

THE INTERACTION OF RYANOIDS WITH THE CARDIAC RYANODINE RECEPTOR

by

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Abstract

The purpose of this thesis was to investigate the interaction of ryanoids, with sheep cardiac intracellular Ca^{2+} -release channels, commonly referred to as the ryanodine receptor (RyR) channel. The rates of association and dissociation of ryanodol (neutral), 8 β -amino-9 α -hydroxyryanodine (+1) and 10-O-succinoylryanodol (-1) to/from single RyR channel incorporated into a planar phospholipid bilayer, demonstrate that all the ryanoids irrespective of net charge, are influenced by transmembrane voltage. The influence of voltage on a neutral ryanoid confirms the proposal that there is a voltage dependent conformational change in the channel protein that switches between a high and low affinity binding site (Tanna *et al*, 1998). However, the voltage dependence of ryanodol is less than that observed for 8 β -amino-9 α -hydroxyryanodine and 21-amino-9 α -hydroxyryanodine yet more than 10-O-succinoylryanodol, suggesting that some of the voltage dependence of the interaction comes from the movement of the charged ligands someway into the voltage drop across the channel. Further studies carried out indicate that the interaction of 21-amino-9 α -hydroxyryanodine with single RyR channels decreases in the presence of the cytosolic blocking cation TEA that binds 90% into the voltage drop. Whilst [^3H]-ryanodine binding studies also demonstrate that TBA (a blocker of the RyR channel, which binds within the voltage drop) reduces ryanodine binding. The studies place the ryanoid-binding site someway within the voltage drop across the channel. The interaction of ryanoids with the RyR reduces the effectiveness (affinity) of TEA as a blocker by altering the location of the blocking site within the voltage drop across the channel. The interaction of the reversible ryanoid 8 β -amino-9 α -hydroxyryanodine with the RyR channel induces a reduced conductance state with excess noise. A closer examination of this reduced conductance state suggested that the excess noise was a result of the RyR channel alternating between two distinct conductance states (α and β) induced by the ryanoid. These observations strengthen the proposal that the interaction of ryanoids with the RyR channel induces structural rearrangement of the conduction pathway of the channel.

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This thesis is dedicated to my Mum and Dad.

Jai Shri Krishna.

Abbreviations

CICR	Ca ²⁺ -induced Ca ²⁺ -release
CoMFA	comparative molecular field analysis
Conduction pathway	passage through the channel from one face to the other
DHPR	dihydropyridine receptor
EC ₅₀	concentration of ligand required producing 50 % activation
EMD 4100	a structural analogue of caffeine
FC	fractional conductance
HSR	heavy sarcoplasmic reticulum
InsP ₃ R	inositol (1,4,5)-trisphosphate receptor
K _d	equilibrium dissociation constant
K _{off}	rate constant for the dissociation of a ligand from the channel
K _{on}	rate constant for the association of a ligand from the channel
P _{mod}	probability a single channel is in the modified state
P _o	channel open probability
QSAR	quantitative structure-activity relationship
RyR	ryanodine receptor
RyR1	mammalian skeletal isoform of the RyR
RyR2	mammalian cardiac isoform of the RyR
RyR3	mammalian brain isoform of the RyR
SR	sarcoplasmic reticulum
TAA	tetraalkylammonium
TBA	tetrabutylammonium
TEA	tetraethylammonium
TMA	tetramethylammonium
TPeA	tetrapentylammonium
TPrA	tetrapropylammonium
Voltage drop	region within the conduction pathway that is influenced by transmembrane holding potential.

Publications

Below I list both my peer reviewed publications and abstracts arising from the work contained in this thesis. In addition, reprints of the peer reviewed publications are included loose in the back cover.

Peer reviewed publications

TANNA, B., WELCH, W., RUEST, L., SUTKO, J. L. & WILLIAMS, A. J. (2000). The interaction of a neutral ryanoid with the ryanodine receptor channel provides insights into the mechanisms by which ryanoid binding is modulated by voltage. *Journal of General Physiology* **116**, 1-9.

TANNA, B., WELCH, W., RUEST, L., SUTKO, J. L., & WILLIAMS, A. J. (2001). Ryanoid modification of the cardiac muscle ryanodine receptor channel results in relocation of the tetraethylammonium binding site. *Journal of General Physiology* **117**, 385-393.

TANNA, B., WELCH, W., RUEST, L., SUTKO, J. L., & WILLIAMS, A. J. (2002). Excess noise in modified conductance states following the interaction of ryanoids with cardiac ryanodine receptor channels. *FEBS Letters* **516**, 35-39.

Three full manuscripts are in preparation

Abstracts

TANNA, B., RUEST, L., WELCH, W., SUTKO, J. L. & WILLIAMS, A. J. (2000). Ryanoid-induced re-location of a blocking site in the RyR2 channel. *Biophys. J.* **78**, A191.

WILLIAMS, A. J., HAJI, N., TANNA, B., WELCH, W., RUEST, L. & SUTKO, J. (2000). Excess noise in the ryanoid-modified conductance state of the RyR2 channel. *Biophys. J.* **78**, A191.

TANNA, B., WELCH, W., RUEST, L., SUTKO, J. & WILLIAMS, A. J. (2001). Further characterisation of the 8 β -amino-9 α -hydroxyryanodine (8 β -amino)-induced subconductance state of sheep cardiac RyR. *Biophys. J.* **80**, A459.

TANNA, B., WELCH, W., SUTKO, J., RUEST, L. & WILLIAMS, A. J. (2002). The interaction of a negatively charged ryanoid with RyR2 is voltage dependent. *Biophys. J.* **82**, A74.

TANNA, B., WEST, D. J. & WILLIAMS, A. J. (2002). The binding of [^{3} H]-ryanodine to RyR2 is influenced by channel blocking cations. *Biophys. J.* **82**, A74.

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Chapter 1

Introduction

a) Structure and function of the RyR channel

The aim of the thesis was to investigate the functional consequences of the interaction of natural congeners and synthetic analogues of ryanodine, with purified sheep cardiac ryanodine receptor (RyR) channels. The first part of the thesis concentrates on the location of the high affinity ryanoid binding site on the RyR channel and the second part pays particular attention to whether the interaction of ryanoids induce a conformational change in the channel protein.

1.1 The identification of ryanodine and the RyR channel

Ryanodine is a naturally occurring plant alkaloid found in the wood of *Ryania speciosa* Vahl (Rogers *et al.*, 1948) that grows as shrubs or slender trees in Central and South America. In the 1940s a survey of plant materials for new insecticides revealed that extracts of the stem and roots of *Ryania speciosa* Vahl had insecticidal activities. Rogers *et al.*, (1948) purified a compound from the stem wood and designated it as ryanodine, which had seven hundred times the insecticidal potency of the starting material. Ryanodine was found to induce flaccid paralysis in many insect species (DeVault, 1983; Edwards *et al.*, 1948; Haslett and Jenden, 1961). Subsequently the effects of low ryanodine concentrations (10 nM) were shown to induce irreversible contractions of vertebrate skeletal muscle resulting in rigid paralysis (Fairhurst, 1974; Fairhurst and Hasselbach, 1970). Similar concentrations of ryanodine led to a progressive flaccid paralysis in vertebrate cardiac muscle (Hillyard and Procita, 1959; Penefsky and Kahn, 1969; Sutko *et al.*, 1979). These paralysing effects of ryanodine suggested that its target proteins might have functional roles in the excitation-contraction coupling (EC-Coupling) process. Further studies revealed ryanodine to be a specific ligand with high affinity for the triad junctional 'foot' or 'feet' protein. The protein was commonly referred to as the SR Ca^{2+} - release channel and because of this high affinity for ryanodine it is now known as the ryanodine receptor (RyR) (Lai *et al.*, 1988; Pessah *et al.*, 1986). The high specificity of ryanodine for RyR channels has made ryanodine an indispensable tool in the investigation of the channel identity, structure and levels of expression in various species and tissues. Ryanodine has also proved

useful in investigation of the functional state of the RyR channel and its modulation by various compounds.

1.2 The role of the RyR channel in EC-Coupling

The Ca^{2+} -dependent contraction of muscle fibres in response to depolarisation of the plasma membrane occurs through EC-Coupling.

1.2.1 Cardiac EC-Coupling

Cardiac excitation-contraction coupling is the process where by electrical excitation of myocytes leads to the contraction of the heart. Ca^{2+} is an essential second messenger in the cardiac electrical activity; it is this second messenger that directly activates the myofilaments for muscle contraction. The central cause of contractile dysfunction and arrhythmias in pathophysiological conditions is the mishandling of Ca^{2+} in myocytes (Bers, 2002). During a cardiac action potential, a wave of depolarisation propagates along the surface of the plasma membrane and transverse-tubules (t-tubules), which overlays the terminal cisternae of the intracellular sarcoplasmic reticulum (SR) (Bers, 1991). Voltage-sensing molecules located in the t-tubules known as the L-type Ca^{2+} channels or the dihydropyridine receptors (DHPRs) are activated. The activation of cardiac DHPRs results in an influx of Ca^{2+} into the cell. Although, this elevation in cytosolic Ca^{2+} is not sufficient to directly activate myofilament contraction, it is enough to stimulate the release of stored Ca^{2+} from the intracellular SR (referred to as the calcium induced calcium release (CICR) process), by activating large intracellular proteins on the SR that span the gap between the two membrane systems termed as the 'feet' or 'foot' protein and now commonly referred to as the RyR channels. Stored Ca^{2+} is released from the SR and binds to the Ca^{2+} -binding subunit on the thin filament protein troponin and initiates muscle contraction (Figure 1.1).

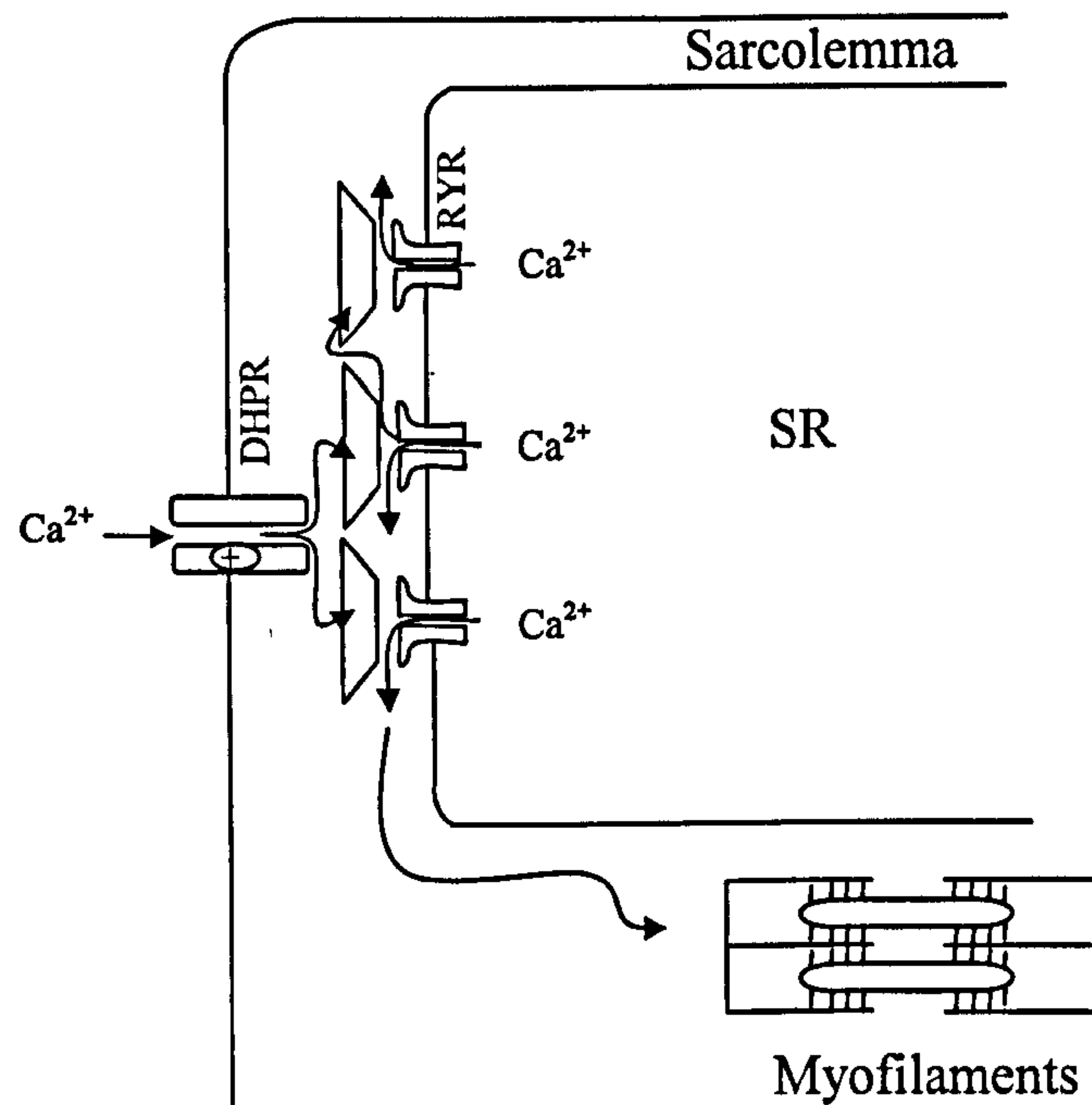


Figure 1.1 Cardiac muscle excitation-contraction coupling. Ca^{2+} entry into the cell via DHPRs activates RyR channels by CICR. Release of stored SR Ca^{2+} initiates muscle contraction by binding to the Ca^{2+} -binding subunit troponin on the thin filament.

1.2.2 Skeletal EC-Coupling

In skeletal muscle, the depolarisation of the t-tubules activates and cause a conformational change in the DHPRs, which translates a signal through direct mechanical interactions via the II-III loop (Lamb *et al.*, 2000; Nakai *et al.*, 1998) to activate the SR Ca^{2+} -release channels (Figure 1.2). Ca^{2+} released from the SR binds to troponin and in turn this initiates muscle contraction. There is some evidence that only a subpopulation of Ca^{2+} -release channels are mechanically linked to t-tubule sensors. Ca^{2+} release by these channels could serve to amplify SR Ca^{2+} release by activating the uncoupled Ca^{2+} -release channels by CICR (Armstrong and Jorgensen, 1994; Block *et al.*, 1988).

Therefore, these systems show that while cardiac muscle contraction depends on both Ca^{2+} entry across the sarcolemma and Ca^{2+} release from the SR. Skeletal muscle contraction depends almost exclusively on the Ca^{2+} released from the SR with insignificant Ca^{2+} entry across the sarcolemma.

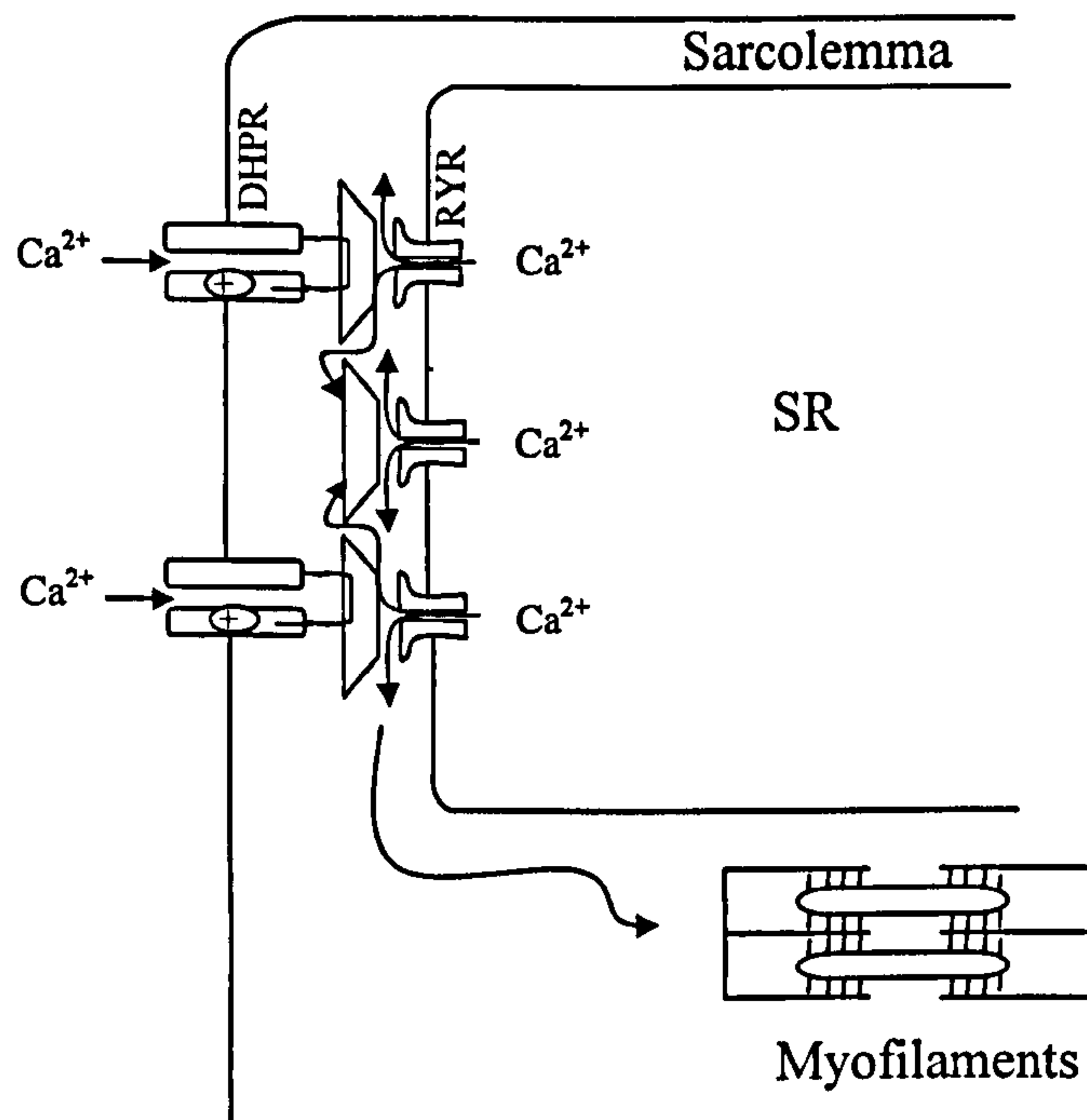


Figure 1.2 Skeletal muscle excitation-contraction coupling. Electrical activity stimulate DHPRs that are physically linked to RyR channels and initiate Ca²⁺-release from the SR. Ca²⁺-release from the SR can then activate uncoupled RyR channels via CICR and lead to muscle contraction by binding to troponin.

1.3 Ryanodine Receptor Isoforms

Based on the ability of ryanodine receptors to bind ryanodine and therefore [³H]-ryanodine with high affinity, it was possible to isolate the protein initially from striated muscle (Sutko and Airey, 1996) and subsequently it has been shown to be distributed widely across species and in different tissues within the same species. Three isoforms of the RyR channel have been cloned and sequenced from mammalian tissues RyR1, RyR2 and RyR3, which are genetically distinct from each other. Although, the isoforms are not tissue specific RyR1 is the primary isoform in skeletal muscles and the Purkinje cells of cerebellum (Ogawa, 1994; Takeshima *et al.*, 1989). RyR2 is the predominant isoform in cardiac muscle and brain (Nakai *et al.*, 1990; Ogawa, 1994; Otsu *et al.*, 1990). RyR3 was first detected in epithelial cells of the lungs (Giannini *et al.*, 1992) and regions of the brain (Hakamata *et al.*, 1992) and was then found to be more widely expressed, for example in smooth and skeletal muscle (Giannini *et al.*, 1995). Two non mammalian vertebrate skeletal isoforms, α and β , RyR channels have been identified (Ogawa *et al.*, 1999; Sutko and Airey, 1996), which are homologues of RyR1 and RyR3

respectively (Ogawa, 1994; Ottini et al., 1996). RyR channel isoforms have also been identified in invertebrate species including insects (Scott-Ward *et al.*, 2001; Sutko and Airey, 1996).

1.4 The Structure of the Ryanodine Receptor

The RyR channel is identified as a high molecular weight homotetramer; each RyR monomer is composed of roughly 5000 amino acid residues and has a molecular weight of approximately 550 kDa (Williams *et al.*, 2001). The overall topology of the various isoforms of the RyR channel is similar. The ion channel forming, membrane-spanning regions are highly conserved and localised to the carboxyl-terminal of the protein (the last 1000 amino acids). As both the amino- and the carboxyl-termini are located in the cytoplasm, only even numbers of membrane-spanning regions are possible. To date 4, 6, 10 and 12 membrane-spanning segments have been suggested (Takeshima *et al.*, 1989; Tunwell *et al.*, 1996; Zorzato *et al.*, 1990). The pore forming components of the protein molecule are suggested to be located in the carboxyl-terminal region, within the luminal loop which links transmembrane domain 3 and 4 in the 4 transmembrane domain model (Balshaw *et al.*, 1999) demonstrated in Figure 1.3; or the loop linking the last two transmembrane domains in other models.

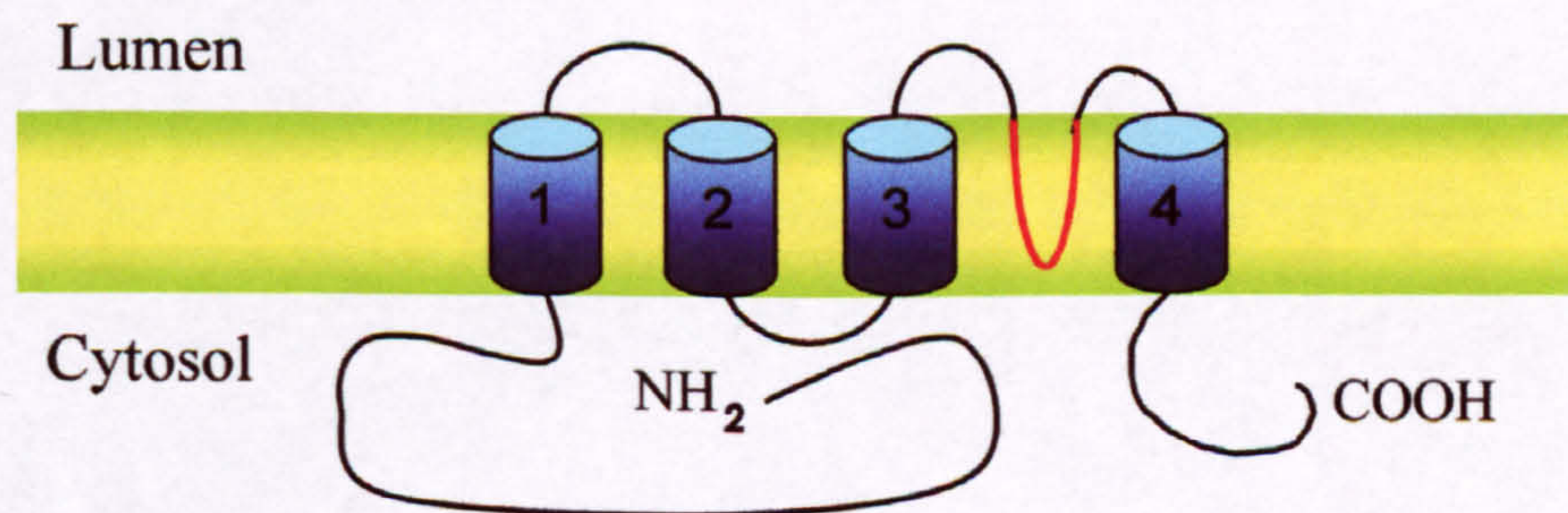


Figure 1.3 A cartoon localising the pore-forming region of the RyR channel, assuming the four transmembrane model proposed by Takeshima *et al.*, (1989). The pore of the channel is formed by the loop dipping into the membrane from the luminal side between the 3rd and 4th transmembrane spanning regions, shown in red (modified diagram taken from Williams *et al.*, 2001).

A marked similarity exists between a sequence of residues present in the luminal loop of both RyR (GIGD) and InsP₃R (GVGD) (Blondel *et al.*, 1993) Ca²⁺-release channels

and the selectivity filter signature sequence in K^+ channels. Homology and secondary structure similarities suggested possible correlation of the KcsA K^+ channel and the RyR channel tertiary structure, for example it was suggested that the luminal loop of the RyR channel might fold back into the membrane and form some part of the channel pore such as the selectivity filter (Figure 1.4), as is the case in the KcsA K^+ channel (Balshaw *et al.*, 1999 and Williams *et al.*, 2001). Further support for this proposal comes from experiments in which residues in and around the RyR luminal loop have been mutated. Substitution of amino acids in this highly conserved sequence produce changes in unitary conductance with both K^+ and Ca^{2+} as the permeant ion under voltage clamp conditions (Zhao *et al.*, 1999).

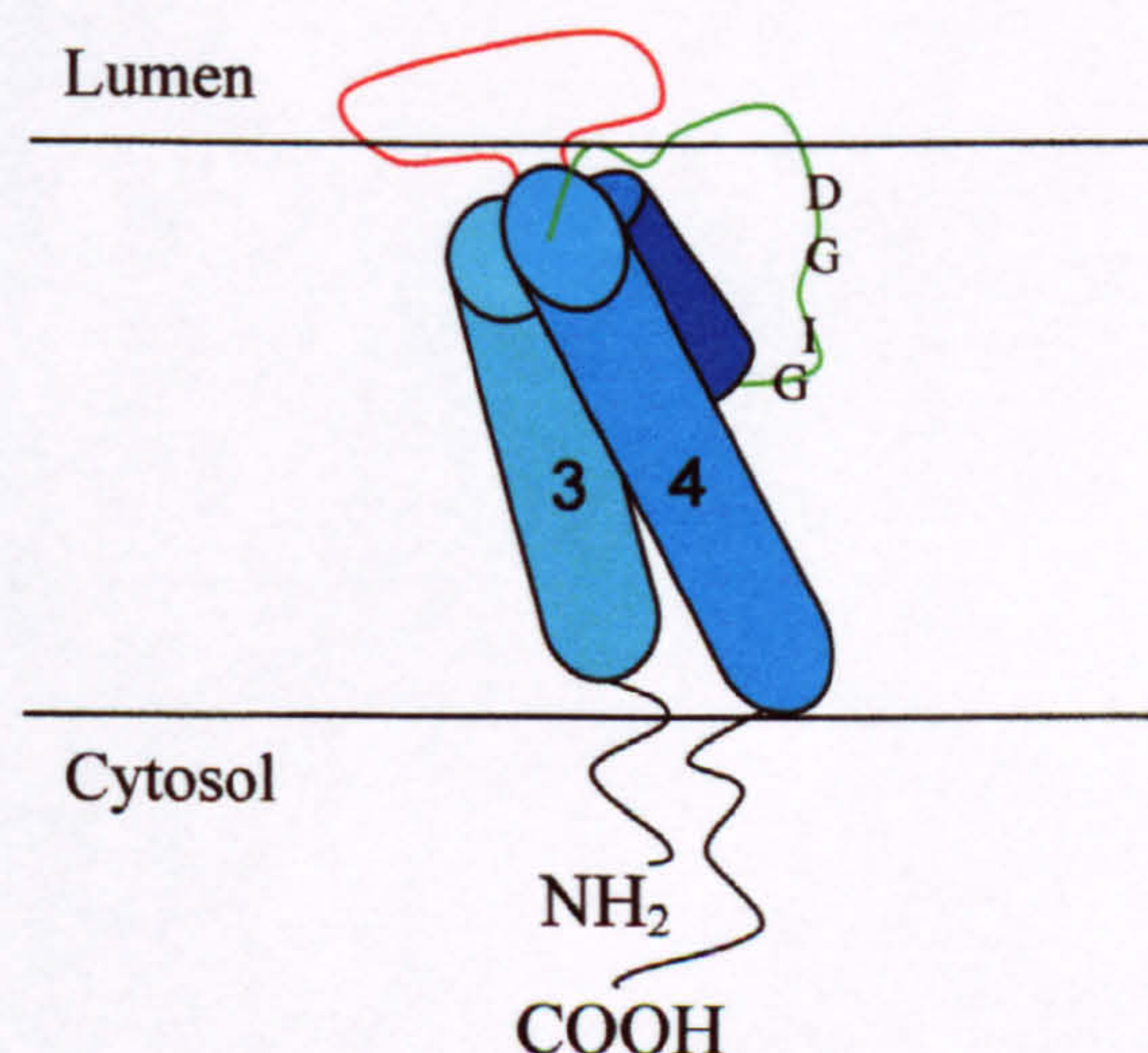


Figure 1.4 A hypothetical arrangement of the pore forming loop of the RyR channel predicted from similarities with the KcsA channel (Williams *et al.*, 2001). The region consists of the last two transmembrane domains (3 and 4) and the loop connecting them including the selectivity filter signature (GIGD) highlighted (modified diagram taken from Williams *et al.*, 2001).

Cryoelectron microscopic images of individual RyR channels have revealed a four fold symmetry with a channel extending as a single pore from the SR luminal end through the middle region of the receptor (Sutko and Airey, 1996). It is believed that the functional RyR channel possesses a single conduction pathway, a component of which is formed from the pore loops of each monomer folded back into the membrane (Williams *et al.*, 2001), a cartoon of two opposing monomers forming the putative pore is shown in Figure 1.5. Site-directed mutagenesis studies have demonstrated an alteration of a single residue (GG**GIGD**) near the conserved sequence results in a

reduction in single channel conductance (by 97%) as mentioned above (Zhao *et al.*, 1998). Co-expression of the mutant and wild type cDNAs, result in the formation of hybrid channels that contain different ratios of wild type and mutant subunits. To determine whether the RyR channel conduction pathway was formed by pore-forming segments of each subunit, co-expression of the mutant and wild type cDNAs to form hybrid channels that contained different ratios of wild type and mutant subunits were carried out in HEK (Human Embryonic Kidney) 293 cells. These hybrid channels demonstrated intermediate unitary conductance that would not come about by a simple additive quarter conductance of the wild type and mutant in any possible ratio for four individual pores from each RyR subunit. These observations are in strong agreement with the proposal that the RyR channel has one conduction pathway, which is formed from the pore loops of each monomer (Zhao *et al.*, 1999). Other correlations between the RyR and the KcsA channel have been suggested, for example a portion of the pore forming loop in the KcsA channel forms a short helical section termed the pore helix (Doyle *et al.*, 1998). The RyR pore-forming loop has also been predicted to have an amino acid sequence, which is analogous to the pore helix, observed in KcsA channels. Williams *et al.*, (2001), have proposed that not only would the pore-forming loops of each monomer form part of the conduction pathway as suggested above, but the putative pore-helix of each monomer would arrange itself, so that the dipoles arising from the pore helices (their partial negative charge) would point towards the cytosol and focus on a water-filled cavity lined with hydrophobic residues. Figure 1.5, shows the analogy made between the KcsA bacterial K⁺ channel and the RyR channel.

The remaining amino-terminal part of the RyR channel comprises approximately 80% of the cytoplasmic protein mass, which is identified with the foot structure that spans the junctional gap between the t-tubules and the terminal cisternae of the SR. Cryoelectron microscopy had identified this as quatrefoil in shape (Serysheva *et al.*, 1995; Wagenknecht and Radermacher, 1995).

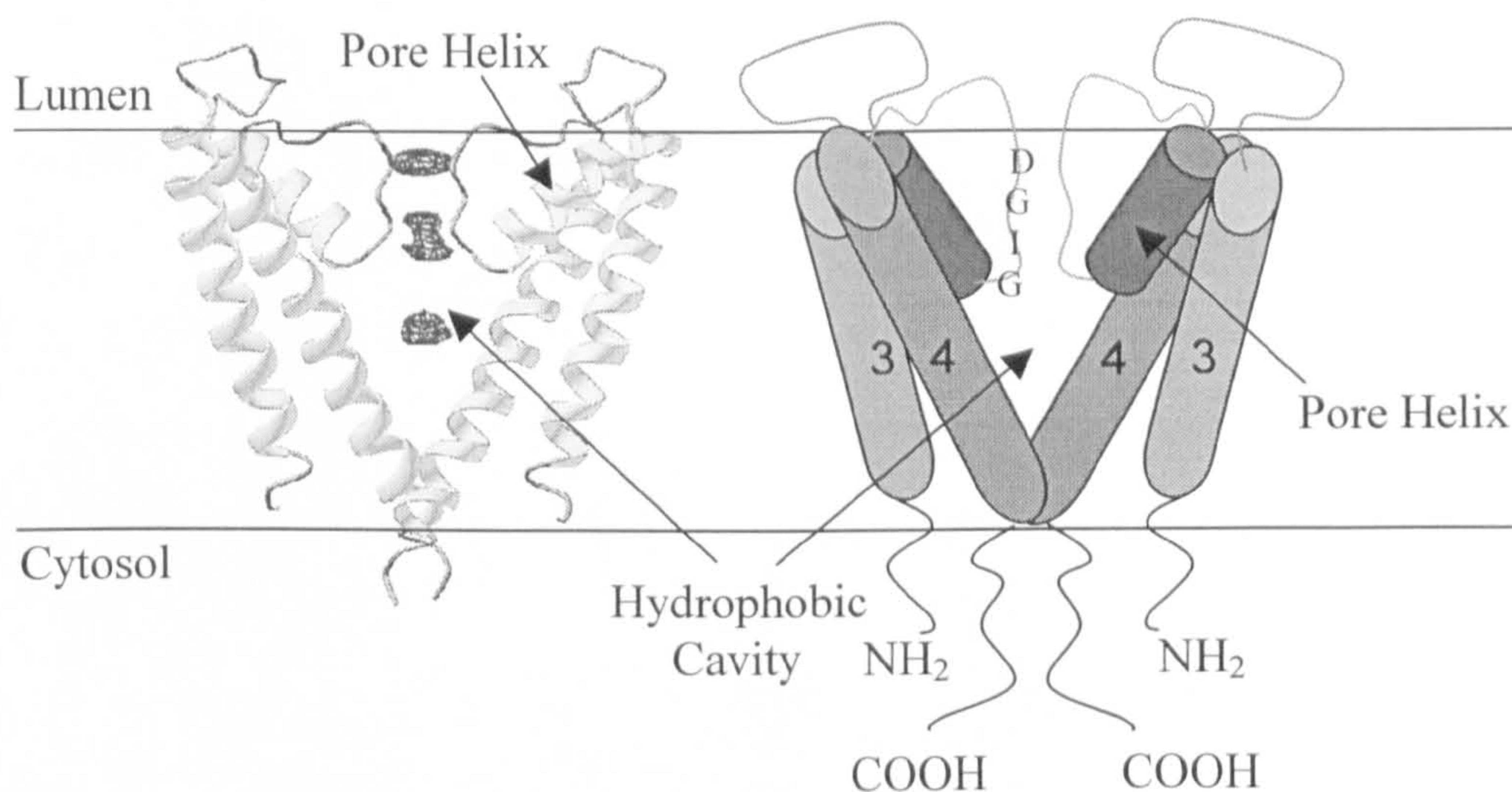


Figure 1.5 (Right) A hypothetical arrangement of two opposing RyR channel subunits (the last two transmembrane domains and the loop connecting them), analogous to the KcsA channel (left) as suggested by Williams *et al.*, (2001). The pore helix of the RyR channel is arranged so that it points towards a water filled cavity lined with hydrophobic residues as observed in the KcsA channel.

1.5 Ion handling in the RyR channel

Although the function of RyR channels is to translocate enormous quantities of stored Ca^{2+} from the SR into the cytoplasm during muscle excitation-contraction coupling, investigations in which ion handling of purified single RyR channels incorporated into planar phospholipid bilayers have been monitored have revealed that the channel is not completely selective for Ca^{2+} (Lindsay and Williams, 1990). The RyR channel is impermeant to anions and can discriminate between inorganic monovalent and divalent cations and organic monovalent cations.

1.5.1 Inorganic monovalent cations

Conductance values for monovalent cations such as K^+ , Na^+ and Li^+ are 723, 446 and 215 pS respectively in 210 mM symmetrical conditions (Lindsay *et al.*, 1991), while with increasing ionic activities of K^+ , Na^+ and Li^+ in the solution, single channel conductance reaches a plateau. The resulting conductance-activity relationships were described as Michaelis-Menten-type saturation curves, which suggested that the channel pore could be occupied by no more than one ion at a time. Although unitary conductance varies considerably with monovalent cations, little variation in permeability is observed in this class of ions relative to K^+ .

1.5.2 Inorganic divalent cations

In comparison, unitary conductance for Ca^{2+} is 93 pS in 210 mM symmetrical conditions. These observations revealed that RyR channels translocated monovalent cations more efficiently than divalent cations. However, in bi-ionic conditions, the RyR channel is more selective for divalent cations than for the monovalent cations, in fact divalent cations are six and a half times more permeant than K^+ and Na^+ ions in the RyR channel (Tinker *et al.*, 1992). However, like the monovalent cations, little difference is observed in the relative permeability of the divalent cations. The affinity of the RyR channel for divalent cations seems to be higher than for the monovalent cations, as 50% saturation for, for example Ba^{2+} , is seen at an activity of approximately 400 μM , whereas 50% saturation for the monovalents is seen at activities of between 9 - 35 mM (Tinker and Williams, 1992).

This suggests that there would be a near maximal rate of Ca^{2+} translocation at the relative low Ca^{2+} (μM) activities found in the lumen of the SR.

1.5.3 Organic permeant cations

The permeability of organic monovalent cations has proven to be more complex than that observed with both inorganic mono- and divalent cations (Tinker and Williams, 1993). Relative permeability (calculated with K^+ in the luminal side and the organic cation in the cytosolic face of the channel) declines as the radius of the organic cation increases; cations that have a minimum circular radius greater than 3.5Å are impermeant. It was therefore, suggested that the sheep cardiac RyR channel conduction pathway had a minimum pore radius of approximately 3.4Å (Tinker and Williams, 1993) and this region is generally referred to as the selectivity filter. The translocation of organic cations through the voltage drop across the channel does not rely exclusively on the size of the ion; hydrophobic interactions of the ion with the channel also play a part in ion permeability, conductance and the affinity of the channel for this class of cations (Tinker and Williams 1993).

1.5.4 Impermeant organic cations

Tetraalkylammoniums (TAA) are classic blocking cations of K^+ channels and have also been shown to block K^+ ion translocation through the RyR channel from the cytosolic

face. The effectiveness of block is dependent on both concentration and transmembrane voltage. Analysis of the voltage dependence of block using the Woodhull method (details given in Chapter 2), demonstrates the presence of two binding site within the voltage drop across the RyR channel for these cations. The smallest TAA, tetramethylammonium (TMA) interacts with a site approximately 50% into the voltage drop from the cytosolic face of the channel. However, the larger TAAs, such as tetraethylammonium (TEA) and tetrapropylammonium (TPrA) do not have access to this site. These larger TAAs are blockers with a greater voltage dependence than that observed for TMA and they interact at a site 90% into the voltage drop from the cytosolic face of the channel (Lindsay *et al.*, 1991; Tinker *et al.*, 1992 a and b). As is the case for K^+ channels, the TAAs that block 90% into the voltage drop across the channel appear to involve hydrophobic interactions. TAAs that are larger still, such as tetrabutylammonium (TBA) and tetrapentylammonium (TPeA), also block K^+ current in the RyR channel in a concentration and voltage dependent manor. The sites of interaction of these TAAs have been located at the extremity of the cytosolic mouth of the voltage drop across the channel and these TAAs act as partial blockers (Tinker *et al.*, 1992a).

1.6 Length of the voltage drop across the RyR channel

The interaction of blocking cations with the RyR channel has been useful in the estimation of the length of the voltage drop across the channel. This is the distance where the ion no longer moves by diffusion as it does within the bulk solution, but is dependent on electrodiffusion as a result of the applied electric field across the channel. Tinker and Williams, (1995), have determined the length of the voltage drop across the RyR channel with the aid of blocking monovalent and divalent derivatives of trimethylammonium of varying carbon chain lengths. The voltage dependent block of K^+ current by the monovalent cations was unaffected by chain length, they all had an effective valence of block of 0.9 irrespective of chain length, consistent with the interaction of these cations at a site 90% into the voltage drop from the cytosolic face of the channel. In contrast, increasing in chain length of the divalent cations from 2 to 7 carbon groups (two TMA groups separated by carbon groups) resulted in a linear decrease in the dependence of block on voltage. The effective valence decreased from 1.5 to a value approaching that obtained with a monovalent derivative. The explanation for this is, that one cationic group of all divalent derivative interacts at a site 90% into

the voltage drop and has an effective valence of 0.9. The high values of effective valence obtained with short chain derivatives reflect the presence of the second trimethylammonium cationic group within the voltage drop. The voltage dependence of a divalent cation with a chain length of 7 carbons groups is equivalent to the effective valence of a monovalent derivative; suggesting the second charged group has dropped out of the voltage drop. Calculating the distance between the two trimethylammonium groups of this divalent derivative and taking into account the last 10% of the voltage drop towards the luminal end permits the determination of the length of the voltage drop. The calculation suggested that applied potential falls over a total length of 10.4 Å in the RyR channel.

The measurements of relative conductance, affinity, permeability and block obtained from a range of permeant and impermeant cations, suggest that the RyR channel is a high conductance, poorly selective ion channel, which has a voltage drop with a length of approximately 10Å with two cation binding sites located 50 and 90% away from the cytosolic entrance of the voltage drop. The narrowest region of the voltage drop has a radius of approximately 3.5Å. Block by TEA (minimum radius of 3.5Å) 90% into the voltage drop, suggests that TEA is too large to cross the narrowest region (the selectivity filter), which occurs over the last 10% (no more than 1Å in length) of the voltage drop at the luminal side of the channel Figure 1.6.

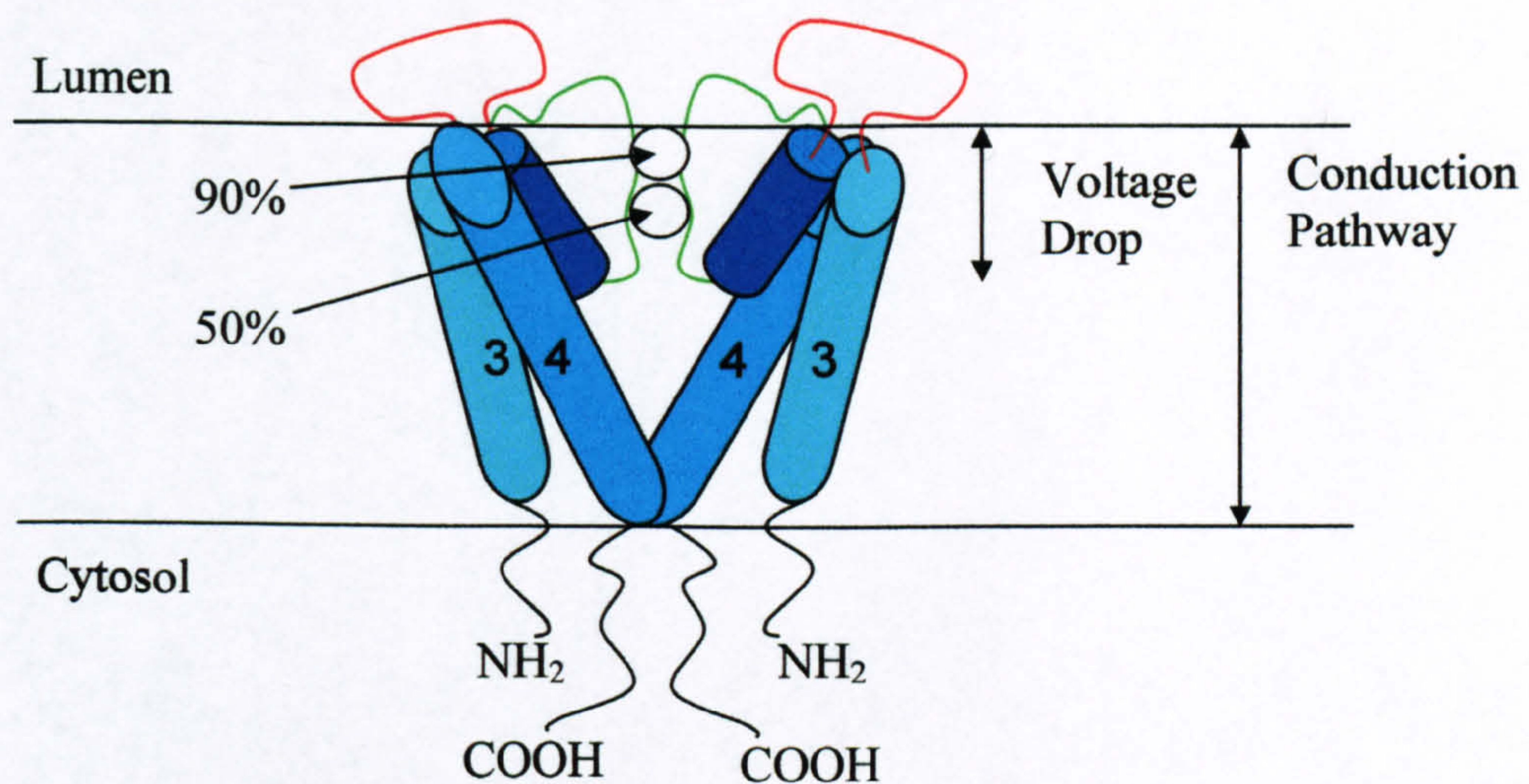


Figure 1.6 A hypothetical diagrammatic representation (suggested by Williams *et al.*, 2001) of the essential features of the RyR channel conduction pathway, obtained from the investigations of permeant and impermeant ion interaction within the RyR channel. The RyR channel has a single conduction pathway formed from components of all four monomers. The pathway has a large water filled cavity, that leads into the putative voltage drop across the channel. The voltage drop is 10Å in length, it has two cation binding sites at 50 and 90% into the channel and a short constriction of approximately 1Å towards the luminal exit that can act as an obstacle for large cations.

1.7 The mechanisms involved with ion discrimination in the RyR channel

Whilst discrimination between cations is relatively weak in the RyR channel, clear differences do exist between the mono- and divalent cations. Tinker *et al.*, (1992c), proposed a single-ion four barrier three binding site Eyring rate theory model of the voltage drop across the channel that suggests mechanism for ion discrimination. TAA blocking experiments provide evidence for two distinct cation binding sites within the voltage drop (at 50 and 90%). As the current voltage relationships of both mono- and divalent cations are linear in symmetrical conditions, this would imply a symmetry in the energy profile through the voltage drop. Therefore, an additional binding site located approximately 10% from the cytosolic face of the channel was suggested, Figure 1.7. This proposed model suggests that overall a combination of two mechanisms favour the translocation of divalent cations over monovalent cations. The peak height over each energy barrier is less for divalent cations than for monovalent cations, whilst the central binding site shows a higher affinity for divalent over monovalent cations. This results in divalent cations having a lower unitary conductance

than monovalent cations. Conductance decreases as affinity increases. The varied conductance values obtained within both classes of cations suggest that each cation has different properties; some interact with the central well with greater affinity. The permeability of organic cations as described previously is slightly more complex. Within the model there is a size discrimination process located 90% into the voltage drop from the cytosolic face of the channel at the selectivity filter (peak 4), which is thought to increase in height and well 3 becomes deeper, as the permeant ion increases in size. This leads to a decline in single channel conductance, until the cation is unable to pass peak 4 (impermeant cations). Tinker and Williams, (1993) also propose that some organic cations may bind to well 3 (a hydrophobic binding site) with greater affinity than others, so that cations that have the same relative permeability may not have the same conductance.

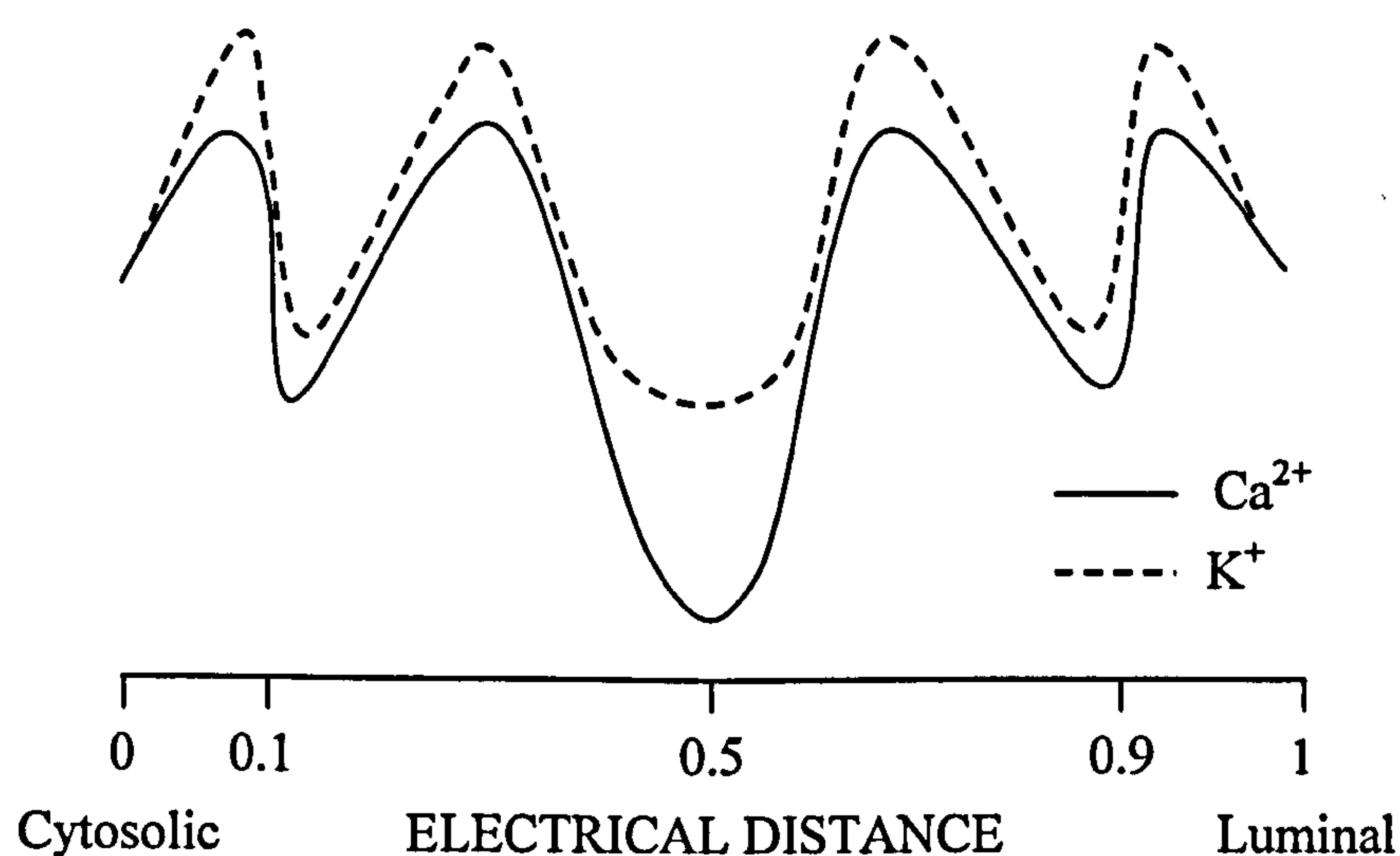


Figure 1.7 A representation of the energy profile that both K^+ and Ca^{2+} cations would face to translocate through the voltage drop across the channel (modified diagram taken from Tinker *et al.*, 1992c).

The high unitary conductance of both mono- and divalent cations observed at high transmembrane potentials indicates that rates of ion entry into the conduction pathway are not limited by diffusion (Tinker *et al.*, 1992c). If the entrance to the voltage drop had a large capture radius, this would facilitate ions leaving the bulk solution and entering an area in the conduction pathway, which can increase the likelihood of the ion entering the region influenced by transmembrane voltage (the voltage drop across the

channel). The accumulation of cations in the putative capture radius can be increased by the presence of fixed negative charge at the entrance of the conduction pathway. This proposal has been supported by experiments which demonstrate that the rate of association of synthetic peptides derived from the N-type inactivation domain of the *Shaker* K⁺ channel, increase approximately 500 fold when the net charge of the peptides is increased from +3 to +7 (Mead and Williams, 1998). The presence of negative charge at the mouth of the channel would also help to concentrate divalent cations over monovalent cations, thus aiding the discrimination process of divalent over monovalent cations.

1.8 Modulation of the RyR channel

There are a number of RyR channel modulators, however, I will only discuss those that are used to activate the RyR channel in the experiments described in this thesis.

Physiologically, the most important ligand that modulates the gating of RyR channels is Ca²⁺. Ca²⁺ has a two-fold action on the RyR channel, it activates the channel at low micromolar concentrations and inactivates the channel at millimolar concentrations. This is observed in isolated SR vesicles (Fleischer and Inui, 1989), single channel (Smith *et al.*, 1986) and ryanodine binding studies (Pessah *et al.*, 1987). These results suggest that the RyR channel possess a high affinity activating and a low affinity inhibitory Ca²⁺ binding site. Occupation of the low affinity Ca²⁺ binding site inhibits ryanodine binding to skeletal muscle SR vesicles (Michalak *et al.*, 1988), Ca²⁺ release from isolated SR vesicles (Meissner, 1984) and the activity of single channels incorporated into planar phospholipid bilayers (Ma *et al.*, 1988). Adenosine triphosphate (ATP) enhances channel open probability in the presence of activating Ca²⁺ concentrations. Ca²⁺ alone does not increase channel open probability (P_o) to 1, but needs another RyR channel activator to fully potentiate channel activity. Therefore, combinations of these two ligands are used to increase channel P_o in binding experiments carried out in Chapter 5. A caffeine analogue EMD 41000 is a potent activator of the RyR channel. It exhibits an EC₅₀ value of 40 µM and is 100 times more potent than caffeine at increasing single channel P_o, without changing unitary channel conductance (McGarry and Williams, 1994). EMD 41000 is used to activate RyR channels in all single channel experiments carried out in this thesis.

Introduction

b) The interaction of ryanodine and ryanodine analogues with the RyR channel

1.9 The interaction of ryanodine with the RyR channel and the functional consequences of this interaction

The effects of ryanodine on muscle are complex and depend on muscle type and ryanodine concentration. For example, in vertebrate skeletal muscle, ryanodine produces an irreversible contraction, as skeletal muscle contraction depends almost exclusively on the Ca^{2+} released from the SR. In cardiac muscle a decline in contractile force is observed, as cardiac muscle contraction depends on both Ca^{2+} entry across the sarcolemma and Ca^{2+} released from the SR as described previously.

The effects of low ryanodine concentrations (nM – low μM) on isolated skeletal SR membrane vesicles results in a slow release of Ca^{2+} , while higher ryanodine concentrations (10 μM and above) prevents Ca^{2+} release. The binding of [^3H]-ryanodine to the RyR channels in both cardiac and skeletal SR vesicles provides the evidence of this dual action of ryanodine. Two classes of ryanodine binding sites were observed; a high affinity and low affinity site. The ratio of high-to-low-affinity binding sites is reported to be 1:3 (Pessah and Zimanyi, 1991). The mechanisms underlying these concentration-dependent events were revealed by the application of ryanodine to individual RyR channels reconstituted into planar phospholipid bilayers. Concentrations of ryanodine that increase vesicle Ca^{2+} permeability modify single channel gating and ion handling. A variable time after addition of nM to low μM ryanodine to the cytosolic face of the channel the channel enters a reduced conductance state which has a high open probability with occasional transitions to the closed state as demonstrated in Figure 1.8 (Lindsay *et al.*, 1994; Tanna *et al.*, 1998 Tinker *et al.*, 1996;). At higher concentrations of ryanodine (100 μM – 2 mM) the RyR channel closes (Meissner, 1994; Tinker *et al.*, 1996).

The association of ryanodine with the RyR channel is slow, however, the dissociation of ryanodine for the RyR channel is even slower and on the time scale of a single channel

experiment the interaction of ryanodine with the RyR channel and the consequent alterations in function are irreversible. The removal of ryanodine from the medium bathing the RyR channel does not bring about the return of normal channel gating and conductance.

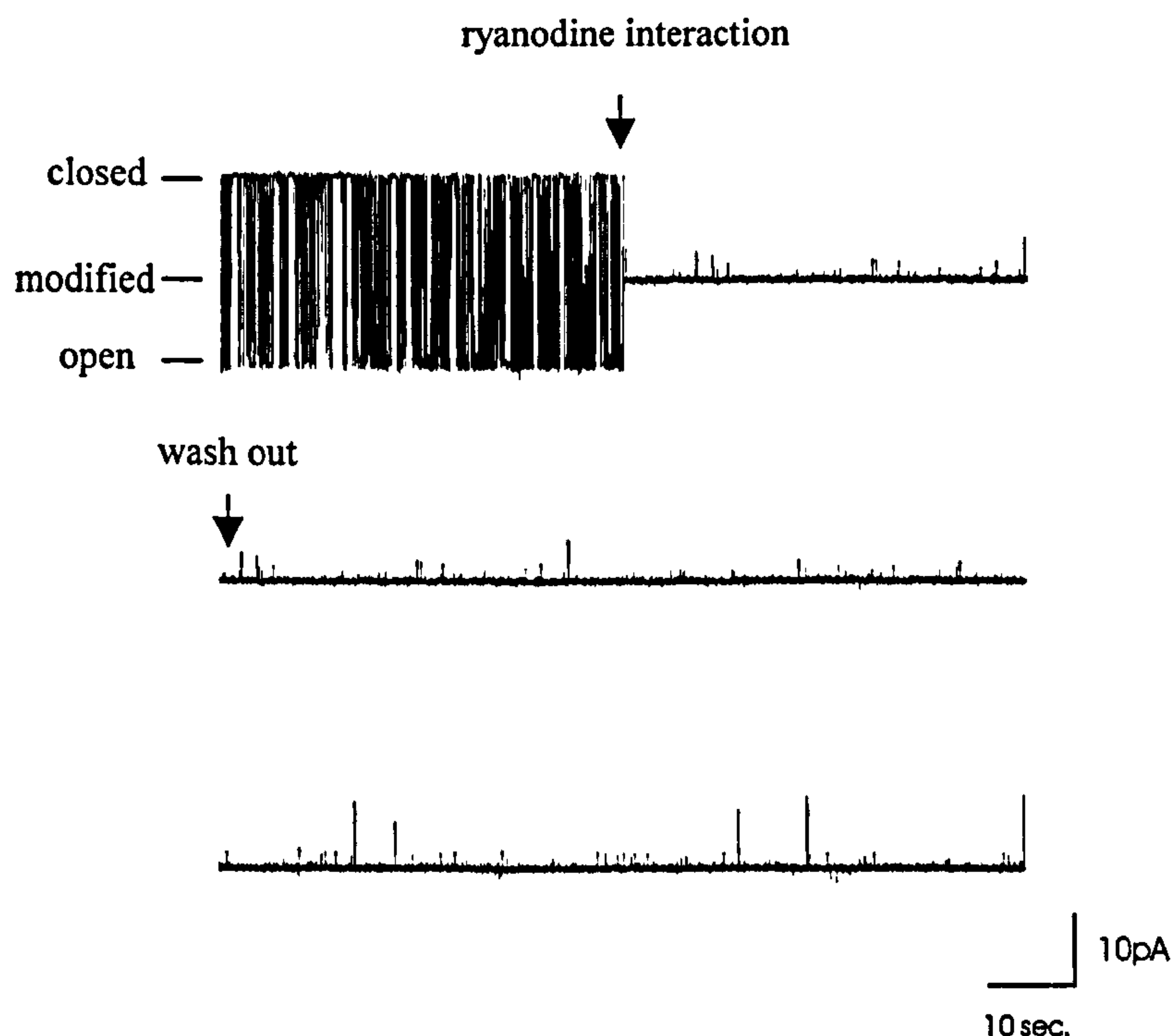


Figure 1.8 A trace of a single cardiac RyR channel in the presence of 200 nM cytosolic ryanodine held at 40 mV. Ryanodine was added to the solutions bathing the channel 4 minutes before it actually interacted with the RyR channel (indicated by the arrow). The medium bathing the RyR channel was then washed out and the channel remained in the reduced conductance state (modified state) until the experiment was stopped approximately 10 minutes later.

1.9.1 Mechanisms that underlie the altered conductance after ryanodine binding to single RyR2 channels

Investigations carried out by Lindsay *et al.*, (1994), looked at the mechanisms that underlie the altered conductance observed after ryanodine binding to the cardiac RyR channel. The conductance of the ryanodine-modified channel was expressed as a fraction of the conductance of the channel in the absence of ryanodine (fractional conductance – FC). The FC brought on by the interaction of ryanodine in symmetrical 210 mM solutions is constant when the channel is held at different transmembrane holding potential, but varies with the nature of the permeant cation. FC values obtained in the presence of symmetrical 210 mM solutions of monovalent cations such as K^+ ,

Na^+ , Cs^+ and Li^+ , are in the range of 0.60 to 0.66. The affinity of the channel for monovalent cations after ryanodine binding is increased, for example the K_D for K^+ is 20 mM in the unmodified channel and 11.6 mM when the channel is modified by ryanodine. The relative permeability of monovalent cations in the channel is unaffected by the interaction of ryanodine.

FC values observed in the presence of divalent cations are considerably lower, FC values of 0.22 and 0.28 were obtained for Ba^{2+} and Sr^{2+} respectively (high mM concentrations of both Ca^{2+} and Mg^{2+} abolish channel openings when present in the cytosolic face of the channel, so no data was obtained for these cations). The affinity of the conductance pathway for divalent cations after the interaction of ryanodine to the RyR channel is also increased, but this is accompanied by a decrease in the relative permeability of the channel for this class of cations, when compared to that observed in the absence of ryanodine.

Lindsay *et al.*, (1994), have explained these findings by suggesting the RyR channel protein goes through a conformational change upon the interaction of ryanodine with the channel. Based on the model that an ion translocating through the conduction pathway of the RyR channel crosses four energy peaks and interacts with three binding sites (Tinker *et al.*, 1992c), the observed reduction in single channel conductance in the presence of all the monovalent cations was explained by an increase in the affinity of the central binding site or the lowering of the central well depth in the energy profile. The increase in the affinity for K^+ ions after ryanodine interaction with the channel is in agreement with this proposal. An increase in the affinity of the central binding site would also explain the reduction in channel conductance in the presence of the divalent cations. This is not surprising, as in the absence of ryanodine, divalent cations are thought to bind more tightly to the central site than the monovalent cations. It had been suggested that a high density of negatively charged residues in the voltage drop would favour divalent over monovalent cation permeability (Tinker *et al.*, 1992c). The interaction of ryanodine could structurally alter the conduction pathway, lowering the density of the negative charge within the voltage drop and so decreasing the permeability of divalent cations in the channel. This redistribution of charge could occur if the surface area of the voltage drop increased resulting in an increase in the minimum pore radius.

Alterations to the surface area of the voltage drop across the channel, which leads to an increase in the minimum pore radius could explain the changes observed in organic cation translocation upon ryanodine binding. A wide variation in FC in the presence of bound ryanodine is observed for permeant monovalent organic cations. FC values are as follows ammonium 0.66, ethylamine 0.76, propanolamine 0.65, diethanolamine 0.92 and diethylamine 1.2. These results demonstrate that the interaction of ryanodine with the RyR channel does not always produce a reduction in channel conductance, but can lead to an increase in single channel conductance depending on the permeant ion. Ryanodine does not simply block the channel. While, the two smaller cations ammonia, radius 1.7Å and ethylamine, radius 2.2 Å, showed small changes in their permeability compared to the values obtained for the unmodified channel, the relative permeability of larger cations such as diethanolamine and diethylamine with radius of 2.8 and 2.5 Å respectively, significantly increased on ryanodine modification. This was thought to be due to a decrease in the energy barriers experienced by the larger cations as they pass through the channel. The lowering of all peak heights and in particular a lowering of peak 4 (the selectivity filter) in the energy profile, while leaving the well depth unaltered, could account for the observed FC for diethylamine. In physical terms, this could be achieved by widening of the filter (minimum pore radius), by 0.2-0.3 Å from the 3.3-3.5 Å determined in the unmodified channel, this would increase the relative permeability of larger organic cations, with little effect on the relative permeability of smaller organic cations. Other factors such as hydrophobic interactions also play a role in the varied FC observed between all organic cations. For example, hydrophobic cations will be influenced by the increase in the affinity of the central site and the hydrophobic site (well 3) in the voltage drop upon ryanodine interaction (Tinker and Williams, 1993).

The binding of ryanodine to the RyR channel induces a conformational change in the protein, which in turn modifies both the relative permeability and the affinity of cations translocating through the conduction pathway. The FC state observed upon binding of ryanodine is therefore dependent on both the size and properties of the permeant cation.

1.10 The Interaction of Natural Congeners and Synthetic Analogues of Ryanodine with the RyR Channel.

Whilst ryanodine has proved to be an extremely useful tool for the study of both the Ca^{2+} -release channel and its role in muscle excitation-contraction coupling, surprisingly little is known about the mechanisms involved in the interaction of ryanodine with its receptor or the functional consequences of this interaction. In recent years these issues have started to be addressed by monitoring the interaction of a large number of natural congeners and synthetic analogues of ryanodine (ryanoids) with the RyR channel. It has been established that altering the steric and electrostatic features of the basic ryanodine structure (Figure 1.9) produce changes in the likelihood of interaction and the functional consequences of the interaction of the ligands with the RyR channel (Sutko *et al.*, 1997). Collaborations with groups in the USA and Canada have given us unique access to a large number of ryanoids produced by simple esterification of both protected and non-protected natural congeners of ryanodine.

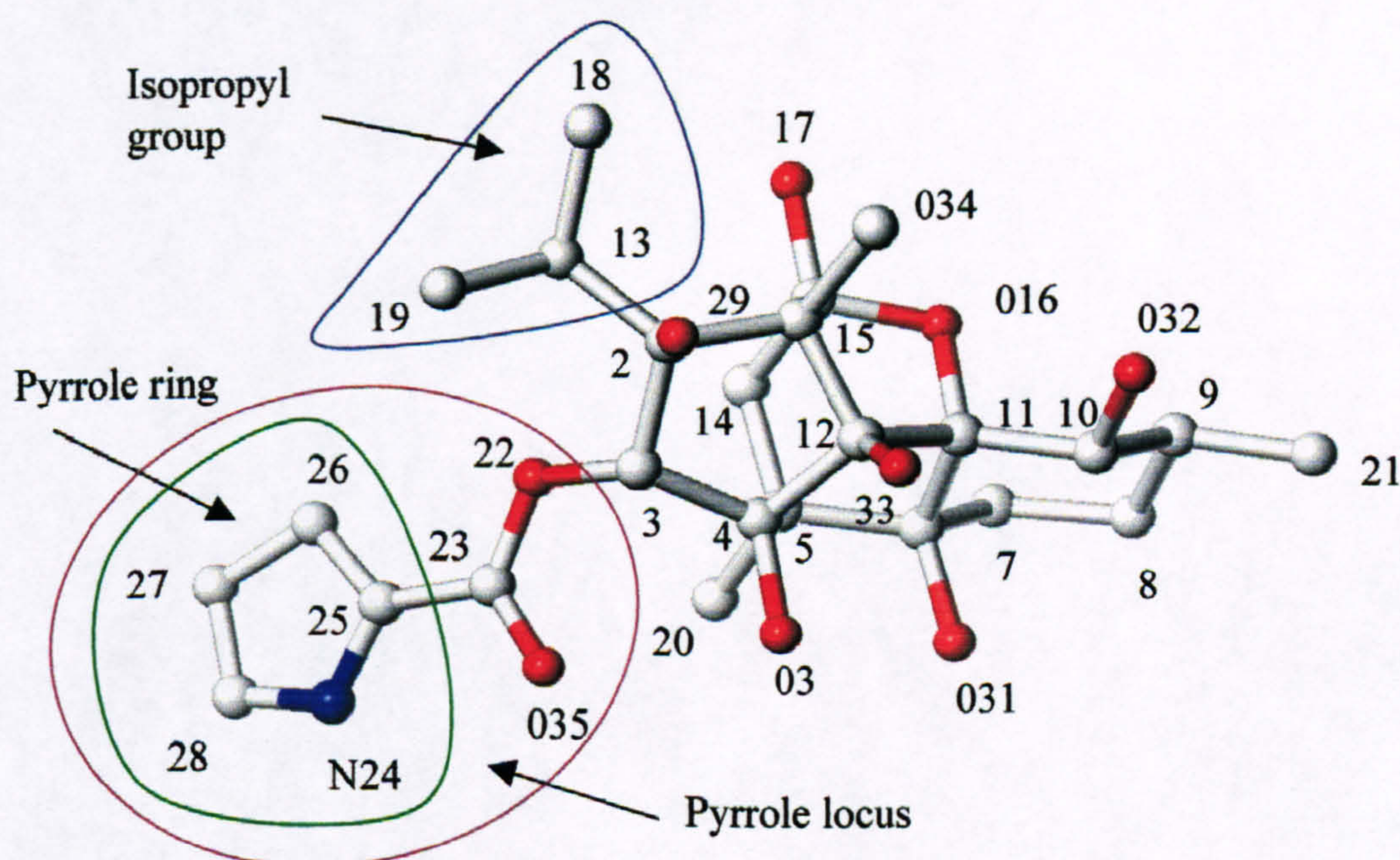


Figure 1.9 The structure of ryanodine with the hydrogen atoms omitted for clarity. The numbering system used for the ryanoids is illustrated. The major groups on the structure are also pointed out.

Initial single channel studies have revealed that the characteristic modification of the RyR channel ion handling and gating by ryanodine is a common property of all the ryanoids. The actions of a large number of ryanoids on single channel function have been investigated and in all cases the interaction of the ryanoid with the channel results

in a dramatic increase in P_o and a reduction in single channel conductance. However, one striking difference is that the amplitude of the ryanoid-induced conductance state is different for each ryanoid, ranging from approximately 0.7 to 0.06 of control conductance with K^+ as the permeant ion (Tinker *et al.*, 1996). The data arising from these experiments have been used to construct a quantitative structure-activity relationship (QSAR), using the principles of comparative molecular field analysis (CoMFA). The CoMFA technique is used to calculate the electrostatic and steric fields at discrete points in the space surrounding each ryanoid. The changes in the steric and electrostatic values at each point is then correlated to measured changes in biological properties of each ryanoid, for example the conductance of the ryanoid-modified state observed in single channel studies. CoMFA predictions have revealed that the amplitude of the ryanoid-induced conductance state correlates strongly to specific loci on the ligand. Change in the electrostatic field (charge) greatly influences the FC than changes in the steric field (molecular size). The presence of steric bulk predominantly at the 2-position (isopropyl position), but also the 4-position correlates with a high FC state, whilst steric bulk at the 10-position is correlated to a decrease in FC. In contrast, increasing positive charge at the 3-position (the pyrrole locus) correlates with an increase in FC, but increasing positive charge at the opposite end of the molecule, at the 10-position, correlates with a decrease in FC. Negative charge proved to have little correlation with FC (Welch *et al.*, 1997).

Ryanoid structure also influences the kinetics of the interaction of the alkaloid with its receptor. In particular the time of residence of the channel in the ryanoid-induced modified conductance state varies. Not all of the ryanoids examined are irreversible like ryanodine, some are reversible on the time scale of a single channel experiment. That is to say in the continued presence of such a ryanoid, a single RyR2 channel alternates between periods of modified conductance and gating, which could last tens of seconds to minutes and periods of unmodified (normal) conductance and gating (Figure 1.10). CoMFA carried out on [^3H]-ryanodine binding experiments to the RyR1 channel demonstrate that increasing negative charge at the 34-position as well as the 32- and 33-position (hydroxyl groups) leads to a marked decrease in ligand binding. However, increasing positive charge at the bay between the 8- and the 21-position, also decreases ligand affinity. Steric bulk can also affect ryanoid binding, increasing steric bulk at the pyrrole, isopropyl locations and position 9 strongly enhances ryanoid binding. In fact

the pyrrole group plays a major role in orientating the ryanoid to its binding site on the RyR channel (Welch *et al.*, 1996) and the region between positions 19 (isopropyl) and 26 (pyrrole) are important for binding. However, steric bulk at a large region extending upwards from position 9 and between the isopropyl group and position 29, inhibit ryanoid binding.

Observation of the interaction of novel ryanoids with single channels provides information on the mechanisms involved in the variation in the affinity with changing ryanoid structure. The interaction of a ryanoid such as ryanodol, with single cardiac RyR channels incorporated into planar phospholipid bilayers, demonstrate exactly these observations. On the time scale of a single channel experiment, ryanodol is a reversible ryanoid (Tinker *et al.*, 1996). The reversibility of this ryanoid is due to the lack of the pyrrole group on this molecule. It could be said that the CoMFA and the biological properties disagree with one another as ryanodol lacks the pyrrole group but can still orientate itself and bind with the RyR channel. However, factors other than the pyrrole group that potentiate ryanoid binding, such as the isopropyl group are still present and so the ryanoid can still bind weakly to the RyR channel. It is proposed that the pyrrole locus (3-position) and the isopropyl group (2-position) interact with the high affinity binding site, while the opposite end of the molecule, such as position 9 and 10 are thought to extend out of the binding site (Welch *et al.*, 1994). This is supported by small changes observed in binding affinity with large increases in steric bulk at these loci. This finding indicates that the loci involved in the modulation of channel ion handling differ from those involved in binding affinity. As a result, ryanoids could be designed to combine a range of attributes so that the ligand can interact reversibly with the RyR channel and either dramatically increase or abolish channel mediated Ca^{2+} -release.

