phosphate on a calcium concentration gradient across the membrane (i.e. a high calcium concentration inside the SR, low calcium concentration outside the SR) (Makinose, 1972). The degree of inhibition by calcium varies with the pH of the medium. At pH 7.0, the calcium concentration required for half maximal inhibition is 100 μM, whereas at pH 6.0 this concentration is 0.1-1.0 μM (de Meis, 1976).

Membrane phosphorylation by phosphate is competitively inhibited by ATP in the external medium (Yamada et al., 1972; de Meis, 1972) and the degree of inhibition is also dependent on the pH of the external medium. However, in contrast to the inhibition by calcium, the degree of inhibition is more pronounced at high pH values (de Meis, 1976). It has been suggested that raising the pH from 6.0-7.5 results in a reciprocal modification of membrane affinity for ATP and phosphate, i.e. as membrane affinity for phosphate decreases, the affinity for ATP increases (de Meis, 1976). It should be noted that the pH optimum for membrane phosphorylation by ATP is pH 7.0 (Tonomura, 1973).

Only phosphate which reacts with the membrane at the outer surface of the membrane is involved in phosphoprotein formation and the reversal of the calcium pump (de Meis and Carvalho, 1976). Phosphorylation by phosphate also requires magnesium in the external medium (de Meis, 1976; Yamada et al., 1972; Makinose, 1972). These observations are consistent with the liberation of phosphate to the outside of the SR during calcium-dependent ATP hydrolysis (Yamamoto and Tonomura, 1968; Tonomura, 1973).

Membrane phosphorylation by phosphate is also inhibited by potassium and sodium ions (Masuda and de Meis, 1973).

(k) Calcium gradient dependent ATP synthesis

The phosphoprotein formed by isolated SR in the presence of a calcium concentration gradient is capable of transferring the phosphate to ADP to form ATP, a process which is coupled with calcium efflux. This is demonstrated by the rapid decrease in the concentration of phosphoprotein formed by phosphorylation with inorganic phosphate (coupled with net synthesis of ATP) when ADP is added to the reaction medium (Yamada et al., 1972; de Meis, 1976; Makinose, 1972). Adenosine triphosphate synthesis and the increase in calcium efflux rate exhibits a different pH profile to phosphoprotein formation. The rate of ATP synthesis and of calcium efflux increases at increasing pH values up to pH 7.5, in contrast to phosphoprotein formation which decreases rapidly at pH values above 6.0 (de Meis, 1976). It has been suggested that the energy produced by the dissipation of the calcium concentration gradient during calcium efflux is utilised for ATP synthesis (Makinose, 1972), However, if the phosphoprotein intermediate is an acyl phosphate compound, containing a high energy bond, it would appear that the energy requiring step in the reversal of the calcium pump is the phosphorylation reaction by phosphate. It is unlikely that the energy requirement for this reaction is supplied by the dissipation of the calcium concentration gradient, because calcium efflux is associated with the subsequent step of ATP synthesis. In addition, membrane phosphorylation by inorganic phosphate can occur in the absence of a calcium concentration gradient.

(l) Calcium gradient independent phosphorylation by phosphate

It was first demonstrated that SR vesicles not previously loaded with calcium could catalyse a rapid $P_i \rightleftarrows HOH$ exchange to the extent of

520 gm atoms oxygen exchanged/$10^6$ gm SR. In vesicles incubated with $H^{18}OH$ and phosphate, the water oxygen atoms were incorporated into the phosphate. This was taken to indicate a dynamic reversal of steps 1 and 8 (Fig.1). The exchange reaction was found to be magnesium-dependent, and was inhibited by free calcium in the medium, by ADP, and by Triton X100 (Kanazawa and Boyer, 1973). Phosphate incorporation into SR in the absence of a calcium concentration gradient was directly demonstrated using SR preparations not previously loaded with calcium (Kanazawa and Boyer, 1973), made leaky by treatment with diethyl ether (Masuda and de Meis, 1973), or in a purified ATPase preparation which cannot transport calcium (Knowles and Racker, 1975). The gradient independent phosphorylation requires magnesium in the external medium, and is inhibited by calcium at the outside surface of the membrane (Knowles and Racker, 1975; Masuda and de Meis, 1973; Kanazawa and Boyer, 1973; Kanazawa, 1975). The reaction was also inhibited by potassium ions (Kanazawa, 1975). The inhibition of gradient-independent phosphorylation by calcium was also pH dependent. The affinity of the membrane for phosphate during gradient independent phosphorylation by phosphate at pH 6.0 is slightly lower than during gradient dependent phosphorylation, with a Km of 3.5 mM. However, the decrease in gradient independent phosphorylation at pH 7.0 is greater than for gradient dependent phosphorylation. The Km for phosphate in leaky vesicles at pH 7.0 is 25.3 mM. Raising the pH from 6.0 to 7.0 in leaky vesicles leads to a 77.5% inhibition of phosphoprotein formation, compared with a 43% inhibition under similar conditions for gradient-dependent phosphorylation (de Meis, 1976). This may account for the relatively low levels of phosphoprotein formed in the absence of a calcium concentration gradient in experiments carried out at pH 7.0

(Kanazawa and Boyer, 1973, Kanazawa, 1975). The maximum level of phosphoprotein formed during gradient-independent phosphorylation by phosphate is just over 2 moles/$10^6$ gm protein (de Meis, 1976).

Inhibition of gradient-independent phosphorylation by calcium exhibited a similar pH dependence to the inhibition of gradient-dependent phosphorylation, the inhibition being more marked at higher pH values (de Meis, 1976).

Calcium gradient-independent membrane phosphorylation by phosphate occurs more rapidly than gradient-dependent phosphorylation (Rauch et al., 1977; Verjovski-Almeida et al., 1978). Steady state levels of phosphoenzyme are reached within 20 milliseconds, suggesting that in the absence of calcium either outside or inside the vesicles, the enzyme resides in a form which can react very rapidly with phosphate (Verjovski-Almeida et al., 1978).

(m) Calcium gradient-independent ATP synthesis

Addition of ADP to SR phosphorylated by phosphate in the absence of a calcium concentration gradient, did not lead to any net synthesis of ATP. This was taken to indicate an absolute requirement for a calcium concentration gradient for ATP synthesis (Beil et al., 1977; Hasselbach, 1978; Hasselbach et al., 1978; de Meis, 1976). It is possible, however, that the apparent requirement for a calcium concentration gradient across the membrane for the synthesis of ATP may only be due to a requirement for the saturation of the low affinity calcium binding sites located at the inside surface of the SR membrane.

Sarcoplasmic reticulum vesicles incubated in a medium containing ATP, magnesium, $^{32}P$ and calcium, catalyse an ATP $\rightleftharpoons$ $P_i$ exchange, which is thought to represent simultaneous ATP hydrolysis and synthesis.

The exchange is stopped when vesicles are made leaky by treatment with phospholipase a, due to the abolition of the calcium concentration gradient caused by the accumulation of calcium phosphate. However, ATP $\rightleftarrows$ $P_i$ exchange could be restored to leaky vesicles by the addition of millimolar concentrations of calcium (de Meis and Carvalho, 1976; de Meis, 1976). Adenosine triphosphate synthesis in the absence of a calcium concentration gradient was directly demonstrated by Knowles and Racker (1975) using a purified ATPase preparation, and by de Meis and Tume (1977) using leaky vesicles reconstituted from a purified ATPase preparation. In both these preparations ATP formation required the addition of a high concentration of calcium following the phosphorylation reaction. It appears, therefore, that phosphorylation of the ATPase by phosphate can occur in the presence or the absence of a calcium concentration gradient across the membrane, provided that the high affinity calcium binding sites at the external surface of the membrane are not occupied by calcium, whereas ATP synthesis requires saturation of the low affinity calcium binding sites at the internal surface of the membrane.

This view is supported by the observation that, following enzyme phosphorylation by phosphate in the absence of a calcium concentration gradient, it is possible to transfer the phosphate from the phosphoenzyme to ADP at progressively lower calcium concentrations, by raising the pH from 6.0 to 8.1 at the time of ADP addition. Phosphorylation of the enzyme by phosphate at pH 5.0 could be followed by ATP synthesis by the simultaneous addition of ADP and potassium hydfoxide to raise the pH to 8.0, at a constant calcium concentration of 0.6 mM for both steps (de Meis and Tume, 1977). At pH 5.0, inhibition of membrane phosphorylation by calcium would be minimal (de Meis, 1976; de Meis

and Tume, 1978), allowing the phosphorylation reaction to occur even in the presence of 0.6 mM calcium, whereas at pH 8.0, ATP synthesis is possible at relatively low calcium concentrations. The effect of the pH change in the above experiments would be to change the calcium affinity of the binding sites. An increase in the affinity of both the low and high affinity sites for calcium, 3-4 orders of magnitude, has been observed on raising the pH from 6.1 to 8.65 (Verjovski-Almeida and de Meis, 1977).

The experiments described above demonstrate that a calcium concentration gradient is not an absolute requirement for ATP synthesis. However, it is likely that in the in vivo situation, at physiological pH, the conditions required for phosphorylation by phosphate and ATP synthesis, i.e. saturation of the internal calcium binding sites and absence of calcium from the external calcium binding sites, would only be satisfied by the presence of a calcium concentration gradient. The demonstration of ATP synthesis in the absence of a calcium concentration gradient indicates that the energy requirement is not derived from the dissipation of the gradient. As discussed previously, it seems likely that the energy-requiring step in the reversal of the calcium pump is the phosphorylation step. The energy for this could be derived from a conformational change in the enzyme molecule, possibly brought about by removal of calcium (Hasselbach, 1978).

(n) Properties of the phosphoprotein intermediates formed during phosphorylation by phosphate

Although the phosphoproteins formed during gradient-dependent and gradient-independent membrane phosphorylation by phosphate are indistinguishable (Beil et al., 1977), the different kinetics and

affinities for phosphate of the two processes have led to the suggestion that two different phosphoproteins are formed depending on whether a calcium concentration gradient across the membrane exists or not. It was not clear whether the two phosphoproteins were intermediates of the same reaction chain, or products of different reaction chains proceeding in parallel (Beil et al., 1977; Hasselbach, 1978).

Stopped-flow and rapid-quench techniques have revealed that phosphate incorporation in the presence of a calcium concentration gradient exhibits a lag phase followed by a linear rise in phosphate incorporation, until steady state is reached about 500 milliseconds after starting the reaction. No lag phase of phosphate incorporation was observed in the absence of a calcium concentration gradient (Verjovski-Almeida et al., 1978). The lag phase of phosphate incorporation in the presence of a calcium concentration gradient occurs only if the reaction is started by the simultaneous addition of EGTA and phosphate to SR vesicles previously exposed to calcium, and was abolished if EGTA was added a few seconds before phosphate, although under these conditions the rate of phosphoprotein formation was still slower than the rate in the absence of a calcium concentration gradient. By suspending unloaded vesicles in a medium containing calcium, followed by the addition of EGTA and phosphate, a lag period occurred before the start of the phosphorylation, and the time course of phosphorylation was identical to that obtained with loaded vesicles (Chaloub et al., 1979).

It is suggested that in the presence of calcium the enzyme resides in the E state which can be phosphorylated by ATP. Removal of calcium would result in the conversion of E to the E* form of the enzyme, which can be phosphorylated by phosphate. It is suggested

that this reaction is relatively slow, and accounts for the lag phase observed during membrane phosphorylation by phosphate using loaded vesicles which had previously been exposed to calcium (Verjovski-Almeida et al., 1978; Chaloub et al., 1979). This is supported by the observation that unloaded vesicles exhibit a lag phase in phosphorylation if they are incubated with calcium prior to the start of the phosphorylation reaction, and by the abolition of the lag phase in loaded vesicles if the vesicles are preincubated with EGTA (Chaloub et al., 1979).

The reverse of this reaction, the conversion of E* to E (step 1, Fig.1) prior to membrane phosphorylation by ATP, is described on page 26. Changes in the conformational state of the enzyme during the calcium transport cycle, based in fluorescence changes, have been described by Dupont and Leigh (1978). The competitive inhibition of membrane phosphorylation by calcium and ATP has also been explained in terms of the two different states of the enzyme. Inhibition of membrane phosphorylation by calcium or ATP would be explained by a shift in the equilibrium of reaction 1 to favour the E form of the enzyme (de Meis and Boyer, 1978).

The observation that membrane phosphorylation by phosphate in the presence and in the absence of a calcium concentration gradient can be obtained with similar kinetics provided evidence that the same phosphoenzyme is formed under both sets of conditions, but that the phosphoenzyme acquires the ability to synthesise ATP only in the presence of a relatively high concentration of calcium.

The sequential formation of ADP-sensitive and ADP-insensitive phosphoenzymes has been described by several authors.

Decomposition of a phosphoenzyme formed in the absence of potassium was not accelerated by ADP, but was accelerated by magnesium

ATP, free magnesium and also by potassium, whereas the phosphoenzyme formed in the presence of potassium was ADP-sensitive, i.e. its decomposition was accelerated by ADP (Shigekawa and Dougherty, 1978a). It was shown that in the absence of potassium salts the ADP-sensitive phosphoenzyme would first be formed and would be converted to the ADP-insensitive form, the hydrolysis of which would be accelerated by magnesium ATP, magnesium and potassium. Millimolar concentrations of calcium were found to favour the formation of ADP-sensitive phosphoenzyme (Shigekawa and Dougherty, 1978b). This could explain the requirement for a relatively high concentration of calcium for ATP synthesis.

A similar scheme has been put forward by Takakawa and Kanazawa (1979), who have also showed the sequential formation of ADP-sensitive and insensitive phosphoenzymes, with the formation of the ADP-sensitive form of phosphoenzyme being favoured in the presence of calcium. However, the scheme differs from that proposed by Shigekawa and Dougherty (1978b) in that decomposition of the ADP-insensitive phosphoenzyme is not accelerated by potassium. These differences may be explained by the demonstration of the sequential formation of at least three forms of phosphoenzyme. One form $EP_{D+}$, would be formed in the presence of a high concentration of calcium, and is 90% ADP-sensitive. Chelation of calcium with EGTA led to the formation of an ADP-insensitive form of phosphoenzyme, $EP_{D-}$. A portion of $EP_{D-}$ was found to be magnesium and potassium-sensitive and was deisgnated $EP_{D-M+}$ (Yamada and Ikemoto, 1980). The ADP-insensitive potassium insensitive phosphoenzyme described by Takakawa and Kanazawa (1979) could correspond to the ADP-insensitive magnesium insensitive phosphoenzyme of Yamada and Ikemoto (1980).

During the reversal of the calcium pump by phosphorylation of the membrane by phosphate, it would be expected that the ADP-insensitive phosphoprotein would be formed before the ADP-sensitve form, whereas in the experiments described above the ADP-sensitive phosphoenzyme is formed first. However, in these experiments, the membrane is first phosphorylated by ATP, so that the pump is operating in the forward mode, allowing the ADP-sensitive phosphoprotein to be formed first. Presumably the phosphoproteins described here, differing in their ADP-sensitivity, correspond to the different phosphoproteins postulated by Froelich and Taylor (1975; 1976) and by Ikemoto (1976), although no attempt appears to have been made to correlate these data.

## SECTION IV

## Reaction Mechanism of the Calcium Dependent ATPase in Cardiac Muscle Sarcoplasmic Reticulum

### (a) Comparison with reaction mechanism in skeletal sarcoplasmic reticulum

As described in Section I, the SR in cardiac muscle is less abundant than in skeletal muscle and, although the properties of the calcium uptake and calcium activated ATPase reactions are similar, the amount of calcium uptake and calcium activated ATPase activity is only about 25-50% of the activity in isolated skeletal muscle SR (Baskin and Deamer, 1969; Chimoskey and Gergely, 1968; Fanburg et al., 1964; Carsten, 1964). The ATPase activity of the SR in the absence of calcium, however, is higher in cardiac, as opposed to skeletal, SR.

Isolated fragments of cardiac SR are also able to catalyse a rapid ADP-ATP phosphate exchange (Suko and Hazzelbach, 1974; 1976) and to incorporate $^{32}P$ ATP into the membrane (Namm et al., 1972; Fanburg and Matsushita, 1973; Suko and Hasselbach, 1974; 1976; Pang and Briggs, 1977; Shigekawa et al., 1976). Polyacrylamide gel electrophoresis has demonstrated that $^{32}P$ is incorporated exclusively into a protein band of molecular weight approximately 100,000 (Fanburg and Matsushita, 1973; Suko and Hasselbach, 1974; 1976). This indicates that the ATPase enzyme itself is the protein that is phosphorylated. The phosphoprotein formed exhibits similar properties to the phosphoprotein formed in skeletal SR, being stable at low temperature and at acid pH, but unstable in hydroxylamine and at alkaline pH, indicating that is is probably an acyl phosphate compound (Shigekawa et al., 1976; Suko and Hasselbach, 1976).

Phosphorylation is calcium-dependent (Namm et al., 1972; Fanburg and Matsushita, 1973; Suko and Hasselbach, 1974;1976; Shigekawa et al., 1976), and the calcium sensitivity at low calcium concentration is similar to that of the calcium uptake and calcium activated ATPase reactions (Namm et al., 1972; Suko and Hasselbach, 1974; 1976; Shigekawa et al., 1976). At high calcium concentrations, at which inhibition of the calcium activated ATPase activity is observed, phosphoprotein levels are maintained at a steady maximum level (Shigekawa et al., 1976; Suko and Hasselbach, 1976). This is similar to the dependence of phosphoprotein formation on calcium concentration reported in skeletal muscle SR. A Km of 0.3 μM for calcium for phosphoprotein formation in cardiac SR has been reported (Suko and Hasselbach, 1976). This is similar to the Km of 0.3-0.5 μM for calcium for the calcium uptake and calcium activated ATPase activities reported by the same authors. However,

a Km of 4.7 μM for calcium for calcium-activated ATPase activity in cardiac SR has also been reported, and compared with a Km of 1.3 μM for calcium for the same reaction in skeletal SR (Shigekawa et al., 1976). The affinity of the cardiac enzyme for calcium may therefore be lower than that of the skeletal enzyme.

Magnesium in the external medium increases the level of phosphorylation, and has a maximum effect when present in amounts equimolar to ATP (Namm et al., 1972; Suko and Hazzelbach, 1976). This indicates that, as in skeletal SR, magnesium ATP is the substrate for the ATPase reaction. Double reciprocal plots of cardiac calcium activated ATPase activity against magnesium ATP concentration are non-linear, indicating a similar biphasic dependence on substrate concentration to that which was observed in skeletal SR (Shigekawa et al., 1976). The pH optimum for cardiac calcium activated ATPase activity is about 7.8-8.3, which is slightly more alkaline than the pH optimum for skeletal SR calcium-activated ATPase activity. The pH optimum for phosphoprotein formation shows a broader optimum between pH 7.0 and 9.0, and is similar in cardiac and skeletal SR (Shigekawa et al., 1976). Reported values for the maximum amount of phosphoprotein formed by cardiac SR are 0.2 moles/$10^6$ gm SR (Namm et al., 1972), 0.05-0.06 moles/$10^6$ gm SR (Fanburg and Matsushita, 1973), 1.2 moles/$10^6$ gm SR (Pang and Briggs, 1977), 1.0-1.3 moles/$10^6$ gm SR (Suko and Hasselbach, 1976), 1.28 moles/$10^6$ SR (Shigekawa et al., 1976) and 1.2 moles/$10^6$ gm SR (Sumida et al., 1980). The maximum amounts of phosphoprotein formed are thus at least 3 to 5 times lower than in skeletal SR. Assuming that the number of phosphorylation sites reflects the number of enzyme active sites, the lower amount of phosphoprotein formed in cardiac SR indicates a lower density of active enzyme sites (Shigekawa et al., 1976). This observation, together with the report that less ATPase protein is present in cardiac SR than

in skeletal SR, probably accounts, at least in part, for the lower ATPase activity of cardiac SR. In cardiac SR, the ATPase protein accounts for 35-40% of the total protein, whereas in skeletal SR it account for 50-90% of the total protein (Suko and Hasselbach, 1976). However, these results assume a similar degree of purity of cardiac and skeletal muscle SR preparation. It is believed that cardiac SR preparations contain a higher proportion of mitochondrial and sacrolemmal contaminants than skeletal SR preparations (Levitsky et al., 1976), which would artificially lower the observed density of enzymic sites/mg protein.

As in skeletal SR, calcium uptake and calcium-activated ATPase activity is stimulated by monovalent cations (Jones et al., 1977).

Dephosphorylation of cardiac SR ATPase is promoted by magnesium (Pang and Briggs, 1978; Shigekawa et al., 1976) and by ADP (Suko and Hasselbach, 1976; Shigekawa et al., 1976). Phosphoprotein hydrolysis has been shown to be the rate-limiting step in the cardiac ATPase reaction (Pang and Briggs, 1973).

Phosphorylation of cardiac SR by orthophosphate has also been demonstrated (Winkler and Suko, 1977; Suko et al., 1977). Phosphorylation by phosphate is strongly inhibited by free calcium in the medium, and requires free magnesium. Preloading of the SR with calcium is also required for phosphorylation with phosphate. The pH optimum for phosphorylation of cardiac SR with phosphate is 6.0-6.2, and the maximum level of phosphoprotein formed is 0.6-0.8 moles/$10^6$ gm SR, which is also lower than the amount formed by skeletal SR. The phosphoprotein formed from phosphate is indistinguishable from that formed by phosphorylation by ATP. Addition of ADP results in dephosphorylation accompanied by ATP synthesis. Adenosine triphosphate synthesis is strongly inhibited by external calcium, but requires magnesium in

the medium (Winkler and Suko, 1977; Suko et al., 1977). An association between stimulation of calcium efflux from cardiac SR and ATP synthesis has also been demonstrated (Suko et al., 1977).

In these experiments, both phosphorylation and ATP synthesis required preloading the SR with calcium (i.e. saturation of the high affinity calcium binding sites inside the SR), but both were strongly inhibited by free calcium in the medium. The reactions were therefore calcium gradient-dependent. As yet there are no reports of either gradient-independent phosphorylation by orthophosphate or gradient independent ATP synthesis in cardiac SR. However, some indication that the requirement for a calcium concentration gradient for ATP synthesis in cardiac SR is due only to saturation of the low affinity binding sites inside the SR, comes from the experiments of Plank et al. (1979), who have measured ATP $\rightleftharpoons$ $P_i$ exchange in cardiac SR. The reactions were carried out in a medium containing SR, ATP, $^{32}P$ phosphate, magnesium ATP and a low concentration of calcium. Adenosine triphosphate $\rightleftharpoons$ $P_i$ exchange exhibited a lag phase, during which calcium was accumulated inside the SR and the calcium concentration in the medium was reduced to a very low level (i.e. a calcium concentration gradient was set up across the membrane). This was followed by an increased rate of ATP $\rightleftharpoons$ $P_i$ exchange, representing net ATP synthesis from ADP and phosphate. Adenosine triphosphate $\rightleftharpoons$ $P_i$ exchange was abolished in cardiac SR vesicles made leaky by treatment with phospholipase a, but was partially restored by the addition of millimolar concentrations of calcium to the medium. The conditions required for ATP $\rightleftharpoons$ $P_i$ exchange in cardiac SR are thus essentially the same as the conditions required for skeletal SR. The exchange reaction can take place in the absence of a calcium concentration gradient, provided that the calcium concentration is high enough.

The reaction mechanism in cardiac and skeletal SR thus appears to be similar. However, the use of quench reagents has revealed kinetic differences in the two types of SR.

Rates of phosphorylation by ATP were found to be similar in cardiac and skeletal SR (Briggs et al., 1978). It has been confirmed that, when ATP is added to SR preincubated with calcium, the rate of phosphoprotein formation is similar in cardiac and skeletal SR. When ATP and calcium are added simultaneously to SR preincubated with EGTA and magnesium, the rate of phosphoprotein formation is reduced (see page 26). However, the reduction in the rate of phosphoprotein formation under these conditions is considerably more marked in cardiac than in skeletal SR. On the basis of this result it has been suggested that the lower rate of phosphorylation in cardiac SR is due to the slower rate of conversion of E* to E (step 1, Fig.1) or to a different equilibrium for this reaction in cardiac SR, favouring E* formation. Since magnesium may affect the equilibrium, favouring E* formation, it is possible that the cardiac enzyme interacts more strongly with magnesium than the skeletal enzyme. This is supported by the results of preliminary experiments which have shown that faster rates of phosphorylation are obtained in cardiac SR if the SR is preincubated with EGTA in the absence of magnesium (Sumida et al., 1978a).

Cardiac SR also exhibits higher levels of phosphoprotein in the early phase of the reaction than in the steady state, as described for skeletal SR (page 39). This overshoot in phosphoprotein formation is more marked in cardiac than in skeletal SR, and could also be explained by a slower conversion of E* to E in cardiac SR. An alternative explanation is that in cardiac SR there is only one predominant form of phosphoprotein, or more than one which decompose at similar rates, whereas in

skeletal SR there may be several phosphoproteins (see pages 35-38) decomposing at different rates (Sumida et al., 1980).

The initial rate of phosphoenzyme hydrolysis is reduced in cardiac SR compared with skeletal SR (Sumida et al., 1978; 1978a; Briggs et al., 1978), and also requires potassium or another monovalent cations (Briggs et al., 1978). The size of the initial phosphate burst is reduced in cardiac SR compared with skeletal SR (Briggs et al., 1978).

The observation that the rate of phosphoprotein decomposition in cardiac SR is slower than in skeletal SR is consistent with recent experiments in which the rates of phosphoprotein decomposition in cat caudiofemoralis (fast twitch) and soleus (slow) muscles were shown to be correlated with relaxation time (Wang et al., 1979).

(b) Phosphorylation of phospholamban

In cardiac SR an additional control mechanism exists, involving cyclic AMP and protein kinase, which regulates the rate of calcium accumulation by cardiac SR. It has been suggested that this mechanism provides a biochemical basis for the mediation by cyclic AMP of the effect of catecholamines to abbreviate systoly (Tada and Kirchberger, 1975).

The initial rate of oxalate supported calcium uptake (Kirchberger et al., 1972; Tada et al., 1974) and of calcium-activated ATPase activity (Tada et al., 1974) in isolated cardiac sarcoplasmic reticulum has been shown to increase more than two-fold when the SR is preincubated with cyclic AMP and protein kinase (Kirchberger et al., 1972). Half maximal activation of calcium uptake (Kirchberger et al., 1972) and of

ATPase activity occurred at the same cyclic AMP concentration of 0.1 μM. The stoichiometric coupling of calcium-activated ATPase and calcium uptake is maintained at 2 moles of calcium taken up/mole ATP hydrolysed (Tada et al., 1974). In the presence of cyclic AMP and protein kinase the Km for calcium of both reactions is reduced, and treatment of cardiac SR with protein kinase and cyclic AMP has also been shown to produce a three-fold leftward shift in the calcium concentration dependency curve for calcium efflux (Kirchberger and Wong, 1978). However, calcium binding, in the absence of precipitating ions, is not affected by preincubation of the SR with cyclic AMP and protein kinase (Kirchberger et al., 1972).

Preincubation of cardiac SR with protein kinase and cyclic AMP stimulated the incorporation of $^{32}P$ from $^{32}P$-labelled ATP into cardiac SR (Kirchberger et al., 1974). However, the phosphoprotein formed is distinct from the acyl phosphate intermediate of the calcium transport ATPase (Kirchberger et al., 1974; Wray et al., 1973), and has been named phospholamban. This protein has a molecular weight of 22,000 Daltons and possesses the stability characteristics of a phosphoester, in which the phosphate is largely incorporated into serine and threonine (Tada et al., 1978). Unlike the phosphorylation of the ATPase, the protein kinase catalysed phosphorylation of phospholamban is independent of calcium concentration in the physiological range (Kirchberger et al., 1974), but is inhibited by millimolar concentrations of calcium (Wray et al., 1973). Protein kinase catalysed phosphorylation of phospholamban is correlated with the protein kinase mediated increase in calcium transport activity (Kirchberger and Chu, 1976). The increase in ATPase activity when cardiac SR is preincubated with cyclic AMP and protein kinase has been shown to be due to an enhancement of the rate of phosphoprotein decomposition (Tada et al., 1979).

Phosphorylation of the 22,000 Dalton protein can be catalysed by cyclic AMP-dependent protein kinase isolated from either skeletal or cardiac muscle, although the cardiac muscle kinase is more effective (Tada et al., 1978). The presence of an endogenous cyclic AMP-stimulated protein kinase in cardiac SR has been demonstrated (Wray et al., 1973) and may account for the increase in microsomal phosphorylation observed on addition of cyclic AMP alone (Kirchberger et al., 1974; Wray et al., 1973).

When phosphorylated SR is incubated with phosphoprotein phosphatase from bovine heart, phospholamban is dephosphorylated (Tada et al., 1975). Phosphoprotein phosphatase stimulated dephosphorylation of phospholamban is stimulated by manganese chloride and, to a lesser extent by magnesium chloride, and is inhibited by inorganic phosphate and sodium fluoride. Dephosphorylation was associated with a decrease in the initial rate of calcium transport (Kirchberger and Raffo, 1977).

These observations support the view that the phosphorylation and dephosphorylation of the 22,000 Dalton protein, phospholamban, catalysed by cyclic AMP-dependent protein kinase and phosphoprotein phosphatase respectively, alter the rate of calcium transport in cardiac SR.

In addition to the phosphorylation of phospholamban by cyclic AMP-dependent protein kinase, this protein is also phosphorylated by a calmodulin-dependent protein kinase (LePeuch et al., 1979), which has been identified as phosphorylase b kinase (Wray and Gray, 1977; LePeuch et al., 1979). This enzyme phosphorylates phospholamban at a site distinct from that phosphorylated by cyclic AMP-dependent protein kinase. Unlike the cyclic AMP-dependent phosphorylation, the

calmodulin-dependent phosphorylation is completely calcium-dependent. In the absence of any phosphorylation by cyclic AMP-dependent protein kinase, calmodulin-dependent phosphorylation of phospholamban induces some activation of calcium uptake by the SR. In the absence of calmodulin, cyclic AMP-dependent phosphorylation does not induce any stimulation of calcium uptake by the SR, but amplifies the stimulation induced by calmodulin-dependent phosphorylation. The activation of calcium uptake by cyclic AMP-dependent phosphorylation alone, reported in the literature, is probably due to insufficient washing of isolated SR preparations to remove endogenous calmodulin (LePeuch et al., 1979).

It is suggested that free cytosolic calcium concentration modulates phosphorylation of phospholamban at the calmodulin-dependent site, inducing a conformational change in phospholamban, which in turn modifies the hydrophobic environment of the ATPase leading to increased calcium uptake. Phosphorylation of phospholamban by cyclic AMP-dependent protein kinase would increase the conformational changes, and lead to a further increase in calcium uptake by the SR (LePeuch et al., 1979).

Using quench-flow kinetic investigations, it has been calculated that, in the presence of cyclic AMP and protein kinase, the amount of calcium accumulated by cardiac SR in the first 200 milliseconds was comparable to the quantity that would need to be accumulated in order to account for relaxation, but that in the absence of cyclic AMP and protein kinase, the rate of calcium uptake by cardiac SR was too slow to account for relaxation (Will et al., 1976). Using isolated perfused rat hearts, it has been shown that fluphenazine, a calmodulin inhibitor, reduced phosphorylation of phospholamban to 37% of control, in the absence of either b-agonists or b-antagonists. These results suggest that the phospholamban may exist in vivo in a phosphorylated form.

Isolated SR preparations would be dephosphorylated *in vitro* by phosphoprotein phosphatase, and would not be rephosphorylated in the absence of exogenous calmodulin. This raises the possibility that figures previously reported for maximal calcium uptake activity of cardiac SR may be lower than the *in vivo* rate, and that cardiac SR would have the capability of transporting sufficient calcium to account for relaxation and contraction of the myofilaments (LePeuch *et al.*, 1980). Calmodulin has been shown to stimulate calcium transport in isolated SR vesicles (Katz and Remtulla, 1978).

Sarcoplasmic reticulum isolated from slow contracting skeletal muscle, has also been shown to contain a protein similar to phospholamban, which can be phosphorylated by cyclic AMP-dependent protein kinase, and is associated with a significant increase in the rate of calcium uptake by the SR (Kirchberger and Tada, 1976). Although some authors have reported an increase in calcium uptake by skeletal muscle SR in the presence of cyclic AMP and protein kinase (Schwartz *et al.*, 1976; Galani-Kranias *et al.*, 1980), other authors report no change in the calcium transport activity of fast skeletal muscle SR in the presence of these compounds (Kirchberger and Tada, 1976). Protein kinase-stimulated phosphorylation of a 22,000 Dalton protein in the membrane has not been observed in fast skeletal SR (Schwartz *et al.*, 1976; Kirchberger and Tada, 1976), although phosphorylation by cyclic AMP dependent protein kinase of a 100,000 Dalton component of the SR has been claimed (Galani-Kranias *et al.*, 1980). A lack of effect of cyclic AMP and protein kinase on isolated fast skeletal muscle SR is consistent with the insensitivity of this type of muscle to catecholamines. However, it is not yet fully resolved whether or not cyclic AMP and protein kinase can cause an increase in calcium transport by fast skeletal SR. In skinned cardiac muscle cells, addition of cyclic AMP

($10^{-6}$ M) caused relaxation of the fibre, attributed to an enhancement of calcium accumulation by the SR. The amplitude of the cyclic contractions elicited by a calcium-induced release of calcium was also increased. This was attributed to a larger release of calcium from more fully loaded SR (Fabiato and Fabiato, 1975). A similar concentration of cyclic AMP ($5 \times 10^{-6}$ M) caused relaxation of tension in skinned fast skeletal muscle fibres. This has been attributed to increased calcium uptake by the SR (Fabiato and Fabiato, 1978). These authors suggested that the lack of effect of catecholamines on intact fast skeletal muscle could be due to optimal calcium accumulation by the SR under physiological conditions in this tissue.

In skeletal SR, as in cardiac SR, it has been shown that phosphorylase b kinase activates (Horl et al., 1978 a and b; Schwartz et al., 1976) and a protein phosphatase inhibites (Horl et al., 1978 a and b) the calcium transport ATPase, providing a regulatory system for the calcium transport process. However, it is not clear whether or not this process is calmodulin-dependent in fast skeletal muscle.

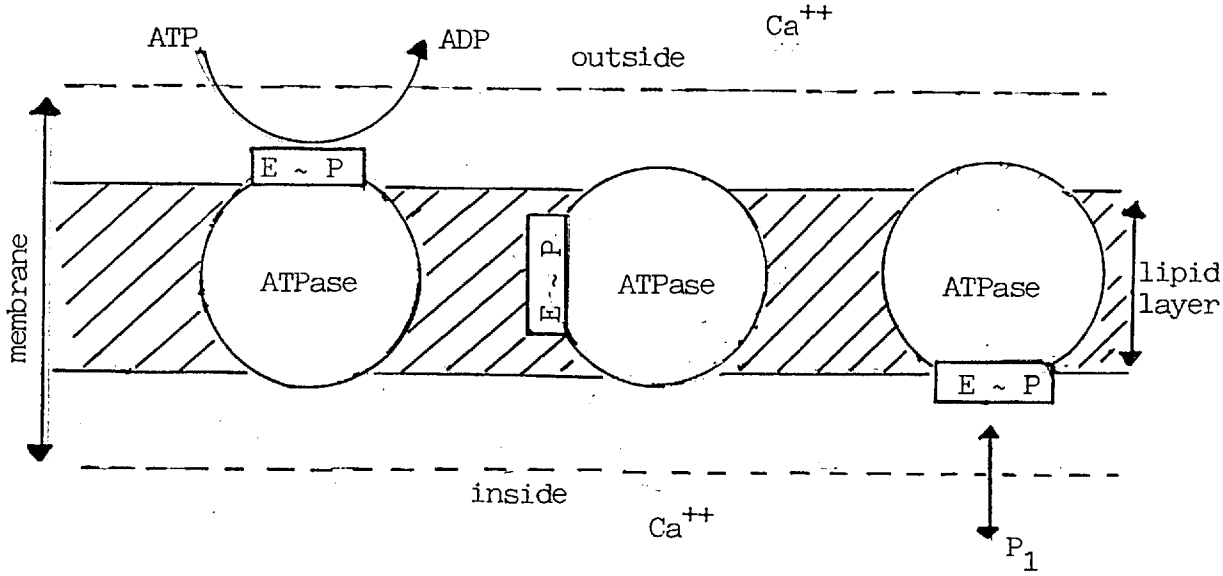
## SECTION V

## Molecular Models of Calcium Transport

The results discussed in Section III show that calcium is transported across the SR membrane coupled with phosphorylation and dephosphorylation of the ATPase enzyme. The molecular mechanisms involved in this process are unclear, but several theoretical models have been put forward to explain it. These are the rotational diffusion of the carrier, transfer of calcium by a mechanochemical process, and conformational change in the ATPase molecule.

(a) Rotational diffusion of the carrier

This is a theoretical model put forward by Martinosi et al. (1976) and is shown schematically below:



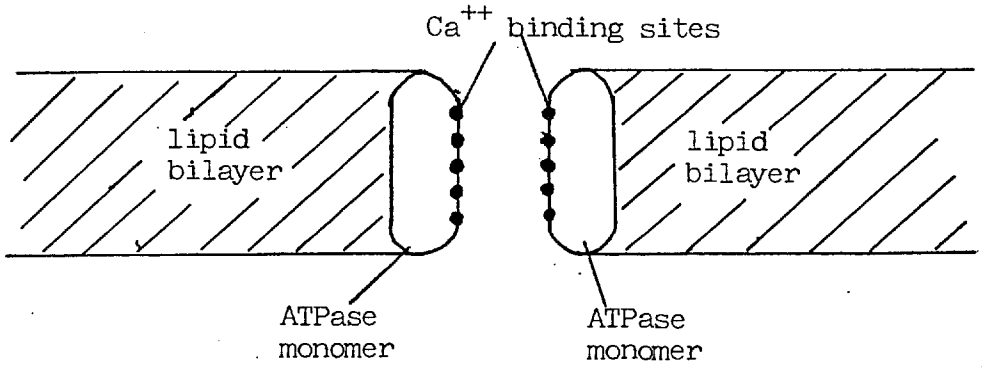

The essential features of this model are that the carrier (ATPase molecule) is embedded in the lipid phase of the membrane with only a small portion of its total surface area exposed to the external and internal surfaces. Phosphorylation and dephosphorylation of the enzyme requires that the active site is exposed to the external or internal surfaces respectively. The initial rapid phase of membrane phosphorylation would involve only those active sites which face the outside. Random rotation of the enzyme in the membrane, defined by its rotary diffusion constant, would result in the transport of the active site, with calcium bound to it, from the outside to the inside of the membrane. The model requires a change in the affinity of the enzyme for calcium at the inner surface, which has been demonstrated experimentally (see Section III). Dephosphorylation would be coupled with the transport of the active site from the inside of the membrane to the outside, but without calcium bound to it. The model does not exclude the counter-transport of magnesium or other cations outwards.

Experimental evidence in support of this model comes from the experiments of Yamamoto and Tonomura (1976) who modified exposed lysine residues of the calcium-dependent ATPase enzyme using the impermeant reagent, nitrobenzene sulfonate, and showed that the number of residues which could be modified by this treatment varied according to the enzymatic state of the ATPase (i.e. whether the enzyme was phosphorylated or not). However, the experiments of Martinosi and Fortier (1974) showing that binding of anti-ATPase antibodies to fragmented SR did not effectively inhibit ATPase activity or calcium transport, suggested that major changes in the orientation of the calcium transport ATPase may not constitute an important feature of calcium transport. The observations of Racker et al. (1979) that oxalate only stimulates calcium transport by purified ATPase preparations reconstituted into liposomes if it is present in high concentrations during the reconstitution process, has also been taken by these authors to exclude the possibility of a rotating carrier model. The reason for this conclusion is, however, unclear, particularly in view of earlier experiments using $^{14}C$-labelled oxalate and fragmented SR, which have shown that oxalate is transported across the SR membrane.

(b) Mechanico-chemical transfer

According to this scheme, put forward by Martinosi et al. (1977), a row of binding sites with decreasing affinities for calcium and increasing affinities for magnesium are arranged in a channel across the membrane. A sequential change in the affinity of the sites for calcium and magnesium produced by phosphorylation of the enzyme by ATP would propel calcium and magnesium from site to site in opposite directions across the membrane. This scheme can be most easily visualised by

the existence of the enzyme in a dimeric form in the membrane, with a channel through which calcium is transported being formed between two enzyme subunits. The scheme is shown diagrammatically below:

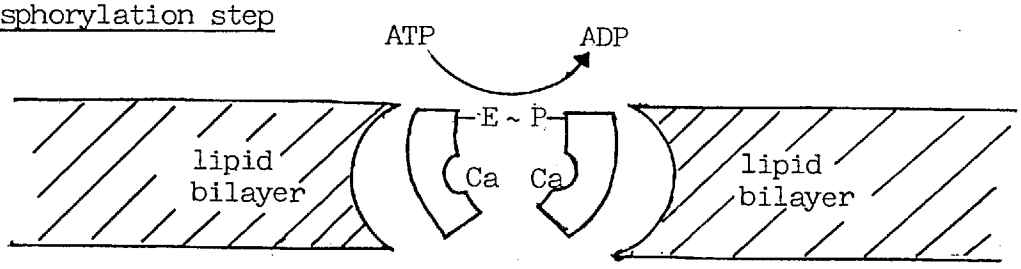



The free energy change between successive steps would be very small. In this respect the mechanism would be analagous to the energy transfer due to the movement of cross bridges during muscle contraction.

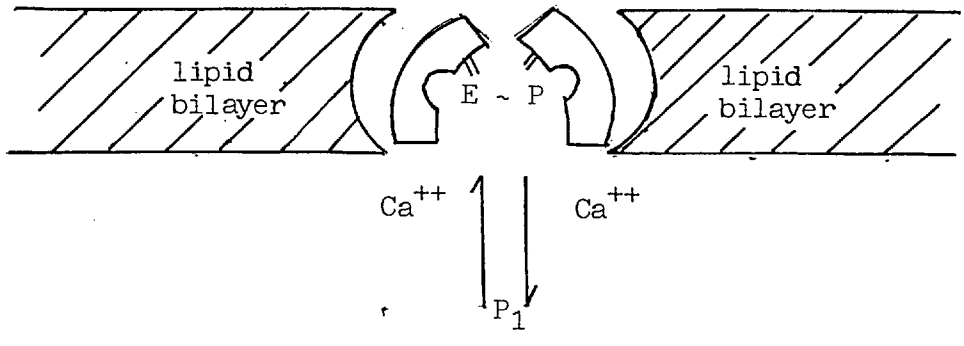
(c) Conformational change in the ATPase molecule

This is an alternative theoretical model put forward by Martinosi et al. (1974), and is shown schematically below:

Phosphorylation step




Dephosphorylation step



Binding of calcium, or enzyme phosphorylation, would lead to a conformational change in the ATPase molecule, and a decrease in the affinity of the enzyme for calcium, so that calcium would be released into the lumen of the SR. This model also allows for the counter-transport of magnesium and potassium ions, due to an increased affinity of the enzyme for magnesium and potassium during the reverse reaction. The model is also consistent with the existence of the enzyme as a dimer (or tetramer) in the membrane with the two halves of the enzyme representing the polypeptide subunits. Similar models have been put forward by Racker (1977) for calcium-activated ATPase, and by Stein et al. (1974) for sodium and potassium-activated ATPase. The latter scheme involves oligomeric interaction of polypeptide chains and results in 'half the sites reactivity' (see Chapter 5).