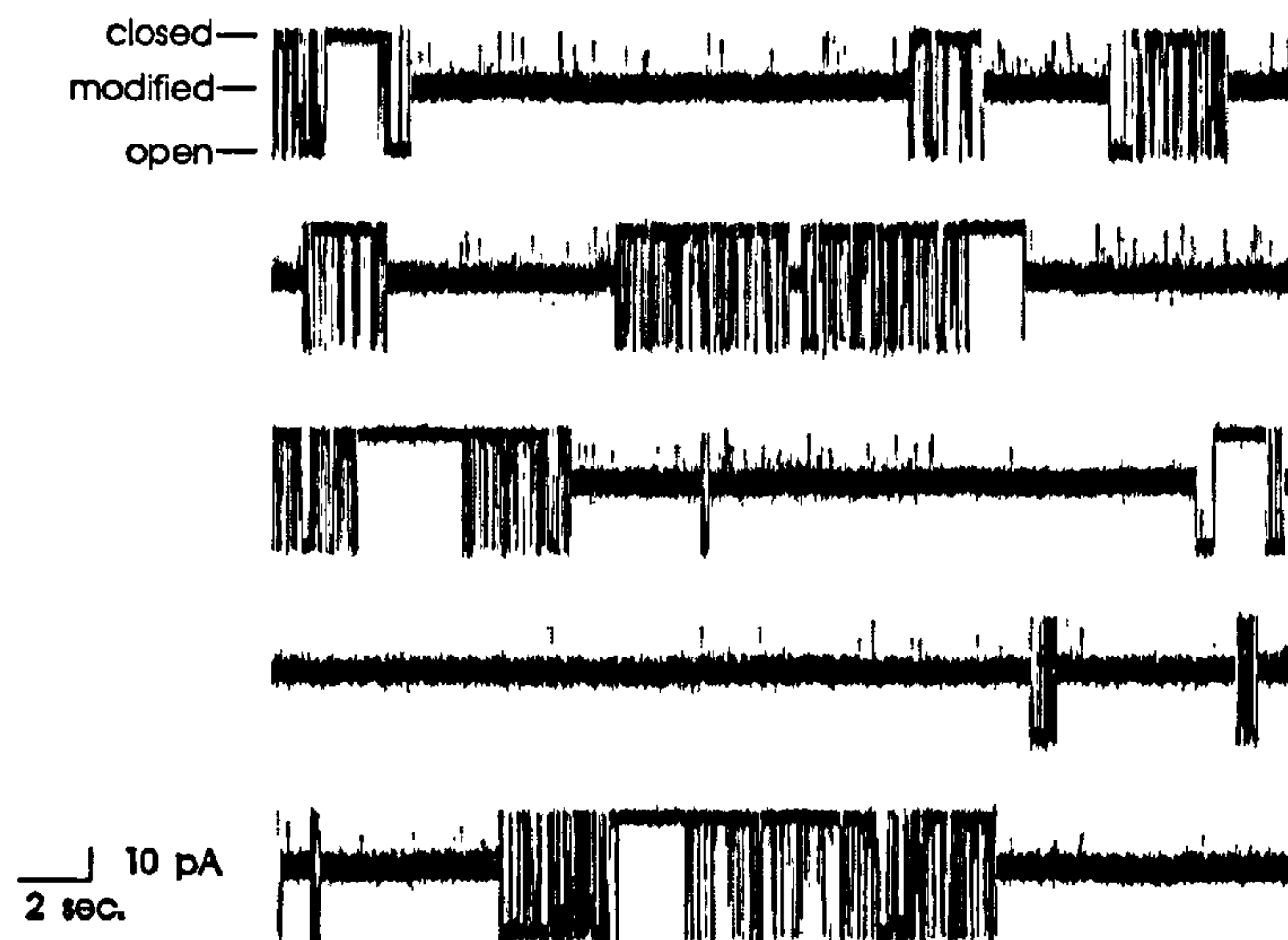


Figure 1.10 Spontaneous transitions of a single sheep cardiac RyR channel from the unmodified (normal channel conductance) to the modified (reduced conductance) conductance state, in the presence of cytosolic 700 nM 21-amino-9 α -hydroxyryanodine. The transmembrane holding potential was held at 40 mV.

1.10.1 The interaction of the reversible ryanoid 21-amino-9 α -hydroxyryanodine with the sheep cardiac RyR channel

The interaction of the reversible synthetic ryanoid, 21-amino-9 α -hydroxyryanodine (which has a net charge of +1) with the sheep cardiac RyR channel induces a characteristic reduced conductance state with a high P_o (Tanna *et al*, 1998), as illustrated in Figure 1.10. Under voltage-clamp conditions, a number of novel features of the interactions of this ryanoid with the RyR channel have been established that could not be obtained from traditional [^{3} H]-ryanodine binding assays. 21-amino-9 α -hydroxyryanodine only acts from the cytosolic face of the channel and increasing ryanoid concentration increases the likelihood of ryanoid interaction with the channel and so the probability of channel modification (P_{mod}) increases. This relationship is described by a simple single-site binding scheme (channel + ryanoid $\rightleftharpoons$ modified channel) (Tanna *et al.*, 1998). Dwell times in both the modified state, that is the time when the ryanoid is bound to the channel and the unmodified state can be described by single exponentials. The rate at which 21-amino-9 α -hydroxyryanodine associates with the channel varies linearly with the ryanoid concentration, however the rate at which 21-amino-9 α -hydroxyryanodine dissociates from the channel is independent of the ryanoid

concentration. These observations are consistent with the mechanism, where the modified state produced by the ryanoid results from the interaction of a single ryanoid molecule with a single RyR channel. Channel P_o also affects the rate of association of the ryanoid to the channel, at zero P_o the association of the ryanoid is effectively zero, as the P_o is raised, the rate of association of the ryanoid with the channel increases linearly. However, the rate at which the ryanoid dissociates from the channel is independent of P_o . This suggests that the ryanoid can only bind to its site when the channel is in an open state. These preliminary observations have also shown that the interaction of 21-amino-9 α -hydroxyryanodine with the RyR is voltage dependent. The association rate increases and the dissociation rate decreases as transmembrane holding potential is made more positive. The influence of transmembrane voltage on the interaction of this charged ryanoid suggests that the ryanoid binding site is located somewhere within the voltage drop across the channel. However, the total voltage dependence (valence) of the interaction of this ryanoid with the RyR channel is greater than 2. As 21-amino-9 α -hydroxyryanodine has a net charge of +1, this suggested either 2 molecules of 21-amino-9 α -hydroxyryanodine interact at a site at the luminal extremity of the voltage drop, or several molecules of 21-amino-9 α -hydroxyryanodine interact at sites near the cytosolic mouth of the voltage drop. However, the interaction of 21-amino-9 α -hydroxyryanodine with the RyR channel was demonstrated as a simple bimolecular reaction (Tanna *et al.*, 1998). It was therefore proposed that most of the voltage dependence of the interaction of 21-amino-9 α -hydroxyryanodine comes from a voltage driven conformational change of the channel protein. While some of it is due to the movement of the charged moiety someway into the voltage drop across the channel (this will be discussed in more detail in Chapter 3).

Chapter 2

Materials and Methods

The vast majority of the experiments described in this thesis involve the observation of single sheep cardiac RyR channel function following the incorporation of individual channels into planar phospholipid bilayers under voltage clamp conditions. This technique will be described in detail below and any further techniques carried in the course of the project will be described in the relevant chapters.

2.1 Isolation of sheep cardiac heavy sarcoplasmic reticulum (HSR) membrane vesicles

Heavy sarcoplasmic reticulum (HSR) membrane vesicles were prepared from sheep cardiac muscle as previously described by Sitsapesan *et al*, (1990). Fresh sheep hearts were collected from an abattoir and transported to the laboratory in ice cold cardioplegic solution containing 102 mM NaCl, 21.4 mM KCl, 16 mM MgCl₂, 28.7 mM Na-lactate, 2.4 mM CaCl₂ and 1 mM procaine. The left ventricle and septum were stripped of fat and connective tissue, blended and further homogenised in a solution containing 300 mM sucrose, 20 mM potassium piperazine-N-N'-bis-2-ethanesulphonic acid (PIPES), 1 mM phenylmethylsulphonylfluoride (PMSF), pH 7.4 with 1 M potassium hydroxide (KOH). The homogenate was centrifuged for 20 minutes at 8,000 r.p.m (10,000g) in a Sorvall GL1500 Superlite rotor at 4°C. The supernatant was then further centrifuged at 36,000 r.p.m (100,000g) in a Sorvall T647.5 rotor for 50 minutes. The resulting pellets, which contained mixed membranes were resuspended in a solution comprised of 400 mM KCl, 0.5 mM MgCl₂, 0.5 mM CaCl₂, 0.5 mM ethylene glycol-bis (-aminoethyl ether)- N, N, N', N'- tetraacetic acid (EGTA), 25 mM PIPES and 10% w/v sucrose, pH 7.0 with 1M KOH. The suspension was layered onto identical salt solutions containing 20, 30 and 40% w/v sucrose and sedimented at 28,000 r.p.m (100,000g) for 2 hours in a Sorvall AH629 rotor at 4°C. The mixed membrane population was separated into different fractions which resulted in an accumulation of sarcolemmal vesicles at the 10 - 20% sucrose interface, the light SR membranes vesicles at the 20 - 30% interface and

HSR membrane vesicles accumulated at the 30 - 40% interface. The HSR was collected and resuspended in 400 mM KCl, pH 7.0 with 1 M KOH before centrifugation at 36,000 r.p.m. (100,000g) for 50 minutes in a Sorvall T647.5 rotor at 4°C. The HSR membrane pellet were resuspended in a solution containing 400 mM sucrose, 5 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES), pH 7.2 with hydroxymethyl-methylamine (Tris) and then snap frozen in liquid nitrogen and stored at -80°C. All the solutions were made using deionised water, filtered (0.45 µm nitrocellulose filters, Millipore (U.K.) Ltd. Watford, Hertfordshire) and kept at +4°C.

2.2 Protein assay

The protein concentration of the HSR preparation was measured using a Bio-Rad reagent, which consisted of an acidic solution of Coomassie Brilliant Blue G-250. This reagent differentially changes colour from an orange-red to a blue in response to various concentrations of protein (Bradford, 1976), this blue species has a maximum absorbance at 595 nm. Protein standards were made up in triplicate using bovine serum albumin (BSA)(Bio-Rad) to concentrations of 20, 40, 60, 80 and 100 mg/ml in 5 ml diluted reagent (1 in 5 dilution of reagent to deionised water). The solutions were gently mixed and left to stand at room temperature for approximately 5 minutes before measuring the absorbance at 595 nm in a spectrophotometer. A plot of the absorbance against the protein concentration of the standards was determined, in the form of a linear relationship. To determine the protein concentration of the HSR preparation, a sample of the HSR (20 µl of a 1 in 10 dilution of HSR to deionised water) was mixed with 5 ml of the reagent as described for the standards above. The protein concentration corresponding to the absorbance value observed for the sample was obtained from the linear standard curve.

2.3 Solubilisation and purification of the ryanodine receptor

RyR channels were solubilised and purified from the HSR membrane vesicles using the method described by Lindsay and Williams (1991). HSR membrane vesicles were diluted to approximately 2mg protein/ml with solubilisation reagent containing 1 M NaCl, 0.1 mM EGTA, 0.15 mM CaCl₂, 25 mM PIPES, pH 7.4 with NaOH in the presence of 0.4% (w/v) of the detergent 3-[(3-cholamidopropyl)-dimethylammonio]-1-

propane sulfonate (CHAPS) and 2 mg/ml L- α -phosphatidylcholine (PC). The sample was divided into two, one fraction was labeled with 20 μ l of [^{3} H]-ryanodine while the other fraction was left in the absence of [^{3} H]-ryanodine before incubation for an hour on ice for solubilisation. At the end of this period, unsolubilised material was sedimented by centrifugation of the samples at 36,000 r.p.m. (100,000g) for 45 minutes in a Sorvall T1270 rotor at 4°C. The supernatants from both the labeled and the non labeled samples containing the solubilised receptor were then carefully placed on top of identical 5-25% (w/v) linear sucrose gradients with a 5 ml 40% sucrose cushion. The sucrose solutions were made up in the solubilisation buffer containing 0.4% CHAPS and 2 mg/ml (PC) to maintain solubility of the protein. The continuous gradient was formed by the gradual mixing of 5 and 25% sucrose using a gradient former (MSE Sanyo Gallenkamp, Loughborough, UK) and peristaltic pump (Masterflex, Cole Parmer Instrument Co., Chicago, IL, USA). The gradients and supernatant were centrifuged at 28,000 r.p.m. (100,000g) for 16-20 hours at 4°C using a swing out Sorvall AH629 rotor.

After centrifugation 2 ml fractions were drawn from the bottom of the tubes using a commercially available tube piercer (MSE, Fisons Instruments) a peristaltic pump and pump controller (built by R.A.P. Montgomery, National Heart and Lung Institute, Imperial College, UK). The fractions containing the unbound receptor were stored on ice whilst 500 μ l of each fraction from the pre-labeled sample were placed in 10 ml of scintillation fluid (UltimaGold MV, Packard, Netherlands) before counting. A peak usually in fraction 3 of the gradient identified the location of RyR protein. 1 ml of the fraction corresponding to the peak was taken from the equivalent unlabeled sample and added to 4 mls of the solubilisation buffer containing 0.4% CHAPS and 2mg/ml PC. The total 5 ml of fraction plus solubilisation reagent was placed in dialysis membrane tubing with a molecular weight cut off of 1000 daltons (Spectra-Pore 6, Spectrum, Perbio Science Ltd., Chester, UK), and dialysed against 5 litres of dialysis buffer containing 0.1 M NaCl, 0.1 mM EGTA, 0.15 mM CaCl₂ and 25 mM PIPES at pH 7.4 with 1 M NaOH for 5 hours at 4°C. During this period detergent was removed from the mixture of protein, lipid and detergent and the purified RyR channel reconstituted into unilamellar liposomes. The reconstituted receptor was then removed from the dialysis tubing and added to an equal volume of 0.5 M sucrose and allowed to stand on ice for 1

hour to allow the vesicles to equilibrate with the sucrose concentration in the solution. The fraction was then aliquoted into 50 µl samples, before snap freezing in liquid nitrogen and storage at -80°C. The sample was now ready for incorporation into planar phospholipid bilayers as described previously (Lindsay and Williams. 1991).

2.4 Phospholipid bilayer technique

To observe single channel gating and ion handling properties RyR channels were incorporated into artificial planar phospholipid bilayers, formed by spreading phospholipid dispersions across a hole that connects two fluid filled chambers as described by Miller (1978).

2.4.1 Bilayer block and cup

The basic bilayer set up consists of two chambers. A styrene copolymer cup, with a cylindrical cavity cut close to one edge giving rise to a thin wall or septum. A 200 µm diameter hole on which bilayers are formed is drilled through the septum, as shown in Figure 2.1. The cup slots into a block made of the same material, so that the hole faces a rectangular chamber in the block, which is referred to as the *trans* chamber. The cylindrical cavity in the cup is termed the *cis* chamber, it is on the *cis* side that bilayers are formed. The front of the block is glass and provides an unobstructed view of the hole once it is positioned in the block. Two smaller cylindrical wells are also drilled at one edge of the block to accommodate an electrolyte solution and provide a means of coupling the *cis* and the *trans* chambers to the head amplifier (discussed below).

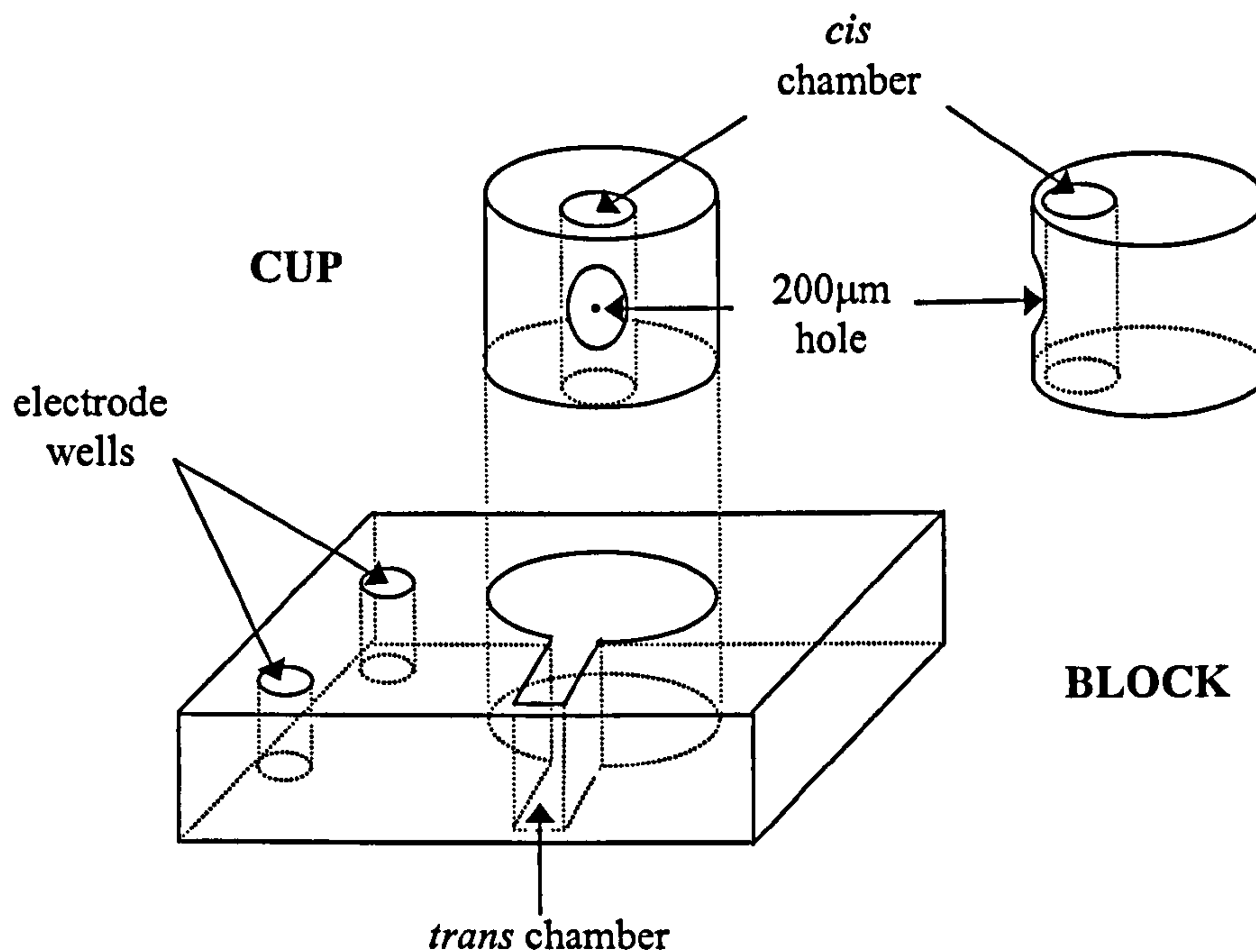


Figure 2.1 A Schematic representation of the block and cup. The cup has a cylindrical cavity termed the *cis* chamber that has a 200 µm diameter hole in the septum. The cup is placed into the block, so that the hole faces the *trans* chamber and connects both chambers. The block also contains two smaller cylindrical wells at the left edge, which accommodate the agar salt bridges and the Ag/AgCl electrodes.

2.4.2 Electrical equipment

The general arrangement of the electrical equipment designed and built by R. A. P. Montgomery (National Heart and Lung Institute, Imperial College, UK) is shown in Figure 2.2. The block and cup were placed on a lead block to reduce mechanical noise. A small magnet stirrer was incorporated into the lead block and by placing the cup and block over the rotating magnet stirrer and adding small magnets into both the *cis* and the *trans* chambers, it was possible to stir the solutions in both compartments. This in turn was set upon an air table to further reduce any mechanical vibrations. The block, cup and the head amplifier mounted on the lead block were enclosed in an insulating aluminum box (Faraday cage), which was closed to reduce electrical noise.

The solutions in the *cis* and the *trans* chamber are separated by the formation of a bilayer (described later in detail) across the 200 µm hole that connects both sides. The solutions in the *cis* and the *trans* chambers are connected to two small wells filled with

3 M lithium chloride (LiCl) by bridges made from glass capillary tubes molded with the aid of a Bunsen burner into a U shape filled with 2% w/v agar dissolved in 3 M LiCl. Silver-silver chloride (Ag/AgCl) electrodes maintain the electrical connection between the wells and the head amplifier. The electrodes were made from silver wire coated with silver chloride by immersion in household bleach.

The head amplifier is an operational amplifier, which is configured as a current-voltage converter. This can voltage clamp the bilayer at a desired holding potential and transform the current flowing through the membrane into an voltage signal. The total membrane current (I_m) of a bilayer containing ion channels arises from the sum of two components, the current due to the movement of ions through the ion channel (I_{ionic}) and the current through the membrane capacitor (C_m) due to the change of charge from one side of the membrane with the other. Therefore, the total membrane current is defined as:

$$I_m = I_{ionic} + C_m \frac{dv}{dt} \quad \text{equation 2.1}$$

During a single channel experiment, the *trans* chamber is always held at ground, while a voltage is applied to the *cis* chamber by means of an external input to the voltage clamp system of the head amplifier. The voltage clamp system employs a negative feed back loop that holds the membrane potential constant to the desired level while the current flowing through the membrane is measured. As the membrane potential is held at a constant value, dv/dt is effectively zero (Eq.2.1) and therefore the measured current would be entirely ionic.

Single channel current is in the picoampere range, to enable us to observe such small current, the measured current flowing through the channel was re-routed through either a 1 or 10 gigohm feedback resistor. The feedback resistors have a parasitic capacitance associated with them. This forms a low pass filter that causes a delay in the response time of the circuit. The rise time of the 10 gigohm feedback resistor to reach 90% of its final value is approximately 200 μ s, while the rise time of the 1 gigohm feedback

resistor is approximately 50 μ s. Thus, they are referred to as the ‘slow’ and ‘fast’ head amplifiers respectively. A compromise is made when using either the slow or the fast head amplifier, the slow head amplifier gives a 10 times gain in the signal output and enables us to view small single channel currents, compared to the fast head amplifier. However, the longer rise time of the slow head amplifier filters out high frequency noise and therefore very brief single channel events are missed. The fast head amplifier resolves fast events but is noisier. The output voltage of the head amplifier is:

$$V_o = -I.R_f + V_c \quad \text{equation 2.2}$$

where V_o and V_c is the output and clamped voltage of the head amplifier respectively, I is the single channel current and R_f is the feedback resistor. In all the experiments carried out in the project a ‘slow’ head amplifier was used, to obtain a clear measurement of the reduced conductance state produced by the interaction of a ryanoid with the RyR channel.

The voltage signal is then passed through a second stage differential amplifier of unitary gain, which subtracts the clamp-voltage (V_c), so that the output of the differential amplifier (V) is:

$$V = -I.R_f \quad \text{equation 2.3}$$

The output is conveyed to an 8-pole Bessel filter (Frequency Devices Inc. Haverhill, MA, USA) before displaying it on an oscilloscope (Gould Instrument Systems, Ilford, Essex, UK). The unfiltered analogue voltage signal was recorded/stored on a Digital Audio Tape (DAT) through a DAT recorder (Intracel, Cambridge, UK).

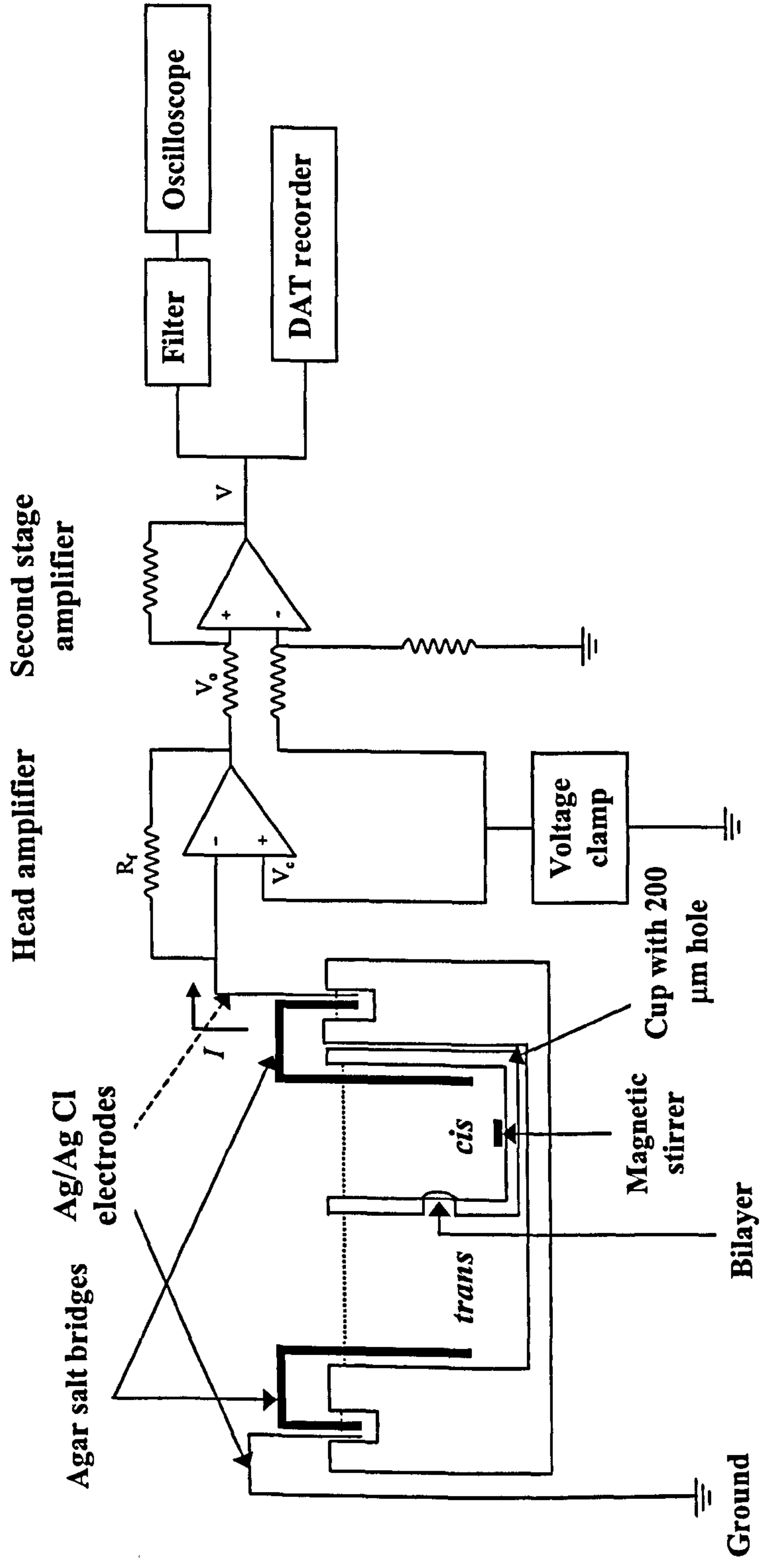


Figure 2.2 A simplified schematic representation of the electronics of the bilayer setup.

2.4.3 Phospholipid

The phospholipid used to form artificial planar bilayers is purified bovine heart phosphatidylethanolamine (PE) (Avanti Polar Lipids, Alabaster, AL, USA). Lipid was stored as a stock solution dissolved in chloroform at a concentration of 50 mg/ml at -80°C. When PE was needed for experiments, aliquots were taken from the stock solution and the chloroform was evaporated under a steady stream of nitrogen prior to addition of a non-polar solvent *n*-decane, to give a final concentration of 35 mg/ml.

2.4.4 Painting bilayers

Before the bilayer can be formed across the hole that connects the *cis* and the *trans* chambers, it is necessary that the surface of the cup around the aperture on the *cis* side is 'primed' with a small amount of the phospholipid and dried with a stream of nitrogen. The phospholipid is spread on using the back of a 'painting stick' made from a plastic pasteur pipette, modified so that the end of the pipette forms a point as shown in Figure 2.3. Once the cup is 'primed' it is placed into the block so that the hole faces the *trans* chamber and then both chambers are filled with the test ionic solution, the *trans* chamber holds 1ml and the *cis* chamber holds 0.5 ml of the solution. In all cases, experiments were conducted in 600 mM KCl, buffered with 20 mM HEPES, in the presence of contaminant 10 μ M Ca^{2+} , pH 7.4 with 1 M KOH, resulting in a solution containing 610 mM K^+ at room temperature. Lipid bilayers were then formed from a suspension of PE drawn across the 200 μ m diameter hole from within the *cis* chamber using the point of the painting stick. The initial priming of the cup provides a hydrophobic environment on the surface of the wall for a second application of PE for the formation of the bilayer. The lipid film formed across the hole would initially be several micrometers thick and will be in equilibrium with a much larger annulus or torus of dispersion formed at the border of the hole. The thick film thins spontaneously to form a bilayer. Initially the thinning process is driven by the Plateau-Gibbs border suction, so that the lipid is drawn towards the border causing the film to thin. As the film thins, London-van der Waals attraction between the aqueous phases on either side of the film provides an additional force for the final thinning process.



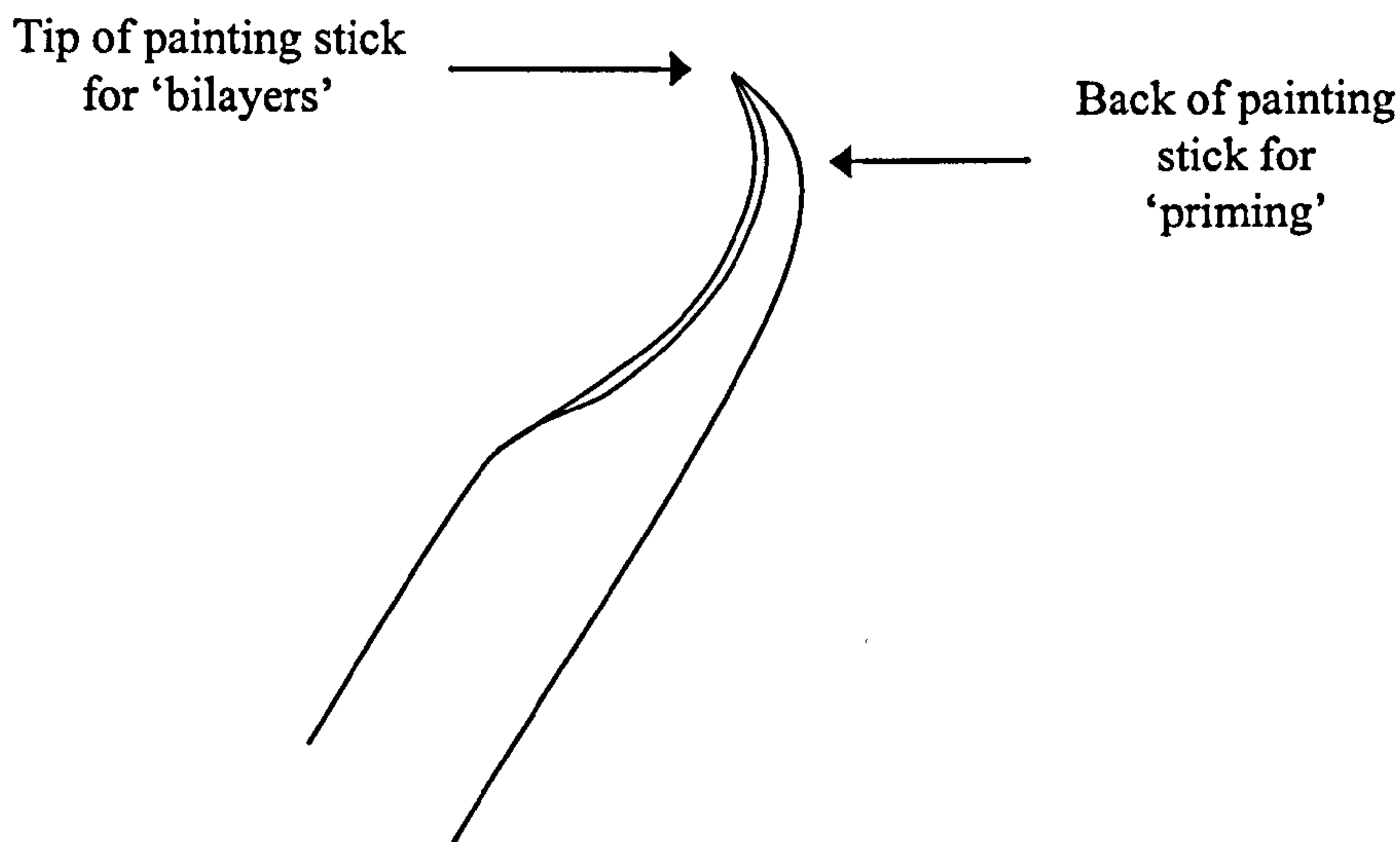


Figure 2.3 A cartoon of the painting stick used to paint planar phospholipid bilayers

The formation of the bilayer could be observed by viewing light reflected from its surface. As the film thins, less light is reflected back from the lipid surface, until no further light reflection is observed and the layer of film becomes completely 'black', at this point the film is a bilayer. Although, this method of assessing the formation of a bilayer is valid, it is very difficult to observe and so generally the thickness of the film across the hole is monitored by measuring the membrane capacitance. Prior to the application of the lipid across the hole, the electrolyte solutions in both the *cis* and *trans* chambers are electrically coupled through the hole, application of a square wave pulse of ± 4 mV to the system produces saturating fluctuations in current. When the hole is covered with a layer of lipid, current flow between the two solutions in the *cis* and the *trans* chamber is prevented, and the film of lipid between the two ionic solutions effectively creates a capacitor. The application of the oscillating voltage results in the occurrence of small deflections (capacitance spike) from zero current with each transition of the voltage clamp, as charge moves from one side of the polar head of the lipid membrane to the other. When the film of lipid is thick, the amplitude of the capacitance spike is small. As the film thins with time, the amplitude of the capacitance spike increases. Although, thinning of the lipid film occurs spontaneously, more often than not, the thinning process is aided with the removal of excess lipid with a clean painting stick until the capacitance spike is sufficiently large enough to indicate the formation of a bilayer. The assessment of the formation of a bilayer in this way relies on

user experience as the capacitance spike varies with different conditions and equipment used, for example changes in head amplifiers. There are times when a sufficient amount of lipid has not been applied across the hole and an incomplete seal is formed across the hole, this results in a deviation in the zero current base line when the input oscillating voltage switches which is known as a 'leak', in this case more lipid is drawn across the hole, until a bilayer is formed.

2.5 Incorporation of purified RyR channels into the bilayer

Purified channel proteins incorporate into bilayers if the membrane vesicles are induced to fuse with the bilayers. Figure 2.4 shows the process of single channel incorporation into bilayers. The purified RyR channels, reconstituted into membrane vesicles, were added to the solution in the *cis* chamber (1-5 μ l). Fusion of the membrane vesicles with the bilayer was encouraged by making the solution in the *cis* chamber hyperosmotic with respect to the solution in the *trans* chamber; this was achieved by the addition of 100-200 μ l of 3 M KCl to the *cis* chamber. Under these conditions there is a net movement of water across the bilayer from *trans* to *cis* chamber. The contents of the *cis* chamber were vigorously stirred to increase the chances of delivering vesicles to the bilayer. Vesicles adhere to the bilayer and swell as water enters the lumen, the rise in the intravesicular hydrostatic pressure causes the vesicles to rupture at the region of contact with the bilayer, this results in the fusion of the membrane vesicle (and so the channel) to the bilayer, the process takes a few minutes. Under these asymmetric conditions the bilayer was held at 0 mV and the occurrence of channel incorporation was monitored by the current fluctuations observed on the oscilloscope, resulting from the movement of K^+ ions down the electro-chemical gradient, from the *cis* to the *trans* chamber. After channel incorporation, further fusion was prevented by perfusion of the *cis* chamber with 610 mM KCl test solution, using a peristaltic pump (Shuco International, London, UK). RyR Channel proteins incorporate into the bilayer in a fixed orientation, so that the cytosolic face of the channel is exposed to the solution in the *cis* chamber and the luminal face of the channel is exposed to the solution in the *trans* chamber (Lindsay and Williams. 1991).

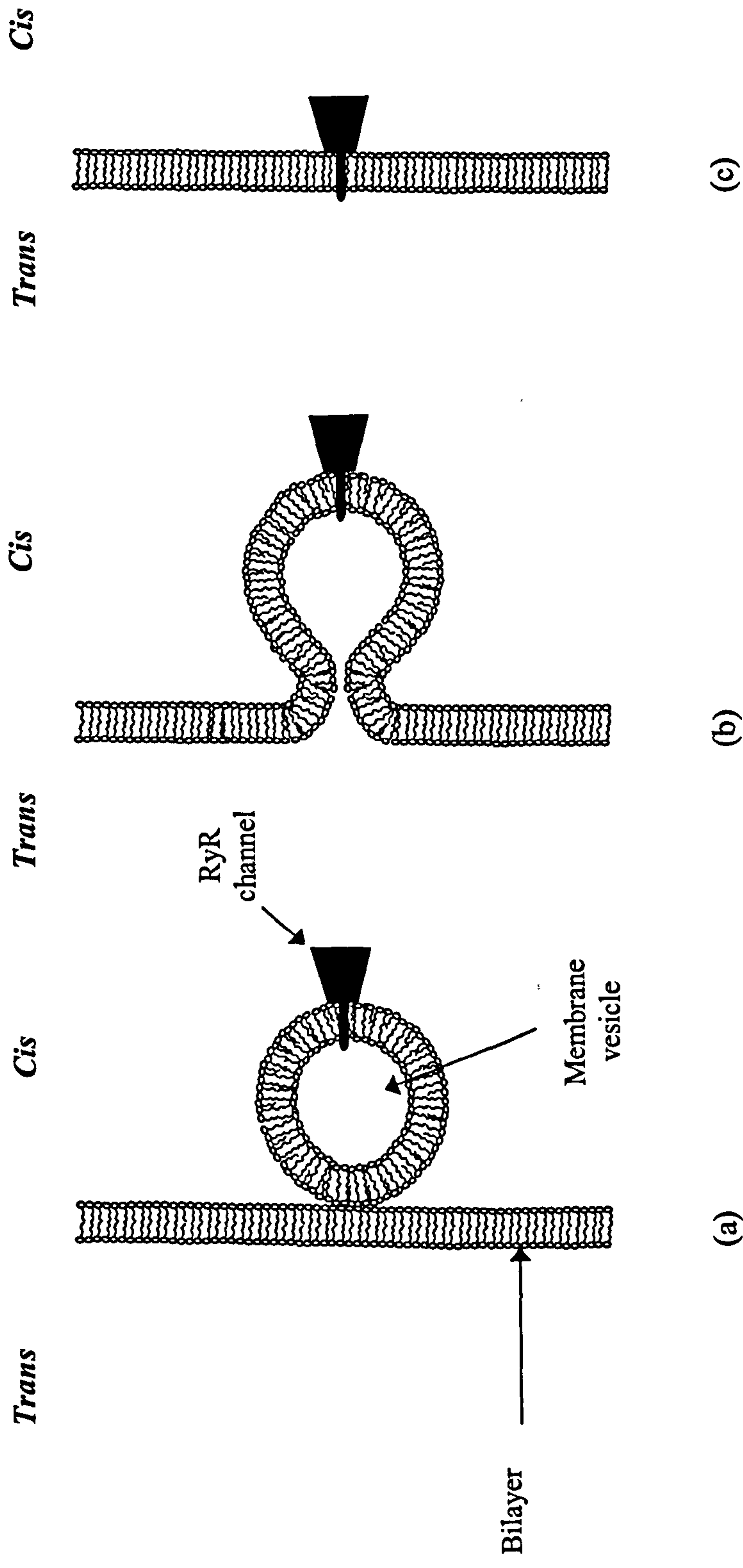


Figure 2.4 A cartoon to illustrate the fusion process that governs the incorporation of single RyR channels into a planar lipid bilayer. Membrane vesicles and salts to initiate fusion are added to the *cis* chamber. The membrane vesicles adhere to the bilayer (a), while the hyperosmotic environment draws water into the *cis* chamber and moves into the lumen of the membrane vesicles. Consequently the membrane vesicles rupture and fuse at the point of contact with the bilayer (b). The membrane vesicle and the ion channel become part of the bilayer so that the cytosolic face of the channel faces the *cis* chamber and the lumen of the channel faces the *trans* chamber.

2.6 Activation of the channel

Single channel experiments were conducted in symmetrical 610 mM KCl solution. K^+ was used as the permeant ion, because of the large conductance observed with this monovalent through RyR channels and the impermeant Cl^- ion was used as this ion has been shown to increase single channel open probability (P_o) (Lui *et al.*, 1998). 10 μ M contaminant Ca^{2+} is also present in the solution, but at this level of Ca^{2+} , single channel P_o is low. Single channel P_o was increased by the addition of 100 μ M of the synthetic caffeine analogue EMD 41000 (McGarry and Williams, 1994; Tanna *et al.*, 1998), (given to us by Merck) (Darmstadt, Germany) to the cytosolic face of the channel. With this combination of ligands, single channel P_o was increased significantly (Tanna *et al.*, 1998) and under these conditions it was immediately apparent if more than one channel was present in the bilayer. Only bilayers containing a single channel were used in the experiments described in this project. Experiments were carried out at room temperature ($21 \pm 2^\circ\text{C}$).

2.7 Ryanoids

The ryanoids used in this project were synthesised and supplied by Professor Luc Ruest (Department of Chemistry, University of Sherbrooke, Sherbrooke, Quebec, Canada) and stored as stock solutions in 50% ethanol at -20°C .

2.8 Single channel data acquisition

Single channel current fluctuations were displayed on an oscilloscope and stored on DAT tape. For analysis, data were filtered at 1kHz through a low-pass Bessel filter to remove high frequency noise. The filtered analogue voltage signal was digitised at 4 kHz with an analogue-to-digital converter (S200 board, Intracel, Cambridge, UK) before storage on a computer hard drive for analysis. Analysis of single channel data were carried out using a commercially available PC-based computer analysis program called Satori V3.2 (Intracel, Cambridge, UK). The analysis program is capable of measuring several parameters of single channel activity, including current amplitude, dwell times in different states, and channel open probability. The representative traces shown in the figures were obtained from digitised data acquired with Satori V3.2 and transferred as

an HPGL graphics file to a graphics software package (Corel Draw; Corel Systems Corporation, Ottawa, Ontario, Canada) for annotation and printing.

2.9 Monitoring the interaction of ryanoids with single RyR channels

The interaction of ryanoids with the RyR channel only occurs from the cytosolic face of the channel (Tanna *et al.*, 1998), therefore, in all single channel experiments carried out in this project, ryanoids were added to the cytosolic face of the channel/*cis* chamber.

The interaction of ryanoids, so far examined, with the high affinity ryanodine binding site on the sheep cardiac RyR channel, induce a modification of channel function. Channel conductance is reduced and channel P_o increases. The interaction of some ryanoids such as ryanodine with the channel is irreversible on the time scale of a single channel experiment, whilst the interaction of other ryanoids for example 21-amino-9 α -hydroxyryanodine with the channel is reversible. In the continued presence a reversible ryanoid, transitions between periods of modified conductance and periods of unmodified (normal channel gating) are observed. Perfusion of a reversible ryanoid from the *cis* chamber with fresh 610 mM KCl solution, results in the occurrence of normal single channel gating from the closed to the open level, with no observable modifications.

2.10 Measurements of single channel current amplitudes and fractional conductance

Single channel current amplitudes were measured by placing cursors at the levels corresponding to the center of the noise for both the closed and the open conductance states, as shown in figure 2.5a. The computer analysis program then calculates the difference between the two states. The fractional conductance (FC) is the amplitude of the ryanoid-induced subconductance state expressed as a proportion of the normal full amplitude of the channel (Lindsay *et al.*, 1994; Tinker *et al.*, 1996). Again the current amplitudes of the ryanoid modified states were measured by placing cursors at levels corresponding to the center of the noise of the closed and the modified state, as shown in Figure 2.5b.

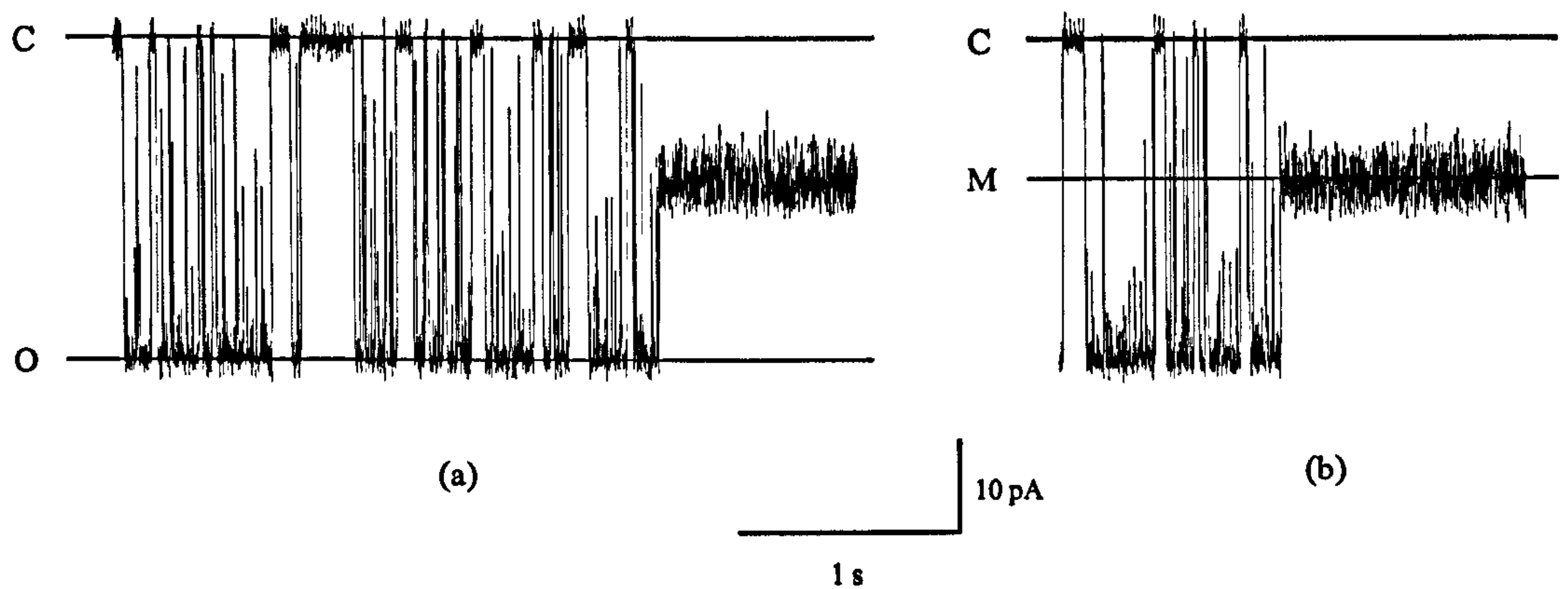


Figure 2.5 Measurement of single channel current amplitude (a) and fractional conductance (b).

2.11 Probability of single channel modification (P_{mod}) and the rate of association and dissociation

Dwell times in both the unmodified and the modified conductance state in the presence of a reversible ryanoid, were determined by using Satori V3.2 as described in Tanna *et al.*, (1998). Sections of the data were defined as the unmodified state (periods where the channel displayed transitions between the open and the closed levels) or the modified state (periods in which the channel displayed transitions between the modified and the closed levels), shown schematically in Figure 2.6. P_{mod} was calculated from the dwell times in the unmodified and modified events as:

$$P_{\text{mod}} = \frac{\text{total time in modified state}}{(\text{total time in modified state} + \text{total time in unmodified state})} \quad \text{equation 2.4}$$

In all experiments reported here P_{mod} was determined from steady state runs lasting at least 6 minutes.

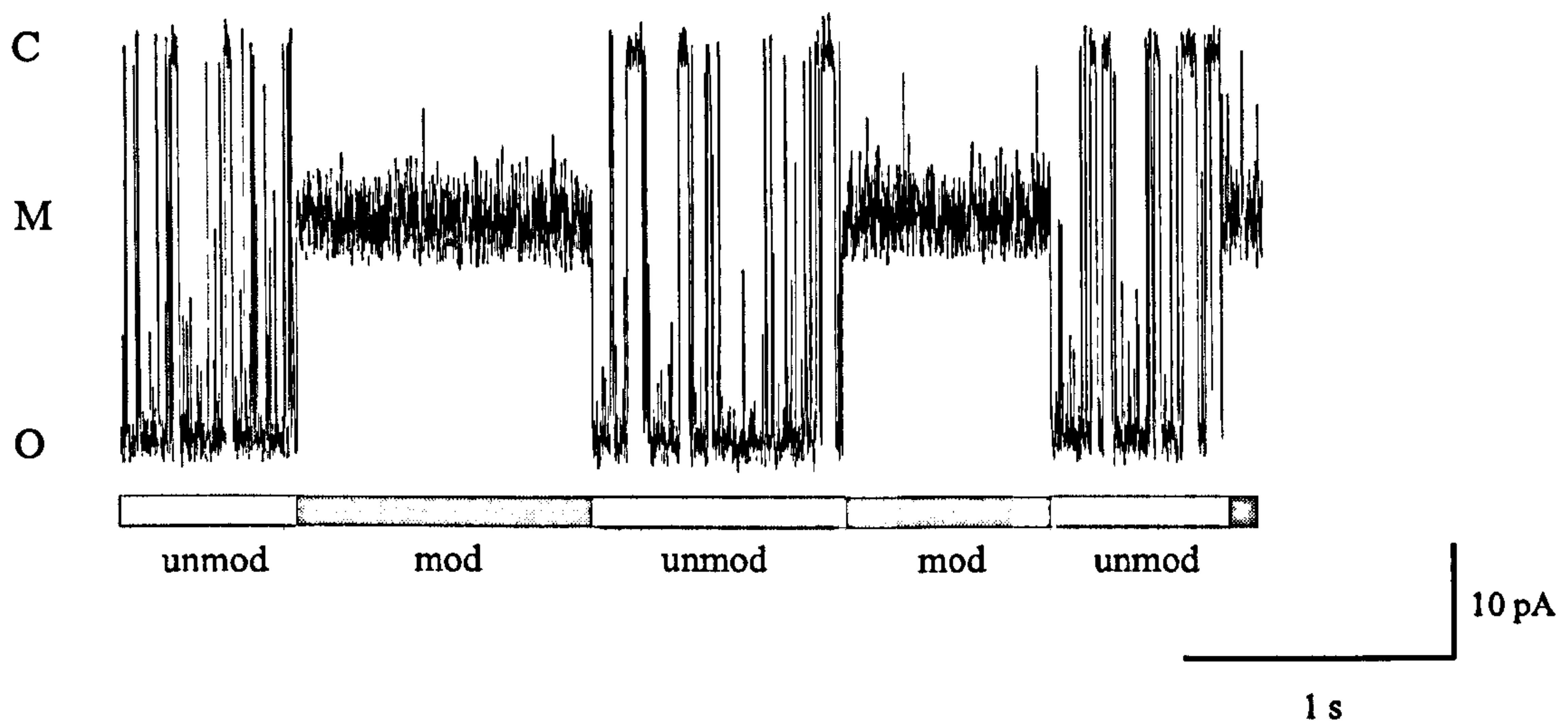


Figure 2.6 Trace of the interaction of a reversible ryanoid (21-amino-9 α -hydroxyryanodine) with the RyR channel. On binding the ryanoid induces a modified conductance state which has a high P_o . With the ryanoid bound, the channel can close but cannot enter the normal open state. When the ryanoid dissociates from its site, channel gating and ion handling return to normal. Dwell times in the unmodified and modified states were monitored by placing a cursor at the beginning of a state (modified or unmodified) and dragging the cursor to the end of the state, the computer automatically calculated the time difference.

The interaction of 21-amino-9 α -hydroxyryanodine with the RyR channel and the resulting modification of channel function are described by a simple bimolecular reaction (Tanna *et al.*, 1998) as described in the introduction. The distribution of dwell times of the RyR channel in the ryanoid modified and unmodified gating states are described by single exponentials. P_{mod} is described by Michaelis-Menten kinetics; that is P_{mod} saturates with respect to ryanoid concentration. The rate of association is dependent on ryanoid concentration, while the rate of dissociation is independent of ryanoid concentration. This is consistent with observations from [^3H]-ryanodine binding studies that the RyR tetramer possesses a single high affinity ryanodine binding site (Valdivia *et al.*, 1995). As predicted for a simple bimolecular reaction scheme, the apparent rate constants for the association (k_{on}) and dissociation (k_{off}) of a ryanoid to and from the channel can be determined from the reciprocal of the mean dwell times in the unmodified and the modified conductance states (Equation 2.5 and 2.6):

$$k_{on} = (\tau_{unmod})^{-1} \quad \text{equation 2.5}$$

and

$$k_{off} = (\tau_{mod})^{-1} \quad \text{equation 2.6}$$

2.12 The influence of transmembrane voltage on the interaction of ryanoids with the RyR channel

The interaction of a reversible ryanoid with the RyR channel and hence the transition between the modified and the unmodified states is dependent on transmembrane holding potential. For such a relationship, the rate constants at a given voltage will be described as follows:

$$k_{on}(V) = k_{on}(0) \bullet \exp^{[z_{on} \bullet (FV/RT)]} \quad \text{equation 2.7}$$

and

$$k_{off}(V) = k_{off}(0) \bullet \exp^{[-z_{off} \bullet (FV/RT)]} \quad \text{equation 2.8}$$

where $k(V)$ and $k(0)$ are the rate constants at a particular voltage and at 0mV respectively, z is the valence of the appropriate reaction, F is the Faraday constant, V is the transmembrane voltage, R is the gas constant, T is temperature ($^{\circ}\text{K}$). Plots of the natural logarithm of k_{on} and k_{off} against holding potential should be linear with slopes $z_{on}F/RT$ and $-z_{off}F/RT$ and intercepts $\text{Ln}[k_{on}(0)]$ and $\text{Ln}[k_{off}(0)]$, respectively. The total voltage dependence (z_{total}) of the reaction is then $z_{on} + z_{off}$.

Analysis of the rate of association and dissociation of the ryanoid to and from the channel was used to ascertain variation of the dissociation constant (K_d) with changing transmembrane holding potential. K_d was calculated from:

$$K_d = \frac{k_{off} (s^{-1})}{k_{on} (\mu M^{-1} \cdot s^{-1})} \quad \text{equation 2.9}$$

2.13 The influence of voltage on block

The reduction in single channel current amplitude observed with the charged blocker TEA at any particular voltage (described in Chapter 5) can be assessed by the model first proposed by Woodhull (1973). The model applies to a charged blocker of valence z , which has access to a site from only one side of the channel and interacts with a single site lying a fraction δ across the voltage drop. The expression of the relative conductance, i.e. the ratio of the conductance with (i) and without the blocker (i_o) at a particular voltage is:

$$i / i_o = \frac{1}{1 + \frac{[B]}{K_{b(0)}} \exp^{(z\delta FV/RT)}} \quad \text{equation 2.10}$$

where $[B]$ is the blocker concentration, $K_{b(0)}$ is the dissociation constant of the blocking cation at 0 mV, $z\delta$ is referred to as the effective valence of the blocker and F , R and T have their usual meanings (as mentioned above).

2.14 Measurement of single channel open probability (P_o)

The dissociation rate of 21-amino-9 α -hydroxyryanodine (Tanna *et al.*, 1998) and all other ryanoids investigated in this project from the RyR channel are independent of channel P_o . However, the rates of association of 21-amino-9 α -hydroxyryanodine (Tanna *et al.*, 1998) and other ryanoids with the RyR channel, are directly proportional to channel P_o . It was therefore necessary to measure channel P_o in all experiments. This was done by monitoring this parameter in the sections of the recorded data during which no ryanoid was bound; i.e. with transitions only between the open and closed conductance levels. P_o was determined by manually placing the cursors at the open and closed channel levels on the single channel record, displayed as digitised data on the computer (as shown in Figure 2.5a for the measurement of single channel current amplitude) and the computer analysis program automatically calculates a point halfway between the two levels, the 50% threshold analysis screen (Sitsapesan and Williams, 1994). The channel P_o was then determined according to the equation below:

$$P_o = \frac{\text{total time in open state}}{\text{total time in open state} + \text{total time in closed state}} \quad \text{equation 2.11}$$

The kinetic parameters determined for each ryanoid tested in this project were compared with equivalent parameters determined for 21-amino-9 α -hydroxyryanodine monitored in earlier investigations (Tanna *et al.*, 1998). To eliminate variation in k_{on} arising from differences in P_o between individual channels, k_{on} values quoted for all ryanoids in this report have been normalised to a P_o of 1.0.

Chapter 3

The influence of net charge of the ryanoid

3.1 Introduction

In Chapter 1, a number of factors governing the interaction of the reversible ryanoid 21-amino-9 α -hydroxyryanodine with the high affinity ryanodine binding site on single RyR channels were reported. The interaction is described as a simple bimolecular association (Tanna *et al*, 1998), the binding site is only accessible from the cytosolic face of the channel and the site is only available when the channel is in the open conformation. This suggests that the ryanoid binding site may be located within the conduction pathway. Alternatively, the site could be located somewhere else on the protein and is only available when the channel is open. It is assumed that upon the interaction of a ryanoid with its binding site on the channel, a conformational change in the channel protein is induced, which results in the observed altered conductance state.

Preliminary observations have also shown that the interaction of 21-amino-9 α -hydroxyryanodine with the RyR is voltage dependent (Tanna *et al*, 1998). The P_{mod} by the ryanoid is high at high positive holding potentials and low at high negative potentials. This reflects the rate of association of the ryanoid to the channel and the dissociation of the ryanoid from the channel. The rate of ryanoid association to the channel increases as transmembrane holding potential is taken to high positive voltages, while the rate of ryanoid dissociation from the channel decreases. Therefore, as transmembrane holding potential increases to high positive voltages the ryanoid binds to the channel faster and stays on for longer. This observed voltage dependence of P_{mod} of the RyR channel by 21-amino-9 α -hydroxyryanodine gives a z_{total} value obtained from the best fit Boltzmann distribution and from variations in k_{on} and k_{off} of 2. As 21-amino-9 α -hydroxyryanodine has a net charge of +1, if all the observed voltage dependence occurs from translocation of the ryanoid into the voltage drop to interact with the high affinity ryanodine binding site, this would mean two molecules of 21-amino-9 α -hydroxyryanodine would need to translocate across the entire voltage drop across the channel. As the binding site is only accessible from the cytosolic face of the channel, this would place the site at the luminal extreme of the voltage drop. Alternatively, several molecules of 21-amino-9 α -hydroxyryanodine could interact with sites at the

cytosolic mouth of the channel. However, as a large body of experimental evidence indicates that the single channel modified state results from the interaction of a single molecule of 21-amino-9 α -hydroxyryanodine with the RyR channel, it is suggested that not all of the measured voltage dependence results from the movement of a charged molecule into the voltage drop. At least some, if not all, of the voltage dependence of the reaction could result from a voltage driven conformational change of the channel protein that switches the ryanoid binding site between a high and low affinity state (Figure 3.1 taken from Tanna *et al*, 1998).

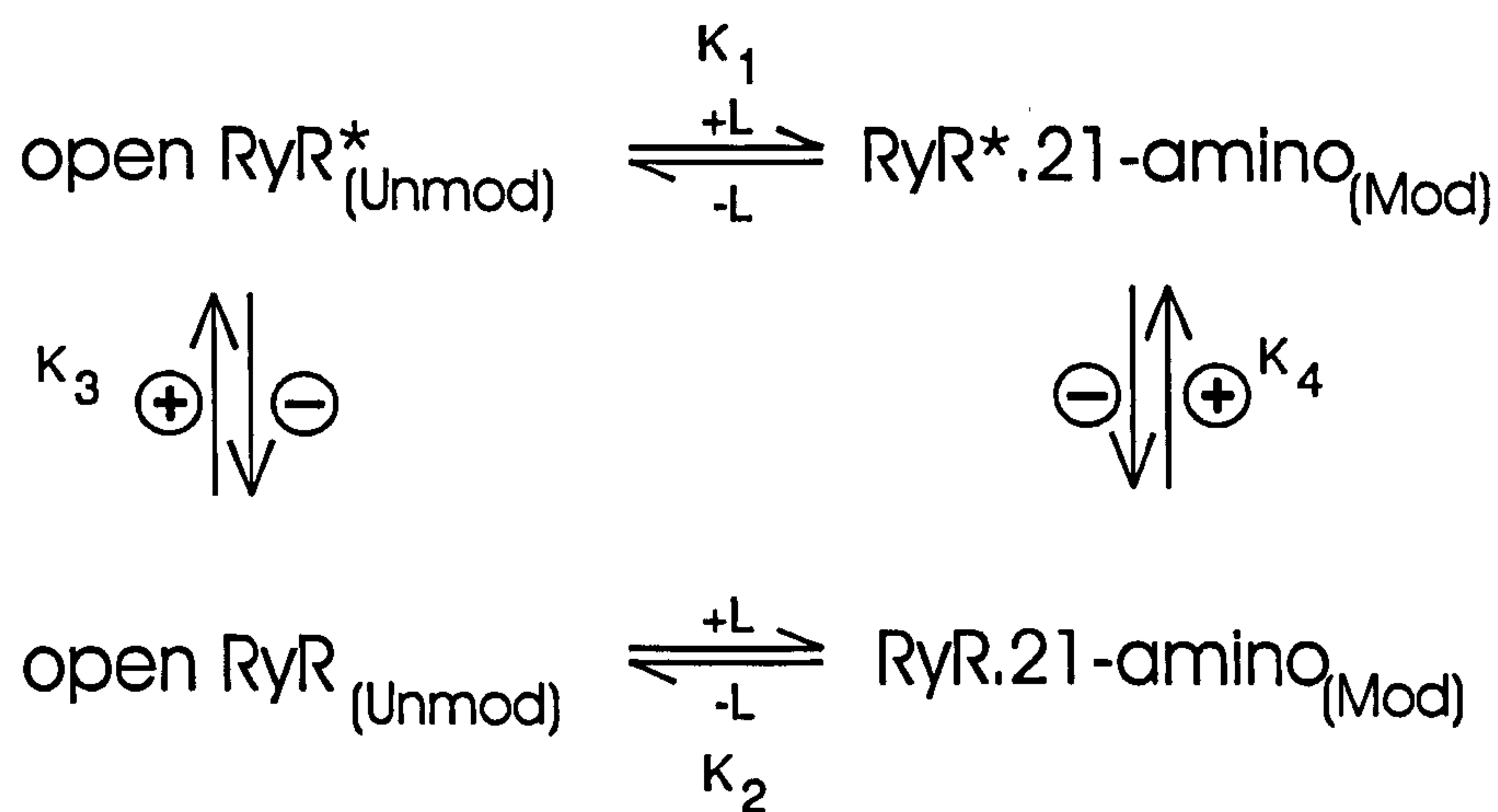


Figure 3.1. Scheme summarising the influence of transmembrane voltage on the interaction of 21-amino-9 α -hydroxyryanodine with the high affinity ryanodine binding site of RyR channel. L represents the ligand (21-amino-9 α -hydroxyryanodine) and + and – indicates the application of positive and negative potential to the cytosolic side of the channel (Tanna *et al*, 1998).

The above scheme, proposes that a minimum of two open conformations of channel exist (open RyR and open RyR*) and the probability of being in either of these conformations is sensitive to the applied electrical field. Increasing positive potential shifts the equilibrium towards open RyR* by increasing the value of the equilibrium constant K₃ and K₄. The open RyR* has a higher affinity for 21-amino-9 α -hydroxyryanodine than the open RyR form, therefore, increasing positive potential encourages the ryanoid to bind and modify the channel.

3.2 Where is the high affinity ryanodine binding site located?

The elucidation of the mechanism underlying the dependence on transmembrane voltage of the interaction of 21-amino-9 α -hydroxyryanodine with the RyR channel may provide information on the location of the ryanodine binding site in RyR channels. For example, translocation of 21-amino-9 α -hydroxyryanodine into the voltage drop across the channel would suggest that the binding site is within the conduction pathway of the RyR channel. I have tried to address this issue by firstly investigating the influence of transmembrane voltage on the interaction of a ryanoid that carries no net charge, ryanodol. Measurements of the kinetics of this ryanoid would establish the general location of the high affinity ryanoid binding site. If all the voltage dependence of the ryanoid binding was derived from a conformational change of the channel protein as described, it would mean the voltage dependence of a charged and neutral ryanoid would be the same. On the other hand, if the voltage dependence of the positively charged ryanoid were greater than that of a neutral ryanoid, this would suggest that the voltage dependence of the charged ryanoid has two components, derived from both a conformational change of the channel protein and the translocation of the ryanoid some way into the voltage drop across the channel. However, if the interaction of ryanodol was independent of holding potential it would suggest that all voltage dependence seen with 21-amino-9 α -hydroxyryanodine was due to translocation of the charged ryanoid into the voltage drop.

The interaction of other charged ryanoids with single RyR channels were also observed with varying transmembrane holding potential, to see if the proposal held true for all ryanoids.

3.3 Experimental procedures

In this chapter all the experiments involve incorporation of single purified RyR channels into artificial planar phospholipid bilayers, using methods described previously in detail in Chapter 2. All the experiments are carried out in symmetrical 610 mM KCl bathing solution in the presence of either 20 μ M ryanodol, 10 μ M 10-O-succinoylryanodol or 10 μ M 8 β -amino-9 α -hydroxyryanodine at the cytosolic face of the channel. Transmembrane holding potential was held at values as described in the text

and each run was recorded for 6 minute periods. Data were recorded and analysed as described in Chapter 2.

3.4 Structural differences between the charged and neutral ryanoids

The electrostatic field of ryanodol (no net charge), 21-amino-9 α -hydroxyryanodine (net charge +1), 8 β -amino-9 α -hydroxyryanodine (+1) and 10-O-succinoylryanodol (-1) together with ball and stick models of the molecules are presented in Figure 3.2. The red wire frames on the structures indicated positive and blue wire frames indicate negative electrostatic fields. 21-amino-9 α -hydroxyryanodine (top right) and 8 β -amino-9 α -hydroxyryanodine (bottom left) are shown with the pyrrole ring at the bottom left of the models, while both ryanodol (top left) and 10-O-succinoylryanodol (bottom right) which lack the pyrrole ring are in the same orientation. The wire frames in all diagrams represent the surface where the electrostatic potential equals 10 Kcal / mol. Note the extensive positive electrical potential of both 21-amino-9 α -hydroxyryanodine (located at the 21-position on the right of the ball and stick figure) and 8 β -amino-9 α -hydroxyryanodine (located at the 8-position in the lower right of the ball and stick figure) due to the ammonium group. While an extensive negative electrical potential is observed on the 10-O-succinoylryanodol due to the succinoyl group (located at the 10-position in the top right of the ball and stick diagram) and the presence of hydroxyl groups creates a nonuniform distribution of potential in the ryanodol molecule.

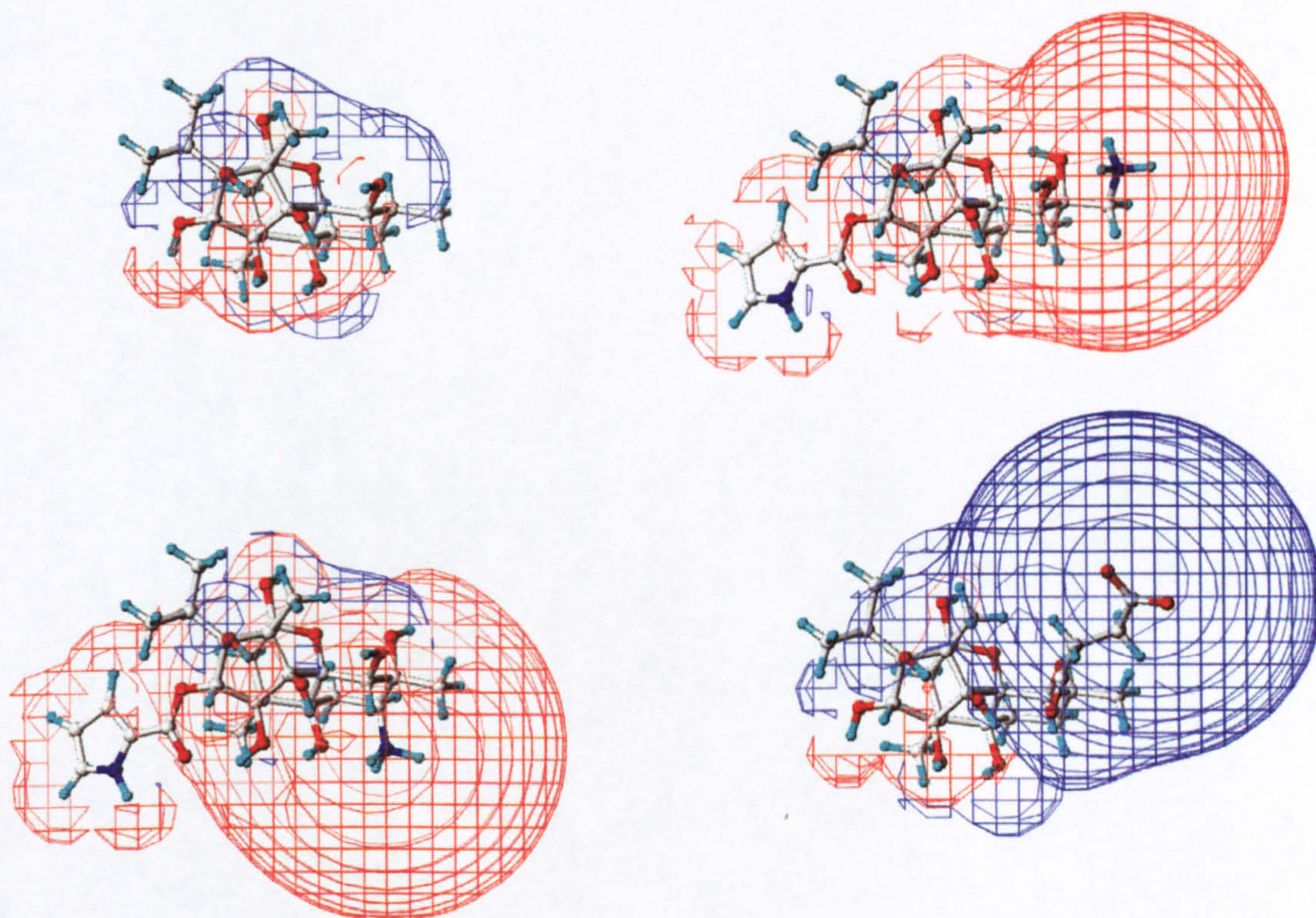


Figure 3.2 The electrostatic fields of ryanodol (top left), 21-amino-9 α -hydroxyryanodine (top right), 8 β -amino-9 α -hydroxyryanodine (bottom left) and 10-O-succinoylryanodol (bottom right). The red wire frames on the structures indicate positive electrostatic fields, while the blue wire frame indicates negative electrostatic fields of the same compounds. In all cases, the wire frames indicate the surface where the electrical field strength is 10 Kcal/mol. Ball and stick models of 21-amino-9 α -hydroxyryanodine (top right) and 8 β -amino-9 α -hydroxyryanodine (bottom left) are shown with the pyrrole ring at the bottom left of the models, while both ryanodol (top left) and 10-O-succinoylryanodol (bottom right) lack the pyrrole ring but are shown in the same alignment.

3.5 Is the interaction of ryanodol with the RyR channel influenced by transmembrane holding potential?

Current fluctuations of a single RyR channel in the presence of 20 μ M ryanodol held at transmembrane holding potentials ranging from -50 to 20 mV are shown in Figure 3.3. Like 21-amino-9 α -hydroxyryanodine, the interaction of ryanodol with the RyR channel is reversible, inducing modifications to both channel gating and ion handling. The interaction of ryanodol also seems to be influenced by transmembrane holding potential. It is clear from Figure 3.3, increasing transmembrane voltage to high positive potentials increases the likelihood of ryanodol interacting with the RyR channel i.e. the time spent

in the modified state increases. However, the FC of the modified state produced by ryanodol is different to that produced by 21-amino-9 α -hydroxyryanodine (Tinker *et al.*, 1996). The FC of ryanodol is independent of holding potential, where mean FC $\pm$ SEM (n) are 0.65 ± 0.01 (5), 0.64 ± 0.02 (6), 0.66 ± 0.01 (8), 0.66 ± 0.01 (5), 0.66 ± 0.01 (5), 0.65 ± 0.02 (5), 0.68 ± 0.02 (5) and 0.68 ± 0.01 (10) at -60, -50, -40, -0, -20, -10, 10, and 20 mV respectively. The FC for 21-amino-9 α -hydroxyryanodine under these conditions is ~ 0.45 (Tanna *et al.*, 1998).