Experimental evidence for a conformational change in the ATPase molecule during the calcium transport cycle, comes from several investigations in which changes in the electron spin resonance spectra of spin-labelled SR were observed.

Landgraf and Inesi (1969) measured the EPR spectrum of spin-labelled SR and demonstrated a change in the EPR spectrum upon addition of ATP. The change was not affected by the presence of magnesium or calcium. They therefore suggested that simple binding of ATP to the membrane produced a conformational change. Nakamura et al. (1972) measured the ESR spectrum of SR labelled with N-(1 oxyl 2,2,$6_1$$6_1$-tetramethyl 1-4-piperidinyl)maleimide, a label directed towards the SH and $NH_2$ groups of the protein. The ESR spectra obtained were altered by ATP in the presence of calcium and magnesium, an effect which could be prevented through the prior incubation of the SR with EGTA. These results suggest a conformational change in the SR membrane accompanied

by the formation and/or decomposition of phosphorylated intermediate. Pang et al. (1974) used three different group specific probes to investigate the mechanisms of ATP-dependent conformational change, and demonstrated that only the sulfhydril groups were involved. These authors confirmed the findings of Landgraf and Inesi (1969) that the ATP binding produces a conformational change, but they also found that propanalol could block such a change. Since propanalol does not block enzyme phosphorylation or ATP binding, these authors concluded that the conformational change must occur at a step further on in the hydrolysis of ATP than EP formation.

Coan and Inesi (1976) also demonstrated a reversible spectral alteration of spin-labelled SR on addition of acetyl phosphate and calcium, and suggested that these changes reflect protein conformational changes related to the transport cycle. Murphy (1978) measured the effect of calcium, magnesium and ATP on the reactivity of the sulfhydril groups of the SR previously reacted with 5,-5'-dithio bis-(2-nitrobenzoate) ($Nbs_2$) and showed decreased reactivity of the sulfhydril groups with $Nbs_2$ as a result of phosphorylation. The decreased reactivity was maintained after disruption of the SR with deoxycholate and was taken to suggest extensive structural changes occurring upon EP formation.

The evidence that conformational changes in the ATPase enzyme are linked to phosphorylation and dephosphorylation is therefore fairly strong, and favours this model of calcium transport. However, the other two models described here are not necessarily incompatible with a concurrent conformational change in the ATPase molecule, and conclusive evidence for any of the three models described here has not yet been obtained.

SECTION VI

## Electrogenicity of Calcium Transport

Considerable speculation has arisen recently as to whether calcium transport by the SR is accompanied or driven by an electrical potential difference across the membrane.

The hypothesis of ion transport driven by an electrical potential difference across the membrane was first elaborated by Mitchell. The basis of the hypothesis is that an electrical potential difference can be generated across a biological membrane by the release of ions to one side of the membrane, provided that the membrane has a restricted permeability to that ion. In the case of mitochondria, protons released by ATP hydrolysis and by electron transport cannot cross the inner mitochondrial membrane, so that a potential difference is created across the membrane, and ions move across the membrane under the influence of this potential difference (Mitchell, 1966). However, Mitchell does not consider proton gradients to be essential for ion transport, and has proposed another mechanism whereby calcium can be transported by an ATPase (Mtichell, 1973). He suggests that ATP is normally hydrolysed within membranes at a site beyond the osmotic barrier. The reaction products, ADP and $P_i$, are ejected on the side on which they entered, but $2H^+$ ions are left behind on the opposite side. In the case of the $Ca^{++}$ ATPase, ATP would enter the site as a calcium complex, and calcium ions rather than protons would be ejected to the interior. The calcium gradient established would be the driving force for resynthesis of ATP. As described in Section III, this has been demonstrated in the SR by several workers, but later experiments have shown that ATP synthesis can occur in the absence of a calcium concentration gradient.

The formation of a proton gradient during calcium transport in the SR has been demonstrated (Madiera, 1978), using the fluorescent dye, 9-amino-6-chloro-2-methoxyacridine. It was shown that a proton gradient was generated during calcium uptake by sarcoplasmic reticulum at the early stages of calcium translocation, reaching a maximum 300-400 milliseconds after triggering the calcium transport. The gradient was not formed in SR made leaky to calcium by treatment with ionophores. Using cyanine dyes, he also demonstrated the slow formation of a positive electrical potential inside the SR during calcium accumulation, and assumed the potential to be the consequence of the inward calcium current, rather than the driving force for it. By incorporating a pH indicator, bromothymol blue, into the SR vesicles, Madiera (1979; 1980) demonstrated a transient alkalinisation inside the SR associated with calcium uptake, which was taken to reflect the formation of a transmembrane proton gradient responsible for sustaining calcium transport.

A sudden ejection of protons from the SR vesicles when calcium uptake is triggered as also been demonstrated (Madiera, 1980). The total amount of protons ejected was found to be a function of the amount of calcium added, and was sufficiently large to create a significant transmembrane pH gradient (Madiera, 1980). The initial rate of proton ejection was three to five times greater than the rate of calcium uptake. The maximal change in pH observed was about 0.15 units (Madiera, 1980). The expected transmembrane change in pH persisting during the uptake of calcium was calculated to be about 0.5 pH units, and it was concluded that the pH inside the vesicles is strongly buffered.

From these experiments, Madiera (1978; 1979; 1980) concluded that formation of a transmembrane proton gradient is a primary event occurring during the transport of calcium in SR and is probably the motive force which sustains the transport.

Using liposomes in which the isolated protein of the SR was incorporated, it is possible to vary the potassium concentration inside the liposomes in a controlled manner (Zimniak and Racker, 1978). Different potassium gradients could then be established across the membrane permitting the creation of controlled membrane potential differences in the presence of valinomycin. By altering the potential difference across the membrane it was possible to either stimulate or inhibit the rate of calcium uptake. The potential difference which did not affect the rate of calcium transport was considered to reflect the membrane potential established by calcium transport. This potential was found to be 61 ± 10 mV. These authors also measured membrane potential changes during calcium uptake directly, using a fluorescent probe, 8-anilo-1-naphthalene sulfonic acid, and obtained a value of + 51 mV by this method. These authors suggested that a potential difference could arise transiently during calcium uptake.

Light absorption changes in the potential sensitive dye, merocyanine oxazoline, have been observed during calcium release from SR induced by caffeine or ionic replacement in skinned skeletal and cardiac muscle fibres (Fabiato and Fabiato, 1977). The direction of the change was the same in both cardiac and skeletal muscle fibres, but the amplitude of the change was nine times larger in skeletal muscle cells. This difference was attributed to the greater abundance of SR in skeletal cells. They concluded that depolarisation of the SR may be the consequence of calcium release even when it is elicited by a calcium or caffeine-induced

process, instead of by ionic modifications that are presumed to cause depolarisation. Alternative, calcium or caffeine could cause calcium release by depolarising the membrane.

However, the change in fluorescence of merocyanine, oxanol and cyanine dyes, during calcium accumulation, is not necessarily related to changes in diffusion potential across the membrane, but could be caused by the response of the dye to changes in electrostatic surface potential caused by the binding of cations and ATP to the $Ca^{++}$ transport ATPase (Russel et al., 1978 a and b). Changes in the absorbance of cyanine dyes during ATP-mediated calcium uptake, and ADP + $P_i$-induced release of calcium could be reproduced in the absence of ATP, or in solubilised SR. The absorbance minimum is different from that observed after membrane potential generation by valinomycin in liposomes. These results provide no indication of changes in membrane potential during calcium uptake or release (Beeler and Martinosi, 1979).

The role of proton gradients and electrical potential differences across the membrane in calcium transport by SR therefore remains in dispute. Much of the work that has been done on the subject of electrical fields across the membrane of the SR has been in relation to calcium release. This will be discussed in Chapter 4.

## SECTION VII

## Calcium Release from the Sarcoplasmic Reticulum

### (a) Source of contractile calcium

The calcium concentration in the sarcoplasm of resting skeletal (Ebashi and Endo, 1968) and cardiac (Nayler and Dunnett, 1974) muscle, is below $10^{-7}$ M. Muscle contraction is activated by a rise in the

intracellular calcium concentration to concentrations between $10^{-7}$ and $10^{-5}$ M (Ebashi and Endo, 1968). This has been demonstrated by the use of the calcium sensitive dye, aequorin. When this dye is injected into skeletal (Blinks et al., 1976) or cardiac (Allen and Blinks, 1978) muscle cells, a transient increase in luminescence, indicating an increased intracellular calcium concentration, was observed on stimulation of the muscle fibre. The increased intracellular calcium concentration preceded tension development. The time course of the change was slower in cardiac than in skeletal muscle fibres and the peak of light intensity for a given amount of aequorin injected was lower (Allen and Blinks, 1978).

Activation of the contraction is brought about by the binding of calcium to troponin c, removing the inhibitory action of troponin I on the magnesium-activated actomyosin ATPase.

In fast skeletal muscle, the origin of the calcium required for contraction is generally accepted to be intracellular. In cardiac muscle, the initial fast sodium current of the action potential is followed by a slow inward current, decaying in 50-300 milliseconds, which is considered to be carried mainly by calcium (Carafoli and Crompton, 1978). Some workers (Rich and Langer, 1975; Shine et al., 1971), believe that the calcium entering the cell during the action potential is the primary source of contractile calcium. In frog heart, a contraction can be induced directly by calcium ions entering the cardiac cells during the action potential, indicating that extracellular calcium may be a major source of contractile calcium in the amphibian heart, in which the SR is anyway poorly developed. In mammalian heart, the calcium entering during the action potential, although essential for the activation of contraction, would saturate only 10% of the binding sites on the troponin molecule (Niedergerke et al., 1976).

The amount of calcium required for full tension development of 1 g of heart muscle has been estimated to be between 50 and 100 μmoles (Solaro et al., 1974; Gertz et al., 1970). The amount of calcium entering the cardiac cell during the action potential has been calculated to be 5-10 μmoles/Kg wet weight, i.e. 5-10% of the calcium required for contraction (Berecewicz and Reuter, 1977). These results all indicate that in mammalian cardiac, as well as in skeletal muscle, the major source of contractile calcium is intracellular.

Both the mitochondria and the SR have been postulated to be the intracellular regulators of the calcium concentration.

(b) The role of the mitochondrial in excitation-contraction coupling

Cardiac muscle is particularly rich in mitochondria. Since mitochondria are known to accumulate large amounts of calcium, it has been proposed that at least in cardiac muscle they play a major role in excitation contraction coupling (Carafoli, 1975). In vitro, the maximum capacity of rat heart mitochondria for calcium is 18 μmoles of calcium accumulated/gm tissue/minute. This is higher than the requirement for relaxation (10 μmoles calcium/gm tissue/minute). However, the affinity of the mitochondria for calcium is inhibited by magnesium at physiological concentration (approximately 1 mM). Half maximal velocity of mitochondrial calcium uptake in the presence of 1 mM magnesium requires 40 μM ionised calcium, which is higher than the physiological concentration of calcium during contraction (Carafoli and Cromptom, 1978). Calcium efflux from heart mitochondria is induced by sodium with a maximum velocity of 15 nmoles/mg protein/minute and with half maximal velocity at 8 mM sodium. The intracellular sodium

activity in heart muscle, as determined by microelectrodes, is 6 mM. At sodium concentrations between 4 and 10 mM the rate of calcium efflux is markedly changed by small changes in sodium concentration, raising the possibility that the influx of sodium during the action potential causes calcium to be released from the mitochondria, which could cause the muscle fibres to contract. However, the increase in the sarcoplasmic sodium concentration that occurs on depolarisation of the sarcolemma is likely to be very small - no more than 0.1 mm - and would not markedly affect calcium efflux from the mitochondria (Carafoli and Crompton, 1978).

These observations make it unlikely that under physiological conditions the mitochondrial play a major role in excitation-contraction coupling.

(c) Role of the sarcoplasmic reticulum in excitation-contraction coupling

Isolated sarcoplasmic reticulum from both skeletal and cardiac muscle is capable of accumulating calcium. The rate and amount of calcium accumulation by skeletal muscle SR has been calculated to be sufficient to cause relaxation of the muscle fibres in vivo (Ohnishi and Ebashi, 1964; Ebashi and Endo, 1968). The yield of SR isolated from cardiac muscle is less than that from skeletal muscle, and the rate of calcium accumulation and the capacity for calcium is reduced compared with skeletal muscle. It was initially thought that the rate of calcium accumulation by cardiac SR might not be sufficient to account for relaxation (Harigaya and Schwartz, 1969; Dunnett, 1976). However, the calculations of Solaro and Briggs (1974), based on a yield of SR from cardiac muscle of 7 mg/gm wet heart, which is

considerably higher than the yields previously reported, indicate that calcium accumulation by cardiac SR is sufficient to account for relaxation. The high estimate of the amount of SR present in cardiac muscle was calculated by taking into account the SR lost during the initial low speed centrifugation steps in the isolation procedure (Solaro and Briggs, 1974). Chapman (1979) has argued that the surface area of the SR is more important than its volume, when estimating its functional capacity, and on this assumption calculated that the capacity of cardiac SR could be similar to that of skeletal SR. Quench-flow measurements of the initial rates of calcium binding have shown that the initial rate of calcium binding is sufficient to cause relaxation, particularly when tension development does not exceed half maximal values, and it was argued that maximum tension is not usually developed in heart muscle (Will et al., 1976). It should be noted that the calcium accumulating capacity of cardiac sarcoplasmic reticulum is increased by cyclic AMP and protein kinase which cause phosphorylation of the 22,000 Dalton protein, phospholamban. It has recently been suggested that phospholamban exists in vivo in the phosphorylated state, but is dephosphorylated in isolated SR preparations, resulting in the artificially low estimates for the calcium binding capacity of cardiac SR (see Section IVb). These observations suggest that in cardiac SR, as in skeletal SR, the rate and extent of calcium accumulation is sufficient to account for relaxation.

Release of calcium from the SR has been less widely studied than calcium acculumation. It is generally accepted that calcium released from skeletal SR provides the calcium required for contraction (Ebashi and Endo, 1968). In cardiac muscle, the observation that the curve of the force frequency relationship of isolated guinea pig papillary muscle

is flattened by ryanodine, a drug which is said exclusively to destroy the connection between the t-tubules and the SR, suggests that the SR is also a source of contractile calcium in cardiac muscle (Rumberger, 1976).

(d) Mechanisms of calcium release from the sarcoplasmic reticulum

Calcium release, coupled with ATP synthesis, occurs in both skeletal (Makinose and Hasselbach, 1971) and cardiac (Suko et al., 1977) SR, during reversal of the calcium pump (see Section III). The maximum rate of this calcium efflux is approximately 50 nmoles/mg protein/minute in skeletal SR (Barlogie et al., 1971) and 20 nmoles/mg protein/minute in cardiac SR (Suko et al., 1977). The calculated rate of calcium release necessary to activate contraction exceeds the rate of carrier mediated calcium release by a factor of $10^2$ to $10^4$, and the release of calcium is complete within a few milliseconds (Martinosi, 1972). This makes it unlikely that carrier-mediated calcium efflux contributes significantly to the calcium involved in excitation-contraction coupling.

The most widely postulated mechanisms for the increases in permeability to calcium that must occur during the activation of contraction, are calcium triggered calcium release, and calcium release by depolarisation of the SR membrane. In this thesis, calcium release from isolated fragments of cardiac SR has been studied, and compared with calcium release from isolated skeletal SR.

# CHAPTER 2

# CALCIUM TRIGGERED CALCIUM RELEASE

SECTION I

Introduction

In cardiac muscle, the small amount of calcium which enters the cell during the plateau phase of the action potential is insufficient in itself to cause contraction. The possibility has been raised that this calcium triggers the release of additional calcium from the SR (Bianchi and Bolton, 1966; Weiss and Bianchi, 1965).

Exposure of skinned frog skeletal fibres, previously loaded with calcium, to calcium-containing solutions, elicited a quick contraction. Since at a calcium concentration of $10^{-4}$ M the rate of development of tension was slower in fibres which had not been preloaded with calcium, it was suggested that in the loaded fibres calcium was released from intracellular stores, probably the SR (Ford and Podolski, 1970; 1972a). That the calcium responsible for causing contraction did, in fact, originate from intracellular stores, was confirmed by experiments in which the fibres were first loaded with labelled calcium. Subsequent immersion of the fibres in unlabelled calcium chloride solutions resulted in a large efflux of $^{45}Ca^{++}$ from the fibres (Ford and Podolski, 1972b).

Using skinned skeletal muscle fibres, Endo et al. (1970) have shown that, in the presence of low concentrations of EGTA, contraction of the myofibrils was observed on addition of caffeine, which, at high concentrations causes calcium release from the SR (Weber and Herz, 1968). No contraction was induced by caffeine at high EGTA concentrations, due to the chelation of the calcium released by EGTA. It was also shown that $10^{-6}$ M free calcium, in itself below the threshold concentration required to cause contraction, evoked a transient contraction in the presence of low concentrations of caffeine, also too low to evoke a contraction in itself. The amount of calcium released was related to

the free calcium concentration in the medium, also suggesting that calcium can trigger the release of additional calcium from internal stores. The experiments also suggest that this effect is enhanced by caffeine (Endo et al., 1970). An increase in the concentration of magnesium reversibly inhibited the contraction induced by calcium (Ford and Podolski, 1972b; Endo, 1975). The inhibitory effect could be overcome by increasing the calcium concentration in the medium (Endo, 1975). It has been demonstrated that, in order for calcium triggered release to occur, the SR must be preloaded with calcium to a certain level, and the concentration of the calcium trigger must also be above a certain level. The concentration of calcium that induced a release of calcium from heavily-loaded SR stimulated calcium uptake in SR only lightly loaded with calcium (Endo, 1975).

Calcium triggered calcium release has also been demonstrated in cardiac cells. In mechanically skinned rat ventricular cells, in the presence of strong buffering of the free calcium with EGTA, tonic tension increased as a function of the free calcium concentration in the medium over the range $10^{-7.65}$ to $10^{-7.4}$ M (Fabiato and Fabiato, 1972; 1975a). When calcium was only slightly buffered by EGTA, cyclic contractions occurred over a wide range of calcium concentrations. These cyclic contractions were induced by slight variations in the free calcium concentration in the buffer between $10^{-7.65}$ and $10^{-7.4}$ M. The amplitude of the contraction at a free calcium concentration of $10^{-7.4}$ M was equal to the tonic tension developed by a free calcium concentration 20 times higher, applied to myofilaments in which the SR had been destroyed. These cyclic contractions were attributed to a calcium-induced release of calcium from internal stores, probably the SR (Fabiato and Fabiato, 1975a). The concentration of free calcium required to trigger the

release of additional calcium from SR was increased when the free magnesium concentration was raised (Fabiato and Fabiato, 1975b). By using skinned cardiac cells labelled with chlorotetracycline, a fluorescent cholate probe, it was possible to measure the amount of calcium bound to the SR, and it was demonstrated directly that the calcium released by the trigger calcium originated from the SR. It was also shown that the calcium triggered release of calcium depends not only of the level of the free calcium trigger, but also on the rate at which the change in the myoplasmic free calcium concentration occurs (Fabiato and Fabiato, 1979). The optimum pH for the calcium-induced release of calcium from both cardiac and skeletal SR was found to be 7.4 (Fabiato and Fabiato, 1978a).

The concentration of free calcium required to trigger the release of additional calcium from the SR, varies according to tissue and according to species (Fabiato and Fabiato, 1978b). In skeletal SR, under physiological conditions, approximately $10^{-4}$ M free calcium is required to trigger the release of additional calcium (Fabiato and Fabiato, 1972; Endo, 1977). This is above the threshold necessary directly to activate contraction. In cardiac muscle, the concentration of free calcium required to trigger release of calcium from the SR is below the concentration required to activate the myofilaments. Adult rat ventricular cells exhibit a highly developed calcium-induced calcium release process. Calcium release from the SR is induced at a free calcium concentration of as low as $3 \times 10^{-8}$ M. Rabbit ventricular and new born rat ventricular cells were the least sensitive. Calcium-induced calcium release was not evident at free calcium concentrations below $3 \times 10^{-7}$ M. Dog ventricular cells showed intermediate sensitivity at $10^{-7}$ M free calcium. In all species studied, atrial tissue required

a lower calcium concentration to trigger calcium release than ventricular tissue (Fabiato and Fabiato, 1978b).

Calcium triggered calcium release has also been demonstrated in isolated SR from skeletal muscle. Addition of a high concentration of calcium chloride (0.5 M) to SR vesicles previously loaded with calcium in the absence of precipitating ions, led to the release of the major portion of accumulated calcium. The release of calcium was prevented by the addition of 5 mM magnesium chloride together with the calcium. However, this concentration of calcium is too high to be of physiological significance (Inesi and Malan, 1976). Using a metallochromic indicator method, Ohnishi (1979) showed that the addition of 20 μM free calcium to calcium-loaded SR led to calcium release.

When calcium is taken up by isolated skeletal SR in the presence of a precipitating ion, and if at the time of steady state the concentration of calcium outside the SR is low (1 μM), addition of a small amount of calcium to a total concentration of 2-4 μM will result in an increase in the rate of net calcium release (Katz et al., 1980).

The data from the literature, reviewed above, raise the possibility that, particularly in cardiac muscle SR, calcium release is triggered by calcium outside the SR. In this section, experiments are described which have been carried out to determine the rate of unidirectional calcium efflux from isolated cardiac sarcoplasmic reticulum at various concentrations of free calcium in the medium. The effects of magnesium, and of variation of the pH of the reaction medium on calcium efflux rate are also described. The results are compared with those obtained from similar experiments which have been carried out using isolated skeletal SR. The significance of the results in relation to the in vivo mechanism for calcium released from the SR is also discussed.

In this section the term 'calcium uptake' refers to net calcium uptake by the SR in the presence of potassium oxalate in the incubation medium. 'Calcium release' refers to the net release of calcium from the SR. 'Calcium influx' refers to the unidirectional movement of calcium into the SR, and 'calcium efflux' refers to the unidirectional movement of calcium outwards.

## SECTION II

## Methods

### (a) Isolation of microsomal fractions

Microsomal fractions rich in sarcoplasmic reticulum were isolated from cardiac and skeletal muscle by the method of Harigaya and Schwartz (1969), as modified by Nayler et al. (1975).

Microsomal fractions were prepared from ventricular muscle of adult male albino guinea pigs. The animals were killed by cervical dislocation and the hearts rapidly excised and immersed in ice-cold 0.9% saline. Fat, connective tissue, and atrial tissue, were removed, and the ventricular muscle was finely chopped and suspended in five volumes of a solution containing 10 mM sodium bicarbonate buffer, pH 7.0, and 5 mM sodium azide. Sodium azide was included in the medium to inhibit mitochondrial respiration. The suspension was homogenised for 20 seconds at $0^{\circ}C$, using an Ultra-Turrax homogeniser with the speed control set at half maximum. The homogenate was spun down at 600 x g for 5 minutes at $4^{\circ}C$ in a Mistral 6L M.S.E. centrifuge to remove cell debris. The supernatant from this spin was decanted and spun at 10,000 x g at $4^{\circ}C$ in an M.S.E. 18 or 21 centrifuge, to sediment mitochondria. The supernatant was again decanted and the mitochondrial pellet discarded.

The supernatant was spun at 104,000 x g at 4°C in an M.S.E. 65 or 50 superspeed centrifuge for 30 minutes. The supernatant from this spin was discarded, and the pellet, consisting of the microsomal fraction, was suspended in a small volume of a solution containing 0.6 M potassium chloride and 10 mM histidine buffer, pH 7.0. The high concentration of potassium chloride in this medium solubilises myofibrillar proteins (Martinosi, 1968). The suspension was again centrifuged at 104,000 x g at 4°C for 30 minutes. The resulting pellet was suspended in a medium containing 50 mM potassium chloride and 10 mM histidine, pH 7.0. The preparation consisted of a microsomal fraction rich in sacroplasmic reticulum, as revealed by electron microscopy, and low in mitochondrial and plasma membrane enzyme markers (Dunnett, 1976). Cardiac fractions were used on the day of preparation. The method used to prepare the microsomal fractions is summarised on page 85.

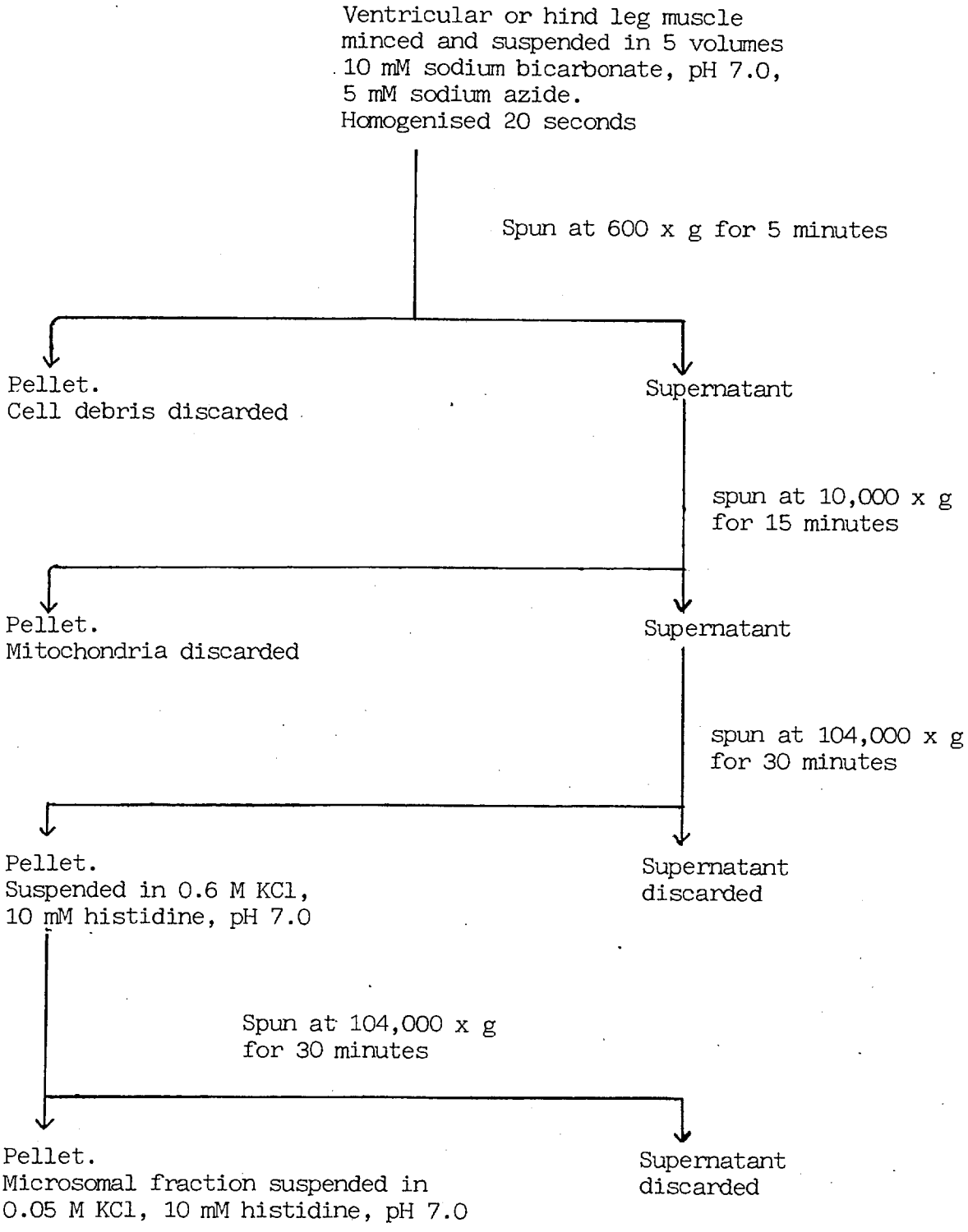
(b) Protein assay

Protein concentrations were determined by the method of Lowry et al. (1951), standardised against crystalline bovine serum albumin, or by the Coomassie blue method (Bradford, 1976), for solutions containing sucrose.

(c) Calcium uptake measurements

Calcium uptake by isolated fragments of SR was measured in a medium containing 120 mM potassium chloride, 40 mM histidine, pH 6.8, 2.5 mM potassium oxalate, 10-20 µg/ml protein, 5 mM disodium ATP, 5 mM magnesium chloride, and calcium chloride labelled with $^{45}Ca^{++}$. The reactions were carried out at 37°C or 25°C as indicated in the legends to the figures. The reactions were started by addition of $^{45}CaCl$ to the concentrations indicated, and the samples were withdrawn

## Preparation of Microsomal Fractions



at appropriate intervals and filtered through millipore filters (0.45 μ diameter pore size) using Swinney adaptors. $^{45}$Calcium remaining in 50 μl samples of the filtrate, was measured by liquid scintillation spectrometry, using either Bray's scintillation fluid or Instagel as scintillant. The composition of the scintillants is shown in Table I. The radioactivity in the filtrate was subtracted from the radioactivity in a 50 μl sample of the total reaction medium before filtration. The difference in the values was used to calculate the amount of calcium taken up by the SR.

In experiments in which the effect of pH was studied, the histidine buffer was replaced by 20 mM Hepes, since at high pH, histidine has been shown to induce contraction in frog auricular trabeculae (Chapman and Miller, 1974). Although frog heart contains only a small amount of SR, a pH-dependent effect of histidine on the SR cannot be excluded.

(d) Calcium efflux measurements

Prior to the measurement of calcium efflux rate, the calcium uptake capacity of the preparations was determined in a pilot experiment in which the SR was allowed to take up calcium to steady state. When the capacity of the preparations for calcium was known, the amount of calcium chloride which should be added to the reaction medium to give a desired concentration of free calcium outside the SR ($Ca_o$) at steady state can be calculated.

Calcium efflux rates were measured using paired experiments. In the first experiment the reaction was started by adding $^{45}Ca^{++}$-labelled calcium chloride. At the time the reaction approached steady state, determined previously in the pilot experiment, samples were

TABLE 1

Composition of scintillants

Bray's Scintillation Mixture

5 parts Toluene
5 parts Dioxan
3 parts Ethanol

containing:
80 gm/litre naphthalene
5 gm/litre PPO
50 mg/litre POPOP

Instagel

1 part Symperonic NP9
2 parts Toluene

containing:
4 gm/litre PPO
0.1 gm/litre POPOP

taken at 30 second intervals for 2 minutes and filtered through millipore filters (0.45 μ diameter pore size) using Swinney adaptors. Radioactivity remaining in the filtrate was measured in the same way as described in the previous section, and used to calculate the calcium content of the SR at each time point. These results were also used to calculate $Ca_o$ at the time that each sample was taken, by subtracting the amount of calcium taken up by the SR from the total calcium added to the medium in each reaction tube. From the difference in the calcium content of the SR at each time point, the rate of net calcium uptake or release over the two minute sampling period, could be calculated. In the other experiment, the reaction was started by adding an equal amount of unlabelled calcium chloride. At the time the first sample was taken from the paired experiment, a trace amount of carrier-free $^{45}CaCl$ was added. Samples were then taken at 15 second intervals and filtered as before. Over a short time scale, the rate of uptake of labelled calcium should represent the unidirectional influx of calcium. These results were therefore used to calculate the rates of unidirectional calcium influx under identical conditions, and at the same concentration of $Ca_o$ as in the paired experiment. The method is explained diagrammatically in Figure 2. Calcium influx rate was calculated according to the equations shown below:

Calcium efflux rate = Calcium influx rate minus net calcium uptake rate

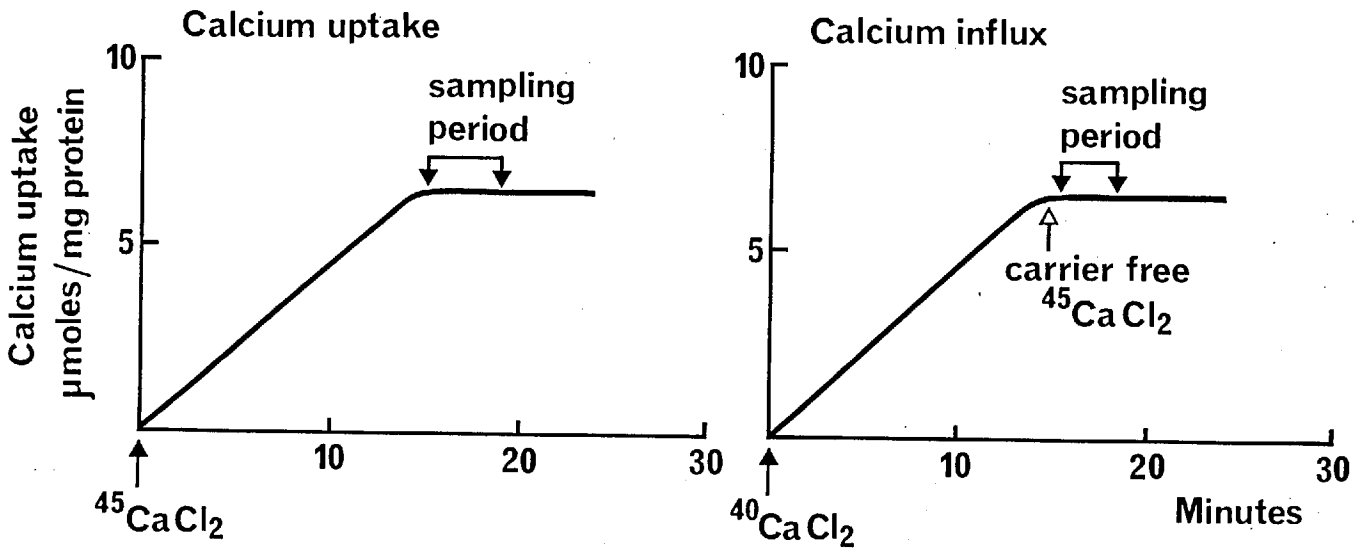
or

Calcium influx rate = Calcium influx rate plus net calcium release rate

If there is no net calcium uptake or release then:

Calcium efflux rate = Calcium influx rate.

Figure 2 Schematic diagram showing method for measuring calcium efflux



The free calcium concentration in the medium ($Ca_o$) was calculated by computer analysis, taking into account the binding of calcium to ATP and the presence of all other ions in the medium. The binding constants were obtained from published data (Chemical Society Special Publication, 1971) and are listed in Table 2. The free calcium concentration inside the SR ($Ca_i$) was maintained constant by presence of oxalate in the medium, which causes calcium oxalate precipitation inside the SR vesicles.

SECTION III

Materials

All solutions were prepared in glass-distilled de-ionised water. ATP, as the disodium salt with a low calcium content. Hepes, Tris, 'Trizma base', Histidine monohydrochloride and Digitonin were obtained from the Sigma Chemical Company Ltd., Poole, Dorset, England. $^{45}CaCl_2$ (CES2) was obtained from the Radiochemical Centre, Amersham, Bucks, England. All other chemicals were obtained from British Drug Houses, Ltd., Poole, Dorset, England.

TABLE 2

Binding Constants used in calculating free calcium concentration.

Ligand adenosine triphosphate ($H_4L$)

| | | |
|---|---|---|
| $H^+$ | Log $K_1$ | 6.53 |
| | Log $K_2$ | 4.06 |
| $K^+$ | Log $K_1$ | 0.99 |
| $Na^+$ | Log $K_1$ | 0.98 |
| $Ca^{++}$ | Log $K_1$ | 3.94 |
| | Log $K_2$ | 2.13 |
| $Mg^{++}$ | Log $K_1$ | 4.22 |
| | Log $K_2$ | 2.24 |

Ligand histidine (HL)

| | | |
|---|---|---|
| $H^+$ | Log $K_1$ | 9.17 |
| | Log $K_2$ | 6.12 |
| | Log $K_3$ | 1.96 |

Ligand oxalate ($H_2L$)

| | | |
|---|---|---|
| $H^+$ | Log $K_1$ | 3.85 |
| | Log $K_2$ | 1.29 |
| $Ca^{++}$ | Log $K_1$ | 1.66 |

SECTION IV

Results

(a) Calcium efflux studies: Effect of external calcium concentration

Rates of calcium influx at various concentrations of $Ca_o$ are shown in Figure 3. The results indicate that the rate of tracer uptake was linear over the sampling period.

Table 3 shows rates of net calcium uptake and release, and rates of calcium influx and efflux at various concentrations of $Ca_o$. The first column of Table 3 shows the concentration of calcium chloride added at the start of the reaction. The second column shows the average of concentrations of $Ca_o$ at the time of all 15 second samples, calculated from the original amount of calcium chloride added as described in 'Methods'. The third column shows the rates of net calcium uptake (U), or release (R), in nmoles/mg protein/minute. At low concentrations of $Ca_o$, slow net calcium uptake occurs, whereas at high concentrations of $Ca_o$ there is a spontaneous net release of calcium, which increases in rate as $Ca_o$ increases further. Column 4 of Table 3 shows the rate of calcium influx in nmoles/mg protein/minute, and column 5 the efflux rate calculated from the rates of calcium influx and net calcium uptake or release, as described in 'Methods'. At low concentrations of $Ca_o$, rates of net calcium uptake and release are relatively slow, and are exceeded by calcium influx rate. However, at very high $Ca_o$ concentrations (e.g. experiment 3, $Ca_o$ = 5.41 µM), the spontaneous release rate is greater than the rate of calcium influx and contributes a greater proportion to the calcium efflux rate. The relative rates of calcium influx and net uptake and release thus vary according to $Ca_o$, but the calcium efflux rate is always stimulated by increasing $Ca_o$. This is shown in Figure 4, which represents results from 10 separate experiments, and shows that calcium efflux rate increases with $Ca_o$, up to $Ca_o$ concentrations of 5-6 µM.

TABLE 3

Rates of net calcium uptake or release and calcium influx and efflux rates at various concentrations of $Ca_o$ in three separate experiments

Protein concentration 10 µg/ml, 5 mM $MgCl_2$, at 37°C. Calcium efflux rates were measured 16 minutes after starting the reaction, as described in 'Methods'.

| | Initial $CaCl_2$ (µM) | $Ca_o$ (µM) | Net calcium uptake rate (U) or release rate (R) (nmoles/mg/minute) | Calcium influx rate (nmoles/mg/minute) | Calcium efflux rate (nmoles/mg/minute) |
|---|---|---|---|---|---|
| Experiment 1 | 35 | 0.14 | 3.00(U) | 18.90 | 15.90 |
| | 45 | 0.40 | 3.7(U) | 51.56 | 47.86 |
| | 55 | 2.19 | 51.3(R) | 168.90 | 220.00 |
| Experiment 2 | 30 | 0.20 | 7.3(U) | 15.81 | 8.51 |
| | 40 | 0.58 | 4.00(R) | 37.73 | 41.73 |
| | 55 | 3.52 | 61.90(R) | 102.14 | 164.04 |
| Experiment 3 | 50 | 0.21 | 3.89(U) | 24.84 | 20.75 |
| | 65 | 0.92 | 13.43(R) | 89.90 | 103.33 |
| | 80 | 5.41 | 144.00(R) | 52.52 | 196.52 |

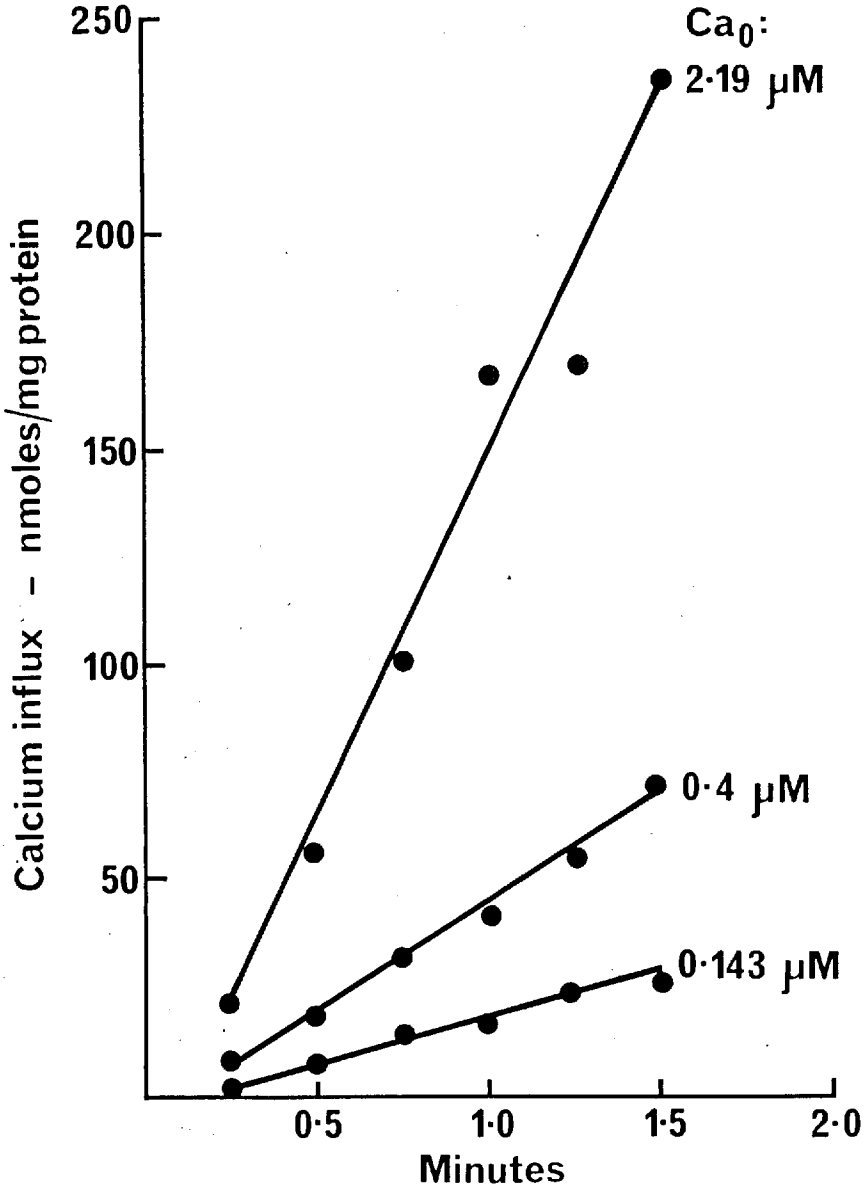



Figure 3

Calcium influx rates into cardiac sarcoplasmic reticulum at various concentrations of $Ca_o$
(0.143 µM, 0.4 µM and 2.19 µM, as shown)

$CaCl_2$ was added at the start of the reaction to final concentrations of 35 µM, 45 µM and 55 µM respectively. Influx rates were measured following addition of carrier free $^{45}CaCl_2$, 16 minutes after starting the reaction with $^{40}CaCl_2$. Protein concentration was 10 µg/ml, $MgCl_2$ concentration was 5 mM. Temperature 37°C.

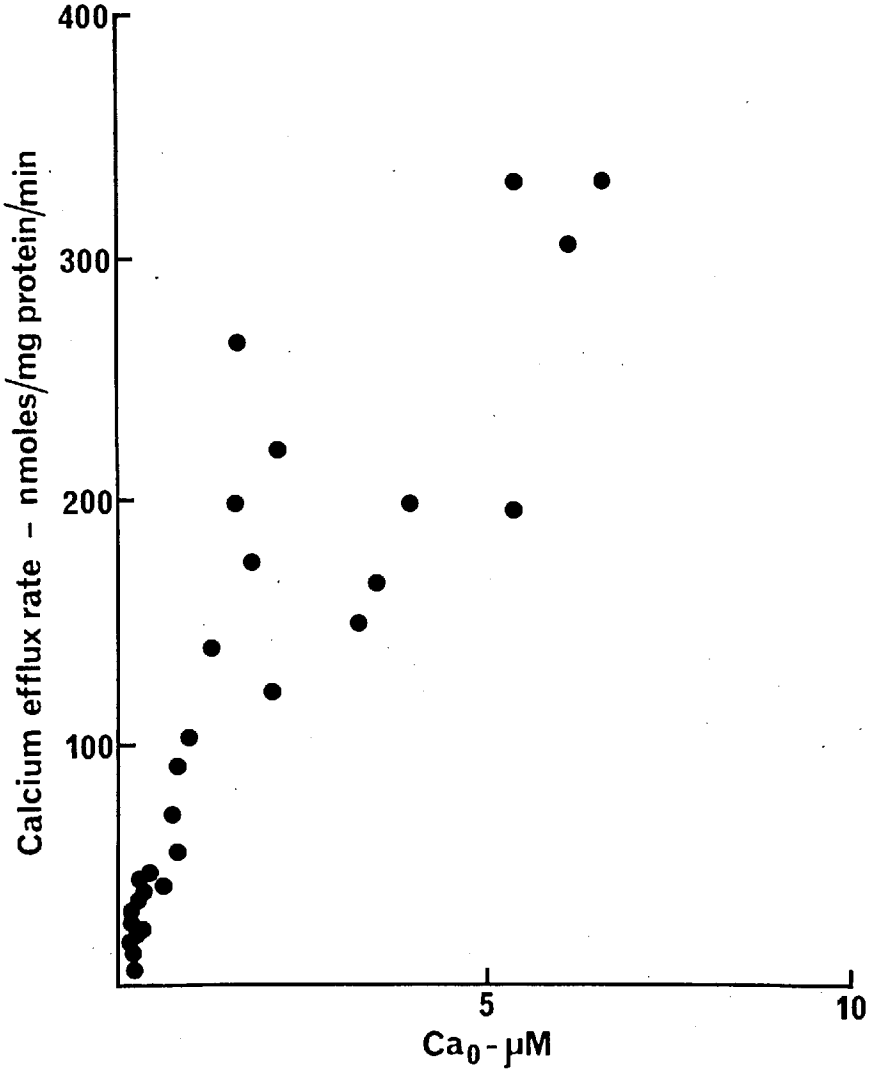



Figure 4

Relationship between calcium efflux rate and $Ca_o$ in cardiac sarcoplasmic reticulum

Reactions were carried out with 10 µg/ml protein, 2.5 mM potassium oxalate and 20-80 µM $CaCl_2$, at 37°C, as described in 'Methods'. Measurements from 10 separate experiments are shown. Calcium efflux rates were measured 12-16 minutes after the start of the uptake reaction.

Figures 5 and 6 show the effect of increasing the magnesium chloride concentration in the medium from 5 mM to 7 mM (Figure 5), or 10 mM (Figure 6) on calcium efflux rate. This represents an increase in the free magnesium concentration from 0.83 mM (5 mM $MgCl_2$), to 2.32 mM (7 mM $MgCl_2$) and 5.12 mM (10 mM $MgCl_2$). Increasing the magnesium chloride concentration also increases the free calcium concentration for any amount of calcium chloride added to the medium.

Calcium efflux rate is clearly inhibited by an increase in magnesium concentration, but there is some indication that at very high concentrations of $Ca_o$ this inhibition can be overcome. For example, in Figure 6, middle panel, calcium efflux in the presence of 10 mM magnesium chloride occurs at rates as fast as those in the control experiment, with 5 mM magnesium chloride, but at high $Ca_o$. From Figures 5 and 6 it appears that calcium efflux rate is inhibited to the same extent by 7 and 10 mM magnesium chloride.

(b) Effect of pH

The time course of net calcium uptake and release at pH 6.4, 6.8 and 7.2, is shown in Figure 7. The amount of calcium initially taken up increases as the pH is raised from 6.4 to 7.2. This seems to be due to the onset of a net release of calcium which begins after 30 minutes incubation at pH 6.8. At pH 7.2, net calcium release occurs earlier and proceeds at a faster rate than at pH 6.8. Under the conditions of the experiments, ATP-independent calcium uptake was low (1-2 nmoles/mg protein) and was not significantly altered by increasing the pH from 6.4 to 7.2.

Figure 8 shows the rate of calcium efflux from cardiac SR measured after 20 minutes incubation at pH 6.4, 6.8 and 7.2, plotted against $Ca_o$.

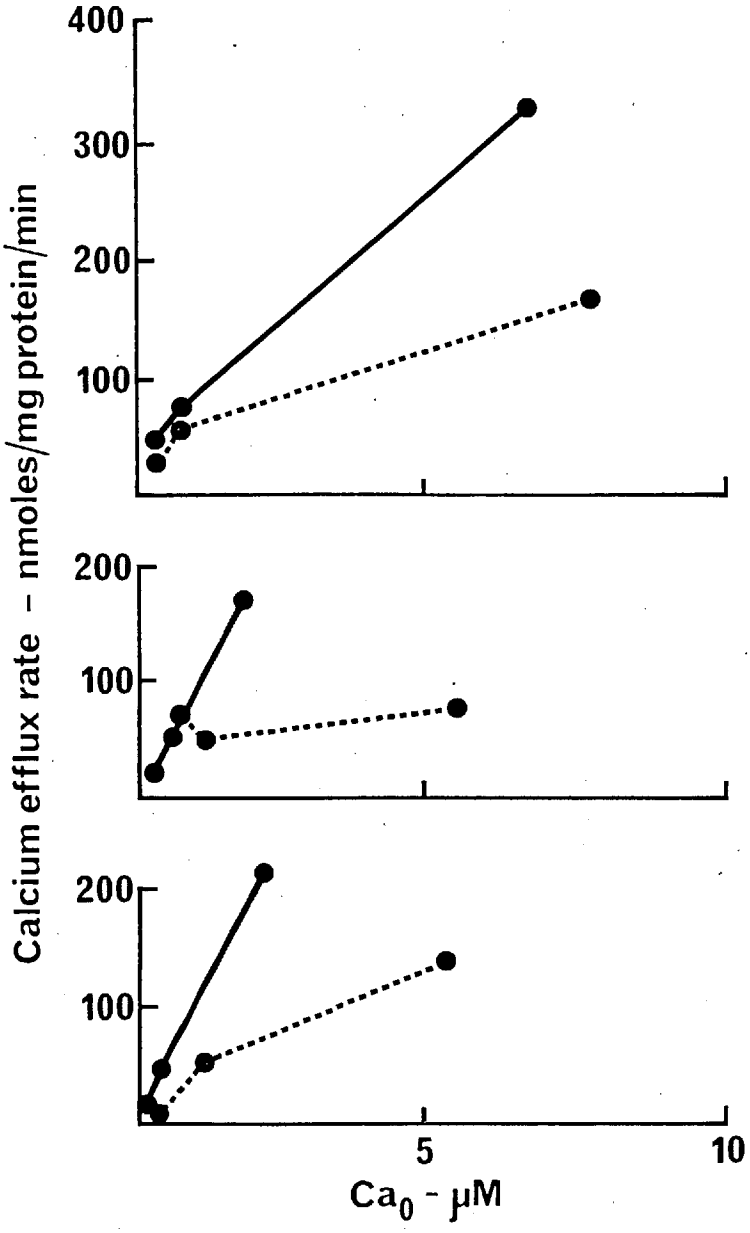



Figure 5

Inhibition of calcium efflux rate from cardiac sarcoplasmic reticulum by 7 mM magnesium chloride

Three separate experiments are shown.
Conditions as in Figure 3.

●——● 5 mM $MgCl_2$,

●-----● 7 mM $MgCl_2$.

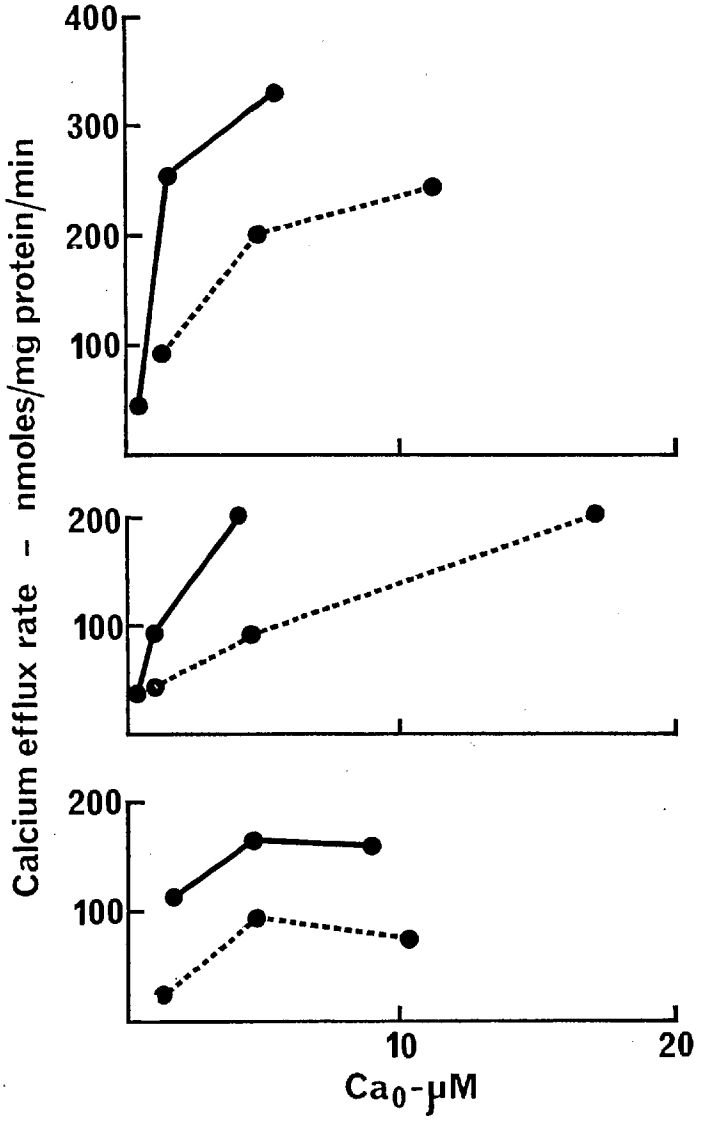



Figure 6

Inhibition of calcium efflux rate from cardiac sarcoplasmic reticulum by 10 mM $MgCl_2$

Three separate experiments are shown.
Conditions as in Figure 3.

●——● 5 mM $MgCl_2$,

●-----● 10 mM $MgCl_2$.

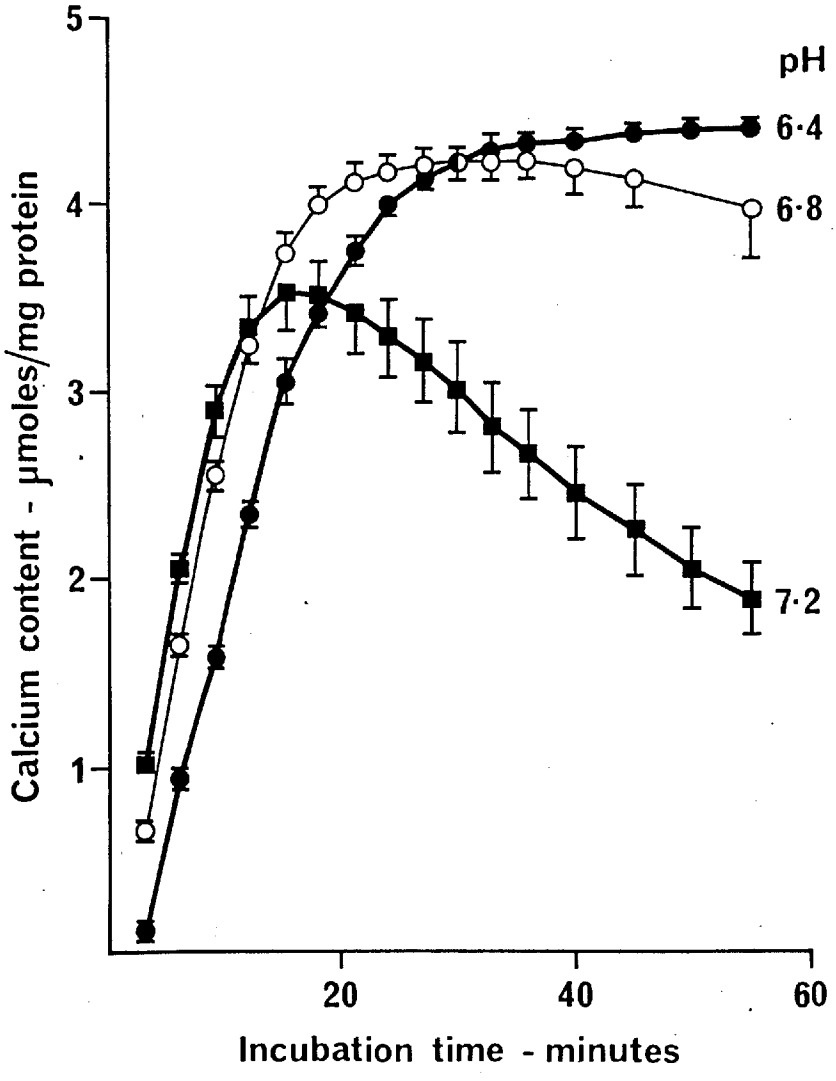



Figure 7

Effect of pH on net calcium uptake and release by cardiac sarcoplasmic reticulum, at an initial $CaCl_2$ concentration of 45 µM

Each point represents the mean of six experiments ± S.E.M. Temperature 25°C. Other conditions as described in 'Methods'.

●——● pH 6.4,

○——○ pH 6.8,

■——■ pH 7.2.

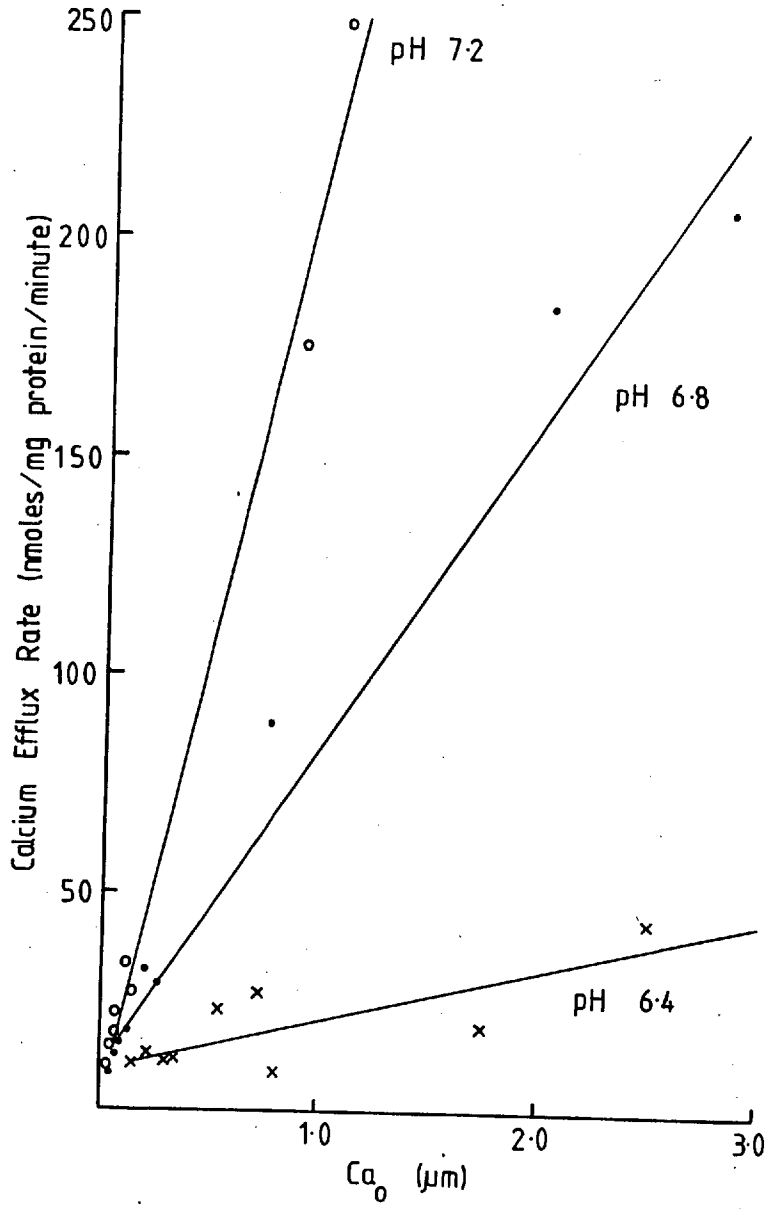



Figure 8

Effect of pH on calcium efflux rates from cardiac sarcoplasmic reticulum

Rates were measured after 20 minutes incubation time and plotted against $Ca_o$, at pH 6.4, 6.8 and 7.2. Data from three separate experiments at each pH are included. Regression lines calculated by computer analysis. Temperature 25°C. Other conditions as described in 'Methods'.

x pH 6.4,

● pH 6.8,

o pH 7.2.

At pH 6.4 the rate of calcium efflux at any $Ca_o$ concentration is substantially depressed compared to that at pH 6.8. At the $Ca_o$ concentrations studied, the rate of calcium efflux at pH 7.2 exceeded that at pH 6.4 or 6.8.

## SECTION V

## Discussion

### (a) Effect of $Ca_o$ on calcium efflux

The data in Table 3 show that a high concentration of $Ca_o$ causes a spontaneous net release of calcium from cardiac sarcoplasmic reticulum. Comparable findings have also been described for skeletal muscle sarcoplasmic reticulum. Entman et al. (1973), using the murexide technique, observed spontaneous calcium release following calcium accumulation in the presence of oxalate. These authors did not, however, correlate the release with a high calcium concentration in the initial medium, but with low ATP concentration. Weber et al. (1971) observed that net calcium release from vesicles that had been preloaded with calcium in the absence of precipitating ions, was inhibited in a calcium-free solution. Makinose (1973) showed that, when skeletal muscle sarcoplasmic reticulum vesicles were loaded with calcium, the addition of a second portion of calcium induced not only a resumption of calcium uptake, but also a simultaneous outflux of calcium.

Detailed studies on the calcium permeability of isolated skeletal SR have been carried out by Katz et al. (1977a). The ability of skeletal SR to retain calcium was influenced by the calcium concentration in the medium at the time that the calcium uptake reaction approached steady state. In reactions started with a high ratio between total calcium

and protein, spontaneous calcium release occurred earlier and more rapidly than in reactions started with a low ratio between calcium and protein (Katz et al., 1977a). Net calcium release was followed by a re-uptake of calcium (Katz et al., 1980). These observations were attributed to a stimulation of calcium efflux from the SR at high levels of $Ca_o$ (Katz et al., 1977a). Stimulation of calcium efflux rate from cardiac SR by increasing concentrations of calcium in the medium is shown in Figure 4. These results also resemble those described for skeletal SR (Katz et al., 1977). Maximum rates of calcium efflux from skeletal SR of 369 nmoles/mg protein/minute have been reported, with half maximal velocity at 0.92 μM $Ca_o$ (Katz et al., 1977b), in experiments in which 50 mM phosphate was used as precipitating anion, and thus at a calculated inside calcium concentration of 150 μM. In cardiac SR a maximum calcium efflux rate velocity of 150-200 nmoles/mg protein/minute has been reported, also in experiments carried out in the presence of 50 mM phosphate, with half maximum stimulation at 2-4 μM $Ca_o$. In experiments in which 2.5 mM potassium oxalate was used as the precipitating anion, resulting in a calculated inside calcium concentration of 8 μM, calcium efflux rate exhibited non-saturation kinetics with respect to $Ca_o$ (Kirchberger and Wong, 1978). This finding is similar to that shown in Figure 4, where saturation of calcium efflux rate was not observed in reactions carried out in the presence of 2.5 mM potassium oxalate. The slightly higher rate of calcium efflux for a given $Ca_o$ concentration in the results shown in Figure 4, could be due to the higher incubation temperature used in this set of experiments. Calcium efflux rates in experiments carried out at 25°C (see Figure 13, control experiment, pH 6.8), were comparable to those reported by Kirchberger and Wong (1978) for a similar $Ca_o$ concentration, and are slightly lower than those reported by Katz et al. (1977a)

for skeletal SR. However, comparison of different sets of data is made difficult by the different methods used to calculate free calcium concentration. In the results presented, free calcium concentrations were calculated by computer analysis, using published binding constants. In published results for skeletal (Katz et al., 1977a) and cardiac (Kirchberger and Wong, 1978) SR, free calcium concentrations were calculated according to the equations described by Katz et al. (1970).

(b) Effect of $Ca_i$ on calcium efflux

The inside calcium concentration of the SR ($Ca_i$) can be calculated from the solubility product of calcium oxalate of $2 \times 10^{-8}$ M (Hasselbach and Makinose, 1963), and of calcium phosphate of $7.5 \times 10^{-6}$ M (W. Hasselbach, unpublished observations). Preliminary experiments in which $Ca_i$ was varied by using different concentrations of oxalate and phosphate in the incubation medium, showed calcium efflux rate to be independent of $Ca_i$ (Katz et al., 1976; 1977a). In these experiments, $V_{max}$ was not reached. It was later shown, in experiments using two different concentrations of phosphate in the incubation medium, that $V_{max}$ was reduced at higher $Ca_i$ concentrations, but no appreciable change in KCa was observed when either phosphate or oxalate concentration in the medium was changed (Katz et al., 1977b). In cardiac SR, replacement of 50 mM phosphate ($Ca_i$, 150 µM) with 2.5 mM potassium oxalate ($Ca_i$, 8 µM) led to an increase in the rate of calcium efflux, which is particularly marked at high external calcium concentrations. The effect of $Ca_i$ on $V_{max}$ was not studied, since in the presence of oxalate a maximum rate of calcium efflux was not obtained (Kirchberger et al., 1978).

In all the experiments described above, the internal calcium concentration was controlled by varying the concentration of oxalate or phosphate in the medium. In the presence of oxalate or phosphate,

the free calcium concentration inside the vesicles increases only until the solubility product of the calcium oxalate, or calcium phosphate, is reached. When the solubility product is reached, calcium oxalate or calcium phosphate precipitates, and the internal free calcium concentration is maintained at a constant level. The internal free calcium concentration at which precipitation starts is lower in the presence of oxalate than in the presence of phosphate, and decreases as the concentration of oxalate or phosphate is increased. Although it is well established that oxalate and phosphate cross the membrane of the SR (Martinosi and Verelos, 1966a) causing calcium precipitates which are visible under EM (Hasselbach and Makinose, 1961; Martinosi and Feretos, 1966a), thus lowering the internal free calcium concentration, it is possible that these ions also have other effects. A calcium-dependent protein kinase which converts phosphorylase a to phosphorylase b has been shown to be associated with the SR, and it was suggested that the protein kinase activates, and a protein phosphatase inhibits, the calcium transport ATPase, providing a regulatory system for the calcium transport process (Horl et al., 1978 a and b). Oxalate has been shown to inhibit phosphatase activity (Horl et al., 1978a), and it has been suggested that oxalate exerts its stimulating effect on calcium transport by its inhibitory effect on the protein phosphatase activity (Fabiato and Fabiato, 1979). Besides acting as a calcium precipitant, phosphate is necessary for the reversal of the calcium pump (Barlogie et al., 1971; Makinose, 1971; 1972; Panet and Selinger, 1972). The concentrations of phosphate used in calcium efflux experiments were 10-50 mM. These concentrations of phosphate in the medium significantly alter the ionic strength of the incubation medium, which in turn alters the free calcium concentration (J. Kentish, personal communication). Binding constants for calcium phosphate are

not available from the published literature, making accurate calculation of the free calcium concentration difficult. For these reasons, the experiments described here were carried out at a constant internal calcium concentration of approximately 8 μM, achieved by the presence of 2.5 mM oxalate in the incubation medium.

(c) Comparison with 'calcium triggered calcium release' from skinned muscle fibres

Experiments with skinned cardiac muscle fibres show that calcium can trigger the release of additional calcium from internal stores (see Section I). The threshold concentration of free calcium for calcium-induced release of calcium from adult mammalian ventricular cells ranges from $5 \times 10^{-8}$ M in the rat, to $3 \times 10^{-7}$ M in the rabbit (Fabiato and Fabiato, 1978b). These concentrations of calcium are substantially lower than the concentrations of free calcium used here and by others to stimulate calcium efflux from isolated cardiac SR, which range from $10^{-6}$ to $10^{-5}$ M. However, similarities can be observed between the two sets of experiments. The effect of magnesium to inhibit calcium efflux from cardiac SR (Figures 5 and 6) is similar to the inhibition by magnesium of cyclic phasic contractions induced by external calcium in skinned cardiac muscle cells (Fabiato and Fabiato, 1975b). The results are also consistent with published data showing inhibition of calcium release from isolated skeletal SR induced by calcium by magnesium (Inesi and Malan, 1976), and with the competitive inhibition by magnesium of calcium-stimulated calcium efflux from skeletal SR (Katz et al., 1977b).

The in vivo concentration of magnesium has been estimated to be approximately 1 mM. The calculated free magnesium concentration in the control experiments described here with 5 mM magnesium chloride,

is similar to this value. This would indicate that the free magnesium concentrations of 2.32 mM (7 mM magnesium chloride) and 5.12 mM (10 mM magnesium chloride) are unphysiologically high, and that the free magnesium concentration in vivo would allow calcium efflux to occur from the SR of cardiac muscle cells in vivo.

(d) Quantitative aspects of calcium efflux from sarcoplasmic reticulum

The yield of SR from guinea pig ventricular muscle isolated by the method reported here, was approximately 0.8 to 1.2 mg/gm ventricular muscle. It has, however, been reported that 70% of cardiac SR is lost in the initial low speed spin following homogenisation, using oxalate supporting calcium uptake as a specific marker for SR. It was calculated that the real SR content of cardiac muscle is 6.8 mg/gm wet weight of heart (Solaro and Briggs, 1974).

At 1 μM outside calcium concentration, calcium efflux rate from cardiac SR is approximately 100 nmoles/mg protein/minute. If 1 gm muscle contains 6.8 mg of SR then the amount of calcium released at 1 μM free calcium would be 680 nmoles/gm muscle/minute, or approximately 11 nmoles/gm muscle/second. It has been calculated that 200 nmoles of calcium are required to saturate the binding sites of troponin (Katz, 1970), and this amount of calcium should be made available within 200 milliseconds. The rate of calcium efflux from cardiac SR reported here is therefore too slow to provide sufficient calcium to cause contraction of the myofilaments. However, if calcium enters the cardiac cell during the action potential via the t-tubules in the region of the diad, local concentrations of calcium around the terminal cisternae could be relatively high, triggering a relatively rapid rate of calcium efflux. The calcium triggered calcium release also requires a rapid

change in myoplasmic calcium concentration, whereas changes in $Ca_o$ in the above experiments are slow.

(e) Effect of pH

The increase in the amount of calcium initially taken up by cardiac SR at pH 7.2, compared with 6.4 (Figure 7), is consistent with pH optimum of 7.4 for the calcium loading of skinned cardiac cells reported by Fabiato and Fabiato (1978a). Since ATP-independent calcium uptake is not affected by alteration of pH over the range studied, the effect is due, at least in part, to an increase in the calcium activated ATPase activity. The pH optimum for cardiac calcium activated ATPase activity has been reported to be rather high at 7.8 - 8.3 (Shigekawa et al., 1976), so that an increase in the calcium-activated ATPase activity of the present preparation would be expected as the pH is raised from 6.4 to 7.2. The increased rate and earlier onset of net calcium release following calcium uptake at pH 7.2, may explain the earlier observations that the pH optimum for calcium uptake shifts toward acidity with increasing incubation time (Streter, 1969), and may also explain the variation in pH optima for calcium uptake reported in the literature (Nakamuru and Schwartz, 1970; 1972; Huxtable and Bressler, 1974; Streter, 1969; Chimoskey and Gergeley, 1968a; Carvalho and Leo, 1967). The increase in rate of net calcium release at high pH is consistent with the increase in the unidirectional calcium efflux rate at pH 7.2, shown in Figure 8, and is probably due to the increased calcium permeability of the SR at alkaline pH, which has been reported by several authors (e.g. Duggan and Martinosi, 1970). This result agrees with the pH optimum of 7.4 for calcium triggered calcium release from skinned cardiac muscle fibres (Fabiato and Fabiato, 1978).

In mammalian cardiac muscle, a fall in pH reduces tension development (McElroy et al., 1958; Lorkovic, 1966; Johannsson and Nilsson, 1975; Cingolani et al., 1975; Williamson et al., 1975). The observation that a metabolic acidosis caused by large anions has less effect than a respiratory acidosis caused by carbon dioxide which diffuses rapidly into the cell (Cingolani et al., 1970; Poole-Wilson and Langer, 1975), has led to the suggestion that the negative inotropic effect of an acidosis is brought about via an action on the subcellular organelles that control excitation-contracting coupling (Williamson et al., 1975; Tsien, 1976). The effect of pH on cardiac SR reported here, to reduce both the amount of calcium taken up, and the rate of calcium efflux at acid pH, could contribute to the observed physiological effect of an acidosis on the heart, although other mechanisms involved in the activation and generation of tension development, such as the slow inward calcium current (Chesnais et al., 1975; Kohlhardt et al., 1976), the calcium sensitivity of the myofilaments (Fabiato and Fabiato, 1978a) and the ATPase activity of the myofibrils (Kentish and Nayler, 1977; Schadler, 1967), have also been shown to be pH sensitive.

(f) Mechanism of calcium efflux

The mechanism of calcium efflux stimulated by external calcium described here, is distinct from the carrier mediated calcium release which occurs during reversal of the calcium pump and involves membrane phosphorylation by phosphate and ATP synthesis coupled with calcium release. Calcium efflux, described here, is stimulated by external calcium, and inhibited by external magnesium, whereas carrier-mediated efflux is inhibited by external calcium and requires magnesium outside the SR. Carrier-mediated calcium release also requires saturation of

the low affinity calcium binding sites inside the SR, whereas calcium stimulated calcium efflux appears to be reduced at higher inside calcium concentrations. Also, calcium stimulated calcium efflux occurs in the presence of ATP, which is inhibitory to carrier-mediated calcium efflux. It seems that the effect of a high concentration of calcium outside the SR must be to increase the permeability of the SR membrane to calcium. An increase in SR permeability accompanying an increase in $Ca_o$ at any given level of $Ca_i$ has been shown by Katz et al. (1977a). An increase in the permeability of the SR membrane to calcium has also been postulated to account for calcium triggered calcium release in skinned muscle cells (Endo, 1977).

It has been observed that calcium triggered calcium release from skinned muscle cells requires preloading of the SR with calcium (Endo, 1975b). Using isolated skeletal muscle sacroplasmic reticulum, it has been shown that, at the start of the reaction, calcium influx rate is high, and calcium efflux rate is minimal. Calcium influx rate slows during the course of the reaction, and calcium efflux rate increases. When calcium efflux rate exceeds calcium influx rate, net calcium release is observed. This shows that, in isolated SR, calcium stimulated calcium efflux also requires preloading of the SR with calcium to a certain level (Katz et al., 1980). These time-dependent changes in calcium influx and efflux rate cannot be related to the calcium concentration inside the SR since there is evidence that both the rate and extent of spontaneous net calcium release (Katz et al., 1980) and of unidirectional calcium efflux (Katz et al., 1977a) are reduced at high levels of $Ca_i$. It is possible that the calcium release phenomenon observed is related to the ratio of inside to outside concentration of calcium. A correlation between this ratio and the calcium permeability of the SR membrane has been reported (Katz et al., 1976; 1977a).

Although the calcium release which occurs during reversal of the calcium pump coupled with ATP synthesis is too small to be of significance in excitation-contraction coupling, the possibility has been raised by several investigators that the calcium pump is still involved in calcium release from the SR by another mechanism. Incorporation of the purified ATPase enzyme into phospholipid vesicles results in an increased passive permeability of these vesicles to calcium (Jilka and Martinosi, 1975). In isolated skeletal muscle SR, the dependence of calcium efflux rate on external calcium concentration and its inhibition by high internal calcium concentration is similar to that of calcium uptake, indicating the possible involvement of a single transport site in the two processes (Katz et al., 1977b). A similar argument applies to calcium efflux from isolated cardiac SR. In addition, in cardiac SR, treatment with cyclic AMP and protein kinase, which increases the rate of calcium uptake by cardiac SR, results in a 2-3 fold decrease in the $KCa_o$ for calcium efflux (Kirchberger and Wong, 1978). Ogawa and Ebashi (1976), using isolated skeletal muscle SR, have shown that calcium release from loaded SR could be induced by the non-hydrolysable ATP substitute, β-γ methylene adenosine triphosphate. The release was competitively inhibited by ATP, and was similar to calcium induced calcium release in that the amount of calcium released was increased in heavily loaded SR, and decreased when the calcium concentration in the medium decreased. This led to the suggestion that the calcium-ATP-enzyme complex is required for calcium release, although the possibility that ATP occupancy of the regulatory site is required was not investigated (Ogawa and Ebashi, 1976).

Martinosi et al. (1977) have put forward a model which associates both calcium uptake and calcium release with the calcium transport ATPase of the SR. The theory assumes that the ATPase occurs in the membrane in

a tetrameric form, but in dynamic equilibrium with the monomeric and dimeric forms. They propose that the increase in the permeability of the SR during excitation is brought about by a potential dependent shift in the equilibrium between monomers and tetramers in favour of tetramer formation. The formation of tetramers is proposed to result in the formation of hydrophilic channels across the membrane permitting the rapid release of calcium. During relaxation, the equilibrium would shift to favour the formation of monomers and dimers, with a decrease in the passive permeability of the membrane to calcium, and active calcium uptake into the SR.

Although there is no direct experimental evidence for this model, fluorescence energy transfer studies of the ATPase labelled with different fluorescent indicators, support the proposition of the reversible formation of ATPase tetramers in the membrane (Martinosi et al., 1977).

Since calcium has been shown to induce conformational changes in the ATPase molecule (see Chapter 1, Section V ), it is possible that, according to the above model, calcium, either at the external or internal surface of the SR, or the gradient of calcium across the membrane, causes the changes in the arrangement of ATPase molecules which would then allow calcium release.

(g) Physiological significance of calcium triggered calcium release

In skeletal muscle, where the threshold concentration of calcium required for the activation of calcium release is higher than the concentration of calcium required for contraction, it seems unlikely that calcium triggered calcium release has a physiological role to play. The mechanism could, however, play a role under certain physiological or pharmacological conditions, such as the contracture induced by

caffeine. In skinned skeletal muscle fibres the concentration of 'trigger' calcium required to induce the release of additional calcium from the SR, is lowered in the presence of caffeine (Endo, 1975b). In isolated skeletal muscle SR, caffeine has been found to be more effective in promoting calcium release at high outside concentrations of calcium (Katz et al., 1977).

In skinned muscle preparations, the concentration of calcium which will trigger the release of additional calcium from the SR is lower in cardiac than in skeletal preparations. In skinned cardiac muscle fibres this 'threshold' concentration of calcium is lower than the concentration of calcium required directly to activate contraction, and it is therefore possible that, in cardiac muscle, calcium triggered release of calcium plays an important physiological role. Thus, in cardiac muscle, the idea that calcium entering the cardiac cell during the action potential, triggers the release of additional calcium from the SR, provides an attractive hypothesis for the mechanism of excitation-contraction coupling. However, in isolated SR preparations, there is no evidence that calcium efflux occurs at lower outside calcium concentrations in cardiac as compared to skeletal preparations.

# CHAPTER 3

# EFFECT OF CARDIOACTIVE DRUGS ON CALCIUM ACCUMULATION AND CALCIUM EFFLUX FROM CARDIAC SARCOPLASMIC RETICULUM

SECTION I

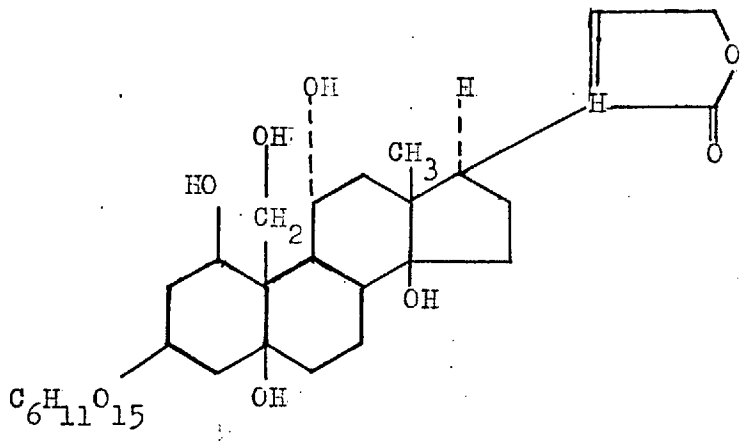
Introduction

A number of cardioactive drugs which either increase (cardiac glycosides), or decrease (calcium antagonists), the force of contraction, are thought to exert their effect by interfering with the calcium regulation of the cell. Due to the importance of the SR in regulating intracellular calcium levels, it is possible that these drugs could act by interfering with the calcium transport system of the SR.

In this thesis, the effect of ouabain, a cardiac glycoside, and of verapamil, a calcium antagonist, on calcium efflux rate from cardiac SR have been studied. The effect of diltiazem, a new drug with some properties of a calcium antagonist, on calcium accumulation and efflux from cardiac SR, has also been investigated.

(a) Ouabain

Ouabain is a cardiac glycoside with the following structure:




The cardiac glycosides have the effect of intensifying ATP utilisation for contraction, so that tension development and oxygen requirement per heart beat rises (Fleckenstein, 1971). Ouabain exerts a positive inotropic effect at a concentration of $2 \times 10^{-7}$ M to $6 \times 10^{-7}$ M and higher concentrations of $2 \times 10^{-6}$ M cause contracture in guinea pig

atria (Lullman and Holland, 1962). Ouabain is a potent inhibitor of the cardiac sodium potassium activated ATPase activity, associated with the sarcolemma (Schwartz et al., 1969). The decrease in sodium potassium activated ATPase activity caused by ouabain has been correlated with the ouabain-induced enhancement of contractile force (Akera et al., 1970; Besch et al., 1970). However, the action of ouabain on cardiac muscle is not thought to be due directly to its inhibitory effect on the sodium potassium activated ATPase enzyme, since the effect of cardiac glycosides is highly sensitive to the concentration of calcium in the medium (Glynn, 1964; Thomas, 1960). It has been suggested that the action of cardiac glycosides is caused by the removal or inactivation of intracellular calcium (Glynn, 1964). This could be secondary to an increase in intracellular sodium, which would compete with intracellular calcium for intracellular binding sites (Glynn, 1964). However, there is little evidence for any change in either intracellular sodium or potassium concentration on treatment with ouabain (Lee and Klaus, 1971).

Cardiac glycosides apparently have no effect on the slow inward current of the cardiac action potential carried by calcium (Josephson and Sperelakis, 1977; Lee and Klaus, 1971). However, an increase in the total exchangeable tissue calcium at concentrations of ouabain exerting a positive inotropic effect has been observed (Lullmann and Holland, 1962).

This raises the possibility that ouabain may exert its effect by altering the calcium transporting properties of intracellular organelles, such as the mitochondria and the SR. There are some reports of a slight reduction in the calcium uptake of cardiac SR in the presence of ouabain (e.g. Carsten, 1967). However, reports show

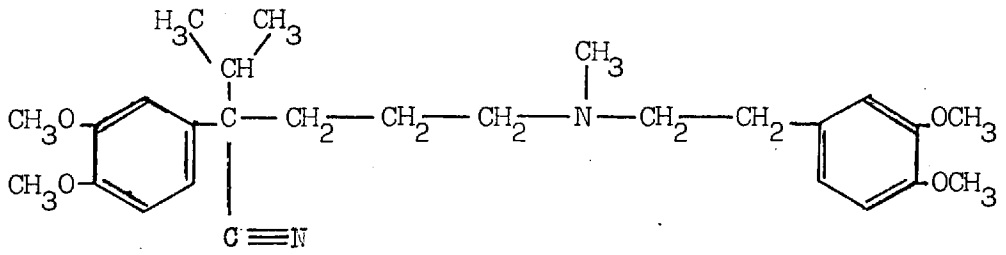
no effect of ouabain on calcium transport by SR isolated from ouabain treated hearts (Besch et al., 1970), or isolated SR treated with ouabain in vitro (Besch et al., 1970; Schwartz et al., 1969; Klaus and Lee, 1969; Entman et al., 1972).

However, Klaus and Lee (1969) showed an increase in the freely releasable calcium of the SR, measured by the change in absorbance beyond the initial baseline on adding EGTA, induced by ouabain. They correlated the increase in the amount of releasable calcium with the positive inotropic effect of ouabain.

Experiments have been undertaken to determine the effect of $10^{-6}$ M ouabain on calcium release from cardiac SR.

(b) Verapamil

Verapamil belongs to the class of drugs known as the calcium antagonists, and has the following structure:



The effect of the calcium antagonist drugs is exactly the same as the withdrawal of calcium, in that they abolish the contractile response, and this effect is counteracted by raising the calcium concentration in the medium. This indicates that the drugs exert their effect on excitation-contraction coupling (Fleckenstein, 1971; Fleckenstein and Spah, 1980). Verapamil has been shown to inhibit the slow influx of calcium into the cardiac cell during the action potential at a concentration of $10^{-6}$ M (Fleckenstein and Spah, 1980),

and has also been shown to inhibit calcium binding to isolated cardiac sarcolemma (Mas-Oliva, 1980). The d-and l-iosmers were found equally effective in inhibiting passive calcium binding to the sarcolemma, but active calcium transport associated with the calcium dependent ATP hydrolysis is only reduced by the l-isomer (Mas-Oliva, 1980).