Traces in Figure 3.3 highlight the first novel finding of these studies. The probability of RyR channel modification by ryanodol, a neutral ryanoid varies with transmembrane holding potential and the effect of voltage is qualitatively the same as that observed with 21-amino-9 α -hydroxyryanodine.

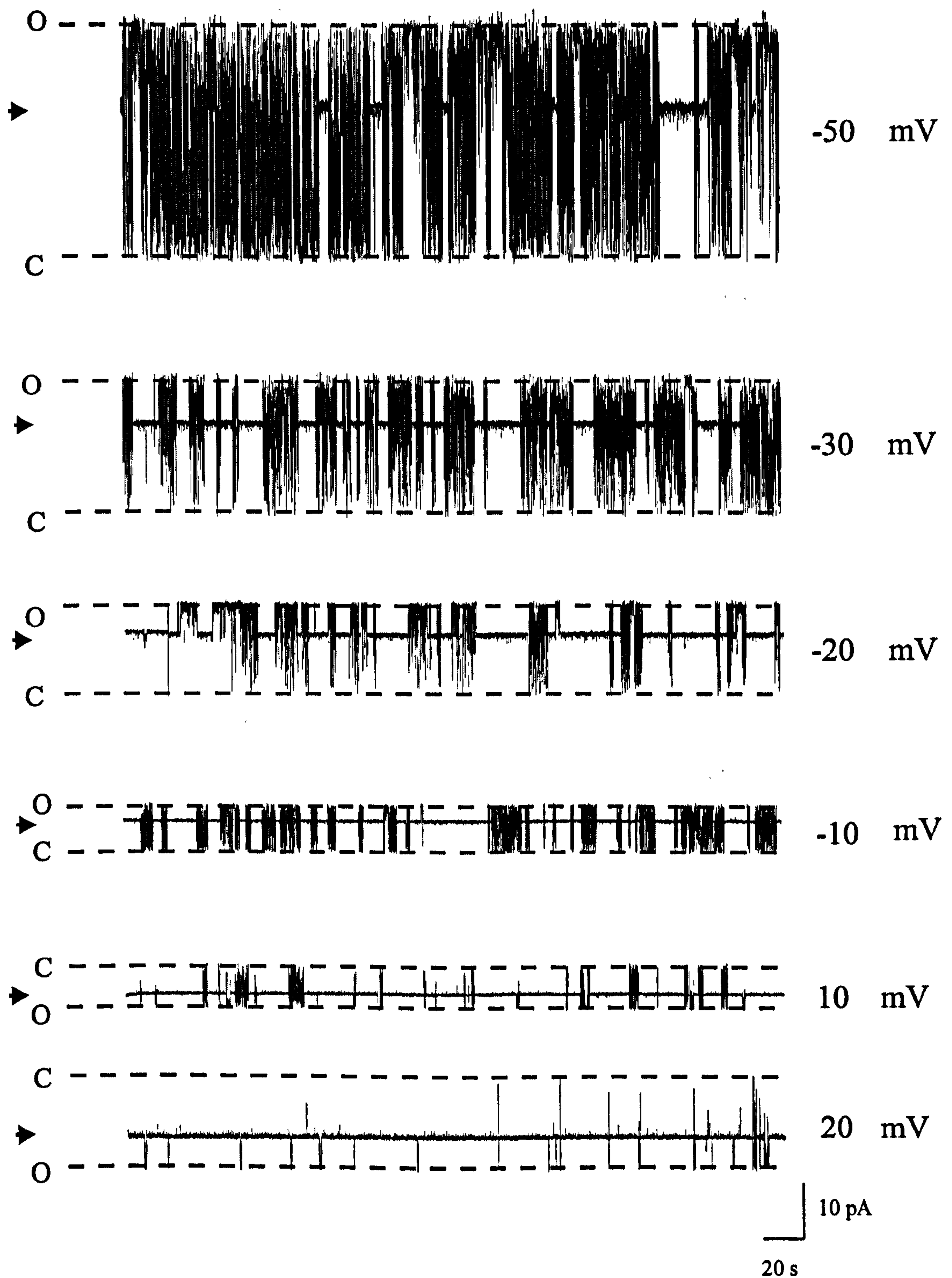


Figure 3.3 The influence of holding potential on the probability of modification of RyR channel by ryanodol. Traces were obtained from a single RyR channel in symmetrical 610 mM K^+ with 20 μ M ryanodol in the solution at the cytosolic face of the channel. O – open, C – closed and → -modified states.

3.6 The interaction of the charged ryanoid 8 β -amino-9 α -hydroxyryanodine with the RyR channel

Current fluctuations of a single RyR channel in the presence of 10 μ M 8 β -amino-9 α -hydroxyryanodine (ryanoid with a net charge of +1) at transmembrane holding potentials ranging from -30 to 30 mV are shown in Figure 3.4. The interaction of 8 β -amino-9 α -hydroxyryanodine with the RyR channel is also reversible and is dependent on voltage. As transmembrane holding potential is taken to high positive voltages, the time the channel resides in the modified state increases. However, the modified state induced by 8 β -amino-9 α -hydroxyryanodine displays excess noise when compared with the other gating states of the channel i.e. closed or opens states. The amplitude of the noise in the modified state shows clear transitions between two states labelled as the α and β state, illustrated in Figure 3.5 and is approximately 9 times larger than that observed when the channel is in the open or closed state. In comparison the excess noise observed in the modified state induced by 21-amino-9 α -hydroxyryanodine and ryanodol is approximately three times and twice the amplitude of that observed when the channel is in the closed or open state, respectively (Tanna *et al.*, 1998). Detailed characterisations of the α and β states observed in the presence of 8 β -amino-9 α -hydroxyryanodine are discussed in Chapter 6. For the purpose of these experiments the current amplitude of the modified state measured for calculating the FC induced by 8 β -amino-9 α -hydroxyryanodine was taken from the middle of the noise. The FC of the modified state induced by 8 β -amino-9 α -hydroxyryanodine is different to that induced by the previous two ryanoids, but is consistent with the FC states being independent of holding potential, where at -60, -50, -40, -30, -20, -10, 10, 20, 30 and 40 mV the mean FC $\pm$ SEM (n) are 0.42 ± 0.01 (5), 0.42 ± 0.01 (7), 0.43 ± 0.00 (6), 0.43 ± 0.00 (4), 0.44 ± 0.01 (5), 0.43 ± 0.01 (4), 0.44 ± 0.01 (4), 0.45 ± 0.01 (6), 0.46 ± 0.01 (5) and 0.44 ± 0.0 (5) respectively.

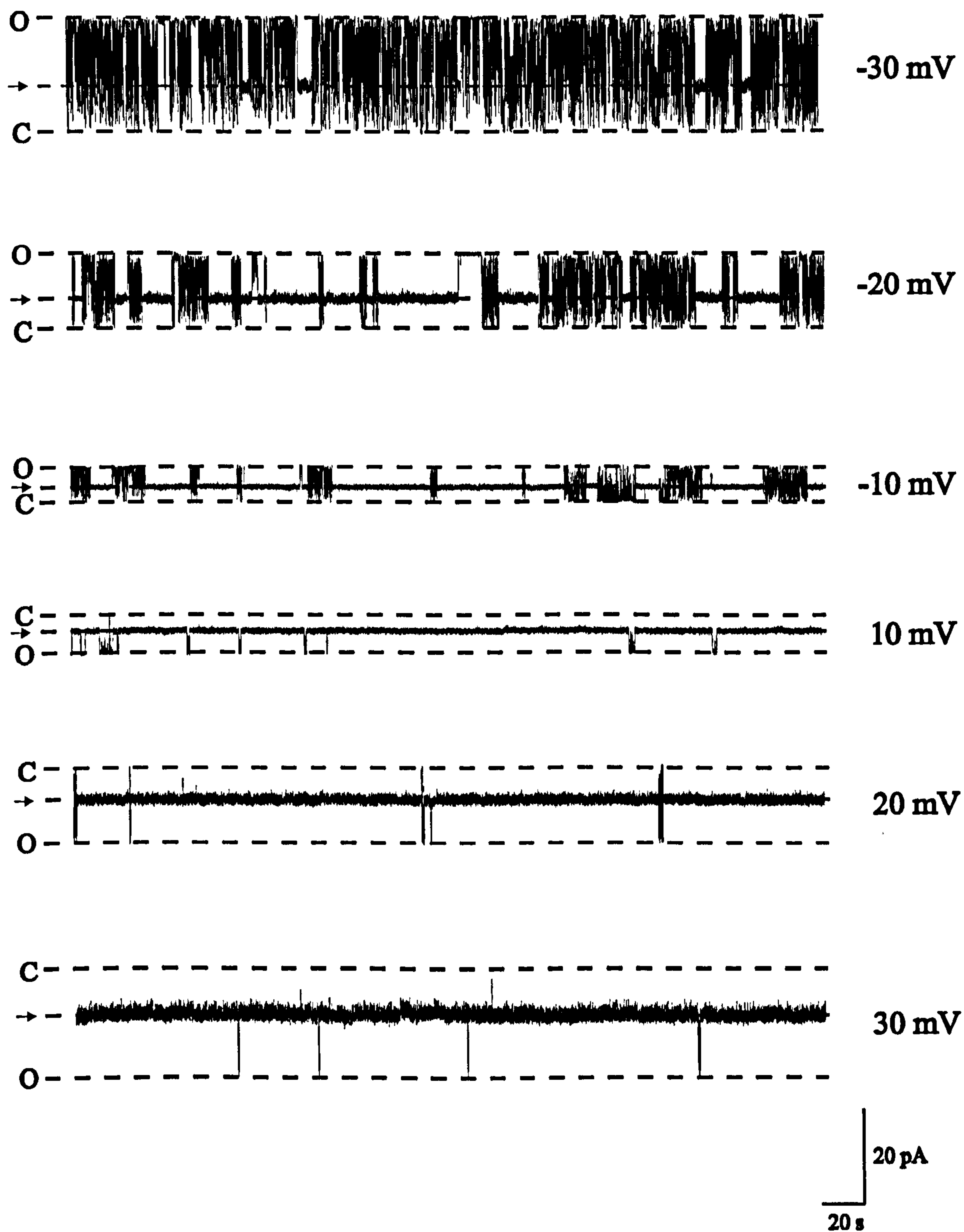


Figure 3.4. The influence of holding potential on the probability of modification of RyR channel function by 8 β -amino-9 α -hydroxyryanodine. Traces were obtained from a single RyR channel in symmetrical 610 mM K⁺ with 10 μ M 8 β -amino-9 α -hydroxyryanodine in the solution at the cytosolic face of the channel. O – open, C – closed and → - modified states.

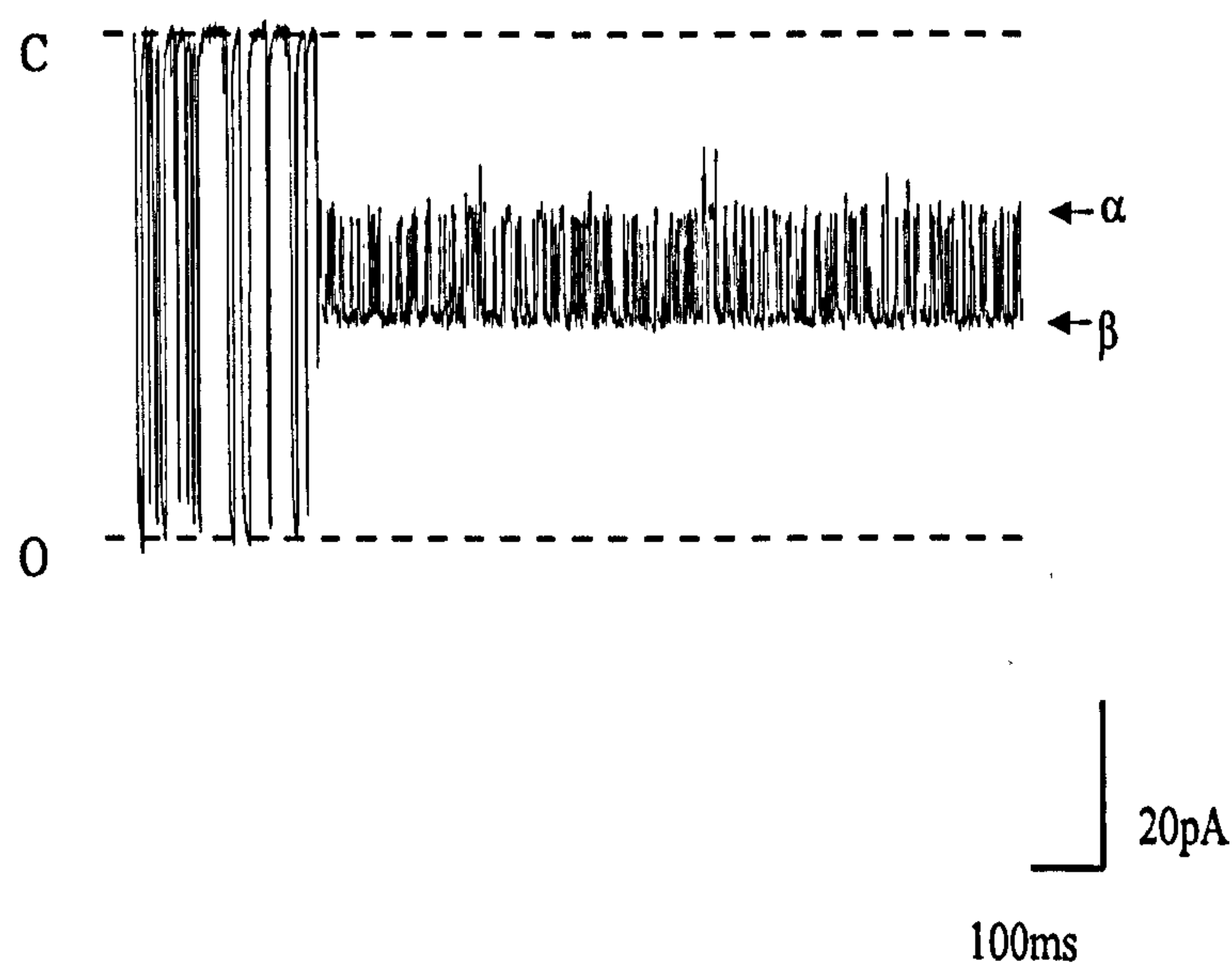


Figure 3.5. The interaction of 8 β -amino-9 α -hydroxyryanodine with the RyR channel. Bound 8 β -amino-9 α -hydroxyryanodine produces the characteristic reduced conductance state with a high P_o . However, the modified state produced by the ryanoid is noisy in comparison to both the open and closed states of the channel and shows clear transitions between two fractional conductance states, labelled here as α and β . O – open and C – closed states.

3.7 The interaction of the net negatively charged ryanoid 10-O-succinoylryanodol with single RyR channels

The interaction of micromolar concentrations of 10-O-succinoylryanodol with single RyR channels results in the modification of channel function that is reversible on the time scale of a single channel experiment as demonstrated in Figure 3.6. However, an inspection of Figure 3.6, illustrates that the interaction of 10-O-succinoylryanodol with the RyR channel results in the occurrence of a number of modified conductance states. A total of 33 different modified conductance states induced by the interaction of 10-O-succinoylryanodol with individual RyR channels were observed with values of FC ranging from 0.20 to 0.93 Figure 3.7. Molecular dynamics simulations have revealed that unlike ryanodine, which has a fixed and rigid structure, the structure of 10-O-succinoylryanodol is highly flexible (at the innermost rotatable bonds of the succinoyl group) in solution and can adopt at least 200 different approximately isoenergetic conformations. The existence of multiple conformations would be consistent with the proposal that each FC state arises from the interaction of a different conformer with the

RyR channel in single channel bilayer studies with this ryanoid. Figure 3.8 a shows superimposed structures that depict 6 local conformational minima of 10-O-succinoylryanodol found in molecular dynamics simulations of the motions of the ryanoid that had at least 1 Angstrom root mean square differences between them. Figure 3.8 b and c are orthogonal views of the ryanoid in which the wire frames are swept volumes of 200 conformers found in an extended molecular dynamics run. The space-fill structures in these figures are the average conformer of the most highly populated group of conformers identified using hierarchical analysis of the 200 conformers (carried out by Professor William Welch). We propose that once the ryanoid binds to the channel the structure of the ryanoid remains in the particular orientation it happens to be in at the time of the interaction. This induces a reduced conductance state that is governed by the structure of the ryanoid. Once the ryanoid dissociates, it and other conformers of the ryanoid molecule are able to bind to the channel again maybe in the same or another configuration. Other configurations will produce different reduced conductance states. This phenomenon is also demonstrated by the interaction of the irreversible ryanoid 21-p-nitrobenzoylamino-9 α -hydroxyryanodine with single RyRs (described in Chapter 5), although, in the case of 21-p-nitrobenzoylamino-9 α -hydroxyryanodine the flexibility of the long floppy *p*-nitrobenzoyl tail is limited to three likely conformers. These internal controls are consistent with the proposal that the reduced conductance state induced by ryanoids is dependent on the structure of the ryanoid and is not dependent upon other interactions between the ligand and receptor that could result from alterations in the covalent structure of the ryanoid.

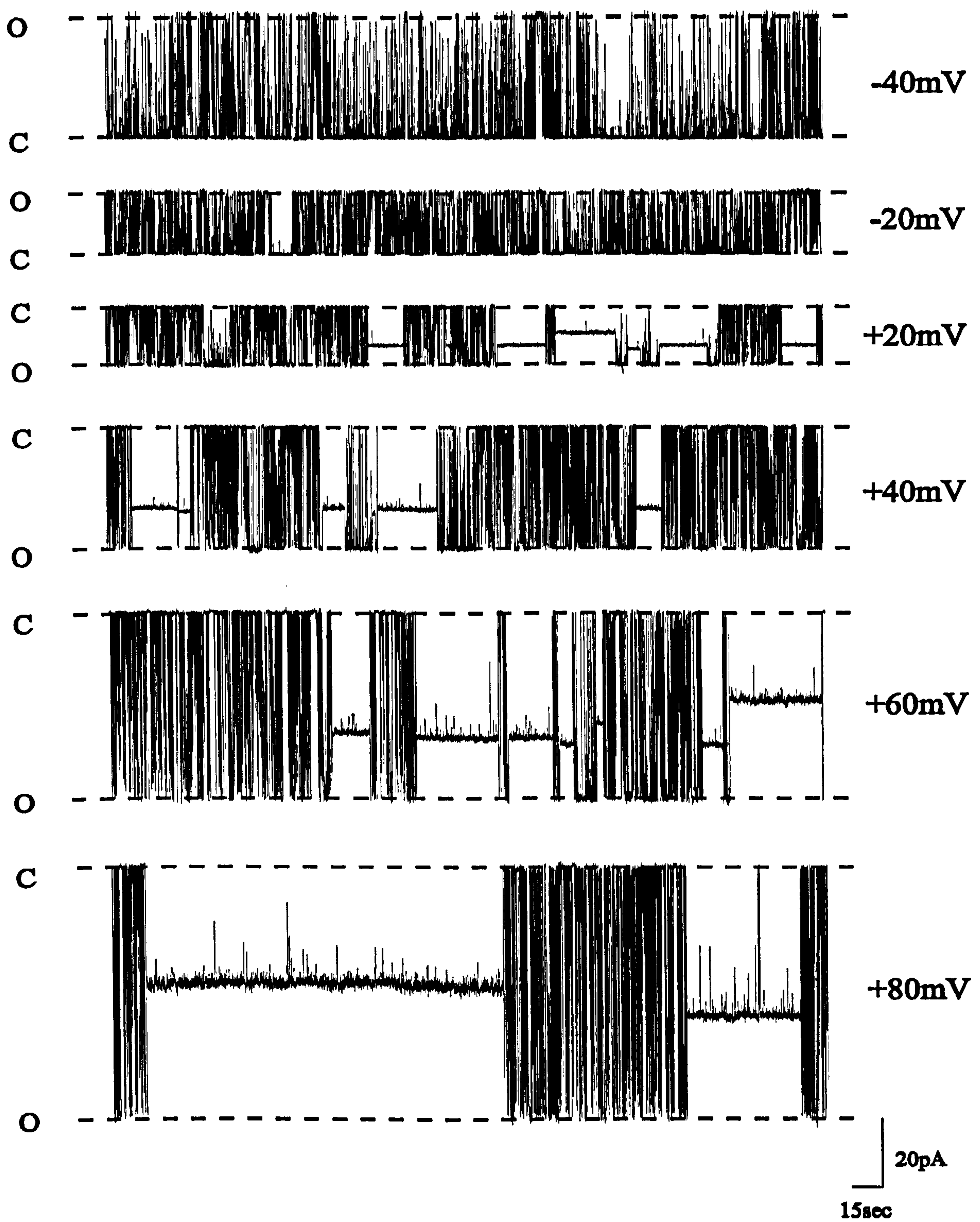


Figure 3.6 The influence of holding potential on the probability of modification of RyR channel function by 10-O-succinoylryanodol. Traces were obtained from a single RyR channel in symmetrical 610 mM K^+ with 10 μ M 10-O-succinoylryanodol in the solution at the cytosolic face of the channel. O – open and C – closed states.

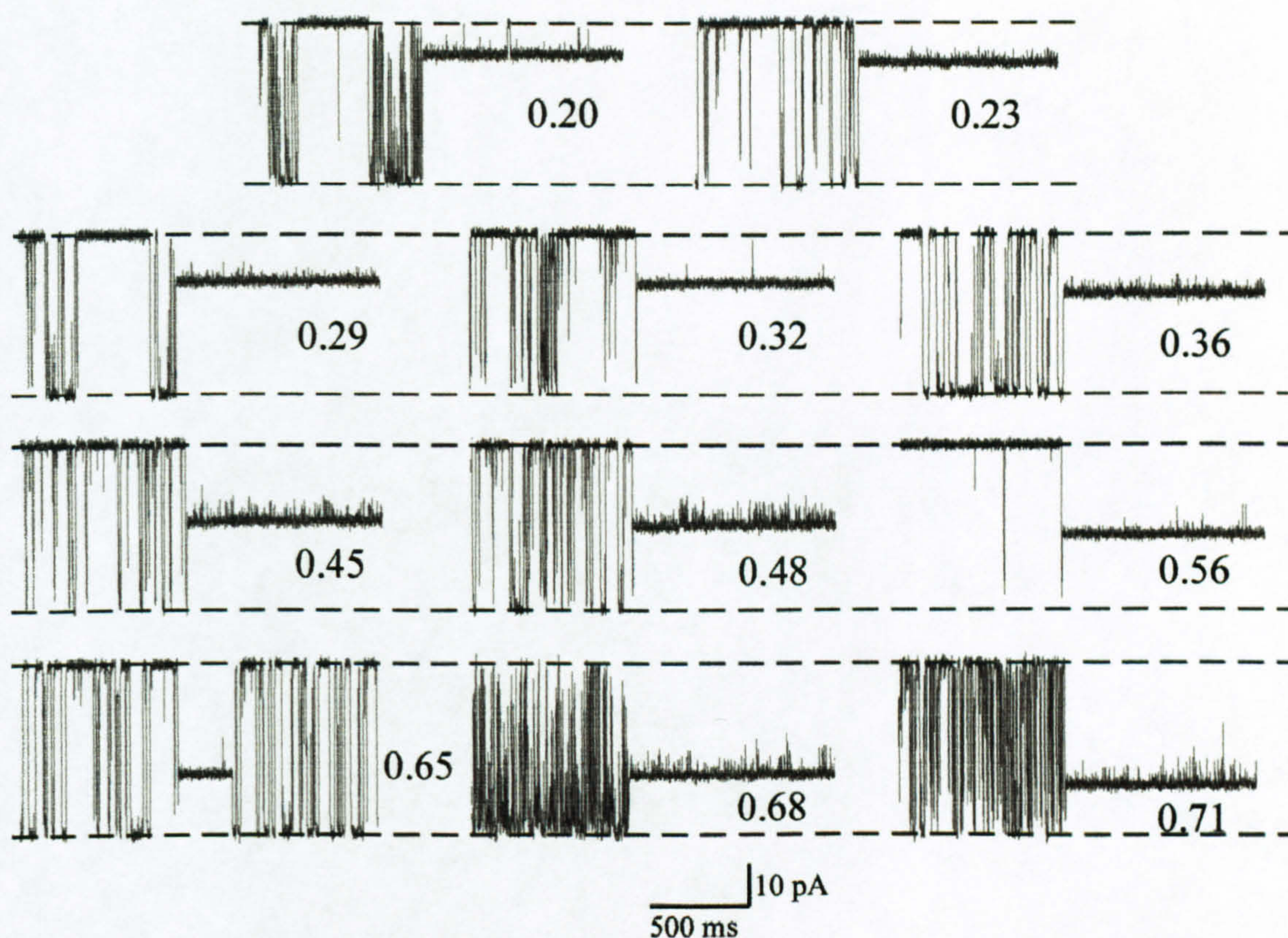


Figure 3.7 Shows a representation of the numerous FC states induce by the interaction of 10-O-succinoylryanodol with a single RyR channel.

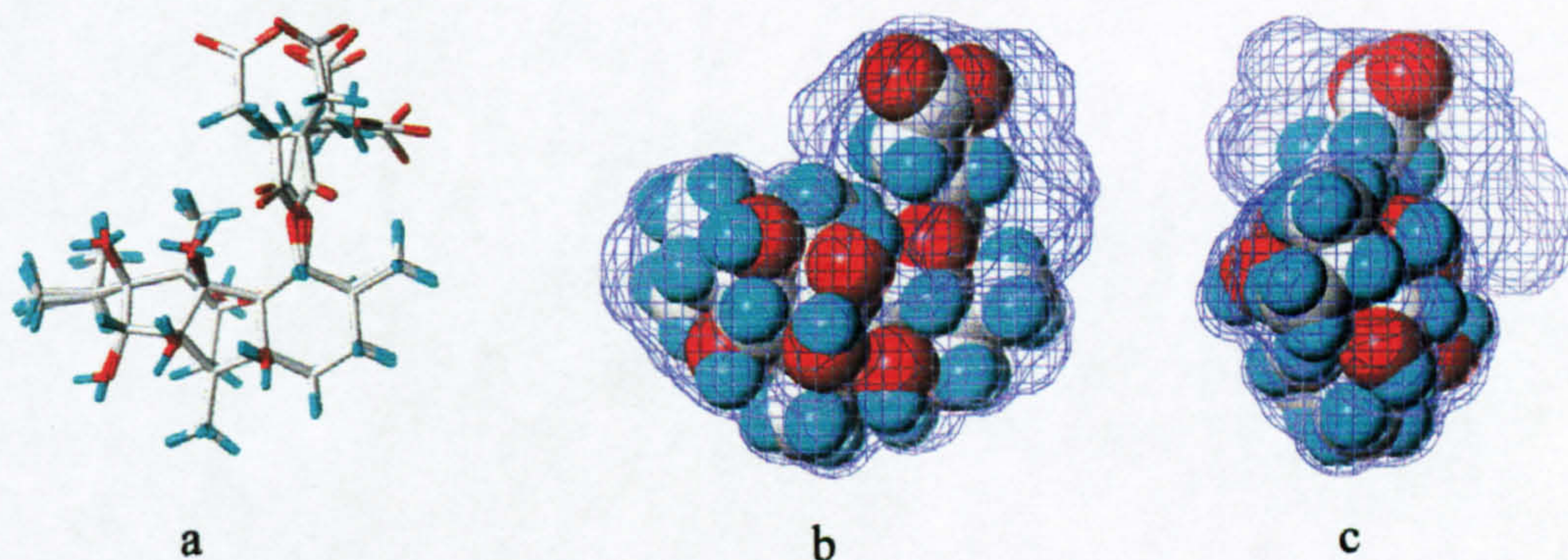


Figure 3.8 Conformations of 10-O-succinoylryanodol. a represents superimposed structures of 6 local conformational minima found in molecular dynamics simulations of the motions of the ryanoid that had at least 1 Angstrom root mean square difference between them. b and c are orthogonal views of the ryanoid. The wire frames are the swept volumes of 200 conformers found in an extended molecular dynamics run. The space-fill structure is the average conformation of the most highly populated group of conformers identified using hierarchical analysis of the 200 conformers.

Figure 3.6 shows current fluctuations of a single RyR channel at holding potentials ranging from -40 to $+80$ mV in the presence of $10\text{ }\mu\text{M}$ 10-O-succinoylryanodol applied at the cytosolic face of the channel. As is the case for both the neutral and cationic ryanoids, the interaction of the anionic ryanoid with the RyR is dependent on voltage. At -40 mV the probability of channel modification is very low; as transmembrane holding potential is made more positive the likelihood that the ryanoid interacts with the channel increases.

Traces in Figure 3.3, 3.4 and 3.6, together with the evidence provided previously with the interaction of 21-amino-9 α -hydroxyryanodine, demonstrate that the interactions of all these ryanoids with the RyR channels irrespective of the total net charge they carry or the FC states induced by the ryanoid have a qualitatively similar dependence on voltage. As transmembrane holding potential is taken to high positive voltages the probability that the ryanoid interacts with the channel increases. However, the FC states induced by the ryanoids are independent of voltage.

3.8 The quantitative differences in the interaction of the ryanoids with the cardiac RyR channel

The apparent rate constants for the association (k_{on}) and dissociation (k_{off}) of ryanoids are determined from the mean dwell times in the unmodified state and the mean dwell times in the induced modified conductance states (as described in section 2.11). For the purpose of this analysis, no distinction between the dwell times in the various 10-O-succinoylryanodol modified states has been made. Variations in k_{on} and k_{off} of ryanodol, 8 β -amino-9 α -hydroxyryanodine and 10-O-succinoylryanodol with voltage are shown in Figures 3.9 and 3.10 respectively. Both the rates of association and the rates of dissociation of all the ryanoids to and from the RyR channel vary with applied holding potential, k_{on} increases, as the holding potential is made more positive, while k_{off} decreases. The lines of best fit obtained by linear regression for the k_{on} plots has a slope of 0.041 ± 0.003 ($r = 1.0$), 0.069 ± 0.003 ($r = 0.96$) and 0.021 ± 0.003 ($r = 0.75$) yielding a z_{on} value of 1.03, 1.75 and 0.54 (Eq. 2.7) for ryanodol, 8 β -amino-9 α -hydroxyryanodine and 10-O-succinoylryanodol respectively. The equivalent plots for k_{off} have slopes of -0.019 ± 0.002 ($r = 1$), -0.037 ± 0.003 ($r = 0.88$) and -0.028 ± 0.005 ($r = 0.75$), yielding a z_{off} value of 0.48 and 0.94 and 0.70 (Eq. 2.8) for ryanodol, 8 β -amino-9 α -hydroxyryanodine and 10-O-succinoylryanodol respectively. Together, these

produce a total valence (z_{total}) of 1.51 for ryanodol, 2.69 for 8 β -amino-9 α -hydroxyryanodine and 1.24 for 10-O-succinoylryanodol. Values for k_{on} and k_{off} at 0 mV, obtained from the lines of best fit in Fig. 3.9 and 3.10, are 0.035 $\mu\text{M}^{-1}\text{s}^{-1}$ and 0.095 s^{-1} for ryanodol, 0.091 $\mu\text{M}^{-1}\text{s}^{-1}$ and 0.036 s^{-1} for 8 β -amino-9 α -hydroxyryanodine and 0.0022 $\mu\text{M}^{-1}\text{s}^{-1}$ and 0.082 s^{-1} for 10-O-succinoylryanodol respectively.

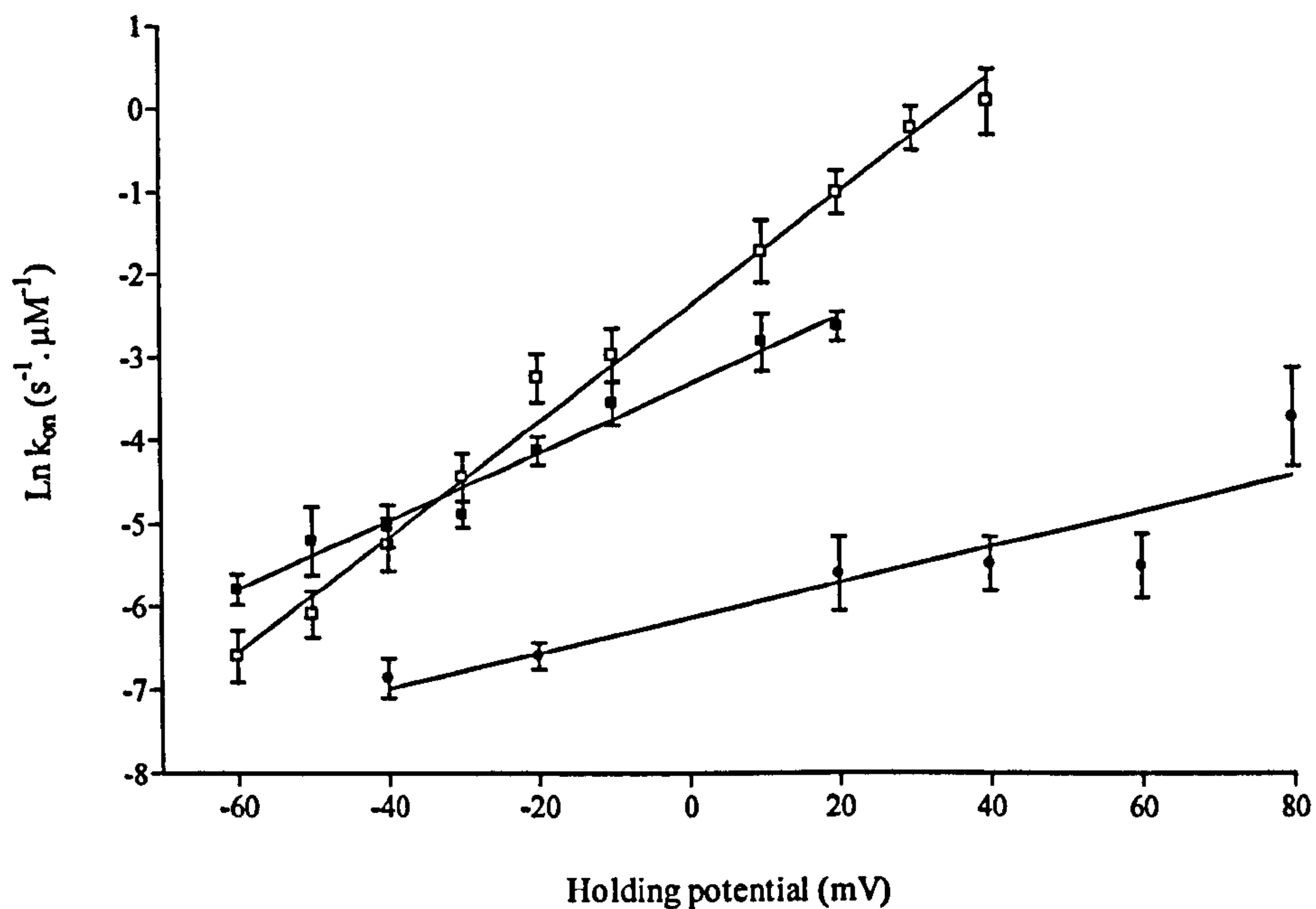


Figure 3.9 The dependence of rates of association of ryanodol, 8 β -amino-9 α -hydroxyryanodine and 10-O-succinoylryanodol on holding potential. 20 μM ryanodol, 10 μM 8 β -amino-9 α -hydroxyryanodine and 10 μM 10-O-succinoylryanodol were applied to the cytosolic face of the channel. Each point is the mean $\pm$ SEM of 4-10 (ryanodol ($\blacksquare$)), 4-7 (8 β -amino-9 α -hydroxyryanodine ($\square$)) and 4-7 (10-O-succinoylryanodol ($\bullet$)) experiments. All values of k_{on} are normalised to a P_o of 1.0 (described in section 2.14). The solid lines are obtained by linear regression with the parameters quoted in the text.

Figure 3.11 shows the relationship of the dissociation constants of the ryanoids (calculated from data in Fig. 3.9 and 3.10 $K_d = k_{\text{off}} (\text{s}^{-1})/k_{\text{on}} (\mu\text{M}^{-1}\text{s}^{-1})$) to transmembrane holding potential. The line of best fit obtained by a linear regression for these plots have slopes of -0.06 ± 0.002 for ryanodol, -0.11 ± 0.004 for 8 β -amino-9 α -hydroxyryanodine, and -0.05 ± 0.007 for 10-O-succinoylryanodol. The extrapolated values of K_d obtained from this data at 0 mV are 2.81, 0.38 and 35.62 μM , for ryanodol, 8 β -amino-9 α -hydroxyryanodine and for 10-O-succinoylryanodol respectively.

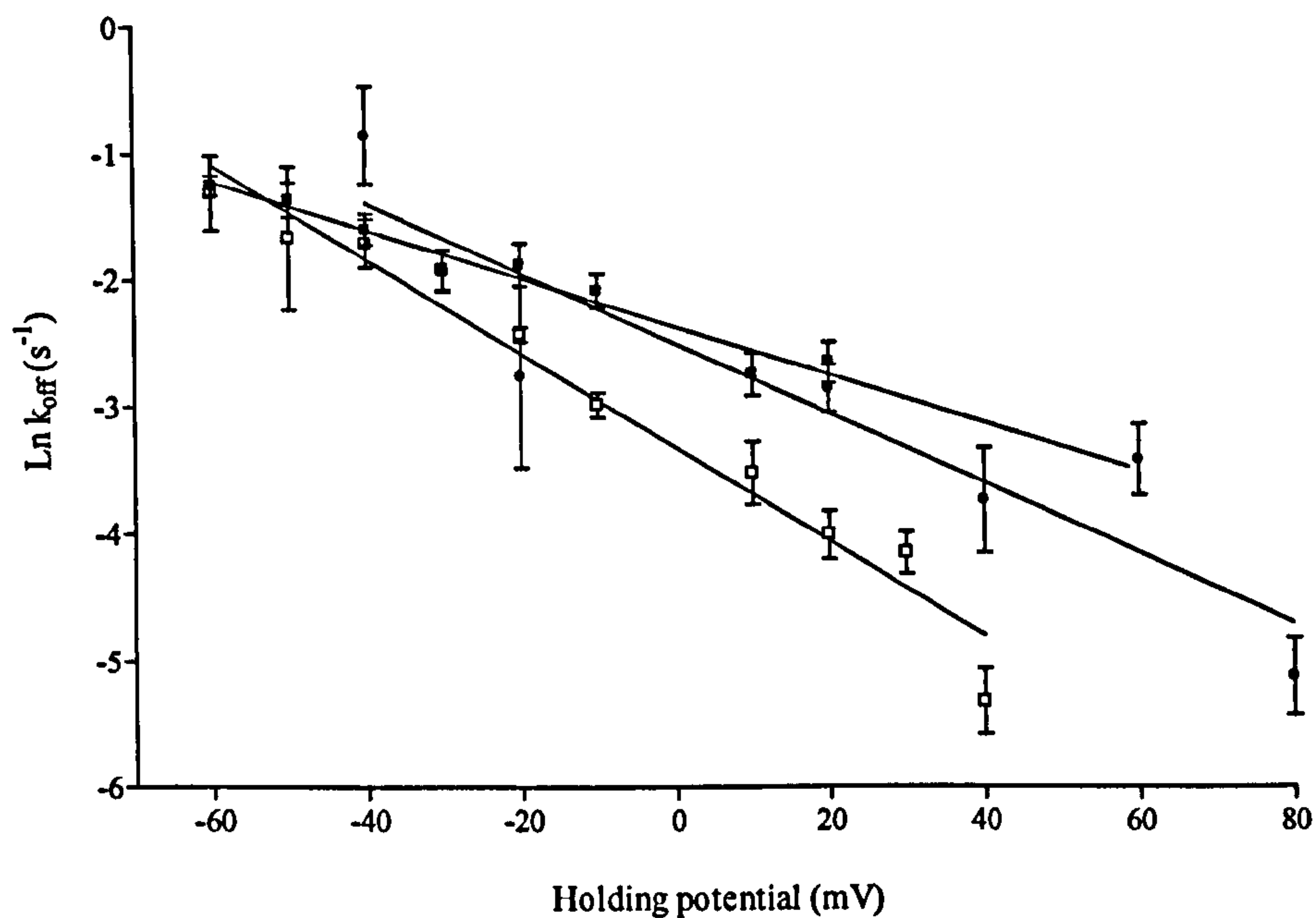


Figure 3.10 The dependence of rates of dissociation of ryanodol, 8 β -amino-9 α -hydroxyryanodine and 10-O-succinoylryanodol on holding potential. 20 μ M ryanodol, 10 μ M 8 β -amino9 α -hydroxyryanodine and 10 μ M 10-O-succinoylryanodol was applied to the cytosolic face of the channel. Each point is the mean $\pm$ SEM of 4-10 (ryanodol ($\blacksquare$)), 4-7 (8 β -amino-9 α -hydroxyryanodine ($\square$)) and 4-7 (10-O-succinoylryanodol ($\bullet$)) experiments. The solid lines are obtained by linear regression with the parameters quoted in the text.

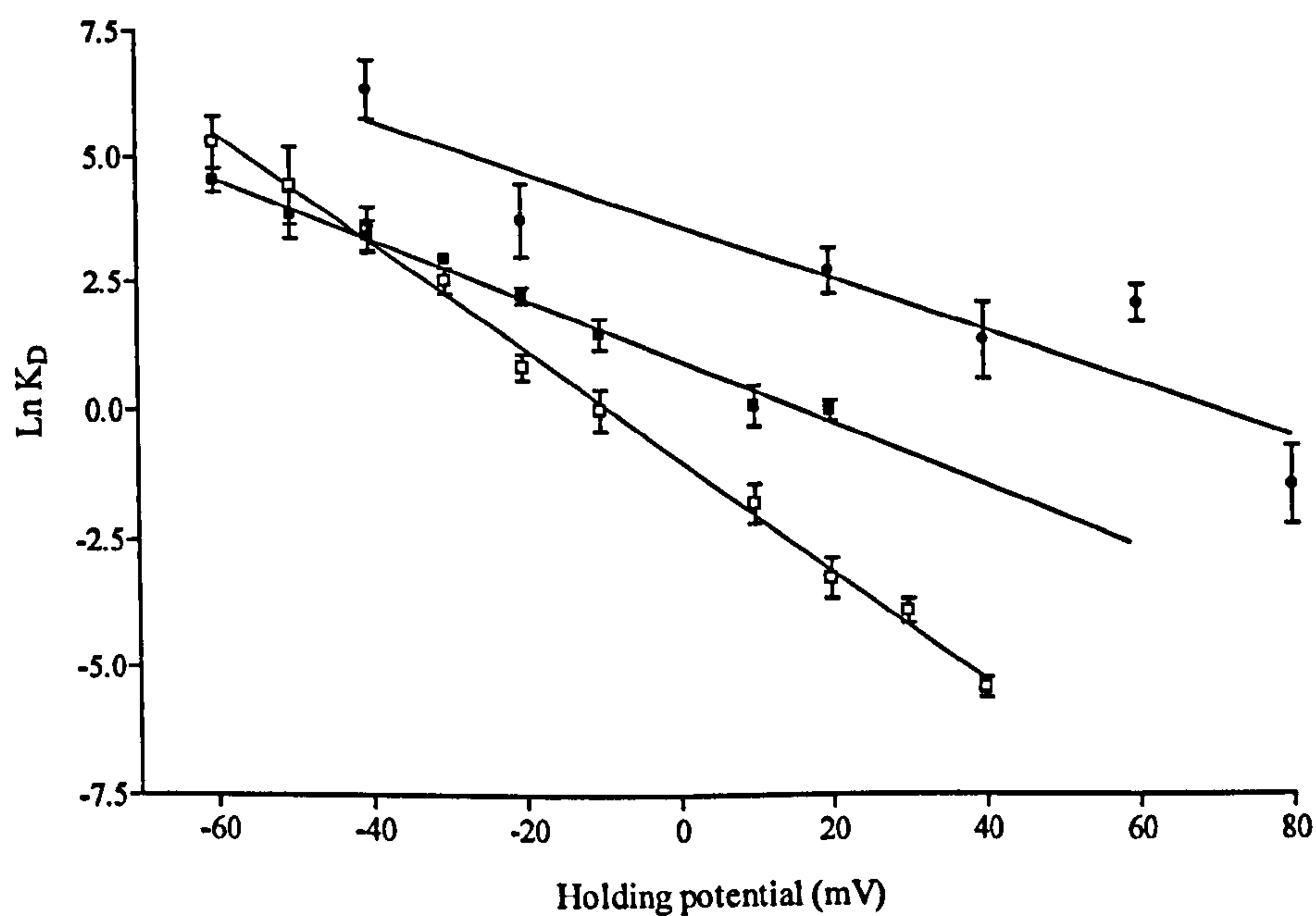


Figure 3.11 The relationship of the dissociation constant [$K_d = k_{off} (s^{-1}) / k_{on} (\mu M^{-1}s^{-1})$] of ryanodol ($\blacksquare$), 8 β -amino9 α -hydroxyryanodine ($\square$) and 10-O-succinoylryanodol ($\bullet$).

For comparison with equivalent parameters obtained for 21-amino-9 α -hydroxyryanodine (Tanna *et al.*, 1998), all data are summarised in Table 3.1.

	Ryanodol	8 β -amino-9 α -hydroxyryanodine	21-amino-9 α -hydroxyryanodine	10-O-succinoylryanodol
Slope ($z_{on}F/RT$)	0.041 ± 0.003 ($r=1$)	0.069 ± 0.003 ($r=0.96$)	0.051 ± 0.002 ($r=1$)	0.021 ± 0.003 ($r=0.75$)
z_{on}	1.03	1.75	1.29	0.54
Slope ($-z_{off}F/RT$)	-0.019 ± 0.002 ($r=1$)	-0.037 ± 0.003 ($r=0.88$)	-0.0344 ± 0.002 ($r=1$)	-0.028 ± 0.005 ($r=0.75$)
z_{off}	0.48	0.94	0.87	0.70
z_{total}	1.51	2.69	2.16	1.24
k_{on} at 0m V ($\mu M^{-1}s^{-1}$)	0.035	0.091	0.365	0.0022
k_{off} at 0mV (s^{-1})	0.095	0.036	0.99	0.082
K_d at 0mV (μM)	2.81	0.38	2.79	35.62

Table 3.1 The influence of transmembrane holding potential on the interaction of ryanodol, 8 β -amino-9 α -hydroxyryanodine, 10-O-succinoylryanodol and 21-amino-9 α -hydroxyryanodine with sheep cardiac RyR channel. Data for 21-amino-9 α -hydroxyryanodine are from Tanna *et al.* (1998). Values of k_{on} are normalised to a P_o of 1.0. Values of k_{on} , k_{off} and K_d at 0mV are calculated from the regression lines fitted to data in Fig. 3.9, Fig. 3.10 and Fig. 3.11.

3.9 Discussion

These results demonstrate that transmembrane holding potential influences the interaction of the neutral ryanoid ryanodol with the cardiac RyR channel. Like the interaction of 21-amino-9 α -hydroxyryanodine with the channel, both the rate of association and the rate of dissociation of ryanodol are dependent on voltage. The data provide evidence that transmembrane holding potential influences the likelihood of a ryanoid interacting with the RyR channel by a mechanism which is independent of a charged moiety translocating into the voltage drop across the channel. Therefore, the likely mechanism that underlies the interaction of ryanoids with the RyR channel would

then involve a voltage-dependent equilibrium between receptor states of different affinities (Tanna *et al.*, 1998). In such a scheme (Figure 3.12), the transmembrane potential would provide an energy source for the movement of a charged component of the RyR within the electric field that would produce a conformational change and result in altered receptor affinity. A degree of asymmetry in the energy profile for the transition between the different affinity states of the channel is indicated by the difference in voltage dependence of the association and dissociation of ryanodol; suggesting the electrical distance covered by the charged component in the forward and reverse reaction may not be the same.

A comparison of the influence of transmembrane holding potential on the probability of RyR channel modification induced by the various ryanoids discussed in this chapter indicated that irrespective of net charge, the probability of occurrence of altered function is dependent on voltage. In all cases an alteration of holding potential from negative to positive values results in an increase in the rate of ryanoid association and a decrease in the rate of ryanoid dissociation. Consistent with the proposal that the high affinity ryanoid binding site is located within the voltage drop across the channel, and that the movement of a charged ryanoid into the voltage drop is likely to contribute to the overall voltage dependence of the interaction of ryanoids with the channel, the observed total voltage dependence of the interaction of the positively charged ryanoids 21-amino-9 α -hydroxyryanodine and 8 β -amino-9 α -hydroxyryanodine are greater than that observed with ryanodol, whilst the total voltage dependence of the interaction of the negatively charged ryanoid 10-O-succinoylryanodol is less than that observed with ryanodol.

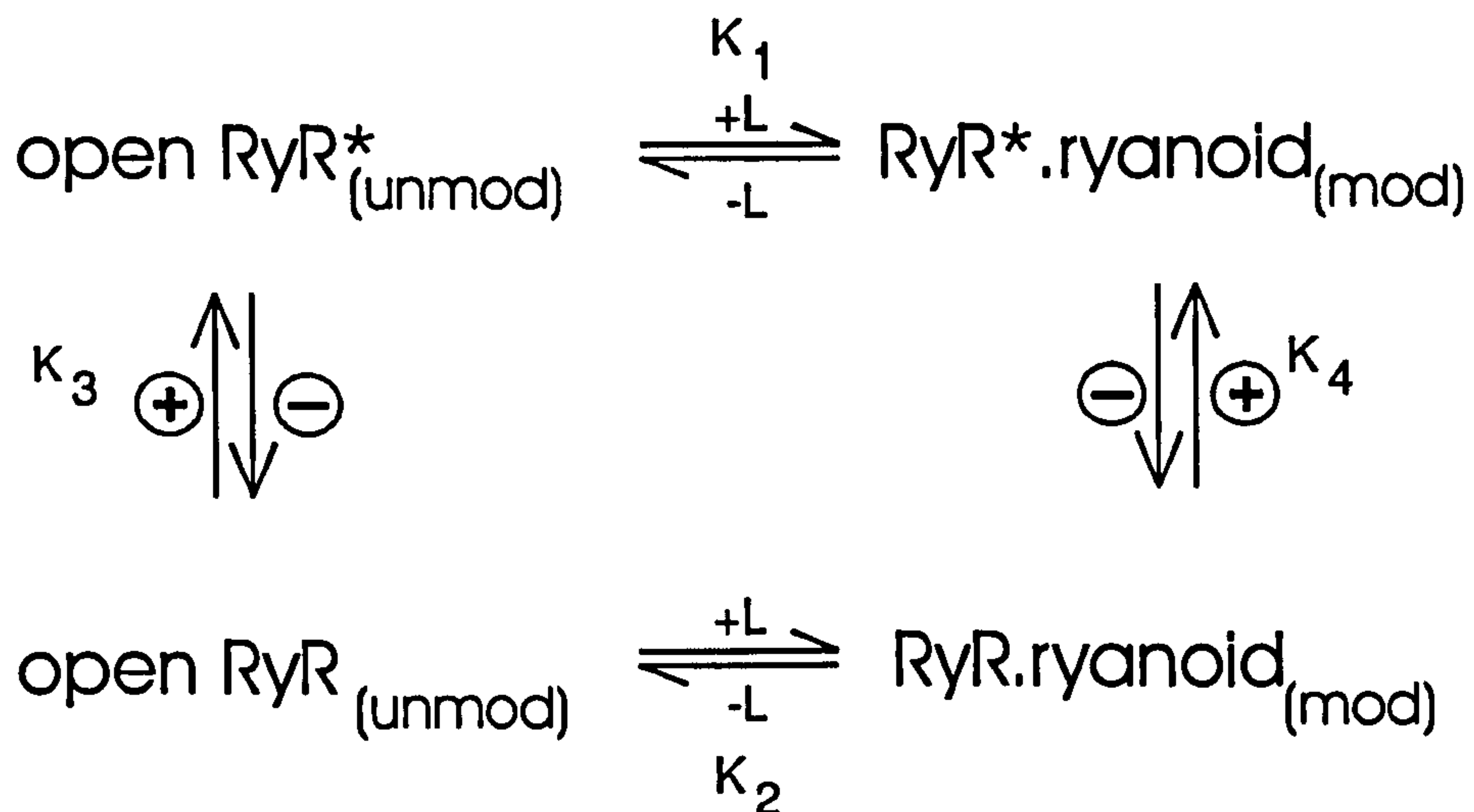


Figure 3.12 Scheme summarising the influence of transmembrane voltage on the interaction of ryanoids with the high affinity binding site of the RyR channel. L represents the ligand (any ryanoid) and + and – indicates the application of positive and negative potential to the cytosolic side of the channel. A minimum of two forms of vacant receptor exist in this model (low affinity, open RyR; high affinity, open RyR*). Increasing positive potential shifts the equilibrium towards open RyR* by increasing the value of the equilibrium constants K₃ and K₄. If the ryanoid binding site is located within the voltage drop across the channel, then charged ryanoids would experience an additional influence of voltage. Figure up dated from Tanna *et al.*, (1998).

However, the voltage dependence of 8β-amino-9α-hydroxyryanodine is greater than that observed with 21-amino-9α-hydroxyryanodine even though they carry the same net charge. This suggests either the transitions between the two modified states (α and β) produced in the presence of 8β-amino-9α-hydroxyryanodine involves a movement of a charge within the ryanoid-RyR channel complex or the charged ammonium group on the 8β-amino-9α-hydroxyryanodine moves further into the voltage drop across the channel than the charged group on the 21-amino-9α-hydroxyryanodine molecule. These voltage dependent effects would be in addition to the voltage-induced transition of the channel between different affinity states.

While a mechanism where the ryanoid binding site is located outside the voltage drop resulting in an allosteric interaction between the binding site and the voltage drop cannot be eliminated, the ryanoid binding site placed within the voltage drop across the channel is more appealing. Under these circumstances, part of the voltage dependence

arises from the movement of a charged ryanoid into and / or out of the electric field. A location of the ryanoid binding site within the voltage drop of RyR would be consistent with the results obtained in this chapter. Experiments indicating that mutations around the probable voltage drop across the channel / pore-forming region (selectivity filter sequence) of the RyR channel produce reductions or abolition of the binding of [³H]-ryanodine and reversible ryanodine modified conductance states in single channel studies, could be interpreted as indicating this region as a component of the ryanoid binding site (Chen *et al.*, 2002; Zhao *et al.*, 1999). The interaction of ryanoids with the open RyR channel is in good agreement with the proposal that the high-affinity binding site for these ligands is located within the conduction pathway of the channel. [³H]-ryanodine binding is altered by interventions that modify RyR channel P_o, ligands that activate the channel such as Ca²⁺, caffeine and ATP increase equilibrium binding while ligands that lower P_o, such as Mg²⁺ and ruthenium red, reduce binding. These observations have given rise to the proposal that the high affinity binding site for [³H]-ryanodine binding is only accessible when the channel is open and as a consequence, equilibrium [³H]-ryanodine binding is used routinely as a method of assessing the P_o of populations of RyR channels. A direct demonstration that the high-affinity binding of ryanoids to the RyR channel requires the channel to be open was provided in the experiments carried out by Tanna *et al.* 1998, in which a linear dependence of the rate of association of 21-amino-9 α -hydroxyryanodine on RyR channel P_o was demonstrated.

The net charge of the ryanoid has a very significant influence on the absolute rate of association of the ligand with the channel. In the absence of a transmembrane holding potential an increase in the rate of association of the ligands is seen as the net charge is increased from -1 through to 0 to +1, Table 3.1. The implication of this observation is that electrostatic forces make a significant contribution to the association of these ligands with their binding site on the RyR channel. A fixed high density of negatively charged sites that are thought to lie close to the cytosolic mouth of the RyR channel (Tinker *et al.*, 1992) could explain this phenomenon. In this case a ryanoid with a net charge of +1 would accumulate in the vicinity of the fixed negatively charged residues, so effectively increasing ryanoid concentration at the mouth of the channel, explaining the faster association rate of 21-amino-9 α -hydroxyryanodine and 8 β -amino-9 α -hydroxyryanodine. A neutral ryanoid would bind to the channel at a rate related to

diffusion, while a ryanoid that has a net charge of -1 would be less attracted to or be repelled from the negative charge on the protein and so a slower association rate of this ryanoid would then be expected. The data is consistent with that observed by Mead *et al.*, (1998). They provided evidence of negative charge at the cytosolic entrance of the conduction pathway of the RyR channel, by experiments in which rates of association of K^+ channel N-type inactivation peptide, added to the solution at the cytosolic face of the channel, were increased dramatically by an increase in net charge of the peptide from $+3$ to $+7$. The net charge of these ligands has little effect on the rate of dissociation from the channel.

In summary, the experiments described in this chapter were designed to examine the mechanisms involved in the striking influence of transmembrane holding potential on the interaction of ryanoids with the high affinity binding site on the RyR channel. The earlier observation of a strong influence of voltage on the interaction of a positively charged ryanoid (21-amino-9 α -hydroxyryanodine) with the RyR channel could be explained either by the movement of the charged ligand into the voltage drop across the channel to reach its binding site and / or a voltage-driven conformational alteration in RyR leading to altered affinity of the receptor. In this chapter I have described experiments that demonstrate that the apparent dissociation constant of neutral ryanoid, ryanodol, and the charged ryanoids (8 β -amino-9 α -hydroxyryanodine ($+1$) and 10-O-succinoylryanodol (-1)) decrease as the potential at the cytosolic face of the channel is made increasingly positive and that this results from alterations in both the rates of ryanoid association with and dissociation from its receptor. This observation provides very strong evidence in support of a voltage-driven alteration in ryanoid receptor affinity as the major determining factor in the influence of transmembrane holding potential on the interactions of ryanoids with the RyR channel (Fig. 3.12).

While this mechanism underlies the influence of voltage on the interaction of all the ryanoids, the experiments indicate that an additional voltage-dependent effect can be observed with the charged ryanoids. This may reflect the movement of the charged moiety from the cytosolic bulk solution to a binding site within the voltage drop across the channel.

Chapter 4

The interaction of ryanoids with the RyR channel in the presence of blocking cations

4.1 Introduction

The location of the high affinity ryanoid binding site in the RyR channel is still a matter for speculation. A considerable amount of information appears to be consistent with the proposal that the site is located in the conduction pathway. The results from the single channel studies carried out in the previous chapter provide strong evidence that ryanoids interact at a site within the conduction pathway that is under the influence of an electrical field. Point mutations made in the putative selectivity filter sequence (which is thought to be within the voltage drop) of the RyR channel, produce alterations in the association of ryanodine with the channel both in ryanodine binding and single channel studies (Chen *et al.*, 2002). Both of these observations suggest that the high affinity ryanoid binding site is located someway within the voltage drop across the channel. If access to the high affinity ryanoid binding site is from the cytoplasmic side of the channel (Tanna *et al.*, 1998) and if this 'site' is located within the voltage drop across the channel (Tanna *et al.*, 1998), it is possible that an impermeant cation that interacts at a site within the voltage drop will impede the passage of a ryanoid to its site. The experiments described in this chapter were designed to test this hypothesis by monitoring the influence of impermeant cations that interact at two distinct sites within the voltage drop, on the interaction of ryanoids with the RyR channel.

4.2 Experimental procedures

4.2.1 [^3H]-ryanodine binding

HSR membrane vesicles (prepared using procedures described in section 2.1 Chapter 2) were diluted to 50-100 μg protein / ml of which up to 10 μl were incubated in buffer medium at 37°C with constant shaking. The buffer medium, designed to optimise full channel opening, contained of 1 M KCl, 1 mM ATP, 100 μM total Ca^{2+} , 10 mM PIPES, pH 7.4, and 5 nM [^3H]-ryanodine. The samples were incubated for the times indicated in the text $\pm$ TEA and TBA at the concentrations indicated, in a final volume of 1 ml. All assays were performed in triplicate and the number of separate HSR preparations

examined is detailed in the results section. Non-specific binding was determined in the presence of a 1000 fold excess (5 μ M) of unlabelled ryanodine. Following incubation, binding was terminated by the addition of 5 ml of ice-cold buffer medium (mentioned above). Bound [^{3} H]-ryanodine was separated from unbound by vacuum filtration through Whatman GF-B filters pre-soaked in buffer medium. Filters were washed a further two times with 5 ml aliquots of buffer kept at room temperature, to remove residual unbound [^{3} H]-ryanodine. Filters were placed in 10 ml of aqueous UltraGold MV scintillant (Packard), vortexed and left to soak for at least 24 hours, before [^{3} H] was quantified by liquid scintillation counting. The filters were soaked for 24 hours to allow the filters to equilibrate with the scintillant. [^{3} H]-ryanodine binding data are displayed as pmoles of [^{3} H]-ryanodine bound per mg of membrane protein.

4.2.2 Variation in binding with increasing blocking cation concentration

The binding activities obtained at increasing blocker concentration were fitted with equation 4.1 describing a sigmoidal inhibition of binding activity:

$$b = \frac{b_0}{1 + ([blocker]/IC_{50})^n} \quad \text{equation 4.1}$$

Where b is the [^{3} H]-ryanodine binding at [blocker] and b_0 represents maximum binding i.e. in the absence of added blocker, IC_{50} represents the concentration of blocker at which b is 50% of the maximum. n is the Hill coefficient.

4.2.3 Single channel experiments

The experiments in this chapter that involve incorporation of single purified RyR channels into artificial planar lipid bilayers were carried out using the methods described previously in Chapter 2. The experiments are carried out in symmetrical 610 mM KCl bathing solution in the presence of cytosolic 500 nM 21-amino-9 α -hydroxyryanodine $\pm$ symmetrical TEA at the concentrations described in the text. All experiments were carried out at room temperature. Transmembrane holding potential was held at 40 mV and each run was recorded for 6 minutes. Data were recorded and analysed as described in Chapter 2.

4.3 The interactions of blocking cations with the RyR channel

4.3.1 Tetraethylammonium (TEA)

Single channel studies have shown that K^+ current through the purified sheep cardiac RyR channel is inhibited from the cytosolic side of the channel by the commonly used K^+ channel blocking cation, TEA. With K^+ as the permeant ion, an increase in the degree of block is observed with rising TEA concentrations and with increasing transmembrane holding potentials to high positive voltages (Tinker *et al.*, 1992b). In each case an increase in block is observed as a reduction in single channel current amplitude, this is consistent with the cation entering the voltage drop of the RyR channel and blocking K^+ conductance. The dwell time of TEA within the voltage drop is so short that individual blocking events can not be resolved and so block appears as a time-averaged reduction in single channel current amplitude. Block observed with increasing TEA concentration is described by Michaelis-Menten saturation kinetics, which suggests that TEA is acting at a single site within the voltage drop across the channel. Consistent with this observation the reduction in single channel current amplitude seen with TEA at any particular voltage can be fitted by the Woodhull equation that also assumes that there is a single TEA binding site accessible from one side of the channel and located within the voltage drop across the channel. These data establish that the blocking cation interacts at a site that is located 90% into the voltage drop from the cytosolic face of the channel.

4.3.2 Tetrabutylammonium (TBA)

TBA modifies K^+ conductance in the RyR channel, however, the block observed by TBA, is not of the form reported above for TEA (Tinker *et al.*, 1992a). Instead of TBA completely blocking current flow, the RyR channel appears to enter a clearly resolved reduced conductance state, in addition of normal full-opening events. The occurrence of the reduced conductance state is both concentration and voltage dependent, this being evident only at positive holding potentials (Tinker *et al.*, 1992a). The voltage dependence of block suggests that more than one TBA molecule interacts with the RyR channel, it has been suggested that two or more TBA ions bind with a site at the cytosolic extremity of the voltage drop. Although, the cation may not fully occlude the channel and block K^+ conductance, the TBA cations could partially occlude the channel

or their presence could modify the surface charge around the entrance of the voltage drop and provide an electrostatic barrier for permeant ion translocation.

[³H]-ryanodine binding studies were carried out in the presence of either TEA and TBA, to observe if their presence influences the interaction of ryanodine with its site.

4.4 The influence of blocking cations on the interaction of [³H]-ryanodine with the high affinity ryanodine binding site of the RyR channel.

The effect of blocking cations on ryanodine binding is shown in Figure 4.1. In order to maximise the open probability of the channel and hence [³H]-ryanodine binding, experiments were carried out in the presence of 100 μ M Ca²⁺, 1 mM ATP and 5 nM [³H]-ryanodine. The results demonstrate that in the presence of 500 mM TEA or 20 mM TBA, [³H]-ryanodine binding is reduced over a period of 90 minutes when compared to binding observed in the absence of a blocker. Since these data demonstrate that the presence of blocking cations inhibit ryanodine binding, the concentration dependence of these phenomena was investigated. For these experiments, samples were incubated with increasing concentrations of blocker for 10 minutes, as this was sufficient time to resolve inhibitory effect (Figure 4.1).

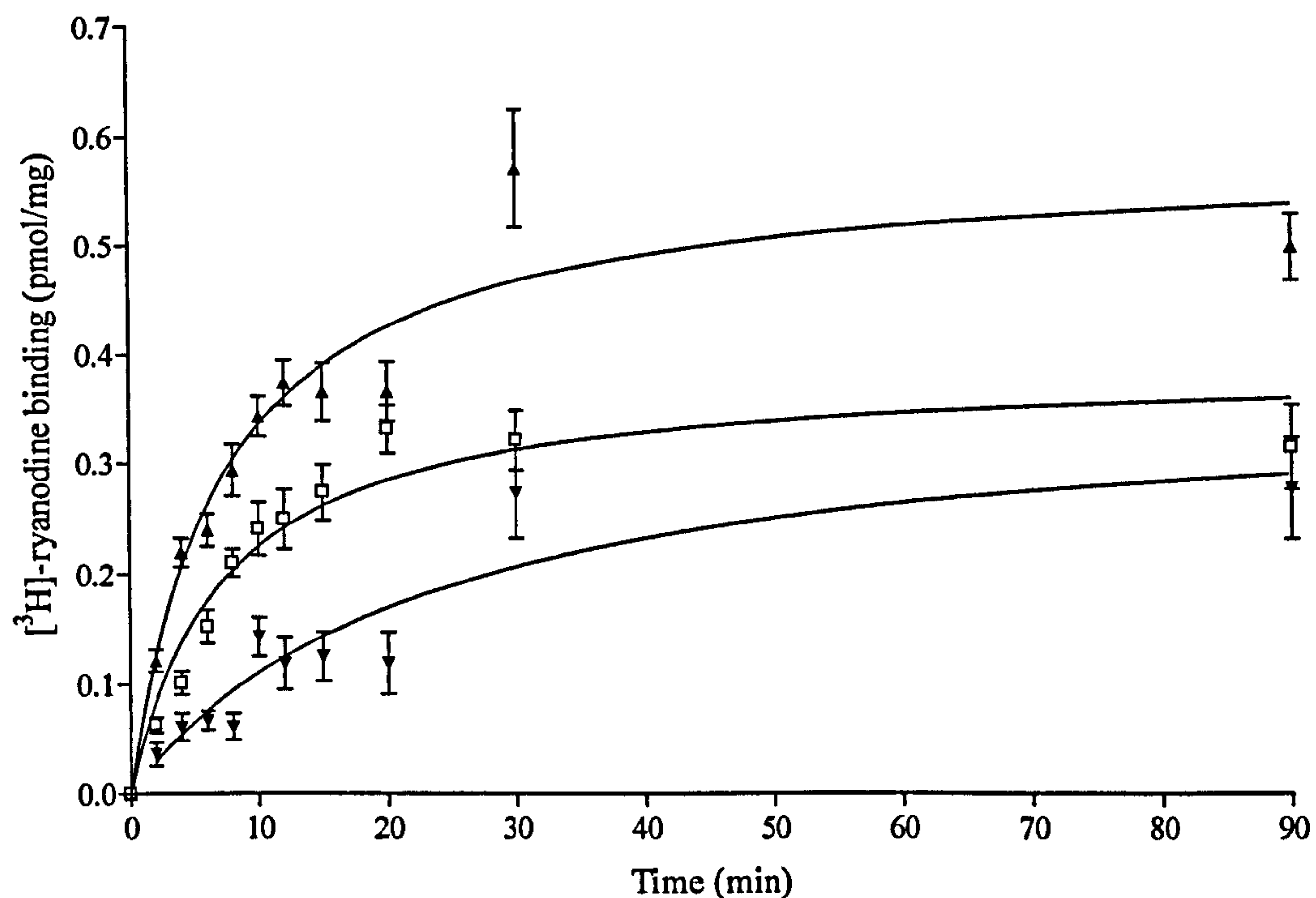


Figure 4.1 The effects of no blocking cation (▲), 500 mM TEA (▼) or 20 mM TBA (□) on the time course of [^{3} H]-ryanodine binding. Each point is the mean and SEM of 11-48 assays from 4-5 preps. Solid lines are best fit rectangular hyperbole obtained by non-linear regression.

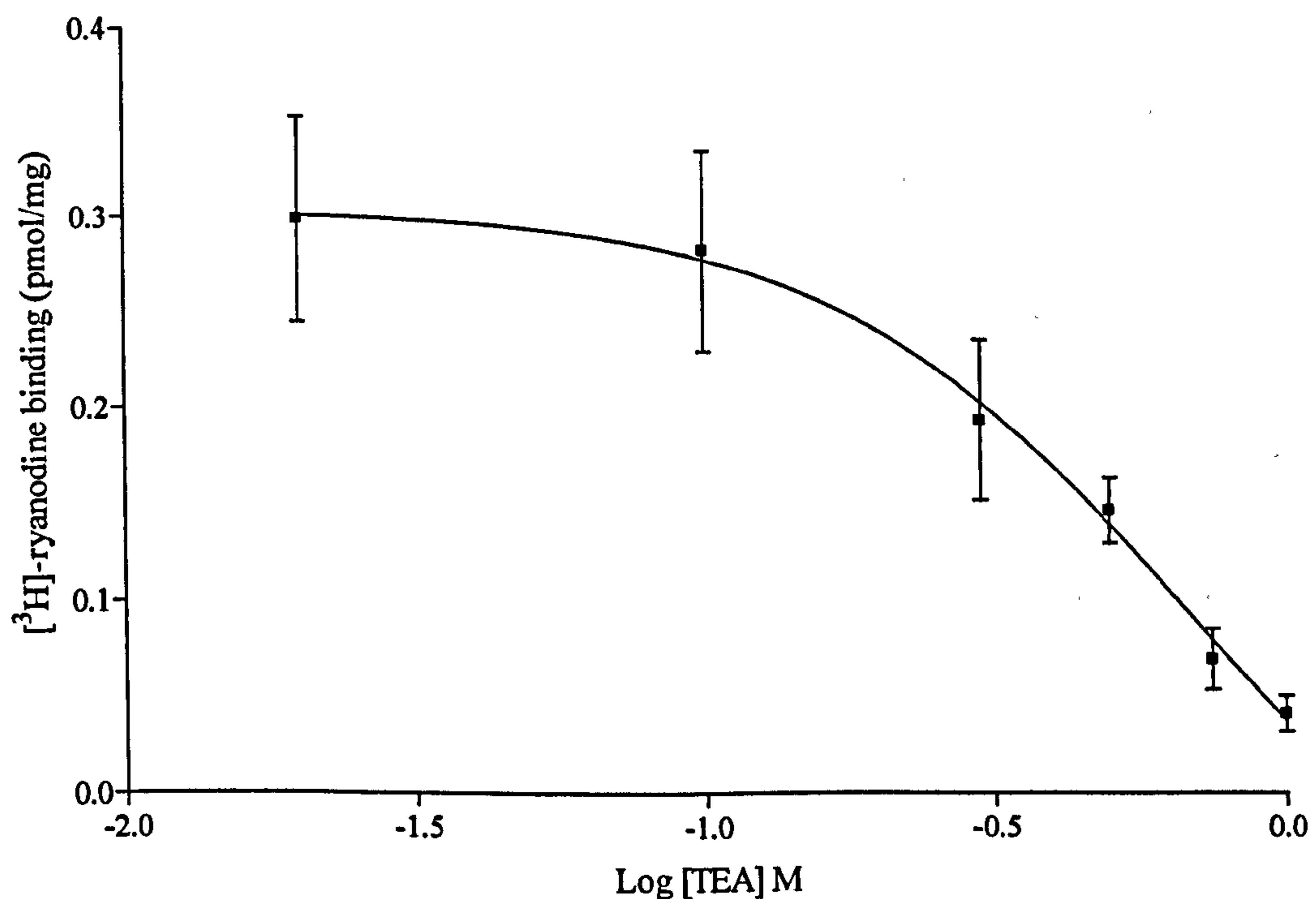


Figure 4.2 The relationship between [^{3} H]-ryanodine binding and [TEA], the samples were incubated for 10 minutes. Each point is the mean $\pm$ SEM of 12-21 assays from 4-5 HSR preps. The solid line is the best fit to Eq. 4.1 obtained by non-linear regression with an IC_{50} of 668 mM.