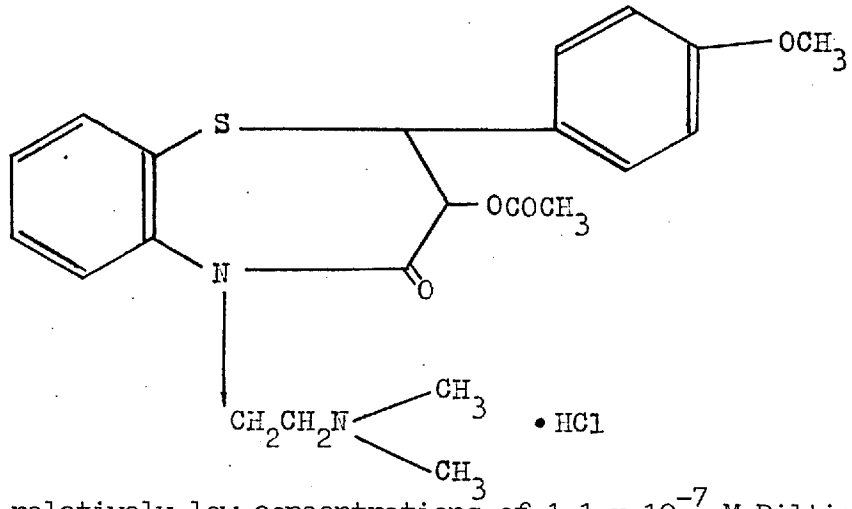
This is consistent with the finding that it is the l-isomer of Verapamil which inhibits the slow calcium channel (Bayer et al., 1975b) and produces depression of the contraction amplitude (Bayer et al., 1975a) in papillary muscles. Although the inhibitory effect of verapamil on the slow inward calcium current is well established, some authors have calculated that this inhibition is insufficient in itself to account for the observed physiological and pharmacological effect of the drug, and an additional effect on the calcium transport system of the SR has been postulated (Bayer et al., 1975).

However, Verapamil has been shown to reduce calcium uptake and binding by isolated cardiac sarcoplasmic reticulum only at very high concentrations of $10^{-3}$ M, and does not effect spontaneous calcium release from the SR, or the release induced by calcium ionophores (Entman et al., 1972a. Calcium triggered calcium release from skinned cardiac muscle cells is also only affected by concentrations of Verapamil of $10^{-3}$ M (Fabiato and Fabiato, 1973).

In this thesis, experiments have been carried out to ascertain the effect of Verapamil on calcium efflux from the SR, at various concentrations of $Ca_o$.

(c) Diltiazem

Diltiazem is another drug which has been classed as a calcium antagonist, and has the following structure:




At relatively low concentrations of $1.1 \times 10^{-7}$ M Diltiazem reduces the contractile force, and this effect is antagonised by calcium. At these low concentrations of the drug, the action potential is not altered (Nakajima et al., 1975; 1976). At higher concentrations of $1.1 \times 10^{-5}$ M there is a reduction in the maximum velocity of contraction, $V_{max}$, and overshoot of the action potential, and also a shortening of the action potential, coupled with the abolition of contractile force. These effects are also antagonised by calcium (Nakajima et al., 1976). Since at low concentrations Diltiazem has no effect on the action potential, it has been suggested that it acts by reducing the amount of calcium available intracellularly, possibly by inhibiting calcium release from the SR (Nakajima et al., 1976).

The effect of Diltiazem on calcium binding, calcium uptake and calcium efflux from isolated cardiac SR is described here.

SECTION II

Methods

(a) Isolation of sarcoplasmic reticulum

Cardiac microsomal fractions rich in sarcoplasmic reticulum were isolated by the method described in Chapter 2. Protein concentration of isolated fractions was determined by the method of Lowry et al. (1951), as described previously.

(b) Calcium binding measurements

Calcium binding was measured in a medium containing 120 mM potassium chloride, 40 mM histidine, pH 6.8, 5 mM magnesium ATP and 500 µg/ml microsomal protein. The reactions were carried out at 25°C and the total reaction volume was 1 ml. Reactions were started by adding calcium chloride labelled with $^{45}Ca^{++}$ to a final concentration of 100 µM. Samples of 100 µl were withdrawn at the times indicated, and filtered through millipore filters of 0.45 µ diameter pore size. The filters were washed with 10 ml of a solution containing 120 mM potassium chloride, 40 mM histidine, pH 6.8. Radioactivity remaining on the filters was measured by liquid scintillation spectrometry using Instagel as scintillant.

(c) Calcium uptake and calcium efflux measurements

Calcium uptake and calcium efflux were measured by the method described in Chapter 2.

SECTION III

Results

(a) Ouabain

Figure 9 shows the rate of calcium efflux from cardiac SR, at various concentrations of $Ca_o$ in the presence and absence of $10^{-6}$ M ouabain. Ouabain at this concentration has no significant effect on calcium efflux rate at any concentration of $Ca_o$ studied.

(b) Verapamil

Figure 10 shows the effect of Verapamil at a concentration of $10^{-5}$ M on calcium efflux rate at various concentrations of $Ca_o$, from isolated cardiac sarcoplasmic reticulum. No significant effect of Verapamil on calcium efflux rate was observed at this concentration.

(c) Diltiazem

Figures 11 and 12 show the effect of $10^{-4}$ Diltiazem on calcium binding in the absence of precipitating ions, and on calcium uptake in the presence of 2.5 mM potassium oxalate, respectively, by isolated cardiac SR. Diltiazem clearly has no effect on either calcium binding or calcium uptake. Figure 13 shows the effect of $10^{-4}$ M Diltiazem on calcium efflux rate from isolated cardiar SR at various concentrations of $Ca_o$. As in the case of calcium binding and calcium uptake, calcium efflux rate is not affected by $10^{-4}$ M Diltiazem.

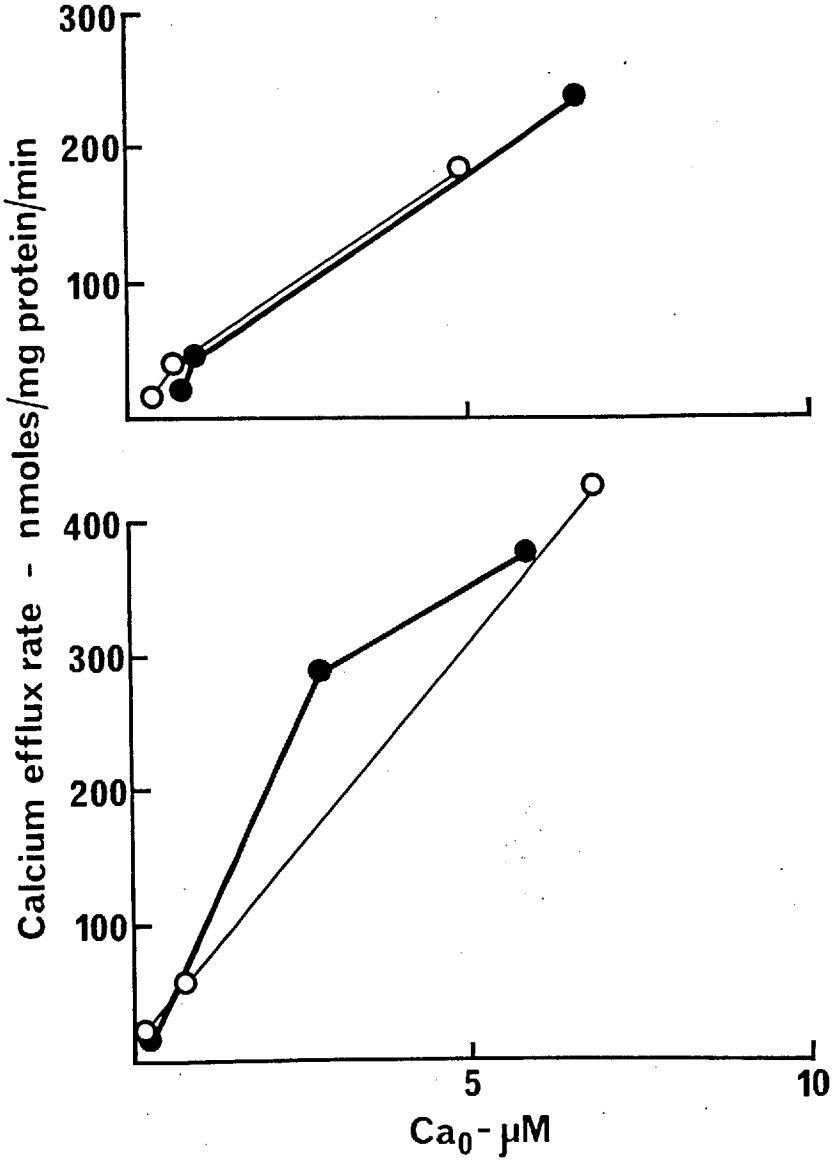



Figure 9

Effect of $10^{-6}$ M ouabain on calcium efflux from cardiac sarcoplasmic reticulum at various concentrations of $Ca_o$

Two separate experiments are shown. Temperature 37°C. Other conditions as in 'Methods'.

o——o control,

●——● $10^{-6}$ M ouabain.

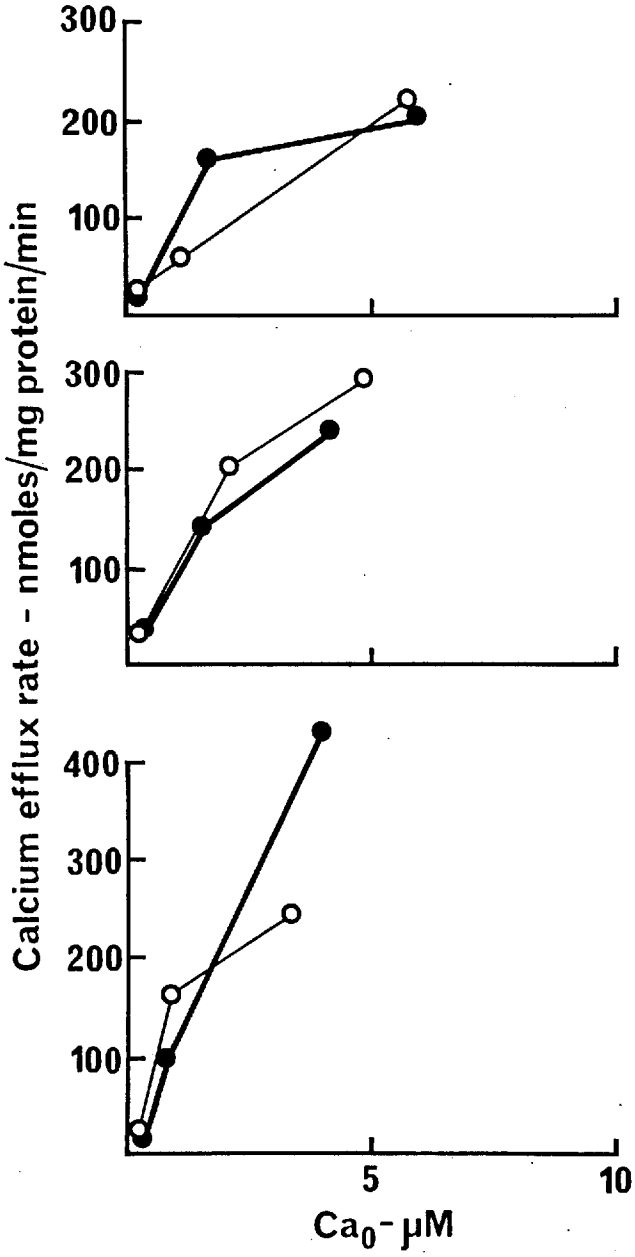



Figure 10

Effect of $10^{-5}$ M verapamil on calcium efflux rate from cardiac sarcoplasmic reticulum at various concentrations of $Ca_o$

Three separate experiments are shown.
Temperature 37°C. Other conditions as in 'Methods'.

o——o control,

●——● $10^{-5}$ M verapamil.

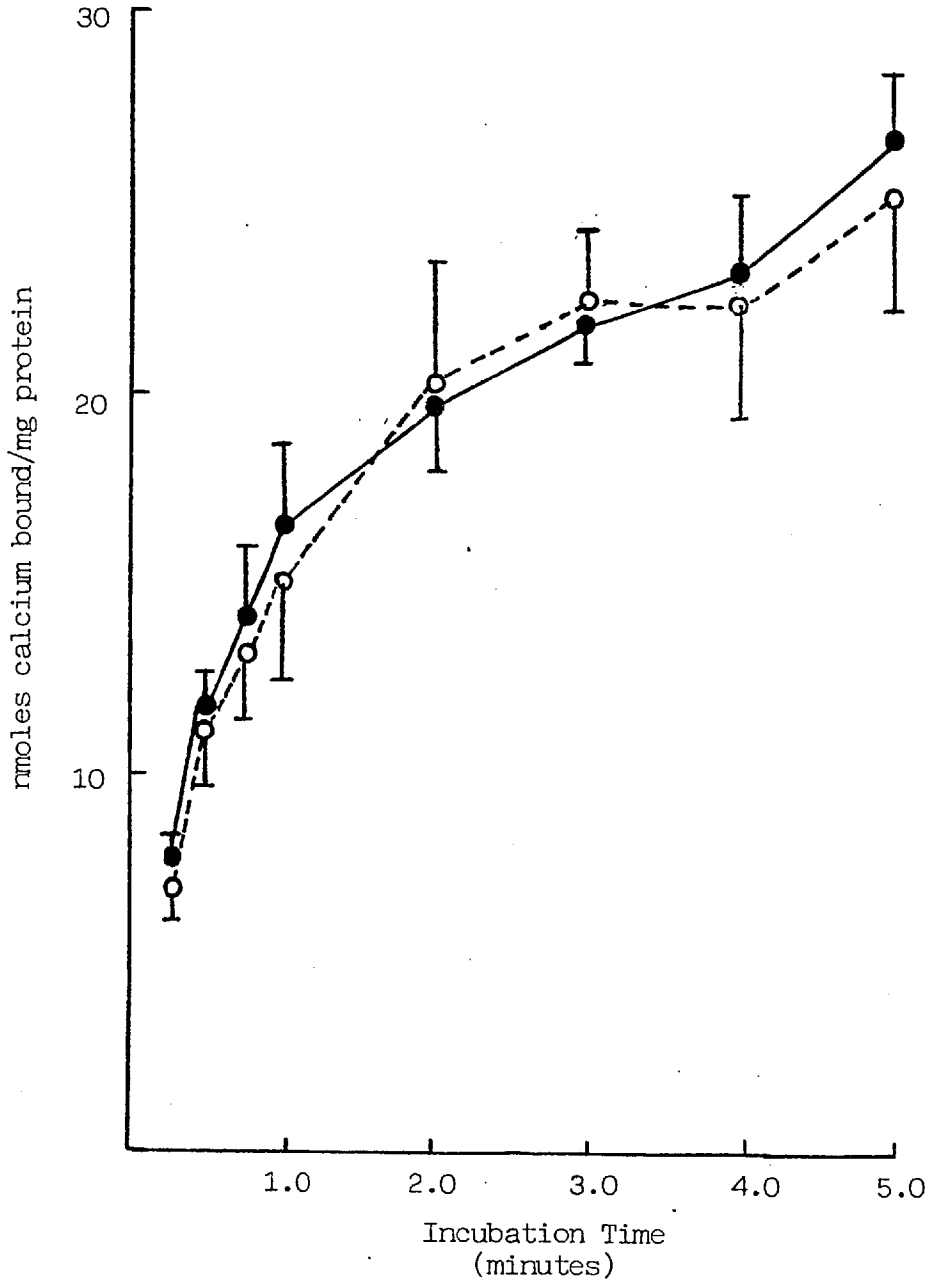



Figure 11

Effect of $10^{-4}$M diltiazem on calcium binding by cardiac sarcoplasmic reticulum

Results are means $\pm$ S.E.M. of three experiments. Temperature 25°C. Other conditions as in Methods.

●——● control o-----o $10^{-4}$M diltiazem

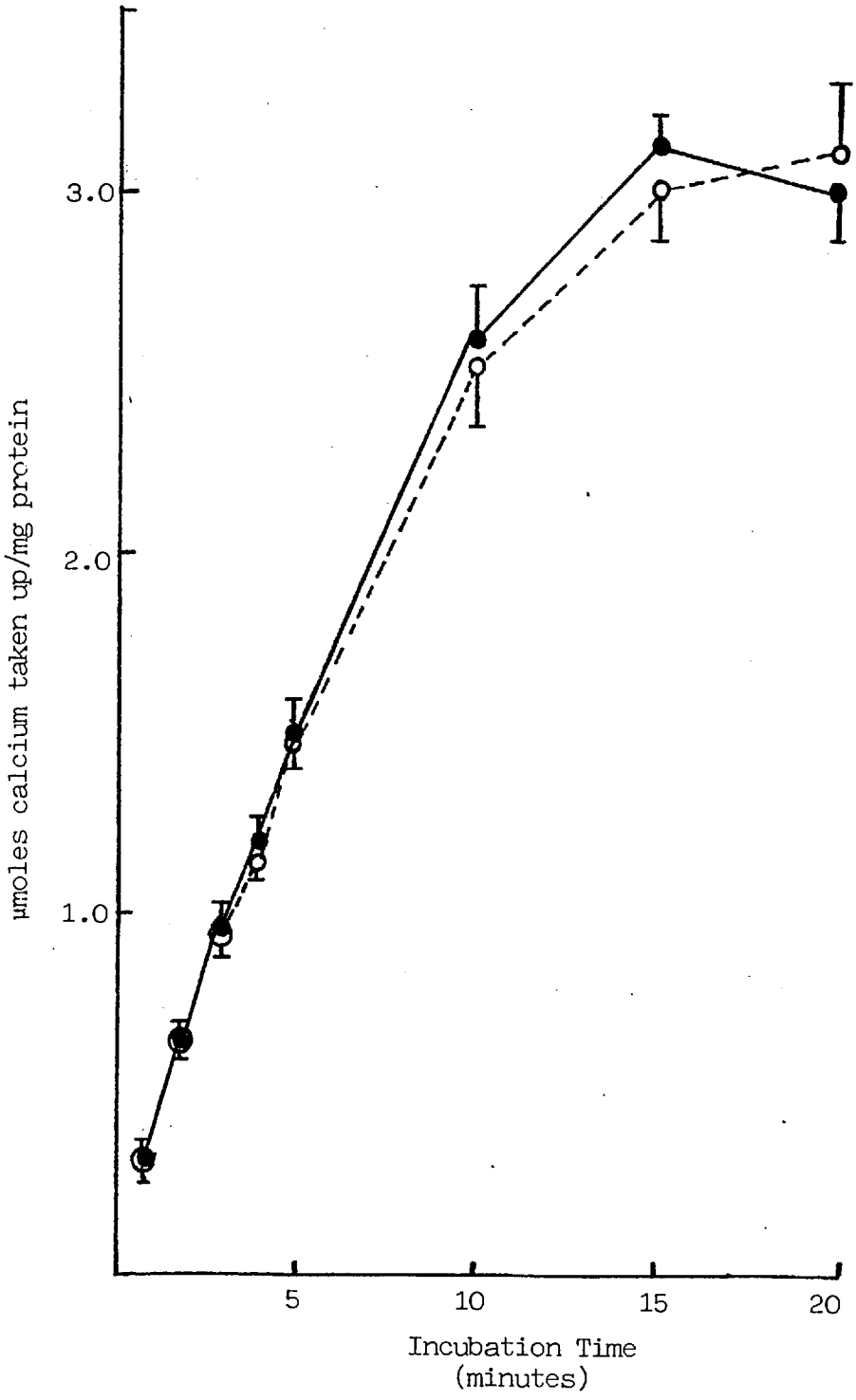



Figure 12

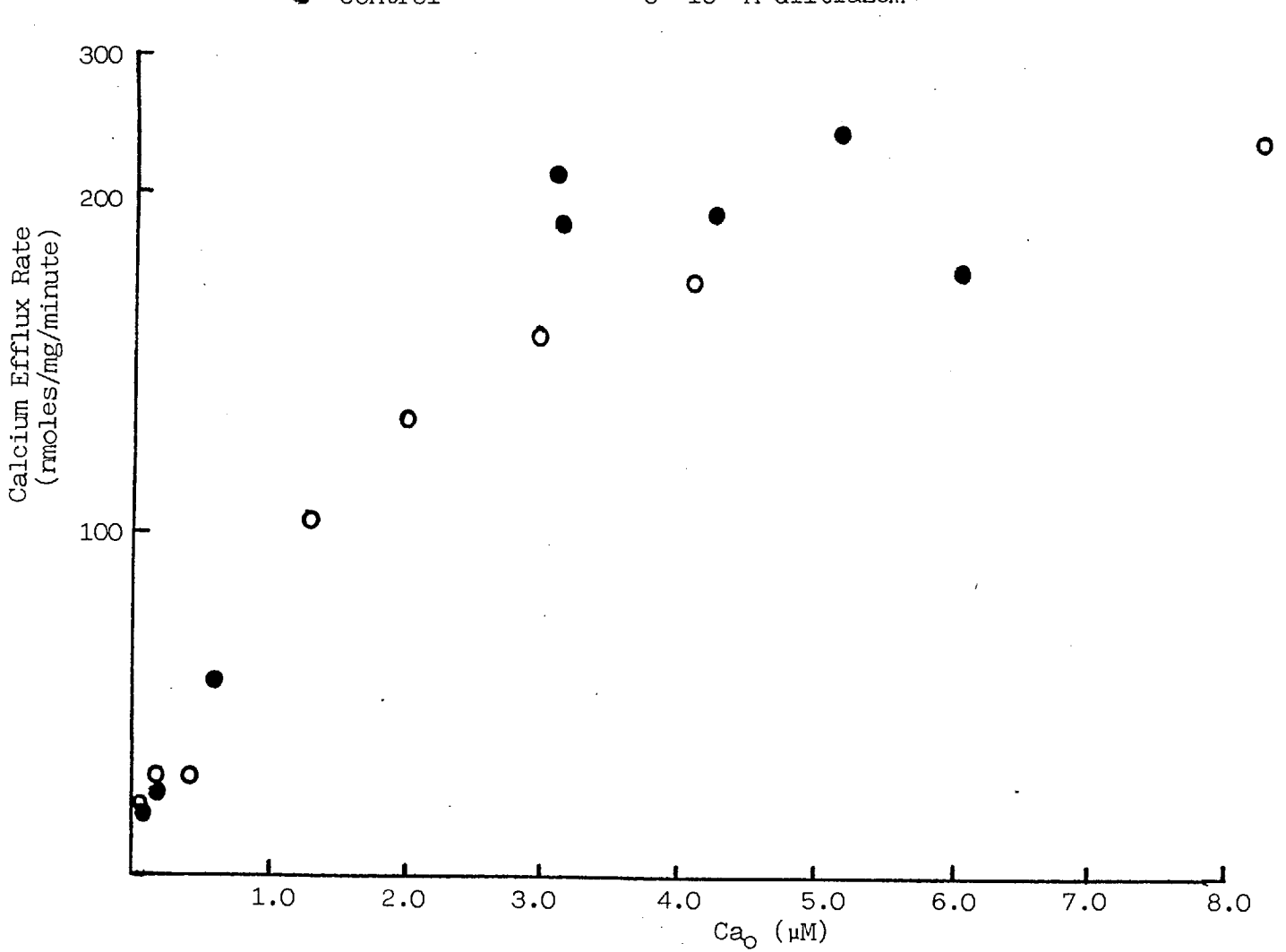
Effect of $10^{-4}$M diltiazem on calcium uptake in the presence of 2.5 mm potassium oxalate by cardiac sarcoplasmic reticulum

Results are means ± S.E.M. of three experiments.
Temperature 25°C. Other conditions as in Methods.

●——● control o-----o $10^{-4}$M diltiazem

Figure 13 Effect of $10^{-4}$M diltiazem on calcium efflux from cardiac sarcoplasmic reticulum

Results from three separate experiments are shown. Temperature 25°C. Other conditions as in Methods.



SECTION IV

Discussion

(a) Ouabain

The lack of effect of ouabain on calcium efflux rate from cardiac SR at concentrations which are inhibitory to the sodium potassium activated ATPase activity, provides further evidence that the SR is not the site of action of ouabain.

Although there is one report of increased calcium release from the mitochondria when treated with ouabain in vitro at $10^{-5}$ to $10^{-6}$ M, the role of the mitochondria in cardiac intracellular calcium regulation is doubtful, making it unlikely that the mitochondria are the site of action of ouabain (see Chapter 1, Section VII).

It seems, therefore, that ouabain must exert its effect by an action on the sarcolemma, although the mechanism of this effect is unknown. Ouabain has been shown to increase $^{45}Ca^{++}$ uptake into rabbit atria (Holland and Sekul, 1959; 1960). This effect is observed in electrically driven, but not in quiescent atria, indicating that ouabain-stimulated entry of calcium is associated with excitation-contraction coupling.

The sodium potassium ATPase enzyme is inhibited by calcium, and it has been suggested that calcium partially substitutes for potassium at one site on the sodium pump, and that the effect of ouabain is mediated by a calcium influx-potassium efflux exchange through the glycoside inhibited sodium pump (Lindenmeyer and Schwartz, 1975). This could explain the sensitivity of the effect of ouabain to the calcium concentration in the medium.

(b) Verapamil

The experiments described here show that Verapamil, at therapeutic concentrations, has no effect on calcium efflux from the SR at any outside calcium concentration. This is consistent with the hypothesis that the site of action of this drug is the sarcolemma. However, if calcium release from the SR is triggered by calcium entering the cardiac cell during the action potential (Bianchi and Bolton, 1966), Verapamil would act indirectly to inhibit calcium release from the SR, by inhibiting the influx of trigger calcium.

(c) Diltiazem

The lack of effect of Diltiazem on calcium transport activity of isolated cardiac SR indicates that the SR is not the site of action of this drug, although, as discussed for Verapamil, inhibition of the slow calcium current could lead to inhibition of calcium release from the SR, if calcium triggered calcium release is the mechanism by which calcium release from the SR is initiated in vivo. The observation that Diltiazem can inhibit contraction at low concentrations which do not affect the action potential is difficult to explain. However, recent experiments have shown that, under the appropriate conditions, Diltiazem can cause some inhibition of the slow calcium current at concentrations as low as $10^{-6}$ M (Clare Harman, personal communication).

# CHAPTER 4

# DEPOLARISATION-INDUCED CALCIUM RELEASE

SECTION I

## Introduction

According to the hypothesis of depolarisation-induced calcium release from the SR, the action potential, travelling in the transverse tubules, transmits an electrical signal to the terminal cisternae of the SR, resulting in depolarisation of the SR membrane and calcium release (Huxley and Taylor, 1958; Huxley and Straub, 1958).

There are several reports of depolarisation of the SR membrane in both isolated SR preparations and in skinned muscle fibres. Depolarisation of the SR membrane has been induced by electrical stimulation, or by ionic replacement in the medium.

### (a) Electrical stimulation

Electrical stimulation of a suspension of skeletal SR led to a reversible release of accumulated calcium. The degree of calcium release was substantially reduced in the presence of oxalate or phosphate. Similarly, electrical stimulation of heart and skeletal muscle glycerol-extracted fibres resulted in the contraction of the fibres, which was attributed to calcium release from the SR induced by electrical stimulation (Lee et al., 1966). However, no release of calcium from isolated skeletal SR was observed when the platinum electrodes were replaced by silver chloride electrodes. The release of calcium induced by stimulation using platinum electrodes was attributed to irreversible denaturation of the SR due to oxidation caused by chloride generated at the platinum electrode. The apparent re-uptake of calcium after cessation of electrical stimulation using platinum electrodes was attributed to binding of calcium to denatured SR (Myamoto and Kasai, 1973).

(b) Ionic replacement

When a less permeant anion such as methanesulfonate, proprionate or sulphate is replaced by a permeant anion, such as chloride, the potential across the SR membrane should be altered so that the inside of the SR becomes more negative with respect to the outside. A similar effect should be achieved by replacing a permeant cation, such as potassium, with a less permeant cation, such as sodium, tris or choline.

Depolarisation of the SR membrane by replacing methanesulfonate with chloride, while keeping the potassium concentration constant, led to a transient increase in the release of calcium from SR previously loaded with calcium (Kasai and Myamoto, 1973).

Using skinned skeletal muscle SR in which the SR was depleted of calcium by treatment with caffeine, and reloaded with calcium in a potassium methanesulfonate or sulphate medium, sudden replacement of potassium methane sulfonate with potassium chloride of equal ionic strength, led to a transient contraction of the fibre due to calcium release (Nakajima and Endo, 1973). That the calcium released from the SR originated from the SR was demonstrated using the calcium sensitive dye, aequorin (Endo and Blinks, 1973).

(c) pH Induced calcium release

On the basis of the observation that intracellular pH is directly related to membrane potential, and to extracellular pH, and that variations in membrane potential produce an almost instantaneous change in the intracellular pH, ranging from 7.69 in the maximally depolarised state, to 3.25 in the maximally hyperpolarised state (Carter et al., 1967), it was postulated that sudden changes in intracellular pH could be the trigger for calcium to be released from the SR (Nakamura and Schwartz, 1970; 1972).

There are several reports of calcium release from isolated skeletal SR when the pH of the medium is suddenly increased following calcium binding in the absence of precipitating ions at relatively low pH (Duggan and Martinosi, 1970; Nakamuru and Schwartz, 1970; 1972). This effect has been demonstrated using both millipore filtration and spectrophotometric techniques, and the rate of calcium release was similar to the initial rate of calcium binding (Nakamuru and Schwartz, 1970; 1972). A similar effect was reported using isolated cardiac SR (Nakamuru and Schwartz, 1970), but Hoeschen and Nayler (1976) were unable to detect any effect of a rapid change in pH on the calcium content of isolated cardiac SR. Similarly, Fabiato and Fabiato (1978a), using skinned muscle fibres, showed that a sudden increase in pH had no effect on calcium loading by cardiac SR, and led to only a slow reduction in the level of calcium loading in skeletal SR.

In view of this conflicting data, the present experiments were undertaken to compare the effect of a sudden increase in pH on isolated cardiac and skeletal muscle SR following calcium binding at pH 6.5.

## SECTION II

## Methods

### (a) Isolation of microsomal fractions

Cardiac microsomal fractions were isolated by the method described in Chapter 2.

Skeletal muscle microsomal fractions were isolated from the hind leg muscle of adult male albino guinea pigs (Dunken-Hartley strain). The muscle was trimmed and finely chopped, and the SR was

isolated in exactly the same way as cardiac microsomal fractions. Skeletal muscle SR fractions were used on the day of preparation, or on the following day.

Protein concentrations were estimated by the method of Lowry et al. (1951), standardised against crystalline bovine serum albumin.

(b) Calcium binding measurements

Calcium binding was measured in a medium containing 120 mM potassium chloride, 40 mM Tris-maleate buffer, pH 6.5, 5 mM magnesium ATP and 500 µg/ml microsomal protein. The reactions were carried out at 25°C and the total reaction volume was 1 ml. Reactions were started by adding calcium chloride labelled with $^{45}Ca^{++}$, to a final concentration of 100 µM. Samples of 100 µl were withdrawn at the times indicated and filtered through millipore filters of 0.45 µ diameter pore size. The filters were washed with 10 ml of a solution containing 120 mM potassium chloride, 40 mM Tris-maleate at the appropriate pH. Radioactivity remaining on the filters was measured by liquid scintillation spectrometry using Instagel as scintillant. In some experiments, the pH was increased to 7.6 at the time specified, by adding 20 µl of 0.8 M Tris.

SECTION III

Results

(a) Effect of pH on calcium release in skeletal muscle sarcoplasmic reticulum

Figure 14 shows calcium binding by skeletal SR at pH 6.5 and 7.6. At pH 6.5, just over 100 nmoles of calcium is bound/mg protein within

1 minute, and this is followed by the spontaneous release of a portion of the bound calcium. At pH 7.6, calcium binding is lower than at pH 6.5, with no spontaneous calcium release. A sudden increase in pH from 6.5 to 7.6, 45 seconds after starting the reaction, led to a rapid release of calcium. A sudden increase in pH at 5.0 or 9.5 minutes after the start of the reaction also led to a release of accumulated calcium (see Figure 15).

(b) Effect of pH on calcium release in cardiac muscle sarcoplasmic reticulum

Figure 16 shows calcium binding at pH 6.5 and 7.6 by isolated cardiac SR. At pH 6.5, less calcium is bound than by skeletal SR at the same pH, and there is no overshoot of the binding. At pH 7.6, less calcium is bound than at pH 6.5. If the pH is suddenly increased from 6.5 to 7.6, after 45 seconds' incubation time, and hence before calcium binding is complete, there is a gradual cessation of calcium binding, sometimes followed by a limited release of accumulated calcium, until intravesicular levels approach the levels observed when the whole reaction is carried out at pH 6.5. However, a sudden increase in pH after calcium binding is complete has no effect on the amount of calcium bound. In the experiments shown in Figure 17, the pH was increased from 6.5 to 7.6, at 5.0 and 9.5 minutes after the start of the reaction, respectively. No significant alterations in the calcium content of the SR was observed.

Similar results were obtained when the initial pH was set with 20 mM Hepes buffer, instead of Tris-maleate, and the pH suddenly increased to 7.6 by adding Tris.

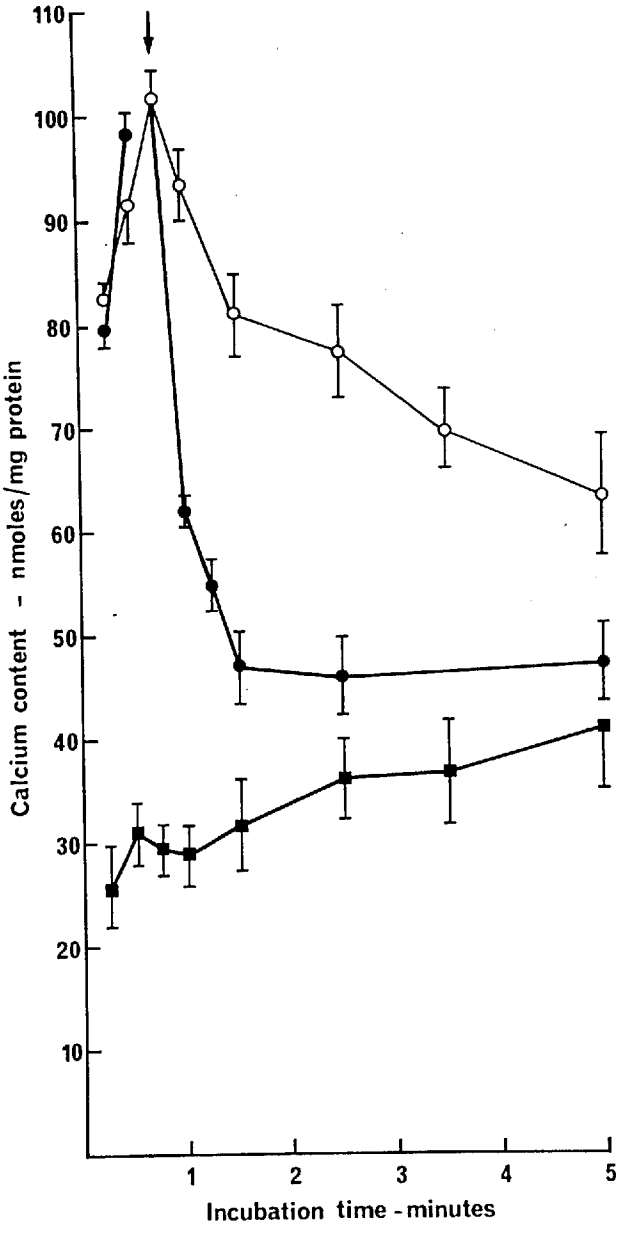



Figure 14

Effect of pH on calcium binding by skeletal sarcoplasmic reticulum

Each point represents mean ± S.E.M. of four or six experiments. Conditions as described in 'Methods'. pH was increased from 6.5 to 7.6 by addition of 20 µl Tris at the time indicated by the arrow.

o——o pH 6.5,

■——■ pH 7.6,

●——● pH increased from 6.5 to 7.6.

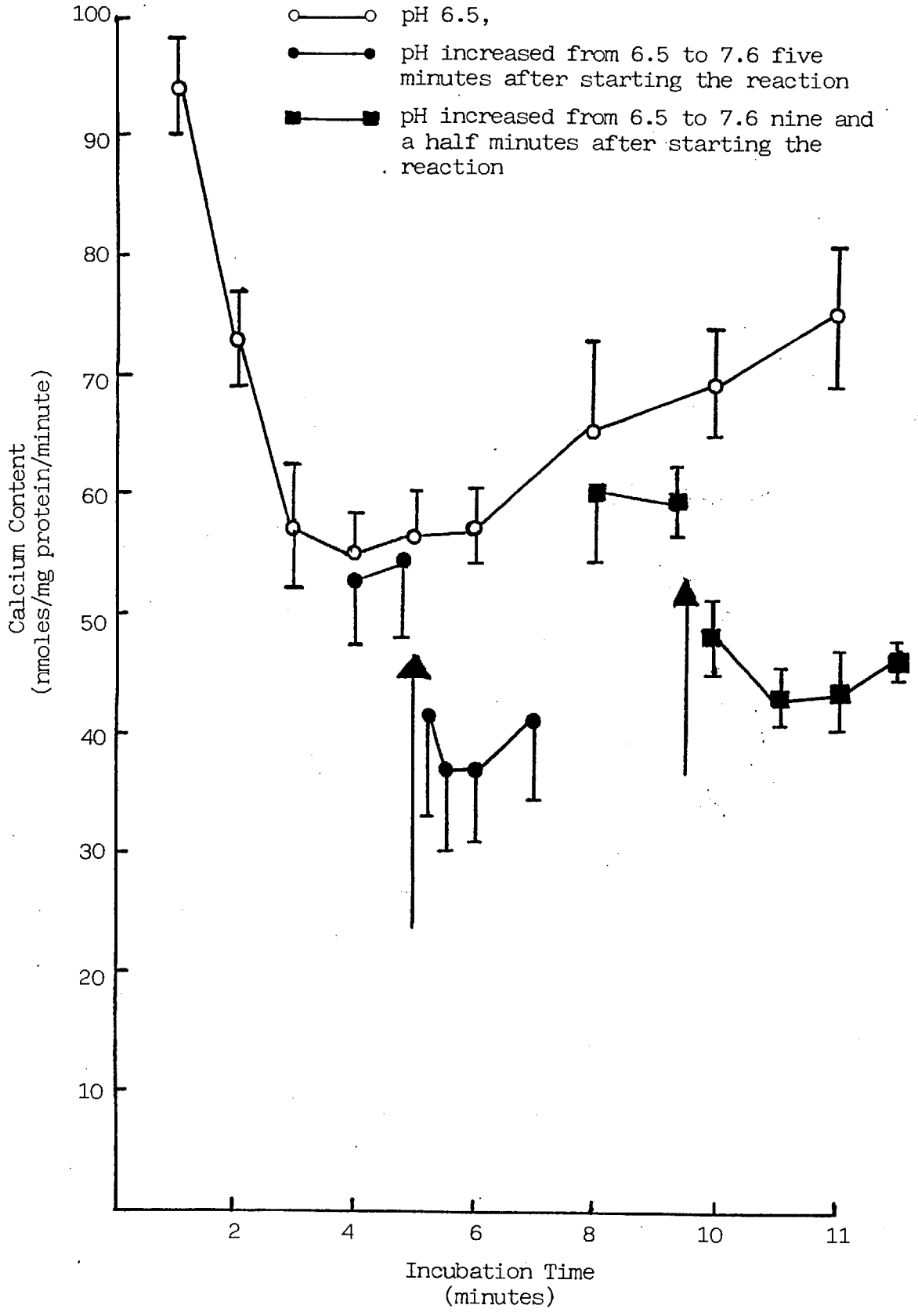



Figure 15

Effect of a sudden pH change on calcium content of skeletal sarcoplasmic reticulum after various incubation times

Each point represents mean $\pm$ S.E.M. of 5 or 6 experiments. Conditions as described in Methods. pH was increased from 6.5 to 7.6 by the addition of 20 µl Tris at the time indicated by the arrow.

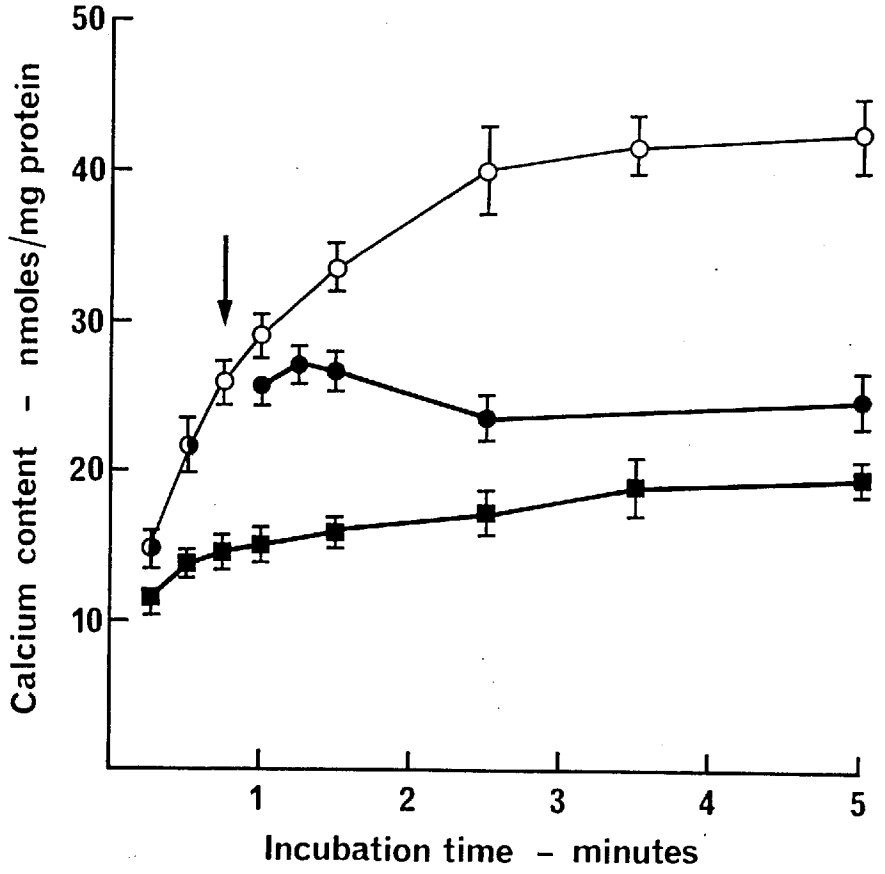



Figure 16

Effect of pH on calcium binding by cardiac sarcoplasmic reticulum

Each point represents mean ± S.E.M. of six experiments.
Conditions as described under 'Methods'.
pH was increased from 6.5 to 7.6 by addition of 20 µl of 0.8 M Tris at the time indicated by the arrow.

o——o pH 6.5,

■——■ pH 7.6,

●——● pH increased from 6.5 to 7.6.