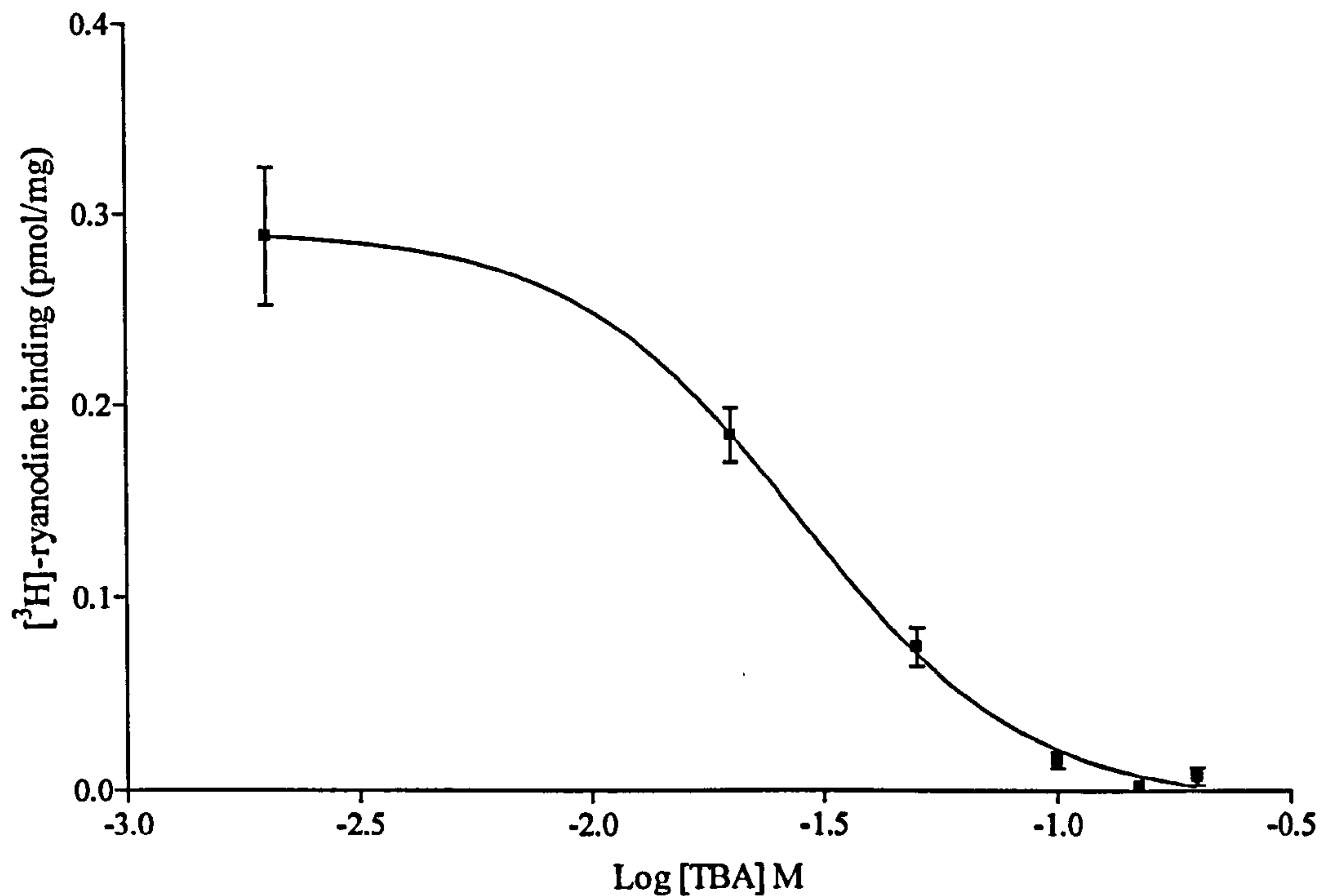


Figure 4.3 The relationship between [³H]-ryanodine binding and [TBA], the samples were incubated for 10 minutes. Each point is the mean $\pm$ SEM of 12-33 assays from 4-5 HSR preps. The solid line is the best fit to Eq. 4.1 obtained by non-linear regression with an IC_{50} of 28 mM.

Figure 4.2 and 4.3 show that TEA and TBA reduce [³H]-ryanodine binding in a concentration-dependent manner. The solid lines in these figures are best fits to Eq. 4.1 obtained by non-linear regression, and gave IC_{50} values of 668 mM for TEA and 28 mM for TBA. These data indicate that the presence of impermeant ions that bind at two distinct sites reduces the interaction of [³H]-ryanodine with the RyR channel. This suggests that both of these blockers and ryanodine compete for access to their respective binding sites. To obtain an insight into the mechanism that governs this process, single channel experiments were carried out to observe the effect of an impermeant ion within the voltage drop on the interaction of a ryanoid with the RyR channel.

4.5 The influence of TEA on the interaction of the positively charged ryanoid 21-amino-9 α -hydroxyryanodine with single RyR2 channels.

The interaction of 21-amino-9 α -hydroxyryanodine with the RyR channel was investigated only in the presence of TEA. TBA only partially blocks the RyR channel and results in a reduced conductance state, this with the additional reduced conductance state observed following the interaction of the ryanoid would make interpretation of single channel results very difficult. As a consequence single channel experiments involving the simultaneous addition of 21-amino-9 α -hydroxyryanodine and TBA were not practical.

The interaction of 21-amino-9 α -hydroxyryanodine with the RyR channel was monitored in the absence and presence of TEA. Figure 4.4 shows the influence of increasing TEA concentrations on the probability of the interaction of 500 nM 21-amino-9 α -hydroxyryanodine with a single RyR channel at a holding potential of 40 mV. In the absence of added TEA the characteristic, reversible modification of the RyR channel gating and ion handling by 21-amino-9 α -hydroxyryanodine is observed (Tanna *et al.*, 1998). The presence of increasing concentrations of TEA modifies various aspects of RyR channel function. As expected, TEA produces a concentration-dependent reduction of K⁺ conductance in RyR channel when 21-amino-9 α -hydroxyryanodine is not bound (Lindsay *et al.*, 1991; Tinker *et al.*, 1992b). TEA produces a qualitative similar reduction in K⁺ conductance in the ryanoid modified state of the channel, however the degree of block is noticeably lower than that seen in the unmodified channel. Clearly, while the binding of 21-amino-9 α -hydroxyryanodine to its binding site on the RyR channel alters the manner in which ions are translocated in the channel, TEA can still enter the voltage drop and reduce K⁺ conductance when the ryanoid is bound. In addition to modifying ion translocation through the RyR channel, TEA also influences the likelihood of the interaction of 21-amino-9 α -hydroxyryanodine with its binding site on the channel. The probability of channel modification by 21-amino-9 α -hydroxyryanodine is markedly reduced by the increasing concentrations of TEA (Figure 4.4). The influence of TEA concentration on the P_{mod} of a number of channels is shown in Figure 4.5. P_{mod} declines from 0.70 ± 0.03 at 0 mM TEA to 0.41 ± 0.06 with 80 mM TEA. An inspection of the apparent rate constants for the association and dissociation of 21-amino-9 α -hydroxyryanodine with increasing concentrations of

TEA (Figure 4.6), indicates that the reduction in P_{mod} illustrated in Figure 4.5 results from a concentration-dependent linear reduction in k_{on} with a slope of $-0.008 \pm 0.001 \text{ s}^{-1} \text{ mM}^{-1}$. TEA has no significant effect on k_{off} (the mean value across the concentration range is $0.24 \pm 0.02 \text{ s}^{-1} \text{ mM}^{-1}$).

In the light of the evidence obtained in the [^3H]-ryanodine binding experiments, the reduction in k_{on} is consistent with competition between 21-amino-9 α -hydroxyryanodine and TEA for access to their respective binding sites.

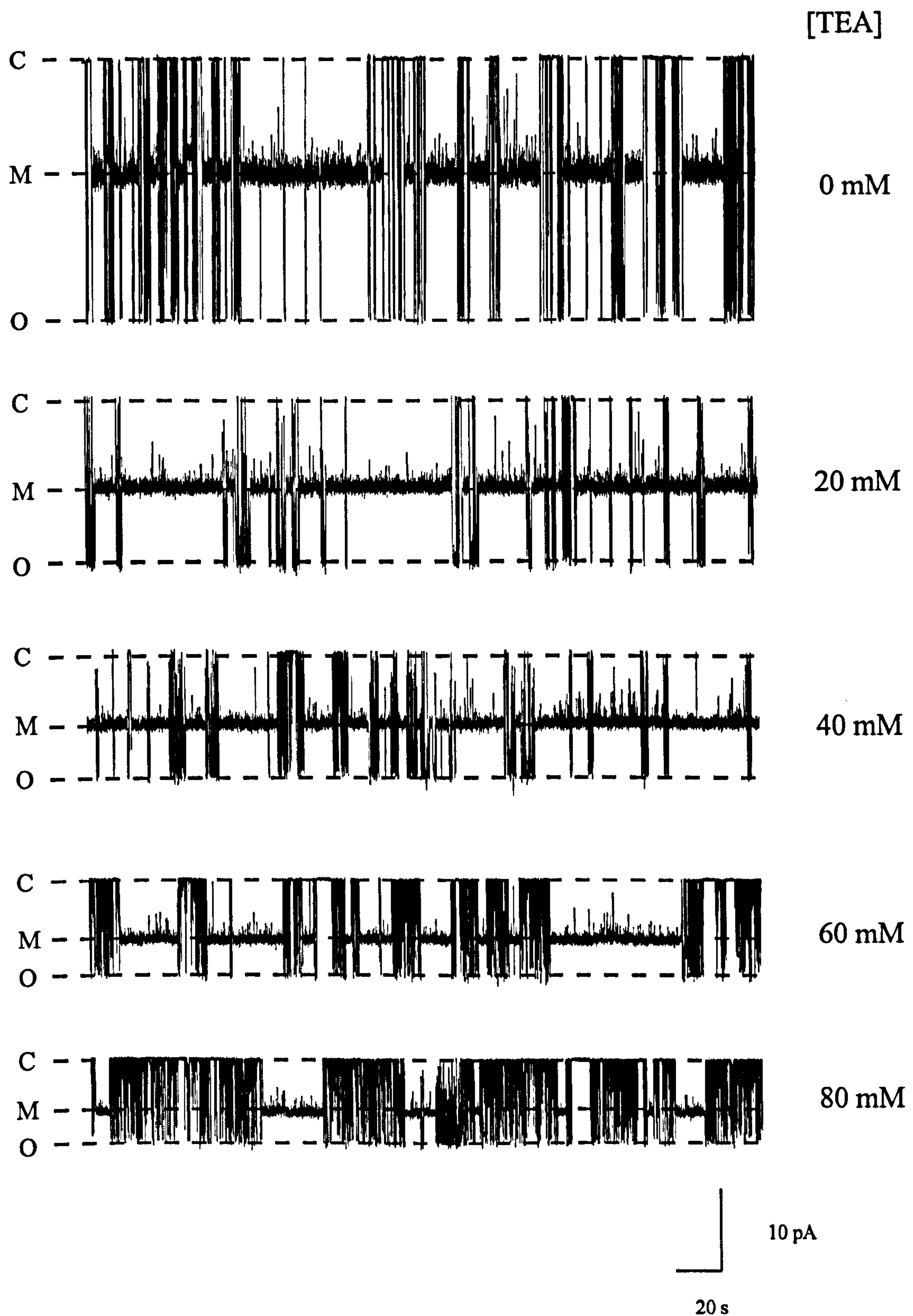


Figure 4.4 Traces obtained from a single RyR channel in symmetrical 610 mM K^+ with 500 nM 21-amino-9 α -hydroxyryanodine in the solution at the cytosolic side of the channel. The indicated concentrations of TEA were added to both the cytosolic and luminal solution. Holding potential is 40 mV. O – open, C – closed and M – modified.

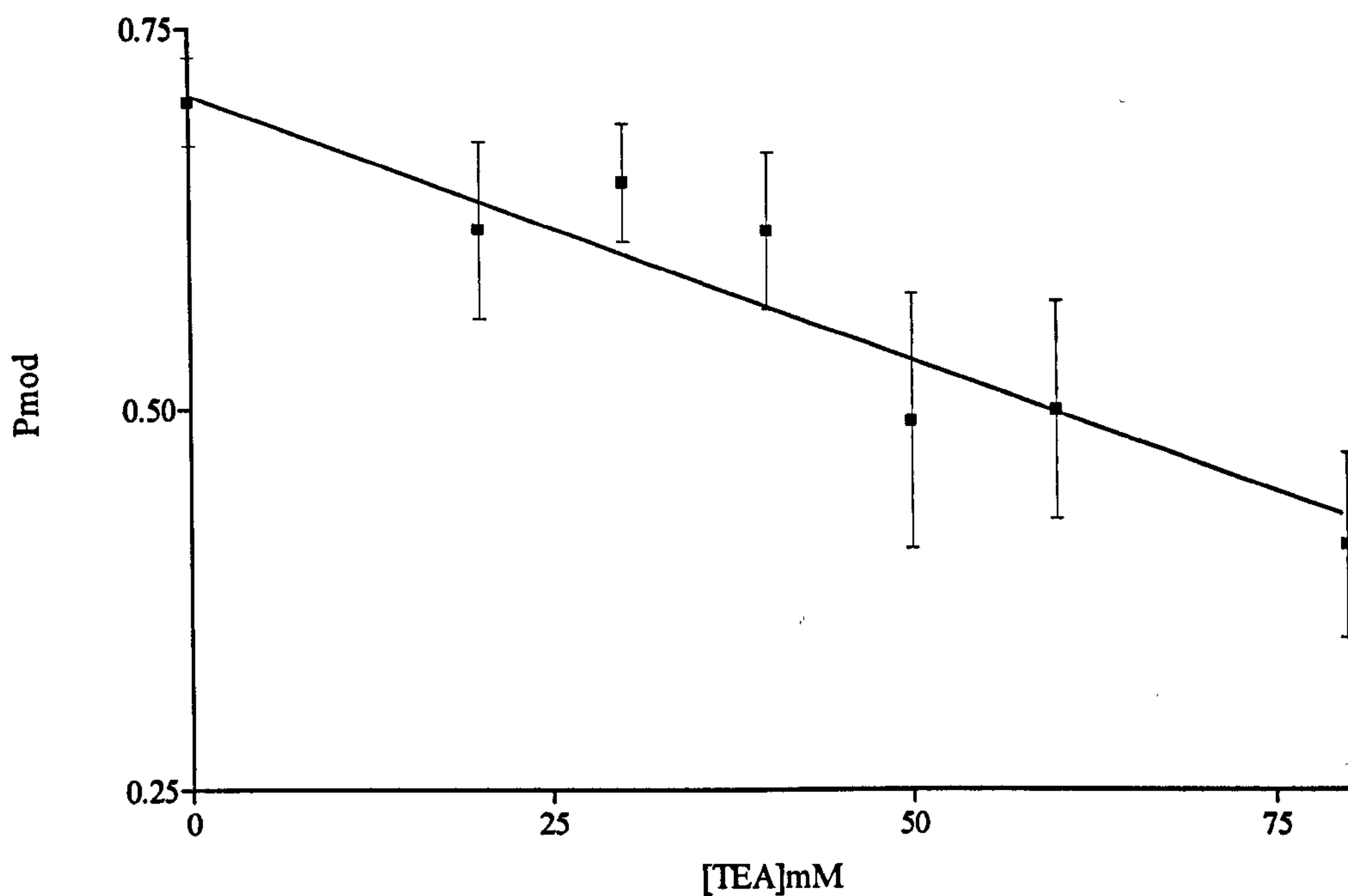


Figure 4.5 The relationship between probability of RyR channel modification by 21-amino-9 α -hydroxyryanodine and [TEA]. P_{mod} is determined by monitoring dwell times in the unmodified and modified conductance states in 6 minute recording with 500 nM cytosolic 21-amino-9 α -hydroxyryanodine at a holding potential of 40 mV. Each point is the mean $\pm$ SEM of 4-18 experiments.

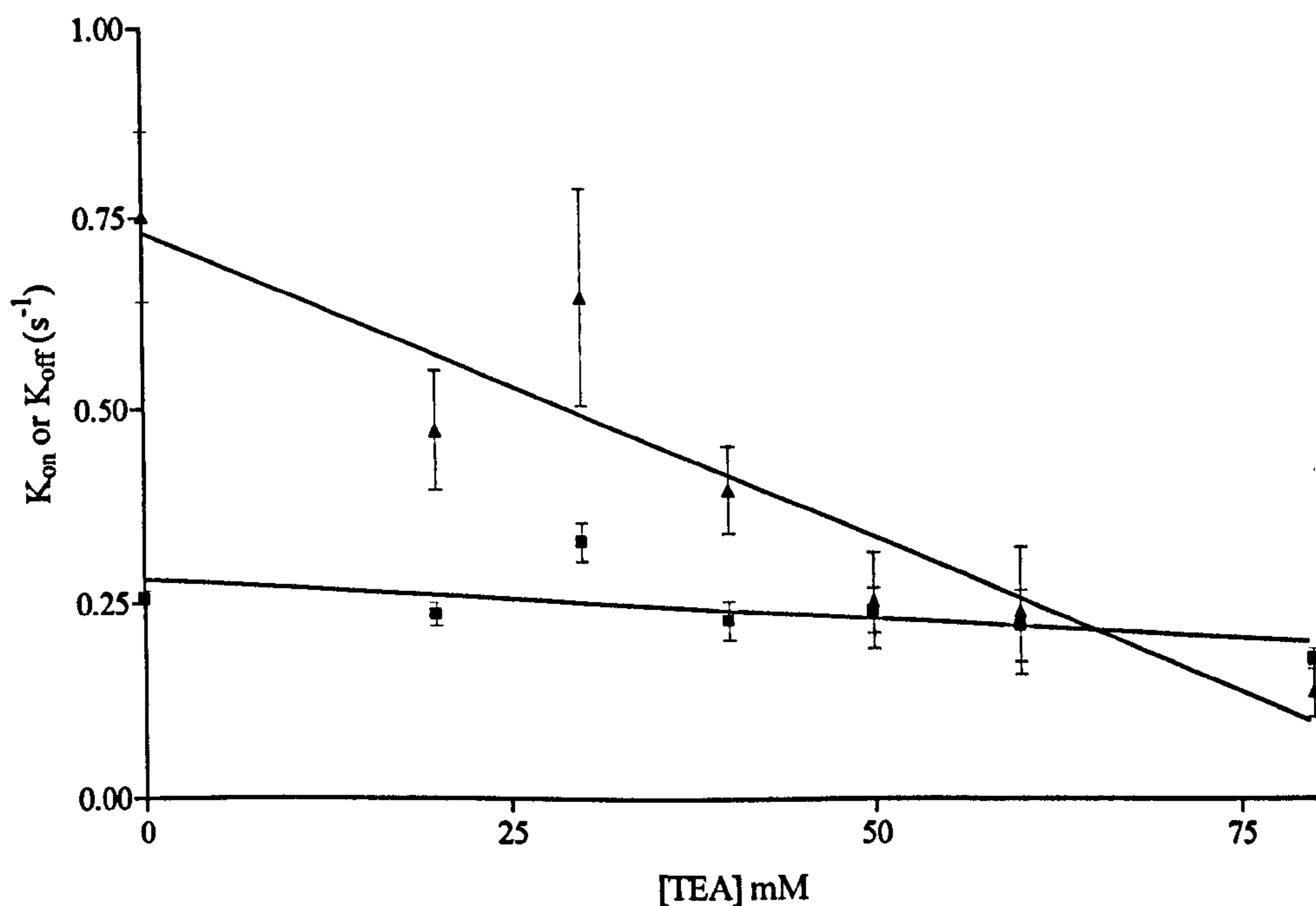


Figure 4.6 The influence of changing [TEA] on rates of association ($\blacktriangle$) and dissociation ($\blacksquare$) of 21-amino-9 α -hydroxyryanodine with the RyR channel. The ryanoid (500 nM) is present in the solution at the cytosolic side of the channel and the holding potential is 40 mV. Each point is the mean $\pm$ SEM of 4-18 experiment. Lines drawn through the points were obtained by linear regression with parameters quoted in the text.

4.6 Discussion

[³H]-ryanodine binding experiments carried out in this chapter demonstrate that the interaction of ryanodine with the RyR channel is affected by the presence of both TEA and TBA blocking cations. Figure 4.1 shows that over a 90 minute period an increase in [³H]-ryanodine binding is observed in the absence of any blocker, however [³H]-ryanodine binding is reduced in the presence of TEA and TBA. Both TEA and TBA are reversible blockers (Lindsay *et al.*, 1991; Tinker *et al.*, 1992 a and b) and the rates of association and dissociation of ryanodine with the RyR channel are very slow so that binding is essentially irreversible. Therefore, it is not surprising that over time an increase of ryanodine binding is observed, once ryanodine interacts with the channel it stays bound, while the blockers dissociate from their respective sites on the channel. Consequently to observe measurable inhibition of [³H]-ryanodine binding in the presence of TEA and TBA samples were incubated for 10 minutes. Increasing blocker concentrations decrease ryanodine binding. The higher affinity of the RyR channel for TBA than for TEA observed in the binding experiments reflects the relative potency of these cations as blockers in single channel experiments. 50% block is observed with μ M and mM concentrations for TBA (Tinker and Williams, 1993) and TEA (Tinker *et al.*, 1992 a and b) respectively in the absence of a ryanoid. The observed reduction in ryanodine binding in the presence of TEA or TBA is consistent with the proposal that some form of competition between ryanodine and both the blockers takes place for access to their respective sites within the voltage drop across the RyR channel. This report is also in agreement with similar competition investigations carried out previously with the RyR channel blocker neomycin (Mead *et al.*, 1998; Mead and Williams, 2002a and 2002b), which demonstrated a dose dependent inhibition of ryanodine binding (Wang *et al.*, 1996).

The interaction of a reversible ryanoid with single RyR channels in both the absence and presence of TEA was monitored in single channel studies, to examine the mechanism involved with the observed decrease in ryanodine binding.

Analogous observations in other channels have demonstrated that the interaction of both charybdotoxin (Miller, 1998) and the *Shaker B* inactivation peptide (Toro *et al.*, 1992) with the Ca²⁺-activated K⁺ channels can be influenced by the presence of voltage-

dependent blocking cations. In both cases it was established that TEA reduced the likelihood of peptide interaction by competing for access to the conduction pore (voltage drop) of the channel. Indeed TEA and charybdotoxin are mutually exclusive; during TEA occupancy charybdotoxin has no access to its binding site. It has been suggested that electrostatic repulsion between TEA, which interacts with a site located at the external mouth of the conduction pore (voltage drop) and charybdotoxin is sufficient to prevent the entry of the toxin into the Ca^{2+} -activated K^+ channel (Miller, 1998). The experiments carried out in this chapter show that RyR channel occupancy by TEA reduces the likelihood of channel modification by 21-amino-9 α -hydroxyryanodine. This observation is superficially similar to the effect of TEA on the interaction of charybdotoxin with Ca^{2+} -activated K^+ channels. However, there are some important differences. TEA and 21-amino-9 α -hydroxyryanodine are not mutually exclusive, certainly TEA can enter the voltage drop of the RyR channel and influence K^+ translocation while 21-amino-9 α -hydroxyryanodine is bound. Nevertheless, TEA does reduce the likelihood of 21-amino-9 α -hydroxyryanodine binding to the channel. The monitored reduction in P_{mod} reflects a lowering of the apparent rate of association of the ryanoid with its receptor while the rate of dissociation of 21-amino-9 α -hydroxyryanodine is unaffected by TEA. These findings suggest that the presence of blocking cations reduce the probability of ryanoid interaction with the high affinity site on the RyR channel. TEA competes with 21-amino-9 α -hydroxyryanodine for access to a region of the RyR channel that contains the binding sites for both ligands. Given that the interaction of both ligands with the RyR is voltage-dependent, it might be proposed that competition occurs within the voltage drop across the channel.

The presence of 21-amino-9 α -hydroxyryanodine at its high affinity binding site reduces the effectiveness of TEA as a blocker of K^+ translocation in the RyR channel. At this stage there is no firm information on the mechanism involved in this process. Bound 21-amino-9 α -hydroxyryanodine might limit the access of TEA to its blocking site by either steric or electrostatic means. Alternatively the properties of the TEA site may be altered by a ryanoid-induced conformational change (Lindsay *et al.*, 1994; Tanna *et al.*, 1998). Investigations in the next chapter describe this phenomenon in greater detail.

Chapter 5

Relocation of the TEA binding site

5.1 Introduction

The reduced conductance state induced by the interaction of ryanodine and other ryanoids with the RyR channel could result from either a simple blocking of the channel by the ryanoid or a conformational change in the channel protein. All evidence suggests that the interaction of ryanodine with the RyR channel result in alterations to many of the processes that underlie single-channel conductance. Alterations occur in the affinity of the conduction pathway of the channel for some permeant ions and the relative permeability of some ions is changed. Ryanoid-induced altered mechanisms of ion handling and the resultant, ryanodine-induced, conductance varies with the nature of the permeant ion. Whilst in the presence of most permeant ions the ryanodine-modified state results in a reduction in current amplitude, the FC state observed in the presence of the organic cation diethylamine (120%) is greater than the single channel current amplitude obtained in the absence of ryanodine. These observations led to the suggestion that it is not simply the blocking or occlusion of the conduction pathway by ryanodine that results in a reduction of current flow through the channel. Rather, the interaction of ryanoids with the RyR channel modifies ion handling in the channel, which results from a conformational change of the channel protein, involving a rearrangement of the conduction pathway (Lindsay *et al.*, 1994).

Single channel studies involving impermeant organic cations, have enabled us to obtain a better insight of the dimensions of the voltage drop across the RyR channel and the location of binding sites within this region (Tinker *et al.*, 1992a; Tinker and Williams, 1993, 1995). For example the site of interaction of the small TAA, TMA is located approximately 50% within the voltage drop from the cytosolic face of the channel, while TEA and TPrA interact at a site approximately 90% within the voltage drop from the cytosolic face of the channel.

Studies on the ryanoid-modified state have revealed changes in ion handling properties of permeant ions translocating the channel as described previously and above. The purpose of this chapter was to examine if the proposed, ryanoid-induced,

conformational alterations of the conduction pathway that results in modified handling of permeant ions produce changes in the way impermeant ions interact with the RyR channel. This issue was addressed by quantitative analysis of single channel voltage-dependent K^+ current block by TEA in the presence of a series of ryanoids.

5.2 Experimental procedures

In this chapter all the experiments involve incorporation of single purified RyR channels into artificial planar phospholipid bilayers, using procedures described previously in detail in Chapter 2. All the experiments are carried out in a symmetrical 610 mM KCl bathing solution $\pm$ 20 mM TEA in the presence of either 500 nM 21-amino-9 α -hydroxyryanodine, 2 μ M ryanodol, 1 μ M 21-azido-9 α -hydroxyryanodine or 100 nM 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine at the cytosolic face of the channel. Channel activity was recorded for 6 minute periods at the transmembrane holding potentials indicated in the text. Data were recorded and analysed as described in Chapter 2.

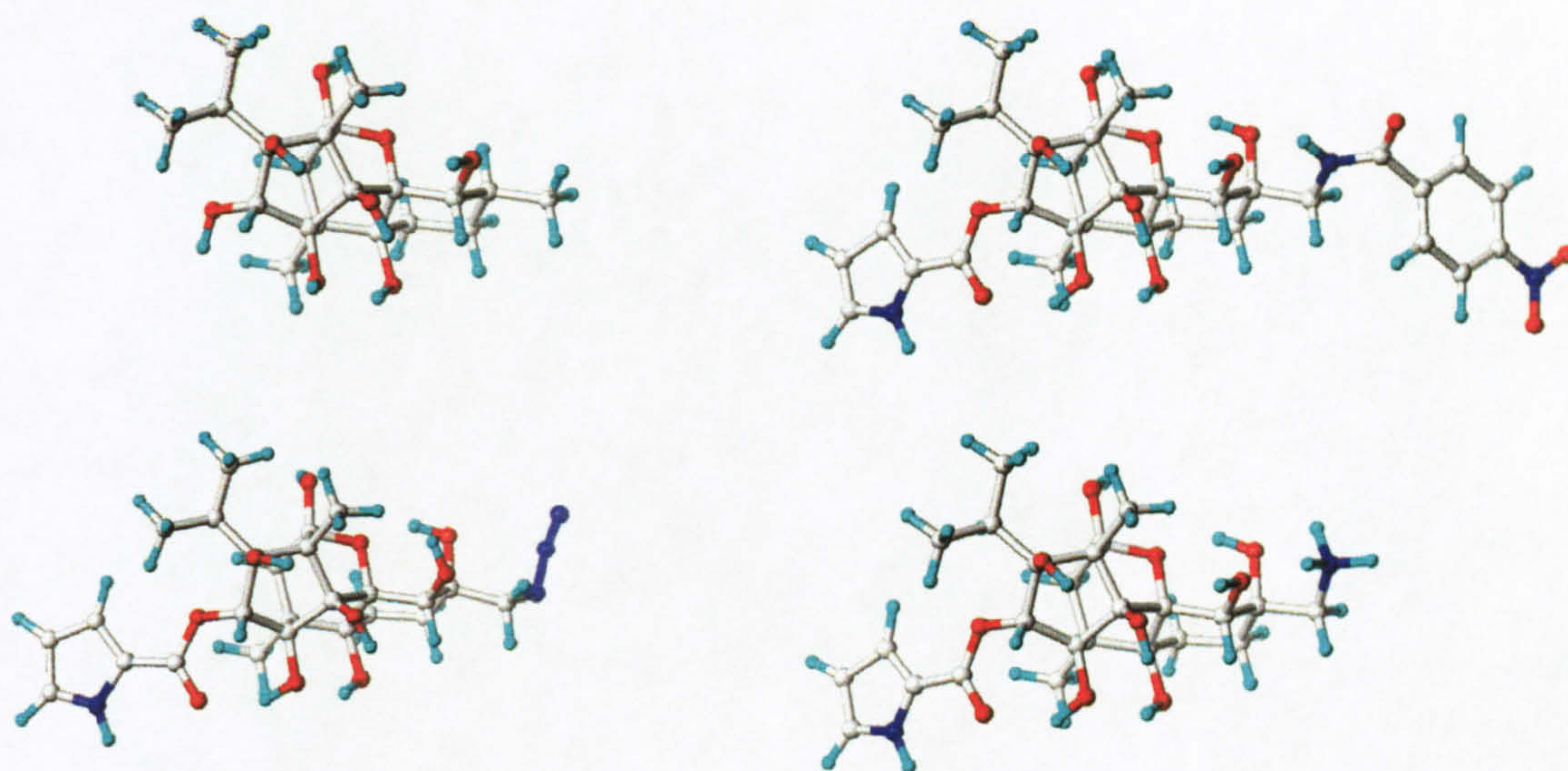
As more than one modified conductance state was observed after the addition of 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine to the RyR channel (see Results), the purity of the compound was reinvestigated to ensure that there had been no decomposition during storage. Samples used in the experiments reported here were found to be homogeneous by TLC (thin layer chromatography) (both charring and fluorescence detection) and by MALDI / TOF (matrix-assisted laser desorption and ionisation / time of flight) mass spectroscopy carried out by J. J. Hull (University of Nevada).

5.3 Molecular Modelling

Figure 5.1 shows ball and stick drawings of a) 21-amino-9 α -hydroxyryanodine (bottom right), 21-azido-9 α -hydroxyryanodine (bottom left), ryanodol (top left), and 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine (top right). All are shown in the conformation corresponding to their respective global minima. Although all of these ryanoids can exist in multiple conformations, the bulk of the long floppy tail of 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine makes the conformers of this ryanoid of experimental interest. Molecular dynamics (SYBYL) simulations indicate that the side

chain exists in three conformational families arising principally from the high rotational energy barriers about the single bond proximal to carbon 9. The local minimum of each of these families is shown in Figure 5.1 b, and the significance of the existence of these conformers is discussed later.

a



b

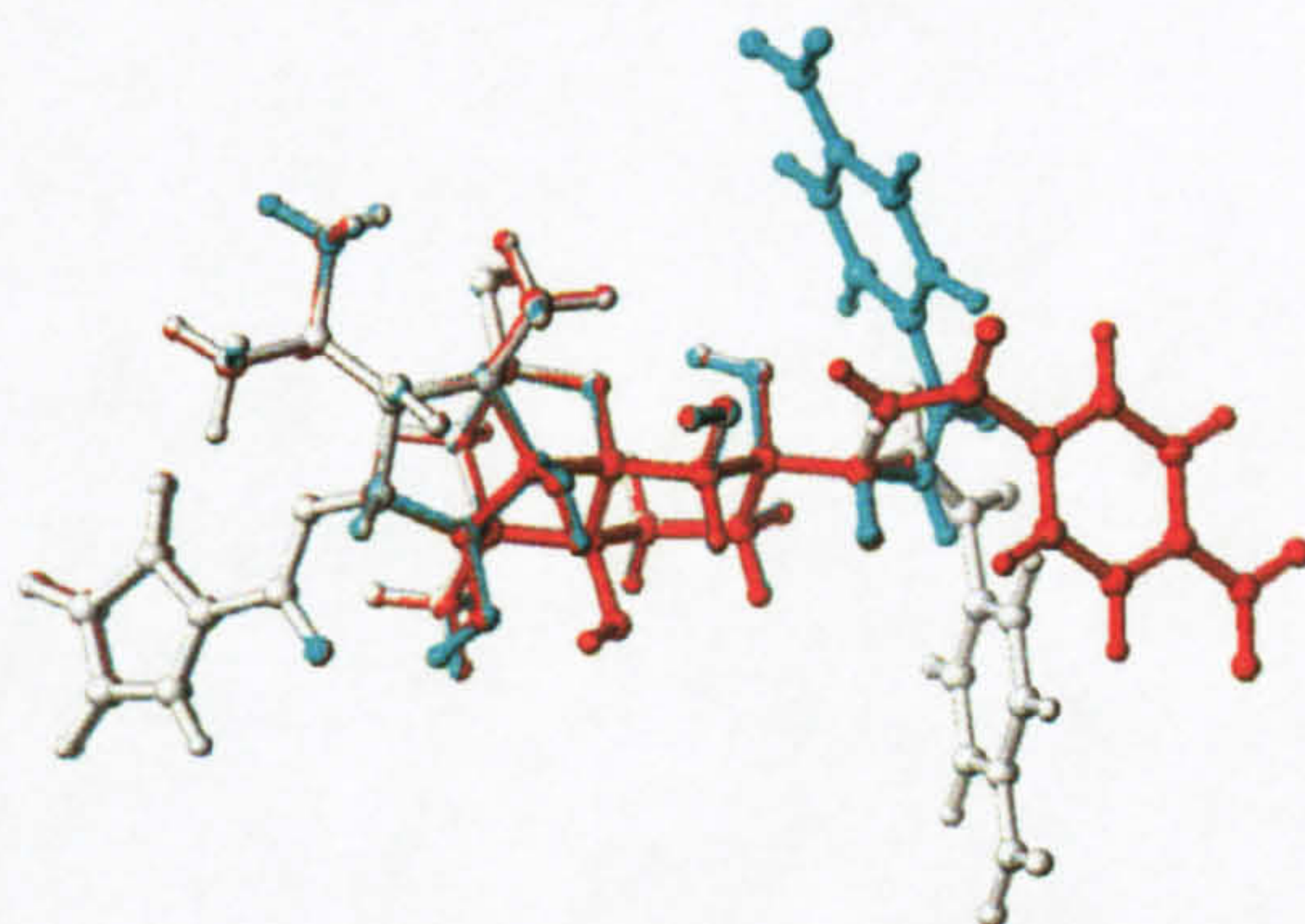


Figure 5.1 a) Ball and stick models of 21-amino-9 α -hydroxyryanodine (bottom right), 21-p-nitrobenzoylamino-9 α -hydroxyryanodine (top right), ryanodol (top left), and 21-azido-9 α -hydroxyryanodine (bottom left) are shown in the conformation corresponding to the respective global energy minimum. All are orientated with the 3-position to the left (note the pyrrole group at the extreme left) and the 21-position to the right (a horizontal line connects these two positions). Ryanodol (top left) is the core structure of all of the other compounds illustrated in this figure. **b)** The local minima of the three most populated conformations of 21-p-nitrobenzoylamino-9 α -hydroxyryanodine are superimposed by the polycyclic fused ring system. The conformational space was investigated by molecular dynamics at a simulated temperature of 25°C. Note the changes in physical shape of the molecule due to large movements of the p-nitrobenzoyl group compared with the other pendent groups.

5.4 Results

The influence of voltage on TEA block of the unmodified and ryanoid-modified RyR channels was monitored in the presence of four reversible ryanoids.

5.4.1 *The influence of 21-amino-9 α -hydroxyryanodine interaction with the RyR channel on TEA block*

Current fluctuations of a single RyR channel in the presence of 21-amino-9 α -hydroxyryanodine as transmembrane holding potential is increased to high positive potentials are shown in the left panel of Figure 5.2. As described before, the probability of 21-amino-9 α -hydroxyryanodine associating with the RyR channel increases while the rate of dissociation of the ryanoid from the channel decreases as the holding potential is made more positive. The right panel of Figure 5.2 shows current fluctuations of the same channel in the presence of 20 mM TEA added to the solution at both sides of the channel. In the previous chapter the interaction of TEA with single RyR channel has already been described as a concentration and a voltage dependent block of the RyR channel. Dwell times in the TEA blocked state are too short to be resolved and, as a consequence, RyR receptor block by this cation appears as a time-average reduction in single-channel current amplitude (Lindsay *et al.*, 1991 and Tinker *et al.*, 1992b). This is evident from the traces in the right panel of Figure 5.2, in the periods of unmodified gating when the ryanoids is not bound to the channel. In agreement with previous observations (Lindsay *et al.*, 1991), the degree of block of the unmodified state of the channel by TEA varies as transmembrane holding potential is changed. Block by TEA increases as the holding potential is made more positive.

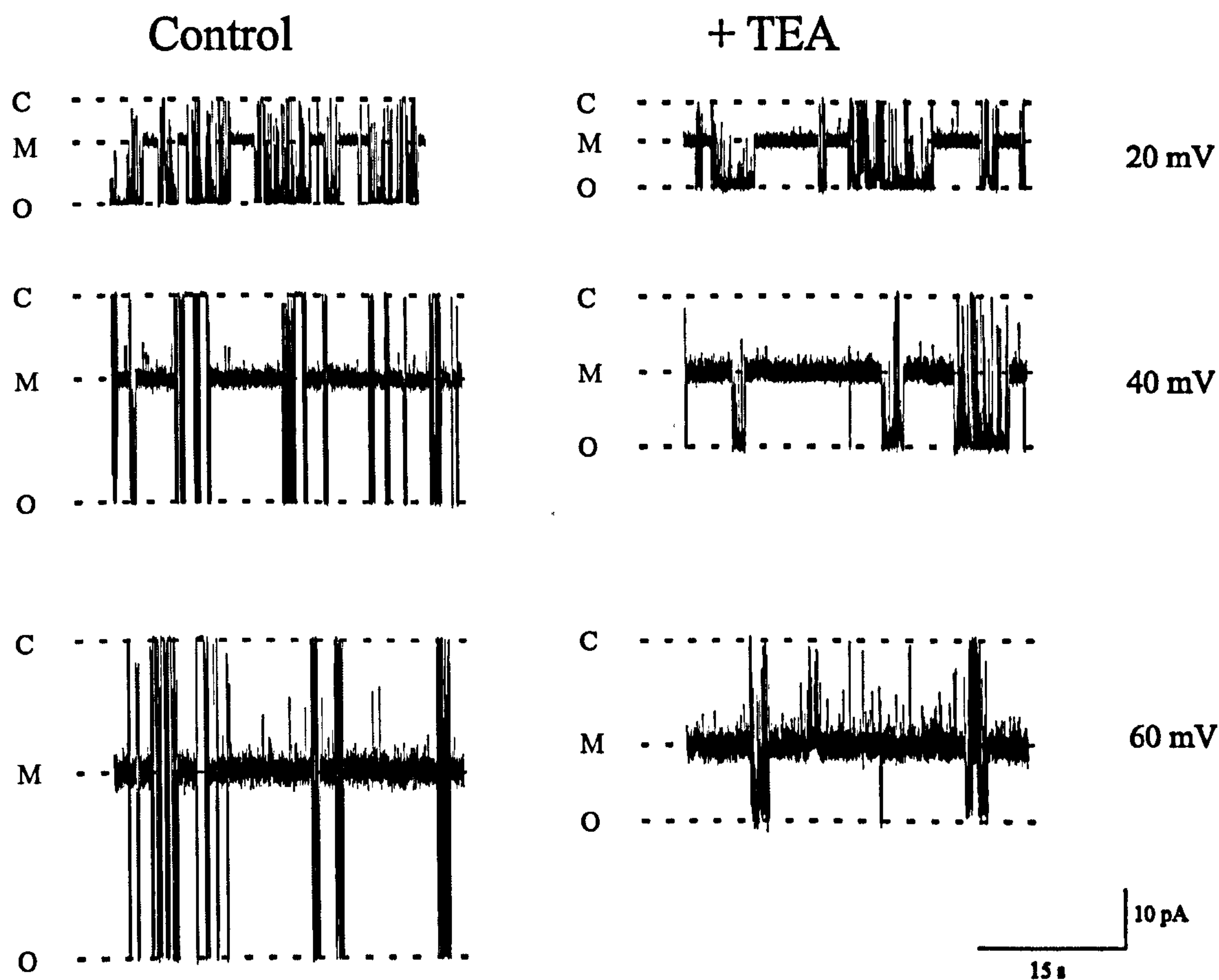
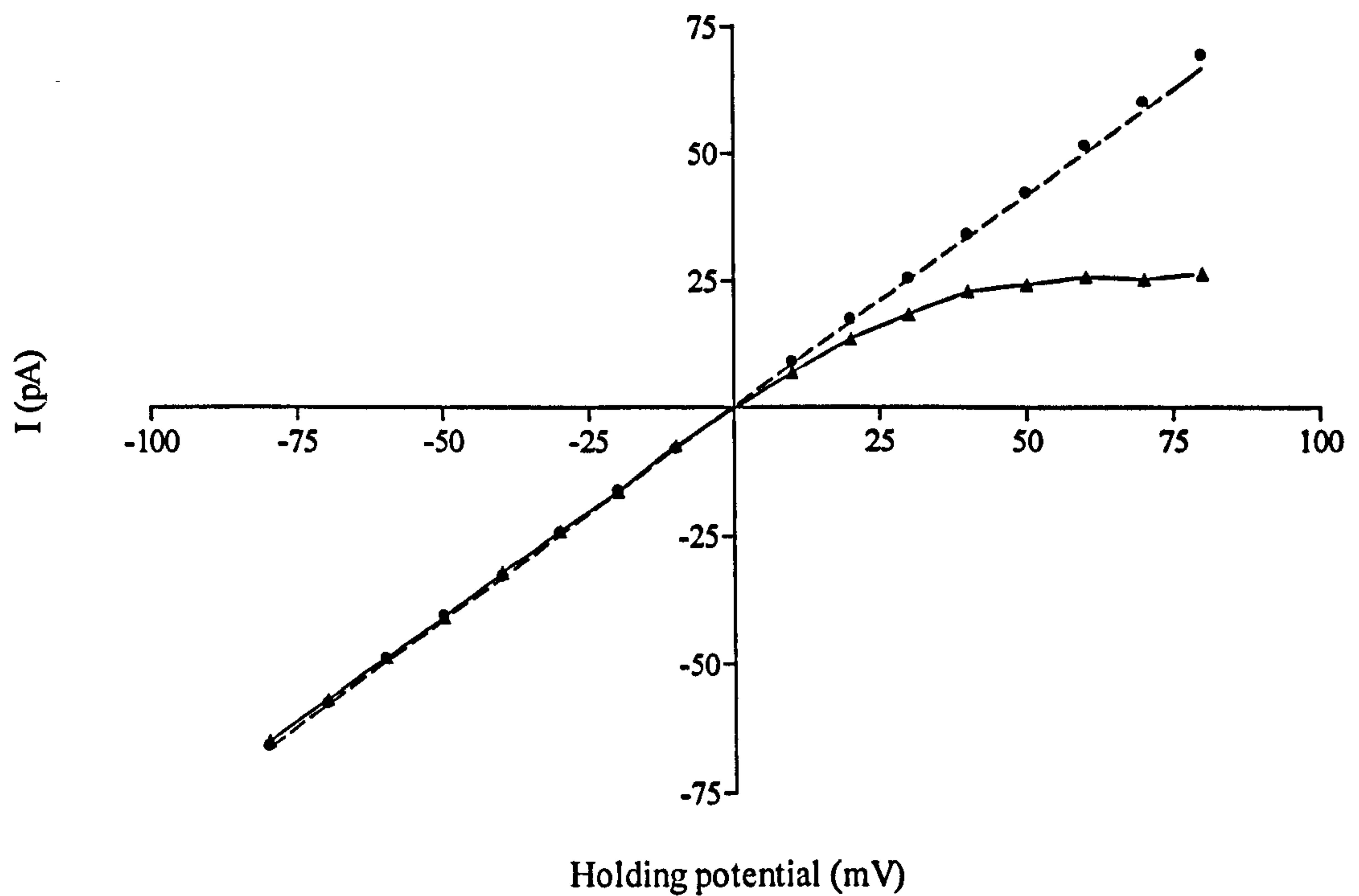


Figure 5.2. The influence of transmembrane holding potential on the single-channel current amplitude of the unmodified and 21-amino-9 α -hydroxyryanodine-modified conductance states of the RyR channel in the absence and presence of 20 mM TEA. O-open, C-closed and M-modified states.

Current-voltage relationships for the unmodified conductance states of several RyR channels $\pm$ TEA are shown in Figure 5.3 a. TEA has no effect on K⁺ translocation at negative holding potentials, however TEA blocks K⁺ current with increasing effectiveness as transmembrane holding potential is taken to positive values. Traces in the right panel of Figure 5.2 suggest TEA also blocks K⁺ current in the 21-amino-9 α -hydroxyryanodine-modified conductance state. The effectiveness of TEA as a blocker seems to be less in the ryanoid-modified conductance state than that

a



b

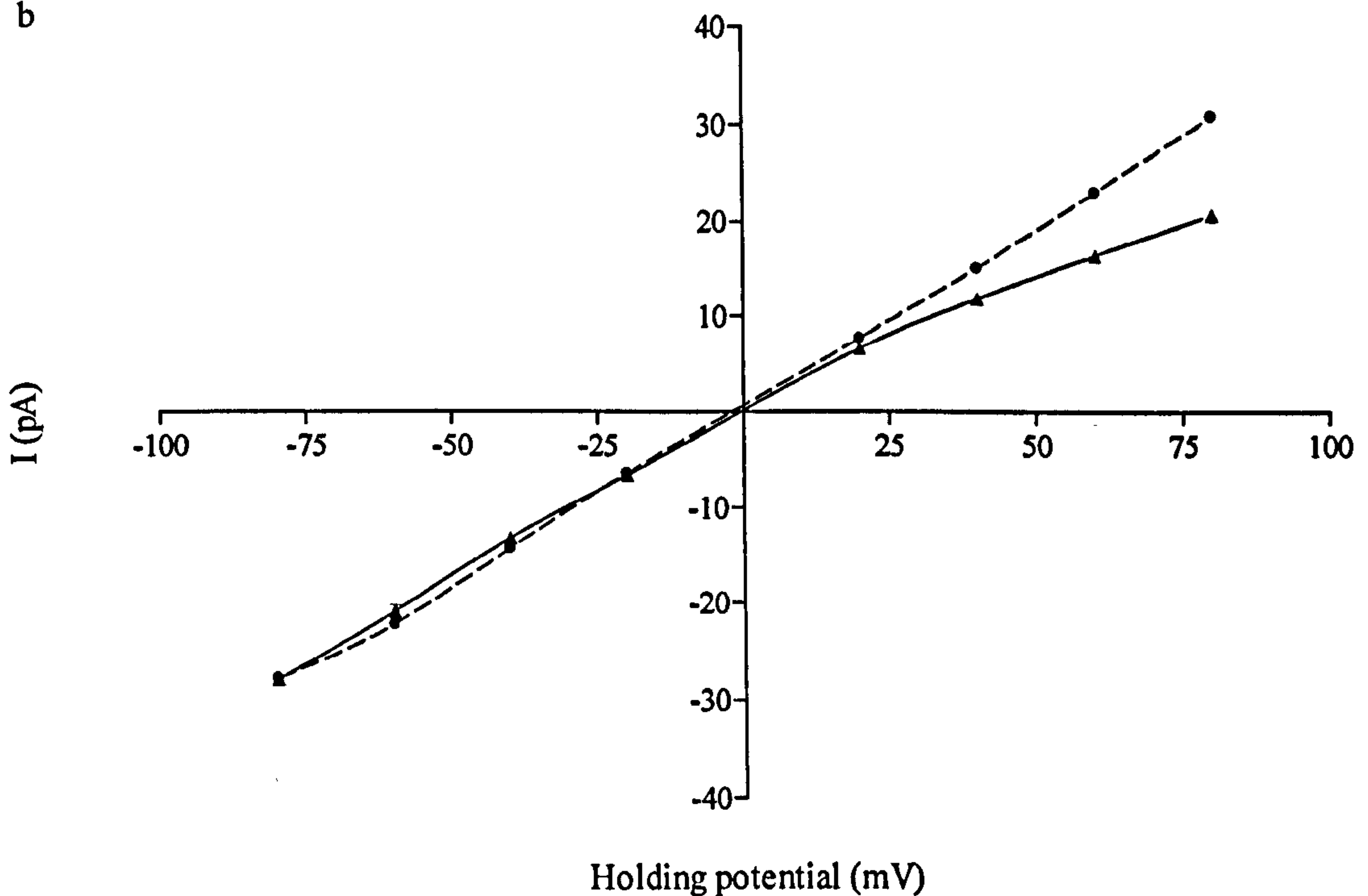


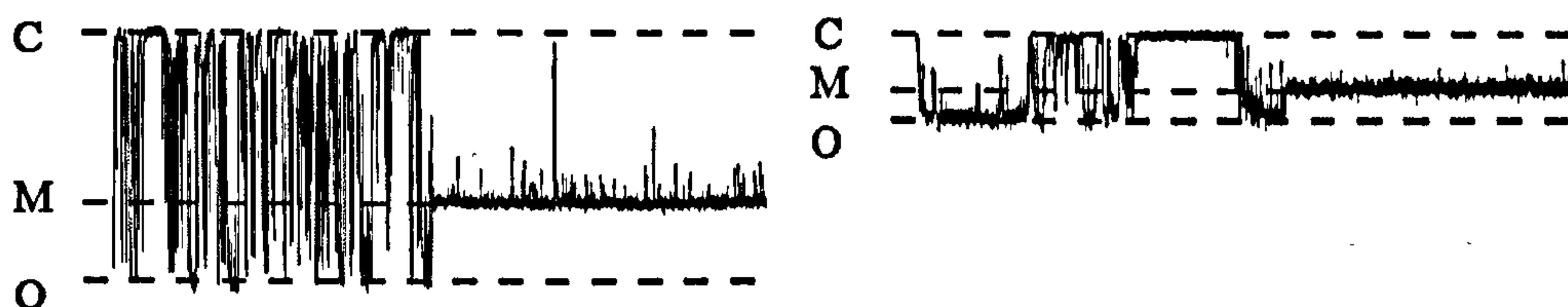
Figure 5.3 a) Single channel current-voltage relationships for unmodified channels in the absence (●; mean $\pm$ SEM of 22-51 experiments) and presence (▲; mean $\pm$ SEM of 4-25 experiments) of 20 mM TEA. b) Single channel current-voltage relationships for 21-amino-9 α -hydroxyryanodine-modified channels in the absence (●; mean $\pm$ SEM of 4-15 experiments) and presence (▲; mean $\pm$ SEM of 4-10 experiments) of 20 mM TEA. Where no error bars are visible they are contained within the symbol.

observed in the unmodified conductance state. The reduction in current amplitude produced by TEA in the absence of the ryanoid and so in the unmodified state (transitions from the C to O) is greater than when the RyR channel is in the ryanoid-modified state (transitions from the C to M). Figure 5.3 b. shows the current-voltage relationship for the 21-amino-9 α -hydroxyryanodine-modified states of several channels in the absence and presence of TEA. As is the case for the unmodified state of RyR, TEA produce no block of K⁺ current at negative holding potentials, but is an increasingly effective blocker of the ryanoid-modified state as holding potential is made more positive. A comparison of the data in Figure 5.3 a and b, confirms the observation made for the representative traces in Figure 5.2, TEA is less effective as a blocker of K⁺ current in the ryanoid-modified conductance state. For comparison, at a holding potential of +80 mV, 20 mM TEA reduces mean single RyR channel current amplitude of the 21-amino-9 α -hydroxyryanodine-modified conductance state by 33%, the equivalent block observed in the unmodified conductance state is 62%. Ryanoid-induced modification of single RyR channels, has previously been demonstrated to alter the permeability of translocated ions through the channel and is strongly correlated with specific structural loci on the ryanoid molecule with contributions from both electrostatic and steric effects (Welch *et al.*, 1997). Do similar structural features of the ryanoids underlie the alteration of the effectiveness of TEA as a blocker of K⁺ ion translocation through the RyR channel?

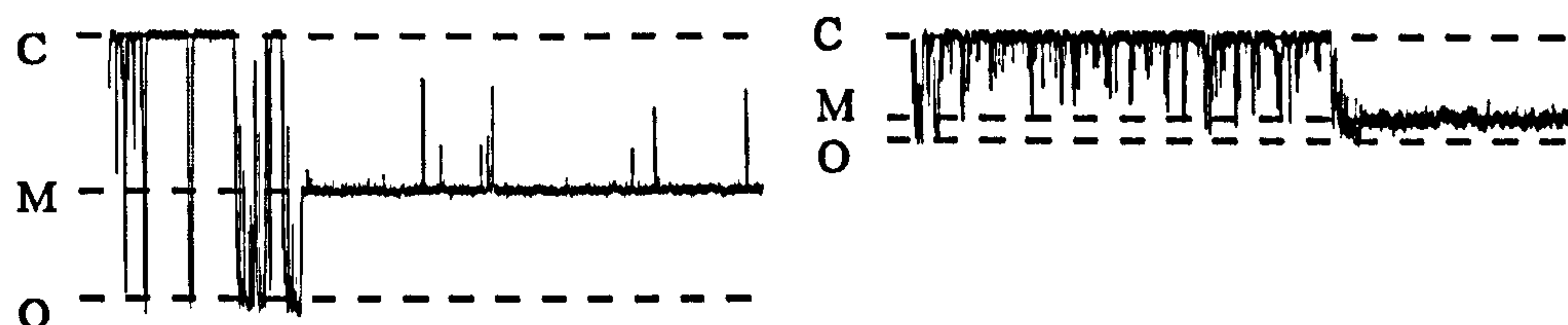
5.4.2 *The influence of the interaction of other ryanoids on TEA block*

The influence of three additional members of the ryanoid group of ligands have been examined; ryanodol, 21-azido-9 α -hydroxyryanodine and 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine. These ryanoids vary in both steric bulk and charge, and induce values of FC states ranging from 0.67 to 0.17. Single channel current amplitude of both the unmodified and ryanoid-modified conductance states were monitored at holding potentials within the range $\pm$ 80 mV in the absence and presence of 20 mM TEA. Examples of current fluctuations of representative channels at a holding potential of +40 mV are given in Figure 5.4. The left panel of Figure 5.4 demonstrates modification of the RyR channel that results from the interaction of the indicated ryanoid. In all cases, the interaction of the ryanoid with the RyR channel results in the occurrence of a modified state with a high P_o. As already described, the amplitude of the modified

ryanodol



21-azido-9 α -hydroxyryanodine



21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine

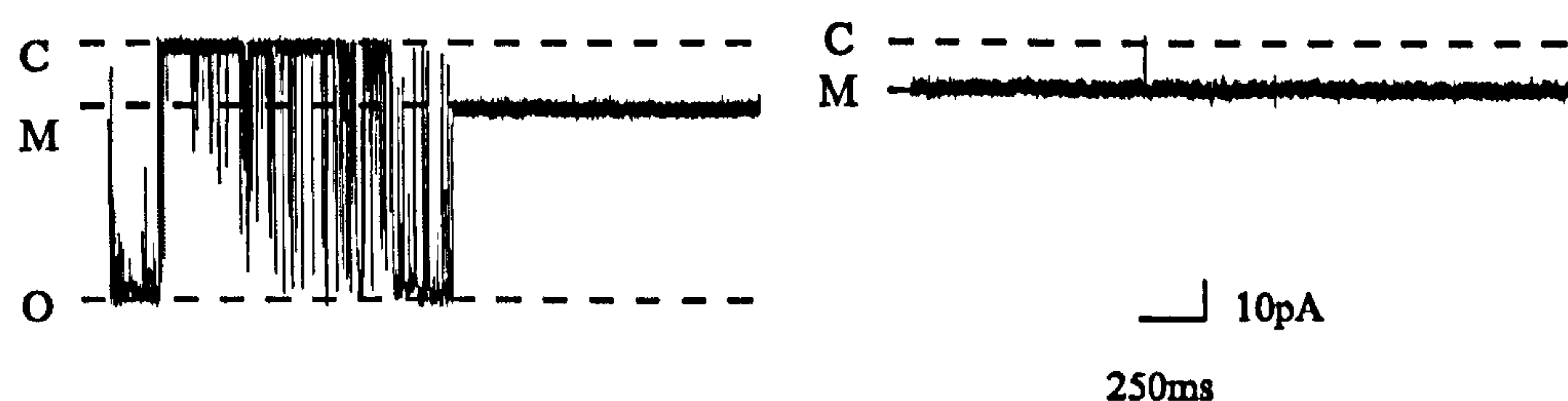


Figure 5.4 Single channel traces showing the influence of 20 mM TEA on the amplitude of ryanoid-modified conductance states at +40 mV. Both ryanodol and 21-azido-9 α -hydroxyryanodine interact reversibly with the RyR channel. For this reason, both unmodified and ryanoid-modified conductance states are shown in the presence of TEA for these ryanoids. In contrast, 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine is an irreversible modifier of the RyR channel function and, as a consequence, traces in the presence of TEA show only the ryanoid-modified conductance state. C – closed, O – open, M – modified states.

conductance state is dependent on the structure of the bound ryanoid (Tinker *et al.*, 1996 and Welch *et al.*, 1997). The values of FC induced by the ryanoids shown in Figure 5.4 are 0.67 for ryanodol, 0.55 for 21-azido-9 α -hydroxyryanodine and 0.27 for 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine. The influence of 20 mM TEA on the current amplitude of these various ryanoid-modified states is shown in the right panel of Figure 5.4. In all cases, the addition of 20 mM TEA leads to a reduction in current

amplitude. However, a comparison of the current amplitudes of the ryanoid-modified conductance states in the absence and presence of the blocking cation demonstrates that the effectiveness of TEA differs in the various ryanoid-modified conductance states. At this holding potential, the magnitude of block produced by TEA of the ryanoid-modified conductance state is in order ryanodol > 21-azido-9 α -hydroxyryanodine > 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine. Current-voltage relationships for the modified state in the absence and the presence of TEA for each ryanoid are shown in Figure 5.5. The mean single channel current amplitude of the ryanodol, 21-azido-9 α -hydroxyryanodine and 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine-modified conductance state at a holding potential of +80 mV is reduced by 65%, 51% and 15% respectively in the presence of 20 mM TEA. This is consistent with the observation made for the representative traces in Figure 5.4. The reduction in amplitude of both the unmodified and ryanoid-modified conductance states of the RyR channel by TEA with varying transmembrane holding potential can be fitted by a model originally described by Woodhull (1973), which is discussed in detail in Chapter 2. From this model it is possible to obtain the dissociation constant ($K_b(0)$) at a holding potential of 0 mV and the $z\delta$ (effective valence) of the blocking cation. Values of $K_b(0)$ and $z\delta$ were determined for 21-amino-9 α -hydroxyryanodine, ryanodol, 21-azido-9 α -hydroxyryanodine and 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine using nonlinear regression to establish the best fit of Eq. 2.10 to mean data from four or more experiments (Figure 5.6). Blocking parameters determined from these plots are presented in Table 5.1.

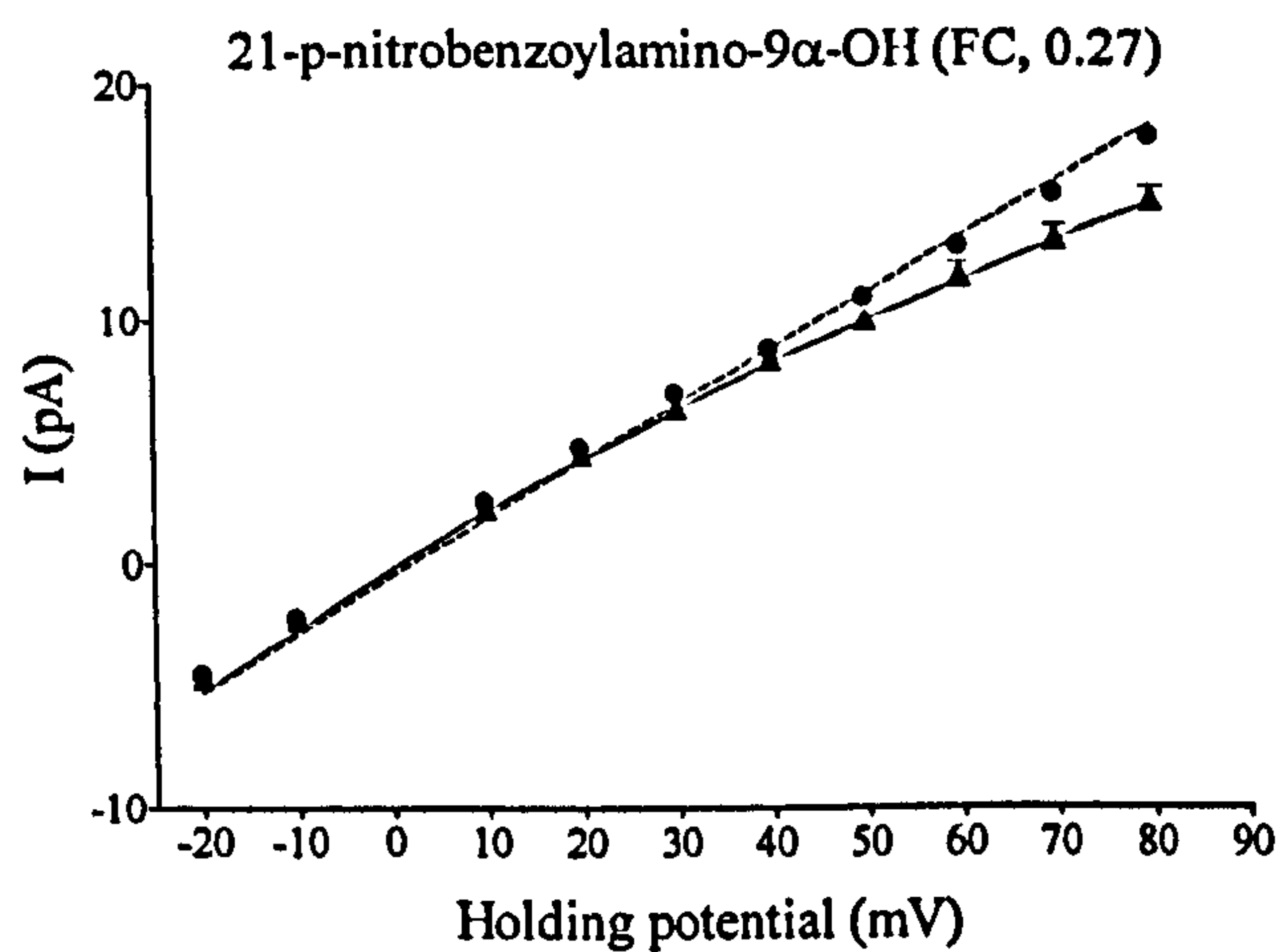
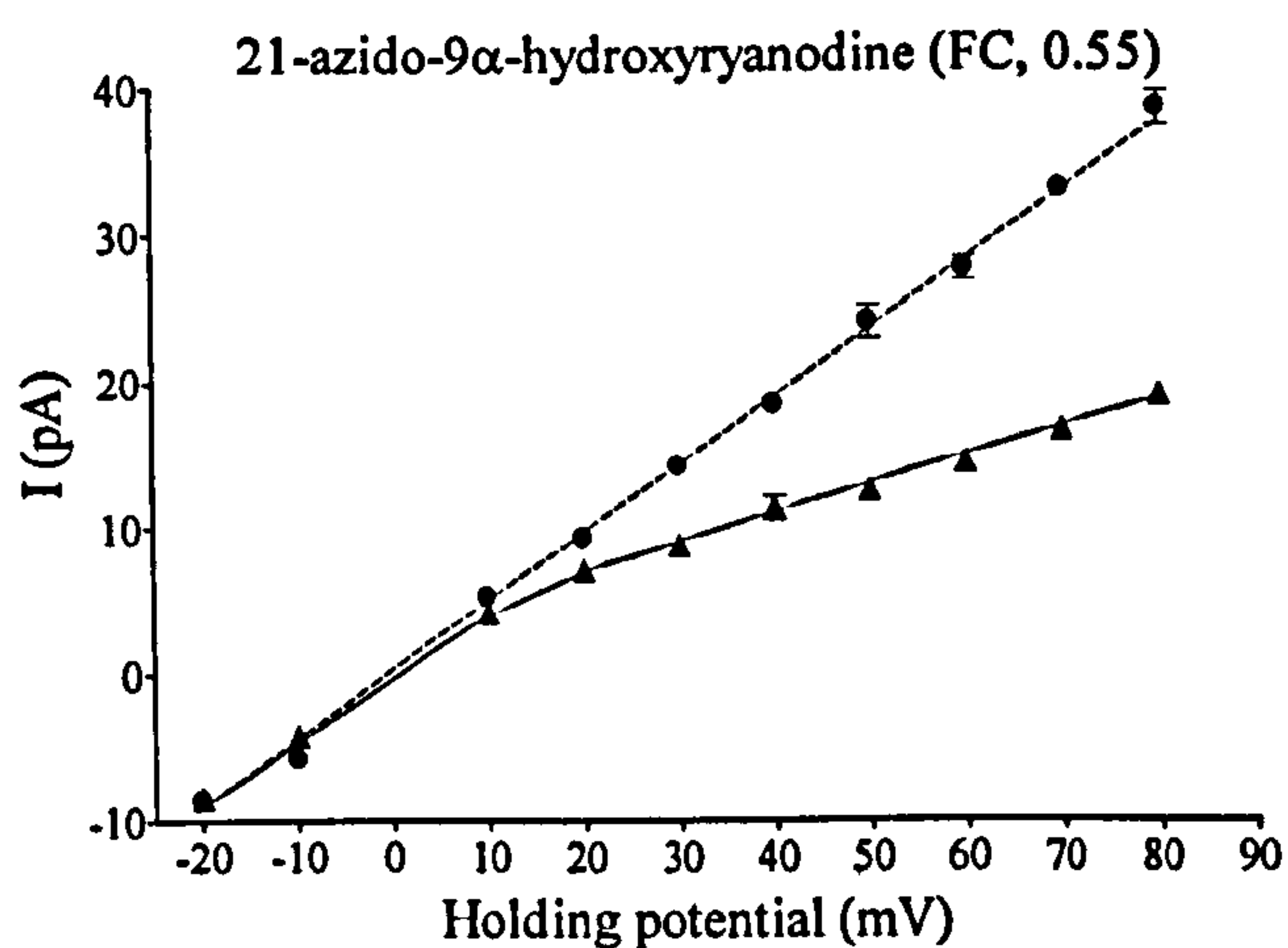
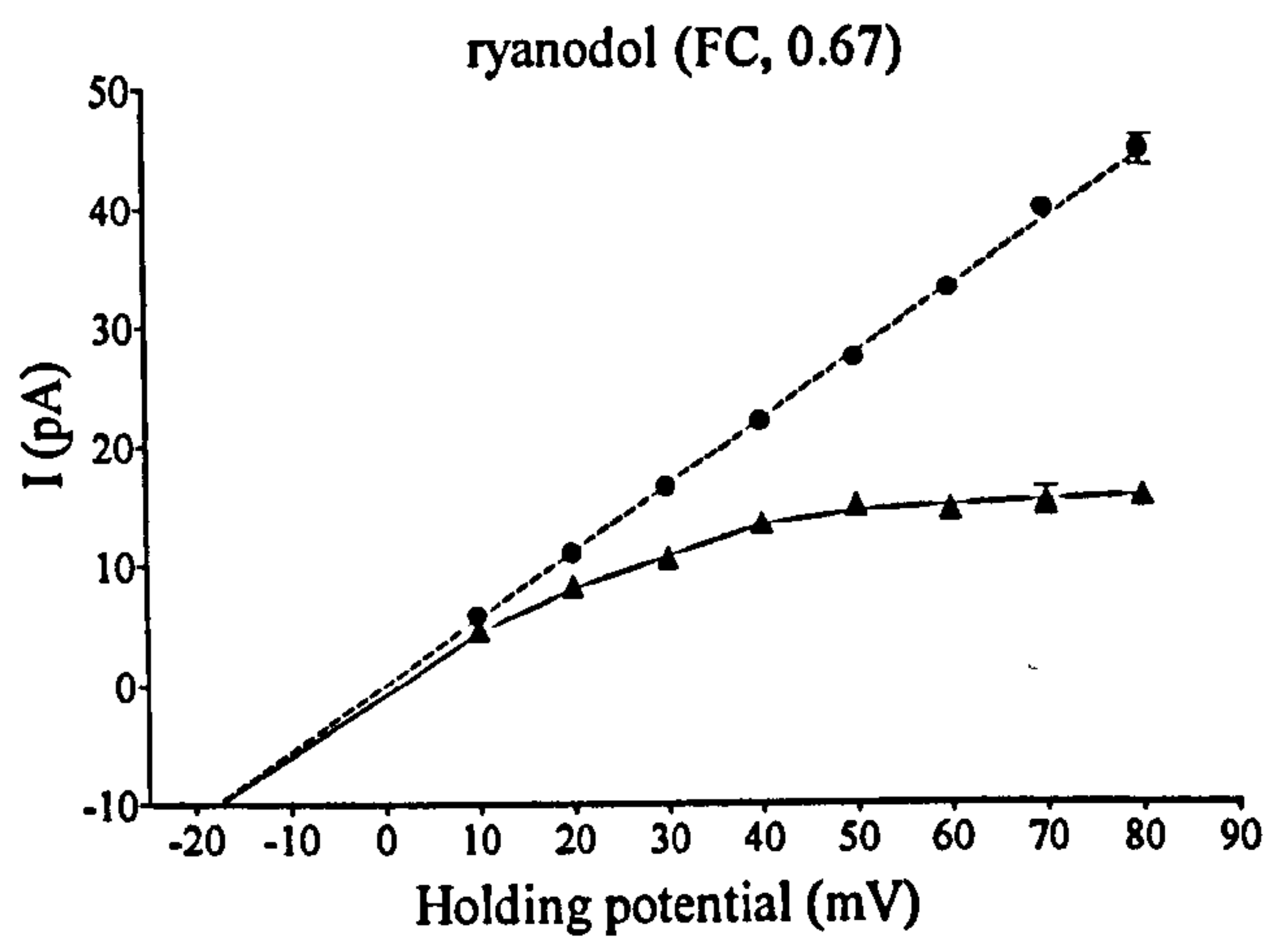


Figure 5.5 Single channel current-voltage relationships for ryanoid-modified conductance in the absence (●) and presence (▲) of 20 mM TEA (mean $\pm$ SEM of ryanodol, $n = 4-11$; 21-azido-9 α -hydroxyryanodine, $n = 4-19$; 21-p-nitrobenzoylamino-9 α -hydroxyryanodine, $n = 10-11$). Where no error bars are visible they are contained within the symbol.