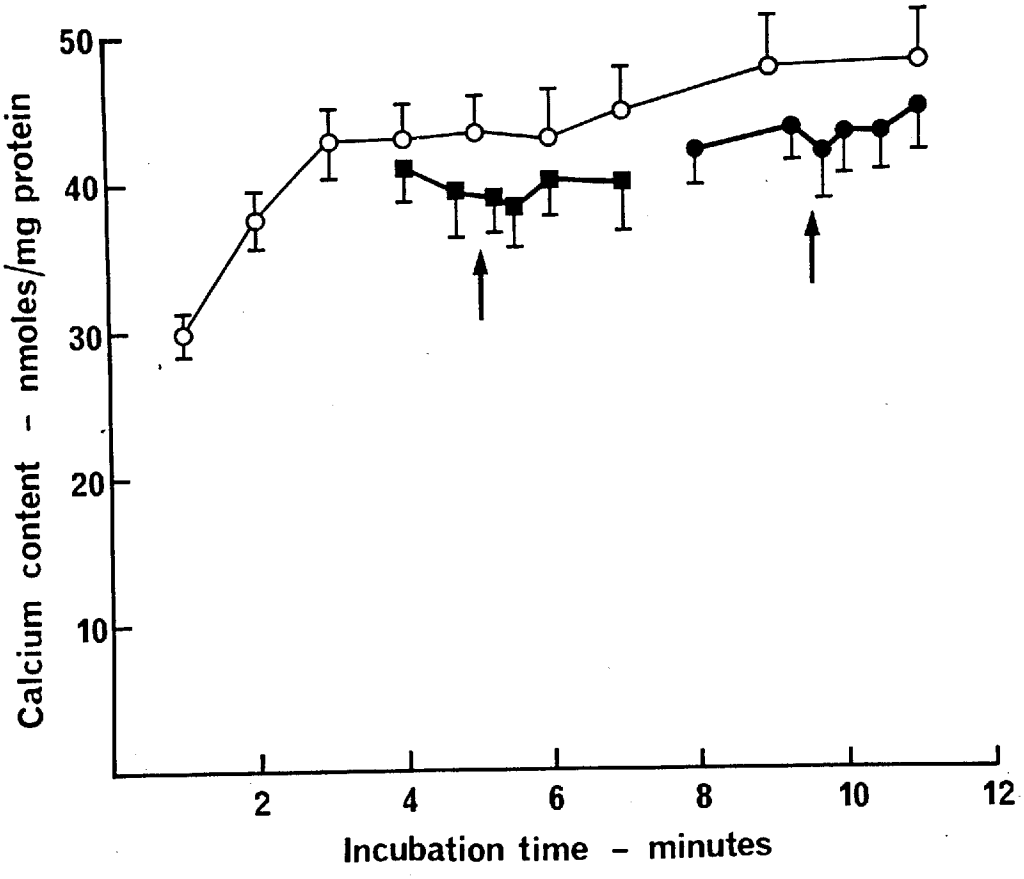



Figure 17

Effect of increasing pH on the calcium content of cardiac sarcoplasmic reticulum

Each point represents mean ± S.E.M. of six experiments.
pH was increased from 6.5 to 7.6 at the times indicated under 'Methods'.

o——o calcium binding at pH 6.5,

■——■ pH change 5 minutes after starting the reaction,

●——● pH change 9.5 minutes after starting the reaction.

SECTION IV

Discussion

(a) Interpretation of data

The data show that a sudden increase in the pH of the incubation medium in which isolated fragments of skeletal SR are suspended, results in the rapid release of previously bound calcium. This release occurs even if the pH is increased 5 or 9.5 minutes after the start of the reaction. These data confirm the observations of Nakamuru and Schwartz (1970; 1972). In cardiac SR, however, the effects of a rapid change in pH are much less marked. This conflicts with the data of Nakamuru and Schwartz (1970), which reported a rapid and relatively large calcium release from cardiac SR induced by an increase in pH, but supports the observations of other investigators. Fabiato and Fabiato failed to show an effect of a rapid pH increase on the calcium loading of cardiac SR, using skinned muscle fibres. Hoeschen and Nayler (1976) found no change in the calcium content of cardiac SR even after only 30 seconds incubation time. This may be accounted for by the fact the measurements were taken for only 90 seconds after changing the pH. Since it is reported here that changes in the calcium content of cardiac sarcoplasmic reticulum following an increase in pH are slow, it is possible that in the experiments of Hoeschen and Nayler the change in the calcium content of the SR had not yet developed to a stage where it could be detected. In addition, the initial pH of the reaction medium used by Hoeschen and Nayler, was 6.8. At this pH, calcium binding would be lower than at the pH of 6.5 used initially in these experiments. An increase in pH to 7.6 would therefore have less effect.

(b) Mechanism of depolarisation-induced calcium release

The electrical influence of depolarisation of the SR membrane would be to make the SR relatively more negative inside. This would be expected to increase calcium uptake into the SR, if calcium accumulation is an electrogenic process (Chapter 1, Section VI). Calcium release due to depolarisation of the membrane must therefore be due to an increase in the permeability of the membrane to calcium induced by depolarisation (Endo et al., 1977).

It has been claimed that calcium release induced by ionic replacement in the medium, is not due to membrane depolarisation, but is simply an osmotic effect. It was argued that replacement of a less permeable ion with a more permeable one would result in a net influx of solute and water, causing swelling of the SR (Meissner and McINley, 1976). In support of this hypothesis, direct measurement of the membrane potential change on replacement of potassium methanesulfonate with potassium chloride, which led to calcium release from the SR, was shown to be accompanied by only a small change in membrane potential. Conversely, large changes in membrane potential obtained by diluting SR equilibrated in potassium methanesulfonate into a medium containing sodium or Tris methanesulfonate and valinomycin, led to only a slight change in calcium release (Beeler et al., 1979). Calcium release from the SR induced by osmotic change has been observed in isolated SR preparations, and this has been attributed to bursting of the SR vesicles. However, evidence that the calcium release induced by ionic replacement is not an osmotic effect comes from the observation that a simple increase in the potassium chloride concentration of the medium did not result in calcium release. This argues against the suggestion of Beeler et al. (1979), that chloride ion, by inhibiting

the calcium pump, causes an apparent release of calcium. However, swelling of the SR may facilitate calcium release (Endo, 1977). This is indicated by the observation that membrane depolarisation caused by cation replacement, which would cause shrinkage of the SR, does not induce calcium release (Kasai and Myamoto, 1976a).

It seems probable that anion replacement in the medium causes depolarisation of the SR membrane, resulting in an increase in the calcium permeability of the membrane and calcium release, although other factors such as osmotic effects, and direct effects of different ions on the membrane, may contribute to the effect.

(c) Mechanism of pH-induced calcium release

As discussed for depolarisation-induced calcium release, a sudden increase in the pH of the medium would have the effect of making the SR relatively more negative inside, which would be expected to increase calcium uptake. In addition, increasing the pH from 6.4 to 7.6 should increase the activity of the calcium-activated ATPase enzyme, since in both skeletal (Martinosi, 1969) and cardiac (Shigekawa et al., 1976) SR preparations, the pH optimum for this enzyme is above 7.5. The increase in calcium release on elevation of the pH must therefore also be due to an increased calcium permeability. The increased permeability of the SR membrane to calcium at high pH has already been referred to (see Chapter 2, Section IV).

(d) Physiological significance of depolarisation-induced calcium release

Some aspects of the depolarisation-induced release of calcium are consistent with its role as the in vivo signal for calcium release from the SR.

Firstly, depolarisation-induced calcium release does not require preloading of the SR with large amounts of calcium, as is the case for calcium-triggered calcium release (Thorens and Endo, 1975). It has been argued that the level of calcium loading of the SR required before calcium-induced calcium release can occur, is unphysiologically high (Endo, 1977), although this estimate does not take into account the possible increase in calcium loading mediated by Calmodulin (see Chapter 1, Section IVb).

Secondly, depolarisation-induced calcium release is not affected by variations in the free magnesium concentration, and could therefore occur at physiological intracellular magnesium concentrations.

Depolarisation-induced calcium release, therefore, overcomes the objections raised to calcium-triggered calcium release, as a calcium release mechanism. Depolarisation of the membrane appears to lead to calcium release in isolated skeletal SR, but little information is available on the effect of membrane depolarisation on the calcium content of cardiac SR.

(e) Physiological significance of pH-induced calcium release

The results presented here show that in cardiac SR the calcium release induced by a rapid increase in pH is too small and too slow to be of importance in excitation-contraction coupling. In skeletal SR, the rate and amount of calcium release induced by a rapid pH increase is much greater. It has been estimated that depolarisation of the SR membrane would cause an increase in pH of 0.5 to 1.0 units under physiological conditions (Nakamuru and Schwartz, 1970; 1972), which would be sufficient to cause substantial calcium release from skeletal SR. However, Kasai and Myamoto (1976b) have shown that the

rate of calcium release, following passive calcium binding in the absence of ATP, is determined by the pH of the medium at which calcium release occurs, and that pH jump in itself has no effect on the rate of calcium release. This was taken as evidence that calcium release from the SR cannot be initiated by a sudden pH change. However this observation does not necessarily rule out pH change as the trigger for calcium release, since, if membrane depolarisation does cause a pH increase, the increased permeability of the SR membrane at the higher pH, following the pH jump, could lead to significant calcium release. However, a change in intracellular pH due to activation of contraction has not been confirmed. Thus, although the role of a pH change in excitation-contraction coupling cannot be entirely ruled out, its contribution remains to be conclusively established.

# CHAPTER 5

# ISOLATION OF THE CALCIUM-DEPENDENT ATPase

SECTION I

Introduction

(a) Protein composition of skeletal muscle sarcoplasmic reticulum

The proteins of skeletal SR may be resolved by polyacrylamide gel electrophoresis. The SR is first treated with sodium dodecyl sulphate (SDS) buffer, containing mercaptoethanol. This treatment solubilises and dissociates the proteins into their constituent polypeptide chains. Sodium dodecyl sulphate binds to all polypeptides in equal amounts on a gram to gram basis (Fish et al., 1970). The electrophoretic mobility of the SDS-polypeptide chain complexes is independent of the amino acid composition and isoelectric point, and is solely determined by the molecular weight of the polypeptide (Weber and Osborn, 1969; Fish et al., 1970; Reynolds and Tanford, 1970; Shapiro et al., 1967).

Although some authors (Yu and Masoro, 1970; Masoro and Yu, 1969) report that rat skeletal muscle SR is composed of a single polypeptide species, in different states of aggregation, most reports show the separation of the SR proteins into several bands. The major protein band consists of a protein of molecular weight approximately 100,000 (Martinosi and Haplin, 1971; Martinosi, 1969; Ikemoto, 1971; Maclennan et al., 1972), which accounts for 70% of the membrane protein (Inesi and Scales, 1974). The proteins thought to be involved in the calcium transport process were identified as the calcium transport ATPase, molecular weight about 100,000, an acidic protein of 54,000 Daltons, calsequestrin, molecular weight 44,000, a series of acidic proteins with molecular weights ranging from 20-32,000, and a proteolipid of molecular weight 6,000-12,000 (Maclennan et al., 1972).

In order to study the properties of the different protein components of the SR, the proteins can be separated following solubilisation by treatment with deoxycholate (Martinosi, 1968). The material solubilised from the SR at low concentrations of deoxycholate contains calsequestrin, the 54,000 Dalton protein, and the low molecular weight acidic proteins (Maclennan et al., 1972). These loosely-bound membrane proteins have been described as extrinsic proteins (Green and Brucker, 1973). The extrinsic proteins can be separated by passage through a DEAE cellulose column, eluted with a potassium chloride gradient. The first peak eluted from the column contains proteins which are not of SR origin. The second peak contains the 54,000 Dalton protein, and the last peak contains calsequestrin and the low molecular weight acidic proteins.

(b) 54,000 Dalton protein

This protein can be purified by sequential passage through DEAE cellulose, Sephadex G200 and hydroxyapatite. The protein contains 32% acidic amino acids and 13% basic amino acids, and is present in lower amounts than calsequestrin (Maclennan et al., 1972). The protein has a relatively low capacity for calcium, about 470 nmoles/mg (Maclennan et al., 1972), but binds calcium with high affinity in the presence of potassium chloride. In the absence of potassium chloride, the dissociation constant for calcium is about 120 μM. In the presence of 0.1 M potassium chloride, this dissociation constant shifts to 2-4 μM (Otswald and Maclennan, 1974).

The 54,000 Dalton protein must play a role in calcium transport, since addition of the protein to particles depleted of calsequestrin restores some of the calcium sequestering activity (Maclennan et al.,

1972), and purified ATPase preparations which retain this protein also retain some calcium sequestering activity (Repke et al., 1976; Meissner and Fleischer, 1973; 1974).

The probable location of this protein on the inside surface of the SR membrane (Maclennan et al., 1972) is supported by the experiments of Yu et al. (1976), who have shown that phospholipase treatment and sonication leads to the labelling of the protein with a non-permeant label.

It has recently been shown that a 55,000 Dalton protein band appearing in SDS polyacrylamide gels, consists of several proteins, but only one of these was a high affinity calcium-binding protein. An intrinsic glycoprotein was also found to be present in this band. Cycloheptamylose-fluorescamine complex (CFC), a non-permeant label, labels the intrinsic glycoprotein heavily, indicating that it is at least partly exposed to the external cytoplasmic surface, and it is suggested that the intrinsic glycoprotein is a transmembrane protein. The high affinity calcium-binding protein is not labelled by CFC, is not affected by proteolytic digestion of the SR, and is not removed by washing the SR with EGTA and Chelex 100. This confirms its location on the internal surface of the SR membrane (Michalek et al., 1980).

(c) Calsequestrin

Calsequestrin has been purified by sequential passage through DEAE cellulose, Sephadex G200 and hydroxyapatite. The molecular weight of calsequestrin has been reported to be 44,000 (Maclennan and Wong, 1971), but there is variation in the molecular weights reported for this protein. A protein of molecular weight 55,000, which precipitated at high calcium concentrations of 4 mM and which

bound calcium with low affinity, has been purified and identified as calsequestrin (Ikemoto et al., 1972; Otswald and Maclennan, 1974; Repke et al., 1976).

Calsequestrin accounts for 7% of the total SR protein, and is highly anionic. It contains 37% acidic amino acids and 3% basic amino acids (Maclennan and Wong, 1971). Calsequestrin has a high capacity for calcium, about twice as high as that reported for the 54,000 Dalton protein, but a low affinity for calcium. The capacity of calsequestrin for calcium is not increased in the presence of 0.1 M potassium chloride, but the dissociation constant is increased (Otswald and Maclennan, 1974). Since calcium binding to calsequestrin was only observed at high calcium concentrations, such as would obtain at the interior of the SR membrane, and since calsequestrin is not released from the SR by sonication, it was suggested that calsequestrin is hydrophobically-bound to the internal surface of the SR (Maclennan and Wong, 1971). This is confirmed by the observations of Yu et al. (1976), who showed that, like the 54,000 Dalton protein, calsequestrin was only labelled by a non-permeant label following phospholipase c treatment and sonication, to disrupt the SR membrane.

It has been observed that some preparations are contaminated with a protein of molecular weight 6% less than that of calsequestrin. It was demonstrated that the contaminant protein was a second form of calsequestrin which replaced the first form in some individual rabbits. In other rabbits, the SR contained the two forms of calsequestrin in equal amounts. Amino acid analysis of the two types of calsequestrin were similar, but differed in the content of tyrosine, cysteine and methionine. Chicken antibodies against calsequestrin 1, also precipitated calsequestrin 2 (Maclennan, 1974).

(d) Low molecular weight acidic proteins

These proteins have a high affinity for calcium, binding 35 nmoles of calcium/mg protein at calcium concentrations as low as $10^{-6}$ M. Twelve nmoles of each of the three proteins may be present in 1 mg SR, and up to three molecules of calcium could be bound/molecule of each of the proteins. This affinity for calcium is similar to the affinity of the calcium-dependent ATPase. Labelling experiments with iodine suggest that these proteins are on the outer surface of the membrane (Maclennan et al., 1972).

However, it was later shown that the high affinity calcium binding by these proteins is abolished in the presence of 0.1 M potassium chloride. The role of these proteins in the calcium transport process is, therefore, open to question (Otswald and Maclennan, 1974). It has been suggested that the low molecular weight acidic proteins, and also part of the 54,000 Dalton protein band, may be proteolytic breakdown products of the high molecular weight ATPase.

(e) Calcium-dependent ATPase

Treatment of SR with low concentrations of deoxycholate (0.1 mg/mg protein) solubilises a large amount of non-ATPase protein, leaving the bulk of the ATPase activity in the insoluble pellet. By this procedure the specific activity of the ATPase is increased 2-4 fold (Maclennan et al., 1970). The ATPase and the proteolipid can be solubilised with a higher concentration of deoxycholate (0.5 mg/mg SR) and fractionated with ammonium sulphate (Martinosi, 1968), or ammonium acetate (Maclennan, 1970). Fractionation with ammonium acetate yields an ATPase preparation with a higher specific activity, probably due to inactivation of the enzyme by ammonium sulphate (Maclennan, 1970). The

ATPase protein has also been solubilised with Triton X100 (Ikemoto et al., 1971). Separation of solubilised protein and removal of detergent can be achieved by passage through a Sepharose 4B column (Ikemoto et al., 1971), dilution (Van Winkle et al., 1978), or sucrose gradient centrifugation (Warren et al., 1974; Van Winkle et al., 1978). Detergent may also be removed by dialysis (Maclennan, 1970; Repke et al., 1976; Ikemoto et al., 1971; Meissner et al., 1973). The purified ATPase forms closed membranous vesicles when deoxycholate is removed (Meissner et al., 1973; Maclennan et al., 1971; Martinosi, 1968; Repke et al., 1976). Although it was at first thought that purified ATPase vesicles were smooth, and that the surface particles characteristic of native SR were absent (Maclennan et al., 1971), it was later shown that the surface particles are retained on both sides of fragments of purified ATPase, and it was concluded that surface particles are structural features of either the ATPase or the proteolipid (Stewart and Maclennan, 1973). Freeze-fracture of the reformed vesicles reveals globular proteins in the range 90-100 Å. Amino acid and phospholipid analysis of the enzyme shows that the phospholipid and neutral lipid content of the ATPase is identical to that of the native SR. Phosphorylation of the enzyme with ATP-$^{32}$P led to a maximum specific phosphoryl content of 0.73 phosphoryl groups/102,000 Daltons. It was therefore concluded that each molecule of 102,000 Daltons contains one active site (Maclennan et al., 1971). The purified ATPase separates electrophoretically as one major band, and retains the same characteristics in isolation as in the native SR, i.e. moderate inhibition by ADP and requirements for both calcium and magnesium. However, the purified enzyme is more specific for ATP than the unfractionated SR (Maclennan, 1970). The purified preparation is unable to transport

calcium to any extent (Maclennan, 1970; Meissner et al., 1973). Purified ATPase preparations which are reported to retain calcium transport activity, have been shown to contain the 54,000 Dalton protein (Repke et al., 1976; Meissner and Fleischer, 1973; 1974). Recently it has been shown that the protein retained by these reconstituted vesicles is not the 54,000 Dalton protein itself, but an intrinsic glycoprotein associated with it (Michalek et al., 1980).

The isolated ATPase contains a proteolipid (Maclennan et al., 1972). It has a molecular weight of 12,000, and it may be involved in the interaction between the globular ATPase and phospholipid (Maclennan et al., 1972). Restoration of calcium transport activity to the purified ATPase by its incorporation into liposomes requires the presence of the proteolipid (Racker and Eytan, 1975).

(f) Subunits of the ATPase

The ATPase of skeletal SR, when treated with low concentrations of trypsin, splits into two fragments of molecular weight 45-55,000 and 55-60,000 (Stewart and Maclennan, 1974; Thorley-Lawson and Green, 1973; Migala et al., 1973; Yu et al., 1976; Stewart et al., 1975; Louis et al., 1974; Inesi and Scales, 1974). Cleavage of the ATPase into two fragments does not lead to any reduction of ATPase activity (Stewart and Maclennan, 1974; Migala et al., 1973; Inesi and Scales, 1974). The 60,000 molecular weight fragment splits slowly into two fragments of molecular weight 33,000 and 24,000 (Thorley-Lawson and Green, 1973; Stewart et al., 1976; Inesi and Scales, 1974). These fragments are extensively iodinated by lactoperoxidase, suggesting that they are located on the outer surface of the membrane. The phosphorylated active site has been located on the 33,000 molecular

weight fragment, i.e. on the outer surface of the membrane (Thorley-Lawson and Green, 1973). Prolonged trypsin digestion leads to cleavage of the ATPase into peptides of molecular weight 10-30,000 (Stewart and Maclennan, 1974; Migala et al., 1973). This is associated with loss of ATPase activity (Stewart and Maclennan, 1974; Migala et al., 1973). The smaller fragment does not contain a phosphorylation site, but showed calcium ionophoric activity, indicating that the ionophoric and active hydrolytic sites are located on separate regions of the ATPase molecule (Shamoo et al., 1976).

Antibodies raised against the two larger fragments of the ATPase show no cross-reactivity, indicating that they arise from a single linear cleavage of the ATPase molecule. The reaction of antibodies raised against the 55,000 molecular weight fragment with the intact membrane is strong, whereas the reaction with anti-45,000 molecular weight fragment antibodies is weak. This indicates that the larger portion of the 45,000 molecular weight fragment lies within the phospholipid bilayer, whereas the 55,000 molecular weight fragment extends to the exterior, and probably makes up a large proportion of the surface particles (Stewart et al., 1976). This is consistent with the more hydrophobic composition of the smaller fragment. It contains 60% non-polar and 40% polar amino acids, whereas the larger fragment has equal proporations of polar and non-polar amino acids (Stewart et al., 1976).

(g) Protein composition of cardiac muscle sarcoplasmic reticulum

Polyacrylamide gel electrophoresis of cardiac SR also reveals a major protein band of molecular weight approximately 100,000, corresponding to the calcium-activated ATPase (Van Winkle et al., 1978;

Levitsky et al., 1976). In addition to the calcium-dependent ATPase, the 54,000 Dalton protein, calsequestrin and low molecular weight acidic proteins have also been identified as components of cardiac SR (Van Winkle et al., 1978), but these have not been purified. A number of other minor protein component bands are also sometimes present, though it is not clear whether these represent extra protein components of cardiac SR, or contaminants resulting from the lower degree of purity of cardiac SR compared with skeletal SR preparations (Levitsky et al., 1976).

The cardiac ATPase has been isolated from pigeon (Levitsky et al., 1976) and canine (Van Winkle et al., 1978) cardiac SR. The method used involved initial solubilisation in deoxycholate followed by ammonium acetate fractionation (Levitsky et al., 1976) or reprecipitation of the ATPase by dilution with a glycerol-containing medium and subsequent purification on a sucrose gradient (Van Winkle et al., 1978). Using both these methods, a 3-5 fold increase in the specific activity of the ATPase was obtained. The ATPase activity was entirely calcium-dependent, and the purified preparation appeared as one band on polyacrylamide gels (Levitsky et al., 1976; Van Winkle et al., 1978).

In the studies reported here the purification of rabbit cardiac SR ATPase was attempted. Due to the shortage of available material, ammonium acetate fractionation was not attempted. The methods used were those of Van Winkle et al. (1978) involving deoxycholate solubilisation and reprecipitation of the ATPase by dilution, and that of Ikemoto et al. (1971) involving solubilisation with Triton X100 and separation of solubilised proteins on a Sepharose 4B column. For comparison, rabbit skeletal SR ATPase was purified by the same method.

The object was to obtain a purified cardiac ATPase which could then be incorporated into artificial phospholipid vesicles, as has been achieved for purified skeletal ATPase preparations (Racker, 1972; Knowles and Racker, 1975). This would enable more accurate control of the internal calcium concentration, since calcium-EGTA buffers could be incorporated into the liposomes. The effect of internal calcium concentration on calcium transport could then be studied. In addition, by incorporating different concentrations of potassium chloride into the liposomes, the role of membrane potential change in calcium transport by cardiac SR could be studied, in a similar way to the experiments carried out with reconstituted skeletal ATPase vesicles (Zimmak and Racker, 1978). By varying the phospholipid composition of the liposomes, the effect of different phospholipids on calcium transport by cardiac ATPase activity could also be studied.

## SECTION II

## Methods

Attempts were made to purify the calcium-activated ATPase enzyme from SR preparations isolated from rabbit or guinea pig hearts by methods adapted from those of Van Winkle *et al.* (1978) using deoxycholate to solubilise the SR and dilution for reprecipitation, or from the method of Ikemoto *et al.* (1971) using Triton X100 for solubilisation and Sepharose 4B chromatography to fractionate solubilised proteins. Solubilisation with digitonin was also attempted.

### (a) Solubilisation with deoxycholate

Deoxycholic acid was purified by dissolving it in a minimal volume of boiling ethanol, and decolourising by shaking with activated

charcoal, followed by filtration. Deoxycholic acid was recrystallised by dilution with an equal volume of distilled water, and the crystals were allowed to form overnight at 4°C, and then collected by filtration and dried overnight at room temperature. A 10% solution of deoxycholate was prepared by suspending 10 gm deoxycholic acid in approximately 70 ml of water, and dissolving it by addition of potassium hydroxide to adjust and maintain the pH at 8.0. The solution was then made up to 100 ml.

Since it was desirable to obtain a large amount of SR for the purification of the ATPase, the SR was isolated from rabbit ventricular or hind leg muscle by the same method as has been described in a previous section for guinea pig ventricular muscle. The final pellet was taken up in a medium containing 50 mM potassium phosphate, pH 8.0, 0.5 M potassium chloride, 1 mM dithiothreitol, 1 mM ATP, 1 mM calcium chloride to give a final protein concentration of approximately 13 mg/ml. The high concentration of potassium chloride increased the amount of protein solubilised (Repke et al., 1976), whilst the presence of calcium and ATP protected the enzyme against inactivation. The 10% deoxycholate solution was added to give a ratio of 0.4 mg deoxycholate/mg SR protein. The mixture was stirred for 5 minutes and spun at 104,000 x g for 30 minutes in an M.S.E. 65 or 50 superspeed centrifuge. The pellet was taken up in 20 mM Tes buffer, pH 6.8. Samples of the supernatant and pellet were retained for protein and ATPase assays, and for gel electrophoresis. The supernatant was then diluted with an equal volume of a solution containing 40% glycerol, 2 mM calcium chloride, 2 mM ATP and 20 mM Tes buffer, pH 6.8. The mixture was stirred for 45 minutes at 4°C. The pellet was taken up in 20 mM Tes buffer, pH 6.8. In some experiments, this suspension was applied to a discontinuous sucrose gradient, with layers of 30, 35, 40 and 45% w/w sucrose, containing

0.5 M potassium chloride and 20 mM Tes buffer, pH 6.8, and spun at 12,000 x g for 1 hour. The top layer of protein was centrifuged at 104,000 x g for 30 minutes and the pellet resuspended in 20 mM Tes buffer, pH 6.8.

(b) Solubilisation with Triton X100

Sarcoplasmic reticulum was isolated from rabbit ventricular or hind leg muscle, and the final pellet was taken up in a solution containing 10% v/v glycerol, 100 mM Tris chloride, pH 8.5, 5 mM dithiothreitol and 4 mM calcium chloride. Triton X100 (10% v/v) was added to give a ratio of 0.7-0.8 mg Triton/SR protein. After stirring for 5 minutes the mixture was centrifuged for 45 minutes at 104,000 x g and 4°C. The supernatant from this spin was applied to a Sepharose 4B column equilibrated with a solution containing 10% glycerol, 100 mM Tris-chloride, pH 8.5, 4 mM calcium chloride and 5 mM DTT. The column was eluted with the same medium. The fraction eluting immediately after the void volume of the column was centrifuged at 104,000 x g for 30 minutes at 4°C and the pellet was taken up in 50 mM potassium chloride, 10 mM histidine, pH 7.0.

(c) Solubilisation with digitonin

Sarcoplasmic reticulum was isolated from guinea pig ventricular muscle and the final pellet was suspended in a medium containing 50 mM Tris-chloride, pH 8.0, 20% v/v glycerol, 1 mM ATP, 1 mM calcium chloride at a protein concentration of approximately 10 mg/ml. Digitonin was added to a final concentration of 2% and the suspension was stirred for 5 minutes and then centrifuged at 104,000 x g for 30 minutes at 4°C. The supernatant was applied to a Sepharose 4B mini-column (9cm x 0.9cm) equilibrated and eluted with a solution containing 10% v/v glycerol,

50 mM Tris-chloride, pH 8.0, 1 mM dithiothreitol, 1 mM ATP. The eluted ATPase was centrifuged and resuspended in 50 mM potassium chloride, 10 mM histidine, pH 7.0, as before.

(d) Assay of adenosine triphosphatase activity

Adenosine triphosphatase activity was measured by following the rate of inorganic phosphate production. Adenosine triphosphatase assays were carried out in a medium containing 120 mM potassium chloride, 40 mM histidine, pH 7.0, 5-20 µg/ml protein, 5 mM magnesium ATP and 2 mM EGTA or 0.06 mM calcium chloride, in a total volume of 1 ml. Reactions were started by addition of magnesium ATP, and were carried out at 37$^{o}$C for 10 minutes. Basal ATPase activity was measured in the presence of 2 mM EGTA, and total ATPase activity was measured in the presence of 0.06 mM calcium chloride. Calcium-activated ATPase activity was calculated as the total activity minus the basal activity. Blanks, which contained no protein, were included in all assays.

Inorganic phosphate was measured by the method of Price and Williams (1977). The reactions were stopped by adding 1 ml of 2.5% w/v ammonium molybdate and 2.5 % w/v cirrasol in 0.9 N sulphuric acid. Fiske Subbarow reagent (0.5 ml) (30 gm sodium metabisulphite, 1 gm sodium sulphite and 0.5 gm 1-amino-2naphthyl sulphonic acid in 200 ml water), was then added. The samples were diluted with 5 ml of distilled water and after 10 minutes the optical density was read at 660 or 600 nm. A calibration curve for phosphate was constructed from solutions of potassium phosphate containing 20, 40 and 60 µmoles phosphate, treated in the same way.

(e) Polyacrylamide gel electrophoresis

Protein samples were hydrolysed overnight, or boiled for 15 minutes in a solution containing 1% sodium lauryl sulphate, 1% 2-mercaptoethanol and 6 M urea. Molecular weight markers (range 14,300-71,500 and 56,000-280,000) were treated in the same way. Samples were run in 7.5% polyacrylamide gels using an LKB multiphor 2117 flat-bed electrophoresis apparatus. The gel buffer contained 4.42 gm sodium dihydrogen orthophosphate, dihydrate, 10.2 gm disodium hydrogen orthophosphate, and 1 gm sodium lauryl sulphate per litre. After electrophoresis, the gels were stained in 0.5% Coomassie blue (100 mg dissolved in 10 ml ethanol and diluted with 190 ml 12.5% trichloroacetic acid) and destained in a solution containing 1 litre methanol, 125 ml acetic acid, 62 ml glycerol and 1312 ml water.

## SECTION III

## Results

### (a) Solubilisation with deoxycholate

#### (i) Skeletal muscle sarcoplasmic reticulum

Figure 18 shows basal and total ATPase activity of the intact SR, and of the pellet obtained on diluting the deoxycholate solubilised enzyme with a glycerol medium. In the glycerol pellet, the amount of basal ATPase activity is considerably reduced, while there is a substantial increase in the total (and accordingly the calcium-activated ATPase activity). Attempts to further purify the enzyme by sucrose density gradient centrifugation led to the majority of protein sedimenting at the interface of the 40 and 45% sucrose gradient layers.

The enzyme activity of this band is shown in the third column of Figure 18. Both the basal and total ATPase activity are slightly reduced, compared to the activities in the glycerol pellet, with no further increase in the specific activity of the calcium-activated ATPase. The specific activity of the calcium-activated ATPase is doubled in the glycerol pellet as compared to that in the intact SR (see Table 4).

(ii) Rabbit cardiac muscle sarcoplasmic reticulum

Figure 19 shows basal and total ATPase activity of intact cardiac SR, and of the pellet obtained following dilution of the deoxycholate-solubilised SR with glycerol-containing medium.

In cardiac SR the total ATPase activity is lower than in skeletal SR and the proportion of the activity that is calcium-independent (basal activity) is higher than in skeletal SR. In addition, in rabbit cardiac SR, the basal ATPase activity is known to be higher than in some other species such as the dog and the guinea pig (Nayler et al., 1975). In the glycerol-precipitated enzyme there is a slight increase in the total ATPase activity as compared with the intact SR, together with a small decrease in the basal activity. The increase in the specific activity of the calcium-dependent ATPase is small compared to the increase in specific activity of the skeletal muscle calcium-dependent ATPase during purification (see Table 5).

The cardiac enzyme appears to be more susceptible to denaturation by deoxycholate than the skeletal enzyme, since in some preparations substantial loss of both basal and total enzyme activity occurred on solubilisation with deoxycholate.

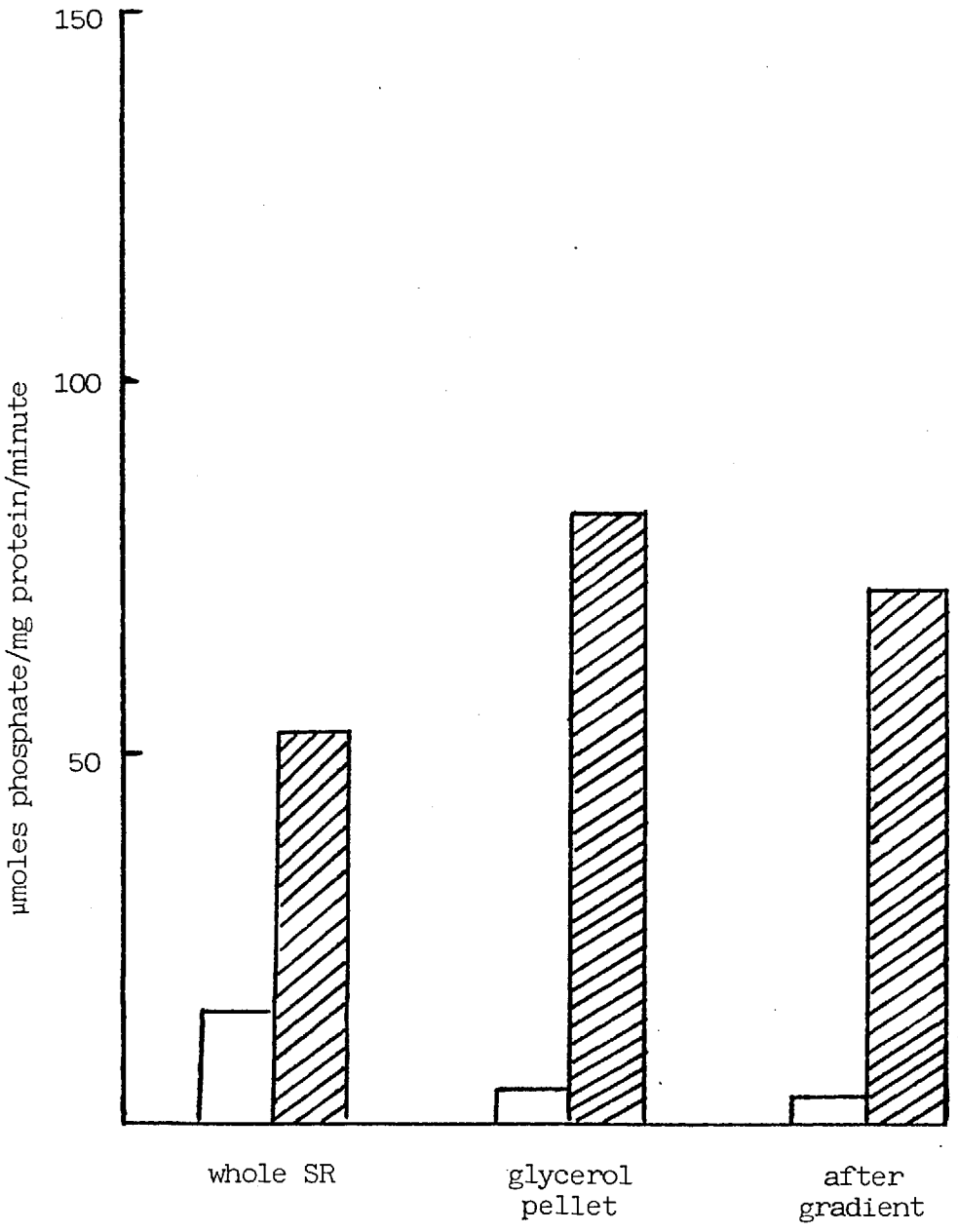



Figure 18

Purification of skeletal ATPase by solubilisation with deoxycholate

Conditions as described in Methods. A single representative experiment is shown.

□ basal ATPase activity ▨ total ATPase activity

TABLE 4

Skeletal sarcoplasmic reticulum adenosine triphosphatase activity
(μmoles phosphate released/mg protein/10 minutes)

Data from experiments shown in Figures 18 and 20.

| | Basal | Total | Calcium activated |
|---|---|---|---|
| Deoxycholate solubilisation | | | |
| Whole SR | 16.9 | 52.8 | 35.9 |
| Glycerol pellet | 5.2 | 82.5 | 77.3 |
| After gradient | 3.9 | 72.0 | 68.1 |
| Triton X100 solubilisation | | | |
| Whole SR | 4.4 | 38.9 | 34.5 |
| Solubilised SR | - | 63.0 | 63.0 |
| Particulate fraction | - | 166.4 | 166.4 |

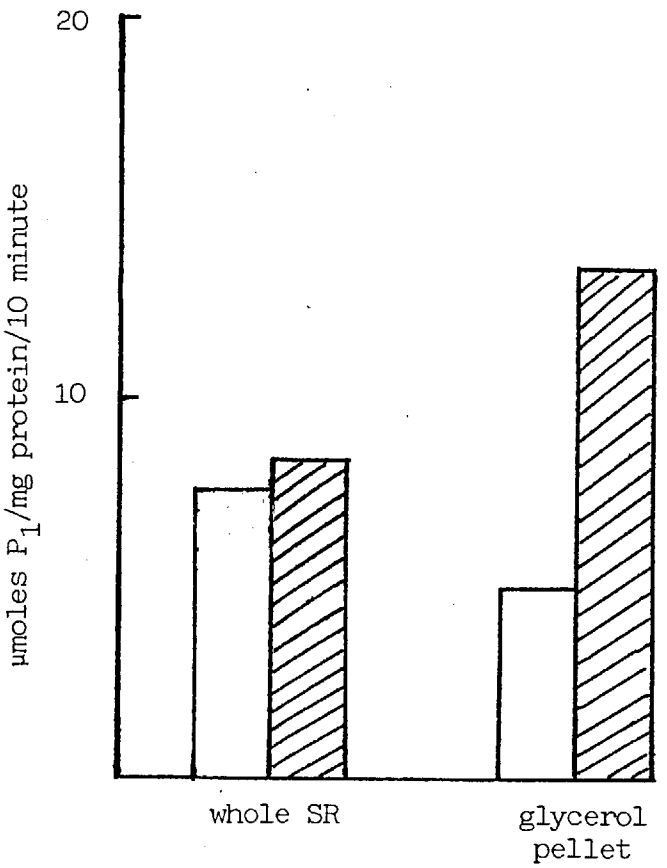



Figure 19

Purification of rabbit cardiac ATPase by solubilisation with deoxycholate

Conditions as described in Methods. A single representative experiment is shown.

basal ATPase activity

total ATPase activity

TABLE 5

Cardiac sarcoplasmic reticulum adenosine triphosphatase activity
(μmoles phosphate released/mg protein/10 minutes)

Data from experiments shown in Figures 19, 22, 24 and 25.

| | Basal | Total | Calcium activated |
|---|---|---|---|
| Deoxycholate solubilisation | | | |
| Whole rabbit SR | 7.45 | 8.4 | 0.95 |
| Rabbit glycerol pellet | 5.1 | 13.45 | 8.35 |
| Triton X100 solubilisation | | | |
| Whole rabbit SR | 11.85 | 16.35 | 4.5 |
| Rabbit solubilised SR | 6.2 | 13.6 | 7.4 |
| Rabbit particulate fraction | 12.65 | 22.3 | 9.65 |
| Guinea pig whole SR | 6.75 | 13.0 | 6.25 |
| Guinea pig solubilised SR | 1.15 | 13.3 | 12.15 |
| Digitonin solubilisation | | | |
| Guinea pig whole SR | 5.15 | 9.9 | 4.75 |
| Guinea pig solubilised SR | 4.28 | 11.18 | 6.9 |

(b) Solubilisation with Triton X100

(i) Skeletal muscle sarcoplasmic reticulum

When Triton-solubilised skeletal SR is passed through a Sepharose 4B column as described in 'Methods', detergent is separated from the protein, and the enzyme is eluted immediately after the void volume of the column in a vesicular form. The enzyme is then recovered as a particulate fraction following centrifugation, and is referred to as the purified ATPase.

Figure 20 shows the basal and total ATPase activity of intact SR, and of the Triton-solubilised SR and the purified ATPase. Solubilisation with Triton X100 results in the disappearance of the basal activity, and an increase in the total enzyme activity. The purified ATPase also showed no calcium-independent ATPase activity, but the specific activity of the calcium-activated enzyme was considerably increased, compared to the intact SR (see Table 4).

The recovery of total protein after Sepharose 4B chromatography was 97% of the total protein applied to the column, but only 39% of the total enzyme activity was recovered in the particulate form, the remainder being eluted from the column later in a soluble form. This must be due to incomplete removal of Triton.