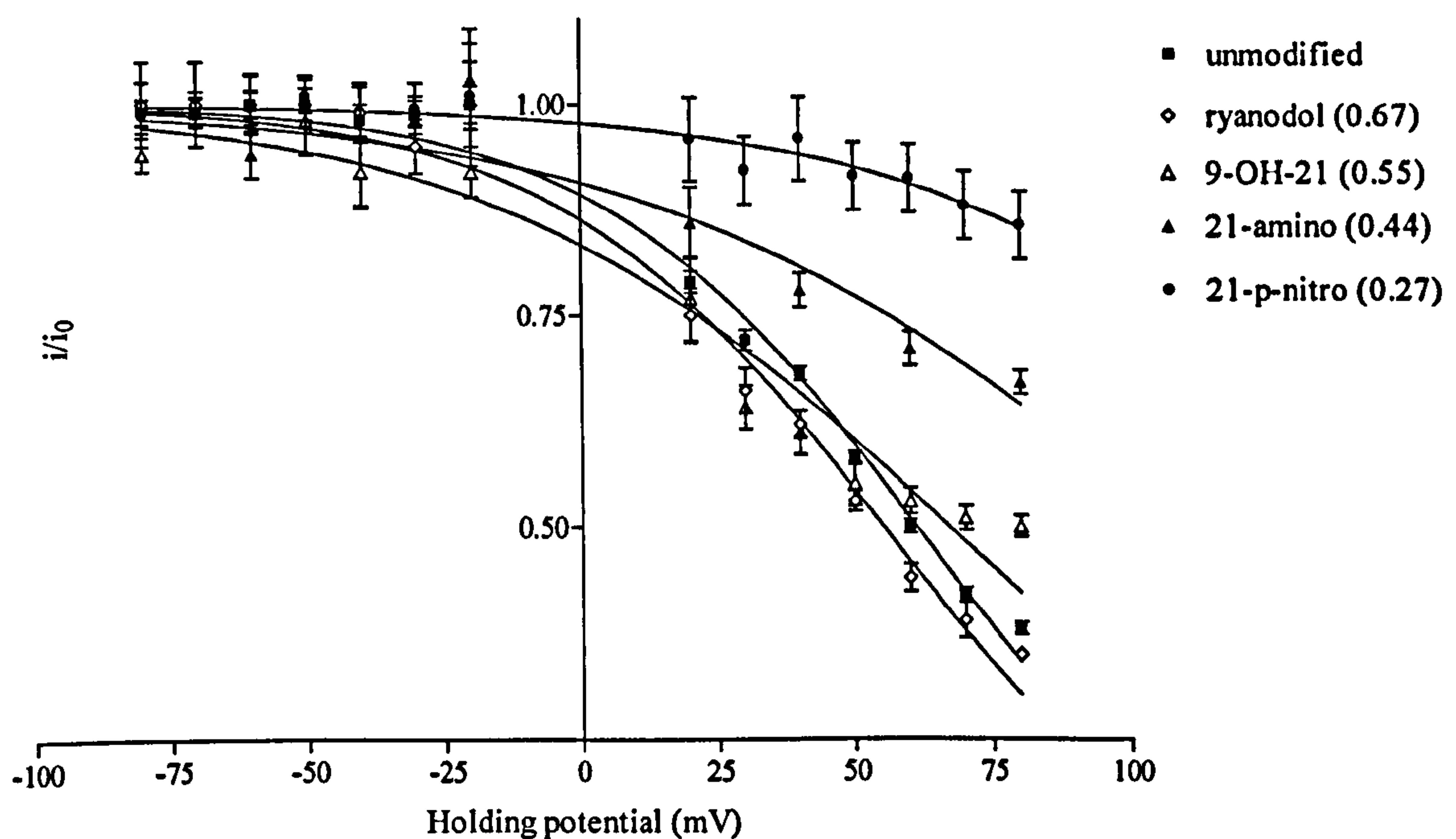


Figure 5.6 Woodhull plots for the indicated unmodified and the indicated ryanoid-modified conductance states in the presence of 20 mM TEA. Points are mean normalised conductance values (i/i_0) $\pm$ SEM (unmodified, $n = 4-51$; ryanodol, $n = 4-11$; 21-azido-9 α -hydroxyryanodine, $n = 4-19$; 21-amino-9 α -hydroxyryanodine, $n = 4-15$; 21-p-nitrobenzoylamino-9 α -hydroxyryanodine (0.27), $n = 10-11$). The solid lines were obtained by non linear regression fits to Eq. 2.10 in Chapter 2 with the parameters quoted in Table 5.1. Figures in brackets indicate the FC induced by the various ryanoids. 21-p-nitro, 21-p-nitrobenoylamino-9 α -hydroxyryanodine; 21-amino, 21-amino-9 α -hydroxyryanodine; 9-OH-21, 21-azido-9 α -hydroxyryanodine.

5.4.3 Multiple conformers of 21-p-nitrobenzoylamino-9 α -hydroxyryanodine affect TEA block

In addition to the FC of 0.27 reported here, two other modified conductance states of the RyR in the presence of 21-p-nitrobenzoylamino-9 α -hydroxyryanodine have been observed (Tinker *et al.*, 1996). In these studies, out of a total of 15 experiments conducted with 21-p-nitrobenzoylamino-9 α -hydroxyryanodine, we observed 11 modified conductance states with an FC of 0.27 (73% of total events), 3 with an 0.17 (20% of total events), and on one occasion a modified state with an FC of 0.59 (7% of total events). This observation of the occurrence of more than one modified conductance state is entirely consistent with the existence of multiple conformations of 21-p-nitrobenzoylamino-9 α -hydroxyryanodine, as determined by molecular dynamics (see Experimental procedures and Figure 5.1 b). Furthermore, the molecular dynamics

determinations demonstrate that the possible conformations of 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine are not isoenergetic. When added to the solution at the cytosolic face of the RyR channel, 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine is present in several conformations with different probabilities of occurrence. The three most common conformers exist in a ratio of 73:24:1% with the remaining 2% corresponding to multiple conformers rarely populated. The striking similarity between the observed probability of occurrence of the three different modified conductance states induced by 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine, and the ratio of the three common conformers of this ryanoid determined by molecular dynamics indicates that on binding to the RyR channel, the different conformers of the bulky *p*-nitrobenzoyl tail (Fig. 5.1 b) produce different FC states. Strong correlations between structure at this locus and FC states have previously been demonstrated (Welch *et al.*, 1997).

ryanoid	FC	K _b (0) (mM)	z δ
none	1.00	167.3	0.88
ryanodol	0.67	126.6	0.84
21- <i>p</i> -nitrobenzoylamino-9 α -hydroxyryanodine	0.59	177.7	0.66
21-azido-9 α -hydroxyryanodine	0.55	100.3	0.60
21-amino-9 α -hydroxyryanodine	0.44	195.1	0.53
21- <i>p</i> -nitrobenzoylamino-9 α -hydroxyryanodine	0.27	853.9	0.63
21- <i>p</i> -nitrobenzoylamino-9 α -hydroxyryanodine	0.17	No Block	No Block

Table 5.1 Blocking parameters for the interaction of TEA with RyR channels in the absence of ryanoid and in the indicated ryanoid-modified states. Parameters were determined using non-linear regression to establish the best fit of equation 2.10 in Chapter 2 for the data displayed in Figure 5.6 and 5.9.

The advantage of the *p*-nitrobenzoyl conformers is that they provide a built-in control. The different values of FC observed after the interaction of 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine are due only to changes in conformation of the ryanoid and not to other ligand-receptor interactions that may arise when the covalent structure of the ryanoid is modified. Examples of the 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine induced 0.17, 0.27 and 0.59 FC states are shown in Figure 5.7. The figure shows the modified conductance states in the absence (Fig. 5.7, left) and presence (Fig. 5.7, right) of 20 mM TEA. The degree of block induced by TEA and the influence of holding potential on block differs for the different FC states as shown in the current-voltage relationship plots in Figure 5.8. Mean single channel amplitude at +80 mV in the presence of symmetrical 20 mM TEA was reduced by 44% in the 0.59 FC state and 14% in the 0.27 FC state, but no reduction in current amplitude was observed under these conditions in the 0.17 FC state. Woodhull analysis of the influence of holding potential on block of K⁺ current by TEA (Eq. 2.10) for all three FC states induced by 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine is shown in Figure 5.9. Blocking parameters for the 0.17 FC state were not determined, as no significant block of K⁺ current by TEA in this ryanoid-modified state at any holding potential was observed.

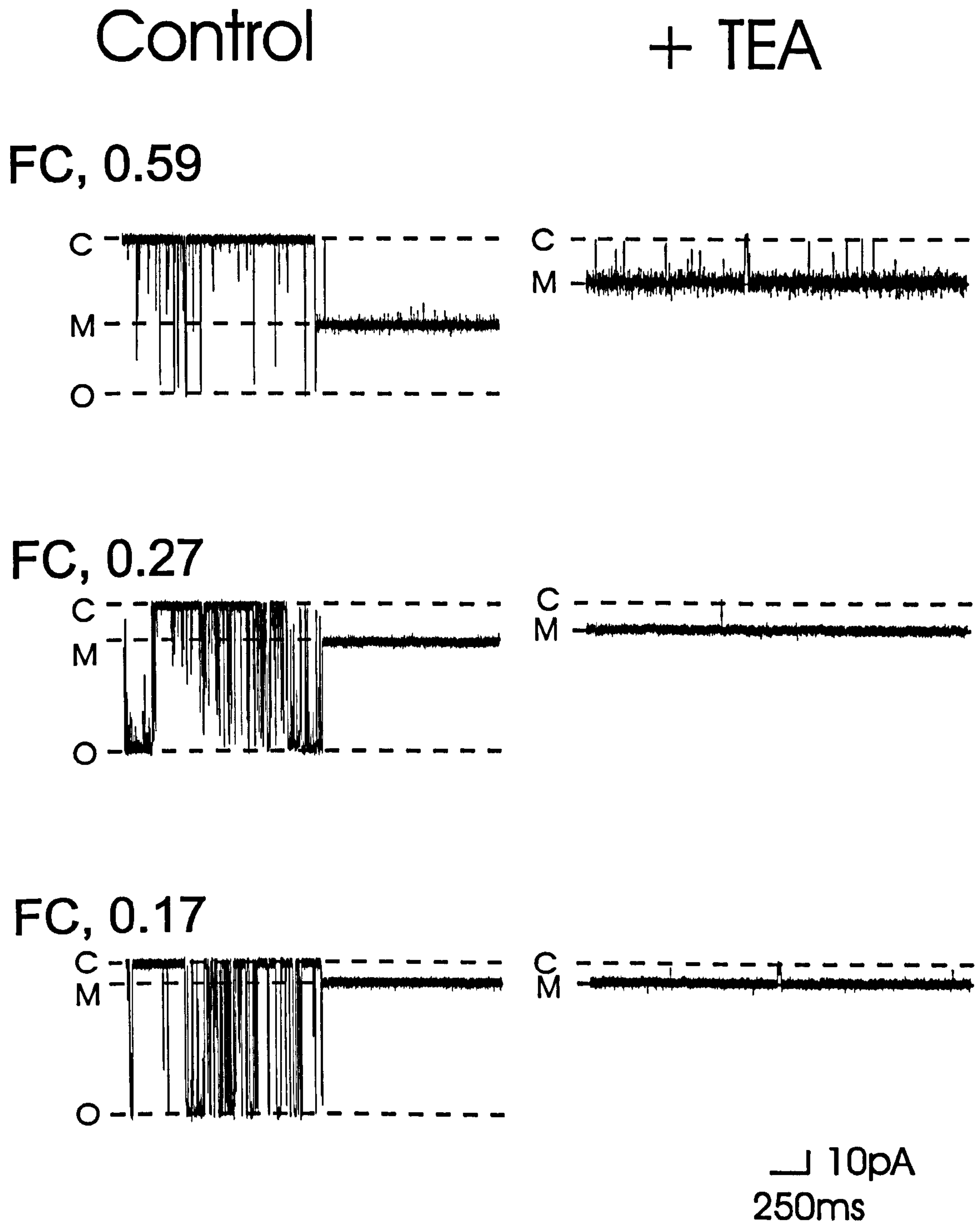


Figure 5.7 Single-channel traces showing the influence of 20 mM TEA on the amplitude of the three 21-p-nitrobenzoylamino-9 α -hydroxyryanodine-modified conductance states held at +40 mV. Irrespective of the FC induced, 21-p-nitrobenzoylamino-9 α -hydroxyryanodine is an irreversible modifier of the RyR channel and as a consequence, traces in the presence of TEA show only the ryanodine-modified conductance state. C – closed, M – modified and O – open states.

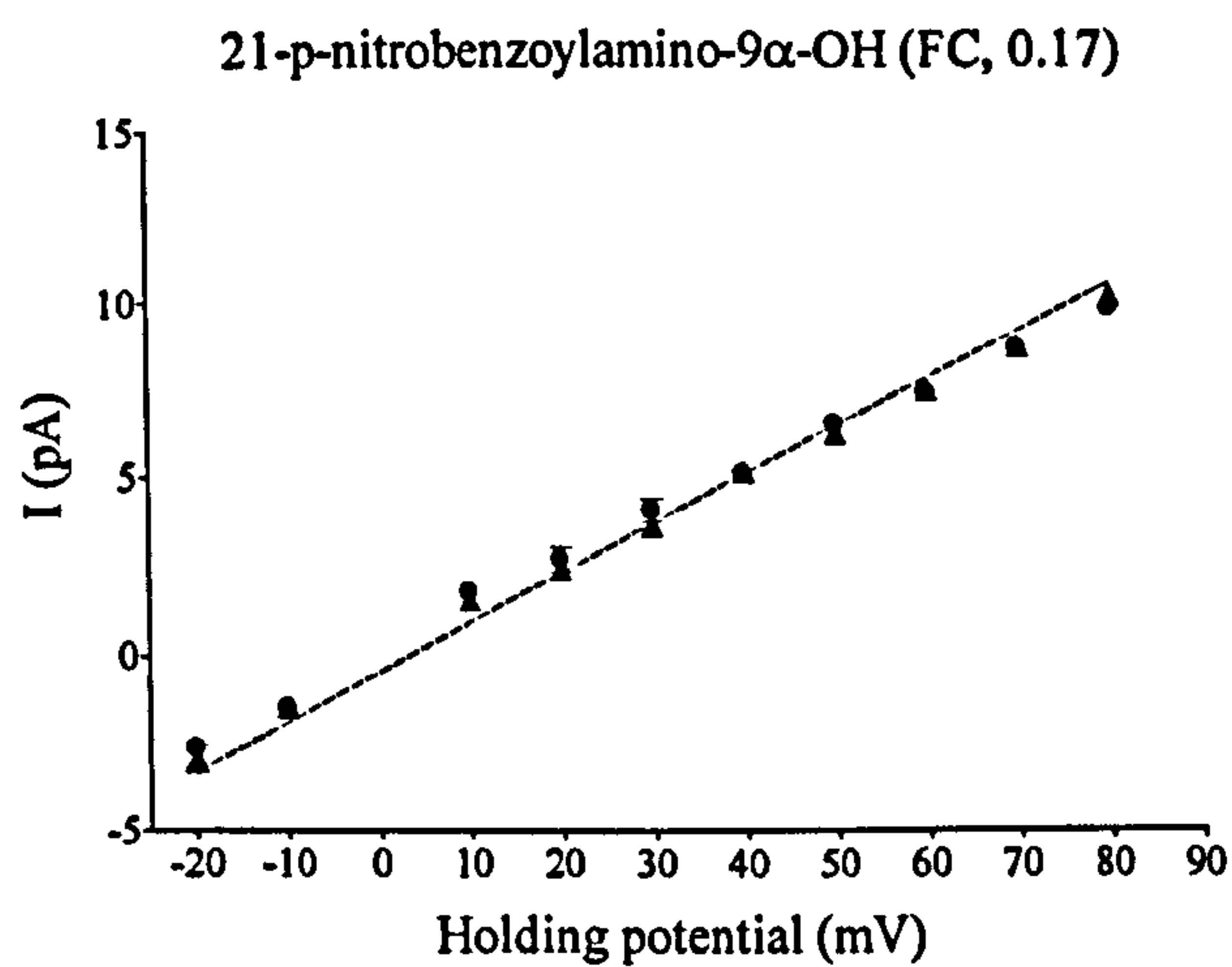
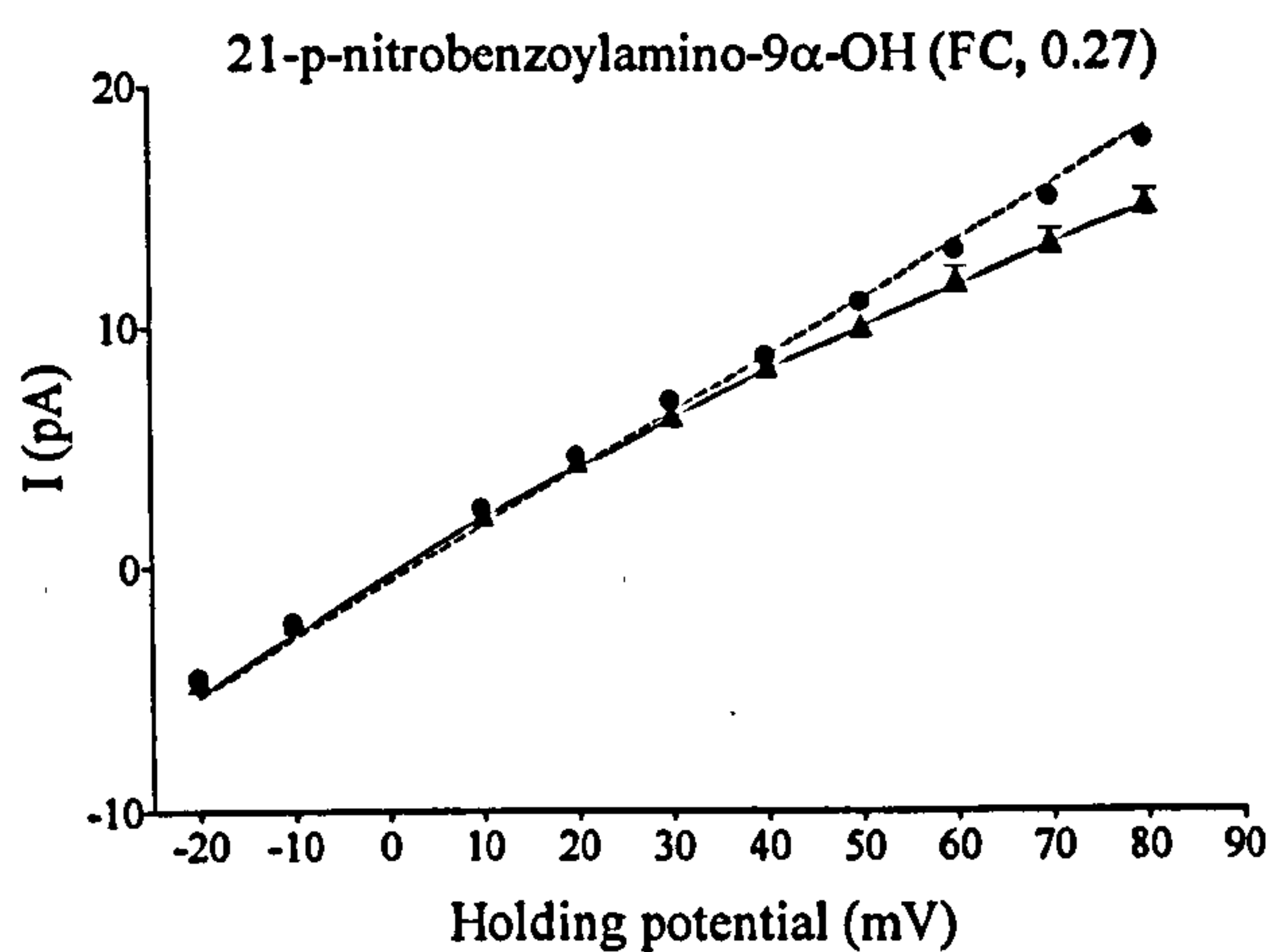
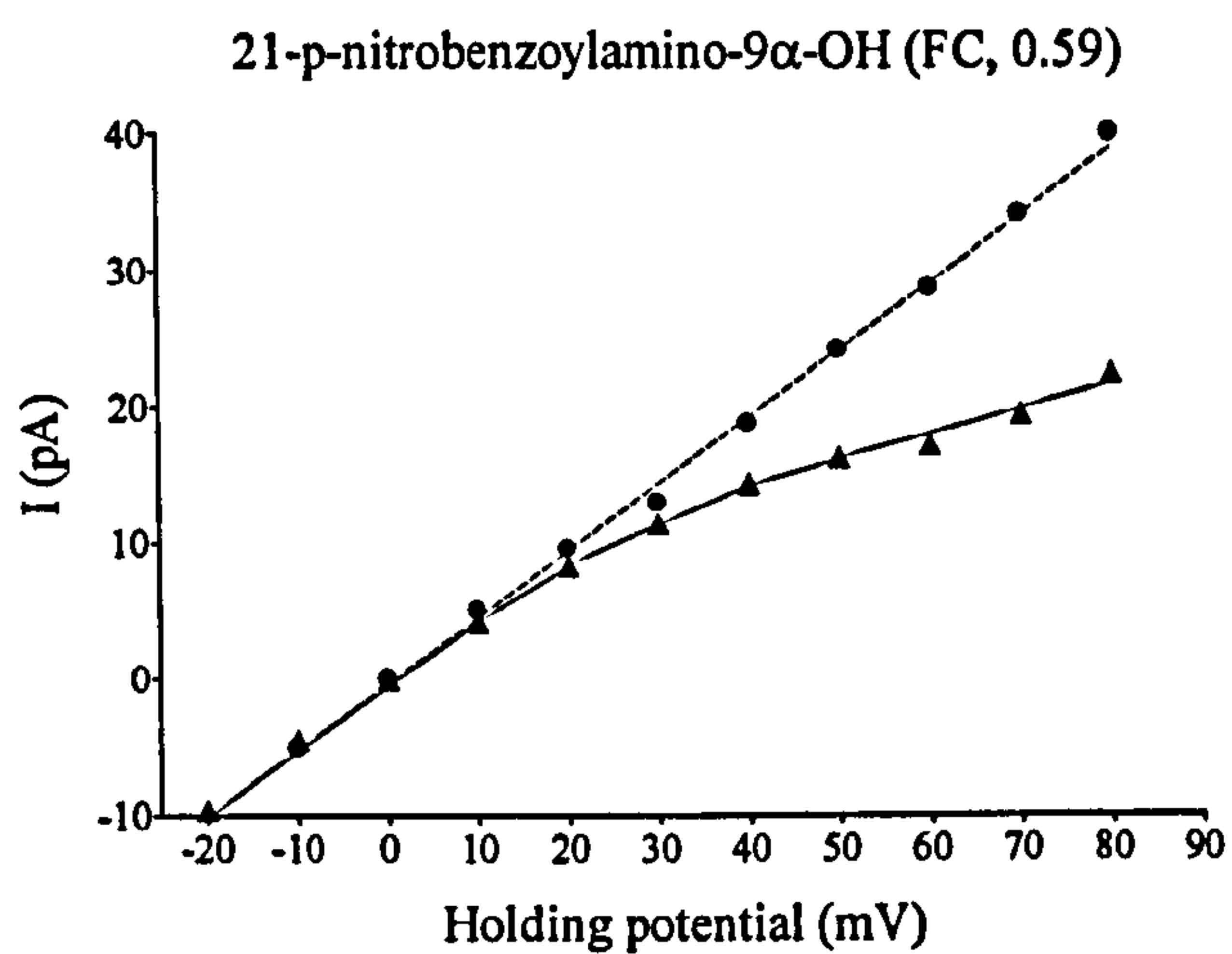


Figure 5.8 Single channel current-voltage relationships for the observed FC states induced by 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine in the absence (●) and the presence (▲) of 20 mM TEA ($n = 1$, FC 0.59; mean $\pm$ SEM $n = 10 - 11$, FC 0.27; mean $\pm$ SD $n = 3$, FC 0.17). Where error bars are not visible they are contained within the symbol.

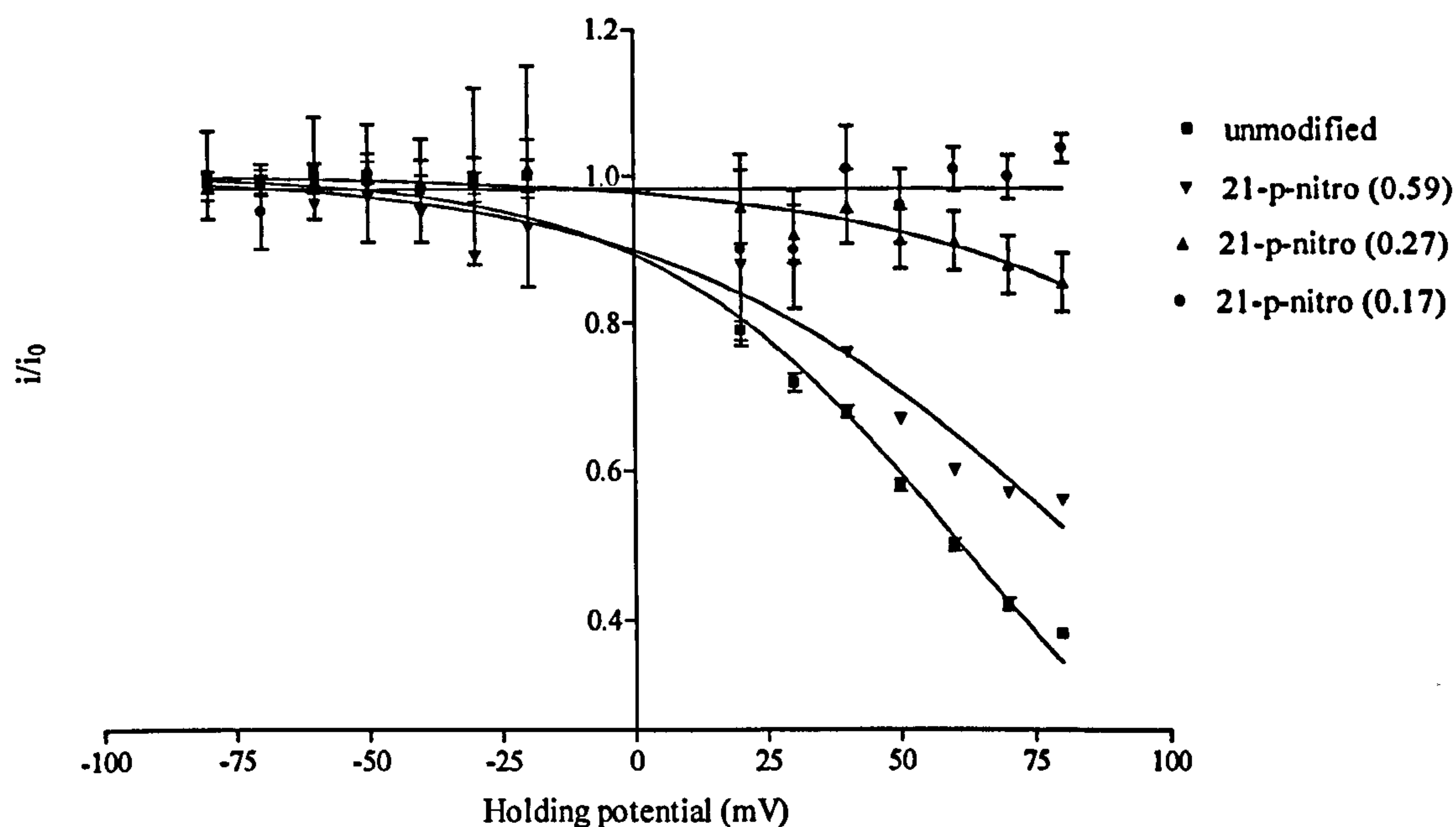


Figure 5.9 Woodhull plots for the indicated unmodified and the indicated 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine-modified conductance states in the presence of 20 mM TEA. Points are of normalised conductance values (i/i_0) (mean $\pm$ SEM unmodified, $n = 4 - 51$; FC 0.59, $n = 1$; mean $\pm$ SEM FC 0.27, $n = 10 - 11$; mean $\pm$ SD FC 0.17, $n = 3$). The solid lines were obtained by nonlinear regression to Eq. 2.10 with the parameters quoted in Table 5.1. Figures in brackets indicate the FC of the various conductance states. 21-*p*-nitro, 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine.

Overall, these investigations of the influence of holding potential on block by TEA of ryanoid-modified conductance states of the RyR channel indicate that there are marked quantitative differences in the degree of block produced by TEA in the modified states induced by the various ryanoids.

The alteration of the effectiveness of TEA as a blocker appears to be correlated to FC of the ryanoid. A comparison of the degree of block (expressed as relative current; single-channel current in the presence of TEA, divided by single-channel current in the absence of TEA) produced by 20 mM TEA at a holding potential of +80 mV of the various ryanoid-modified conductance states monitored in this study is presented in Figure 5.10. The degree of block induced by TEA increases as FC rises from 0.17 to 0.67; block in the ryanodol-modified state is essentially the same as that observed in the RyR channel in the absence of a ryanoid (FC = 1.0).

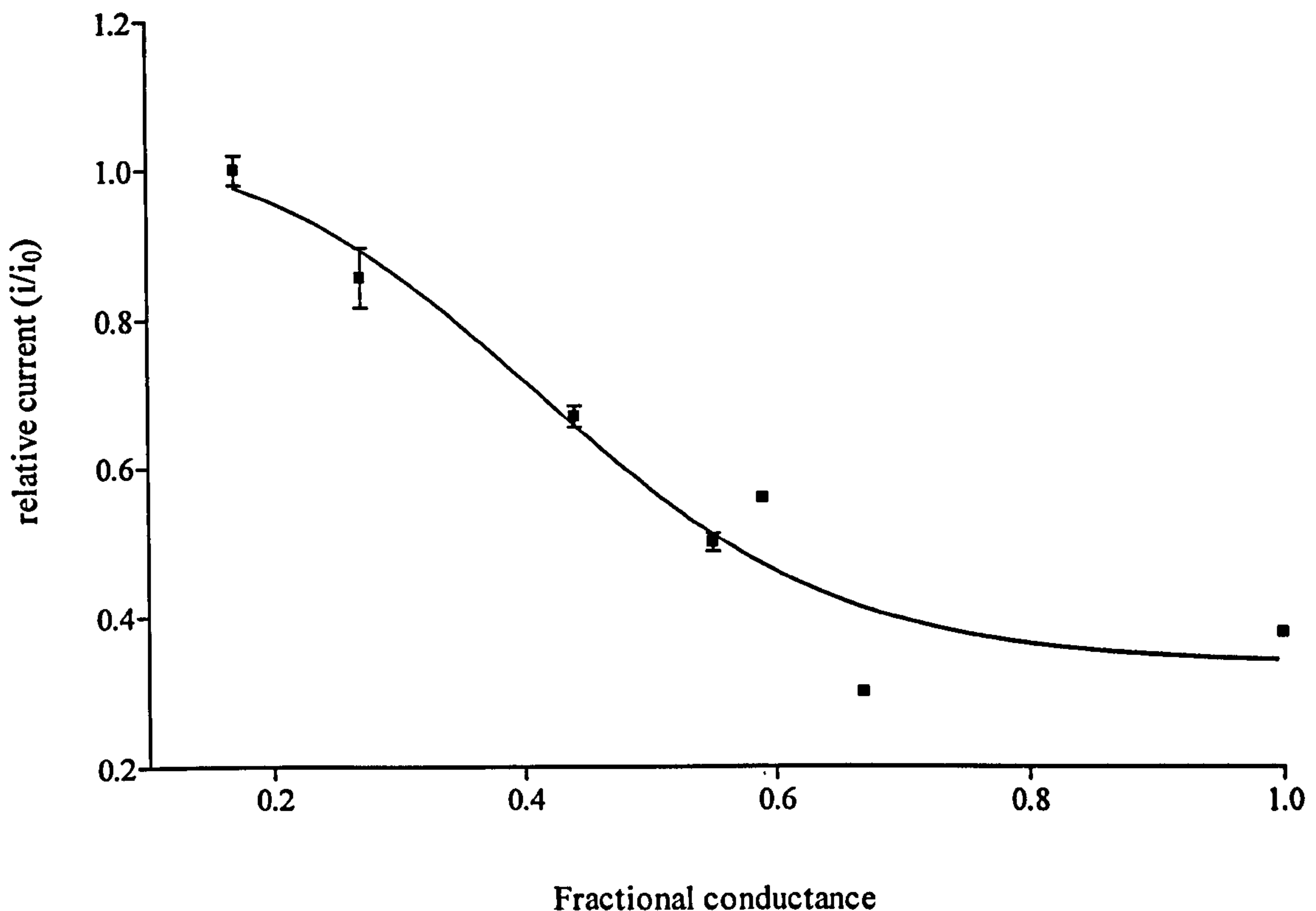


Figure 5.10 Relationship between relative conductance (i/i_0) produced by TEA in the ryanoid-modified state at +80 mV and FC of the ryanoid-modified state. The line plotted through the data is of no theoretical significance.

Quantitative analysis of the influence of holding potential on block by TEA provides additional information. The relationships between FC in the range 0.27 to 1.0 and the blocking parameters derived from the Woodhull analysis are shown in Figure 5.11 (no block was observed in the 0.17 FC state of 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine). The values of $z\delta$ determined for TEA block of K^+ conductance of the channels in the absence of a ryanoid, and channels modified with various ryanoids, show a broad correlation with relative K^+ conductance (FC; Figure 5.11, a). Ryanoid-modified conductance states with values of FC in the range of 0.67 to 0.44 yield dissociation constants for TEA at 0 mV that show little or no variation from the value determined in the absence of ryanoid. Dissociation constants increased markedly at FCs below 0.44 (Figure 5.11, b).

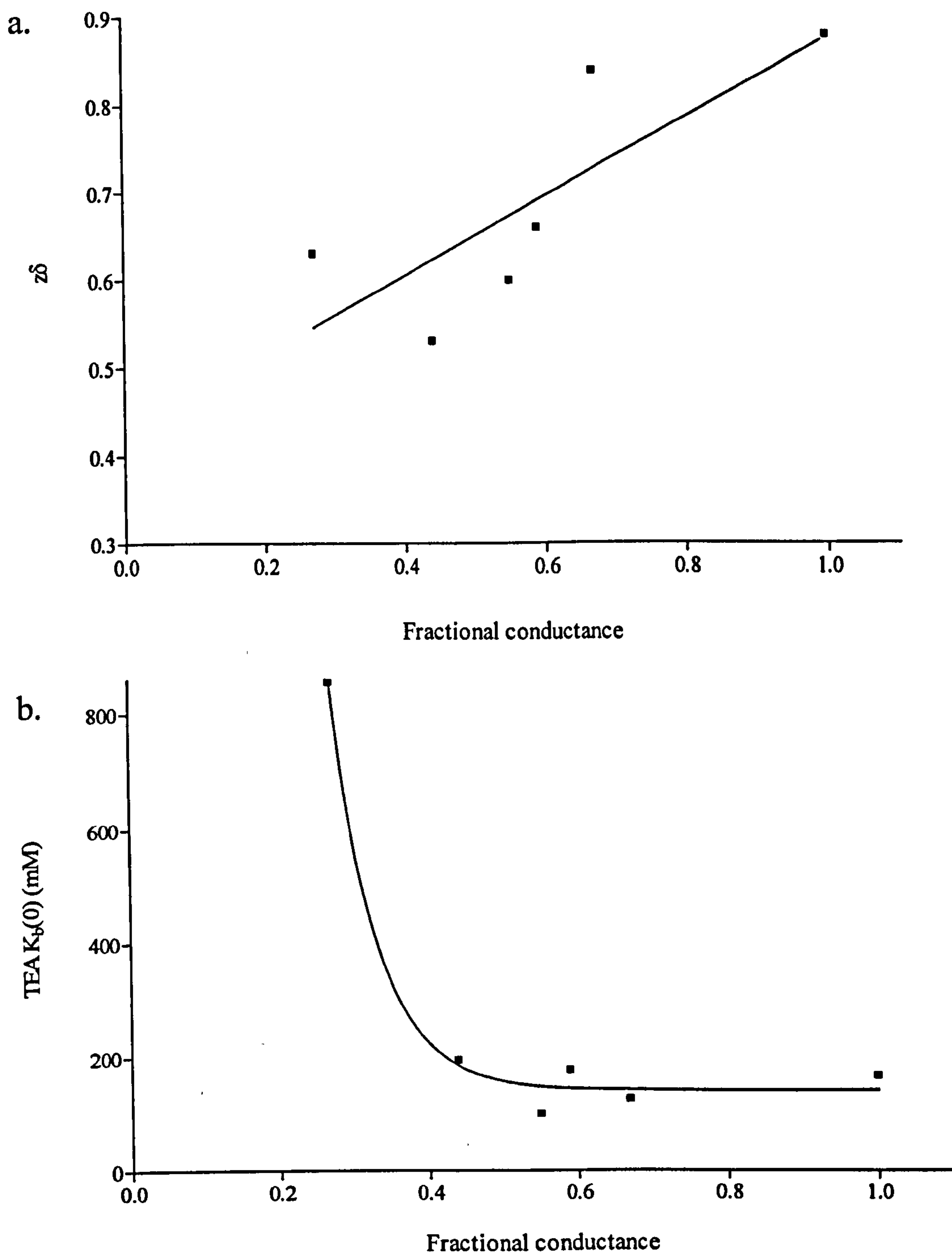


Figure 5.11 a) Variation in $z\delta$ of TEA in the unmodified RyR channel and ryanoid-modified states with FCs in the range 0.27 – 0.67. It should be noted that no measurable block by TEA was seen, and therefore no value of $z\delta$ was determined, for the 0.17 FC state induced by 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine. The solid line is the best-fit line obtained by linear regression. b) Variation in $K_b(0)$ of TEA in the unmodified RyR channel and ryanoid-modified states with FCs in the range 0.27 – 0.67. Again, in the absence of measurable block, no value of $K_b(0)$ could be determined for the 0.17 FC state induced by 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine. The line plotted through the data has no theoretical significance.

5.5 Discussion

This study has demonstrated that the interaction of a range of ryanoids with the RyR channel alters the blocking characteristics of TEA in the channel. An inspection of the influence of voltage on block by TEA in the unmodified and the ryanoid-modified conductance states reveals something of the mechanism involved in this action. The interaction of ryanoids with the RyR channel can affect both the $z\delta$ of the blocking cation and the affinity of the conduction pathway for the blocker ($K_b(0)$). For a cation, such as TEA, that blocks by entering the electric field across the voltage drop of the RyR channel, δ is a measure of the relative depth of penetration of the blocking cation into the field (French and Shoukimas, 1985). As z , the valence of TEA, is constant, a change in the $z\delta$ after channel modification by a ryanoid implies that the location of the site of interaction of TEA within the voltage drop across the channel is altered. This relocation of the TEA binding site within the voltage drop is likely to be the consequence of a reorganisation of the RyR channel conduction pathway induced by the binding of the ryanoid. Previous studies have demonstrated that a reduction in the rate of association of TBA with the RyR channel underlies a reduction in affinity of the channel for this cation after the interaction of ryanodine. Structural rearrangement leading to a decrease in the hydrophobicity of the TBA binding site upon the interaction of ryanodine were thought to influence the decrease in the affinity of the binding site for this cation. Hydrophobic interactions are also likely to contribute to the strength of binding at the TEA site. In the absence of ryanoids, single channel studies have shown an increase in affinity as the hydrophobicity of cations interacting with this site is increased (Tinker *et al.*, 1992b). If as suggested, the binding of ryanoids bring about conformational changes in the RyR channel, then alterations in $K_b(0)$ are likely to reflect the rearrangement of groups in and around the TEA binding site. Decreased access to the site, changes in electrical potential, or lowering of hydrophobicity of the site could all contribute to the observed reduction in affinity.

Quantitative differences in blocking parameters were monitored in the various ryanoid-modified states. This is reminiscent of the influence of ryanoids on the conductance of permeant cations such as K^+ . On binding to the high affinity ryanodine binding site, all ryanoids examined so far reduce single-channel K^+ current amplitude, but the relative amplitude of the ryanoid-modified conductance state (FC) varies and is dependent on

the electrostatic and steric features of the bound ryanoid (Tinker *et al.*, 1996; Welch *et al.*, 1997). By monitoring the influence of voltage on TEA block of K^+ conductance in RyR channels modified with ryanoids inducing different FC states, the possibility that the mechanisms underlying the alteration of TEA block might also be responsible for the alterations of K^+ conductance induced by these ligands can be examined. The results of these experiments are consistent with the proposal that the interaction of a ryanoid with the RyR channel induces structural rearrangements in the channel. In effect, the position of the TEA binding site is relocated relative to the voltage drop across the channel. In each case, the TEA site is shifted towards the cytosolic limit of the voltage drop.

When considering how a binding site might move within the voltage drop of the RyR channel, it is relevant to discuss what constitutes the TEA binding site. Does TEA simply transverse a portion of the RyR channel voltage drop until it jams in a narrow region of the conduction pathway or are more specific interactions involved? The evidence that has emerged from previous investigations of ion translocation and block in the RyR channel indicates that small TAA cations do not block the RyR channel by simply jamming in a constriction in the conduction pathway. Studies with these cations, trimethylammonium derivatives, and charged local anaesthetics have identified two regions within the voltage drop across the RyR channel with which blocking cations can interact. TMA blocks K^+ in the RyR channel by binding at a site located 50% into the voltage drop from the cytosolic face of the channel (Tinker *et al.*, 1992b). Larger TAA and related cations are excluded from the TMA site and bind further into the voltage drop (80-90%; Tinker *et al.*, 1992b; Tinker and Williams, 1993). As described above, that the affinity of the site located 90% into the voltage drop for blocking cation increases in proportion to the hydrophobicity of the cations, indicating that hydrophobic forces are likely to be involved in this interaction. The exclusive association of the smallest TAA, TMA, with the 50% site has been interpreted as indicating that this site is likely to be a small, relatively rigid structure from which larger blocking cations are excluded by steric restrictions (Tinker *et al.*, 1992b).

Within the context of the picture of the RyR channel conduction pathway set out above, it is probable that the observed relocation and changes in affinity of the TEA blocking site within the voltage drop result from rearrangement in this region of the channel after

ryanoid interaction. We can envisage movement of the relative position of the TEA site within the voltage drop across the RyR channel as a physical relocation of the site; alternatively, the monitored variations in $z\delta$ could arise from a change in the dimensions or position of the voltage drop across the channel. In such a scheme, the interaction of a ryanoid with the RyR channel would induce a structural reorganisation within the channel that results in the elongation of the voltage drop or a shift of the region of the channel over which transmembrane voltage drops towards the luminal face of the channel. Assuming that the physical location of the TEA site within the conduction pathway of the channel is unchanged, either of these manoeuvres would result in a lowering of $z\delta$.

The absence of voltage-dependent block of K^+ current by TEA in the 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine-modified state with an FC of 0.17 is likely to result from either, or both, a relocation of the blocking site outside the voltage drop across the channel or a dramatic decrease in the affinity of the TEA blocking site in the conduction pathway of the RyR channel. The trends in the data observed in Figure 5.11, b. indicate that alterations in affinity of the TEA binding site are likely to be the dominant factor.

Previous investigations, have suggested that modifications of permeant ion conductance in the ryanoid-modified channel arise from structural reorganisation of the conduction pathway of the RyR channel (Lindsay *et al.*, 1994; Tinker *et al.*, 1996; Welch *et al.*, 1997). The experiments described in this Chapter provide direct evidence for this postulated ryanoid-induced restructuring. Structural rearrangements within the conduction pathway of the RyR channel are unlikely to have exactly equivalent effects on the interactions of permeant and blocking cations. However, the broad correlation of TEA blocking parameters with the FC induced by individual ryanoids with different steric and electrostatic properties indicates that the structural reorganisations identified here are also likely to underlie ryanoid-induced alterations in permeant ion translocation in the RyR channel.

Chapter 6

Excess noise in the ryanoid modified state

6.1 Introduction

The functional consequence of the interaction of a ryanoid with the high affinity binding site on the RyR channel is the production of a fractional conductance (FC) state. The amplitude of the FC state is dependent on the permeant ion and the structure of the ryanoid. With alkaline earth divalent cations as the permeant ion, the FC state induced by ryanodine is less than 0.40, while with the group 1a monovalent cations values of FC are within the range 0.60-0.66 (Lindsay *et al.*, 1994). Altered unitary conductance reflects changes in both the affinity of the conduction pathway of the RyR channel for cations and the relative permeability of cations within the channel. It has been proposed that these changes occur as the result of a conformational alteration in the conduction pathway of the channel induced by the binding of the ryanoid (Lindsay *et al.*, 1994). The application of ryanoids of varying structure to individual RyR channels in previous studies (Tinker *et al.*, 1996) and as described in chapters prior to this, has revealed that the FC induced by the binding of these molecules to the channel is governed by structural features of the ligand. Comparative molecular field analysis has revealed strong correlations between FC and specific structural loci on the ryanoid molecule (Welch *et al.*, 1997). It has been assumed that the interaction of a single ryanoid molecule with the high affinity ryanoid binding site on the RyR channel gives rise to a single modified conductance state. However, detailed inspection of the modified conductance states induced by a large number of ryanoids indicates that some of these ryanoid-induced states display excess noise when compared with the normal open-channel conducting state. In this chapter I will describe examples of such 'noisy' ryanoid-induced states and investigate the mechanisms responsible for their occurrence.

6.2 Experimental procedures

The experiments in this chapter involve incorporation of single purified RyR channels into artificial planar phospholipid bilayers, using methods described in detail in chapter 2. All experiments were carried out in symmetrical 610 mM KCl bathing solution in the presence of a ryanoid (as stated in the text) at the cytosolic face of the channel $\pm$ symmetrical TEA both at concentrations indicated in the text. Transmembrane holding

potential was held at voltages as described in the text and each run was recorded and analysed using the Satori computer analysis program as described in chapter 2.

6.2.1 Probability that the RyR channel resides in the α ($P\alpha$) or β ($P\beta$) subconductance state

The interaction of 8 β -amino-9 α -hydroxyryanodine with the single RyR channel induces two fractional conductance states. The time the channel resides in each state was measured in the same manner as single channel P_o using the Satori analysis program. However, in this instance transitions between the α and β subconductance state levels was determined by manually placing the cursors at the α and β subconductance levels on the single channel recorded data. The computer analysis program automatically calculates a point halfway between the two levels and calculates the time the channel spends in each subconductance state (see Chapter 2). The probability of the channel being in either subconductance state was determined according to the equations below:

$$P\alpha = \frac{\text{total time in } \alpha \text{ state}}{\text{total time in } \alpha \text{ state} + \text{total time in } \beta \text{ state}} \quad \text{equation 6.1}$$

and

$$P\beta = \frac{\text{total time in } \beta \text{ state}}{\text{total time in } \beta \text{ state} + \text{total time in } \alpha \text{ state}} \quad \text{equation 6.2}$$

6.2.2 The influence of transmembrane holding potential on the probability of channel residing in the α and β subconductance state.

The transitions between the α and β subconductance states are dependent on transmembrane holding potential. For such a relationship $P\alpha$ and $P\beta$ can be described by a Boltzmann distribution as shown below:

$$\frac{P\alpha}{1 - P\alpha} \text{ or } \frac{P\beta}{1 - P\beta} = \exp \frac{[zFV - G_i]}{RT} \quad \text{equation 6.3}$$

where F is the Faraday constant, V is the transmembrane voltage, z is the voltage dependence of the occurrence of the α or β state and G_i / RT is an expression of the equilibrium of the reaction at the holding potential of 0 mV.

6.3 Results

Examples of altered channel function following the interaction of various ryanoids with individual RyR channels are shown in Figure 6.1. In all cases, ryanoid binding results in the occurrence of a high P_o , reduced conductance state. However, significant differences in channel activity when the ryanoid is bound to the channel are observed and are visible on the traces in Figure 6.1. The modified conductance states induced by ryanodine and 21-azido-9 α -hydroxyryanodine are 'quiet'; that is the current noise in the ryanoid-modified state is comparable to that of the normal open state of the channel. In contrast, it is clear that the current noise associated with the modified conductance state induced by both 21-amino-9 α -hydroxyryanodine and 8 β -amino-9 α -hydroxyryanodine is significantly greater than that of the normal open state of the channel. With closer inspection it can be seen that with these ryanoids bound, the RyR channel fluctuates between two modified conductance states. Rates of transitions between these states are very fast in the case of 21-amino-9 α -hydroxyryanodine and as a consequence the individual dwell times in each state are poorly resolved. However, on occasions, modified events induced by 21-amino-9 α -hydroxyryanodine show clear transitions between two conductance levels. These rare events seem to occur on occasions distinct from the usual noisy events, that is to say the ryanoid has to dissociate from the channel and associate with the channel again before it induces this distinctive state, as shown in Figure 6.2. These events are very brief and the channel resides predominantly in the subconductance state closest to the closed level (α) (Figure 6.2). In the presence of 8 β -amino-9 α -hydroxyryanodine (Ball and stick figure shown in Figure 6.3) transition rates are slower and dwell times in the two states (α and β) are clearly resolved (Figure 6.1).

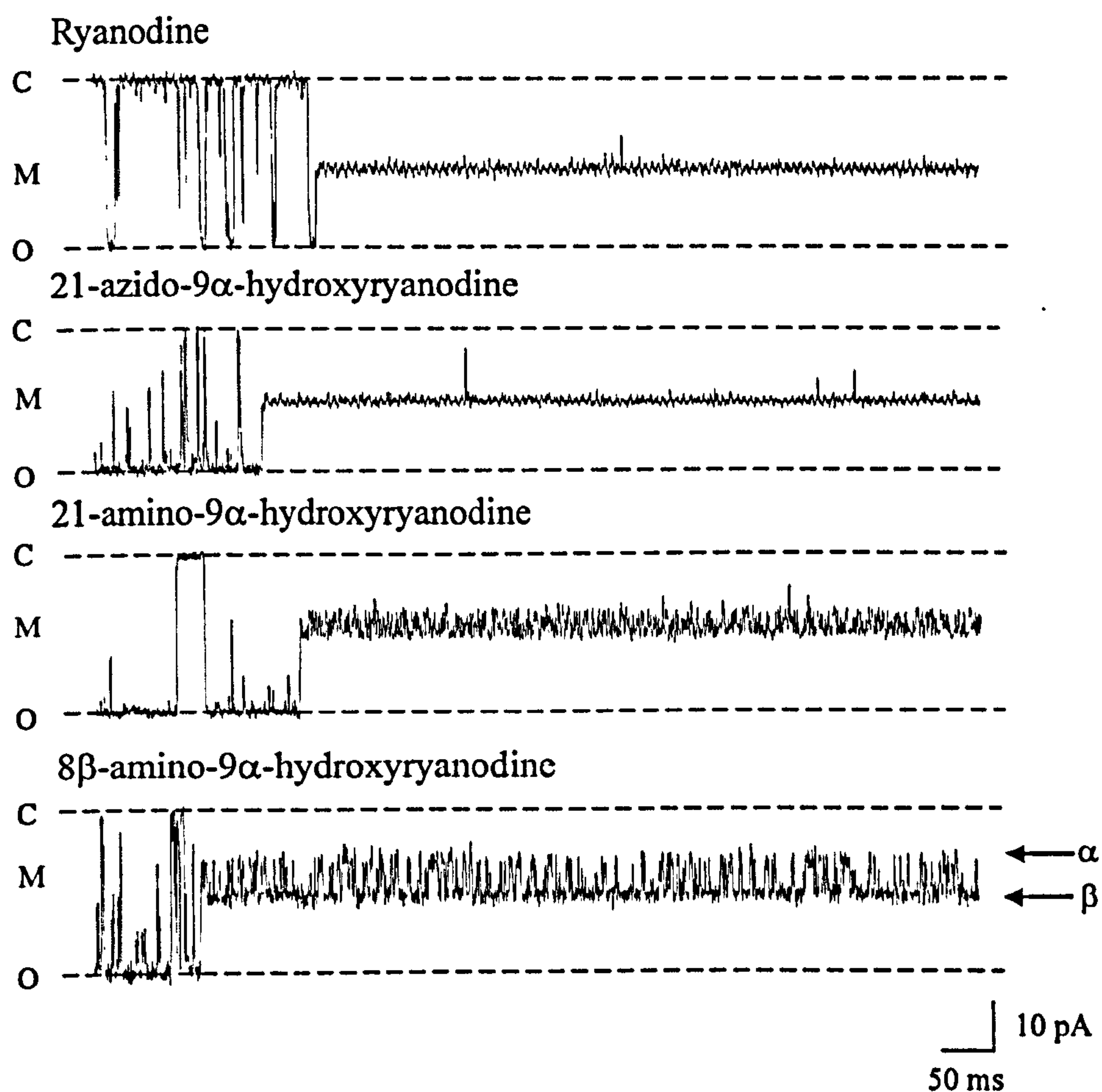


Figure 6.1 Modification of function following the interaction of the indicated ryanoids with single RyR channels. In all cases K^+ is the charge carrying species and the transmembrane holding potential is +40 mV.

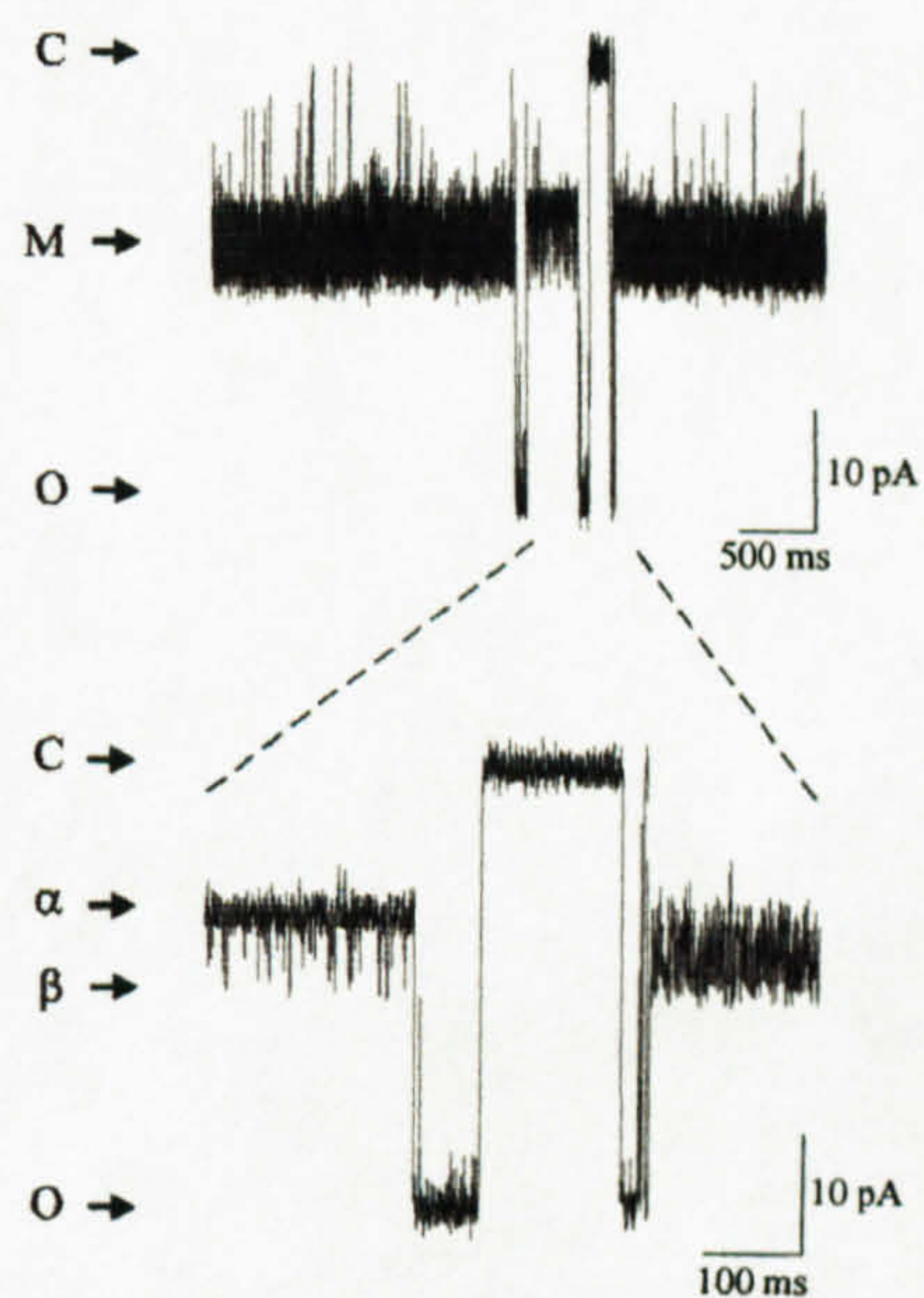


Figure 6.2 The interaction of 21-amino-9 α -hydroxyryanodine with a single RyR channel illustrating the occasional induced subconductance state which alternates between two distinct subconductance levels.

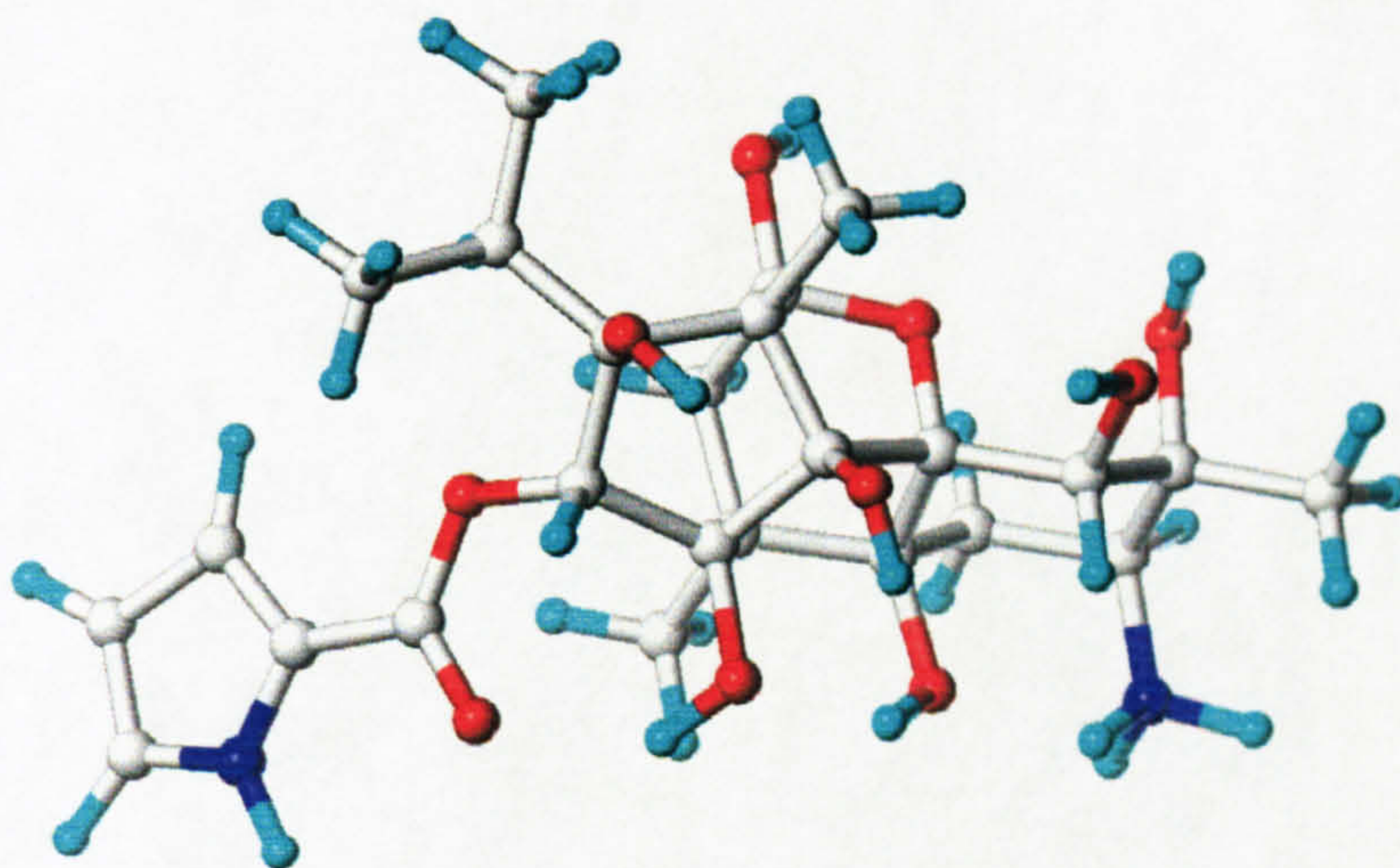


Figure 6.3 Ball and stick structure of 8 β -amino-9 α -hydroxyryanodine

6.3.1 *What mechanisms underlie this apparent fluctuation between different conductance states following the interaction of some ryanoids with the RyR channel?*

Two possible explanations for the observed behaviour come to mind. The first of these is that following the interaction of a ryanoid with the RyR channel, and the subsequent induction of a modified conductance state, unbound ryanoid in the bulk solution enters the conduction pathway of the ryanoid-modified channel and acts as a partial blocker of K^+ translocation through the channel. Both 21-amino-9 α -hydroxyryanodine and 8 β -amino-9 α -hydroxyryanodine have a net positive charge and there are examples in the literature of partial block of RyR channels by large cations including tetraalkylammoniums (Tinker *et al.*, 1992 a and b) and local anaesthetics (Tinker and Williams, 1993). Under this circumstance the β -subconductance state observed in the RyR channel in the presence of 8 β -amino-9 α -hydroxyryanodine would be the modified conductance state induced by the interaction of the ryanoid with the channel and the α -subconductance state would represent a partial blocked state of the ryanoid-modified state. The feasibility of this mechanism was investigated by simply varying the concentration of 8 β -amino-9 α -hydroxyryanodine in the solution at the cytosolic face of the channel. If the observed α -subconductance state is a partial blocked state of the ryanoid-modified conductance state, an increase in ryanoid concentration will increase the probability of occurrence of the blocked (α) state, whilst decreasing the probability of occurrence of the β state. Figure 6.4 shows the probability of occurrence of the α - and β -subconductance states during modified conductance events in the presence of cytosolic 8 β -amino-9 α -hydroxyryanodine at concentrations ranging from 10 nM to 10 μ M at a holding potential of +60 mV. There is no variation in the probability of occurrence of either the α or the β states within a 1000-fold concentration range of the ryanoid. These observations indicate that the α -subconductance state is not a partially blocked state of a single modified conductance state induced by 8 β -amino-9 α -hydroxyryanodine.

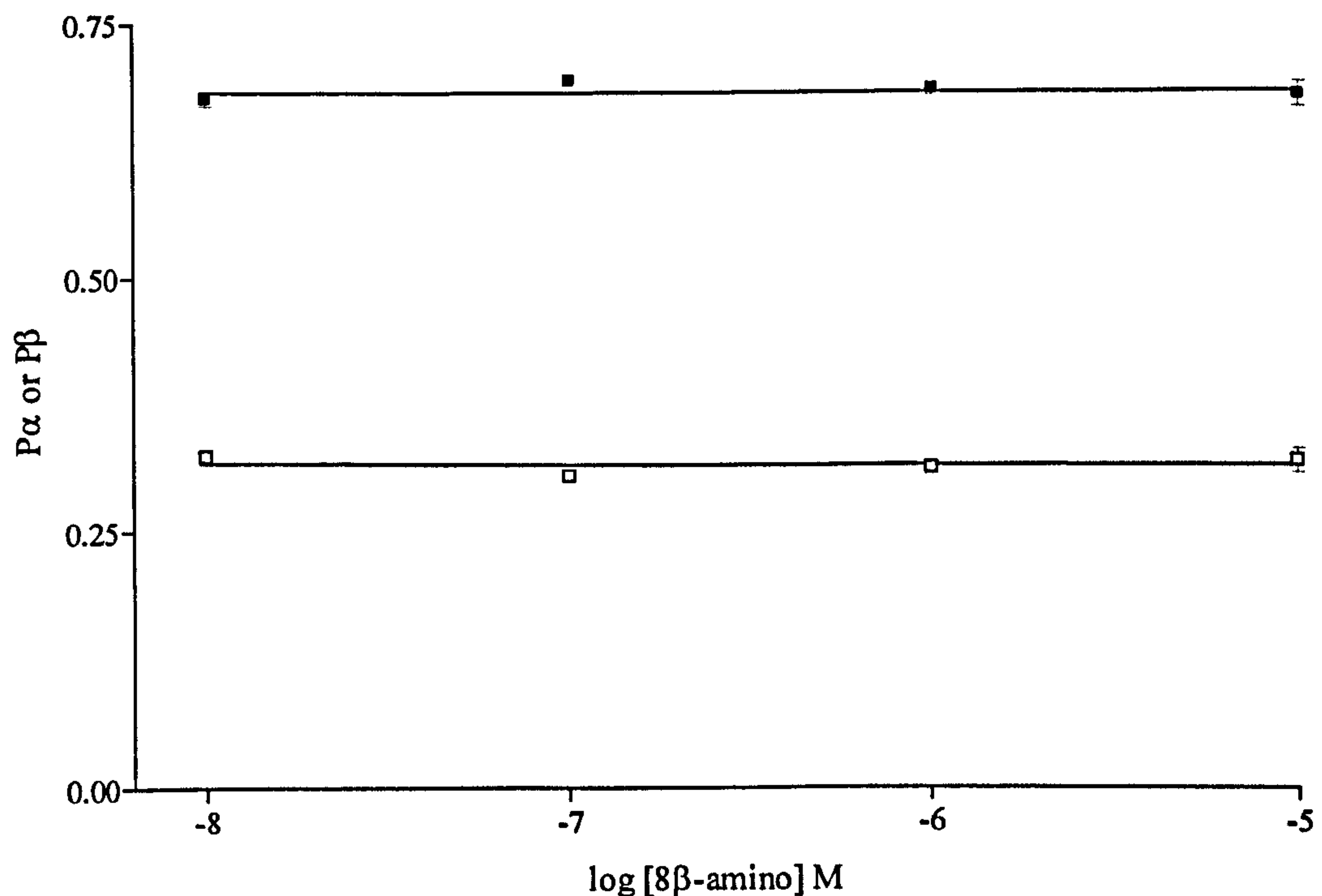


Figure 6.4 Relationship between the probability of occurrence of the α (□) and β (■) subconductance states and concentration of 8 β -amino-9 α -hydroxyryanodine in the solution at the cytosolic face of the RyR channel. Transmembrane holding potential is +60 mV. Points are mean $\pm$ SEM., $n = 4-6$.

The alternative explanation for the observed behaviour is that with the ryanoid bound, the RyR channel fluctuates between two conductance states (α and β). This in turn suggests that with a ryanoid, such as 8 β -amino-9 α -hydroxyryanodine occupying the high affinity ryanoid binding site on the RyR channel, the conduction pathway of the channel fluctuates between two conformations with slightly different ion-handling properties. Consistent with this proposal, the unitary current amplitudes of both the α and β states vary linearly with holding potential as shown in Figure 6.5. The α and β states have values of FC of 0.31 ± 0.01 and 0.56 ± 0.01 respectively ($n = 4-9$).

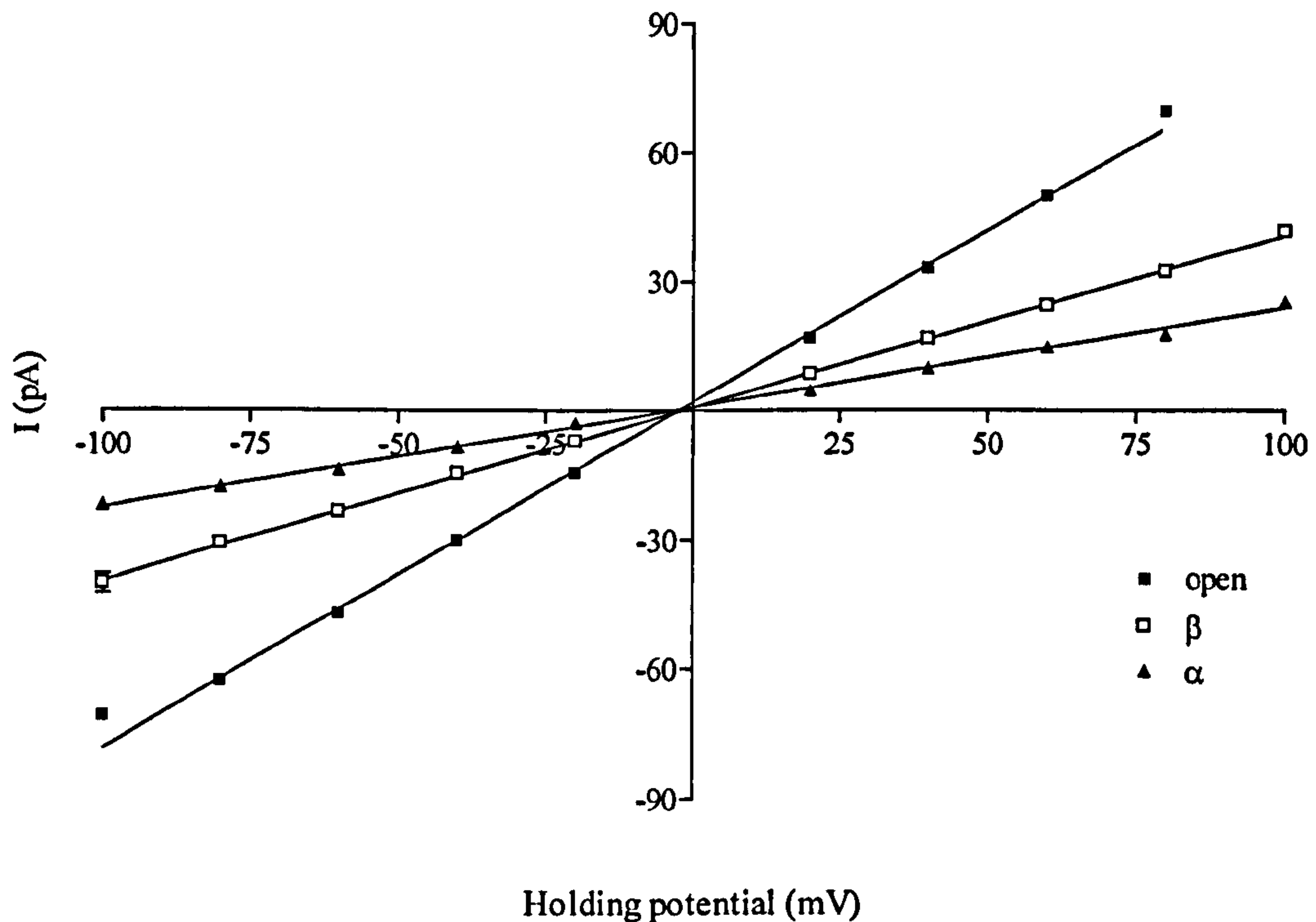


Figure 6. 5 Variations in unitary current amplitude with transmembrane holding potential of the full open conductance state and the α and β modified subconductance state induce by 8 β -amino-9 α -hydroxyryanodine. Points are mean $\pm$ SEM. Open, $n = 6-18$; α , $n = 4-9$; β , $n = 4-9$.

Investigations in the previous chapters demonstrate that, in addition to modifying rates of permeant ion translocation, the interaction of a ryanoid with the RyR channel alters the effectiveness of TEA as a concentration- and voltage-dependent blocker of K^+ translocation. Broadly, the effectiveness of TEA as a blocker of a ryanoid-modified RyR channel correlates with the FC of the modified state induced by the ryanoid; the smaller the FC induced by a ryanoid, the greater the concentration of TEA is required to produce block. These ryanoid-induced changes result from alterations in the voltage dependence of the TEA blocking reaction and in the affinity of the conduction pathway for the blocking cation. TEA was used to investigate the nature of the α and the β conductance states induced by 8 β -amino-9 α -hydroxyryanodine. Figure 6.6 shows current fluctuations of a single RyR channel in the presence of 10 μ M 8 β -amino-9 α -hydroxyryanodine at holding potentials ranging from -80 to $+80$ mV. The left hand panel shows control traces whilst the right-hand panel shows representative traces from the same channel following the addition of 20 mM TEA to the solutions at both the cytosolic and luminal faces of the channel. Relationships between unitary current

amplitude and holding potential for the full open conductance state and the α - and β -subconductance states in the absence and presence of 20 mM TEA are shown in Figure 6.7 (n = 4-9).