(ii) Rabbit cardiac muscle sarcoplasmic reticulum

Since cardiac SR showed a tendency to be inactivated by treatment with Triton X100, and since difficulty was experienced in obtaining a particulate fraction following Sepharose 4B chromatography, an experiment was carried out to ascertain the minimum concentration of Triton which could be used to solubilise the enzyme. Figure 21 shows the protein concentration and ATPase activity in a supernatant fraction obtained

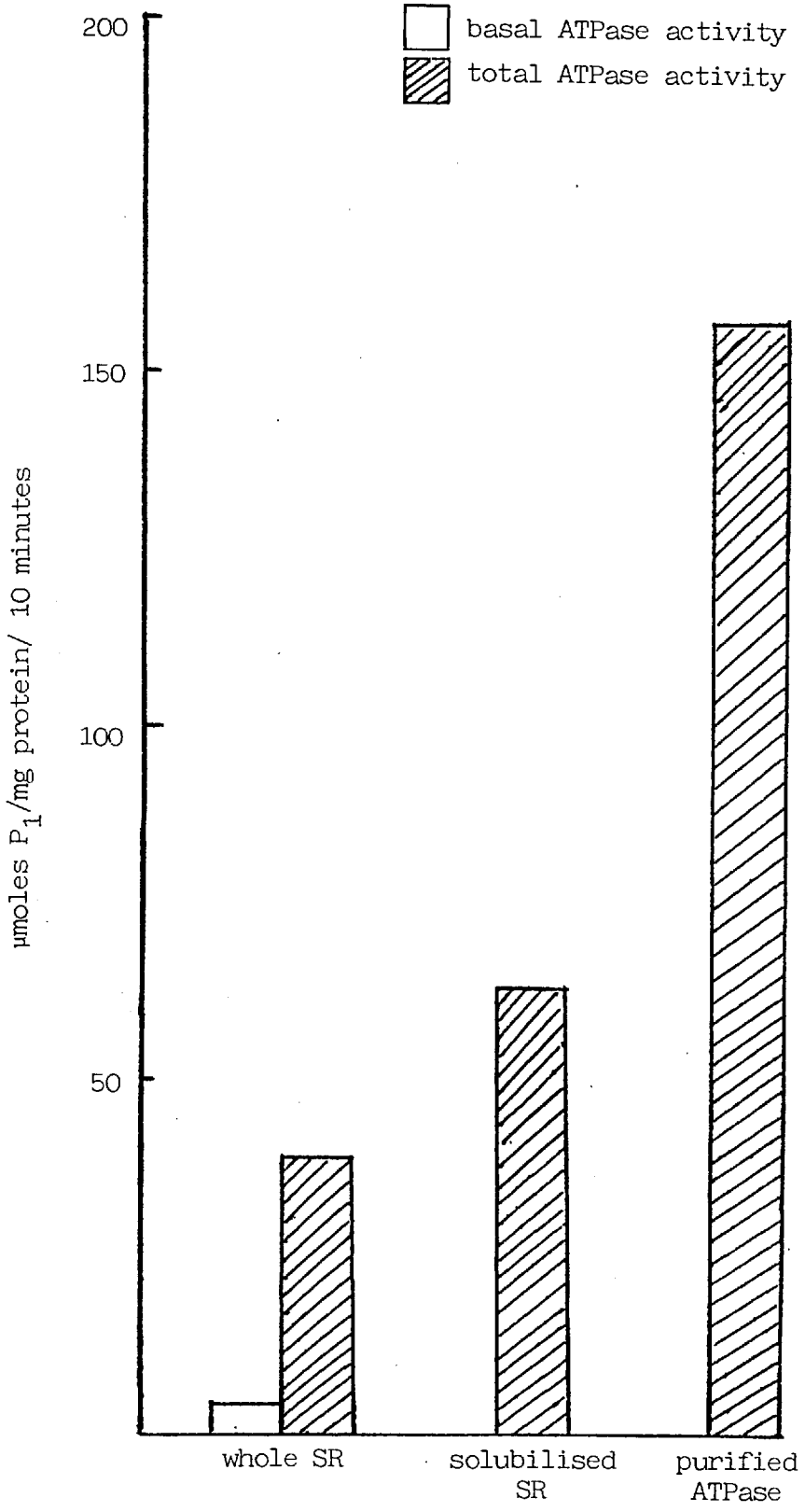



Figure 20

Purification of rabbit skeletal ATPase by solubilisation with Triton X100

Conditions as described in Methods. A single representative experiment is shown.

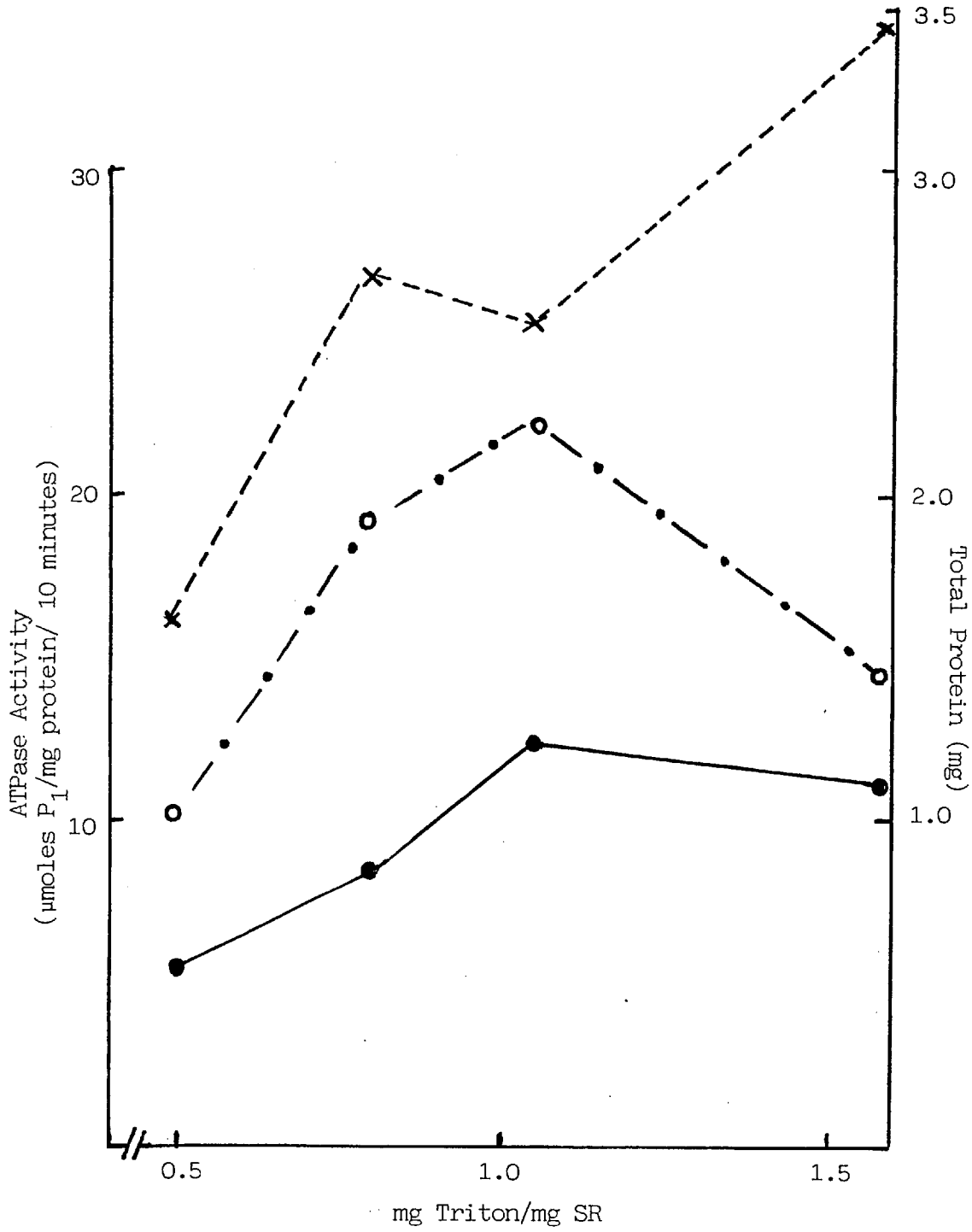



Figure 21

Effect of Triton concentration on solubilisation of rabbit cardiac ATPase

Conditions as described in Methods. A single experiment is shown.

●———● basal ATPase activity; o-•-•o total ATPase activity

x-----x total protein concentration

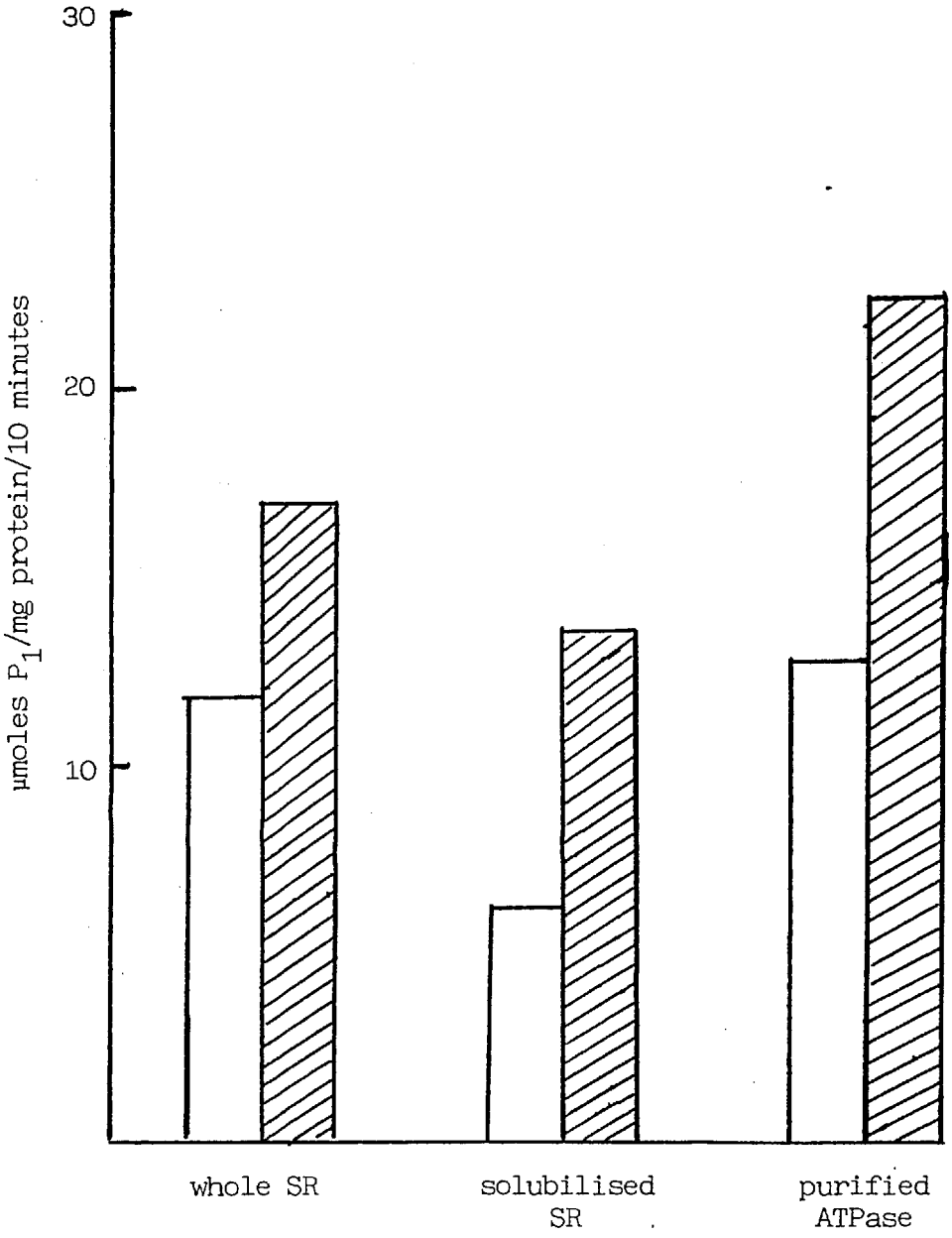



Figure 22

Purification of cardiac ATPase by solubilisation with Triton X100

Conditions as described in Methods. A single representative experiment is shown.

basal ATPase activity  total ATPase activity

after treatment of rabbit cardiac SR with Triton at concentrations of 0.5, 0.8, 1.06 and 1.6 mg/mg SR protein. From Figure 22 it appears that the optimal concentration of enzyme activity is obtained when the SR is solubilised with Triton at a concentration of 0.8-1.0 mg Triton/mg SR. At higher concentrations of Triton, although more protein is solubilised, enzyme activity is lower, probably due to inactivation of the cardiac enzyme at high detergent concentrations. In order to obtain the maximum yield of enzyme in a particulate form following Seoharose 4B chromatography, Triton was used to solubilise cardiac SR at a concentration of 0.7-0.8 mg/mg SR protein. Using this amount of Triton, a small amount of enzyme was eluted from the column in a particulate form. Figure 22 shows basal and total ATPase activity in intact and Triton-solubilised SR, and in the particulate fraction. Solubilisation of the SR results in a decrease of both basal and total activity, although calcium-activated ATPase activity (total minus basal activity) is increased slightly. In the particulate enzyme fraction, specific activity of both basal and total activity is increased relative to the unfractionated enzyme (see Figure 22 and Table 5).

(c) Comparison of purification obtained by solubilisation with deoxycholate and Triton X100

Table 4 shows basal, total and calcium-activated ATPase activity at various stages of the purification of the skeletal SR enzyme. Using deoxycholate to solubilise the enzyme, a two-fold increase in the specific activity of the calcium-activated ATPase activity was obtained in the glycerol pellet, as compared with the intact SR. It should be noted that the basal ATPase activity is reduced, but not completely

absent in the glycerol pellet, although in this particular preparation the basal ATPase activity of the intact SR was unusually high.

Using Triton X100 to solubilise the enzyme, the basal activity disappears on solubilisation, and the specific activity of the calcium activated ATPase activity enzyme is increased by a factor of approximately two, compared to the intact SR. In the particulate fraction there is a further increase in the specific activity of the calcium-activated enzyme to a value approximately five times higher than those observed in the intact SR.

Figure 23 is a photograph of a polyacrylamide gel of intact rabbit skeletal SR and of the enzyme preparations obtained by solubilisation with deoxycholate and with Triton X100.

In the SR a major protein band is observed, and several smaller protein bands are also present. In both the purified enzyme preparations the minor protein bands have largely disappeared, leaving only the major protein band, which probably corresponds to the ATPase enzyme.

Difficulty was experienced in subjecting cardiac preparations to polyacrylamide gel electrophoresis, largely because of the small amounts of the purified enzyme which were obtained, but also because of difficulties encountered in the pre-treatment of the cardiac SR with protein hydrolysing solutions, which led to the formation of precipitates. The reason for this is not known.

(d) Purification of adenosine triphosphatase from guinea pig cardiac sarcoplasmic reticulum

Rabbit cardiac SR exhibits a high basal ATPase activity, which was found to persist after purification of the enzyme by solubilisation with deoxycholate or Triton X100. Attempts were made to purify the guinea pig

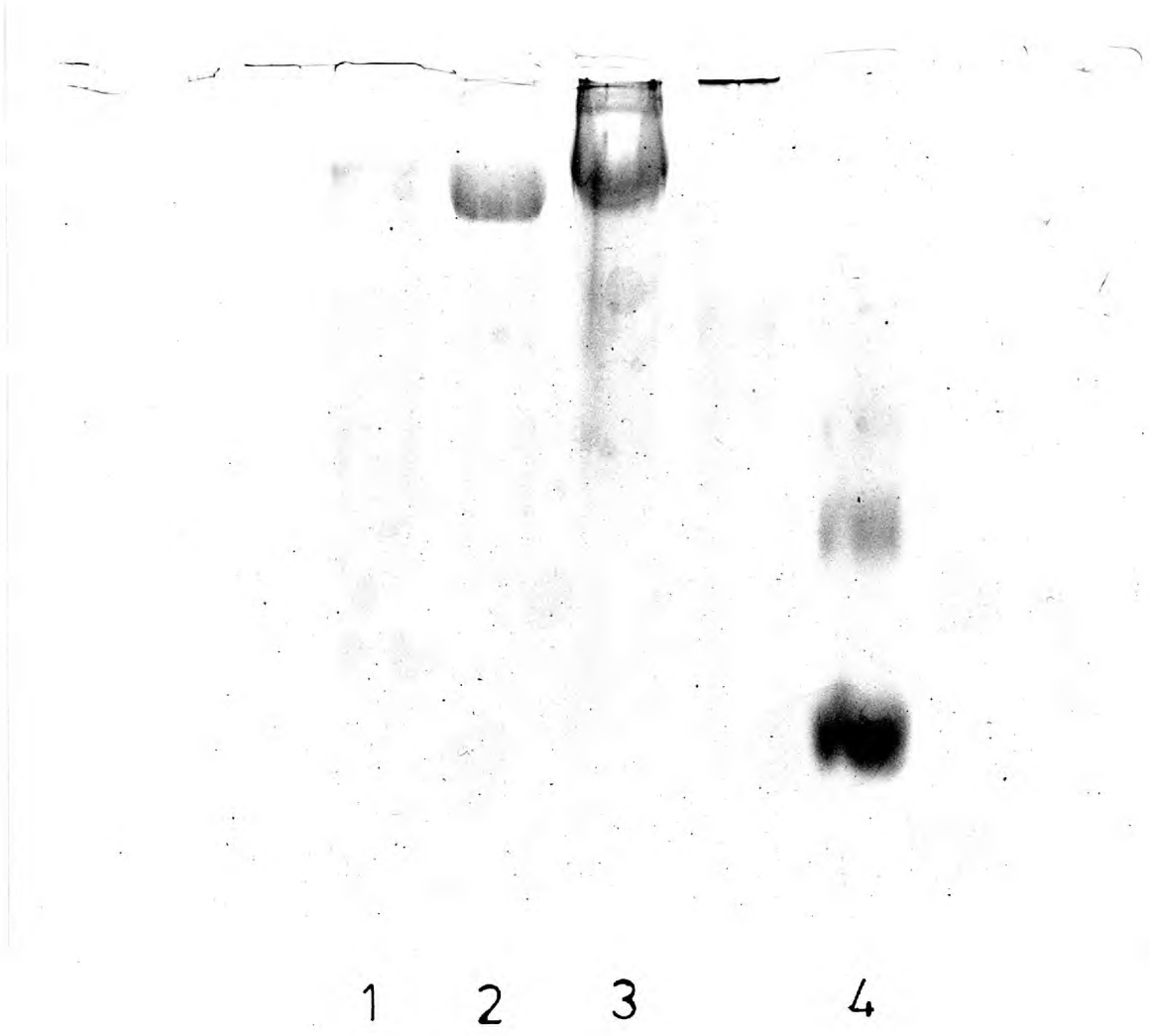



Figure 23

Polyacrylamide gel electrophoresis of skeletal muscle sarcoplasmic reticulum

1 ATPase purified by solubilisation with deoxycholate

2 ATPase purified by solubilisation with Triton X100

3 Intact sarcoplasmic reticulum

4 Molecular weight marker

cardiac ATPase, since in guinea pig cardiac SR the basal ATPase activity is relatively low (Nayler et al., 1975). The purification obtained in skeletal SR was more satisfactory when Triton was used to solubilise the enzyme. This method was, therefore, also used to purify guinea pig cardiac SR ATPase, the only difference in the method being that the pH of the solubilisation medium was 8.0, not 8.5. Figure 24 shows basal and total ATPase activity in intact SR, and in the supernatant fraction following Triton solubilisation. Triton solubilisation resulted in a slight increase in the total activity, and the basal activity, though not completely abolished, is more markedly reduced than in rabbit cardiac SR. However, due to the small amount of material available using guinea pig SR, the quantity of material obtained as a particulate fraction after Sepharose 4B chromatography was too little to perform any enzyme assays or for polyacrylamide gel electrophoresis.

(e) Solubilisation with digitonin

Digitonin is a detergent which binds specifically to βOH-steroids, especially cholesterol (Elias et al., 1979). It has been used to extract monoamine oxidase from the outer mitochondrial membrane (Erwin and Hellerman, 1967; Schnaltman et al., 1967), which can be selectively solubilised with digitonin.

Figure 25 shows basal and total ATPase activity of intact guinea pig cardiac SR, and of the supernatant fraction obtained after solubilisation with 2% digitonin. Although in the solubilised SR basal activity is reduced, it is not abolished, and no increase in total ATPase activity is observed. Solubilisation with digitonin does not, therefore, have any advantage over solubilisation with Triton X100.

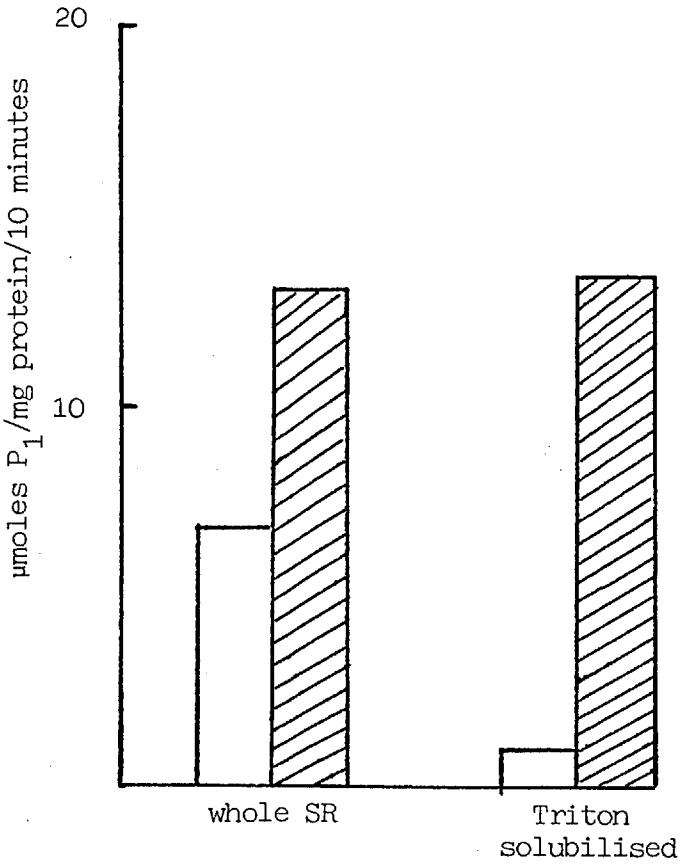



Figure 24

Purification of guinea pig cardiac sarcoplasmic reticulum by solubilisation with Triton X100

Conditions as described in Methods. A single representative experiment is shown.

□ basal ATPase activity

▨ total ATPase activity

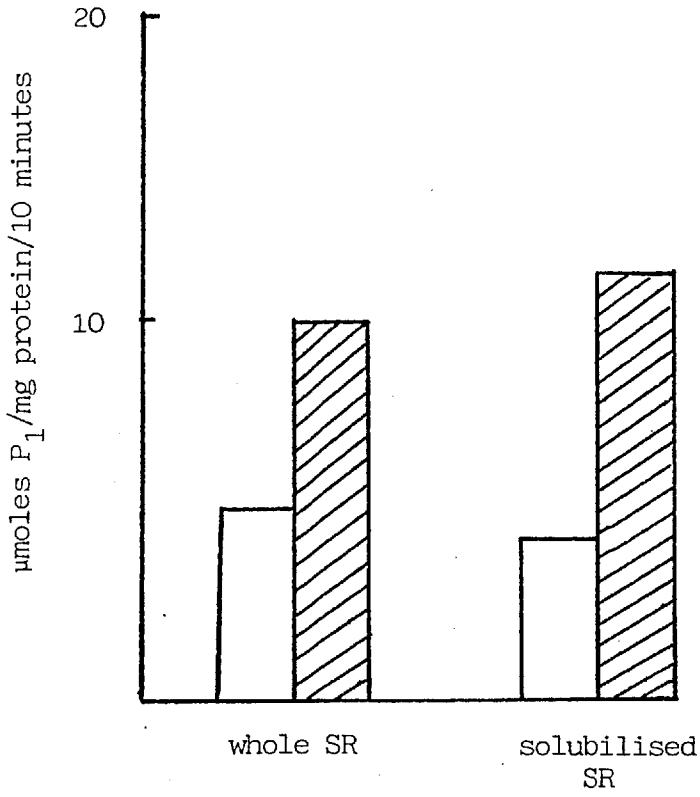



Figure 25

Solubilisation of guinea pig cardiac sarcoplasmic reticulum with digitonin

Conditions as described in Methods. A single representative experiment is shown.

basal ATPase activity

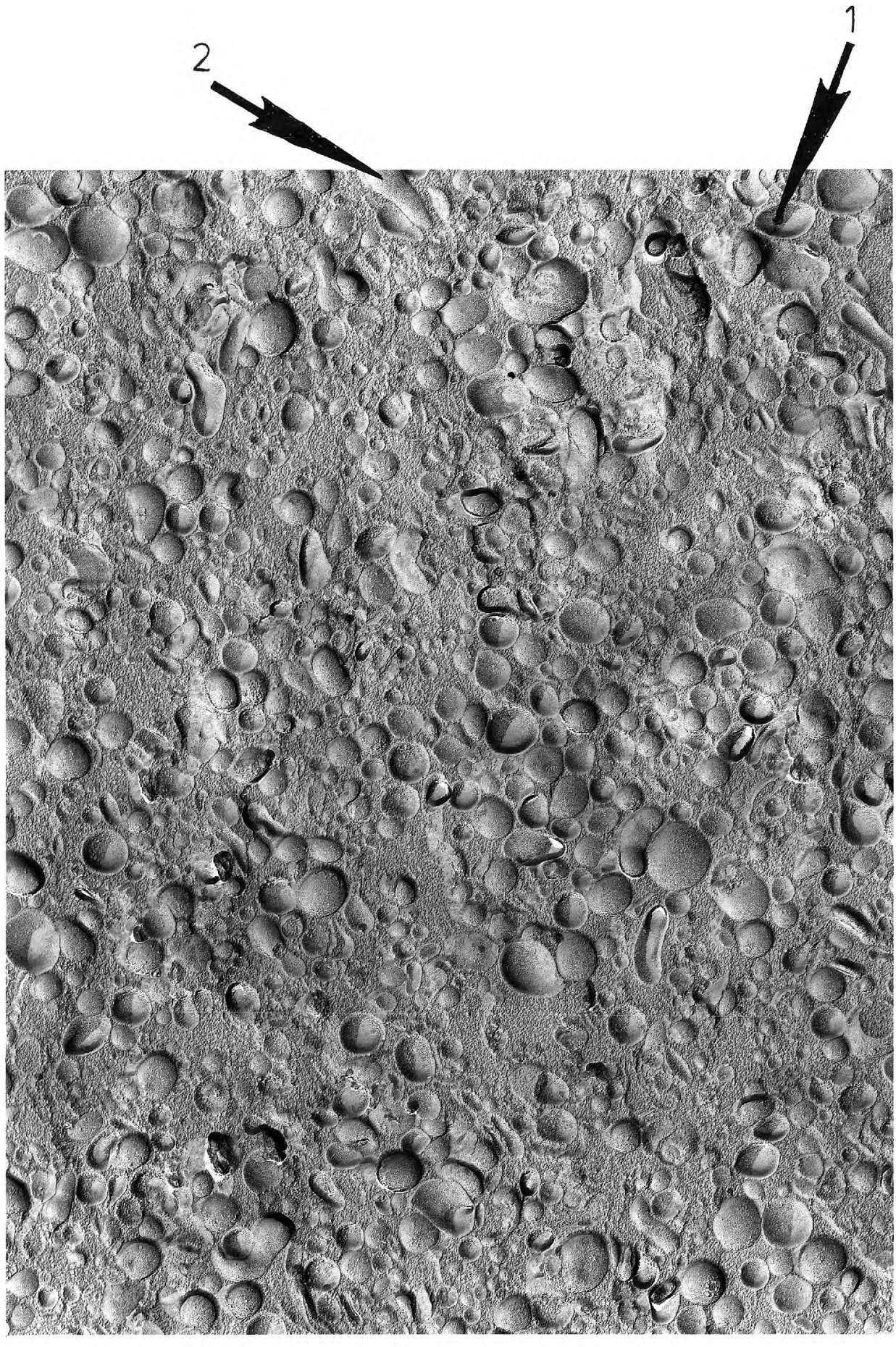
total ATPase activity

Figure 26

Appearance of guinea pig cardiac microsomal preparation as revealed by freeze-fraction electron microscopy

Magnification x 54,000.

The arrow in the bottom right-hand corner indicates the shadow direction. The other arrows indicate the intramembranous particles appearing on both convex (1) and concave (2) fracture faces.


1
2

(f) Appearance of the guinea pig microsomal fraction

Figure 26 is an electron micrograph showing the appearance of the cardiac microsomal preparation used in the present study as seen by freeze-fracture electron microscopy. The preparation can be seen to consist of a population of vesicles ranging in size between 0.05-0.2 μ diameter, and are either fractured in a convex or concave manner, to reveal the hydrophobic interior of the membrane. Intramembranous particles are present on both fracture faces of the vesicles (examples indicated by arrows), though many other vesicles appear smooth. These particles probably represent the ATPase of the SR, and in the SR of intact cells are distributed asymmetrically on the 'P' and 'E' fracture faces of the membrane (see Chapter 1, Section I). The micrograph shown in Figure 26 does not permit analysis of the different types of particle, but the heterogenous appearance of the vesicles suggests the possibility that both right side out and inside out vesicles are present in the preparation.

## SECTION IV

## Discussion

(a) Skeletal muscle sarcoplasmic reticulum

Using either of the methods described, a satisfactory purification of the calcium-dependent ATPase from skeletal muscle SR was obtained. However, the method of Ikemoto et al. (1971) utilising Triton X100 and a Sepharose 4B column, was found to be superior, since preparations obtained using this method exhibited a higher specific activity, the enzyme was 100% calcium sensitive, and a higher degree of purity was revealed by polyacrylamide gel electrophoresis.

(b) Cardiac muscle sarcoplasmic reticulum

A major difficulty in isolating the purified calcium-dependent ATPase from cardiac SR was the small amount of material available. This, together with the smaller amount of the enzyme present in cardiac, as compared with skeletal SR (Shigekawa et al., 1976), led to a very low yield of the enzyme after purification.

Although some increase in the specific activity of the calcium-activated enzyme was obtained, the increase was generally rather small. In addition, the purified preparation retained rather high basal ATPase activity. This is in contrast to the results obtained by Van Winkle et al. (1978), and Levitsky et al. (1976), who obtained preparations which showed 100% calcium-dependent ATPase activity. This may partly be due to species differences. Using guinea pig cardiac SR, which has a lower basal activity in the intact SR, solubilised SR preparations exhibited lower basal activity, but this was not completely abolished.

(c) Nature of the calcium-independent adenosine triphosphatase activity

The question arises as to whether the basal ATPase activity represents a separate and contaminating enzyme, or whether it represents a different form of the calcium-dependent enzyme.

It was originally thought that the basal and calcium-activated ATPase activities represented two separate enzymes. This was based on the observation that the two activities exhibit different Kms for ATP (Weber et al., 1966; Yamamoto and Tonomura, 1967). However, the calcium-dependent ATPase itself exhibits a biphasic dependence on the concentration of ATP (Yamamoto and Tonomura, 1967; Inesi et al., 1967; Vianna, 1975; Moller et al., 1980) and others (see Chapter 1, Section III).

Separation of skeletal SR into light and heavy fractions by sucrose density gradient centrifugation shows that the light fraction exhibits higher basal ATPase activity than the heavy fraction, although the total ATPase activity of the different fractions is similar (Inesi et al., 1976b; Sarzala and Michalek, 1978). Only the heavier fractions exhibit calcium transport activity (Inesi et al., 1976; Sarzala and Michalek, 1978; Chevalier et al., 1977). Polyacrylamide gel electrophoresis of the different fractions shows that all the fractions have a similar protein composition. All fractions exhibit a major band of approximate molecular weight 100,000, corresponding to the ATPase (Inesi et al., 1976b; Sarzala and Michalek, 1978; Chevalier et al., 1977). The different fractions vary slightly in the ratios of acidic and binding proteins, the heavy fraction containing a greater proportion of the protein calsequestrin (Sarzala and Michalek, 1978; Inesi et al., 1976b). The 100,000 Dalton protein of the lighter fraction cross-reacts immunologically with the calcium dependent ATPase protein of the heavy fraction (Sarzala and Michalek, 1978).

The SR fractions exhibiting high and low basal ATPase activity therefore have similar protein composition, but differ in the expression of enzyme activity. Under certain conditions, basal ATPase activity in the lighter fractions can be converted to calcium-dependent activity. Treatment with Triton X100 converts all the ATPase activity in the light fraction to calcium-dependent activity, with little change in total activity. Calcium-independent ATPase activity observed at low temperatures (5-10$^{o}$C) can also be converted to calcium-dependent activity by raising the temperature to 37$^{o}$C (Inesi et al., 1976).

(d) Functional state of the adenosine triphosphatase

It has been proposed that the basic and calcium-activated ATPase activity corresponds to the enzyme in two different states. In one state the enzyme shows calcium-dependent ATPase activity. Adenosine triphosphate hydrolysis by this form of the enzyme proceeds by enzyme phosphorylation as an intermediate step, and is coupled with calcium transport. In native SR at sufficiently high ATP concentrations, the enzyme can also exist in a state in which ATP hydrolysis is 'uncoupled' from calcium transport and a phosphorylated intermediate cannot be isolated (Inesi et al., 1976). This is similar to the scheme put forward by Froelich and Taylor (1976), discussed in Chapter 1, Section III. These authors propose that in the absence of calcium and at relatively high ATP concentrations and an acid labile phosphorylated intermediate is formed via calcium-independent ATP hydrolysis. An acid labile intermediate would be broken down by the normal method of T.C.A. precipitation used to isolate phosphorylated intermediates, and would account for the observation that a phosphorylated intermediate could not be isolated in the absence of calcium. However, Froelich and Taylor (1976) postulated that in vivo a coupling mechanism exists, and that acid stable and acid labile intermediates are formed sequentially, and that conversion of acid stable intermediates to the acid labile form is accelerated by high concentrations of ATP (Froelich and Taylor, 1975). The mechanism of the ATPase could then be described in terms of 'half the sites reactivity' or 'flip-flop' mechanism. According to these hypotheses, the activity of only one of two apparently identical subunits is expressed in a given reaction. This may arise from 'negative co-operativity', i.e. binding of substrate at one subunit is coupled to a reduction of affinity at the second subunit (Stein et al.,

1974). In terms of the SR ATPase, the enzyme would alternate between states of high calcium affinity (acid stable intermediate) and low calcium affinity (acid labile intermediate) out of phase with each other. The ATPase is thought to exist in the membrane in an oligomeric form, and it is likely that an ATP regulatory site, activated by high ATP concentrations, is situated on a different polypeptide chain from the active site (see Chapter 1). Basal ATPase activity could thus represent ATP hydrolysis at the regulatory site.

(e) Effect of lipid environment

Phospholipid environment is known to affect ATPase activity, since treatment of SR membranes with phospholipase c leads to inhibition of calcium transport and ATPase activity parallel with hydrolysis of membrane lecithin to diglycerides (Martinosi et al., 1979; Yu et al., 1968). This is thought to be due to an inhibition of phosphoprotein hydrolysis (Martinosi et al., 1971; Hidalgo et al., 1976).

The phospholipid pattern and cholesterol content is similar in both light and heavy SR fractions. However, the ratio of phospholipid to protein is almost one and a half times higher in light than in heavy vesicles. A possible association of cholesterol with particles exhibiting high basal ATPase activity is thought to be due to contamination of this fraction with t-tubules (Barret et al., 1980; Flaherty et al., 1975). This is consistent with the experiment shown in Figure 25, in which digitonin was used to solubilise cardiac SR membranes. The decrease in basal ATPase activity was no greater than the decrease in the activity observed using Triton X100 to solubilise the membrane. This result is expected, since SR membranes contain only a small amount of cholesterol compared to the sarcolemma (Barret et al., 1980; Flaherty et al., 1975).

(f) Orientation of vesicles

The SR membrane is known to be asymmetric. The 90 Å particles occur on the E face of freeze-fractured preparations, whilst the 35 Å particles appear on the P face of the membrane (see Chapter 1). It has also been shown that in membranous ATPase preparations purified by deoxycholate solubilisation and removal of detergent by dialysis, the asymmetric distribution of surface particles is lost, and this is correlated with loss of calcium transport activity in purified preparations (Packer et al., 1974).

Freeze-fracture electron microscopy of light and heavy SR fractions shows that light vesicles exhibiting low calcium transport activity are largely inside out (Chevalier et al., 1977). This observation that light vesicles may be inside out is supported by studies that show that treatment of light vesicles with EDTA to render them leaky, increased the calcium-activated ATPase activity of the vesicles, and also increased the amount of phosphatidylethanolamine and phosphatidylserine which is digested with phospholipase c or $a_2$ (Sarzala and Michalek, 1977). However, although labelling studies with non-permeant and permeant labels have shown that phosphatidylethanolamine is located at the outside surface of the membrane, phosphatidylserine has been shown to be located at the inside surface of the membrane (Vale, 1977; Hidalgo and Ikemoto, 1977). If the light vesicles represent inside out vesicles, then it would be expected that phosphatidylserine would be readily hydrolised by phospholipase c in intact vesicles. It is therefore more likely that the light fraction contains vesicles of mixed orientation.

(g) Aggregational state of the adenosine triphosphatase

Calcium-activated ATPase activity is associated with the active site of the ATPase molecule. This is thought to be located at the outer surface of the SR membrane, probably associated with the 35 Å surface particles. If basal ATPase activity is caused by ATP hydrolysis at the regulatory site, this could occur at the inner surface of the membrane, particularly if the active site requires dimeric enzyme interaction, since the 90 Å particles at the inner surface of the membrane are thought to be the result of oligomeric interaction between the hydrophobic ends of 3-4 polypeptide chains (Scales and Inesi, 1976 a and b). This would also lead to the appearance of high basal ATPase activity in the lighter SR fractions, if these fractions contain a high proportion of inside out vesicles. This could also explain the loss of basal ATPase activity in detergent-treated SR, since the ATPase solubilises mainly as a monomer when treated with deoxycholate (le Maire et al., 1976a; Jorgensen et al., 1978), or Triton X100 (Moller et al., 1980); Neet and Green, 1977). However, chromatography on a Sepharose 4B column led to the elution of the enzyme as monomers, dimers and high molecular weight oligomers, and the monomeric form was enzymatically inactive, while the oligomeric species retained calcium-dependent ATPase activity (Le Maire et al., 1978). Basal ATPase activity in these preparations was not recorded.

(h) Possible reasons for high basal ATPase activity in cardiac sarcoplasmic reticulum preparations

The presence of high basal ATPase activity in isolated cardiac SR could indicate a greater degree of 'uncoupling' of acid labile phosphoprotein formation and acid stable phosphorylation. Alternatively,

it could reflect a different equilibrium of the reaction converting acid stable to acid labile intermediates. The kinetics of inter-conversion of different phosphoenzymes have been shown to differ in cardiac and skeletal muscle preparations (Sumida et al., 1980) (see Chapter 1, Section IVa).

The higher basal activity in cardiac SR could also be due to a higher ratio of phospholipid to protein in cardiac, as compared to skeletal, SR preparations. This is consistent with the lower amount of ATPase protein which is present in cardiac SR (Shigekawa et al., 1976).

Alternatively, isolated cardiac SR preparations could contain a higher proportion of inside out vesicles, though it is difficult to visualise why this should arise.

(i) Purity of isolated cardiac adenosine triphosphatase preparations

The presence of a high basal activity in cardiac SR vesicles following purification procedures, is not therefore in itself an indication of impurity of the preparation, but may simply indicate that the enzyme is present in two different functional states, or it may be due to the presence of vesicles of mixed orientation, or to a relatively high phospholipid content of the preparations. It could also be due to the use of a relatively low concentration of Triton X100 used to solubilise the enzyme. The ratio of Triton to protein used is half to one-third of that used by Ikemoto et al. (1971) for skeletal SR, and could lead to the retention of relatively high amounts of lipid in the solubilised preparations. Solubilisation of skeletal SR at low detergent to protein ratios has been shown to lead to the formation of monomeric ATPase preparations which retain the biphasic substrate dependence of the intact SR (Moller et al., 1980; Neet and Green, 1977;

Taylor and Hatton, 1979), and this has been explained by the existence of two regulatory ATP binding sites, one on the same polypeptide chain as the active site, and the other on a separate polypeptide chain, which only exerts its effect through dimeric enzyme interaction of the two polypeptide chains (see Chapter 1, Section IIIc) (Moller et al., 1980). If basal ATPase activity represents ATP hydrolysis at the regulatory site, retention of high basal activity in solubilised cardiac ATPase preparations could be due to a greater contribution of the regulatory sites on the same polypeptide chain as the active site compared to skeletal preparations.

The isolation of purified cardiac ATPase preparations which exhibit 100% calcium dependence, by Levitsky et al. (1976), may be due to the fact that prior to enzyme purification these authors isolated a heavy SR fraction exhibiting calcium transport activity. This fraction was used for subsequent enzyme purification. The vesicles containing the major part of the basal activity were therefore removed prior to enzyme purification. However, this does not explain the 100% calcium dependence of the cardiac ATPase isolated by Van Winkle et al. (1978).

The purity and functional state of the isolated SR preparations could be further assessed by polyacrylamide gel electrophoresis to ascertain the protein composition of native SR and purified preparations, by further freeze-fracture electron microscopy to determine the distribution of surface particles, analysis of phospholipid content of native SR and purified preparations and kinetic analysis of the substrate dependence of intact and solubilised cardiac SR. However, all these studies would require isolation of the ATPase in larger quantities than was achieved in this study.

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## REFERENCES

Akera, T., Larson, F.S. and Brody, T.N. (1970).
J. Pharmacol. Exp. Ther. 173, 145-151.

Allen, D.G. and Blinks, J.R. (1978).
Nature 273, 509-513.

Baird, G.D. and Perry, S.V. (1960).
Biochem. J. 77, 262-271.

Barlogie, B., Hasselbach, W. and Makinose, M. (1971).
FEBS Lett. 12, 267-268.

Barrett, E.J., Ryan, N.M. and Headon, D.R. (1980).
Biochem. J. 188, 569-572

Baskin, R.J. and Deamer, D.W. (1969).
J. Cell Biol. 43, 610-617.

Bayer, R., Hennekes, R., Kaufmann, R. and Mannhold, R. (1975).
Naunyn-Schneideberg's Arch. Pharmacol. 290, 49-68.

Bayer, R., Kaufman, R., and Mannhold, R. (1975a).
Naunyn-Schmeideberg's Arch. Pharmacol. 290, 69-80.

Bayer, R., Kalusche, D., Kaufmann, R. and Mannhold, R. (1975b).
Naunyn-Schmeideberg's Arch. Pharmacol. 290, 81-97.

Beeler, T., Russel, J.T. and Martinosi, A. (1979).
Eur. J. Biochem. 95, 579-591.

Beeler, T. and Martinosi, A. (1979).
FEBS Lett. 98, 173-176.

Beil, F.U., von Chak, D. and Hasselbach, W. (1977).
Eur. J. Biochem. 81, 151-164.

Bendall, J.R. (1952a).
Nature 19 170, 1058-1060.

Bendall, J.R. (1952b).
Proc. Roy. Soc. B. 124, 523-525.

References (continued)...

Bendall, J.R. (1953).

J. Physiol. 2 121, 232-254.

Bendall, J.R. (1954).

Proc. Roy. Soc. B. 142, 409-426.

Bendall, J.R. (1958).

Nature 181, 1188-1190.

Beresewicz, A. and Reuter, H. (1977).

Naunyn-Schmeiderberg's Arch. Pharmacol. 301, 99-107.

Besch, H.R., Allen, J.C., Glick, G. and Schwartz, A. (1970).

J. Pharmacol. Exp. Ther. 171, 1-12.

Bianchi, C.P. and Bolton, T.C. (1966).

J. Pharmacol. Exp. Ther. 151, 456-463.

Blinks, J.R., Prendergast, F.G. and Allen, D.G. (1976).

Pharmacol. Rev. 28, 1-93.

Bozler, E. (1954).

J. Gen. Physiol. 38, 149-159.

Bradford, M.M. (1976).

Anal. Biochem. 72, 248-254.