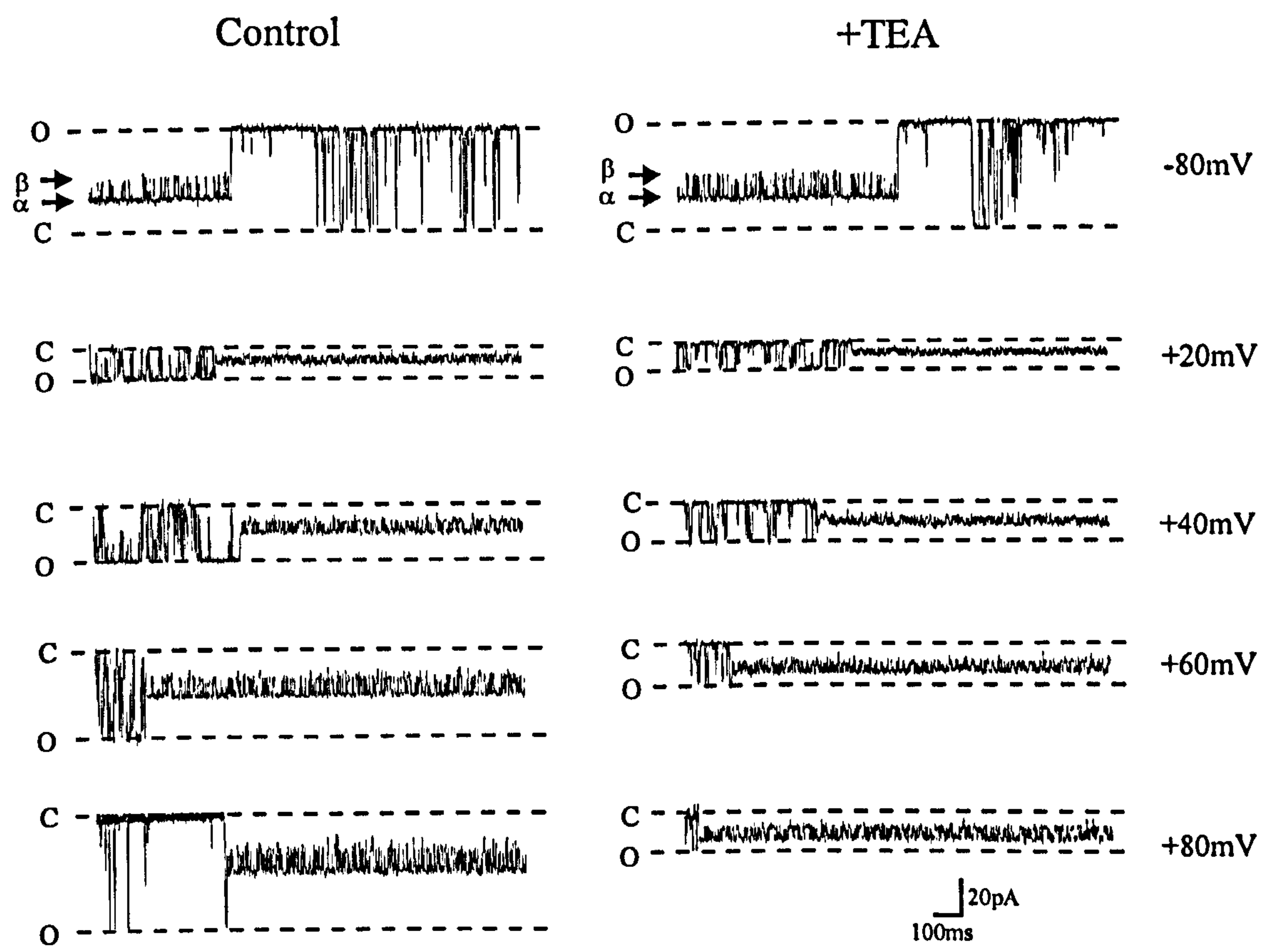


Figure 6. 6 Block of full open conductance state and the α and β 8 β -amino-9 α -hydroxyryanodine-modified subconductance states of a representative channel by symmetrical 20 mM TEA. Left panel shows variation in unitary current amplitude at the indicated transmembrane holding potentials in the absence of TEA. The right panel shows equivalent current fluctuations in the presence of TEA.

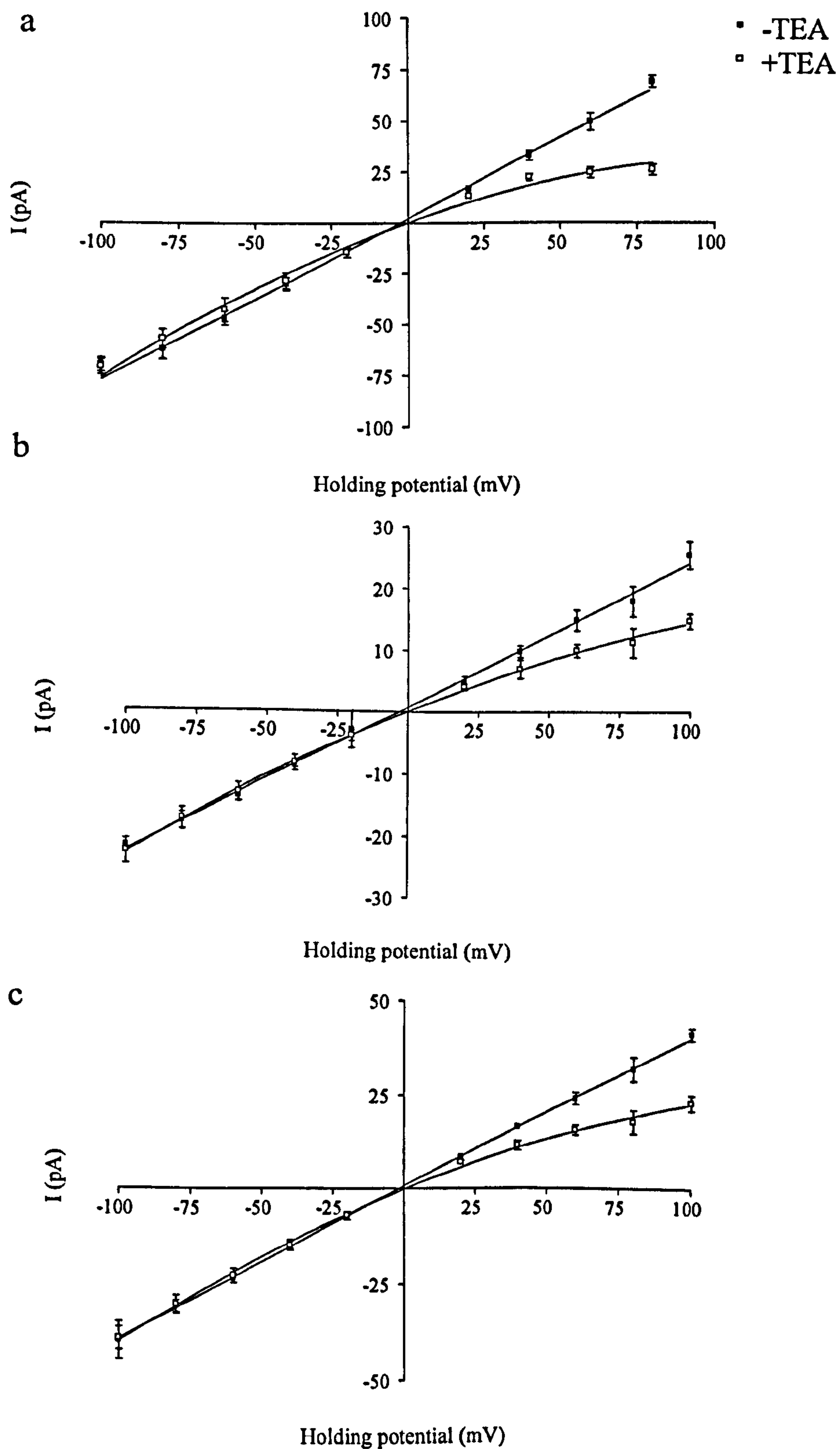


Figure 6. 7 Current-voltage relationships for the full open conductance state (a) and the α (b) and β (c) 8 β -amino-9 α -hydroxyryanodine-modified subconductance states of the RyR channel in the absence ($\blacksquare$) and presence ($\square$) of symmetrical 20 mM TEA. Points are mean $\pm$ SEM. Open $\pm$ TEA, $n = 6-18$; α -TEA, $n = 4-9$; α + TEA, $n = 6-8$; β - TEA, $n = 4-9$; β + TEA, $n = 6-8$.

In the absence of ryanoids, TEA is a concentration- and voltage-dependent blocker of K^+ translocation when present at the cytosolic face of the RyR channel (Lindsay *et al.*, 1991). As can be seen in Figure 6.7a, the degree of block produced by TEA increases as transmembrane voltage is taken to more positive potentials; at a holding potential of 80 mV, 20 mM TEA reduces unitary current amplitude of the full open state of the RyR channel from 69.65 ± 0.86 pA to 25.94 ± 0.73 pA (a reduction of 63%). TEA also blocks K^+ translocation in the α and β -subconductance states induced by 8 β -amino-9 α -hydroxyryanodine, however, the degree of block is significantly less than that seen in the full open state. At a holding potential of 80 mV, 20 mM TEA reduces the unitary current amplitude of the α state from 17.77 ± 1.21 pA to 11.13 ± 0.89 pA (a reduction of 37%) (Figure 6.7b) and that of the β state from 32.24 ± 1.44 pA to 18.13 ± 1.16 pA (a reduction of 44%) (Figure 6.7c). Woodhull analysis of the data presented in Figure 6.6 demonstrates that the reduced effectiveness of TEA in the α and β states induced by 8 β -amino-9 α -hydroxyryanodine results from the alterations in the voltage dependence and affinity of the TEA blocking reaction. Values of $z\delta$ and $K_b(0)$ for block of the open, α and β states are: $z\delta = 0.88$, $K_b(0) = 167.3$ mM; $z\delta = 0.45$, $K_b(0) = 139.3$ mM; $z\delta = 0.52$, $K_b(0) = 147.8$ mM respectively. These observations indicate that both the α and β -subconductance states have properties that characterise them as separate ryanoid-modified subconductance states and result from rearrangements in the structure of the conduction pathway of the RyR channel following the binding of 8 β -amino-9 α -hydroxyryanodine.

6.3.2 *The influence of transmembrane holding potential on the probability of occurrence of the α and β -subconductance states induced by 8 β -amino-9 α -hydroxyryanodine*

The interaction of 8 β -amino-9 α -hydroxyryanodine with the RyR channel results in the occurrence of two subconductance states, the α and β states. Altering transmembrane holding potential dramatically influences the probability of the channel residing in either the α or β states. Traces in Figure 6.8 show the occurrence of either the α or the β state induced by the interaction of 8 β -amino-9 α -hydroxyryanodine with a single RyR channel while transmembrane holding potential is altered. At +80 mV the channel resides mostly in the β state, as transmembrane holding potential is made more negative

the probability that the channel resides in the β state decreases whilst the probability of occurrence of the α state is increased. The relationship between the probability that the channel is in the α or β state ($P\alpha$ or $P\beta$) and transmembrane holding potential is shown in Figure 6.9. The solid line is the best fit Boltzman distribution (equation 6.3) obtained by nonlinear regression. The value of z_α and z_β derived from the slopes of these lines are 0.96 and 1, respectively.

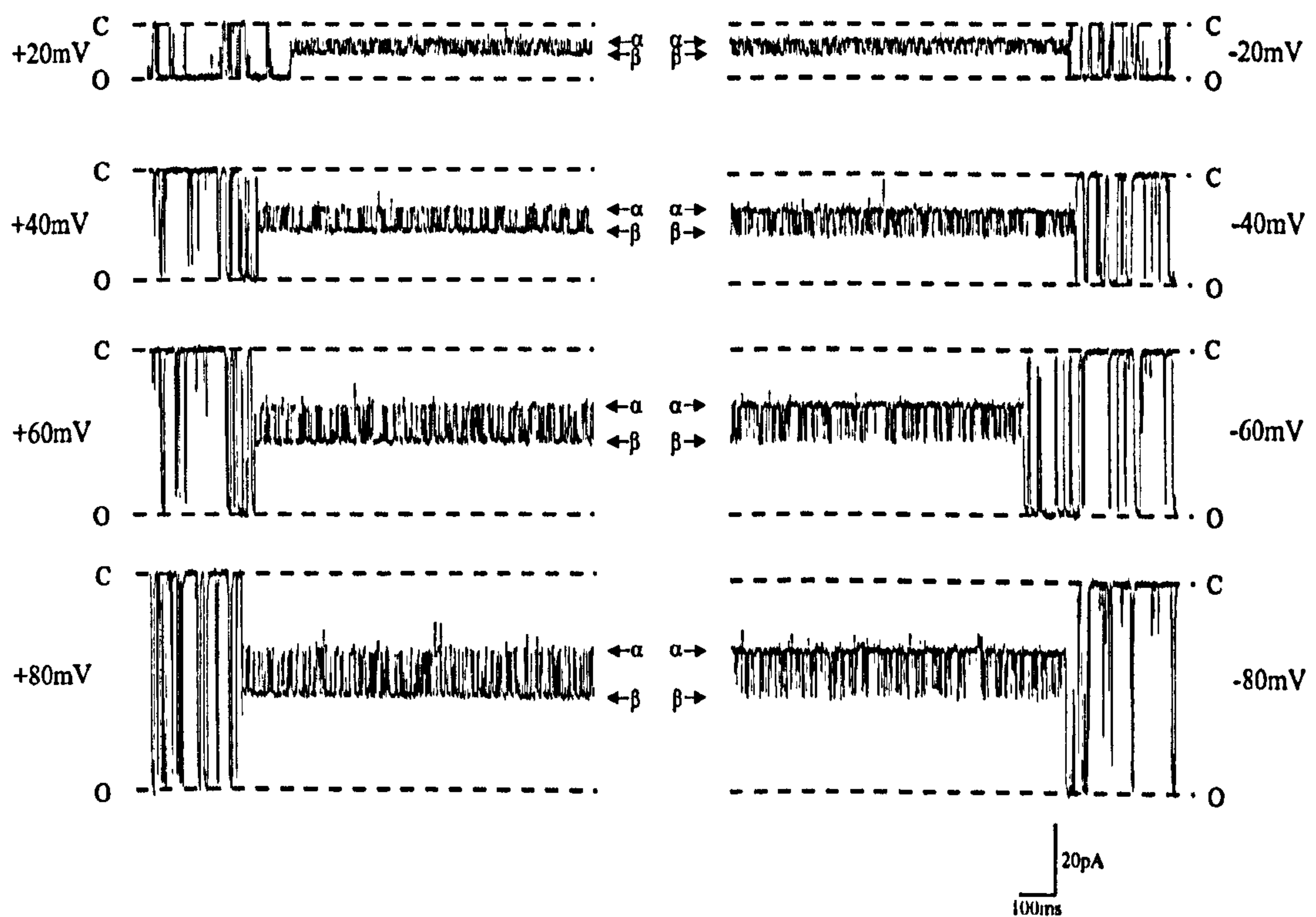


Figure 6.8 Representative traces of 8 β -amino-9 α -hydroxyryanodine-modified subconductance states at different transmembrane holding potentials.

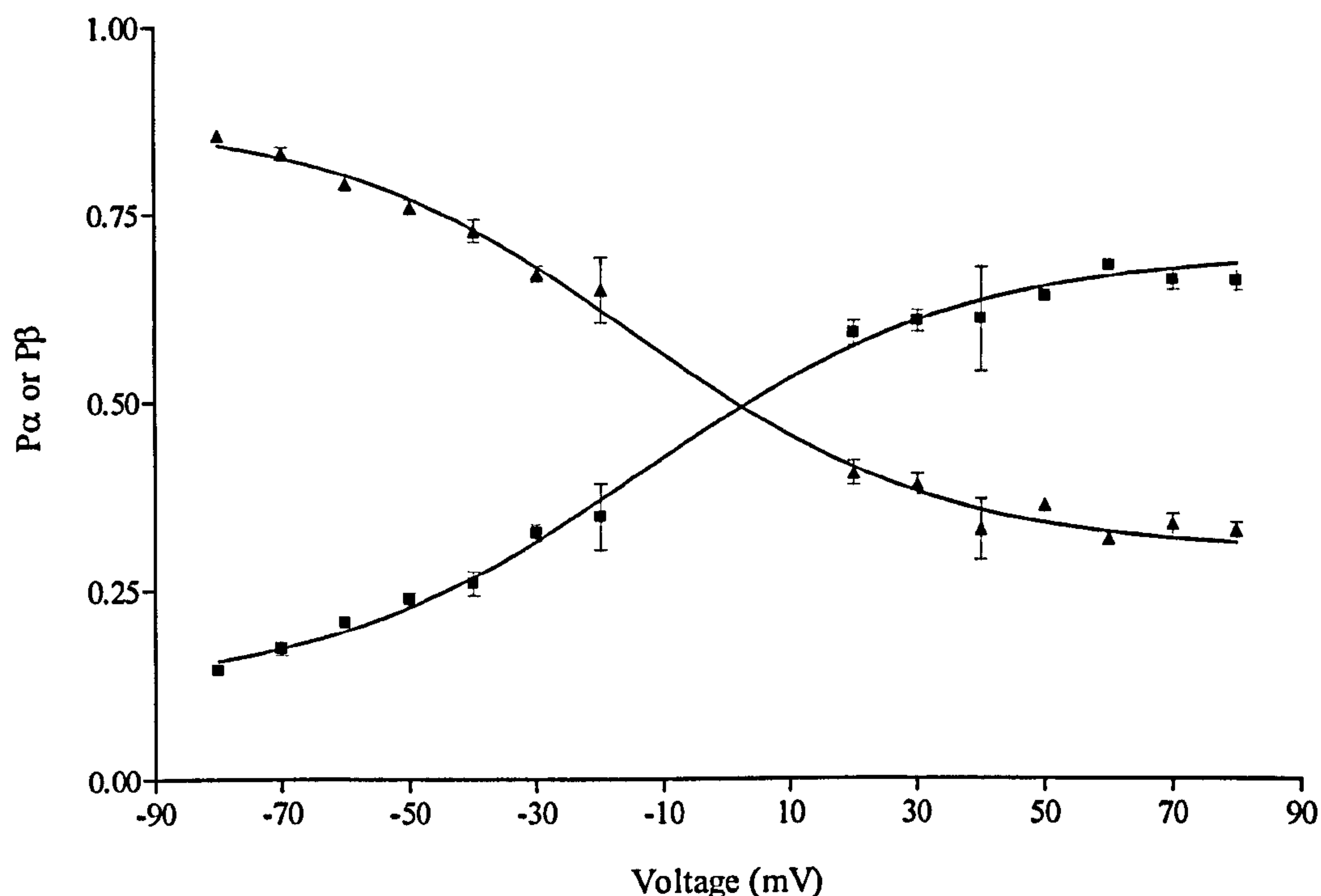


Figure 6. 9 The relationship between the probability of occurrence of the α (▲) and β (■) states of the 8 β -amino-9 α -hydroxyryanodine-modified subconductance state and transmembrane holding potential. Solid lines are best fit Boltzmann distributions (equation 6.3) obtained by non linear regression. Points are mean $\pm$ SEM. P α and P β n = 4-23.

Lifetime analysis of dwell times in the α and β state was carried out for all 8 β -amino-9 α -hydroxyryanodine-modified subconductance states held at all transmembrane holding potentials observed using the analysis program Satori. The distribution of dwell times in the α and β state in all cases were best described by single exponentials. Figure 6.10 shows typical non-cumulative histograms of dwell times in the 8 β -amino-9 α -hydroxyryanodine-induced α and β subconductance state of a single channel, monitored at a transmembrane holding potential of +30 mV. In both plots, the solid line is a probability density function (pdf) with a single exponential term fitted by maximum likelihood to individual dwell times. A reduced number of events in the first bin of the histograms in Figure 6.10 would have been expected, due to the limitations of the recording and analysis system not being able to resolve very brief events. These experiments were filtered using a low pass Bessel filter (mentioned in chapter 2) at 1 kHz prior to analysis, at this frequency the minimum time resolution is approximately 1.2 ms, so any events below this would not be resolved. Therefore, for lifetime analysis,

any events below 1.2 ms were stripped from the data. A missed events correction was then employed, (Colquhoun and Sigworth. 1983). The mean dwell time in the α state (τ_α) is 2.14 ms, while that of the β state (τ_β) is 3.05 ms at +30 mV.

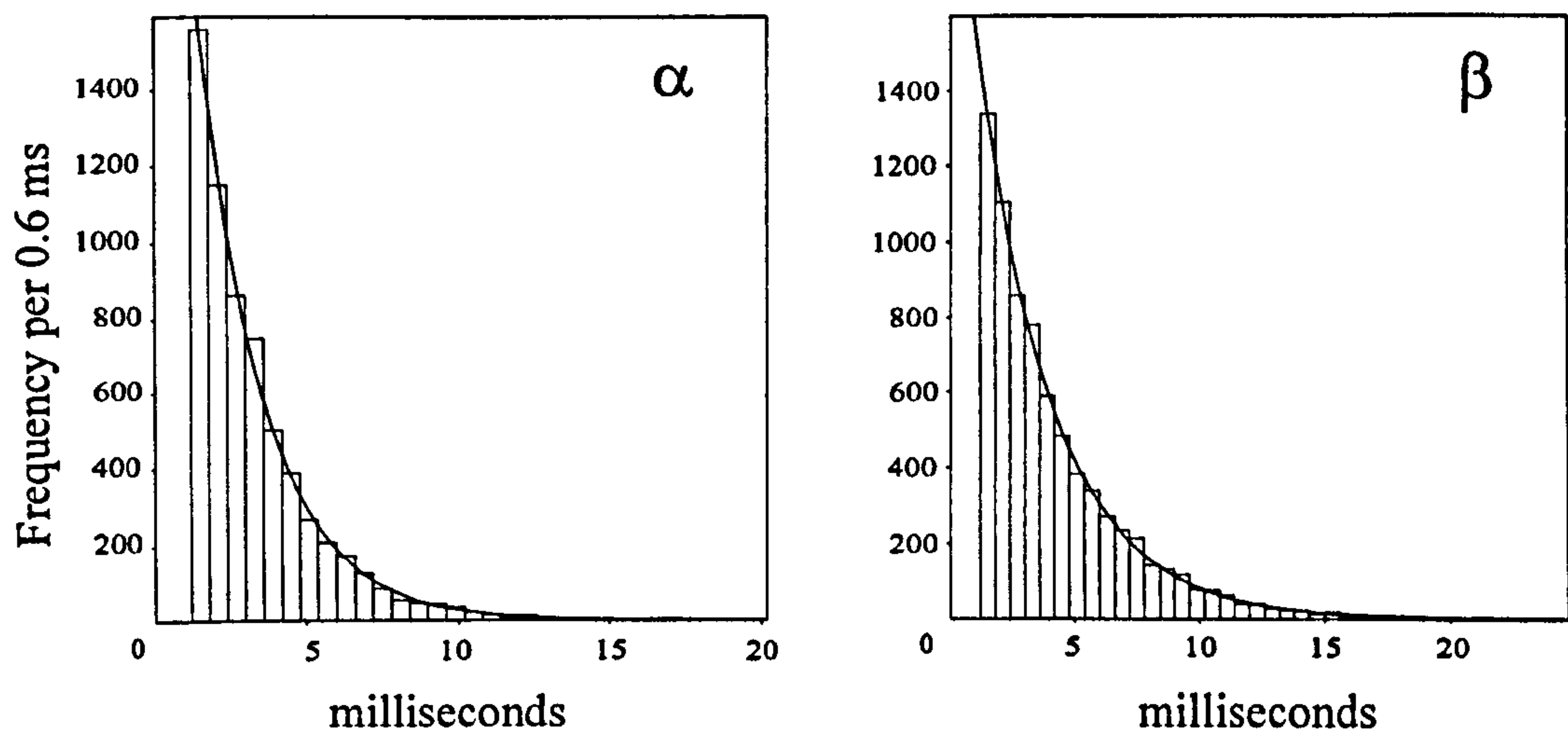
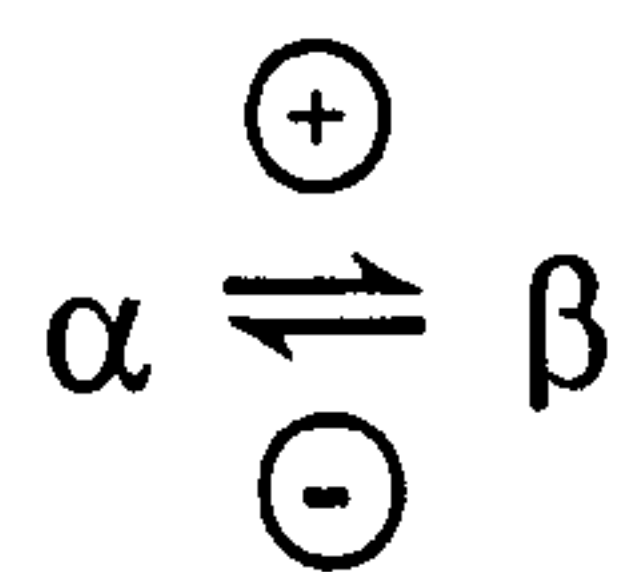


Figure 6. 10 Non-cumulative histograms of α and β dwell times of an 8 β -amino-9 α -hydroxyryanodine-modified subconductance state at a transmembrane holding potential of +30 mV. Solid lines are pdfs fitted by maximum likelihood to individual dwell times. Both distributions are best described by single exponential fits (τ_α is 2.14 ms; τ_β is 3.05 ms).

The data are consistent with a simple kinetic scheme such that the 8 β -amino-9 α -hydroxyryanodine-modified conductance state is derived from a voltage dependent equilibrium between a single α and a single β subconductance state as shown below:



Therefore the apparent rate constants for the transitions between the α and β -subconductance states ($K_{\alpha\beta}$) and the β and α subconductance states ($K_{\beta\alpha}$) of the 8 β -amino-9 α -amino-hydroxyryanodine-modified state can be determined from the mean dwell times obtained from lifetime analysis in the α and β -subconductance states, as shown below:

$$K_{\alpha\beta} = (\tau_{\alpha})^{-1} \quad \text{equation 6.4}$$

and

$$K_{\beta\alpha} = (\tau_{\beta})^{-1} \quad \text{equation 6.5}$$

The rate constants at a given voltage can be described as follows:

$$K_{\alpha\beta}(V) = K_{\alpha\beta}(0) \cdot \exp^{[z_{\alpha\beta} \cdot (FV / RT)]} \quad \text{equation 6.6}$$

and

$$K_{\beta\alpha}(V) = K_{\beta\alpha}(0) \cdot \exp^{[-z_{\beta\alpha} \cdot (FV / RT)]} \quad \text{equation 6.7}$$

were $K(V)$ and $K(0)$ are the rate constants at a particular voltage and at 0 mV, respectively, and z is the valence of the appropriate reaction. z may then be determined as the slope of the plot of the natural logarithm of the rate constant against transmembrane holding potential ($z_{\alpha\beta}F/RT$ and $-z_{\beta\alpha}F/RT$) and $K(0)$ may be determined from the intercept ($\ln[K_{\alpha\beta}(0)]$ and $\ln[K_{\beta\alpha}(0)]$). The total voltage dependence of the reaction is then given by $z_{\alpha\beta} + z_{\beta\alpha}$. A plot of this form is shown in Figure 6.11.

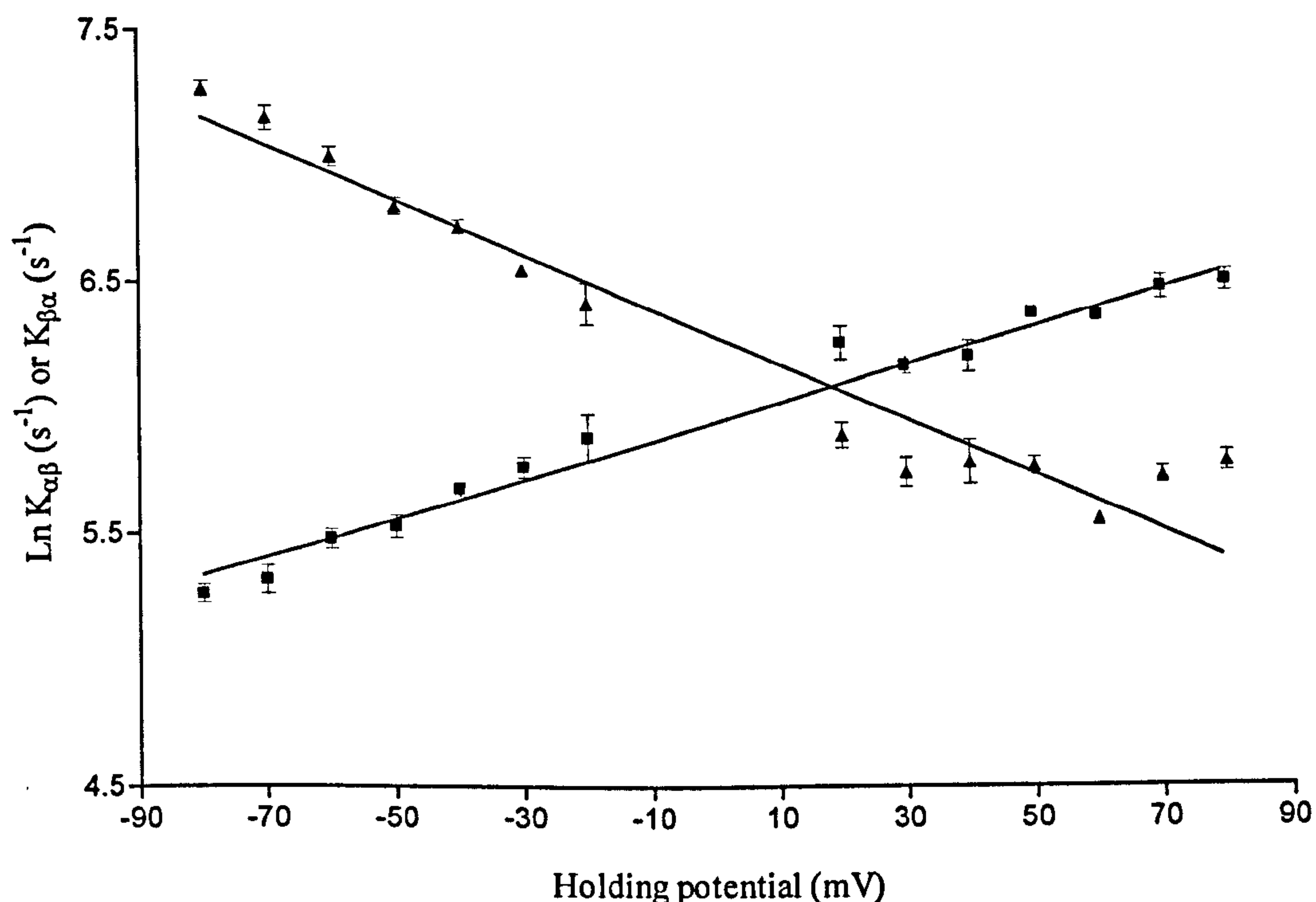


Figure 6.11 Variation in the transition rates between α and β (■) and β and α (▲) subconductance states of the 8 β -amino-9 α -hydroxyryanodine-modified state with transmembrane holding potential. Each point is the mean $\pm$ SEM of 4-23 experiments. The solid lines were obtained by linear regression with parameters quoted in the text.

$K_{\alpha\beta}$ and $K_{\beta\alpha}$ both vary with applied transmembrane holding potential. $K_{\alpha\beta}$ increases, as transmembrane holding potential is made more positive, while the opposite is true for $K_{\beta\alpha}$. The solid lines drawn through the points in Figure 6.11 were obtained by linear regression and the values of $z_{\alpha\beta}$ and $z_{\beta\alpha}$ obtained from the slopes of these lines are 0.19 and 0.28, respectively giving a total valency of 0.47.

There seems to be some discrepancy between the z values obtained from Figure 6.11 (0.47) and Figure 6.9 (~ 1.0). In both cases the measurements were carried out independently, yet the z values are somewhat different. An inspection of the data in Figure 6.11, also suggests that the rate of transition between the β and α subconductance state deviates from the linear regression after trans membrane holding potentials is taken above +30 mV. This indicates that there is another voltage dependent effect happening at high positive voltages, but the reason for this phenomenon is still unclear.

The association and dissociation of *8 β -amino-9 α -hydroxyryanodine* with the single RyR channel was closely investigated for a better insight of the occurrence of the α and β subconductance states, as the ryanoid binds and unbinds to and from the channel. These investigations were carried out on individual *8 β -amino-9 α -hydroxyryanodine*-modified events for every experiment mentioned in this chapter. In all cases the association and the dissociation of *8 β -amino-9 α -hydroxyryanodine* to and from the channel occurs from the α -subconductance state (94% of the time) and very rarely to and from the β -subconductance state (6%). Figure 6.12A (top trace) displays traces of both the association and dissociation of the ryanoid to and from the channel at +60 mV, while Figure 6.12B (bottom trace) displays traces of both the association and dissociation of *8 β -amino-9 α -hydroxyryanodine* to and from the channel at -60 mV. The association and the dissociation of the ryanoid to and from the channel were observed by eye from the recorded data displayed on the computer screen, therefore, it is possible that the association and the dissociation of the ryanoid to and from the channel does always occur from the α -subconductance state. However, due to the limitations of the recording and analysis program on resolving very brief events it is not always possible to observe these transition and so a small percentage of these transitions seem to go to and from the β -subconductance state.

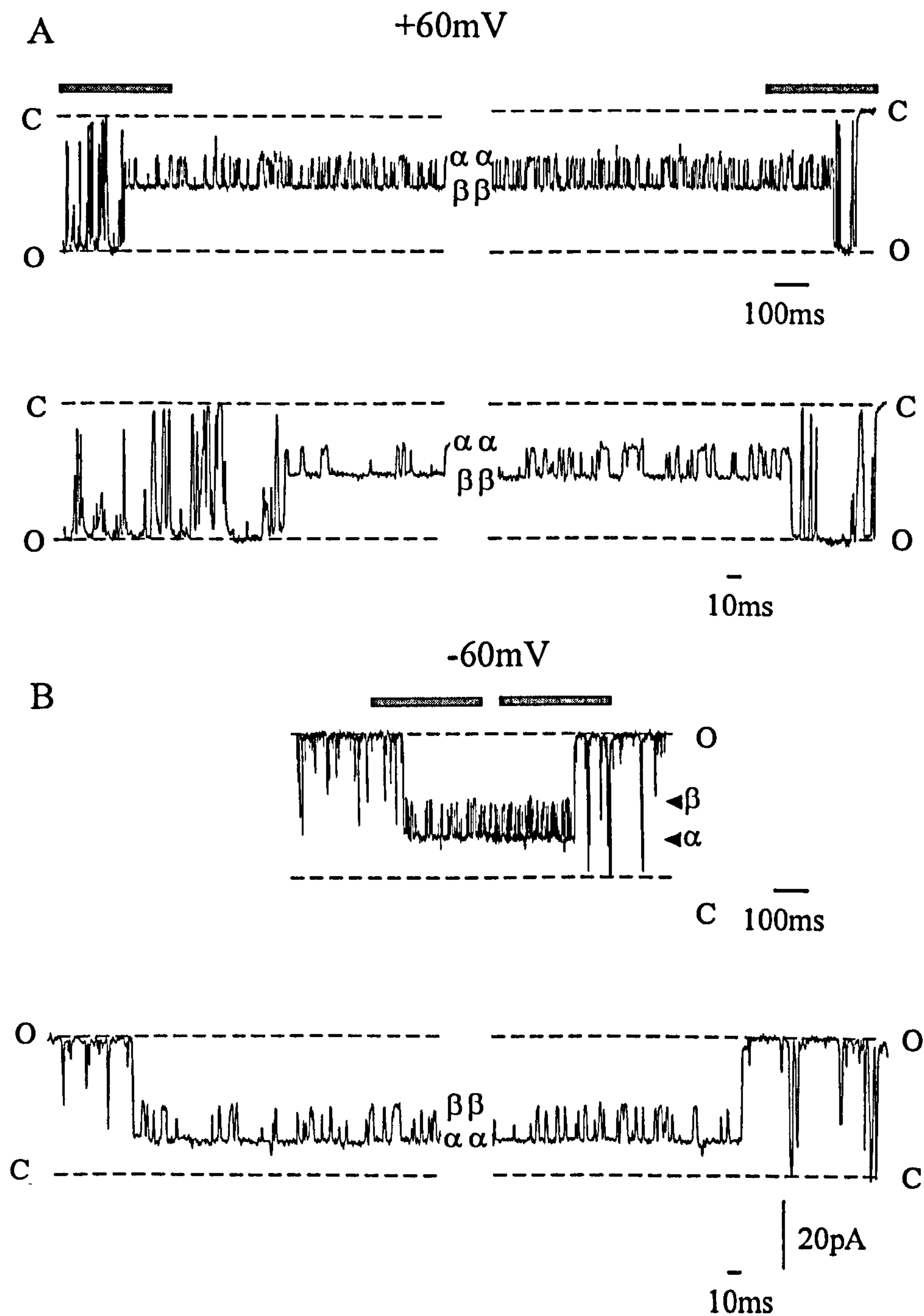


Figure 6.12 A trace of the association and dissociation of 8β-amino-9α-hydroxyryanodine to and from the RyR channel at +60 mV (A, top trace) and at -60 mV (B, bottom trace).

6.4 Discussion

The data presented in this chapter suggest that the α and β -subconductance states observed with 8β-amino-9α-hydroxyryanodine bound to the RyR channel have properties that characterise them as separate ryanoid-modified states, which would

result from a rearrangement in the structure of the conduction pathway of the RyR channel following the binding of this ryanoid with the channel. When the actions of the blocking tetraalkylammonium cation TEA on the α and β states were compared with the control full open state of the channel, TEA was a significantly less effective blocker of the two ryanoid-induced α and β -subconductance states. The small difference in effectiveness of TEA in the α and β -subconductance states is entirely consistent with observations made in the previous chapter with ryanoids that induce single 'quiet', modified conductance state. Previously, the effectiveness of TEA was shown to reduce with lowered channel FC, as a result of the rearrangement of the conduction pathway of the channel by the interaction of a ryanoid that altered the position of the TEA binding site within the voltage drop across the channel and the affinity of the channel for TEA. In this instance the FC of the β state (0.56) is larger than that observed with the α state (0.31), and overall the effectiveness of TEA as a blocker of the RyR channel would have been predicted be greater when the channel was in the β rather than the α -subconductance state. The results presented here do indeed agree with this hypothesis, the effectiveness of TEA, as a blocker is greater when the channel is in the β -subconductance state. These observations agree with the proposal that both the α and β subconductance states are different conductance states reflecting different conformations of the conduction pathway of the RyR channel with different ion-handling properties induced by 8 β -amino-9 α -hydroxyryanodine. The small difference in the FC of α and β -subconductance states, suggests little difference in the conformations of the conduction pathway between the two states, and is reflected by the small shift in both the TEA binding site within the voltage drop across the channel and the effectiveness of TEA when the channel is in either of the two subconductance states.

Altering transmembrane holding potential proved to have a significant effect on the probability of the channel being in either the α and β -subconductance state. The probability the channel is in the β -subconductance state is high at high positive potentials and low at the high negative potentials. While the opposite is true for the α -subconductance state. Lifetime analysis carried out on the 'noisy' 8 β -amino-9 α -hydroxyryanodine-modified state at all transmembrane holding potential, demonstrated that the modified state resulted from transitions between a single α and a single β -subconductance state. This raised the question, is the probability the channel residing in

either the α and β -subconductance state in anyway related to the association and dissociation of 8 β -amino-9 α -hydroxyryanodine to and from the channel? A closer inspection of both the association and the dissociation of 8 β -amino-9 α -hydroxyryanodine to and from the channel revealed that the majority of the time it was the α -subconductance state the ryanoid preferentially induced upon the association of the ryanoid with the channel. Similarly, the ryanoid dissociated from the α -subconductance state. In other words, the association of the ryanoid induces the α -subconductance state prior to the β -subconductance state, and the dissociation of the ryanoid only occurs from the α -subconductance state, so if the channel was in the β -subconductance state the ryanoid could only dissociate from the channel after a transition from the β to the α -subconductance state. Would this explanation of the interaction of 8 β -amino-9 α -hydroxyryanodine with the RyR channel fit in with the previous observations (Tanna *et al.*, 1998; see Chapter 3) made with the interaction of ryanoids with the RyR channel? Earlier investigations of the interaction of 8 β -amino-9 α -hydroxyryanodine and other ryanoids described in chapter 3, with individual RyR channels, demonstrated the association of a ryanoid with the channel was dependent on the channel being in the open conformation, the net charge of the ryanoid and a voltage dependent conformational change of the channel protein, resulting in the transition from a low to a high affinity binding site for the ryanoids. Both the association and dissociation of these ryanoids to and from its high affinity binding site on the channel are sensitive to transmembrane holding potential. A kinetic scheme was proposed for this reaction and is shown in Figure 6.13 (Tanna *et al.*, 1998; Chapter 3 of this thesis).

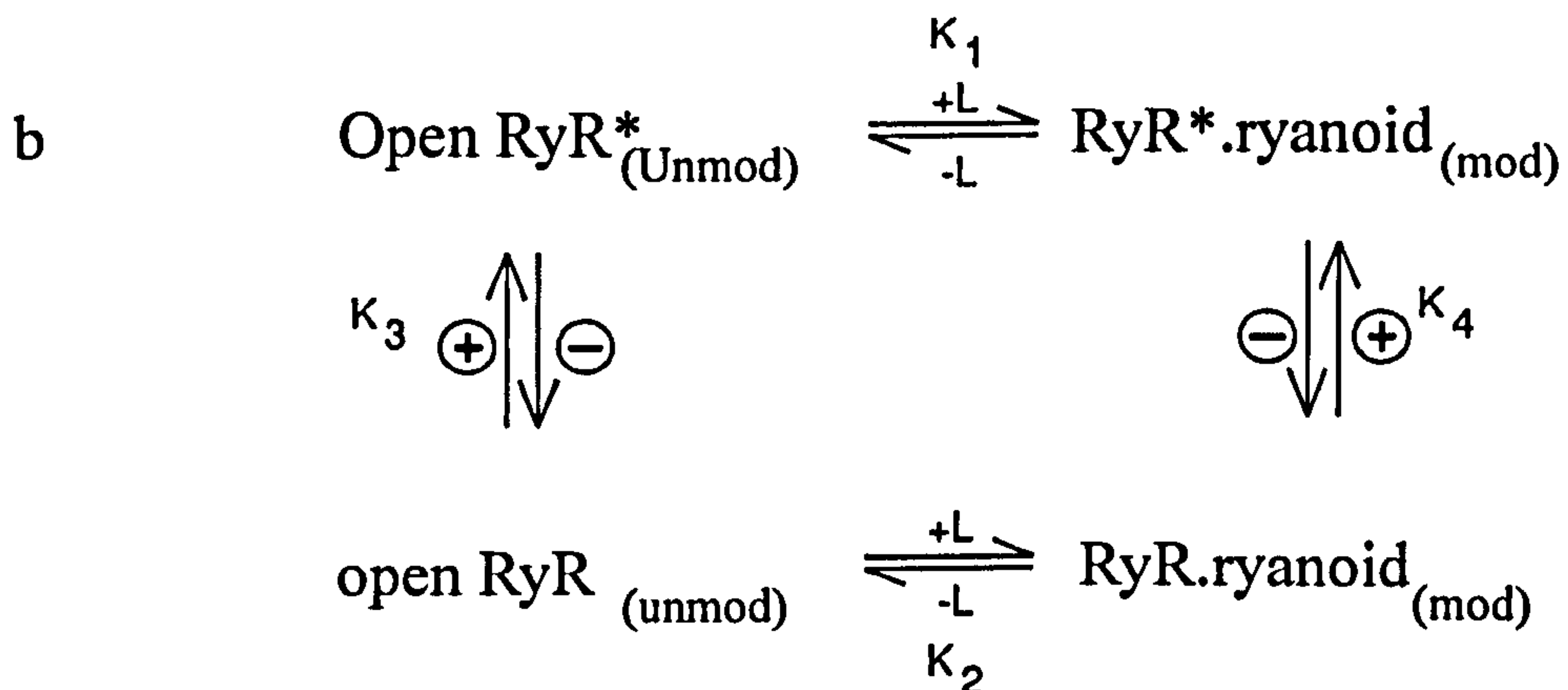
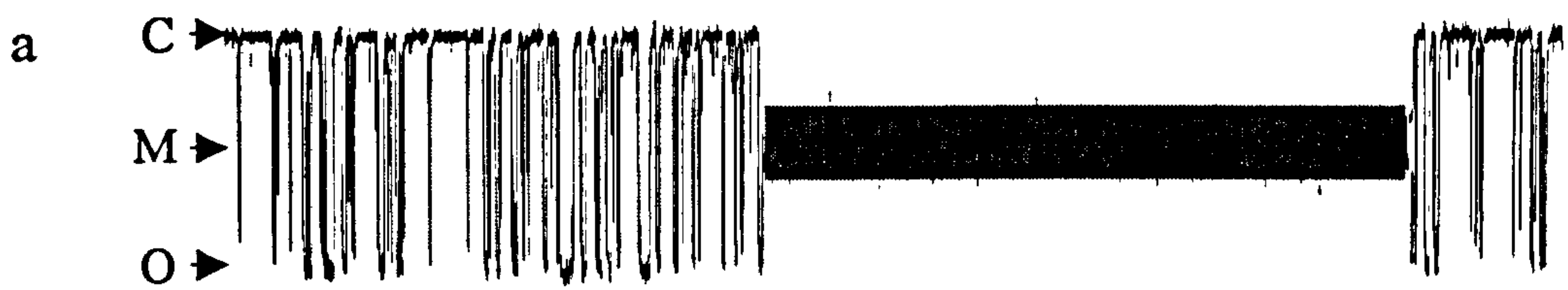


Figure 6.13 b) Scheme summarising the influence of transmembrane holding potential on the interaction of a ryanoid with the high affinity binding site of the RyR channel as shown in a) the modified state induced by all ryanoids is measured as just one subconductance state indicated by the grey block. The ryanoid binding site is only available when the channel is open and only accessible from the cytosolic side of the channel. L represents the ligand (any ryanoid), and + and – indicate the application of positive and negative potentials to the cytosolic side of the channel. A minimum of two forms of vacant receptors exist in this model (low affinity, open RyR: high affinity, RyR*). Increasing positive potential shifts the equilibrium towards open RyR* by increasing the value of the equilibrium constants K_3 and K_4 . The ryanoid binding site is located someway into the voltage drop across the channel, and so a charged ryanoid will experience an additional influence of voltage.

With the additional information observed in this chapter, the proposed kinetic scheme is further developed for the interaction of 8β-amino-9α-hydroxyryanodine and possibly 21-amino-9α-hydroxyryanodine as that shown below in Figure 6.14.

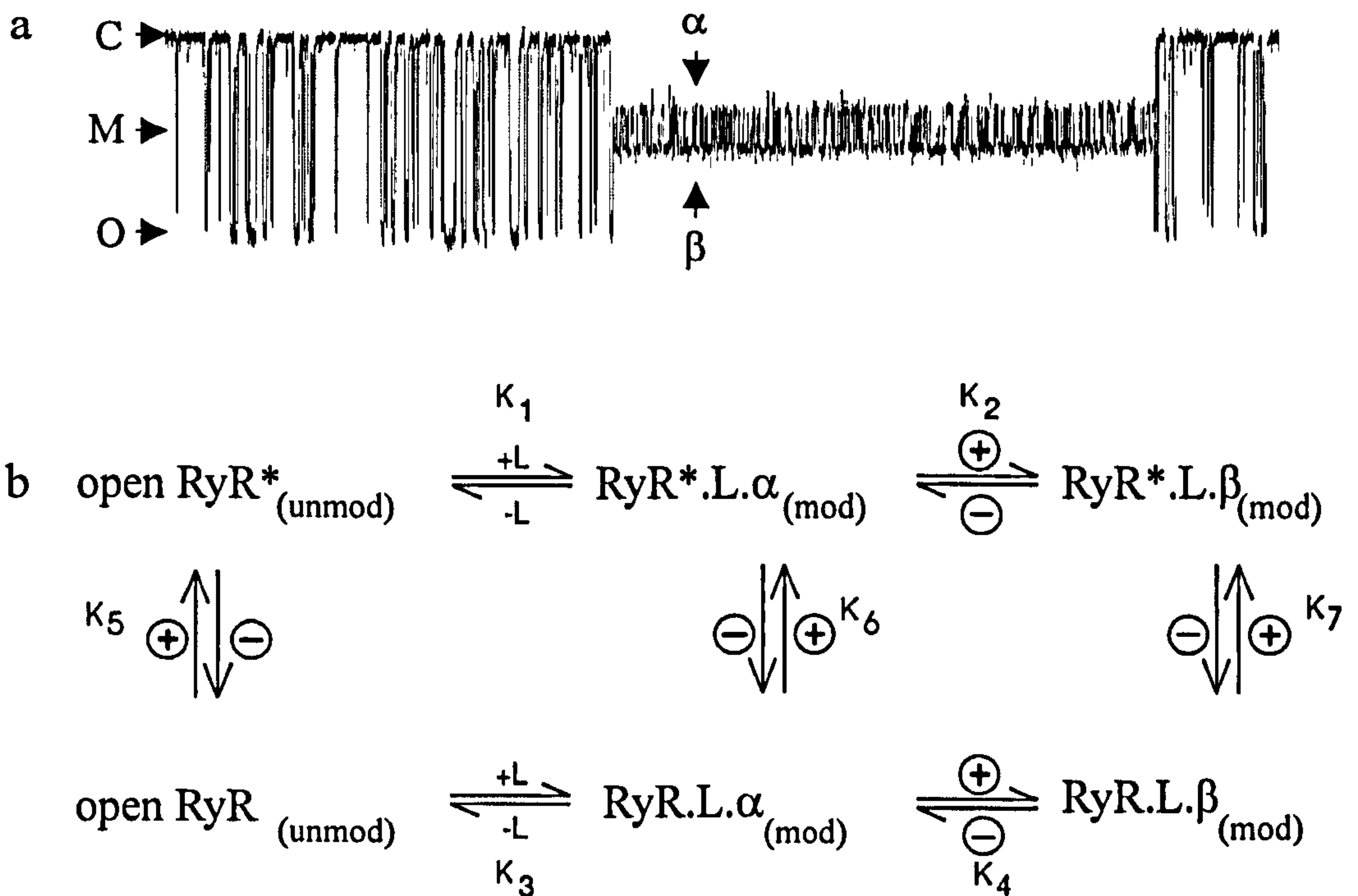


Figure 6.14 b) Scheme summarising the influence of transmembrane holding potential on the interaction of ryanoids that induce clearly resolvable α and β -subconductance states such as 8 β -amino-9 α -hydroxyryanodine shown in a (top). The ryanoid binding site is only available when the channel is open and only accessible from the cytosolic side of the channel. L represents the ligand (i.e. 8 β -amino-9 α -hydroxyryanodine), and + and – indicate the application of positive and negative potentials to the cytosolic side of the channel. A minimum of two forms of vacant receptors exist in this model (low affinity, open RyR: high affinity, RyR*). Increasing positive potential shifts the equilibrium towards open RyR* by increasing the value of the equilibrium constants K_5 , K_6 and K_7 . The ryanoid binding site is located someway into the voltage drop across the channel, and so a charged ryanoid will experience an additional influence of voltage.

The association of 8 β -amino-9 α -hydroxyryanodine with the channel is dependent on channel P_o , the voltage dependent switch in the affinity of the channel for the ryanoid as mentioned above and also the net charge of the ryanoid (+1). However, the scheme proposed in Figure 6.14 constructed from the data obtained in this chapter suggests, that upon the association of 8 β -amino-9 α -hydroxyryanodine to the channel, the ryanoid initially induces the α -subconductance state prior to transitions to and from the β -subconductance state. The probability the channel resides in either subconductance states is influenced by transmembrane holding potential as described in Figure 6.9. At

high positive potentials the probability the channel is in the β -subconductance state is high, while a shift of voltage to high negative potential increases the likelihood the channel resides in the α -subconductance state. Analysis of the voltage dependence of the transitions between the α and β -subconductance states indicate an increase in the transition from the α -subconductance state to the β -subconductance state at high positive potentials, whilst the opposite is true for the transition from the β to the α -subconductance. A change in voltage to negative potentials would shift the equilibrium to the left so that the probability of occurrence of the α -subconductance state increases and the probability that the ryanoid dissociates from the channel would be raised. Therefore, although, the occurrence of either one of the two subconductance states is subject to the association of the ryanoid with the channel, the dissociation of the ryanoid from the channel is dependent on the occurrence of the α -subconductance state.

The overall voltage dependence of the interaction of 8 β -amino-9 α -hydroxyryanodine (2.69, observed in chapter 3) with the RyR channel is greater than that observed for the transitions between the two subconductance states (1-0.5) induced by the ryanoid. This is consistent with the scheme proposed in Figure 6.14, which suggests that the rate of association of a ryanoid to its high affinity binding site is dependent on voltage dependent conformational change in the channel protein. While the dissociation is reliant on the occurrence of the α -subconductance state, which is also influenced by voltage. Therefore the overall voltage dependence of the interaction of the ryanoid with the channel would be the sum of the two voltage dependent components of the scheme and to some extent the net charge of the ryanoid, if the high affinity binding site is located someway into the voltage drop.

Is there a unified theory for ryanoid reversibility? Ryanoids that interact with the channel and induce a 'noisy' modified state are generally those that are reversible i.e. 8 β -amino-9 α -hydroxyryanodine and 21-amino-9 α -hydroxyryanodine. Is the occurrence of a 'noisy' modified state, due to the occurrence of two subconductance states, which in the case of 21-amino-9 α -hydroxyryanodine are not readily resolvable, whilst irreversible ryanoids such as 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine and ryanodine, induce a single 'quiet' modified state? A possible explanation for this phenomenon could be that all ryanoids can induce two subconductance states, however

the occurrence of both in the presence of an irreversible ryanoid may not be as readily observable as those observed with reversible ryanoids. This would suggest that the 'quiet' modified subconductance state observed with the irreversible ryanoids would be the equivalent to the β -subconductance state seen in the presence of 8 β -amino-9 α -hydroxyryanodine. The scheme proposed in Figure 6.14 demonstrates that for the ryanoid to dissociate from the channel, the channel has to be in the α -subconductance state, if however the channel resided in the β -subconductance state, the probability that the ryanoid could dissociate from the channel would be effectively zero and the ryanoid-modified conductance state would appear to be 'quiet' and irreversible. The transitions between the α and β -subconductance states are voltage dependent, and for reversible ryanoids both are apparent over the transmembrane holding potential range (± 80 mV) the planar phospholipid bilayer can withstand before breaking, although they are not always clearly distinguishable. However, to observe the α -subconductance state in the presence of an irreversible ryanoid, may require transmembrane holding potential to be taken to negative voltages above (-80 mV) that a planar phospholipid bilayers can withstand and the probability of occurrence of the α state in the voltage range normally employed in a single channel experiment is very low. Alternatively, this proposal also suggests as shown in Figure 6.15C, the probability of the occurrence of the β -subconductance state induced by a reversible ryanoid could be very low at all workable holding potentials, therefore the ryanoid-induced state will, in effect, always be in the α -subconductance state and as a consequence the ryanoid is very likely to dissociate from the high affinity binding site. Such a ryanoid would not cause modifications. The rare brief events in which the channel is found predominantly in the α -subconductance state observed in the presence of 21-amino-9 α -hydroxyryanodine (Figure 6.2) appear to be consistent with this theory. Figure 6.15 describes the proposed hypothesis.

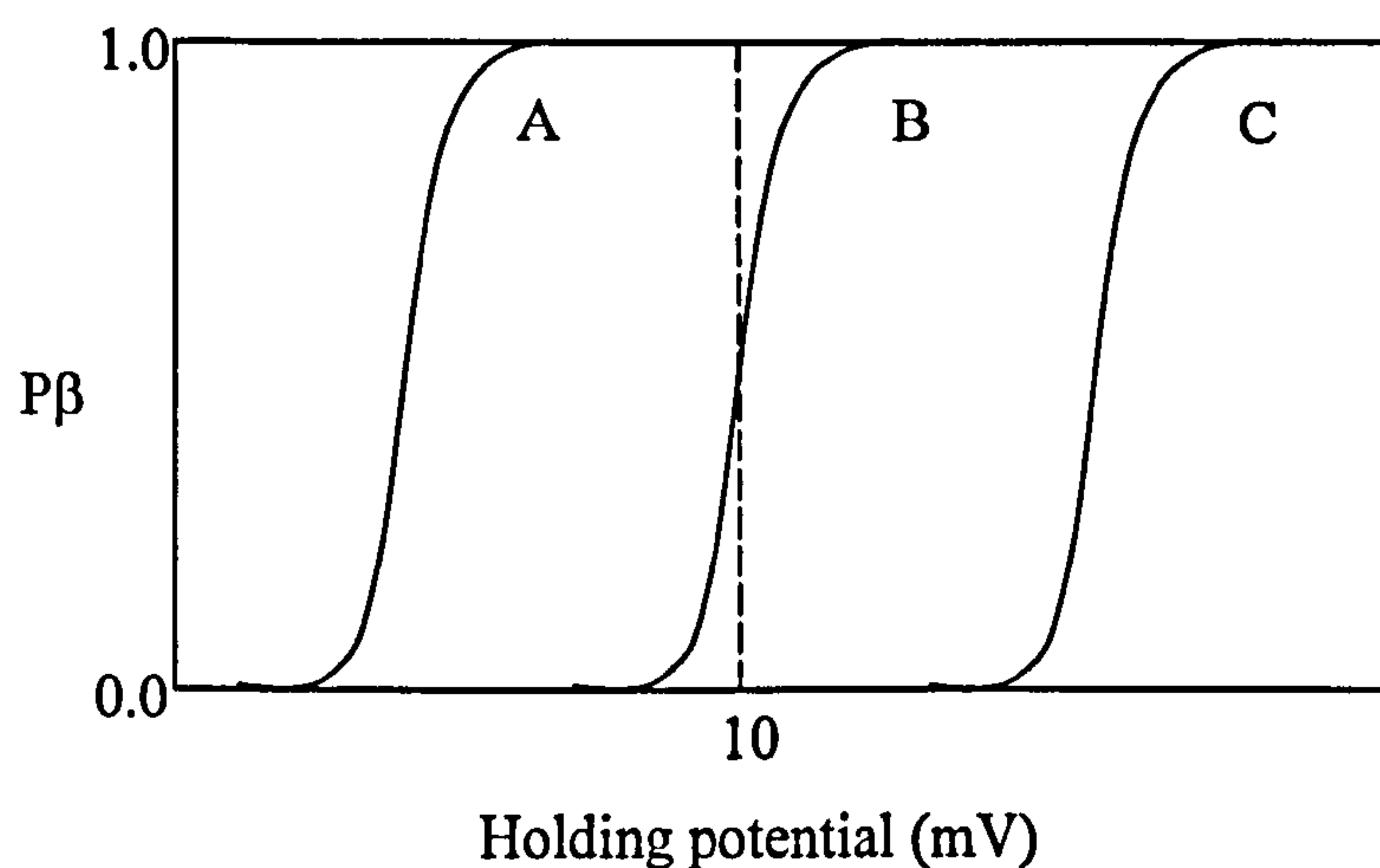


Figure 6.15 A unified theory for ryanoid reversibility. A) Represents the interaction of an irreversible ryanoid with the RyR channel, over the voltage range normally employed in a single channel experiment the channel resides in the β -subconductance state. **B)** Represents the interaction of a reversible ryanoid such as 8 β -amino-9 α -hydroxyryanodine with the RyR channel, the probability of the occurrence of the α and β -subconductance state is observed as a noisy substate with a change in transmembrane holding potential. **C)** Represents a ryanoid that has a very fast rate of dissociation over the voltage range normally used in a single channel experiment, ryanoid-modified events are not resolvable.

In summary, the interaction of most ryanoids with the high affinity binding site on the RyR channel produces changes in the conformation of the conduction pathway of the channel that results in altered ion handling and yields a single observable modified conductance state the amplitude of which is dependent upon the structure of the ligand. Some ryanoids can induce a number of resolvable single FC states. In the case of 21-p-nitrobenzoylamino-9 α -hydroxyryanodine, three modified conductance states associated with the interaction of three different conformers of this flexible ryanoid have been observed. In each case the modified state is induced by the interaction of a conformer of the ryanoid with the open RyR channel, there is no transition between conductance states whilst the ryanoid is bound over the voltage range normally used in a single channel experiment. In contrast, the novel data presented in this chapter demonstrates that the interaction of 8 β -amino-9 α -hydroxyryanodine and 21-amino-9 α -hydroxyryanodine with the channel produces RyR channels that display transitions between two modified subconductance states whilst the ryanoid is bound.

Chapter 7

Conclusion

7.1 Background

It had previously been established that the interaction of ryanoids with the high affinity binding site on the RyR channel induces a conformational change in the channel protein, which alters both the ion handling and gating properties of the channel. The channel maintains a high open probability, while a modified conductance state is observed. Analysis of the occurrence of the modified conductance state by comparative molecular field analysis demonstrated this to be dependent on the structure of the ryanoid. Investigations with the charged ryanoid (+1) 21-amino-9 α -hydroxyryanodine revealed that the high affinity binding site was only accessible from the cytosolic face of the channel and only when the channel was in an open conformation, suggesting the binding site was located within the conduction pathway of the channel (Tanna *et al.*, 1998). Altering transmembrane holding potential influenced both the association and the dissociation of 21-amino-9 α -hydroxyryanodine to and from the channel. The total voltage dependence of this reaction involved the translocation of more than 2 charges across the voltage drop across the channel; however, we had previously demonstrated that the interaction of 21-amino-9 α -hydroxyryanodine with the channel followed a simple bimolecular reaction (Tanna *et al.*, 1998). With this in mind it was proposed that most if not all of the voltage dependence of the reaction was due to a voltage driven conformational change in the channel protein itself. A component of the voltage dependence of the reaction could come from the possible movement of the charged ryanoid somehow within the voltage drop, assuming the high affinity binding site was located within the voltage drop across the channel. Therefore, although, these experiments revealed yet another factor governing the interaction of a ryanoid with the RyR channel, this interaction and the location of the high affinity ryanoid binding site on the channel is still unclear. I have tried to address both these issues in this thesis. First of all I have tried to address the location of the high affinity ryanoid binding site on the channel and secondly I have looked closely at the effects of the ryanoid-induced modified-conductance state on both the voltage drop across the channel with the aid of an impermeant cation and on the actual interaction of the ryanoid with the channel.

7.2 The location of the high affinity ryanoid binding site on the RyR channel

The influence of transmembrane holding potential on the interaction of a readily reversible neutral ryanoid with the channel was observed to determine if a component of the voltage dependence of the interaction of 21-amino-9 α -hydroxyryanodine with the channel involved movement of the charged ryanoid into the voltage drop. The results of these studies demonstrated that the voltage dependence of the interaction of ryanodol with the channel was less than that observed for 21-amino-9 α -hydroxyryanodine. This suggested a component of the voltage dependence of the interaction of 21-amino-9 α -hydroxyryanodine with the channel was due to the movement of the charged ryanoid within the voltage drop across the channel. This data strengthens the proposal that the high affinity binding site is located within the voltage drop across the channel. Further evidence came from the reduction in the probability of channel modification by 21-amino-9 α -hydroxyryanodine in the presence of increasing concentrations of the tetraalkylammonium cation TEA, which interacts at a site deep within the voltage drop across the channel accessible only from the cytosolic face of the channel. The data demonstrated that the rate of association of the ryanoid with the channel was decreased in the presence of increasing concentrations of TEA, this led to a reduction in P_{mod} of the channel, suggesting the high affinity binding site was not readily accessible for the charged ryanoid in the presence of TEA within the voltage drop. The effects most likely resulted from an electrostatic repulsion between TEA and the charged ryanoid, placing the ryanoid in close proximity to TEA and so the logical location for the high affinity binding site would be within the voltage drop. To further support this proposal a reduction in [^3H]-ryanodine binding was observed in the presence of TEA and TBA, another blocking cation that interacted at the cytosolic extremity of the voltage drop across the channel. The results from both the voltage dependence study and the blocking experiments suggest that the high affinity binding site is located someway into the voltage drop. Since TEA can still reach its binding site deep within the voltage drop in the presence of 21-amino-9 α -hydroxyryanodine and most of the voltage dependence of ryanodol is due to a voltage driven conformational change in the channel protein, it is probable that the high affinity binding site is not located deep within the voltage drop across the channel, but most likely at the cytosolic extremity. A paper published recently demonstrates that mutations of amino acid residues within and near the probable selectivity filter signature sequence (GIGD) of the RyR2, alters ryanodine

interaction with the channel and the functional consequences of bound ryanodine (Chen *et al.*, 2002). Point mutations at the G4828A and D4829A (GIGD), decreased [³H]-ryanodine binding affinity by approximately 2 and 12-fold, respectively, when compared to wild type. The dissociation rate of bound [³H]-ryanodine increased by approximately 4 and 13-fold for G4828A and D4829A respectively, when compared with wild type. In single channel experiments, the effects of ryanodine on single G4828A and D4829A mutant channels were reversible on the time scale of single channel experiment, unlike the irreversible interaction of ryanodine observed in wild type channels. The mutant D4829A also exhibited a reduced single channel conductance. A further point mutation on the selectivity filter signature sequence at G4826 to a cysteine residue (GIGD-G4826C), eliminated the functional effect of ryanodine on Ca²⁺ release in Human Embryonic Kidney 293 (HEK293) cells. These observations strongly suggest that the selectivity filter signature sequence plays an essential role in the determination of the ryanodine interaction, and supports the data obtained in this thesis that the high affinity binding site is located at the cytosolic extremity of the voltage drop across the channel. Figure 7.1, demonstrates the proposed ryanoid-RyR channel interaction within the conduction pathway of the channel (modified from Chen *et al.*, 2002). CoMFA analysis on ryanoids has shown that the features of the ryanoid structure that are essential for tight binding of the ligand to the high affinity binding site are the 3-position with the hydrophobic edge of the pyrrole and the 2-position with the isopropyl group (Welch *et al.*, 1994). Therefore, while the hydrophobic parts of the ryanoid interact with the binding site, the more polar regions such as the 9 and 10-positions would face the aqueous cavity displayed in Figure 7.1.

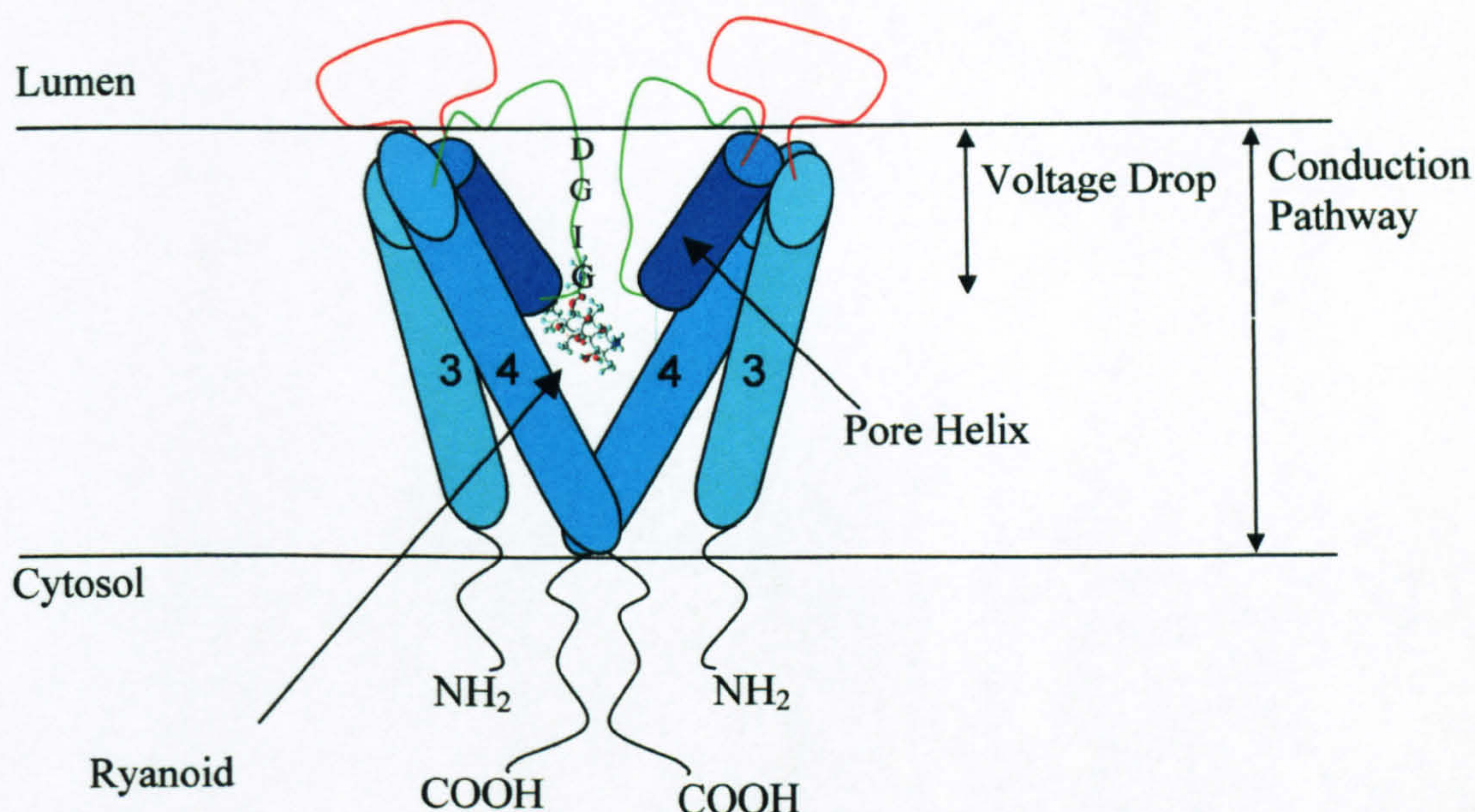


Figure 7.1 The proposed site of interaction of ryanoids to the RyR channel. A hypothetical arrangement of two opposing RyR channel subunits (the last two transmembrane domains and the loops connecting them), analogous to the KscA channel as suggested by Williams *et al.*, (2001).

7.3 Conformational change in the channel protein after ryanoid interaction

The interaction of a range of ryanoids with the RyR channel alters the blocking characteristics of TEA in the channel. In the presence of a bound ryanoid the affinity of the channel for TEA is altered and the TEA binding site within the voltage drop across the channel is relocated. The likely explanation for the relocation of the TEA binding site would arise from a change in the dimensions or position of the voltage drop across the channel. These observations, provide strong evidence that, as previously proposed (Lindsay *et al.*, 1994) structural reorganisation of the voltage drop across the channel occurs after ryanoid interaction. In most cases the interaction of a ryanoid conformer with the channel produces changes in the conformation of the voltage drop across the channel that leads to an alteration of ion handling, which induces a single modified conductance state. However, in the case of 8 β -amino-9 α -hydroxyryanodine and possibly all reversible ryanoids, transitions between two modified conductance states are observed, whilst the ryanoid is bound to the channel. The data suggests that the conduction pathway of the RyR channel can exist in two conformations with different ion-handling properties.

As outline above, the reduced conductance state observed following the interaction of a ryanoid with the RyR channel, is due to a conformational change of the voltage drop across the channel that alters both the affinity of permeant and impermeant cation and the relative permeability of ions translocating through the channel. However, the mechanism that increases channel open probability observed after ryanoid interaction with the channel has yet to be discussed.

7.4 An analogy of the gating mechanism between K^+ channels and the RyR channel

On the basis of the structure of the closed KcsA (channel from the *Streptomyces lividans*, discussed in detail in the Chapter 1) and the open MthK (channel from *Methanobacterium thermoautotrophicum*) K^+ channels and amino-acid sequence analysis, a structural basis for gating transitions was proposed (Jiang *et al.*, 2002). For the channel to exist in the closed state, the four inner helices are thought to come together at the intracellular opening to form a teepee like structure as seen in the crystal structure of the closed KcsA K^+ channel (Figure 7.2a, taken from Jiang *et al.*, 2002). However, according to the MthK open channel, the inner helix bends causing the top of the teepee like structure to splay open as shown in Figure 7.2b (also taken from Jiang *et al.*, 2002).

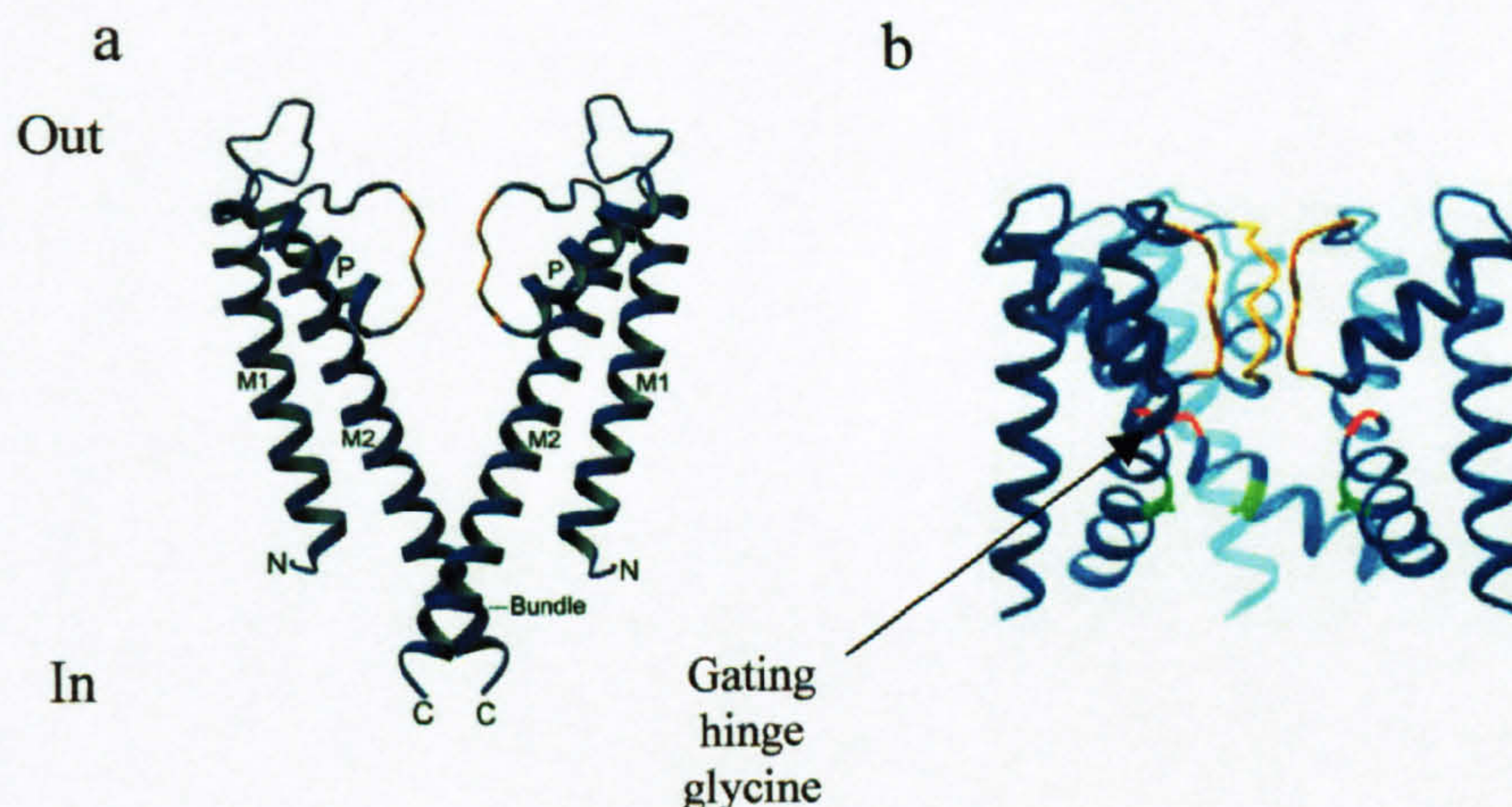


Figure 7.2 (a) Two subunits of the KcsA K^+ channel are shown with the extracellular side at the top. This includes the selectivity filter (orange), three helical segments -from N to C terminus, the outer helix (M1) , the inner helix (M2) and the pore helix (P). The gate is formed by the inner helix bundle, the channel is in the closed conformation. (b) Three subunits of the MthK pore with the corresponding segments to the KcsA K^+ channel, the channel is in the open conformation with the inner helix bent so the channel is splayed opened. Figures taken from Jiang *et al.*, (2002).

The structural feature that is thought to permit the conformational change is the presence of a 'gating hinge' located deep within the membrane below the selectivity filter sequence. When structure-based sequence alignment was carried out on a wide range of K⁺ channels, a strong amino-acid conservation at two positions in the inner helix was found. The first was a glycine residue that is thought to be the gating hinge, which was present in all the K⁺ channels observed. The second position was an alanine or glycine residue five amino acids C-terminal to the gating hinge. Although, the conformational changes to the open and closed states would be very large, the gating mechanisms are thought to be confined to the intracellular half of the channel and it is thought that all K⁺ channels go through similar changes in pore conformation (Jiang *et al.*, 2002).

The structure of the conduction pathway of the RyR channel has been proposed to be closely related to the structure of the KcsA channel as described by Williams *et al.*, (2001). The question is does the 'gating hinge' mechanism observed in the K⁺ channels apply to the RyR channel? The amino-acid sequence of all three mammalian isoforms of the RyR channel was reviewed and a 'gating hinge' motif was observed (Prof. Alan Williams personal communication). A glycine residue was located within the putative inner helix (Transmembrane 4 region (TM 4)), while an alanine residue positioned five amino acid C-terminal from the glycine residue lay just outside of the TM 4 sequence as proposed Williams *et al.*, (2001). Recently our collaborator Prof. W. Welch has carried out homology modelling of the putative pore region of the RyR channel based on the known structure of the KcsA K⁺ channel. This study revealed that the TM 4 region proposed by Williams *et al.*, (2001) can be extended as the following sequence of the TM 4 region was helical (Prof. Alan Williams personal communication), shown in Figure 7.3a. Therefore, the homology modelling between the KcsA K⁺ channel and the RyR channel indicated that with the extended RyR channel TM 4 helix, the motif equivalent to the K⁺ channel gating hinge was present in the TM 4 region of the RyR channel. The gating hinge motif was located approximately at the same position for both channels as shown in Figure 7.3b.

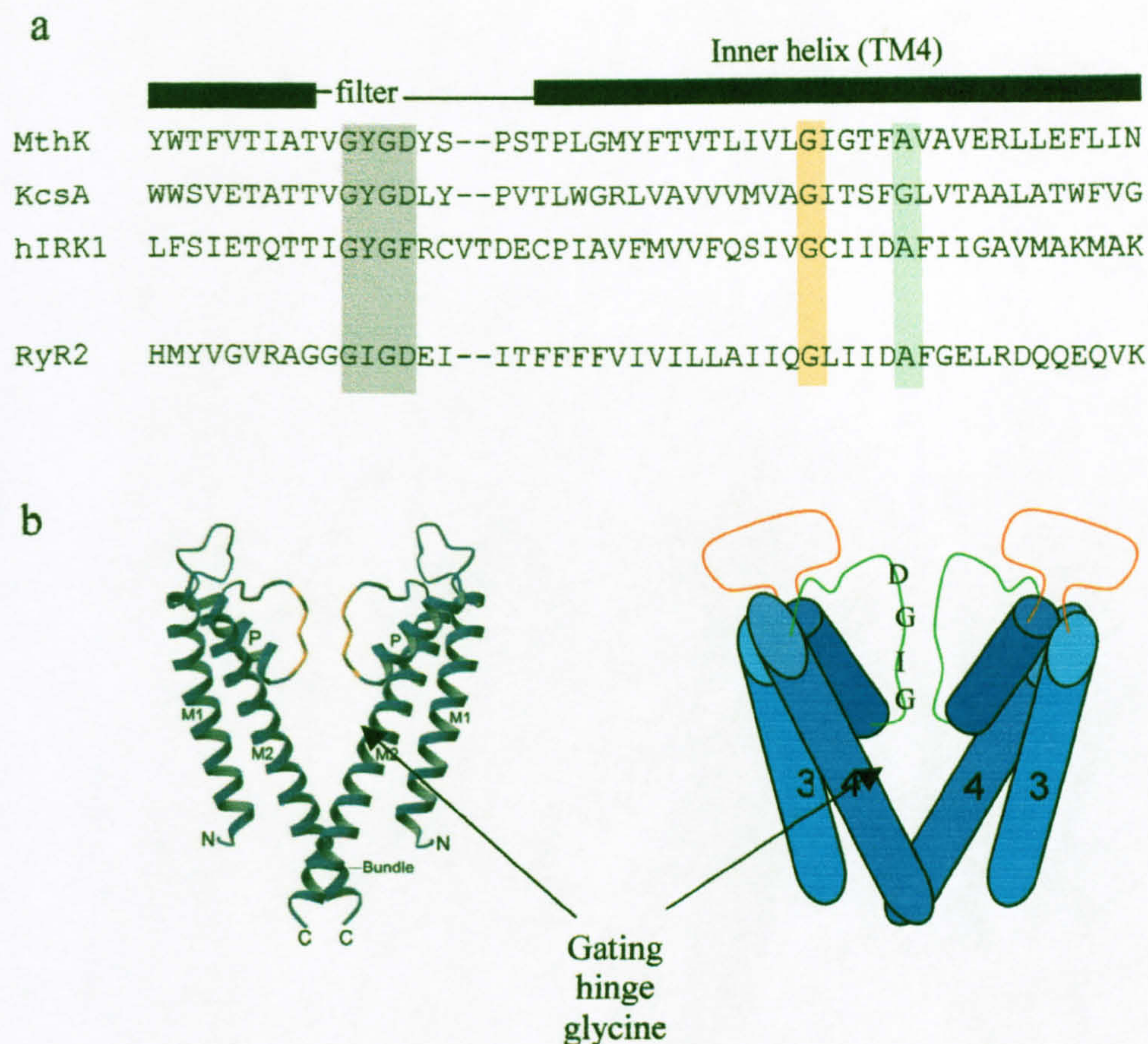


Figure 7.3 Structure based sequence analysis suggests conserved gating conformations. (a) Sequences from the inner helices of various K^+ channels and the proposed extended RyR2 channel TM 4 helix. The selectivity filter sequence is coloured in grey, the gating hinge glycine is shown in brown and the amino acid five amino acids C-terminal to the hinge located at the narrowest point of the MthK intracellular conduction pathway is in green. (b) The approximate location of the gating hinge glycine motif in the KcsA K^+ channel (left) and the RyR2 channel (right), suggested by homology modelling.

The amino acid residues within the gating hinge motif varies between the K^+ channels and so it is not surprising that the amino acid sequence of the putative RyR channel gating motif is not identical to the KcsA K^+ channel, but relates closely with the inward rectifier K^+ channel (hIRK1) (Figure 7.3a). These observations suggests that like K^+ channels, it is possible that the RyR channel could also undergo similar conformational changes between the closed and the open states. The high open probability of the channel in the presence of a ryanoid indicates some alteration in the transition of this gate between the open and closed state of the channel.

7.5 Gating mechanism is hindered by the presence of a ryanoid

The interaction of a ryanoid with the high affinity binding site thought to be located at the cytosolic extremity of the voltage drop, would place the ryanoid in very close proximity to the proposed gating hinge. Therefore, it could be possible that the ryanoid affects channel gating by either physically sitting on the proposed gating hinge or by ryanoid binding energy modifying the hinge region preventing the gate from moving and so the channel would be 'locked' in the open state. This theory is consistent with previous observations, since it was thought that the binding of ryanodine with the RyR channel 'locked' the channel in a very long lived modified conductance state with a very high P_o . The ryanoid-modified conductance state was also thought to be insensitive to modulations by other ligands such as Ca^{2+} , Mg^{2+} and ruthenium red (Xu *et al.*, 1998). However, recently we have demonstrated with the aid of the reversible ryanoid 21-amino-9 α -hydroxyryanodine that the P_o of the channel before ryanoid modification influenced the P_o of the ryanoid-modified state. Channels with low P_o displayed more channel closing events than channels with high P_o after ryanoid-modification (Tanna *et al.*, 1998). These observations suggested that the ryanoid does not simply 'lock' the channel into an open state.

7.6 Sensitisation of the ryanoid-modified channel to Ca^{2+}

Subsequently, studies have shown that the interaction of ryanodine with the cardiac RyR is actually able to increase the sensitivity of RyR channels to Ca^{2+} activation by approximately 1,000-fold (Masumiya *et al.*, 2001). The ryanodine-modified channels were also modulated with Mg^{2+} at low nM Ca^{2+} concentrations. The same study also demonstrated that ryanodine-modification also restored the responsiveness of a Ca^{2+} insensitive (a channel that exhibited a 1,000-fold reduction in Ca^{2+} sensitivity compared to wild type channel) RyR2 mutant (E4032A) to Ca^{2+} and caffeine. This data indicates that the interaction of ryanodine with the channel that leads to a conformational change in the channel may alter the affinity of the channel for Ca^{2+} . Therefore, whilst the contaminant 10 μ M Ca^{2+} present in the solutions bathing the channels in the single channel experiments carried out in this thesis has little effect on channel P_o in the unmodified state, this concentration would keep the ryanoid-modified channel permanently in a high open probability state. This data again provides good evidence that the interaction of ryanoid with the RyR channel induces a conformational change in

the channel protein. If it is possible for the affinity of the channel for TEA and the location of the TEA binding site to change, then it certainly is plausible for an alteration in the channel to occur that changes the affinity of the channel for channel modulators. For future study, it would be interesting to see if the affinity of the channel for Ca^{2+} altered to the same degree for all ryanoid-modified states.

7.7 A prior isomerisation model

Discussions with Prof. W. Welch led to the proposal that the RyR channel may exist in many 'open' configurations and each of these configuration had somewhat different properties manifested by, for example, open probability and conductance. It would therefore seem that the normal open state of the channel that we observe is merely the most energetically stable open state. The unmodified channel in the absence of any activating ligands such as Ca^{2+} or EMD 41000, resides in the closed state, because this is the most energetically favoured state in these conditions. Application of an activating ligand to the channel in the closed state would induce the channel to 'open' a mechanism termed as 'induced fit'. However, the interaction of a ryanoid with the channel acting as an allosteric modulator, could stabilise an open configuration of the channel that we would not normally observe because it is so energetically unfavourable and normal dwell times in this state are below the limits of our resolution. This mechanism can be thought of as a 'prior isomerisation' model. Each conformation of a ryanoid would stabilise a different 'open configuration' of the channel with a different conducting configuration of the channel, hence the observed difference in ion handling properties. In the case of ryanoids that induce two observable subconductance states, the ryanoid in effect stabilises two conducting configurations that are almost isoenergetic. With the ryanoid bound, the RyR channel can be in either conducting configuration, but transmembrane potential can determine which is most likely to occur. Stabilisation of different open configurations of the channel, which have unique ion handling properties, could also explain the altered affinity of the channel for TEA and the relocation of the TEA binding site within the voltage drop with different ryanoids. In a similar fashion, each open configuration of the channel stabilised by a ryanoid may have different affinities for channel modulators such as Ca^{2+} , as observed by Masumiya *et al.*, (2001).

Therefore, whilst the precise mechanisms governing the altered ion handling and gating properties observed in the presence of a ryanoid is unclear, we now have better working hypotheses on which to base future experiments.

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44-444

Excess noise in modified conductance states following the interaction of ryanoids with cardiac ryanodine receptor channels

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Abstract The interaction of ryanodine with the ryanodine receptor (RyR) produces profound changes in channel function. Open probability increases dramatically and conductance is reduced. In this report we describe differences in the properties of reduced conductance states produced by the interaction of ryanodine derivatives with RyR channels. Some reduced conductance states are considerably noisier than the normal open state of the RyR channel. Inspection and analysis of these events reveals that the excess noise arises from transitions between two conductance states. Following the interaction of certain ryanodine derivatives, RyR channels undergo transitions between two conformations with slightly different ion-handling properties. © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Calcium-release channel; Sarcoplasmic reticulum; Ryanodine receptor; Ryanodine

1. Introduction

Ryanodine receptors (RyRs) are cation-selective membrane ion channels that provide pathways for the regulated release of Ca^{2+} from intracellular storage organelles such as the endoplasmic or sarcoplasmic reticulum [1–3]. This class of channels are termed RyRs because each functional channel possesses a single high affinity binding site for this plant alkaloid.

The specificity of the ryanodine binding site, and the regions and physical properties of the ryanodine molecule that are responsible for its high affinity interactions, have been investigated by monitoring and analysing the binding of natural congeners and synthetic derivatives of ryanodine (ryanoids) to the receptor sites on populations of channels in isolated membrane vesicles [4]. The functional consequence of the interaction of ryanodine with the high affinity binding site on RyR channels in native membrane vesicles is an increase in the Ca^{2+} permeability of the membrane [5]. The mechanisms underlying this increased permeability were revealed when micromolar concentrations ryanodine were applied to individual RyR channels reconstituted into planar phospholipid bilayers. Under these conditions the interaction

of ryanodine with RyRs results in marked alterations in function; channel open probability (P_o) increases dramatically and channel unitary conductance is reduced [6]. With alkaline, earth divalent cations as the charge carrying species the fractional conductance (FC; single channel conductance of the modified state expressed as a proportion of the conductance in the absence of ryanodine) is less than 0.4, while with the group 1a monovalent cations values of FC are within the range 0.60–0.66 [7]. Altered unitary conductance reflects changes in both the affinity of the conduction pathway of the RyR channel for cations and the relative permeability of cations within the channel. These changes are thought to occur as the result of conformational alterations in the conduction pathway of the channel induced by the binding of ryanodine [7]. The application of ryanoids of varying structure to individual RyR channels has revealed that the FC induced by the binding of these molecules to the channel is governed by structural features of the ligand [8] and comparative molecular field analysis has revealed strong correlations between FC and specific structural loci on the ryanoid molecule [9].

It has been assumed that the interaction of a single ryanoid molecule with the high affinity ryanoid binding site on RyR gives rise to a single modified conductance state. However, detailed inspection of modified conductance states induced by a large number of ryanoids reveals that some of these ryanoid-induced states display excess noise when compared with the normal open-channel conducting state. Such events could result from transitions between more than one conductance state. In this communication we describe examples of such ‘noisy’ ryanoid-induced states and investigate the mechanisms responsible for their occurrence.

2. Materials and methods

Phosphatidylethanolamine was purchased from Avanti Polar Lipids Inc. and phosphatidylcholine from Sigma-Aldrich. Standard chemicals were obtained as best available grade from BDH Ltd. or Sigma-Aldrich. Ryanodine was supplied by Agrisystems International, and 21-azido-9 α -hydroxyryanodine, 21-amino-9 α -hydroxyryanodine and 8 β -amino-9 α -hydroxyryanodine were synthesised as described earlier [9]. Ryanoids were stored as stock solutions in 50% ethanol at -20°C .

Heavy sarcoplasmic reticulum membrane vesicles were prepared from sheep cardiac muscle [10] and RyR channels isolated and reconstituted into unilamellar liposomes [11] as described previously.

Planar phospholipid bilayers were formed from suspensions of phosphatidylethanolamine in *n*-decane and individual RyR channels

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incorporated into the bilayer following previously described methods [12]. Single channel current fluctuations were monitored with K^+ as the charge carrying species and data stored on Digital Audio Tape. Single channel current amplitudes were measured from digitised data [12] and data are quoted as mean values $\pm$ S.E.M.

3. Results and discussion

(Fig. 1) shows examples of altered channel function following the interaction of various ryanoids with individual RyR channels. In all cases ryanoid binding results in the occurrence of a high P_o , reduced conductance, state. However, inspection of these traces indicates significant differences in channel activity when the ryanoids are bound to the channel. The modified conductance states induced by ryanodine and 21-azido-9 α -hydroxyryanodine are 'quiet'; that is the current noise in the ryanoid-modified state is comparable to that of the normal open state of the channel. In contrast, it is clear that the current noise associated with the modified conductance states induced by both 21-amino-9 α -hydroxyryanodine and 8 β -amino-9 α -hydroxyryanodine is significantly greater than that of the normal open state of the channel. With closer inspection it can be seen that with these ryanoids bound, the RyR channel fluctuates between two modified conductance states. Rates of transition between these states are very fast in the case of 21-amino-9 α -hydroxyryanodine and as a consequence the individual dwell times in each state are poorly resolved. With 8 β -amino-9 α -hydroxyryanodine transition rates are slower and dwell times in the two states (α and β) are clearly resolved.

What mechanisms underlie this apparent fluctuation between different conductance states following the interaction

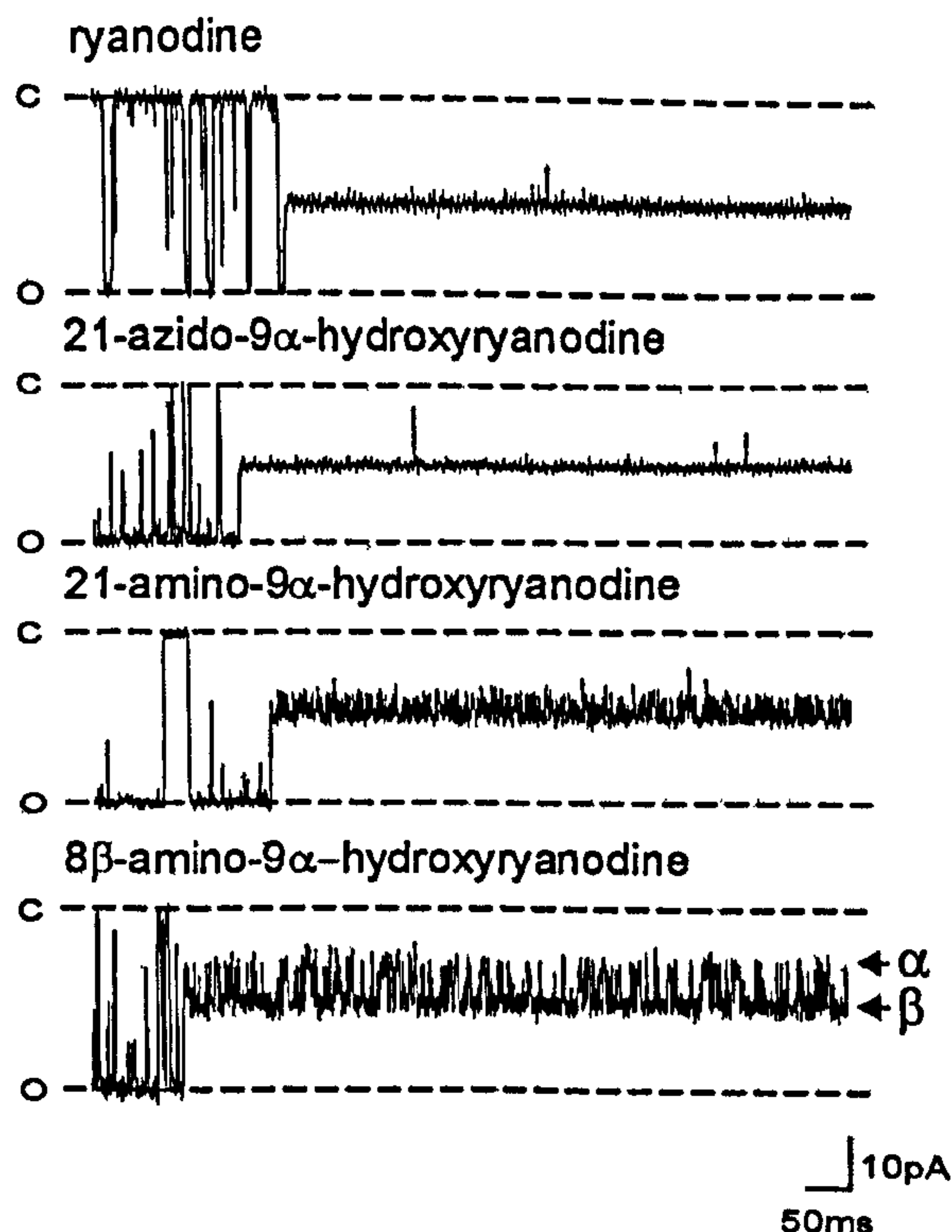


Fig. 1. Modification of function following the interaction of the indicated ryanoids with single RyR channels. In all cases K^+ is the charge carrying species and the holding potential is +40 mV.

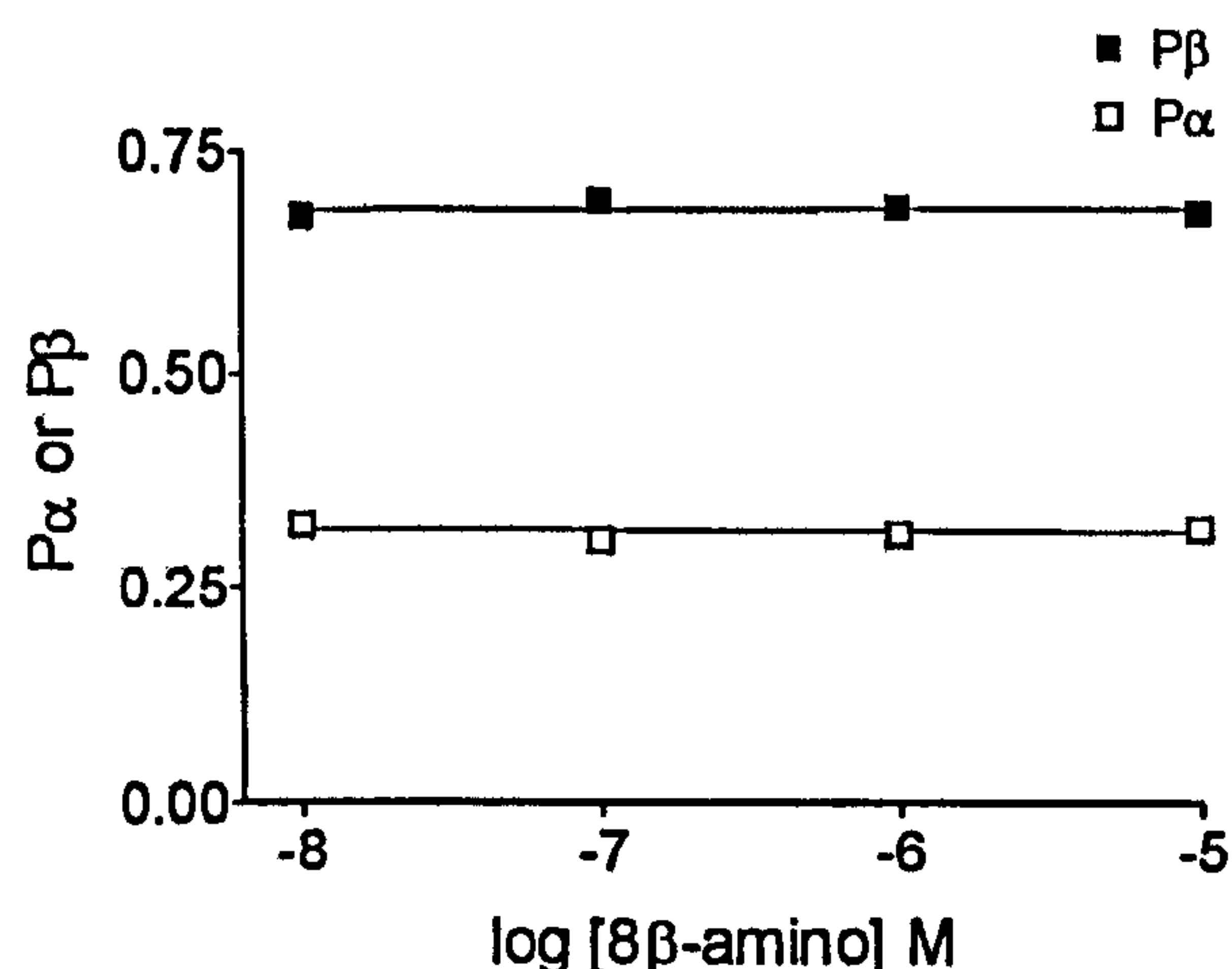


Fig. 2. Relationship between the probability of occurrence of α and β modified conductance states and concentration of 8 β -amino-9 α -hydroxyryanodine in the solution at the cytosolic face of the RyR channel. Points are mean $\pm$ S.E.M., $n=4-6$. Holding potential is +60 mV.

of some ryanoids with RyR? We consider there to be two possible explanations for the observed behaviour. The first of these is that following the interaction of a ryanoid with the RyR channel, and the subsequent induction of a modified conductance state, unbound ryanoid in the bulk solution enters the conduction pathway of the ryanoid-modified channel and acts as a partial blocker of K^+ translocation through the channel. Both 21-amino-9 α -hydroxyryanodine and 8 β -amino-9 α -hydroxyryanodine have a net positive charge and there are examples in the literature of partial block of RyR channels by large cations including tetraalkylammoniums [13] and local anaesthetics [14]. Under these circumstances the β -conductance state observed in RyR in the presence of 8 β -amino-9 α -hydroxyryanodine would be the modified conductance state induced by the interaction of the ryanoid with the channel and the α -conductance state would represent a partial blocked state of the ryanoid-modified state. The feasibility of this mechanism can be investigated by simply varying the concentration of 8 β -amino-9 α -hydroxyryanodine in the solution at the cytosolic face of the channel. If the observed α -subconductance state is a partial blocked state of the ryanoid-modified state, an increase in ryanoid concentration will increase the probability of occurrence of the blocked (α) state whilst decreasing the probability of occurrence of the β state. We have investigated this possibility by monitoring the probabilities of occurrence of the α - and β -conductance states during modified conductance events in the presence of cytosolic 8 β -amino-9 α -hydroxyryanodine at concentrations ranging from 10 nM to 10 μ M at a holding potential of +60 mV. The results of these investigations are shown in Fig. 2. Clearly there is no variation in the probability of occurrence of either the α or β states within a 1000-fold concentration range of the ryanoid. These observations indicate that the α -subconductance state is not a partial blocked state of a single modified conductance state induced by 8 β -amino-9 α -hydroxyryanodine.

The alternative explanation for the observed behaviour is that with the ryanoid bound, RyR fluctuates between two conductance states (α and β). This in turn suggests that

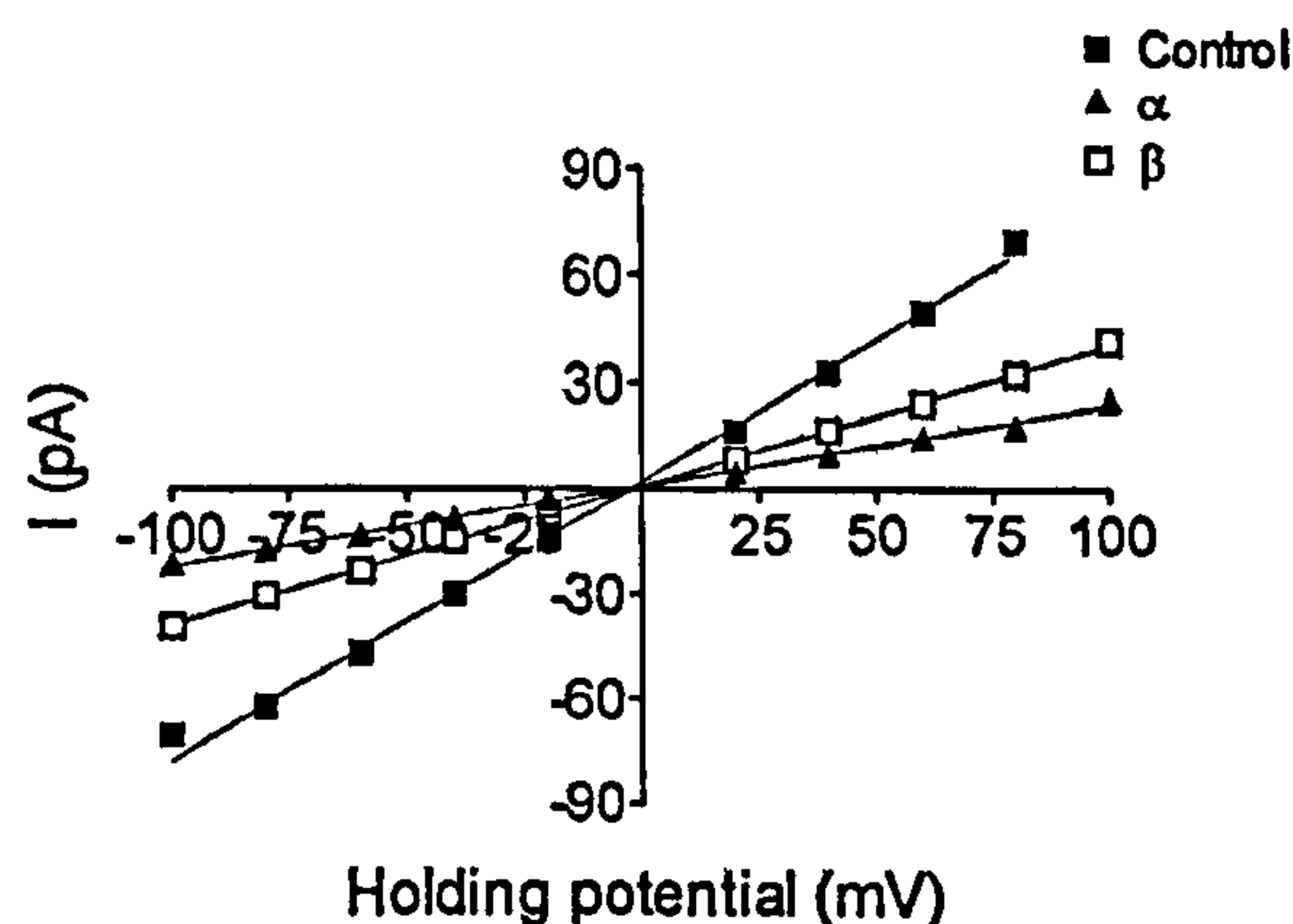


Fig. 3. Variations in unitary current amplitude with holding potential for the full open conductance state (control) and the α and β modified conductance states induced by 8 β -amino-9 α -hydroxyryanodine. Points are mean $\pm$ S.E.M. Control, $n=6-18$; α , $n=4-9$; β , $n=4-9$.

with a ryanoid, such as 8 β -amino-9 α -hydroxyryanodine occupying the high affinity ryanoid binding site on RyR, the conduction pathway of the channel fluctuates between two conformations with slightly different ion-handling properties [7,15]. Consistent with this proposal, the unitary current amplitudes of both the α and β states vary linearly with holding potential (Fig. 3). The α and β states have values of FC of 0.31 ± 0.01 and 0.56 ± 0.01 respectively ($n=4-9$).

In previous investigations we have demonstrated that, in addition to modifying rates of permeant ion translocation, the interaction of a ryanoid with the RyR channel alters the effectiveness of tetraethylammonium (TEA⁺) as a concentration- and voltage-dependent blocker of K⁺ translocation. Broadly, the effectiveness of TEA⁺ as a blocker of a rya-

noid-modified RyR channel correlates with the FC of the modified state induced by the ryanoid; the smaller the FC induced by a ryanoid, the greater the concentration of TEA⁺ that is required to produce block. These ryanoid-induced changes result from alterations in the voltage dependence of the TEA⁺ blocking reaction and in some cases the affinity of the conduction pathway for the blocking cation [15].

We have used TEA⁺ to investigate the nature of the α and β conductance states induced by 8 β -amino-9 α -hydroxyryanodine. Fig. 4 shows current fluctuations of a single RyR channel in the presence of 10 μ M 8 β -amino-9 α -hydroxyryanodine at holding potentials ranging from -80 to $+80$ mV. The left-hand panel shows control traces whilst the right-hand panel shows representative traces from the same channel following the addition of 20 mM TEA⁺ to the solutions at both the cytosolic and luminal faces of the channel. Relationships between unitary current amplitude and holding potential for the full open conductance state and the α - and β -conductance states in the absence and presence of 20 mM TEA⁺ are shown in Fig. 5 ($n=4-9$).

In the absence of ryanoids, TEA⁺ is a concentration- and voltage-dependent blocker of K⁺ translocation when present at the cytosolic face of the RyR channel [15,16]. As can be seen in Fig. 5a, the degree of block produced by TEA⁺ increases as trans-membrane voltage is taken to more positive potentials; at a holding potential of 80 mV, 20 mM TEA⁺ reduces unitary current amplitude of the full open state of RyR from 69.65 ± 0.86 pA to 25.94 ± 0.73 pA (a reduction of 63%). TEA⁺ also blocks K⁺ translocation in the α and β conductance states induced by 8 β -amino-9 α -hydroxyryanodine, however, the degree of block is significantly less than that seen in the full open state. At a holding potential of 80 mV, 20 mM TEA⁺ reduces the unitary current amplitude of the α state from 17.77 ± 1.21 pA to 11.13 ± 0.89 pA (a reduc-

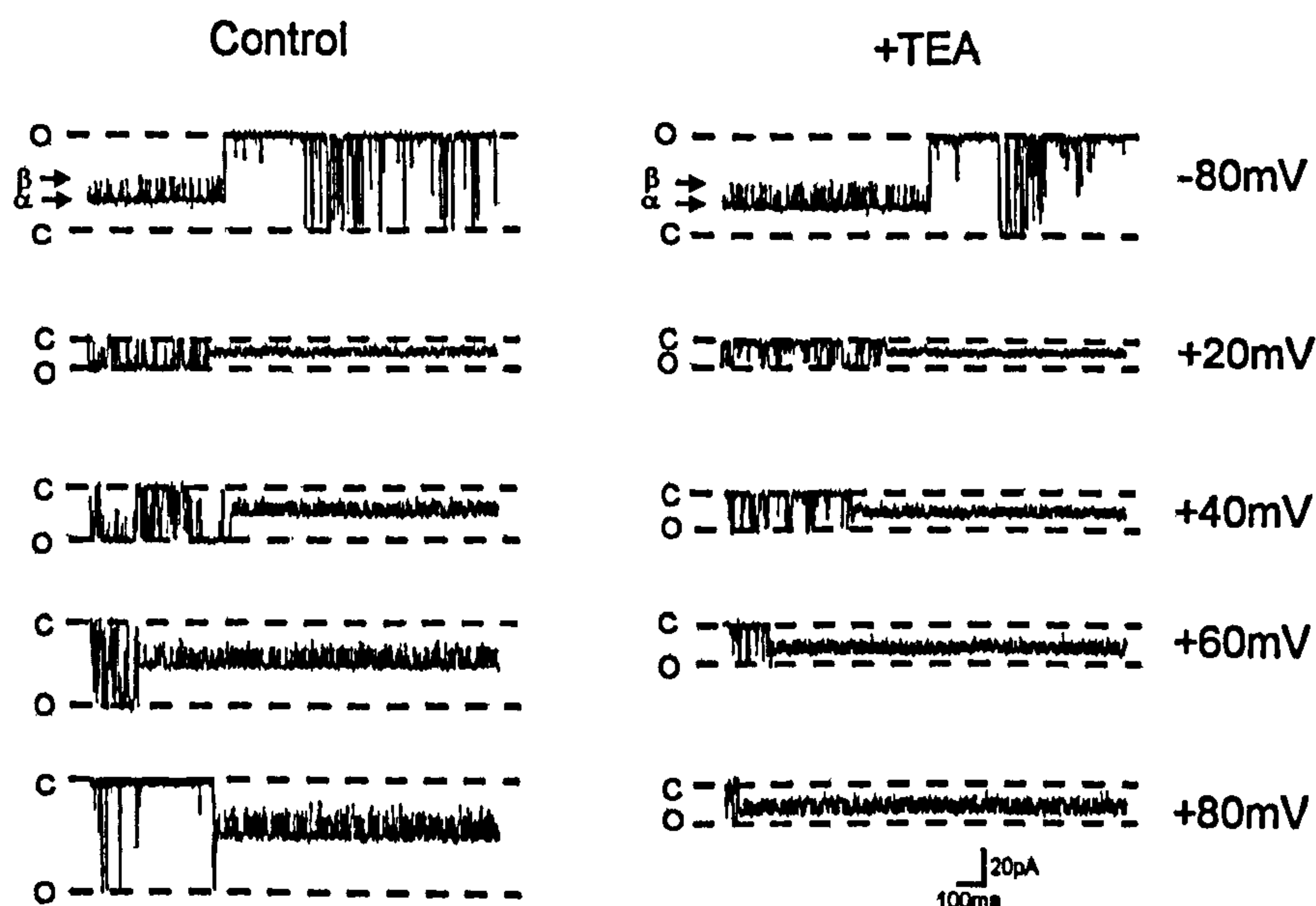


Fig. 4. Block of full open conductance state and the α and β 8 β -amino-9 α -hydroxyryanodine-induced modified conductance states of a representative channel by symmetrical 20 mM TEA⁺. Left panel shows variation in unitary current amplitude at the indicated holding potentials in the absence of TEA⁺. The right panel shows equivalent current fluctuations in the presence of TEA⁺.

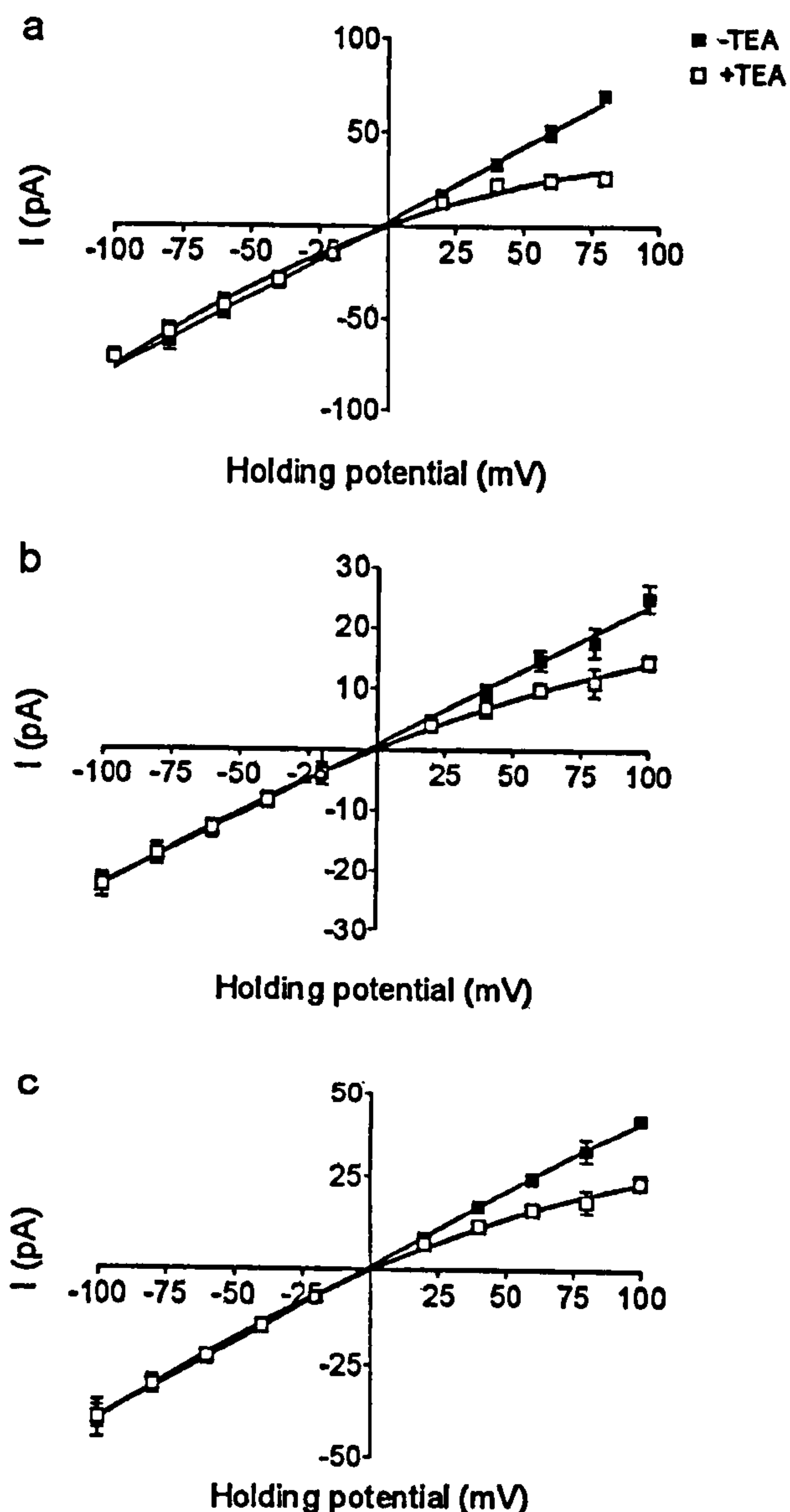


Fig. 5. Current-voltage relationships for the full open conductance state (a) and the α (b) and β (c) 8 β -amino-9 α -hydroxyryanodine-induced modified conductance states of RyR in the absence (filled symbols) and presence (open symbols) of symmetrical 20 mM TEA⁺. Points are mean $\pm$ S.E.M. Control $\pm$ TEA⁺, $n=6-18$; α -TEA⁺, $n=4-9$; α +TEA⁺, $n=6-8$; β -TEA⁺, $n=4-9$; β +TEA⁺, $n=6-8$.

tion of 37%) (Fig 5b) and that of the β state from 32.24 ± 1.44 pA to 18.13 ± 1.16 pA (a reduction of 44%) (Fig 5c). These data are consistent with the proposal that the α and β states seen in the presence of 8 β -amino-9 α -hydroxyryanodine are different conductance states reflecting different conformations of the RyR conduction pathway induced by the interaction of this ryanoid with the channel. When compared with its action on the control full open state of the channel, TEA⁺ is a significantly less effective blocker of the ryanoid-induced α and β states. The small difference in effectiveness of TEA⁺ in the α and β states is entirely consistent with observations made previously with ryanoids that induce single, 'quiet', modified

conductance states [15]. Similarly, Woodhull analysis [17] of the data presented in Fig. 5 demonstrates that the reduced effectiveness of TEA⁺ in the α and β states induced by 8 β -amino-9 α -hydroxyryanodine results from alterations in the voltage dependence and affinity of the TEA⁺ blocking reaction (values of $z\delta$ and $K_b(0)$ for block of the open, α and β states are respectively: $z\delta=0.88$, $K_b(0)=167.3$ mM; $z\delta=0.45$, $K_b(0)=139.3$ mM; $z\delta=0.52$, $K_b(0)=147.8$ mM). These observations indicate that both the α and β conductance states have properties that characterise them as separate ryanoid-modified states and result from rearrangements in the structure of the conduction pathway of the RyR channel following the binding of 8 β -amino-9 α -hydroxyryanodine.

We conclude that the interaction of ryanoids with the high affinity binding site on the RyR channel protein induces profound effects on channel function and that the ryanoid-induced alteration in ion handling by the channel is dependent upon the structure of the ligand. In most cases ryanoid interaction produces changes in the conformation of the conduction pathway of the channel that results in altered ion handling and yields a single modified conductance state [7]. Some ryanoids can induce more than one FC state. In the case of 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine we have observed three modified conductance states associated with the interaction of three different conformers of this flexible ryanoid [15]. In each case the modified state is induced by the interaction of a conformer of the ryanoid with the open RyR channel; there is no transition between conductance states whilst the ryanoid is bound. In contrast, the novel data presented in this communication demonstrate that with ryanoids such as 21-amino-9 α -hydroxyryanodine and 8 β -amino-9 α -hydroxyryanodine, binding produces RyR channels that display transitions between two states whilst the ryanoid is bound. Each state possesses properties characteristic of a ryanoid-modified conductance state. With these ryanoids bound, the conduction pathway of the RyR channel can exist in conformations with slightly different ion-handling properties. The factors governing the conductance properties of these states and the rates of transition between the states remain to be established.

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Ryanoid Modification of the Cardiac Muscle Ryanodine Receptor Channel Results in Relocation of the Tetraethylammonium Binding Site

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ABSTRACT The interaction of ryanodine and derivatives of ryanodine with the high affinity binding site on the ryanodine receptor (RyR) channel brings about a characteristic modification of channel function. In all cases, channel open probability increases dramatically and single-channel current amplitude is reduced. The amplitude of the ryanoid-modified conductance state is determined by structural features of the ligand. An investigation of ion handling in the ryanodine-modified conductance state has established that reduced conductance results from changes in both the affinity of the channel for permeant ions and the relative permeability of ions within the channel (Lindsay, A.R.G., A. Tinker, and A.J. Williams. 1994. *J. Gen. Physiol.* 104:425–447). It has been proposed that these alterations result from a reorganization of channel structure induced by the binding of the ryanoid. The experiments reported here provide direct evidence for ryanoid-induced restructuring of RyR. TEA⁺ is a concentration- and voltage-dependent blocker of RyR in the absence of ryanoids. We have investigated block of K⁺ current by TEA⁺ in the unmodified open state and modified conductance states of RyR induced by 21-amino-9 α -hydroxyryanodine, 21-azido-9 α -hydroxyryanodine, ryanodol, and 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine. Analysis of the voltage dependence of block indicates that the interaction of ryanoids with RyR leads to an alteration in this parameter with an apparent relocation of the TEA⁺ blocking site within the voltage drop across the channel and an alteration in the affinity of the channel for the blocker. The degree of change of these parameters correlates broadly with the change in conductance of permeant cations induced by the ryanoids, indicating that modification of RyR channel structure by ryanoids is likely to underlie both phenomena.

KEY WORDS: ryanodine receptor • sarcoplasmic reticulum • ryanodine • ryanoids • block

INTRODUCTION

The specific, high affinity interaction of the plant alkaloid ryanodine with one class of intracellular membrane Ca²⁺ release channel defines these channels as ryanodine receptors (RyRs).¹ On binding, ryanodine and derivatives of ryanodine (ryanoids) induce profound alterations in RyR channel function. Single-channel open probability (P_o) rises dramatically and rates of ion translocation are reduced, giving rise to the occurrence of characteristic subconductance states (Rousseau et al., 1987; Ashley and Williams, 1990). Ryanodine-induced modification of permeant ion translocation in RyR cannot be explained by a simple blocking mechanism; rather, the interaction of ryanodine with

RyR results in alterations to many of the processes that underlie single-channel conductance. Alterations occur in the affinity of the conduction pathway of the channel for some permeant ions and the relative permeability of some ions is changed. Altered mechanisms of ion handling and the resultant, ryanodine-induced conductance vary with the nature of the permeant ion. These observations led to the suggestion that the ryanodine-induced modification of ion handling in RyR results from a conformational change of the channel protein, involving a rearrangement of the conduction pathway (Lindsay et al., 1994).

Information on the dimensions of the conduction pathway of RyR and the location of sites within this region of the channel have emerged from studies involving channel block by impermeant cations (Tinker et al., 1992; Tinker and Williams, 1993c, 1995). In this report we have examined the consequences of the modification of RyR channel function by a series of ryanoids on block by TEA⁺, a concentration- and voltage-dependent blocker of K⁺ current in this channel (Lindsay et al., 1991; Tinker et al., 1992).

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¹Abbreviations used in this paper: FC, fractional conductance; P_o , open probability; RyR, ryanodine receptor; TMA, tetramethylammonium; $z\delta$, effective valence.

Quantitative analysis of voltage-dependent block of K^+ current in RyR indicates that the effective valence ($z\delta$) of TEA^+ is altered in the ryanoid-modified channel, which would be consistent with a relocation of the TEA^+ binding site within the voltage drop across the channel. Ryanoid modification also produces alterations in the affinity of the conduction pathway for the blocking cation. These observations are consistent with the proposal that the interaction of a ryanoid with the high affinity binding site on RyR produces a conformational alteration in the channel protein resulting in modification of conduction pathway structure and a consequent change in the handling of both permeant and impermeant cations.

MATERIALS AND METHODS

Materials

Phosphatidylethanolamine was purchased from Avanti Polar Lipids, Inc., and phosphatidylcholine from Sigma-Aldrich. [^3H]Ryanodine was supplied by New England Nuclear Ltd. Aqueous counting scintillant was purchased from Packard Instrument Co. Other reagents were obtained as the best available grade from BDH Ltd. or Sigma-Aldrich. The ryanoids used in these studies were synthesized as described previously (21-amino-9 α -hydroxyryanodine, 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine, 21-azido-9 α -hydroxyryanodine [Welch et al., 1997]; ryanodol [Deslongchamps et al., 1990]) and stored as stock solutions in 50% ethanol at $-20^\circ C$.

As more than one modified conductance state was observed after the addition of 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine to RyR (see RESULTS), the purity of the compound was reinvestigated to ensure that there had been no decomposition during storage. Samples used in the experiments reported here were found to be homogeneous by TLC (both charring and fluorescence detection) and by MALDI/TOF mass spectroscopy.

Molecular Modeling

The conformations of the ryanoids shown in Fig. 1 were analyzed by molecular mechanics using the Tripos force field (SYBYL; Tripos Inc.) and semiempirical quantum mechanics (MOPAC). In Fig. 1 A, ball and stick drawings of 21-amino-9 α -hydroxyryanodine (bottom left), 21-azido-9 α -hydroxyryanodine (bottom right), ryanodol (top right), and 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine (top left) are shown. All are shown in the conformation corresponding to their respective global minima. Although all of these ryanoids can exist in multiple conformations, the bulk of the long floppy tail of 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine makes the conformers of this ryanoid of experimental interest. Molecular dynamics (SYBYL) simulations indicate that the side chain exists in three conformational families arising principally from the high rotational energy barriers about the single bond proximal to carbon 9. The local minimum of each of these families is shown in Fig. 1 B, and the significance of the existence of these conformers is discussed later.

Isolation of Sheep Cardiac Heavy Sarcoplasmic Reticulum Membrane Vesicles and Solubilization and Separation of the RyR

Sheep hearts were collected from a local abattoir in ice-cold cardioplegic solution and heavy sarcoplasmic reticulum membrane

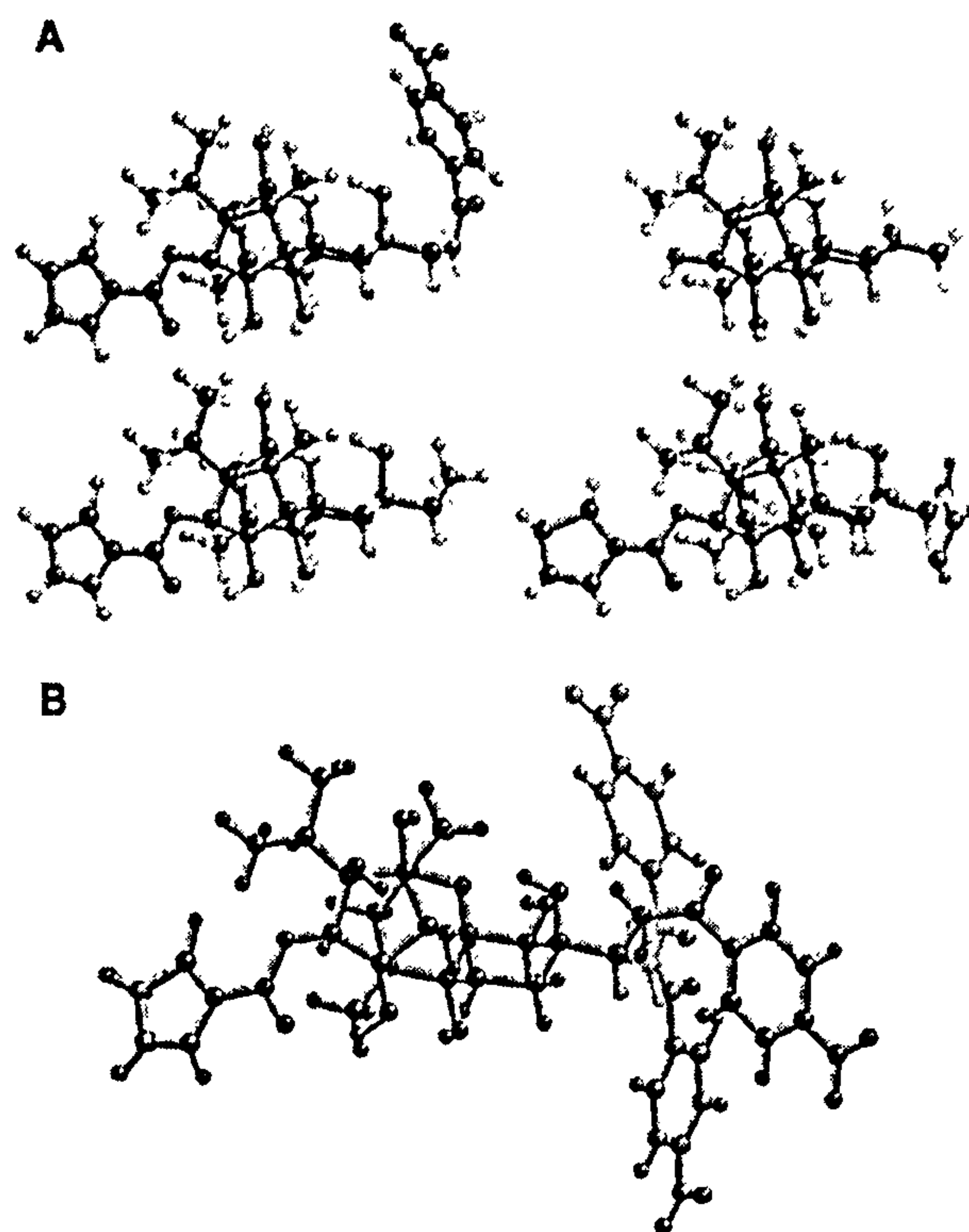


FIGURE 1. (A) Ball and stick models of 21-amino-9 α -hydroxyryanodine (bottom left), 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine (top left), ryanodol (top right), and 21-azido-9 α -hydroxyryanodine (bottom right) are shown in the conformation corresponding to the respective global energy minimum. All are oriented with the 3-position to the left (note the pyrrole group at the extreme left) and the 21-position to the right (a horizontal line connects these two positions). Ryanodol (top right) is the core structure of all of the other compounds illustrated in Fig. 1. (B) The local minima (differing shades of gray) of the three most populated conformations of 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine are superimposed by the polycyclic fused ring system. The conformational space was investigated by molecular dynamics at a simulated temperature of $25^\circ C$. Note the changes in physical shape of the molecule due to large movements of the *p*-nitrobenzoyl group compared with the other pendant groups.

vesicles prepared using procedures described previously (Sitsapesan and Williams, 1990).

Heavy sarcoplasmic reticulum membrane vesicles were solubilized with 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS) and RyR isolated and reconstituted into unilamellar liposomes for incorporation into planar phospholipid bilayers, as described previously (Lindsay and Williams, 1991).

Planar Phospholipid Bilayers

Phospholipid bilayers were formed from suspensions of phosphatidylethanolamine in *n*-decane (35 mg/ml) across a 200- μm diam hole in a styrene copolymer partition which separated two chambers referred to as *cis* (volume 0.5 ml) and *trans* (volume 1.0 ml). The *trans* chamber was held at virtual ground, whereas the *cis* chamber could be clamped at holding potentials relative to ground. Current flow across the bilayer was monitored using an

operational amplifier as a current-voltage converter (Miller, 1982). Bilayers were formed with solutions containing 600 mM KCl, 20 mM HEPES, titrated to pH 7.4 with KOH, resulting in a solution containing 610 mM K^+ in both chambers. An osmotic gradient was created by the addition of an aliquot (50–100 μ l) of 3 M KCl to the cis chamber. Proteoliposomes were added to the cis chamber and stirred. Under these conditions, channels usually incorporated into the bilayer within 2–3 min. If channels did not incorporate, a second aliquot of 3 M KCl could be added to the cis chamber. After channel incorporation, further fusion was prevented by perfusion of the cis chamber with 610 mM K^+ . Channel proteins incorporate into the bilayer in a fixed orientation so that the cytosolic face of the channel is exposed to the solution in the cis chamber and the luminal face of the channel to the solution in the trans chamber. Single-channel P_o was increased by the addition of 100 μ M EMD 41000 to the cytosolic face of the channel (McGarry and Williams, 1994; Tanna et al., 1998). Experiments were carried out at room temperature ($21 \pm 2^\circ\text{C}$).

Modification of RyR channel function by 21-amino-9 α -hydroxyryanodine, ryanodol, 21-azido-9 α -hydroxyryanodine, and 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine was achieved by adding an appropriate concentration of the ryanoid to the solution at the cytosolic face of the bilayer. Although TEA $^+$ is an effective blocker of the RyR channel only from the cytosolic face of the channel (Lindsay et al., 1991), it was added symmetrically to the solutions at both sides of the bilayer to avoid the possibility of asymmetric surface potentials arising from the binding of the cation to the bilayer.

Single-channel Data Acquisition and Analysis

Single-channel current fluctuations were displayed on an oscilloscope and stored on Digital Audio Tape. For analysis, data were replayed, filtered at 1 kHz with an 8-pole Bessel filter, and digitized at 4 kHz using Satori v3.2 (Intracel). Single-channel current amplitudes were measured from digitized data. The representative traces shown in the figures were obtained from digitized data acquired with Satori v3.2 and transferred as an HPGL graphics file to a graphics software package (CorelDraw; Corel Systems Corporation) for annotation and printing.

RESULTS

The high affinity association of a ryanoid with the RyR channel produces a dramatic alteration in channel function. With a ryanoid bound, rates of Ca^{2+} and K^+ translocation through RyR are reduced and the channel enters a characteristic modified conductance state. The fractional conductance (FC) of the modified state (the amplitude of the ryanoid-modified state expressed as a proportion of the full amplitude of the channel; Lindsay et al., 1994) is determined by structural features of the ryanoid (Tinker et al., 1996; Welch et al., 1997). Dwell times in the various ryanoid-modified states differ considerably, lasting for seconds with, for example, 21-amino-9 α -hydroxyryanodine, to hours with ryanodine (Tanna et al., 1998, 2000).

The Influence of Voltage on TEA $^+$ Block of Unmodified and Ryanoid-modified RyR Channels

The left panel of Fig. 2 shows current fluctuations of a single RyR channel in the presence of 500 nM 21-amino-9 α -hydroxyryanodine with K^+ as the permeant

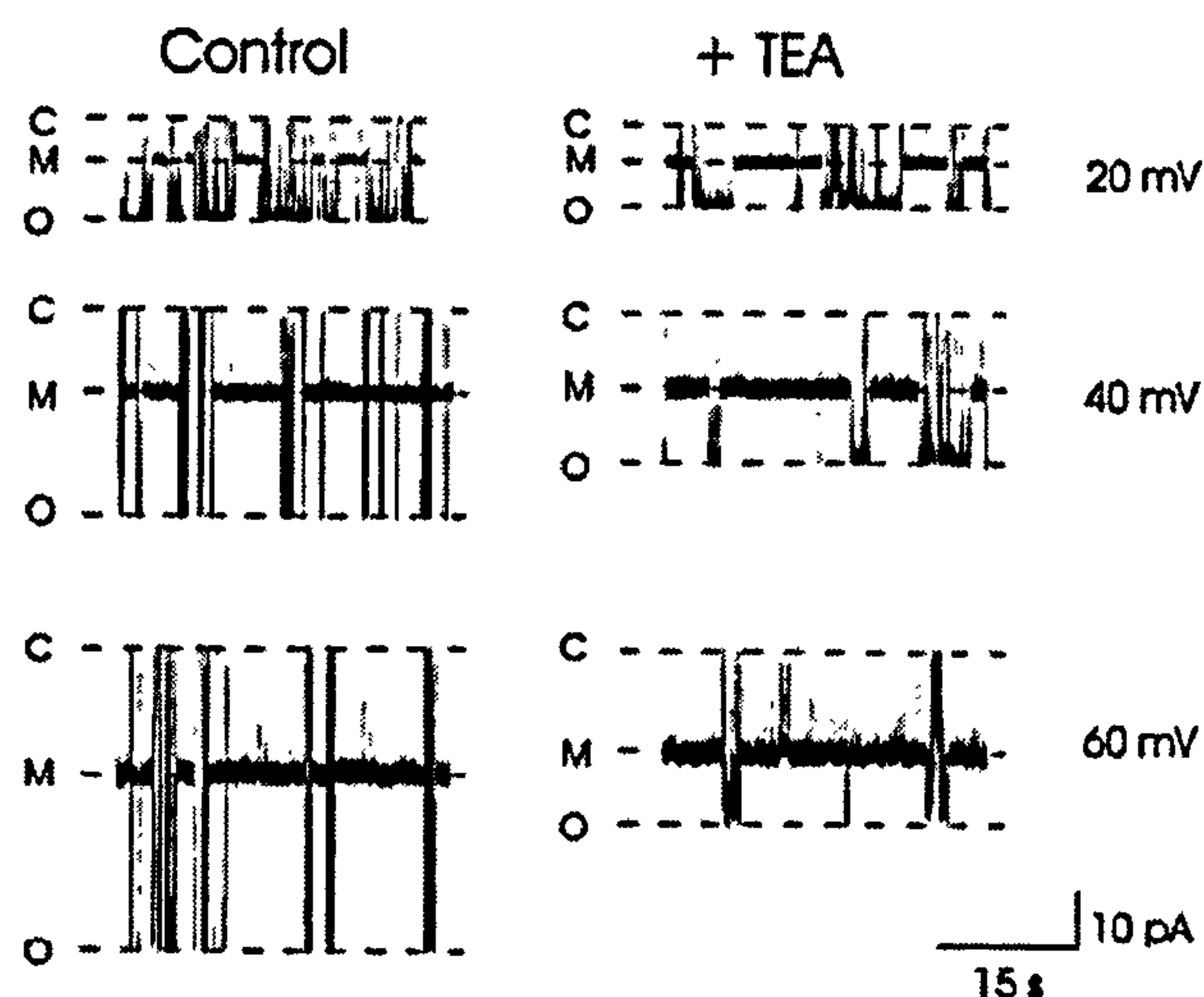


FIGURE 2. The influence of transmembrane holding potential on the single-channel current amplitude of the unmodified and 21-amino-9 α -hydroxyryanodine-modified conductance states of the RyR channel in the absence and presence of 20 mM TEA $^+$. C, closed conductance level; M, modified conductance level; O, fully open conductance level.

ion. Under these conditions, this ryanoid associates with and readily dissociates from the channel. With the ryanoid bound, RyR channel P_o increases markedly and single-channel conductance is reduced to a modified level (M) with an FC of 0.44. After dissociation of the ryanoid, the channel gates normally with fluctuations between the closed (C) and fully open conductance level (O). The probability of 21-amino-9 α -hydroxyryanodine associating with the channel increases and the probability of the ryanoid dissociating from the channel decreases as the holding potential is made more positive (Tanna et al., 1998).

The right panel of Fig. 2 shows current fluctuations of the same channel after the addition of 20 mM TEA $^+$ to the solutions at both sides of the channel. In the absence of ryanoid, TEA $^+$ is a concentration- and voltage-dependent blocker of K^+ conductance in the RyR channel. Dwell times in the TEA $^+$ blocked state are too short to be resolved and, as a consequence, RyR channel block by this cation appears as a time-averaged reduction in single-channel current amplitude (Lindsay et al., 1991; Tinker et al., 1992). Block of this form can be seen in Fig. 2 in the periods of unmodified gating when the ryanoid is not bound to the channel. In agreement with previous observations (Lindsay et al., 1991), the degree of block of this unmodified state of the channel by TEA $^+$ varies as transmembrane potential is changed; block increases as the holding potential is made more positive. Current-voltage relationships for unmodified conductance states of several RyR channels in the absence and presence of 20 mM TEA $^+$ are shown in Fig. 3 A. TEA $^+$ has no effect on K^+ transloca-

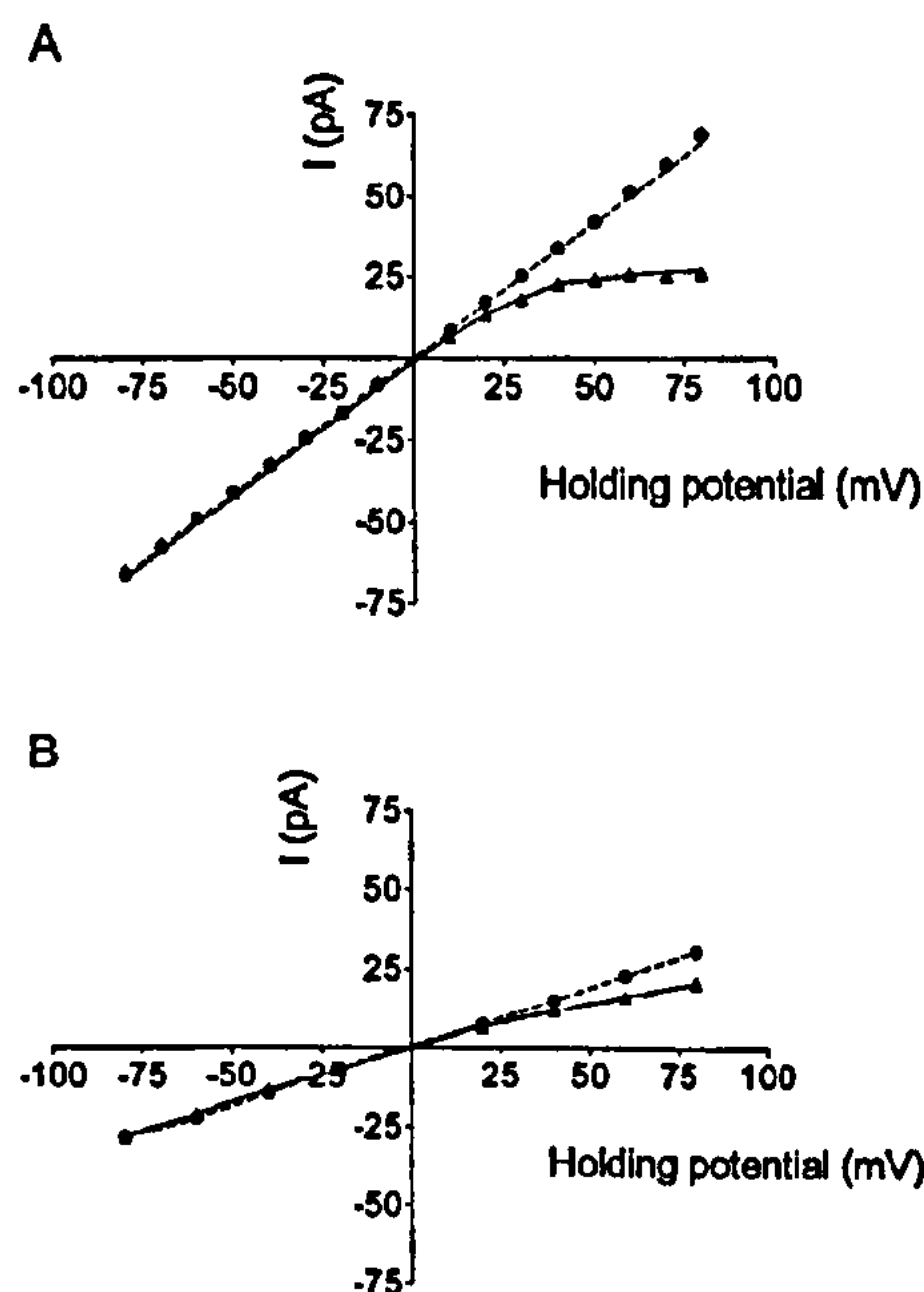


FIGURE 3. (A) Single-channel current-voltage relationships for unmodified channels in the absence (●; mean $\pm$ SEM of 22–51 experiments) and presence (▲; mean $\pm$ SEM of 4–25 experiments) of 20 mM TEA⁺. (B) Single-channel current-voltage relationships for 21-amino-9 α -hydroxyryanodine-modified channels in the absence (●; $\pm$ SEM of 4–15 experiments) and presence (▲; $\pm$ SEM of 4–10 experiments) of 20 mM TEA⁺. Where no error bars are visible they are contained within the symbol.

tion at negative holding potentials but blocks K⁺ current with increasing effectiveness as the holding potential is shifted to more positive values.

TEA⁺ also blocks K⁺ current in the 21-amino-9 α -hydroxyryanodine-modified conductance state. However, TEA⁺ is a less effective blocker of the ryanoid-modified conductance state of RyR than the unmodified conductance state. At all three holding potentials shown in Fig. 2, the reduction in current amplitude produced by TEA⁺ is greater for transitions in the unmodified state (transitions from C to O) than in the ryanoid-modified state (transitions from C to M). Fig. 3 B shows current-voltage relationships for the 21-amino-9 α -hydroxyryanodine-modified states of several channels in the absence and presence of 20 mM TEA⁺. As is the case for the unmodified state of RyR, TEA⁺ produces no block of K⁺ current at negative holding potentials, but is an increasingly effective blocker of the ryanoid-modified state as holding potential is made more positive. However, a comparison of the data in Fig. 3 (A and B) establishes the observation made for the representative channel in Fig. 2; TEA⁺ is less effective as a blocker of K⁺ current in the ryanoid-modified conductance state of RyR. As a point of comparison, at a holding potential of +80 mV, 20 mM TEA⁺ reduces mean single-channel current amplitude of the 21-amino-9 α -hydroxyryanodine-modified

conductance state of RyR by 33%; the equivalent block of the unmodified conductance state is 62%.