Briggs, F.N. and Fuchs, F. (1960).

Biochim. Biophys. Acta 42, 519-527.

Briggs, F.N., Wise, R.M. and Hearn, J.A. (1978).

J. biol. Chem. 253, 5884-5885.

Carafoli, E. (1975).

J. Mol. Cell. Cardiol. 7, 83-89.

Carafoli, E. and Crompton, M. (1978).

Current Topics in Membrane Transport 10, 151-216.

Carsten, M.E. (1964).

Proc. Natl Acad. Sci. 52, 1456-1462.

References (continued)...

Carsten, M.E. (1967).

Circ. Res. 20, 599-605.

Carter, N.W., Rector, F.C., Campion, D.S. and Seldin, D.W. (1967).

J. Clin. Invest. 46, 920-933.

Carvalho, A.P. and Leo, B. (1967).

J. Gen. Physiol. 50, 1327-1352.

Chaloub, R.M., Guimaraes-Motta, H., Verjovski-Almeida, S. and de Meis, L. (1979). J. biol. Chem. 254, 9464-9468.

Chapman, R.A. (1979).

Prog. Biophys. Mol. Biol. 35, 1-52.

Chapman, R.A. and Miller, D.J. (1974).

J. Physiol. 242, 615-634.

Chimoskey, J.E. and Gergely, J. (1968a).

Arch. Int. Pharmacodyn. 176, 289-297.

Chesnais, J.M., Coraboeuf, E., Sauviat, M.P. and Vassais, J.M. (1975).

J. Mol. Cell. Cardiol. 7, 627-642.

Chevalier, J. and Butow, R.A. (1971).

Biochemistry 10, 2733-2737.

Chevallier, J., Bonnet, J-P, Galante, M., Tenu, J-P. and Gulik-Krzywicki, T. (1977). Biol. Cellulaire 30, 103-110.

Chimoskey, J.E. and Gergely, J. (1968)

Arch. Biochem. Biophys. 128, 601-605.

Chui, U.C.K. and Haynes, D.H. (1977).

Biophys. J. 18, 3-22.

Cingolani, H.E., Mattiazzi, A.R., Blesa, E.S. and Gonzales, N.C. (1970).

Circ. Res. 26, 269-278.

Coan, C.R. and Inesi, G. (1976).

Biochem. Biophys. Res. Commun. 71, 1283-1288.

References (continued)...

Deamer, D.W. and Baskin, R.J. (1969).

J. Cell. Biol. 42, 296-307.

De Meis, L. (1972).

Biochemistry 11, 2460-2465.

De Meis, L. (1976).

J. biol. Chem. 251, 2055-2062.

De Meis, L. and Boyer, P.D. (1978).

J. biol. Chem. 253, 1556-1559.

De Meis, L. and Carvalho, M.G.C. (1976).

J. biol. Chem. 251, 1413-1417.

De Meis, L. and De Mello, M.C.F. (1973).

J. biol. Chem. 248, 3691-3701.

De Meis, L. and Tume, R.K. (1977).

Biochemistry 16, 4455-4463.

Dupont, Y. (1977).

Eur. J. Biochem. 72, 185-190.

Dupont, Y. and Leigh, J.B. (1978).

Nature 273, 396-398.

Duggan, P.F. and Martinosi, A. (1970).

J. Gen. Physiol. 56, 147-167.

Dunnett, S.J. (1976).

M.Phil. Thesis, University of London.

Ebashi, S. (1958).

Arch. Biochem. Biophys. 76, 410-423.

Ebashi, S. (1961a).

J. Biochem. 50, 236-244.

Ebashi, S. (1961b).

Supplement of the Progress in Theoretical Physics 17, 35-40.

References (continued)...

Ebashi, S. and Endo, M. (1964).

In 'Biochemistry of Muscle Contraction' (Ed. J. Gergely), Little, Brown & Co., Boston, pp.199-206.

Ebashi, S. and Endo, M. (1968).

In 'Progress in Biophysics and Molecular Biology' (Eds Butler, J.A.V. and Noble, D.), Pergamon Press, Oxford, p.123-183.

Ebashi, S. and Lippmann, F. (1962).

J. Cell. Biol. 14, 389-400.

Elias, P.M., Goerke, J., Freund, D.S. and Brown, B.E. (1978).

J. Cell. Biol. 78, 577-596.

Endo, M. (1975a).

Proc. Japan Acad. 51, 467-472.

Endo, M. (1975b).

Proc. Japan Acad. 51, 497-484.

Endo, M. (1977).

Physiol. Rev. 57, 71-108.

Endo, M. and Blinks, J.R. (1973).

Nature New Biol. 246, 218-221.

Endo, M., Tanaka, M. and Ogawa, Y. (1970).

Nature 228, 34-36.

Entman, M.L., Bornet, E.P. and Schwartz, A. (1972).

J. Mol. Cell. Cardiol. 4, 155-169.

Entman, H.L., Allen, J.C., Bornet, E.P., Gillette, P.C., Wallick, E.T. and Schwartz, A. (1972). J. Mol. Cell. Cardiol. 4, 681-687a.

Entman, M.L., Snow, T.R., Freed, D. and Schwartz, A. (1973).

J. biol. Chem. 248, 7762-7772.

Erwin, V.G. and Hellerman, L. (1967).

J. biol. Chem. 242, 4230-4238.

References (continued)...

Fabiato, A. and Fabiato, F. (1972).
Circ. Res. 31, 293-307.

Fabiato, A. and Fabiato, F. (1973).
Eur. J. Cardiol. 1, 143-155.

Fabiato, A. and Fabiato, F. (1975).
Nature 253, 556-558.

Fabiato, A. and Fabiato, F. (1975a).
J. Physiol. 244, 469-495.

Fabiato, A. and Fabiato, F. (1975b).
J. Physiol. 249, 497-517.

Fabiato, A. and Fabiato, F. (1977).
J. Gen. Physiol. 70, 6a.

Fabiato, A. and Fabiato, F. (1978).
Biochim. Biophys. Acta 539, 253-260.

Fabiato, A. and Fabiato, F. (1978a).
J. Physiol. 276, 233-255.

Fabiato, A. and Fabiato, F. (1978b).
Annals N.Y. Acad. Sci. 307, 491-522.

Fabiato, A. and Fabiato, F. (1979).
Ann. Rev. Physiol. 41, 473-484.

Fanburg, B., Finkel, R.M. and Martinosi, A. (1964).
J. biol. Chem. 239, 2298-2306.

Fanburg, B.L. and Matsushita, S. (1973).
J. Mol. Cell. Cardiol. 5, 111-115.

Fawcett, D.W. (1961).
Circulation 24, 336-348.

Fawcett, D.W. and McNutt, N.S. (1969).
J. Cell. Biol. 42, 1-45.

References (continued)...

Fish, W.W., Reynolds, J.A. and Tanford, C. (1970).
J. biol. Chem. 245, 5166-5168.

Flaherty, J.O., Barrett, E.J., Bradley, D.P. and Headon, D.R. (1975).
Biochim. Biophys. Acta 401, 177-183.

Fleckenstein, A. (1970-71).
In 'Calcium and the Heart' (Eds. Harris, P. and Opie, L.),
Academic Press, London, New York, pp.135-138.

Fleckenstein, A. and Spah, F. (1980).
In '$Ca^{++}$-Antagonism. Mechanics of Action and Clinical Implications',
(Ed. Richardson, R.G.), Abbott Laboratories Ltd., pp.5-21.

Ford, L.E. and Podolski, R.J. (1970).
Science 167, 58-59.

Ford, L.E. and Podolsky, R.J. (1972a).
J. Physiol. 223, 1-19.

Ford, L.E. and Podolsky, R.J. (1972b).
J. Physiol. 223, 21-33.

Froelich, J.P. and Taylor, E.W. (1975).
J. biol. Chem. 250, 2013-2021.

Froelich, J.P. and Taylor, E.W. (1976).
J. biol. Chem. 251, 2307-2315.

Fuchs, F. and Briggs, F.N. (1961).
Biochim. Biophys. Acta 51, 423-425.

Fuchs, F. amd Briggs, F.N. (1962-63).
J. Gen. Physiol. 46, 893-904.

Galani-Kranias, E., Bick, R. and Schwartz, A. (1980).
Biochim. Biophys. Acta G83, 438.

Gertz, E.W., Hess, M.L., Lain, R.F. and Briggs, F.N. (1967).
Circ. Res. 20, 477-484.

References (continued)...

Glynn, I.M. (1964).

Pharmacol. Rev. 16, 381-407.

Goodall, M.C. and Szeat-Gyorgyi, A.G. (1953).

Nature 172, 84-85.

Green, D.E. and Brucker, R.F. (1972).

Bioscience, 22, 13

Harigaya, S. and Schwartz, A. (1969).

Circ. Res. 25, 781-794.

Harigaya, S, and Schwartz, A. (1969).

Circ. Res. 25, 781-794.

Hasselbach, W. (1964a).

Proc. Roy. Soc. B. 160, 501-504.

Hasselbach, W. (1964b).

Prog. Biophys. Mol. Biol. 14, 167-222.

Hasselbach, W. (1978).

Biochim. Biophys. Acta 515, 23-53.

Hasselbach, W. and Makinose, M.B. (1961).

Biochem. Z. 333, 518-528.

Hasselbach, W. and Makinose, M.(1962).

Biochem. Biophys. Res. Commun. 7, 132-136.

Hasselbach, W. and Makinose, M. (1963).

Biochem. Z. 339, 94-111.

Hasselbach, W. and Elfvin, L.G. (1967).

J. Ultrastruct. Res. 17, 598-622.

Hasselbach, W., Beil, F.V. and Rauch, B. (1978).

In 'The Proton and Calcium Pumps' (Eds. Azzone, G.K. et al.),

Elsevier/North Holland Biomedical Press, pp. 3-16.

References (continued)...

Hidalgo, C., Ikemoto, N. and Gergely, J. (1976).
J. biol. Chem. 251, 4226-4232.

Hidalgo, C. and Ikemoto, N. (1977).
J. biol. Chem. 252, 8446-8454.

Hoeschen, R.J. and Nayler, W.G. (1976).
Clin. Res. 24, 647a.

Holland, W.C. and Sekul, A.A. (1959).
Amer. J. Physiol. 197, 757-760.

Horgan, D.J. (1974).
Arch. Biochem. Biophys. 162, 6-11.

Horl, W.H., Jennissen, H.P. and Heilmayer, L.M.G. Jr. (1978a).
Biochemistry 17, 759-766.

Horl, W.H. and Heilmeyer, L.M.G. Jr. (1978b).
Biochemistry 17, 766-772.

Huxley, A.F. and Straub, R.W. (1958).
J. Physiol. (London) 143, 40-41P.

Huxley, A.F. and Taylor, R.E. (1958).
J. Physiol. (London) 144, 426-441.

Huxtable, R. and Bressler, R. (1974).
J. Membrane Biol. 17, 189-197.

Ikemoto, N. (1974).
J. biol. Chem. 249, 649-651.

Ikemoto, N. (1975).
J. biol. Chem. 250, 7219-7224.

Ikemoto, N. (1976).
J. biol. Chem. 251, 7275-7277.

Ikemoto, N., Bhatnagar, G.M., Nagy, B. and Gergely, J. (1972).
J. biol. Chem. 247, 7835-7837.

References (continued)...

Ikemoto, N., Bhatnagar, G.M. and Gergely, J. (1971).
Biochem. Biophys. Res. Commun. 44, 1510-1517.

Ikemoto, N., Streter, F.A., Nakamura, H. and Gergely, J. (1968).
J. Ultrastruct. Res. 23, 216-232.

Inesi, G., Ebashi, S. and Watanabe, S. (1964).
Amer. J. Physiol. 207, 1339-1344.

Inesi, G. and Asai, H. (1968).
Arch. Biochem. Biophys. 126, 469-477.

Inesi, G. and Almendarer, J. (1968).
Arch. Biochem. Biophys. 126, 733-735.

Inesi, G., Maring, E., Murphy, A.T. and MacFarland, B.H. (1970).
Arch. Biochem. Biophys. 138, 285-294.

Inesi, G. and Scales, D. (1974).
Biochemistry 13, 3298-3306.

Inesi, G., Goodman, J.J. and Watanabe, S. (1967).
J. biol. Chem. 242, 4637-4643.

Inesi, G., Cohen, J.A. and Coan, C.R. (1976).
Biochemistry 15, 5293-5298.

Inesi, G. and Malan, N. (1976).
Life Sci. 18, 773-780.

Jilka, R.L., Martinosi, A. and Tillack, T.W. (1975).
J. biol. Chem. 250, 7511-7524.

Johannsson, M. and Nilsson, E. (1975).
Acta Physiol. Scand. 93, 295-309.

Jones, L.R., Besch, H.R. and Watanabe, M. (1977).
J. biol. Chem. 252, 3315-3323.

Jorgensen, K.E., Lind, K.E., Zøigaard-Petersen, H. and Møller, J.V.
(1978). Biochem. J. 169, 489-498.

References (continued)...

Josephson, I. and Sperelakis, N. (1977).
J. Mol. Cell. Cardiol. 9, 409-418.

Kalbitzer, H.R., Stehlik, D. and Hasselbach, W. (1978).
Eur. J. Biochem. 82, 245-255.

Kanazawa, T. (1975).
J. biol. Chem. 250, 113-119.

Kanazawa, T., Yamada, S. and Tonomura, Y. (1970).
J. Biochem. 68, 593-595.

Kanazawa, T., Yamada, S., Yamamoto, T. and Tonomura, Y. (1971).
J. Biochem. 70, 95-123.

Kanazawa, T. and Boyer, P.D. (1973).
J. biol. Chem. 248, 3153-3172.

Kasai, M. and Miyamoto, H. (1973).
FEBS Lett. 34, 299-301.

Kasai, M. and Miyamoto, H. (1976a).
J. Biochem. 79, 1053-1066.

Kasai, M. and Miyamoto, H. (1976b).
J. Biochem. 79, 1067-1076.

Katz, A.M. (1970).
Physiol. Rev. 50, 63-158.

Katz, A.M., Repke, D.I., Upshaw, J.E. and Polascik, M.A. (1970).
Biochim. Biophys. Acta 205, 473-490.

Katz, A.M., Dunnett, J., Repke, D.I. and Hasselbach, W. (1976).
FEBS Lett. 67, 207-208.

Katz, A.M., Repke, D.I. and Hasselbach, W. (1977).
J. biol. Chem. 252, 1938-1949.

Katz, A.M., Repke, D.I., Dunnett, J. and Hasselbach, W. (1977a).
J. biol. Chem. 252, 1950-1956.

References (continued)....

Katz, A.M., Repke, D.I., Fudyma, G. and Shigekawa, M. (1977b).
J. biol. Chem. 252, 4210-4214.

Katz, A.M., Louis, C.F., Repke, D.I., Fudyma, G., Nash-Adler, P.A., Upsaw, R. and Shigekawa, M. (1980). Biochim. Biophys. Acta 596, 94-107.

Katz, S. and Remtulla, M.A. (1978).
Biochem. Biophys. Res. Commun. 83, 1373-1379.

Kentish, J. and Nayler, W.G. (1977).
J. Physiol. 265, 18P.

Kirchberger, M.A., Tada, M., Repke, D.I. and Katz, A.M. (1972).
J. Mol. Cell. Cardiol. 4, 673-680.

Kirchberger, M.A., Tada, M. and Katz, A.M. (1974).
J. biol. Chem. 249, 6166-6173.

Kirchberger, M.A. and Wong, D. (1978).
J. biol. Chem. 253, 6941-6945.

Kirchberger, M.A. and Chu, G. (1976).
Biochim. Biophys. Acta 419, 559-562.

Kirchberger, M.A. and Tada, M. (1976).
J. biol. Chem. 251, 725-729.

Kirchberger, M.A. and Raffo, A. (1977).
J. Cyclic Nucleotide Research 3, 45-53.

Klaus, W. and Lee, K.S. (1969).
J. Pharmacol. Exp. Ther. 166, 68-76.

Knowles, A.I. and Racker, E. (1975).
J. biol. Chem. 250, 1949-1951.

Kohlhardt, M., Haap, R. and Figulla, H.R. (1976).
Pflugers Arch. 366, 31-38.

References (continued)...

Kumagai, H., Ebashi, S. and Takeda, F. (1955).
Nature 176, 166.

Kurzmack, M., Verjouski-Almeida, S. and Inesi, G. (1977).
Biochem. Biophys. Res. Commun. 78, 772-776.

Kurzmack, M. and Inesi, G. (1977).
FEBS Lett. 74, 35-37.

Landgraf, W.C. and Inesi, G. (1969).
Arch. Biochem. Biophys. 130, 111-118.

Lee, K.S., Ladiwsky, H., Choi, S.J. and Kasuya, Y. (1966).
J. Gen. Physiol. 49, 689-715.

Lee, K.S. and Klaus, W. (1971).
Pharmacol. Rev. 23, 193-261.

LeMaire, M., Lind, K.E., Jorgensen, K.E., Roigaard, H. and Møller, J.V.
(1978). J. biol. Chem. 253, 7051-7060.

LeMaire, M., Jorgensen, K.E., Røigaard-Petersen, H. and Møller, J.V.
(1976 ). Biochemistry 15, 5805-5812.

LePeuch, C.J., Haiech, J. and Lemaille, J.G. (1979).
Biochemistry 18, 5150-5157.

LePeuch, C.J., Guilleux, J-G. and Domaille, G. (1980).
FEBS Lett. 114, 165-168.

Levitsky, D.O., Aliev, M.K., Fuzmin, A.V., Levchenko, T.S., Smirnov, V.H.
and Chazov, E.I. (1976). Biochim. Biophys. Acta 443, 468-484.

Lindenmeyer, G.E. and Schwartz, A. (1975).
J. Mol. Cell. Cardiol. 7, 591-612.

Lipmann, F. and Tuttle, L.C. (1944).
J. biol. Chem. 153, 571-582.

Lorand, L. (1953).
Nature 172, 1181-1183.

References (continued)...

Lorand, L. and Moos, L. (1956).

Fed. Proc. 15, 121.

Lorkovic, H, (1966).

Circ. Research 19, 711-720.

Louis, C.F., Buonaffina, R. and Binks, B. (1974).

Arch. Biochem. Biophys. 161, 83-92.

Lowry, O.H., Rosebrough, N.T., Farr, A.L. and Randall, R.J. (1951).

J. biol. Chem. 193, 265-275.

Lullmann, H. and Holland, W. (1962).

J. Pharmacol. Exp. Ther. 137, 186-192.

McCollum, W.B., Besch, H.R., Entman, M.L. and Schwartz, A. (1972).

Amer. J. Physiol. 223, 608-614.

McElroy, W.T., Gerdes, A.J. and Brown, E.B. (1958).

Amer. J. Physiol. 195, 412-416.

MacLennan, D.H. (1970).

J. biol. Chem. 245, 4508-4518.

MacLennan, D.H. (1974).

J. biol. Chem. 249, 980-984.

MacLennan, D.H. and Wong, P.T.S. (1971).

Proc. Natl Acad. Sci. (U.S.A.) 68, 1231-1235.

MacLennan, D.H., Seeman, P., Iles, G.H. and Yip, C.C. (1971),

J. biol. Chem. 246, 2702-2710.

MacLennan, D.H., Yip, C.C., Iles, G.H. and Seeman, P. (1972).

Cold Spring Harbor Symposium on Quantitative Biology 37, 469-477.

Madiera, V.M.L. (1978).

Arch. Biochem. Biophys. 185, 316-325.

Madiera, V.M.L. (1979).

Arch. Biochem. Biophys. 193, 22-27.

References (continued)...

Madiera, V.M.L. (1980).

Arch. Biochem. Biophys. 200, 319-325.

Makinose, M. (1969).

Eur. J. Biochem. 10, 74-82.

Makinose, M. (1971).

FEBS Lett. 12, 269-270.

Makinose, M. (1972).

FEBS Lett. 25, 113-115.

Makinose, M. (1973).

FEBS Lett. 37, 140-143.

Makinose, M. and The, R. (1965).

Biochem. Z. 343, 383-393.

Makinose, M. and Hasselbach, W. (1965).

Biochem. Z. 343, 360-382.

Makinose, M. and Hasselbach, W. (1971).

FEBS Lett. 12, 271-272.

Marsh, B.B. (1951).

Nature 167, 1065-1066.

Marsh, B.B. (1952).

Biochim. Biophys. Acta 9, 246-260.

Mas-Oliva, J. (1980).

In '$Ca^{++}$ Antagonism. Mechanism of Action and Clinical Implications'
(Ed. Richardson, R.G.) Abbott Laboratories Ltd., pp.67-70.

Martinosi, A. (1967).

Biochem. Biophys. Res. Commun. 29, 753-757.

Martinosi, A. (1968).

J. biol. Chem. 243, 71-81.

References (continued)...

Martinosi, A. (1968a).

Biochim. Biophys. Acta 150, 694-704.

Martinosi, A. (1969).

Biochem. Biophys. Res. Commun. 36, 1039-1046.

Martinosi, A. (1969a).

J. biol. Chem. 244, 613-620.

Martinosi, A. (1972).

In 'Current Topics in Membranes and Transport'.

Vol.3 (Eds Kleinzeller, A. and Bronner, F.).

Academic Press Inc., New York, pp.83-197.

Martinosi, A., Lagwinska, E. and Oliver, M. (1974).

Ann. N.Y. Acad. Sci. 227, 549-567.

Martinosi, A. and Feretos, R. (1964).

J. biol. Chem. 239, 648-658.

Martinosi, A. and Haplin, R.A. (1971).

Arch. Biochem. Biophys. 144, 66-77.

Martinosi, A., Donley, J.R., Pucell, A.G. and Haplin, R.A. (1971).

Arch. Biochem. Biophys. 144, 529-540.

Martinosi, A. and Fortier, F. (1974).

Biochem. Biophys. Res. Commun. 60, 382-389.

Martinosi, A., Nakamura, H., Jilka, R.L. and Vanderkooi, J.H.

(1977). In 'Biochemistry of Membrane Transport', FEBS

Symposium no.42 (Eds Semenza, G. and Carafoli, E.),

pp. 401-415.

References (continued)...

Masoro, E.J. and Yu, B.P. (1969).

Biochem. Biophys. Res. Commun. 34, 686-693.

Masuda, H. and de Meis, L. (1973).

Biochemistry 12, 4581-4585.

Meissner, G. (1973).

Biochim. Biophys. Acta 298, 906-926.

Meissner, G., Connor, G.E. and Fleischer, S. (1973).

Biochim. Biophys. Acta 298, 246-269.

Meissner, G. and Fleischer, S. (1973).

Biochem. Biophys. Res. Commun. 52, 913-920.

Meissner, G. and Fleischer, S. (1974).

J. biol. Chem. 249, 302-309.

Meissner, G. and McKinley, D. (1976).

J. Membrane Biol. 30, 79-98.

Michalek, M., Campbell, K.P. and MacLennan, D.H. (1980).

J. biol. Chem. 255, 1317-1326.

Migala, A., Agostini, B. and Hasselbach, W. (1973).

Z. Naturforsch Tech. B. 28, 178-182.

Mitchell, P. (1966).

Biol. Rev. 41, 445-502.

Mitchell, P. (1973).

FEBS Lett. 33, 267-274.

Moller, J.V., Lind, K.E. and Andersen, J.P. (1980):

J. biol. Chem. 255, 1912-1920.

Molnar, J. and Lorand, L. (1959).

Nature 183, 1032-1034.

Murphy, A. (1978).

J. biol. Chem. 253, 385-389.

References (continued)...

Muscatello, V., Anderson-Cadergren, E. and Azzone, G.F. (1961).
Biochim. Biophys. Acta 51, 426-428.

Myamoto, H. and Kasai, M. (1973).
J. Gen. Physiol. 62, 773-786.

Myamoto, H. and Kasai, M. (1979)
J. Biochem. 85, 765-773.

Nagai, T., Makinose, M. and Hasselbach, W. (1960).
Biochim. Biophys. Acta 43, 223-238.

Nakajima, Y. and Endo, M. (1973).
Nature New Biol. 246, 216-218.

Nakajima, H., Hoshiyama, M., Yamashita, K. and Kyamoto, A. (1975).
Japan J. Pharmacol. 25, 383-392.

Nakajima, H., Hoshiyama, M., Yamashita, K. and Kiyamoto, A. (1976).
Japan J. Pharmacol. 26, 571-580.

Nakamuru, Y. and Schwartz, A. (1970).
Biochem. Biophys. Res. Commun. 41, 830-836

Nakamuru, Y. and Schwartz, A. (1972).
J. Gen. Physiol. 59, 22-32.

Nakamura, H., Hori, H. and Mitsui, T. (1972).
J. Biochem. 72, 635-646.

Namm, D.H., Woods, E.L. and Zucker, J.L. (1972).
Circ. Res. 31, 308-316.

Nayler, W. (1975).
In 'Contraction and Relaxation in the Myocardium' (Ed. Nayler, W.G.)
Academic Press, London and New York, pp. 1-28.

Nayler, W.G. and Dunnett, J. (1974).
Adv. Cardiol. 12, 45-58.

References (continued)...

Nayler, W.G., Dunnett, J. and Berry, D. (1975).
J. Mol. Cell. Cardiol. 7, 275-288.

Neet, K.E. and Green, N.M. (1977).
Arch. Biochem. Biophys. 178, 588-597.

Niedergerke, R., Goden, D.C. and Page, S. (1976).
Society for Experimental Biology Symposium no.30, Cambridge University Press, pp.381-395.

Ogawa, Y. (1970).
J. Biochem. 67, 667-683.

Ogawa, Y. and Ebashi, S. (1976).
J. Biochem. 80, 1149-1157.

Ohnishi, S.T. (1979).
J. Biochem. 86, 1147-1150.

Ohnishi, T. and Ebashi, S. (1963).
J. Biochem. 54, 506-511.

Ohnishi, T. and Ebashi, S. (1964).
J. Biochem. 55, 599-603.

Otswald, T.J. and MacLennan, D.H. (1974).
J. biol. Chem. 269, 974-979.

Packer, L., Mehard, C.W., Meissner, G., Zahler, W.L. and Fleischer, S. (1974). Biochim. Biophys. Acta 363, 159-181.

Panet, R. and Selinger, Z. (1972).
Biochim. Biophys. Acta 255, 34-42.

Panet, R., Pick, U. and Selinger, Z. (1971).
J. biol. Chem. 246, 7349-7356.

Pang, D.C. and Briggs, F.N. (1973).
Biochemistry 12, 4905-4911.

Pang, D.C., Briggs, F.N. and Rogowski, R.S. (1974).
Arch. Biochem. Biophys. 164, 332-340.

References (continued)...

Parker, J.R. and Berger, C.K. (1963).

Biochim. Biophys. Acta 74, 730-734.

Parker, C.J. and Gergely, J. (1960).

J. biol. Chem. 235, 3449-3433.

Parker, C.J. and Gergely, J. (1961).

J. biol. Chem. 236, 411-415.

Plank, B., Hellmann, G., Punzengruber, C. and Suko, F. (1979).

Biochim. Biophys. Acta 550, 259-268.

Poole-Wilson, P.A. and Langer, G.A. (1975).

Amer. J. Physiol. 299, 570-581.

Porter, K.R. and Palade, G.E. (1957).

J. Biophys. Biochem. Cytol. 3, 269-299.

Price, N.R. and Williams, A.J. (1977).

Biochem. Med. 17, 153-157.

Racker, E. (1972).

J. biol. Chem. 247, 8198-8200.

Racker, E. (1977).

In 'Calcium-Binding Proteins and Calcium Function' (Eds Wasserman, R.H., Corradine, R.A., Carafoli, E., Kretsenger, R.H., MacLennan, D.H. and Snegal, F.L.) Elsevier/North Holland Publishing Inc.

Racker, E. and Eytan, E. (1975).

J. biol. Chem. 250, 7533-7534.

Racker, E., Violand, B., O'Neal, S., Alfonso, M. and Telford, J. (1979).

Arch. Biochem. Biophys. 198, 470-477.

Rauch, B., Chak, D. and Hasselbach, W. (1977).

Z. Naturforsch. 32c, 828-834.

Repke, D.I., Spivak, J.C. and Katz, A.M. (1976).

J. biol. Chem. 251, 3169-3175.

References (continued)...

Reynolds, J.A. and Tanford, C. (1970a).

Proc. Natl Acad. Sci. (U.S.A.) 66, 1002-1007.

Rich, T.L. and Langer, G.A. (1975).

J. Mol. Cell. Cardiol. 7, 767-765.

Rumberger, E. (1976).

Pflugers Arch. 364, 203-204.

Russel, J.T., Beeler, T. and Martinosi, A. (1978a).

J. biol. Chem. 254, 2040-2046.

Russel, J.T., Beeler, T. and Martinosi, A. (1978b).

J. biol. Chem. 254, 2047-2052.

Sarzala, M.G. and Michalek, M. (1978).

Biochim. Biophys. Acta 513, 221-235.

Scales, D.J. and Inesi, G. (1976a).

Arch. Biochem. Biophys. 176, 392-394.

Scales, D.J. and Inesi, G. (1976b).

Biophys. J. 16, 735-751.

Schadler, M. (1967)

Pflugers Arch. 296, 70-90.

Schnaitman, C., Erwin, V.G. and Greenawalt, J.U. (1967).

J. Cell. Biol. 32, 719-735.

Schwartz, A., Allen, J.C. and Harigaya, S. (1969).

J. Pharmacol. Exp. Ther. 168, 31-41.

Schwartz, A., Entman, M.L., Kaniike, K., Lane, L.K., VanWinkle, B. and Bornet, E.P. (1976). Biochim. Biophys. Acta 426, 57-72.

Sekul, A.A. and Holland, W.C. (1960).

Amer. J. Physiol. 199, 457-459.

Shammoo, A.E., Ryan, T.E., Stewart, P.S. and MacLennan, D.H. (1976).

J. biol. Chem. 251, 4147-4154.

References (continued)...

Shapiro, A.L., Vinuela, E. and Maizel, J.V. (1967)
Biochem. Biophys. Res. Commun, 28, 815-820.

Shigekawa, M. and Pearl, L.J. (1976).
J. biol. Chem. 251, 6947-6952.

Shigekawa, M., Finegan, J.M. and Katz, A.M. (1976).
J. biol. Chem. 251, 6894-6900.

Shigekawa, M. and Dougherty, J.P. (1978a).
J. biol. Chem. 253, 1451-1457.

Shigekawa, M. and Dougherty, J.P. (1978b).
J. biol. Chem. 253, 1458-1464.

Shine, K.I., Serena, S.D. and Langer, G.A. (1971).
Amer. J. Physiol. 221, 1408-1417.

Solaro, R.J., Wise, R.M., Shiner, J.S. and Briggs, F.N. (1974).
Circ. Res. 34, 525-530.

Solaro, R.J. and Briggs, F.N. (1974).
Circ. Res. 34, 531-540.

Stability Constants. Supplement no.I. Special Publication 25,
London, The Chemical Society (1971).

Stein, W.D., Eilam, Y. and Lieb, W.R. (1974).
Ann. N.Y. Acad. Sci. 227, 328-336.

Stewart, P.S. and MacLennan, D.H. (1974).
J. biol. Chem. 249, 985-993.

Stewart, P.S., MacLennan, D.H. and Shamoo, A.E. (1976).
J. biol. Chem. 251, 712-719.

Streter, F.A. (1969).
Arch. Biochem. Biophys. 134, 25-33.

References (continued)...

Suko, J. and Hasselbach, W. (1974).

Naunyn Schmeideberg's Arch. Pharmakol. 282, R97.

Suko, J. and Hasselbach, W. (1976).

Eur. J. Biochem. 64, 123-130.

Suko, J., Hellmann, G. and Winkler, F. (1977).

Basic Research in Cardiology 72, 147-152.

Sumida, M. and Tonomura, Y. (1974).

J. Biochem. 75, 283-297.

Sumida, M., Schwartz, A. and Froelich, J.P. (1978).

Ann. N.Y. Acad. Sci. 307, 228.

Sumida, M., Wang, T., Mandel, F., Froelich, J.P. and Schwartz, A. (1978a).

J. biol. Chem. 253, 8772-8777.

Sumida, M., Wang, T., Schwartz, A., Younkin, C. and Froelich, J.P. (1980).

J. biol. Chem. 255, 1497-1503.

Tada, M. and Kirchberger, M.A. (1975).

Acta Cardiologica 30, 231-237.

Tada, M., Kirchberger, M. and Li, H.C. (1975).

J. Cyclic Nucleotide Research 1, 329-338.

Tada, M., Kirchberger, M.A., Repke, D.I. and Katz, A.M. (1974).

J. biol. Chem. 269, 6176-6180.

Tada, M., Ohmori, F., Kinoshita, N. and Abe, H. (1978).

Adv. Cyc. Nuc. Res. 9, 355-369.

Tada, M., Yamamoto, Y. and Tonomura, Y. (1978).

Physiol. Rev. 58, 1-79.

Takakuwa, Y. and Kanazawa, T. (1979).

Biochem. Biophys. Res. Commun. 88, 1209-1216.

Taylor, J.S. and Hattan, D. (1979).

J. biol. Chem. 254, 4402-4407.

References (continued)...

Thomas, L.J. Jr. (1960).

Amer. J. Physiol. 199, 146-150.

Thorens, S. and Endo, M. (1975).

Proc. Japan Acad. 51, 473-478.

Thorley-Lawson, D.A. and Green, N.M. (1977).

Biochem. J. 167, 739-748.

Tillack, T.W., Boland, R. and Martinosi, A. (1976).

J. biol. Chem. 249, 624-633.

Tonomura, V. (1973).

In 'Muscle Proteins, Muscle Contraction and Cation Transport'

Univ. of Tokyo Press, Tokyo, pp. 381-407.

Tsein, R.W. (1976).

Circulation 53(1), 1-16.

Vale, M.G.P. (1977).

Biochim. Biophys. Acta 471, 39-48.

VanWinkle, W.B., Pitts, B.J.R. and Entman, M.L. (1978).

J. biol. Chem. 253, 8671-8673.

Verjovski-Almeida, S. and de Meis, L. (1977).

Biochemistry 16, 329-334.

Verjovski-Almeida, S., Kurzmack, M. and Inesi, G. (1978).

Biochemistry 17, 5006-5013.

Verjovski-Almeida, S. and Inesi, G. (1979).

J. biol. Chem. 254, 18-21.

Vianna, A.L. (1975).

Biochim. Biophys. Acta 410, 389-406.

Vieyra, A., Scofano, H.M., Guimaraes-Motta, H., Tume, R.K. and de Meis, L.

(1979). Biochim. Biophys. Acta 568, 437-445.

References (continued)...

Wang, T., Grassi, A.O. and Schwartz, A. (1979).
J. biol. Chem. 254, 10675-10678.

Watanabe, S. (1955).
Arch. Biochem. Biophys. 54, 559-562.

Weber, A. (1971).
J. Gen. Physiol. 57, 50-63.

Weber, K. and Osborn, M. (1969).
J. biol. Chem. 244, 4406-4412.

Weber, A. and Herz, R. (1968).
J. Gen. Physiol. 52, 760-772.

Weber, A., Herz, R. and Reiss, I. (1963).
J. Gen. Physiol. 46, 679-702.

Weber, A., Herz, R. and Reiss, I. (1964).
Proc. Roy. Soc. Lond. Series B 60, 489-501.

Weber, A., Herz, R. and Reiss, I. (1966).
Biochem. Z. 345, 329-369.

Weiss, G.B. and Bianchi, C.P. (1965).
J. Cell. Comp. Physiol. 65, 385-392.

Will, H., Blanck, J., Smettan, G. and Wollenberger, A. (1976).
Biochim. Biophys. Acta 449, 295-303.

Williamson, J.R., Safer, B., Rich, T., Schaffer, S. and Kobayashi, K.
(1975). Acta Med. Scand. Suppl. 587, 95-111.

Winkler, F. and Suko, J. (1977).
Eur. J. Biochem. 77, 611-619.

Wray, H.L., Gray, R.R. and Olsson, R.A. (1973).
J. biol. Chem. 248, 1496-1498.

Wray, H.L. and Gray, R.R. (1977).
Biochim. Biophys. Acta 461, 441-459.

Yamada, S., Yamamoto, T. and Tonomura, Y. (1970).
J. Biochem. 67, 789-794.

Yamada, S. and Tonomura, Y. (1972a).
J. Biochem. 72, 417-425.

Yamada, S. and Tonomura, Y. (1972b).
J. Biochem. 71, 1101-1104.

Yamada, S. and Ikemoto, N. (1980).
J. biol. Chem. 255, 3108-3119.

Yamada, S., Sumida, M. and Tonomura, Y. (1972).
J. Biochem. 72, 1537-1548.

Yamamoto, T. and Tonomura, Y. (1967).
J. Biochem. 62, 558-575.

Yamamoto, T. and Tonomura, Y. (1968).
J. Biochem. 64, 137-145.

Yamamoto, T. and Tonomura, Y. (1976).
J. Biochem. 79, 693-707.

Yamamoto, T., Yoda, A. and Tonomura, Y. (1971).
J. Biochem. 69, 807-809.

Yu, B.P. and Masoro, E.J. (1970).
Biochemistry 9, 2909-2917.

Yu, B.P., Masoro, E.J. and Morley, T.F. (1976).
J. biol. Chem. 251, 2037-2043.

Yu, B.P., DeMartinis, F.D. and Masoro, E.J. (1968).
J. Lipid Res. 9, 492-500.

Zimniak, P. and Racker, E. (1978).
J. biol. Chem. 253, 4631-4637.

# CONTROL OF CALCIUM PERMEABILITY IN THE SARCOPLASMIC RETICULUM

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## 1. Introduction

Several reports indicate that the calcium permeability of sarcoplasmic reticulum vesicles isolated from skeletal muscle is not constant, but is influenced by the $Ca^{2+}$ concentrations outside ($Ca_o$) and inside ($Ca_i$) the vesicles. The level of $Ca_o$ at which oxalate-supported calcium uptake reached steady state was found by Weber et al. [1] to increase when more calcium was presented to these vesicles, findings that were interpreted to mean that the leakiness of the vesicles was increased. These investigators also reported that calcium exchange diminished at the start of the calcium uptake reaction, when $Ca_i$ increased [1]. Hasselbach et al. [2] and Makinose [3] found a marked acceleration of calcium efflux from vesicles preloaded with $^{40}Ca$ when addition of $^{45}Ca$ to the medium outside the vesicles increased $Ca_o$ and led to renewed calcium uptake.

The present study was undertaken to define the effects of varying $Ca_o$ on the calcium permeability of sarcoplasmic reticulum vesicles under conditions where $Ca_i$ was maintained at different levels by calcium oxalate or calcium phosphate precipitates.

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## 2. Experimental

Sarcoplasmic reticulum vesicles were prepared according to Makinose and Hasselbach [4]. Calcium uptake by 10 μg protein/ml in 0.12 M KCl, 5 mM MgATP, 40 mM histidine buffer (pH 6.8) and varying concentrations of oxalate or phosphate was measured by the Millipore Filtration method as described previously [5]. $Ca_o$ was calculated from measurements of total calcium outside the vesicles, taking into account the binding of calcium to ATP [6]. $Ca_i$ was calculated from the solubility product of calcium oxalate ($2.0 \times 10^{-8}$ $M^2$, 7) or calcium phosphate ($7.5 \times 10^{-6}$ $M^2$ at pH 6.8; W. Hasselbach, unpublished observations).

Calcium permeability was calculated from the equation: Calcium Efflux = Calcium Permeability × $Ca_i$. Calcium efflux was measured by the use of paired identical reaction mixtures, started concurrently with $^{45}Ca$ and $^{40}Ca$. The former allowed measurement of the slow calcium uptake or calcium release at a time, 10–20 min after the start of the reaction, when calcium uptake approached steady state. Calcium influx at this time was measured by addition of carrier-free tracer $^{45}Ca$ (1 mCi per μmol, total added Ca < 1 nM) to the reaction mixture started with $^{40}Ca$. Tracer uptake, measured at 15 sec intervals, was linear with time for 60–90 sec. after the initial 15 sec interval. Calcium influx was calculated from the rate of tracer influx, adjusted to reflect uptake of total Ca outside the vesicles. The latter was obtained from analysis of the data from the reaction started with $^{45}Ca$. Calcium efflux was calculated as the sum of calcium influx *plus* calcium release, or calcium influx *minus* calcium uptake at the time of tracer addition.

## 3. Results and discussion

Calcium efflux rates at any level of $Ca_i$ were found to increase with increasing $Ca_o$, as suggested by earlier reports [1–3]. At any level of $Ca_o$, calcium permeability decreased markedly as $Ca_i$ increased, and a close correlation was found between calcium permeability and the $Ca^{2+}$ gradient across the membrane ($Ca_i/Ca_o$) (fig.1). This relationship was not affected significantly when phosphate instead of oxalate was used as the calcium precipitating anion.

These findings indicate that the membranes of the sarcoplasmic reticulum contain a 'gating mechanism' that controls calcium efflux rate, and an almost 1000-fold change in calculated calcium permeability was found to accompany a 3000-fold change in the ratio $Ca_i/Ca_o$. The maximum calcium permeability of 100 nmol/mg min/$\mu$M $Ca_i$ would allow a calcium efflux rate of approximately $2 \times 10^{-7}$ mol/mg sec at a $Ca_i$ concentration of 100 $\mu$M, which corresponds to a calcium efflux rate of $\sim 10^{-10}$ mol/cm$^2$ sec. This value is approximately 1 order of magnitude less than that of $10^{-9}$ mol/cm$^2$ sec calculated for calcium efflux from the sarcoplasmic reticulum of activated skeletal muscle [8–10], and would allow efflux of sufficient calcium to bind to 2 of the troponin–calcium binding sites of muscle ($\sim$ 0.2 $\mu$mol/g muscle) in approx. 400 msec at a $Ca_i$ level of 100 $\mu$M. It is possible that the 'gating mechanism' observed in the present study may participate in the physiological control of calcium fluxes by the sarcoplasmic reticulum of intact muscle.

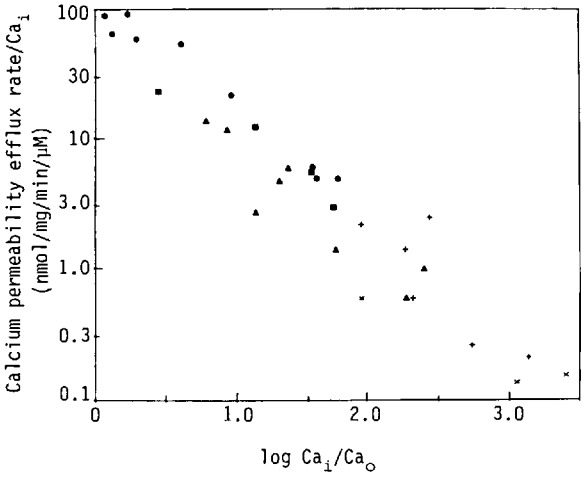



Fig.1. Relationship between calcium permeability and the $Ca^{2+}$ gradient across the sarcoplasmic reticulum. All data obtained in 5 series of experiments are plotted. $Ca_i$ was 4 $\mu$M (5.0 mM oxalate, ●), 8 $\mu$M (2.5 mM oxalate, ■), 20 $\mu$M (1.0 mM oxalate, ▲), 150 $\mu$M (50 mM phosphate, +) or 750 $\mu$M (10 mM phosphate, X).

## References

[1] Weber, A., Herz, R. and Reiss, I. (1966) Biochem. Z. 345, 329.
[2] Hasselbach, W., Fiehn, W., Makinose, M. and Migala, A. J. (1969) in: The Molecular Basis of Membrane Function, p. 299 (D. C. Tosteson, ed.) Prentice Hall, Englewood Cliffs, N. J.
[3] Makinose, M. (1973) FEBS Lett. 37, 140.
[4] Makinose, M. and Hasselbach, W. (1965) Biochem. Z. 343, 360.
[5] Katz, A. M. and Repke, D. I. (1973) Biochim. Biophys. Acta 298, 270.
[6] Katz, A. M., Repke, D. I., Upshaw, J. E. and Polascik, M. (1970) Biochim. Biophys. Acta 205, 473.
[7] Hasselbach, W. and Makinose, M. (1963) Biochem. Z. 338, 94.
[8] Jobsis, F. F. and O'Connor, M. J. (1966) Biochem. Biophys. Res. Commun. 25, 246.
[9] Ashley, C. C. and Ridgway, E. B. (1970) J. Physiol. (London) 209, 105.
[10] Martonosi, A. (1972) in: Current Topics in Membrane and Transport, Vol. 3, p. 83 (F. Bronner and A. Kleinzeller, eds.) Academic Press, New York.

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# Dependence of Calcium Permeability of Sarcoplasmic Reticulum Vesicles on External and Internal Calcium Ion Concentrations*

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**The ability of sarcoplasmic reticulum vesicles to retain calcium following ATP-supported calcium uptake in the presence of the calcium-precipitating anions oxalate and phosphate depends on $Ca_o$ (calcium ion concentration outside the vesicles) and $Ca_i$ (calcium ion concentration within the vesicles). Calcium efflux rates at any level of $Ca_i$ are accelerated when $Ca_o$ is increased. Higher $Ca_o$ at the time that calcium uptake reactions reach steady state is associated with a spontaneous calcium release that reflects this effect of increased $Ca_o$. Increasing $Ca_i$ at any level of $Ca_o$ causes little or no acceleration of calcium efflux rate so that calcium permeability coefficients, estimated by dividing calcium efflux rates by $Ca_i$, the "driving force," are inversely proportional to $Ca_i$. Calcium permeability coefficients thus correlate, as a first approximation, with the ratio $Ca_i/Ca_o$, decreasing 1000-fold as this ratio increases over a 3000-fold range ($Ca_o$ = 0.1 to 3.3 $\mu$M, $Ca_i$ = 4 to 750 $\mu$M).**

**Oscillations in both the calcium content of the vesicles and $Ca_o$ are seen as calcium uptake reactions approach steady state, suggesting that calcium permeability undergoes time-dependent variations. Sudden reduction of $Ca_o$ to levels that markedly inhibit calcium influx via the calcium pump unmasks a calcium efflux that decreases slowly over 60 to 90 s.**

**The maximal calcium permeability observed in the present study would allow the calcium efflux rate from the sarcoplasmic reticulum at a $Ca_i$ of 100 $\mu$M to be approximately $10^{-10}$ mol/cm$^2$/s, which is about 1 order of magnitude less than that estimated for the sarcoplasmic reticulum of activated skeletal muscle *in vivo*. The release of most of the stored calcium in some experiments indicates that the observed permeability changes can occur over a large portion of the surface of the sarcoplasmic reticulum.**

A number of observations regarding the ability of sarcoplasmic reticulum vesicles to transport and retain calcium suggest that the calcium permeability of these membranes is not constant. Weber *et al.* (2) found that the level of ionized calcium outside the vesicles at which oxalate-supported calcium uptake reaches a steady state is markedly increased when larger amounts of calcium are presented to the vesicles, a finding that was suggested to mean that vesicles of increasing leakiness participated in calcium uptake as the total load of calcium was increased. These investigators also found that the rate of calcium exchange by vesicles that had reached a steady state of calcium storage in the absence of calcium-precipitating anions increased with increasing $Ca_o$,[1] and that the rate of $Sr^{2+}$ uptake was markedly reduced at high levels of $Ca_i$, after vesicles were preloaded with calcium (2). Further evidence that calcium permeability of sarcoplasmic reticulum vesicles may be influenced by $Ca_o$ was obtained by Hasselbach *et al.* (3), who found that when $Ca_i$ is held constant by a calcium oxalate precipitate, calcium efflux rate increases 50- to 100-fold immediately after an elevation of $Ca_o$ that also leads to renewed calcium uptake. These findings were confirmed by Makinose (4), who reported that elevation of $Ca_o$ after a small initial calcium load has been taken up by sarcoplasmic reticulum vesicles evokes the spontaneous release of a portion of the previously accumulated calcium at the same time that the added calcium is taken up.

The dependence of calcium permeability on the concentrations of $Ca_i$ and $Ca_o$ was examined further in the present study. Evidence has been obtained that calcium permeability increases when $Ca_o$ is increased, and decreases when $Ca_i$ is increased.

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[1] The abbreviations used are: $Ca_i$, calcium ion concentration inside the microsomal vesicles; $Ca_o$, calcium ion concentration in the medium outside the microsomal vesicles, calculated as described previously (5); EGTA, ethylene glycol bis($\beta$-aminoethyl ether)$N,N'$-tetraacetic acid. As used in this article, "calcium uptake" and "calcium release" refer to the rate of net gain or loss of calcium by the vesicles, "calcium influx" and "calcium efflux" refer to rates of unidirectional calcium fluxes into and out of the vesicles, respectively.

## MATERIALS AND METHODS

Preparation of microsomes and measurements of calcium uptake and release were as described in Ref. 5. All experiments were carried out in 0.12 M KCl, 40 mM histidine buffer at pH 6.8, and 5 mM MgATP at 25°. Calcium influx was measured by addition of tracer amounts of high specific activity, carrier-free $^{45}CaCl_2$ (1 mCi/$\mu$mol) to a reaction mixture in which $^{40}CaCl_2$ was used for the initial calcium uptake reaction. The added tracer calcium increased total $CaCl_2$ concentration in the medium by <0.02 nmol/liter, which could be ignored. A duplicate reaction was started with $^{45}CaCl_2$ instead of $^{40}CaCl_2$ to permit assessment of net calcium uptake and release prior to and after addition of the tracer calcium, as well as to allow calculation of the total calcium concentration outside the vesicles at the time of tracer addition. All measurements were initiated at the time that calcium uptake reached steady state, as determined in a series of pilot reactions.

The rate of uptake of the tracer calcium was measured with serial samples taken at 15-s intervals and analyzed by the Millipore filtration method (5). Calcium influx rates were calculated from the rate of tracer uptake, adjusted to reflect the average calcium concentration outside the vesicles during each 15-s interval, and expressed as nanomoles of influx per mg of protein. The apparent rate of total calcium influx measured by the tracer experiment was high during the first 15-s interval, due possibly to exchange with traces of calcium in the glassware. Over the subsequent 60 to 90 s, however, the rate of disappearance of the tracer became linear with time. Calcium influx rates were thus calculated from curves obtained from tracer uptake during four to six consecutive 15-s intervals.

## RESULTS

*Effects of $Ca_o$ on Spontaneous Calcium Release after Anion-supported Calcium Uptake*—The level of $Ca_o$ at the time that calcium uptake in the presence of calcium-precipitating anions approached steady state influenced the ability of the sarcoplasmic reticulum to retain calcium. When reactions were started with a high ratio between total calcium and protein, spontaneous calcium release began earlier and was much greater in extent than in reactions started with a low total calcium:protein ratio (Fig. 1). The more rapid spontaneous release of calcium can be seen in Fig. 1 to result from the higher level of $Ca_o$ at the time that calcium uptake reached a steady state, and not to be dependent on the absolute calcium or protein concentration at the start of the reaction.

As shown in the preceding paper (5), an increase in calcium permeability will lead to greater calcium release when $Ca_o$ is high because more calcium must be added to the external medium from stores in the vesicles to restore the equilibrium between calcium influx and the increased calcium efflux rate than when $Ca_o$ is low (Fig. 15 in Ref. 5). This relationship cannot, however, explain fully the findings in Fig. 1 because calcium release began after $t$ = 10 min in the presence of high $Ca_o$, in contrast to the continued uptake of calcium without spontaneous release until $t$ = 30 min when $Ca_o$ was low. This finding suggests that high $Ca_o$ has an additional effect to increase calcium permeability. A similar spontaneous calcium release at high $Ca_o$ has also been reported in cardiac sarcoplasmic reticulum vesicles (6), and spontaneous calcium release by cardiac preparations is remarkably reduced at low concentrations of $Ca_o$.[2] High $Ca_o$ also was noted to promote spontaneous calcium release in studies of calcium storage in the absence of calcium-precipitating anions (7).

*Spontaneous Changes in Calcium Uptake "Capacity"*—Analysis of the behavior of sarcoplasmic reticulum vesicles in the presence of high $Ca_o$ consistently showed instability in calcium uptake capacity as calcium uptake approached an initial steady state. These changes, which can be seen in many of the control reactions at high $Ca_o$ in Ref. 5 (*e.g.* Figs. 9*A* and 10*C*), were associated with large changes in $Ca_o$ when measurements were made as calcium uptake in 50 mM phosphate approached a steady state. As seen in Fig. 2, where mean values for five similar reactions carried out concurrently are plotted, $Ca_o$ first reached a minimum, then increased and finally decreased again as calcium uptake approached steady state. The differences between $Ca_o$ at $t$ = 22 min from those at $t$ = 18, 19, and 26 min (Fig. 2) were highly significant ($p < 0.01$) and arise from oscillations in the calcium content of the vesicles.

*Effects of $Ca_o$ and $Ca_i$ on Calcium Permeability*—The effects of $Ca_o$ on calcium permeability were studied in paired experiments in which initiation of the calcium uptake reaction with $^{45}Ca$ allowed calcium uptake or release rates to be measured, while simultaneous measurements of tracer uptake after addition of small amounts of $^{45}CaCl_2$ to identical reactions initiated with $^{40}Ca$ allowed calcium influx rates to be calculated (see "Materials and Methods"). Calcium efflux rates could then be calculated from those of calcium influx, from which calcium uptake rate during this time was subtracted or calcium release rates added. Results in a typical experiment are shown in Fig. 3, in which calcium influx is plotted at three levels of $Ca_o$. Calcium influx rates decreased as $Ca_o$ was lower at the time, 15 min after the start of the reaction, when these measurements were initiated.

Calcium efflux rates, calculated from measurements of calcium content and of calcium influx, are shown in Fig. 4 as a function of $Ca_o$ at two levels of $Ca_i$. The latter were maintained at 20 or 4 $\mu$M by the presence of 1.0 or 5.0 mM oxalate as the calcium-precipitating anion. Calcium efflux rate at any level of $Ca_o$ was not increased significantly at the higher $Ca_i$ (*i.e.* at the lower oxalate concentration) even though the higher $Ca^{2+}$

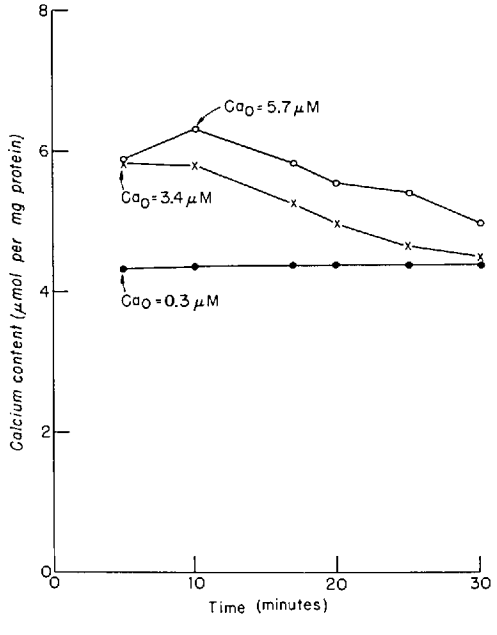



FIG. 1. Effects of $Ca_o$ on spontaneous calcium release. Calcium release after calcium uptake at a high total calcium/protein ratio of 9 $\mu$mol/mg of protein (×, ○) is compared to that at a low total calcium/protein ratio of 4.5 $\mu$mol/mg of protein (●). Initial total $^{45}CaCl_2$ was 100 $\mu$M (○) and 50 $\mu$M (●, ×); protein concentrations were 5.5 (×) and 11.1 (○, ●) $\mu$g/ml. Reactions were carried out as described under "Materials and Methods," in the presence of 5 mM oxalate. Estimations of minimal levels of $Ca_o$ were 0.3 $\mu$M at the low total calcium/protein ratio (●), and 3.4 and 5.7 $\mu$M at the high total calcium/protein ratio (× and ○, respectively).

[2] A. M. Katz and S. Corkedale, unpublished observations.

concentration within the vesicles might be expected to accelerate calcium efflux.

Calcium permeability coefficients ($K_e$) were calculated from data such as those shown in Fig. 4 with the assumption that calcium efflux rate is a direct linear function of $Ca_i$, according to the equation:

$$\text{Calcium efflux rate} = K_e \times Ca_i \quad (1)$$

Levels of $Ca_i$ were calculated from the solubility products of calcium oxalate and calcium phosphate as described in Ref. 5. As shown in Fig. 5, the calcium permeability coefficient increased with increasing $Ca_o$ and decreased with increasing $Ca_i$.

The effects of both $Ca_i$ and $Ca_o$ on the calcium permeability coefficient reflect a close relationship between this coefficient and the ratio $Ca_i/Ca_o$. The data from Fig. 5, obtained in a series of experiments in which $Ca_i$ was varied by the use of two oxalate concentrations, fell on a single line when the calcium permeability coefficients were plotted as a function of the ratio $Ca_i/Ca_o$ (Fig. 6). When data from eight series of experiments carried out with varying oxalate or phosphate concentrations were plotted in this manner, the calcium permeability coefficient was found to correlate with the ratio $Ca_i/Ca_o$ over a more than 3,000-fold range (Fig. 7).

*Effects of Sudden Reduction of $Ca_o$ on Calcium Permeability*—Sudden reduction of $Ca_o$ following $^{45}Ca$ uptake, by greatly slowing calcium influx via the calcium pump, unmasked a rapid calcium efflux that caused the rapid release of a small amount of calcium. The extent of the initial calcium release during the first 15-s interval increased only slightly when the final level of $Ca_o$ was lowered in the range between 1.0 to 0.05 $\mu$M by addition of varying amounts of EGTA (Fig. 8). After 60 to 90 s, calcium release slowed (Fig. 8*A*) or, following addition of smaller amounts of EGTA, was followed by reuptake of the previously released calcium (Fig. 8, *B* and *C*). These findings indicate that calcium permeability slowly decreases following addition of EGTA, which by chelating $Ca^{2+}$ outside the vesicles, increased the ratio $Ca_i/Ca_o$. When only small amounts of EGTA were added, the lower level of $Ca_o$ appeared to reduce calcium permeability to a greater extent than it inhibited the

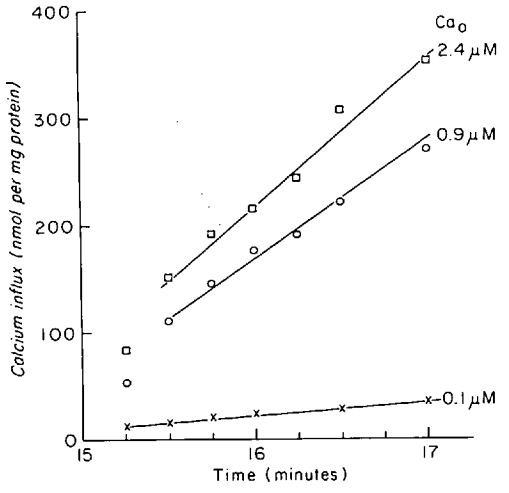



FIG. 3. Calcium influx rates, measured after tracer $^{45}CaCl_2$ addition to a reaction 15 min after its initiation with $^{40}CaCl_2$ as described under "Materials and Methods." Protein concentration was 10 $\mu$g/ml and 1.0 mM oxalate was the calcium-precipitating anion. Final $Ca_o$ concentrations at the time of tracer addition were 2.4 $\mu$M (□), 0.88 $\mu$M (■), and 0.10 $\mu$M (○) following addition of $CaCl_2$ at the start of the reactions to final concentrations of 37, 26, and 18 $\mu$M, respectively.

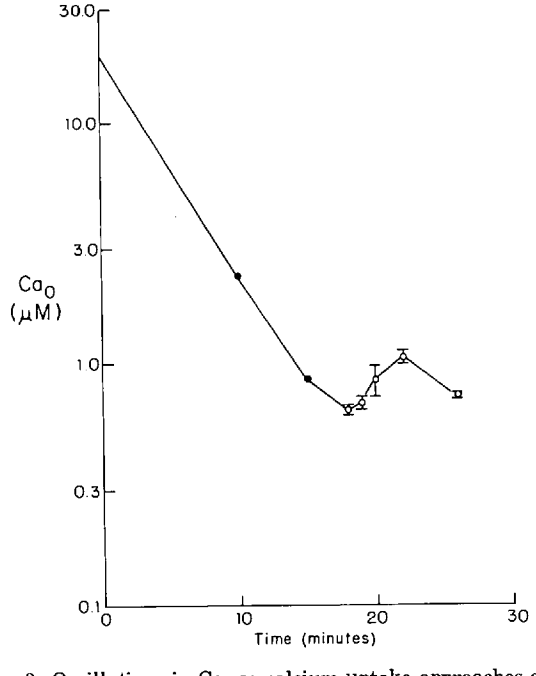



FIG. 2. Oscillations in $Ca_o$ as calcium uptake approaches steady state at high $Ca_o$. Data from five independent reactions in which calcium uptake by 51 $\mu$g of protein/ml was measured at a total calcium concentration of 100 $\mu$M in the presence of 50 mM phosphate as described under "Materials and Methods" are shown (○——○). The *horizontal bars* represent ±S.D. Also plotted for reference is the initial calcium uptake reaction (●——●) taken from a pilot run.

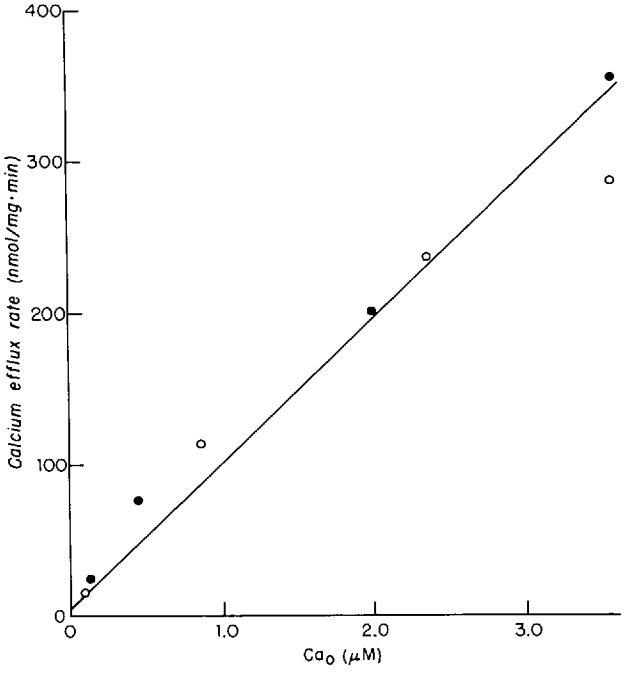



FIG. 4. Dependence of calcium efflux rate on the level of $Ca_o$. Calcium uptake reactions were carried out with 10 $\mu$g of protein/ml and 18 to 80 $\mu$M $CaCl_2$ as described under "Materials and Methods." Calcium efflux rates were measured, beginning at $t$ = 10 min in experiments with 5.0 mM oxalate (●), and at $t$ = 12 min when oxalate was 1.0 mM (○).

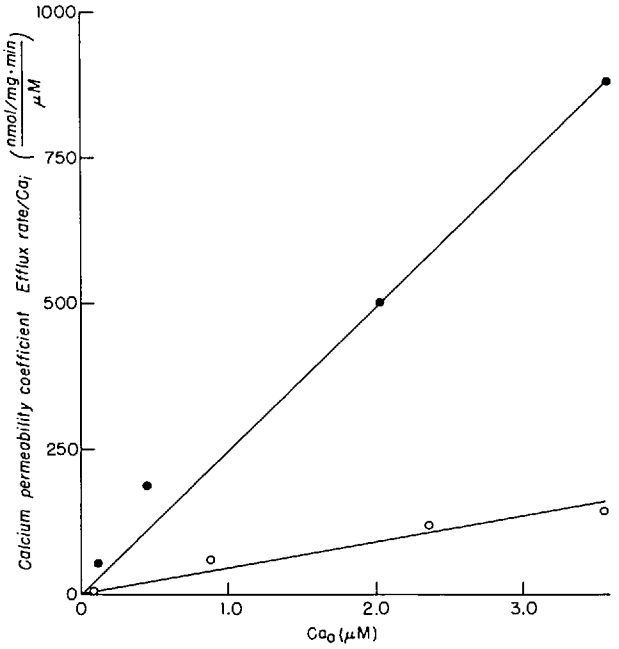



FIG. 5. Dependence of the calcium permeability coefficient on $Ca_o$. The calcium permeability coefficient, calculated as the ratio calcium efflux rate/$Ca_i$, was measured at 20 and 4 $\mu$M $Ca_i$, maintained by 1.0 mM (○) and 5 mM (●) oxalate, respectively (data from Fig. 4).

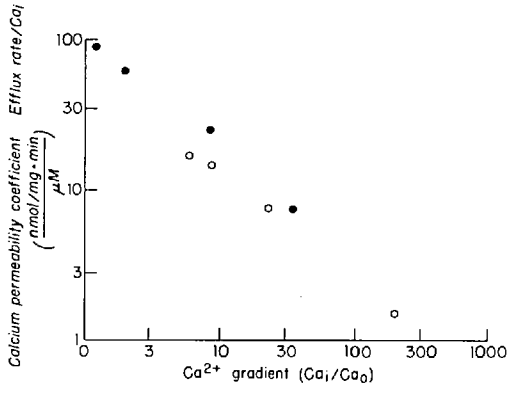



FIG. 6. Relationship between calcium permeability coefficient and the ratio $Ca_i/Ca_o$. Data and symbols shown in Figs. 4 and 5.

calcium pump, thereby allowing for a slow renewal of calcium uptake (Fig. 8*C*). Addition of larger amounts of EGTA reduced $Ca_o$ to levels where calcium uptake became minimal (Fig. 8*A*). Under these conditions, marked slowing of the rate of calcium release indicated that calcium permeability gradually decreased at the lower $Ca_o$.

*Effects of X537A on Calcium Permeability*—Although it is well established that the ionophore X537A is able to induce a cation-selective leak in lipid membranes, and that this ionophore can increase calcium fluxes across the membranes of the sarcoplasmic reticulum (see Ref. 5), the effects of low concentrations of X537A on calcium release after oxalate- and phosphate-supported calcium uptake were quite complex. In addition to its ability to induce a small, rapid calcium release, the ionophore greatly slowed the rate of the subsequent spontaneous calcium release seen in the presence of high $Ca_o$ (Fig. 9, see also Figs. 9*A*, 11*A*, and 13 in Ref. 5). Furthermore, in some experiments of the series presented in Ref. 5 (*e.g.* Figs. 4 to 7, 11*A*, 13*A*, 15), where $Ca_o$ was low, the calcium initially released after addition of X537A was subsequently taken up again. Similar phenomena have been seen in cardiac sarcoplasmic reticulum vesicles.[3]

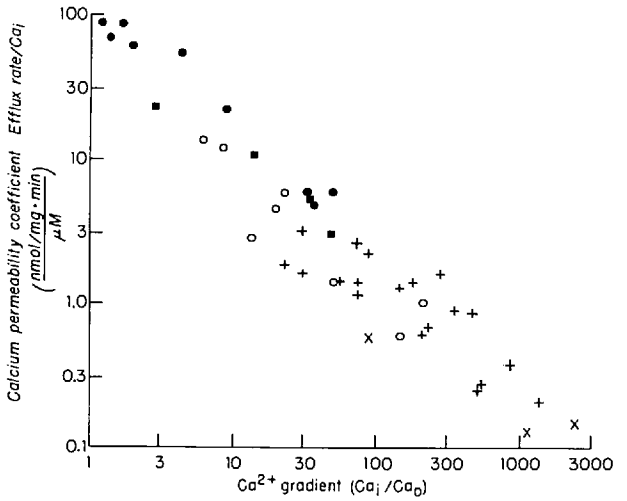



FIG. 7. Relationship between calcium permeability coefficient and the ratio $Ca_i/Ca_o$. All data obtained in eight series of experiments, carried out as described under "Materials and Methods," are plotted. Concentrations of $Ca_i$ were 4 $\mu$M (5.0 mM oxalate, ●), 8 $\mu$M (2.5 mM oxalate, ■), 20 $\mu$M (1.0 mM oxalate, ○), 150 $\mu$M (50 mM phosphate, +), and 750 $\mu$M (10 mM phosphate, ×).

Reduction of the rate of spontaneous calcium efflux at high levels of $Ca_o$ after addition of X537A could be shown to result from a paradoxical effect of the ionophore to decrease the rate of calcium efflux. Following its effect to promote a rapid calcium release, 10 $\mu$M X537A markedly reduced the rates of both spontaneous calcium release and influx at high $Ca_o$ (Fig. 9). Calcium efflux rate calculated as described under "Materials and Methods," fell from approximately 310 to 160 nmol/mg of protein/min 30 s after the initial X537A-induced calcium release shown in Fig. 9. Because $Ca_i$ was maintained constant by the calcium oxalate precipitate within the vesicles, these findings indicate that after causing an initial calcium release, the ionophore reduced the high calcium permeability seen at high $Ca_o$. When similar studies were carried out at lower $Ca_o$, where initial calcium permeability was much lower, the ionophore had the expected effect to increase calcium efflux rate, which at a constant $Ca_i$ indicates that calcium permeability was increased. A decrease in spontaneous calcium release where calcium permeability was high, *i.e.* at high levels of $Ca_o$, was also seen to follow the initial calcium release that was induced by caffeine.

## DISCUSSION

The present findings indicate that as calcium uptake in the presence of a constant level of $Ca_i$ approaches steady state, spontaneous calcium release begins sooner, and is greater in magnitude when the levels of $Ca_o$ are high (1 to 5 $\mu$M) than when $Ca_o$ is low (0.1 to 0.5 $\mu$M) (Fig. 1). This spontaneous calcium release cannot be attributed simply to deterioration of the membranes as it can be halted when $Ca_o$ is suddenly lowered by addition of EGTA (Fig. 8, *B* and *C*). Furthermore, the findings of significant oscillations in calcium uptake "capacity" (5) and in the level of $Ca_o$ at the time that the reaction approaches a steady state (Fig. 2) are difficult to reconcile with the view that this spontaneous calcium release is due to a

[3] J. Dunnett and W. Nayler, personal communication.

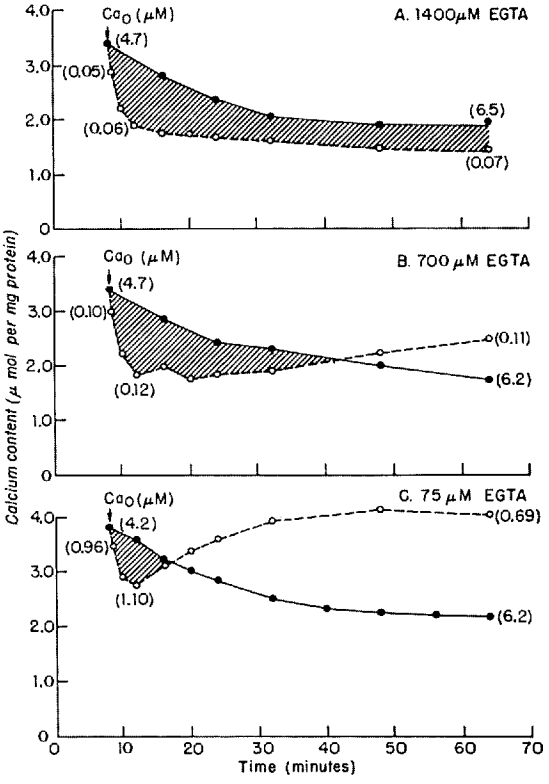



FIG. 8. Effects of EGTA addition on calcium release. Calcium uptake was carried out with 7 μg of protein/ml in 2.5 mM Tris oxalate as described under "Materials and Methods." After 8 min, EGTA was added to final concentrations of 1.4 mM. (*A*), 0.7 mM (*B*), and 75 μM (*C*). Subsequent calcium release (○) and the calcium retained by an aliquot of the control reaction to which no EGTA was added (●) were followed. The *numbers in parentheses* on the graphs are the calculated $Ca_0$ levels.

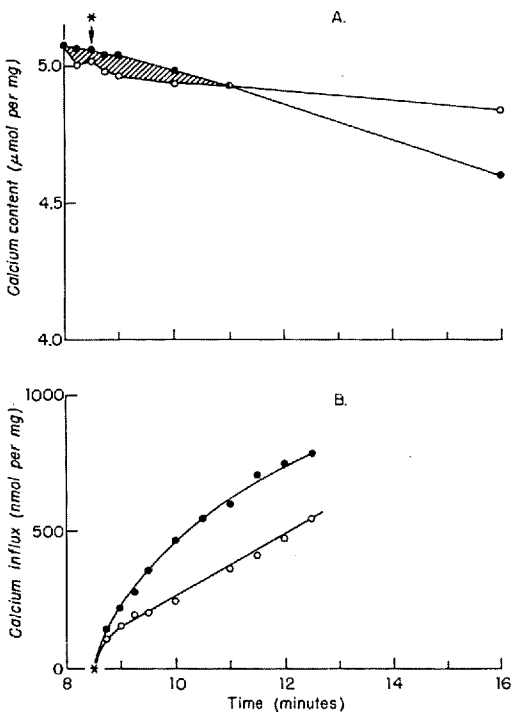



FIG. 9. Effects of X537A on calcium content and calcium influx. Effects of 10 μM X537A on calcium release by 13.4 μg of protein/ml in the presence of 50 μM total $^{45}CaCl_2$ and 2.5 mM oxalate were measured as described under "Materials and Methods" (control, ●; X537A, ○; difference *shaded*). X537A was added at $t$ = 8 min. Calcium influx was measured in identical reaction mixtures to which X537A was added as in *Panel A*, except that calcium uptake reactions were started with $^{40}CaCl_2$ (*B*) and at $t$ = 8 1/2 min. *, a small amount of $^{45}CaCl_2$ was added as described under "Materials and Methods." Calcium influx was calculated from measurements of total calcium outside the vesicles obtained from the data shown in *Panel A*, and plotted for the control (●) and X537A (○) reactions.

nonspecific deterioration of the membranes. Instead, it appears that these spontaneous changes in calcium uptake "capacity" reflect the operation in these vesicles of a mechanism that controls the membrane permeability to calcium.

The findings described above and in the preceding paper (5) indicate that the level of $Ca_0$ has two important effects on calcium release from sarcoplasmic reticulum vesicles containing an insoluble calcium precipitate. The first arises from the relationship between the $Ca^{2+}$ concentration gradient, $Ca_i/Ca_0$, and $Ca_0$ in which higher levels of $Ca_0$ increase the amount of calcium released following a given increase in calcium permeability (5). This effect, which is shown diagrammatically in Fig. 10 of Ref. 5, reflects the ability of increased $Ca_0$ to amplify the net calcium release caused by a given increase in membrane permeability to calcium. Amplification of calcium release by high $Ca_0$ is due to the constraints of an experimental design in which most of the calcium in the reaction mixture comes to be stored within the vesicles and so is unavailable to the calcium pump sites, which are stimulated only by $Ca^{2+}$ at the outside of the vesicles. A second effect of $Ca_0$, which is described in the present report, can be attributed to an increase in calcium permeability that accompanies an increase in $Ca_0$ at any given level of $Ca_i$ (Fig. 5). These latter findings are in accord with previously reported data (2–4) which indicate that elevation of $Ca_0$ increases the rate of calcium exchange.

Evidence that calcium permeability decreases when $Ca_i$ is increased is found in Fig. 15 of Ref. 5, which illustrates the finding that both the rate and extent of spontaneous calcium release can decrease when oxalate concentration is reduced, or when phosphate instead of oxalate is used as the calcium-precipitating anion. These findings appear paradoxical as the rate of net calcium release from vesicles of constant leakiness might be expected to increase when $Ca_i$ is increased from 4 to 150 μM, when 50 mM phosphate is substituted for 5 mM oxalate (Fig. 15 of Ref. 5). Thus, the phenomena illustrated in this figure may be manifestations of the same mechanism observed by Weber *et al.* (2) whereby an increase in $Ca_i$ slows $Sr^{2+}$ influx. The present findings are thus in accord with those reported earlier, and indicate that calcium permeability decreases when $Ca_i$ is increased, an effect that is independent of changes in $Ca_0$ (Fig. 5). Additional evidence that calcium permeability decreases following an increase in $Ca_i$ is seen in Ref. 5, where the effects of X537A on vesicles before and after the initiation of oxalate-supported calcium uptake are contrasted. The apparent "protective" effect of prior calcium uptake (Fig. 4 of Ref. 5) may arise from the ability of the increased $Ca_i$ to reduce calcium permeability, and thus to slow the rate of calcium efflux following addition of X537A to levels below those of the calcium influx which can be achieved by the calcium pump.

The relative independence of calcium efflux rate and $Ca_i$ (Fig. 4) might reflect the existence of a calcium efflux channel that is controlled by a carrier with a $Ca^{2+}$ affinity sufficiently

high to remain saturated at $Ca_i$ = 4 $\mu$M, the lowest level achieved in this study. In view of the evidence discussed above, which indicates that higher levels of $Ca_i$ reduce calcium efflux rate, it appears more likely that this finding arises from a direct effect of $Ca_i$ to reduce calcium permeability. Furthermore, the $Ca^{2+}$ sensitivity of the receptor inside the vesicles which mediates the inhibitory effects of $Ca_i$ on the calcium pump is very low (8).

Calcium permeability, as estimated from Equation 1, bears a close relationship to the $Ca^{2+}$ concentration gradient, $Ca_i/Ca_o$ (Figs. 6 and 7). This relationship holds over a greater than 3000-fold range of $Ca_i/Ca_o$ in experiments where $Ca_i$ ranges between 4 and 750 $\mu$M, and $Ca_o$ varies between 0.1 and 3.3 $\mu$M. Substitution of phosphate for oxalate as the calcium-precipitating anion is without significant effects on this relationship, showing that these effects do not arise from changing $Mg^{2+}$ concentration such as might result from complexation of this ion by oxalate.

Findings similar to those described here have been reported by Portzig (9), who studied passive calcium influx into human erythrocyte ghosts. Portzig found that passive calcium influx into these cells, which is in some ways analogous to passive calcium efflux from the sarcoplasmic reticulum, is reduced by high levels of $Ca^{2+}$ outside the cells and increased by high levels of internal $Ca^{2+}$. These findings were interpreted as reflecting a role of membrane-bound calcium in regulating the passive flux of calcium across these membranes (9).

The present findings can be interpreted in terms of an hypothetical calcium carrier whose mobility is determined by the $Ca^{2+}$ concentrations on both sides of the sarcoplasmic reticulum membrane. Under conditions where $Ca_i$ and $Ca_o$ are approximately equal, the calcium binding site on this carrier would be able to approach the inner and outer faces of the membrane with similar probabilities, whereas when $Ca_i$ is much greater than $Ca_o$, the ability of the carrier to carry calcium from the inner surface of the membrane might be impaired by the tendency of the calcium binding site to remain in the region of higher $Ca^{2+}$ concentration. However, the finding that the calcium efflux which is mediated by this permeability control mechanism can effect spontaneous changes in the total calcium content of the vesicles (Figs. 1 and 2), and that the calcium release which begins after addition of EGTA slows significantly with time (Fig. 8) indicate that these phenomena are not due simply to a variable rate of calcium-calcium exchange, as has been described recently by Beirão and de Meis (10). Instead, this permeability control mechanism is able to mediate a large net calcium movement out of the sarcoplasmic reticulum. As the retention of a large portion of the calcium stores of sarcoplasmic reticulum vesicles is influenced by the permeability control mechanism described in this report, these studies provide evidence that the calcium permeability of a significant fraction of the sarcoplasmic reticulum is sensitive to $Ca_i$ and $Ca_o$. Although the calcium release in activated muscle may begin at specialized sites adjacent to the t-system (11), it is possible that other surfaces of the sarcoplasmic reticulum participate in a $Ca^{2+}$-controlled excitation process.

The findings that the calculated calcium permeability coefficient is inversely proportional to $Ca_o$ and is markedly reduced after the sudden calcium release caused by EGTA (Fig. 8), or caffeine (5) are similar to observations with partially "skinned" muscle fibers where sudden elevation of $Ca_o$ ("calcium-triggered calcium release," Refs. 12 and 13), or caffeine (14) causes a transient calcium release. The ability of elevated $Ca_o$ to promote calcium release in "skinned fibers" may reflect the relationship between $Ca_o$ and both calcium efflux (Fig. 4) and the calculated calcium permeability coefficient (Fig. 5), while the transient nature of both calcium- and caffeine-induced calcium release seen in "skinned" fibers may reflect time-dependent changes in calcium permeability such as shown in Figs. 2 and 8. Caffeine-induced oscillations in the tension developed by intact muscle fibers (15) may also be manifestations of these time-dependent permeability changes.

Calcium release from "skinned" muscle fibers (13, 14) and sarcoplasmic reticulum vesicles (16) has been observed after replacement of a nonpermeant anion with a permeant anion. This calcium release, which is believed to be associated with the entry of anions into the interior of the sarcoplasmic reticulum, has been postulated to result from depolarization of a positive potential within this structure that maintains calcium permeability at a low level. The present finding that calcium permeability coefficients correlate with the calcium concentration gradient (Figs. 6 and 7) might be interpreted to reflect an effect of an electrical potential associated with the $Ca^{2+}$ concentration gradient across these membranes. Recently, however, we have found that the ability of increasing $Ca_o$ to enhance calcium efflux rate at a given $Ca_i$ is limited, and that this effect of $Ca_o$ resembles a saturable process.[4] The relationships shown in Figs. 6 and 7, therefore, probably reflect the operation of opposing effects of $Ca_i$ and $Ca_o$ on calcium permeability, rather than a direct response of the vesicles to the calcium concentration gradient *per se*.

The maximum calcium efflux rate observed in this study was ~400 nmol/mg/min, which corresponds to a rate of ~2 × $10^{-12}$ mol/cm$^2$/s as each milligram of these vesicles contains approximately 3000 cm$^2$ of membrane surface. This value is more than $10^4$- to $10^6$-fold greater than the rates of passive calcium efflux from phospholipid vesicles (16, 17) and similar to that of the passive calcium efflux from reconstituted ATPase and sarcoplasmic reticulum vesicles (18).

The maximum calcium permeability of ~100 nmol/mg/min/$\mu$M $Ca_i$ (Fig. 7) would allow a calcium efflux rate of ~$10^{-10}$ mol/cm$^2$/s if this high permeability became manifest in an intact muscle where the $Ca^{2+}$ concentration within the sarcoplasmic reticulum was 100 $\mu$M. This value is approximately an order of magnitude less than that of $10^{-9}$ mol/cm$^2$/s calculated for calcium efflux from the sarcoplasmic reticulum of activated skeletal muscle *in vivo* (19–21). Furthermore, the observed rate of change in the calcium permeability coefficient following a decrease in $Ca_o$ (Fig. 8) is quite slow. However, the ability of the calcium permeability control mechanism observed in the present study to effect a 1000-fold change in the calculated calcium permeability coefficient suggests that it may be of some physiological significance in the intact muscle.

## REFERENCES

1. Katz, A. M., Dunnett, J., Repke, D. I., and Hasselbach, W. (1976) *FEBS Lett.* 67, 207–208
2. Weber, A., Herz, R., and Reiss, I. (1966) *Biochem. Z.* 345, 329–369
3. Hasselbach, W., Fiehn, W., Makinose, M., and Migala, A. J. (1969) in *The Molecular Basis of Membrane Function* (Tosteson, D. C., ed) pp. 299–316, Prentice-Hall, Inc., Englewood Cliffs, N.J.
4. Makinose, M. (1973) *FEBS Lett.* 37, 140–143
5. Katz, A. M., Repke, D. I., and Hasselbach, W. (1977) *J. Biol. Chem.* 252, 1938–1949

[4] A. M. Katz, D. I. Repke, G. Fudyma, and M. Shigekawa, unpublished observations.

6. Entman, M. L., Snow, T. R., Freed, D., and Schwartz, A. (1973) *J. Biol. Chem.* **248**, 7762–7772
7. Katz, A. M., and Repke, D. I. (1973) *Biochim. Biophys. Acta* **298**, 270–278
8. Makinose, M., and Hasselbach, W. (1965) *Biochem. Z.* **343**, 360–382
9. Portzig, H. (1972) *J. Membrane Biol.* **8**, 237–298
10. Beirão, P. S., and de Meis, L. (1976) *Biochim. Biophys. Acta* **433**, 520–530
11. Winegrad, S. (1965) *Fed. Proc.* **24**, 1146–1152
12. Ford, L. E., and Podolsky, R. J. (1970) *Science* **167**, 58–59
13. Endo, M., Tanaka, M., and Ogawa, Y. (1970) *Nature* **228**, 34–36
14. Nakajima, Y., and Endo, M. (1973) *Nature New Biol.* **246**, 216–218
15. Marco, L. A., and Nastuk, W. L. (1968) *Science* **161**, 1357–1358
16. Kasai, M., and Miyamoto, H. (1976) *J. Biochem.* (*Tokyo*) **79**, 1053–1066
17. Vanderkooi, J. M., and Martonosi, A. N. (1971) *Arch. Biochem. Biophys.* **147**, 632–646
18. de Boland, A. R., Jilka, R. L., and Martonosi, A. N. (1975) *J. Biol. Chem.* **250**, 7501–7510
19. Jilka, R. L., Martonosi, A. N., and Tillack, T. W. (1975) *J. Biol. Chem.* **250**, 7511–7524
20. Jobsis, F. F., and O'Conner, M. J. (1966) *Biochem. Biophys. Res. Commun.* **25**, 246–252
21. Ashley, C. C., and Ridgway, E. B. (1970) *J. Physiol.* (*Lond.*) **209**, 105–130
22. Martonosi, A. (1972) in *Current Topics in Membranes and Transport* (Bronner, F., and Kleinzeller, A., eds) Vol. 3, pp. 83–197, Academic Press, New York