What mechanisms are involved in the alteration in effectiveness of TEA⁺ as a blocker of RyR after the interaction of 21-amino-9 α -hydroxyryanodine with the channel? In previous investigations, we have demonstrated that ryanoid-induced modification of permeant ion handling in RyR is strongly correlated to specific structural loci on the ryanoid molecule with contributions from both electrostatic and steric effects (Welch et al., 1997). To investigate the possibility that similar structural features underlie the alteration of the effectiveness of TEA⁺ as a blocker of K⁺ current in RyR, we have examined the influence of three additional members of the ryanoid group of ligands: ryanodol, 21-azido-9 α -hydroxyryanodine, and 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine. These ryanoids vary in both steric bulk and charge and, after interaction with the channel, yield values of FC ranging from 0.67 to 0.17. Single-channel current amplitude of both unmodified and ryanoid-modified conductance states were monitored at holding potentials within the range ± 80 mV in the absence and presence of 20 mM TEA⁺. Examples of current fluctuations of representative channels at a holding potential of +40 mV are given in Fig. 4. The left panel of Fig. 4 demonstrates modification of the RyR channel that results from the interaction of the indicated ryanoid. In all cases, the interaction of the ryanoid with the RyR chan-

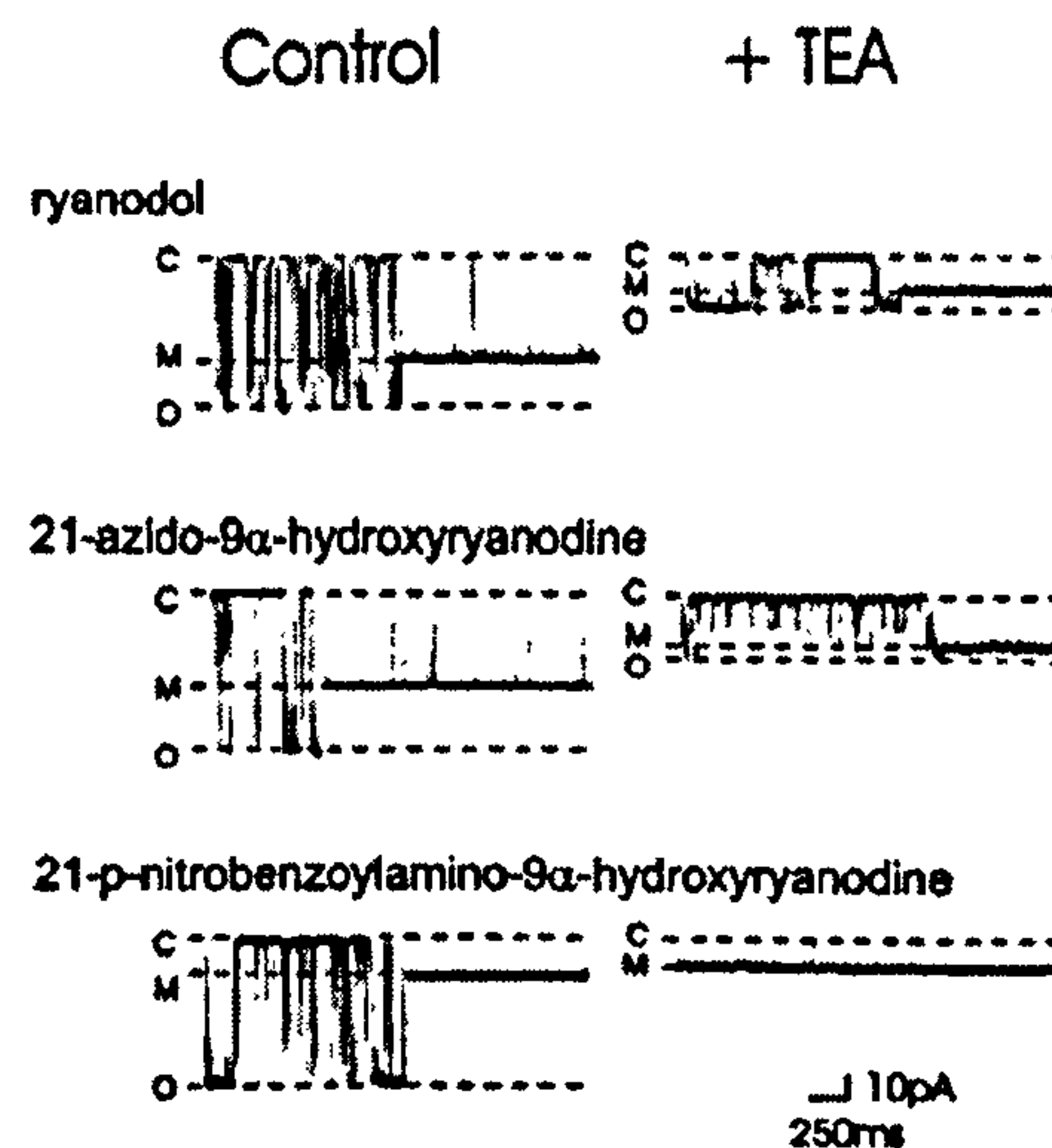


FIGURE 4. Single-channel traces showing the influence of 20 mM TEA⁺ on the amplitude of ryanoid-modified conductance states at +40 mV. Both ryanodol and 21-azido-9 α -hydroxyryanodine interact rapidly with RyR. For this reason, both unmodified and ryanoid-modified conductance states are shown in the presence of TEA⁺ for these ryanoids. In contrast, 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine is a slow-binding modifier of RyR channel function and, as a consequence, traces in the presence of TEA⁺ show only the ryanoid-modified conductance state. C, closed conductance level; M, modified conductance level; O, fully open conductance level.

nel results in the occurrence of a modified conductance state with high P_o . As outlined above, the amplitude of the modified conductance state is dependent on the structure of the ryanoid bound to the channel (Tinker et al., 1996; Welch et al., 1997). The values of FC induced by the ryanoids shown in Fig. 4 are 0.67 for ryanodol, 0.55 for 21-azido-9 α -hydroxyryanodine, and 0.27 for 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine. The influence of 20 mM TEA⁺ on the current amplitude of these various ryanoid-modified states is shown in the right panel of Fig. 4. In all cases, the addition of 20 mM TEA⁺ leads to a reduction in current amplitude. However, a comparison of the current amplitudes of the ryanoid-modified conductance states in the absence and presence of the blocking cation demonstrates that the effectiveness of TEA⁺ differs in the various ryanoid-modified conductance states. At this holding potential, the magnitude of block produced by TEA⁺ of the ryanoid-modified conductance state is in order

ryanodol > 21-azido-9 α -hydroxyryanodine > 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine.

The reduction in amplitude of both the unmodified and ryanoid-modified conductance states of RyR by TEA⁺ with varying holding potential can be fitted by a model originally described by Woodhull (1973). This scheme envisages a single site, accessible to a blocking ion of valence z from only one side of the channel and located a fraction (δ) into the voltage drop across the channel. Under these conditions, the relationship between relative conductance (i/i_0 ; the current in the presence of TEA⁺ divided by the current in the absence of the blocking cation) and holding potential is:

$$\frac{i}{i_0} = \left(1 + \frac{[B]}{K_{b(0)}} e^{z\delta(FV/RT)}\right)^{-1} \quad (1)$$

where $[B]$ is the concentration of TEA⁺ (20 mM), $K_{b(0)}$ is the dissociation constant at a holding potential of 0 mV, and $z\delta$ is conventionally referred to as the effective valence. F , R , and T have their usual meanings, and RT/F is 25.2 mV at 20°C. Values of $K_{b(0)}$ and $z\delta$ were determined for 21-amino-9 α -hydroxyryanodine, ryanodol, 21-azido-9 α -hydroxyryanodine, and 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine using nonlinear regression to establish the best fit of Eq. 1 to mean data from four or more experiments (GraphPad Prism v2.01; Fig. 5). Blocking parameters determined from these plots are shown in Table I.

In addition to the FC of 0.27 reported above, we have observed two other modified conductance states of RyR in the presence of 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine (Tinker et al., 1996). In these studies, out of a total of 15 experiments conducted with 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine, we observed 11 modified conductance states with an FC of 0.27 (73%

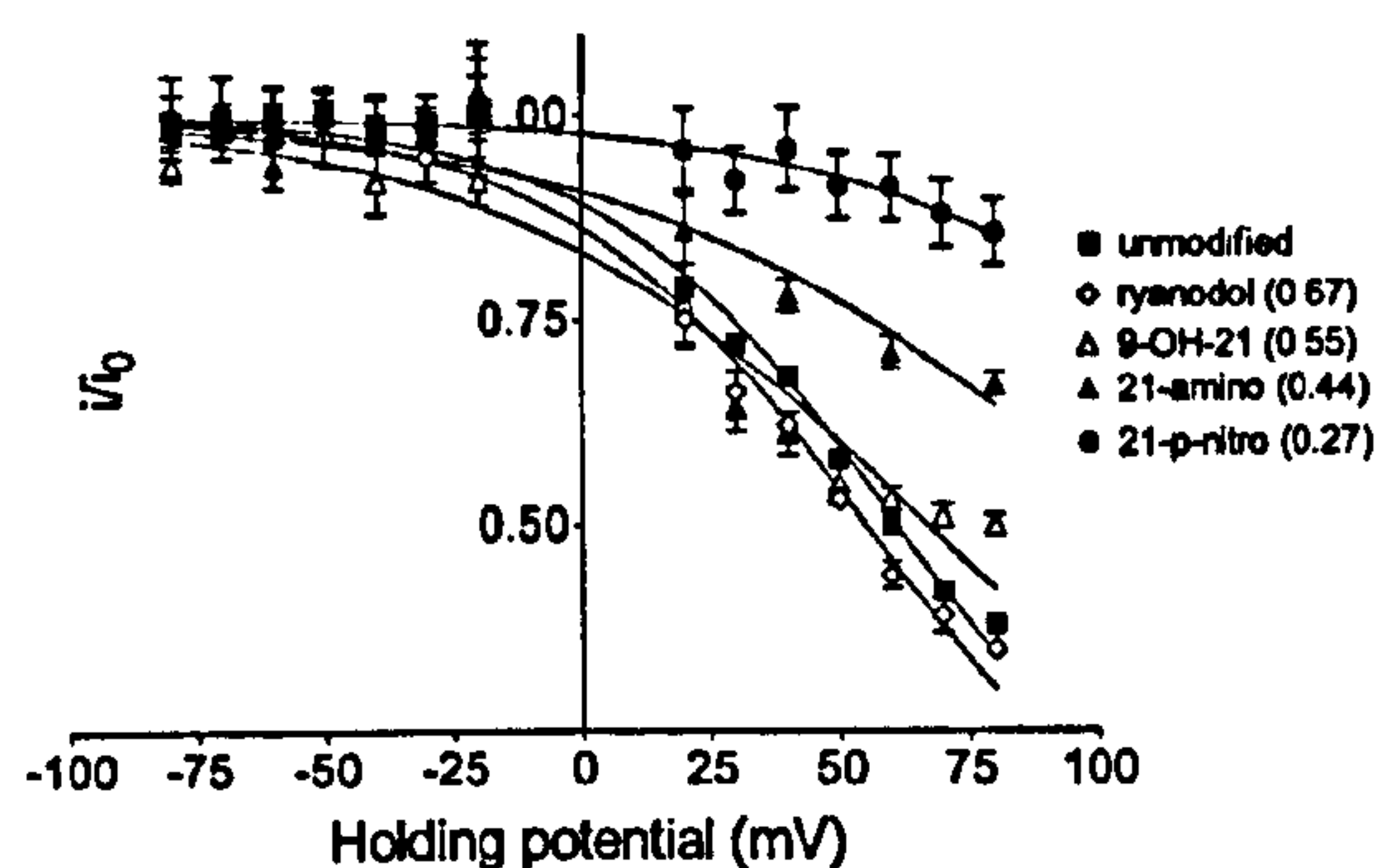


FIGURE 5. Woodhull plots for the indicated unmodified and ryanoid-modified conductance states in the presence of 20 mM TEA⁺. Points are mean normalized conductance values (i/i_0) $\pm$ SEM (unmodified, $n = 4-51$; ryanodol, $n = 4-11$; 21-azido-9 α -hydroxyryanodine, $n = 4-19$; 21-amino-9 α -hydroxyryanodine, $n = 4-15$; 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine [0.27], $n = 10-11$). The solid lines were obtained by nonlinear regression fits to Eq. 1 with the parameters quoted in Table I. Figures in brackets indicate the FC induced by the various ryanoids. 21-*p*-nitro, 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine; 21-amino, 21-amino-9 α -hydroxyryanodine; 9-OH-21, 21-azido-9 α -hydroxyryanodine.

of total events), 3 with an FC of 0.17 (20% of total events), and on one occasion a modified state with an FC of 0.59 (7% of total events). This observation of the occurrence of more than one modified conductance state is entirely consistent with the existence of multiple conformations of 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine, as determined by molecular dynamics (see MATERIALS AND METHODS and Fig. 1 B). Furthermore, these determinations demonstrate that the possible conformations of 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine are not isoenergetic. When added to the solution at the cytosolic face of RyR, 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine will be present in several conformations with different probabilities of occurrence. The three most common conformers exist in a ratio of 73:24:1%, with the remaining 2% corresponding to multiple conformers rarely populated. The striking similarity between the observed probability of oc-

TABLE I
Blocking Parameters for the Interaction of TEA⁺ with RyR Channels in the Absence of Ryanoid and in the Indicated Ryanoid-modified States

Ryanoid	FC	$K_{b(0)}$ (mM)	$z\delta$
None	1.00	167.3	0.88
Ryanodol	0.67	126.6	0.84
21-azido-9 α -hydroxyryanodine	0.55	100.3	0.60
21-amino-9 α -hydroxyryanodine	0.44	195.1	0.53
21- <i>p</i> -nitrobenzoylamino-9 α -hydroxyryanodine	0.27	853.9	0.63
21- <i>p</i> -nitrobenzoylamino-9 α -hydroxyryanodine	0.17	No block	No block

Parameters were determined using nonlinear regression to establish the best fit of Eq. 1 for the data displayed in Fig. 5. No block was seen in the 0.17 FC state of 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine. Blocking parameters determined for the 0.59 FC state induced by 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine ($n = 1$) are $K_{b(0)} = 177.7$ mM and $z\delta = 0.66$.

currence of the three different modified conductance states induced by 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine and the ratio of the three common conformers of this ryanoid determined by molecular dynamics indicates that on binding to the RyR channel, the different conformers of the bulky *p*-nitrobenzoyl tail (Fig. 1 B) produce different FC states. We have demonstrated previously a strong correlation between structure at this locus and FC (Welch et al., 1997). The advantage of the *p*-nitrobenzoyl conformers is that they provide a built-in control. The different values of FC observed after the interaction of 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine are due only to changes in conformation of the ryanoid and not to other ligand-receptor interactions that may arise when the covalent structure of the ryanoid is modified. Examples of the 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine-induced 0.27 and 0.17 FC states are shown in Fig. 6. The figure shows the modified conductance states in the absence (Fig. 6, left) and presence (Fig. 6, right) of 20 mM TEA⁺. The influence of TEA⁺ on the 0.59 FC induced by 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine is not included in Fig. 6, as only one such event occurred during the course of this investigation. The low probability of occurrence of a third 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine-induced modified conductance state is not surprising in light of the ratio of possible conformers of this ryanoid determined by molecular dynamics. TEA⁺ blocking parameters of the 0.59 FC state are quoted in the text. The degree of block induced by TEA⁺ and the influence of holding

21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine

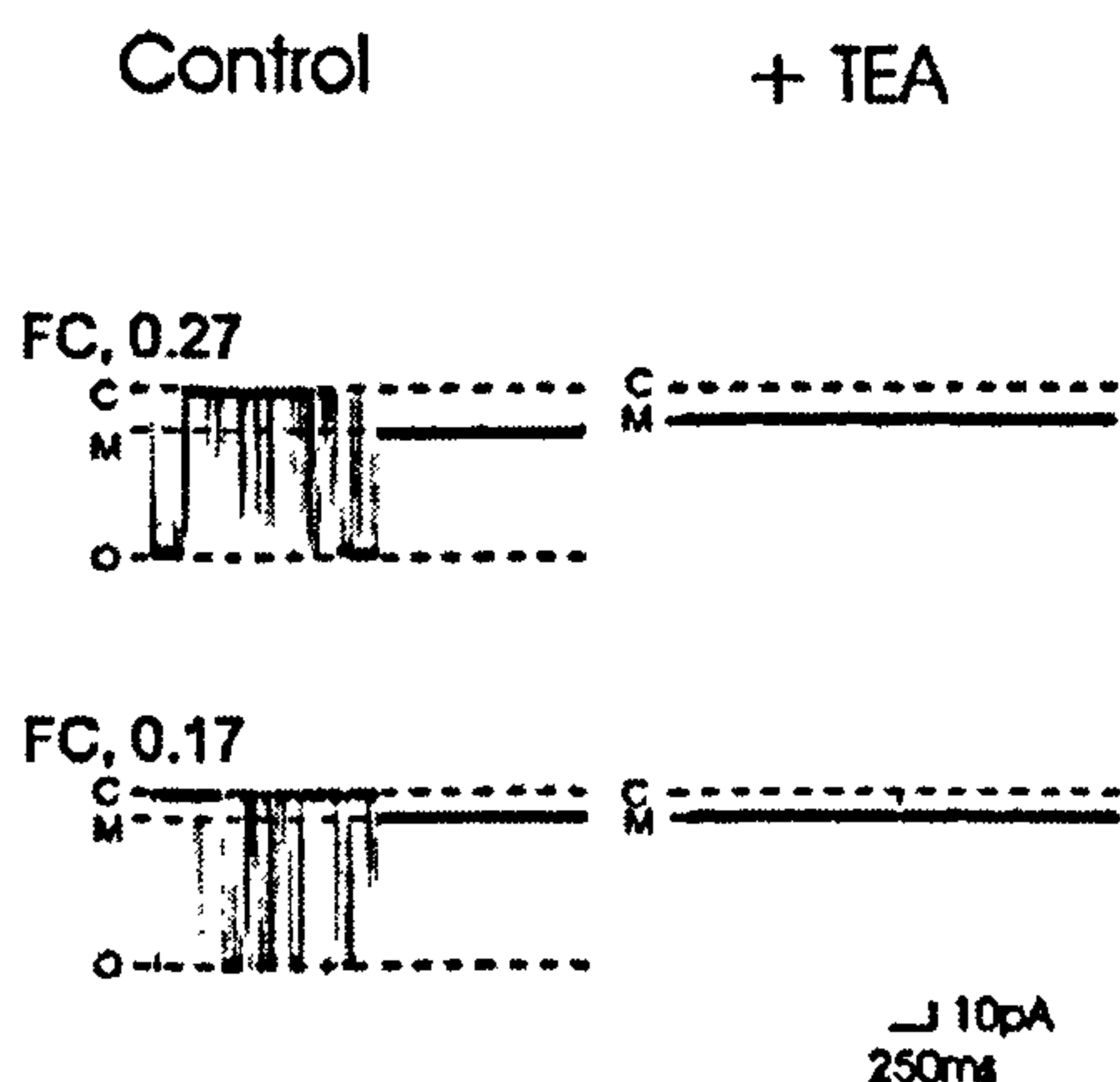


FIGURE 6. Single-channel traces showing the influence of 20 mM TEA⁺ on the amplitude of two 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine-modified conductance states at +40 mV. Irrespective of the FC induced, 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine is an irreversible modifier of RyR and, as a consequence, traces in the presence of TEA⁺ show only the ryanoid-modified conductance state. C, closed conductance level; M, modified conductance level; O, fully open conductance level.

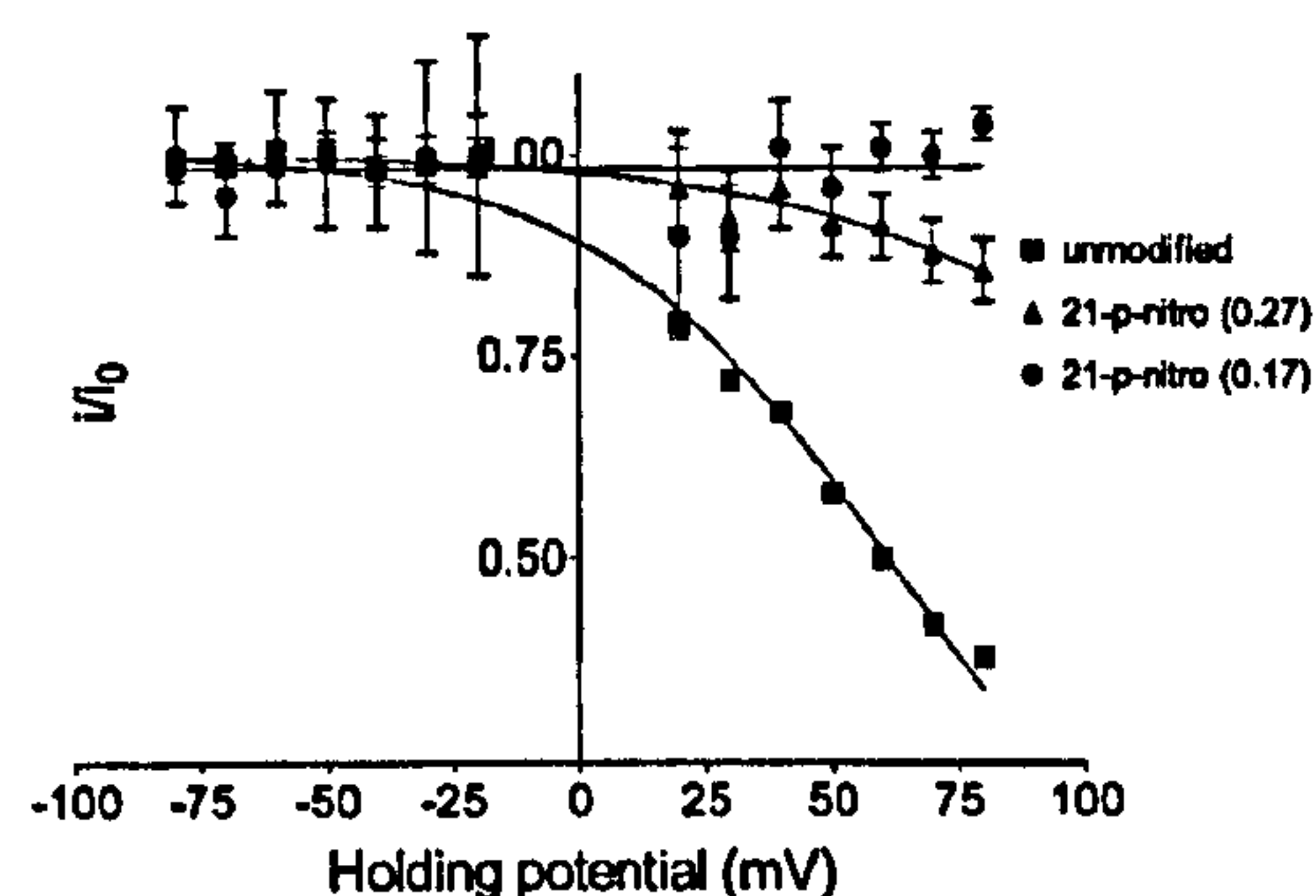


FIGURE 7. Woodhull plots for the indicated unmodified and 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine-modified conductance states in the presence of 20 mM TEA⁺. Points are mean normalized conductance values (i/i_0) $\pm$ SEM (unmodified, $n = 4-51$; FC 0.27, $n = 10-11$; FC 0.17, $n = 3$). The solid lines were obtained by nonlinear regression to Eq. 1 with the parameters quoted in Table I. Figures in brackets indicate the FC of the various conductance states. 21-*p*-nitro, 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine.

potential on block differs for the different FC states. Mean single-channel current amplitude at +80 mV in the presence of symmetrical 20 mM TEA⁺ was reduced by 14% in the 0.27 FC state, but no reduction in current amplitude was seen under these conditions in the 0.17 FC state. Single-channel current amplitude at +80 mV was reduced by 44% in the 0.59 FC state (data not shown). Woodhull analysis of the influence of holding potential on block of K⁺ current by TEA⁺ (Eq. 1) of the 0.27 and 0.17 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine-induced FC states is shown in Fig. 7. Blocking parameters for the 0.17 FC state were not determined, as we observed no significant block of K⁺ current by TEA⁺ in this ryanoid-modified state at any holding potential. Blocking parameters for the 0.59 FC state are quoted in the legend of Table I.

Overall, our investigations of the influence of holding potential on block by TEA⁺ of ryanoid-modified conductance states of RyR indicate that there are marked quantitative differences in the degree of block produced by TEA⁺ in the modified states induced by the various ryanoids. The alteration of the effectiveness of TEA⁺ as a blocker appears to be correlated with the FC of the ryanoid. A comparison of the degree of block (expressed as relative current; single-channel current in the presence of TEA⁺ divided by single-channel current in the absence of TEA⁺) produced by 20 mM TEA⁺ at a holding potential of +80 mV of the various ryanoid-modified conductance states monitored in this study is presented in Fig. 8. The degree of block induced by TEA⁺ increases as FC rises from 0.17 to 0.67; block in the ryanoid-modified state is essentially the same as that in the RyR channel in the absence of ryanoid (FC = 1.0).

Quantitative analysis of the influence of holding potential on block by TEA⁺ provides additional information. The relationships between FC in the range 0.27 to

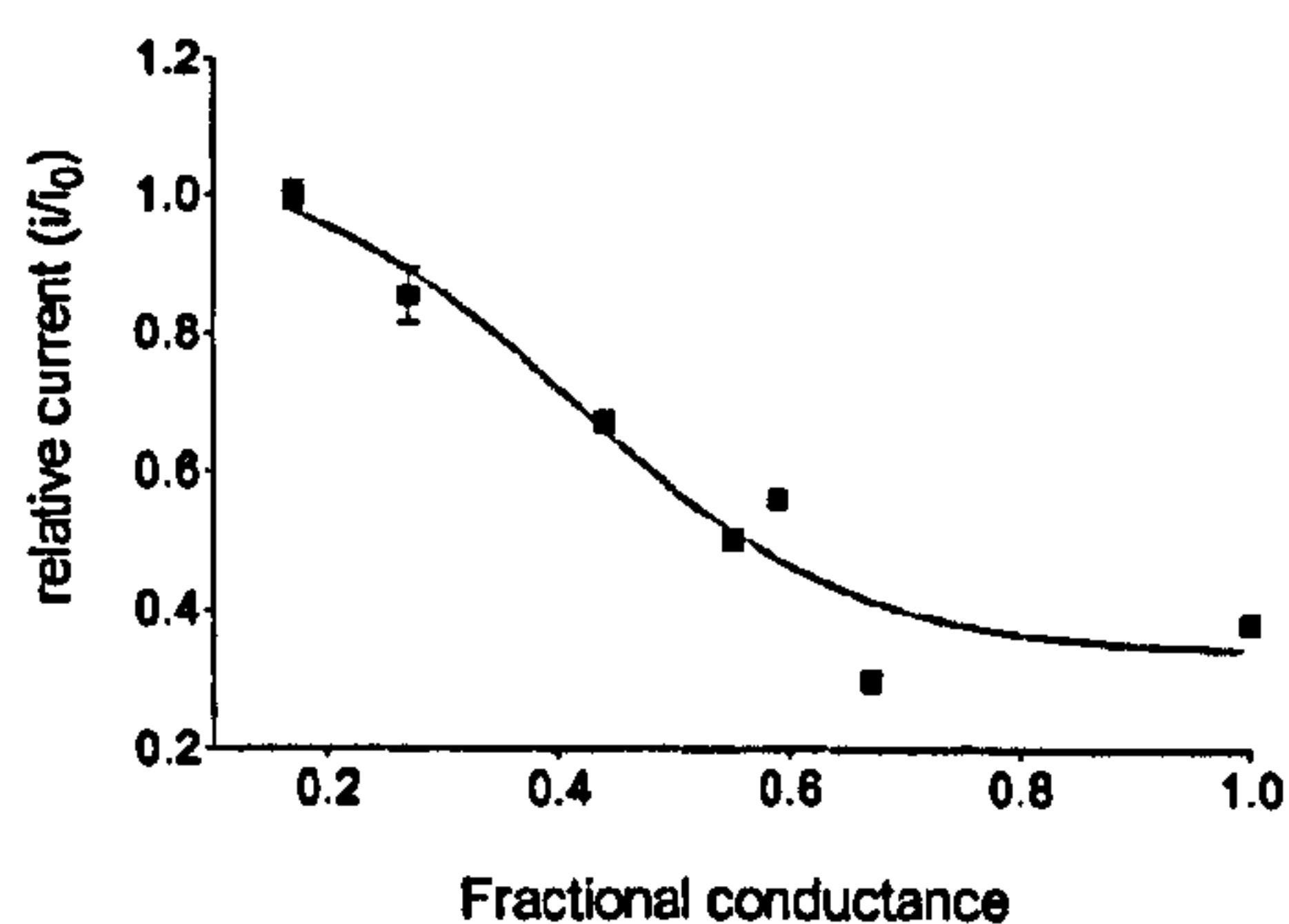


FIGURE 8. Relationship between relative conductance (i/i_0) produced by TEA^+ in the ryanoid-modified state at +80 mV and FC of the ryanoid-modified state. The line plotted through the data is of no theoretical significance.

1.0 and the blocking parameters derived from the Woodhull analysis are shown in Fig. 9 (no block was seen in the 0.17 FC state of 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine). The values of $z\delta$ determined for TEA^+ block of K^+ conductance of channels in the absence of ryanoid, and channels modified with various ryanoids, show a broad correlation with relative K^+ conductance (FC; Fig. 9 A). Ryanoid-modified conductance states with values of FC in the range 0.67 to 0.44 yield dissociation constants for TEA^+ at 0 mV that show little or no variation from the value determined in the absence of ryanoid. Dissociation constants increased markedly at FCs below 0.44 (Fig. 9 B).

DISCUSSION

We have demonstrated that the interaction of a range of ryanoids with RyR alters the blocking characteristics of TEA^+ in the channel. An inspection of the influence of voltage on block by TEA^+ in the unmodified and ryanoid-modified conductance states reveals something of the mechanism involved in this action. The interaction of ryanoids with RyR can affect both the $z\delta$ of the blocking cation and the affinity of the conduction pathway for the blocker ($K_{b(0)}$).

For a cation, such as TEA^+ , that blocks by entering the electric field across the RyR channel, δ is a measure of the relative depth of penetration of the blocking cation into the field (French and Shoukimas, 1985). As z , the valence of TEA^+ , is constant, a change in $z\delta$ after channel modification by a ryanoid implies that the location of the site of interaction of TEA^+ within the voltage drop across the channel is altered. This relocation of the TEA^+ binding site within the voltage drop is likely to be the consequence of a reorganization of the RyR conduction pathway induced by the binding of the ryanoid.

The interaction of some of the ryanoids investigated produces a concomitant alteration of the affinity of the TEA^+ site in RyR. In previous studies we have demonstrated that a reduction in the rate of association of a

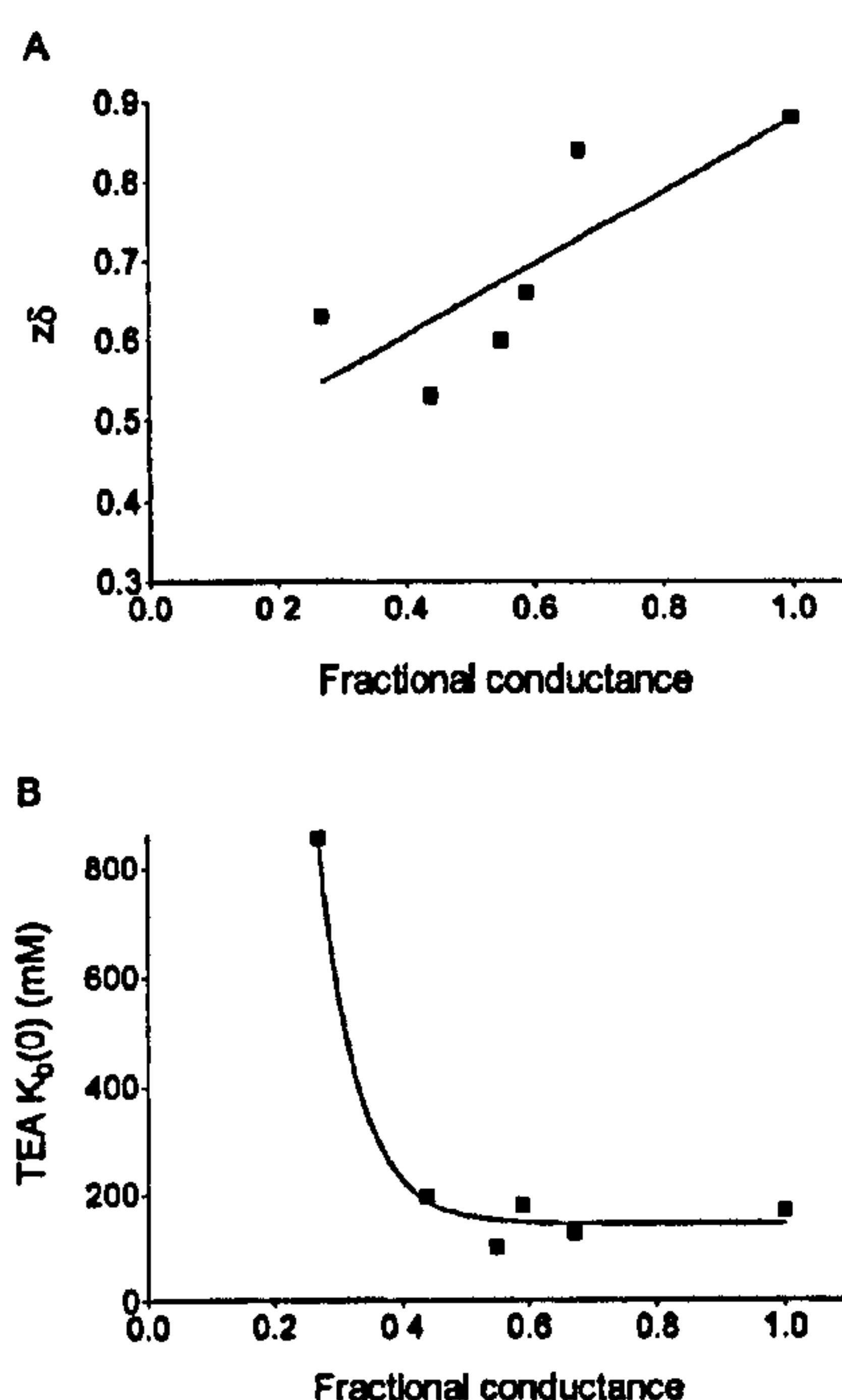


FIGURE 9. (A) Variations in $z\delta$ of TEA^+ in the unmodified RyR channel and ryanoid-modified states with FCs in the range 0.27–0.67. It should be noted that no measurable block by TEA^+ was seen, and therefore no value of $z\delta$ was determined, for the 0.17 FC state induced by 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine. The solid line is the best-fit line obtained by linear regression. (B) Variations in $K_{b(0)}$ of TEA^+ in the unmodified RyR channel and ryanoid-modified states with FCs in the range 0.27–0.67. Again, in the absence of measurable block, no value of $K_{b(0)}$ could be determined for the 0.17 FC state induced by 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine. The line plotted through the data has no theoretical significance.

large organic blocking cation (tetrabutylammonium) with RyR underlies a reduction in affinity of the channel for this cation after the interaction of ryanodine (Tinker and Williams, 1993b). In addition, hydrophobic interactions are likely to contribute to the strength of binding at the TEA^+ site. In the absence of ryanoids, $K_{b(0)}$ decreases as the hydrophobicity of cations interacting with this site is increased by the substitution of methyl and methylene groups (Tinker et al., 1992). If, as we suggest, the binding of ryanoids brings about conformational changes in RyR, then alterations in $K_{b(0)}$ are likely to reflect the rearrangement of groups in and around the TEA^+ site. Decreased access to the site, changes in electrical potential, or a lowering of the hydrophobicity of the site could all contribute to the observed reductions in affinity.

Quantitative differences in blocking parameters were monitored in the various ryanoid-modified states. This is reminiscent of the influence of ryanoids on the conductance of permeant cations such as K^+ . On binding to the high affinity site on RyR, all ryanoids so far exam-

ined reduce single-channel K^+ current amplitude, but the relative amplitude of the ryanoid-modified conductance state (FC) varies and is dependent on the electrostatic and steric features of the bound ryanoid (Tinker et al., 1996; Welch et al., 1997). By monitoring the influence of voltage on TEA⁺ block of K^+ conductance in RyR channels modified with ryanoids yielding different FCs, we have examined the possibility that the mechanisms underlying the alteration of TEA⁺ blocking parameters by ryanoids might also be responsible for the alterations in K^+ conductance induced by these ligands.

The results of these experiments are consistent with the proposal that the interaction of a ryanoid with RyR can induce structural rearrangements in the channel. In effect, the position of the TEA⁺ binding site is relocated relative to the voltage drop across the channel. In each case, the TEA⁺ site is shifted towards the cytosolic limit of the voltage drop.

When considering how a binding site might move within the voltage drop of the RyR channel, it is relevant to discuss what constitutes the TEA⁺ binding site. Does TEA⁺ merely traverse a portion of the RyR voltage drop until it jams in a narrow region of the conduction pathway, or are more specific interactions involved? The evidence that has emerged from previous investigations of ion translocation and block in RyR indicates that small tetraalkylammonium cations do not block RyR by simply jamming in a constriction in the conduction pathway. Studies with these cations, trimethylammonium derivatives, and charged local anesthetics have identified two regions within the voltage drop across RyR with which blocking cations can interact. Tetramethylammonium (TMA⁺) blocks K^+ conductance in RyR by binding at a site located ~50% into the voltage drop from the cytosolic face of the channel (Tinker et al., 1992). Larger tetraalkylammoniums and related cations are excluded from the TMA⁺ site and bind further into the voltage drop (80–90%; Tinker et al., 1992; Tinker, 1993; Tinker and Williams, 1993a). As outlined above, the affinity of this site for blocking cations increases in proportion to the hydrophobicity of the cations, indicating that hydrophobic forces are likely to be involved in this interaction. The exclusive association of the smallest tetraalkylammonium, TMA⁺, with the 50% site has been interpreted as indicating that this site is likely to be a small, relatively rigid structure from which larger blocking cations are excluded by steric restrictions (Tinker et al., 1992).

Within the context of the picture of the RyR conduction pathway set out above, it is probable that the observed relocation and changes in affinity of the TEA⁺ blocking site within the voltage drop result from rearrangement in this region of the channel after ryanoid interaction. We can envisage movement of the relative position of the TEA⁺ site within the voltage drop across

the RyR channel as a physical relocation of the site; alternatively, the monitored variations in $z\delta$ could arise from a change in the dimensions or position of the voltage drop across the channel. In such a scheme, the interaction of a ryanoid with RyR would induce a structural reorganization within the channel that results in the elongation of the voltage drop or a shift of the region of the channel over which transmembrane voltage drops towards the luminal face of the channel. Assuming that the physical location of the TEA⁺ site within the conduction pathway of the channel is unchanged, either of these maneuvers would result in a lowering of $z\delta$.

The absence of voltage-dependent block of K^+ current by TEA⁺ in the 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine-modified state with an FC of 0.17 is likely to result from either, or both, a relocation of the blocking site outside the voltage drop across the channel or a dramatic decrease in the affinity of the TEA⁺ blocking site in the conduction pathway of RyR. The trends in the data observed in Fig. 9 indicate that alterations in affinity of the TEA⁺ binding site are likely to be the dominant factor.

In previous investigations, we reported modifications of permeant ion conductance in ryanoid-bound channels and suggested that these variations were likely to result from ryanoid-induced structural reorganization of the conduction pathway of RyR (Lindsay et al., 1994; Tinker et al., 1996; Welch et al., 1997). The experiments reported in this communication provide direct evidence for this postulated ryanoid-induced restructuring. Although structural rearrangements within the conduction pathway of the RyR channel are unlikely to have exactly equivalent effects on the interactions of permeant and blocking cations, the broad correlation of TEA⁺ blocking parameters with the FC induced by individual ryanoids with different steric and electronic properties indicates that the structural reorganizations identified here are likely to underlie ryanoid-induced alterations in permeant ion translocation in RyR.

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The Interaction of a Neutral Ryanoid with the Ryanodine Receptor Channel Provides Insights into the Mechanisms by which Ryanoid Binding Is Modulated by Voltage

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ABSTRACT In an earlier investigation, we demonstrated that the likelihood of interaction of a positively charged ryanoid, 21-amino-9 α -hydroxyryanodine, with the sarcoplasmic reticulum Ca²⁺-release channel (ryanodine receptor, RyR) is dependent on holding potential (Tanna, B., W. Welch, L. Ruest, J.L. Sutko, and A.J. Williams. 1998. *J. Gen. Physiol.* 112:55–69) and suggested that voltage dependence could result from either the translocation of the charged ligand to a site within the voltage drop across the channel or a voltage-driven alteration in receptor affinity. We now report experiments that allow us to assess the validity of these alternate mechanisms. Ryanodol is a neutral ryanoid that binds to RyR and induces modification of channel function. By determining the influence of transmembrane potential on the probability of channel modification by ryanodol and the rate constants of ryanodol association and dissociation, we demonstrate that the influence of voltage is qualitatively the same for both the neutral and positively charged ryanoids. These experiments establish that most, if not all, of the modification of ryanoid interaction with RyR by transmembrane holding potential results from a voltage-driven alteration in receptor affinity.

KEY WORDS: ryanodine receptor • sarcoplasmic reticulum • calcium channel • ryanodine • ryanodol

INTRODUCTION

Ryanodine is a plant alkaloid that binds with high affinity and specificity to a class of intracellular membrane Ca²⁺-release channels (Sutko et al., 1997). As a consequence of this interaction, these channels are commonly referred to as ryanodine receptors (RyRs).¹ Ryanodine has proven to be a useful tool in establishing the molecular identity of the Ca²⁺-release channel (Lai et al., 1988a,b), in the characterization of the function of the receptor as a channel, and in the elucidation of its role in Ca²⁺ signaling processes in a wide range of systems (Bazotte et al., 1991; Foskett and Wong, 1991; Swann, 1992; Walz et al., 1995; Ullmer et al., 1996). The binding of ryanodine to its high affinity site on RyR initiates a marked change in channel function; rates of permeant ion translocation are altered and open probability (P_o) is increased dramatically. The modification of RyR ion handling by ryanodine is the consequence of the alteration of a number of processes governing cation translocation. The reduction in single-channel conductance observed on the binding of ryanodine to the RyR channel with either divalent or monovalent in-

organic cations as the permeant species reflects alterations in both the relative permeability of ions and the affinity of sites within the conduction pathway for these ions (Lindsay et al., 1994). It seems likely that the binding of ryanodine to the channel induces a conformational change in the protein that in turn modifies the manner in which permeant ions interact with the conduction pathway (Lindsay et al., 1994).

The kinetic parameters of the ryanodine–RyR interaction are traditionally determined by monitoring the binding of [³H]ryanodine to populations of receptors, either in intact membrane vesicles, or after receptor purification (Fleischer et al., 1985; Pessah et al., 1986; Lai et al., 1989; Chu et al., 1990; Holmberg and Williams, 1990; Needleman and Hamilton, 1997; Murayama et al., 1999). We have recently developed methods that permit the investigation of the kinetic parameters of the interaction of ryanoids with individual RyR channel proteins under voltage clamp conditions. These experiments are based on the observation that (a) the interaction of a ryanoid with the channel induces a distinct modification of channel function and (b) alterations in ryanoid structure produce variations in binding kinetics. Consistent with the very slow rates of dissociation monitored for [³H]ryanodine in binding assays, the interaction of ryanodine with a single RyR channel is irreversible on the timescale of a single-channel experiment (Rousseau et al., 1987; Lindsay et al., 1994; Tinker et al., 1996); ryanod-

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¹Abbreviation used in this paper: RyR, ryanodine receptor.



dine "locks" the channel in a high- P_o , modified-conductance state. Modification of the structure of ryanodine yields ryanoids that compete with ryanodine for the high affinity site on the RyR channel (Welch et al., 1994, 1996; Bidasee and Besch, 1998; Humerickhouse et al., 1994) and induce high- P_o , modified-conductance states. However, the kinetics of the interaction of some of these ligands with single RyR channels is much faster than those of ryanodine. In the continued presence of the ryanoid, we observe transitions between periods of normal channel gating and ion translocation and periods of modified channel function during which the ryanoid is bound (Tinker et al., 1996; Tanna et al., 1998). We have characterized the interaction of one such ryanoid, 21-amino-9 α -hydroxyryanodine, with the sheep cardiac isoform of the RyR channel and have identified a number of novel features (Tanna et al., 1998). Variations in 21-amino-9 α -hydroxyryanodine binding to single channels with changing concentrations of the ryanoid indicate that modifications of RyR channel function induced by this ryanoid result from the interaction of a single molecule of the ryanoid with each channel protein. Related experiments demonstrated that 21-amino-9 α -hydroxyryanodine has access to its binding site only in open conformations of the channel protein, that the ryanoid binding site can only be reached from the cytosolic side of the channel, and that the interaction of the ryanoid with its binding site is influenced strongly by transmembrane voltage (Tanna et al., 1998).

The quantitative determination of the voltage dependence of the interaction of 21-amino-9 α -hydroxyryanodine with RyR indicates that approximately two positive charges move through the voltage drop across the channel during the activation of 21-amino-9 α -hydroxyryanodine binding by voltage (Tanna et al., 1998). Both the rate of association of the ryanoid with the receptor and the rate of dissociation from the receptor are influenced by voltage. A likely source of the voltage dependence of this reaction would involve the translocation of 21-amino-9 α -hydroxyryanodine (net charge +1) to a binding site within the voltage drop across the channel. If all the voltage dependence of the interaction arises in this way, either two molecules of the ryanoid would need to be translocated across the entire voltage drop, or several ryanoid molecules could interact with sites located nearer the cytosolic entry to the voltage drop. Neither of these possibilities is consistent with our observation that other aspects of the interaction of 21-amino-9 α -hydroxyryanodine with RyR can be described in terms of a bimolecular reaction (Tanna et al., 1998). An alternative explanation is that the measured voltage dependence results not from the movement of the charged ryanoid into an electric field, but from a voltage-driven movement of charge within the RyR channel protein that produces a conformational change in the channel protein and switches

the ryanoid binding site between two states with different affinities (Tanna et al., 1998). The elucidation of the mechanism underlying the dependence on transmembrane voltage of the interaction of 21-amino-9 α -hydroxyryanodine with RyR is of interest in its own right, but may also provide information on the location of the ryanodine binding site in RyR. For example, translocation of 21-amino-9 α -hydroxyryanodine into the voltage drop across the channel would suggest that the binding site is within the conduction pathway of the RyR channel.

In this communication we have addressed these issues by investigating the influence of transmembrane voltage on the interaction of a neutral ryanoid, ryanodol, with single RyR channels. Our experiments demonstrate that ryanodol displays the same qualitative voltage dependence as the positively charged 21-amino-9 α -hydroxyryanodine and are consistent with the proposal that most, if not all, of the influence of voltage on the interaction of ryanoids with the RyR channel is derived from a voltage-driven alteration in the affinity of the receptor.

MATERIALS AND METHODS

Materials

Phosphatidylethanolamine was supplied by Avanti Polar Lipids, Inc. and phosphatidylcholine by Sigma-Aldrich. [^3H]Ryanodine was purchased from New England Nuclear Ltd. Aqueous counting scintillant was purchased from Packard. Standard chemicals were obtained as the best available grade from BDH Ltd. or Sigma-Aldrich. Ryanodol was synthesized as described earlier (Wiesner, 1972; Deslongchamps et al., 1990) and stored as a stock solution in 50% ethanol at -20°C .

Isolation of Sheep Cardiac Heavy Sarcoplasmic Reticulum Membrane Vesicles and Solubilization and Separation of the Ryanodine Receptor

Heavy sarcoplasmic reticulum (HSR) membrane vesicles were prepared using procedures described earlier (Sitsapesan and Williams, 1990). Sheep hearts were collected from a local abattoir in ice-cold cardioplegic solution (Sitsapesan and Williams, 1990). A mixed membrane fraction was obtained by differential centrifugation after homogenization of the ventricular septum and left ventricle free wall. The mixed membrane vesicles were further fractionated by sucrose density gradient centrifugation and the HSR fraction was collected at the 30/40% (wt/vol) interface. The HSR fraction was resuspended in 0.4 M KCl before sedimentation at 100,000 g . The resulting pellet was resuspended in 0.4 M sucrose, 5 mM HEPES, titrated to pH 7.2 with hydroxymethyl methylamine (Tris). HSR membrane vesicles were solubilized with 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS) and RyR was isolated and reconstituted into unilamellar liposomes for incorporation into planar phospholipid bilayers, as described previously (Lindsay and Williams, 1991).

Planar Phospholipid Bilayers

Phospholipid bilayers were formed from suspensions of phosphatidylethanolamine in n -decane (35 mg/ml) across a 200- μm diameter hole in a polystyrene copolymer partition that separated two chambers referred to as cis (0.5 ml) and trans (1.0 ml). The trans chamber was held at virtual ground while the cis cham-

ber could be clamped at holding potentials relative to ground. Current flow across the bilayer was monitored using an operational amplifier as a current-voltage converter (Miller, 1982). Bilayers were formed with solutions containing 600 mM KCl, 20 mM HEPES, titrated to pH 7.4 with KOH, resulting in a solution containing 610 mM K⁺ in both chambers. An osmotic gradient was created by the addition of an aliquot (50–100 μ l) of 3 M KCl to the cis chamber. Proteoliposomes were added to the cis chamber and stirred. Under these conditions, channels usually incorporated into the bilayer within 2–3 min. If channels did not incorporate, a second aliquot of 3 M KCl could be added to the cis chamber. After channel incorporation, further fusion was prevented by perfusion of the cis chamber with 610 mM K⁺. Channel proteins incorporate into the bilayer in a fixed orientation so that the cytosolic face of the channel is exposed to the solution in the cis chamber and the luminal face of the channel to the solution in the trans chamber. Single channel P_o was increased by the addition of up to 200 μ M EMD 41000 to the cytosolic face of the channel (McGarry and Williams, 1994; Tanna et al., 1998). Only bilayers containing a single channel were used in the experiments described in this communication. Experiments were carried out at room temperature ($21 \pm 2^\circ\text{C}$). The interactions of ryanodol with the channel were studied by adding the indicated concentration to the solution at the cytosolic face of the bilayer.

Single Channel Data Acquisition

Single channel current fluctuations were displayed on an oscilloscope and stored on Digital Audio Tape. For analysis, data were replayed, filtered at 1 kHz with an eight-pole Bessel filter, and digitized at 4 kHz using Satori V3.2 (Intracel). Single channel current amplitudes and lifetimes were measured from digitized data. The representative traces shown in the figures were obtained from digitized data acquired with Satori V3.2 and transferred as an HPGL graphics file to a graphics software package (CorelDraw; Corel Systems Corp.) for annotation and printing.

Monitoring the Interaction of Ryanodol with Single Channels

Ryanodol binds to the high affinity ryanodine binding site on the SR Ca²⁺-release channel and induces modifications of channel function; channel conductance is reduced and P_o increases (Tinker et al., 1996). The interaction kinetics of ryanodol are sufficiently rapid that, in the continued presence of the ryanoid, we observe repeated transitions between periods of modified channel function and periods of normal gating and conductance. In previous studies, we have established that the interaction of 21-amino-9 α -hydroxyryanodine and the resulting modification of channel function can be described by a simple bimolecular reaction scheme (Tanna et al., 1998). Consistent with this scheme, dwell times of the RyR channel in the ryanodol-modified and -unmodified gating states are described by single exponentials, the rate of association of ryanodol with its receptor varies with ryanodol concentration while the rate of dissociation of ryanodol from its receptor is independent of ligand concentration (data not shown). As a consequence, apparent rate constants for the association (k_{on}) and dissociation (k_{off}) of ryanodol can be determined from the mean dwell times in the unmodified and modified conductance states (Eqs. 1 and 2):

$$k_{on} = (\tau_{unmod})^{-1} \quad (1)$$

and

$$k_{off} = (\tau_{mod})^{-1}. \quad (2)$$

Dwell times and the probability that the channel is in the ryanoid-modified state (P_{mod}) were determined using a pattern recognition program as described in Tanna et al. (1998). To obtain sufficient events, these parameters were obtained from steady state recordings lasting at least 6 min.

The Modification of the Interaction of Ryanodol with RyR by Voltage

If the transition between the normal gating state of the RyR and the modified state resulting from the interaction of ryanodol is dependent on holding potential, P_{mod} will be determined by the Boltzmann distribution,

$$\frac{P_{mod}}{1 - P_{mod}} = \exp \frac{[z_1 FV - \Delta G_1]}{RT}, \quad (3)$$

where F is the Faraday constant, V is the transmembrane voltage, R is the gas constant, T is temperature ($^\circ\text{K}$), z_1 is the voltage dependence of the occurrence of the ryanoid modified state, ΔG_1 is the difference in free energy of the unmodified and ryanoid-modified states, and $\Delta G_1/RT$ is an expression of the equilibrium of the reaction at a holding potential of 0 mV.

For such a relationship, the rate constants at a given voltage will be described as follows:

$$k_{on}(V) = k_{on}(0) \cdot \exp^{[z_{on} \cdot (FV/RT)]} \quad (4)$$

$$k_{off}(V) = k_{off}(0) \cdot \exp^{[-z_{off} \cdot (FV/RT)]}, \quad (5)$$

where $k(V)$ and $k(0)$ are the rate constants at a particular voltage and at 0 mV, respectively, and z is the valence of the appropriate reaction. Plots of the natural logarithm of k_{on} and k_{off} against holding potential should be linear with slopes $z_{on}F/RT$ and $-z_{off}F/RT$ and intercepts $\ln[k_{on}(0)]$ and $\ln[k_{off}(0)]$, respectively. The total voltage dependence (z_{total}) of the reaction is then $z_{on} + z_{off}$.

The Probability of the Channel Being Open

While the rates of dissociation of ryanodol and 21-amino-9 α -hydroxyryanodine from RyR are independent of P_o , the rates of association of both ryanodol (data not shown) and 21-amino-9 α -hydroxyryanodine (Tanna et al., 1998) are directly proportional to channel P_o . For this reason, it is necessary to measure P_o in all experiments. This was done by monitoring this parameter in the sections of the recorded data during which no ryanoid was bound; i.e., with transitions only between the open and closed conductance levels. P_o was determined by 50% threshold analysis as described previously (Sitsapesan and Williams, 1994). To minimize variability in P_o , all experiments were carried out in the presence of cytosolic EMD 41000. As kinetic parameters determined for ryanodol are to be compared with equivalent parameters determined in an earlier investigation for 21-amino-9 α -hydroxyryanodine (Tanna et al., 1998), it is important to correct for variations in k_{on} arising from unavoidable differences in P_o between the populations of channels used in the two sets of experiments. The k_{on} values quoted for both ryanodol and 21-amino-9 α -hydroxyryanodine have been normalized to a P_o of 1.0.

RESULTS

Is the Interaction of Ryanodol with the RyR Channel Influenced by Transmembrane Holding Potential?

In previous studies, we demonstrated that the interaction of 21-amino-9 α -hydroxyryanodine with the high af-

finity ryanodine binding site on the RyR channel is influenced by transmembrane voltage (Tanna et al., 1998). We observed a marked dependence of P_{mod} on voltage with a value of z obtained from best fit Boltzmann distributions (Eq. 3) and from variations in k_{on} and k_{off} (Eqs. 4 and 5) of ~ 2 . As outlined in the INTRODUCTION, this dependence on holding potential could arise from the translocation of the positively charged ryanoid into the voltage drop across the RyR channel. Alternatively, voltage dependence could be derived from a voltage-driven movement of charge within the RyR channel that results in a conformational change that switches the ryanoid binding site between two states of different affinity (Tanna et al., 1998). In such a scheme, the location of the ryanoid binding site could influence the measured voltage dependence of a charged ryanoid such as 21-amino-9 α -hydroxyryanodine. If the site were located outside the voltage drop across the channel, all of the voltage dependence would be derived from the proposed voltage-driven conformational change. However, if the site were located within the voltage drop, a proportion of the total voltage dependence would arise from the movement of the charged ryanoid into and out of the voltage drop. These alternatives can be distinguished by monitoring the voltage dependence of a ryanoid such as ryanodol that carries no ionic groups.

Fig. 1 presents the electrical potential of 21-amino-9 α -hydroxyryanodine (right, net charge +1) and ryanodol (left, no ionic groups) together with ball and stick models of the molecules. The top shows positive and the bottom shows negative electrostatic fields. 21-amino-9 α -hydroxyryanodine is shown with the pyrrole ring at the bottom left. Ryanodol, which lacks the pyrrole ring, is in the same orientation. The wire frames in all diagrams represent the surface where the electrostatic potential equals 10 kcal/mol. Note the extensive electrical potential of 21-amino-9 α -hydroxyryanodine (top right) due to the ammonium ion (located in the upper right of the ball and stick diagram). In comparison with ryanodol (top left), the far more extensive electrical interactions of 21-amino-9 α -hydroxyryanodine with other molecules is clearly visible. While ryanodol is neutral (has no net charge), the presence of hydroxyl groups creates a nonuniform distribution of potential in the molecule. The hydroxyl groups on one surface mean that there will be excess negative electrostatic potential localized to a specific surface region of ryanodol. The resulting microscopic dipoles sum to yield the electrical fields shown on the left hand figures. Note that, as for the positive electrostatic field, the distribution of the negative electrostatic potential (bottom) is much different for 21-amino-9 α -hydroxyryanodine and ryanodol. Fig. 1 visualizes the large difference in the macroscopic dipole moments of these two

molecules. The dipole (24.2 Debye) of 21-amino-9 α -hydroxyryanodine runs along the long axis of the cationic ryanoid (the long axis is parallel to the plane of Fig. 1). In contrast, the dipole (3.3 Debye) of the neutral ryanodol is at right angles to that of 21-amino-9 α -hydroxyryanodine. Therefore, these two ryanoids will experience considerably different torsional forces within any electrical potential gradient, including the applied transmembrane voltage. The large permanent dipole will tend to orient 21-amino-9 α -hydroxyryanodine with the long axis parallel to the electric field.

Fig. 2 shows current fluctuations of a single RyR channel at holding potentials ranging from -50 to 20 mV in the presence of $20 \mu\text{M}$ ryanodol. As is the case with 21-amino-9 α -hydroxyryanodine, ryanodol interacts reversibly with the RyR channel, inducing modifications to both channel gating and ion handling; however, the fractional conductance of the modified state (Tinker et al., 1996) differs for the two ryanoids. The fractional conductance of ryanodol is independent of holding potential, being 0.65 ± 0.01 , 0.64 ± 0.02 , 0.66 ± 0.01 , 0.66 ± 0.01 , 0.66 ± 0.01 , 0.65 ± 0.02 , 0.68 ± 0.02 , and 0.68 ± 0.01 at -60 , -50 , -40 , -30 , -20 , -10 , 10 , and 20 mV. Fractional conductance for 21-amino-9 α -hydroxyryanodine under these conditions is ~ 0.45 (Tanna et al., 1998).

Inspection of the traces in Fig. 2 highlights the first novel finding of these studies. The probability of RyR channel modification by a neutral ryanoid, ryanodol, varies markedly with holding potential, and the effect of voltage is qualitatively the same as that observed with 21-amino-9 α -hydroxyryanodine: P_{mod} for both ryanoids rises as holding potential is shifted to more positive values. The relationship between P_{mod} and holding potential, in the range -60 to 20 mV, for several channels in the presence of $20 \mu\text{M}$ ryanodol is shown in Fig. 3. The solid line is the best fit Boltzmann distribution (Eq. 3) obtained by nonlinear regression with a value for z of 1.54 ($r = 1.0$).

Fig. 4 shows the influence of holding potential on k_{on} (a) and k_{off} (b) of ryanodol together with equivalent data for 21-amino-9 α -hydroxyryanodine taken from Tanna et al. (1998). Both the rates of association and dissociation of ryanodol vary with applied holding potential; k_{on} increases and k_{off} decreases as the holding potential is shifted to more positive values. The line of best fit obtained by linear regression for the k_{on} plot has a slope of 0.041 ± 0.003 ($r = 1.0$), yielding a value of z_{on} of 1.03 (Eq. 4). The equivalent plot for k_{off} has a slope of -0.019 ± 0.002 ($r = 0.98$), yielding a value for z_{off} of 0.48 (Eq. 5). Together, these produce a z_{total} of 1.51 . Values for k_{on} and k_{off} at 0 mV, obtained from the lines of best fit in Fig. 4, a and b, are $0.035 \mu\text{M}^{-1} \text{s}^{-1}$ and 0.095s^{-1} , respectively. Fig. 5 shows the relationship of the ryanodol dissociation constant [$K_d = k_{\text{off}} (\text{s}^{-1}) / k_{\text{on}} (\mu\text{M}^{-1} \text{s}^{-1})$] to trans-

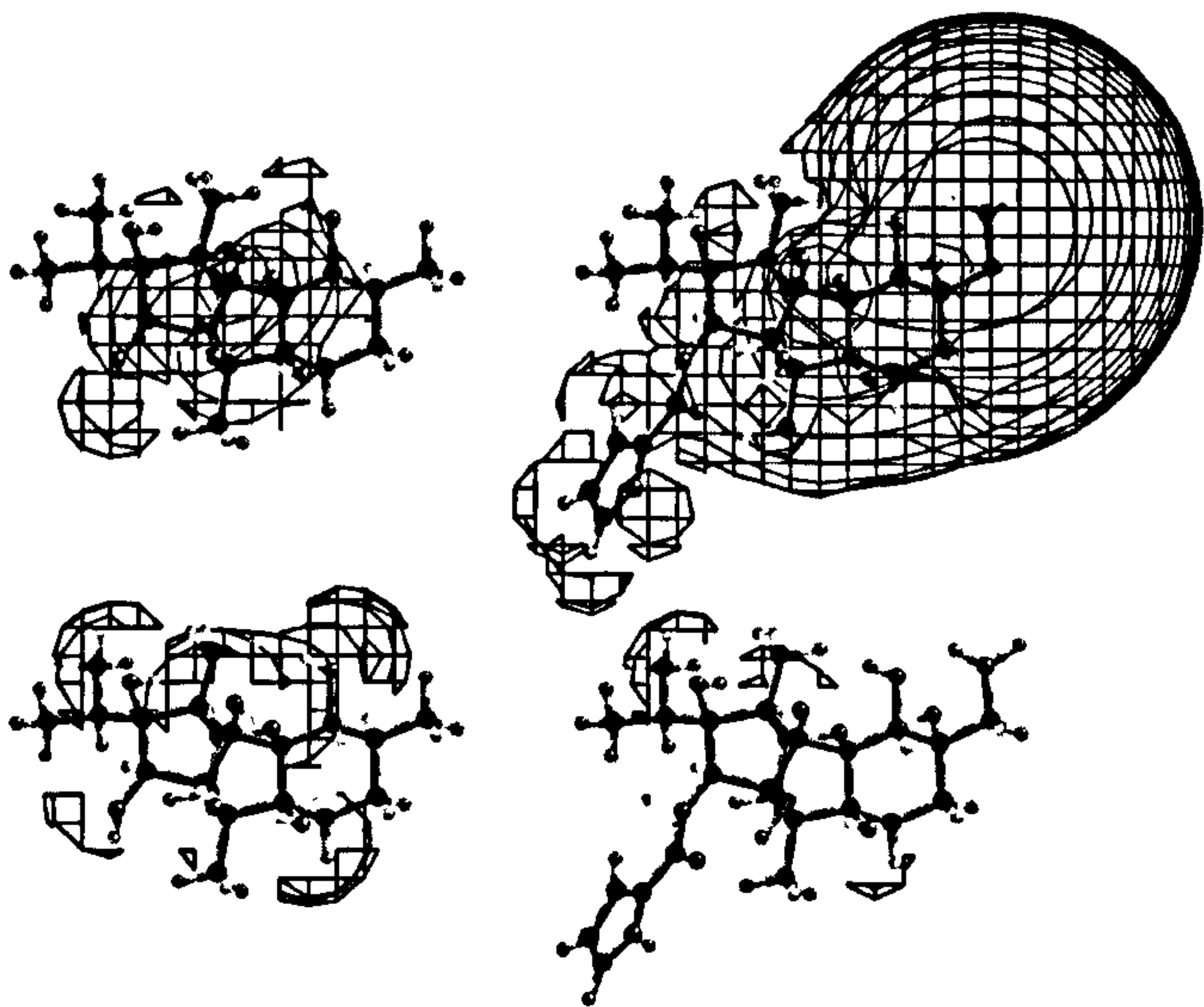


FIGURE 1. (Top) The positive electrostatic fields of 21-amino-9 α -hydroxyryanodine (right) and ryanodol (left). (Bottom) The negative electrostatic fields of the same compounds. In all cases, the wire frames indicate the surface where the electrical field strength is 10 kcal/mol. Ball and stick models of 21-amino-9 α -hydroxyryanodine (right) and ryanodol (left) are shown in the same alignment. Referring to the models of 21-amino-9 α -hydroxyryanodine, the pyrrole group is at the bottom left and the 21-amino group is at the top right.

membrane holding potential; 21-amino-9 α -hydroxyryanodine data are again included for comparison.

The ryanodol data are summarized, together with equivalent parameters for 21-amino-9 α -hydroxyryanodine (Tanna et al., 1998), in Table I.

DISCUSSION

Our earlier investigations of the interaction of 21-amino-9 α -hydroxyryanodine with individual cardiac muscle RyR channels demonstrated for the first time that both the association of a ryanoid with its receptor and dissociation of a ryanoid from its receptor could be sensitive to transmembrane voltage (Tanna et al., 1998). While the mechanism underlying the observed voltage dependence was not established, we proposed that it might result from either the translocation of the charged ryanoid into the voltage drop across the channel or a voltage-driven conformational change of RyR resulting in altered receptor affinity. We have tested these proposals in this report by monitoring the influence of voltage on the likelihood of interaction of ryanodol, a neutral ryanoid, with the RyR channel.

These experiments establish that transmembrane voltage influences the likelihood of a ryanoid being bound to the receptor on the RyR channel by a mechanism that is independent of the translocation of a charged moiety into the voltage drop across the channel.

A likely mechanism for the modulation of ryanoid in-

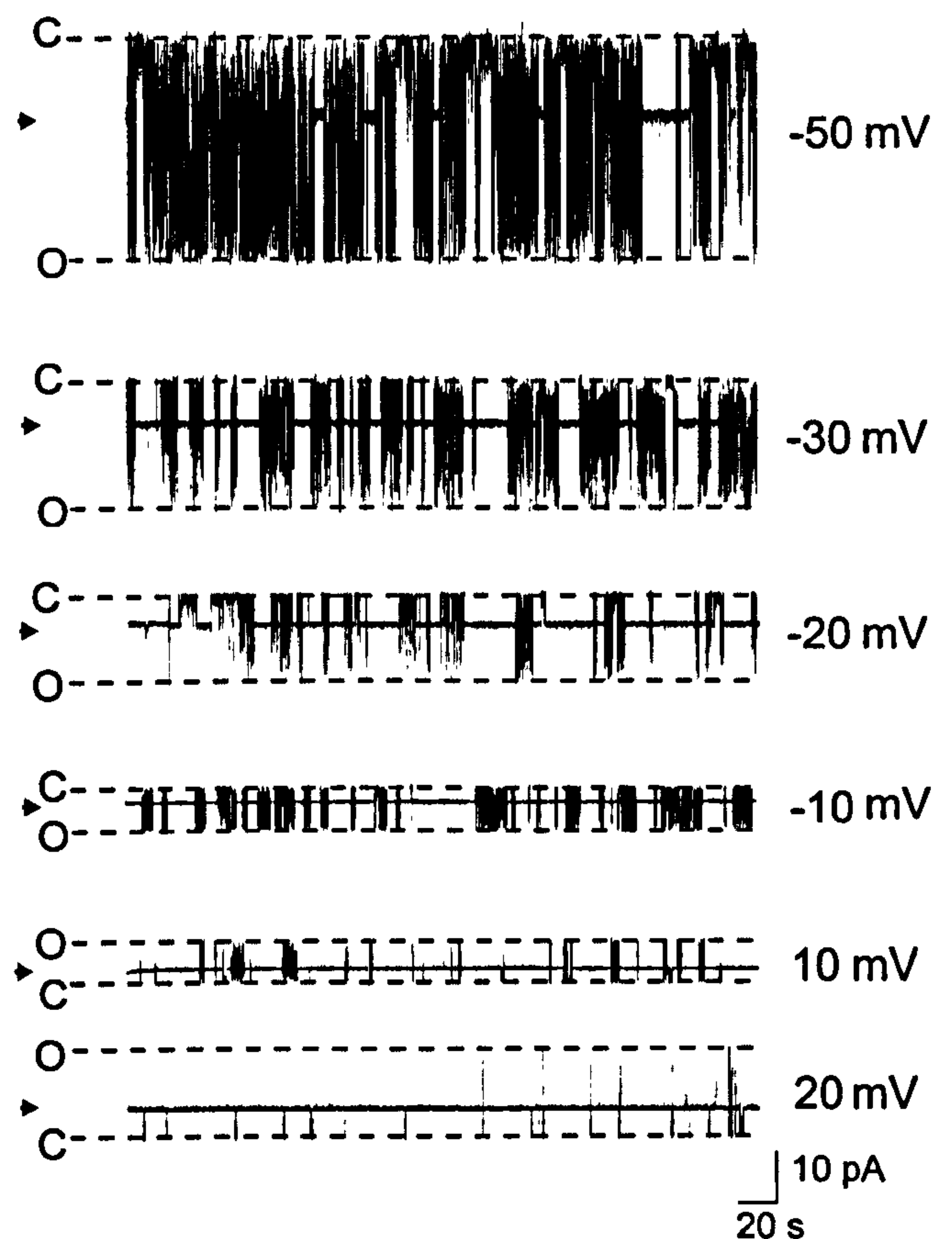


FIGURE 2. The influence of holding potential on the probability of modification of RyR channel function by ryanodol. Traces were obtained from a single RyR channel in symmetrical 610 mM K⁺ with 20 μ M ryanodol in the solution at the cytosolic face of the channel. O, open; C, closed; $\rightarrow$, modified.

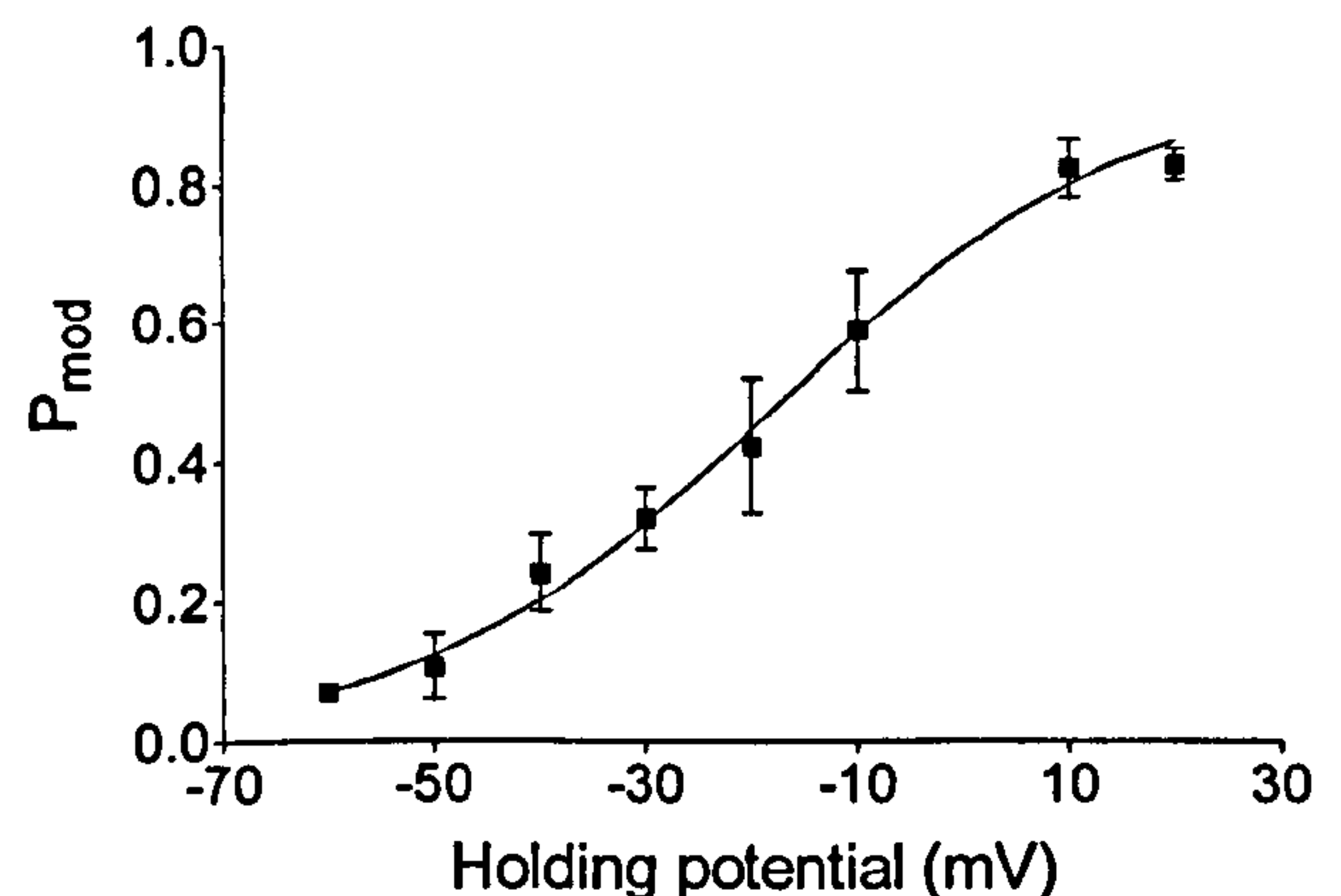


FIGURE 3. The relationship between P_{mod} by ryanodol and holding potential. P_{mod} was determined by monitoring dwell times in the unmodified and modified conductance states in 6-min recordings with 20 μ M ryanodol in the solution at the cytosolic face of the channel. Each point is the mean $\pm$ SEM of 4–10 experiments. The curve is the best fit Boltzmann distribution obtained by non-linear regression with the parameters quoted in the text.

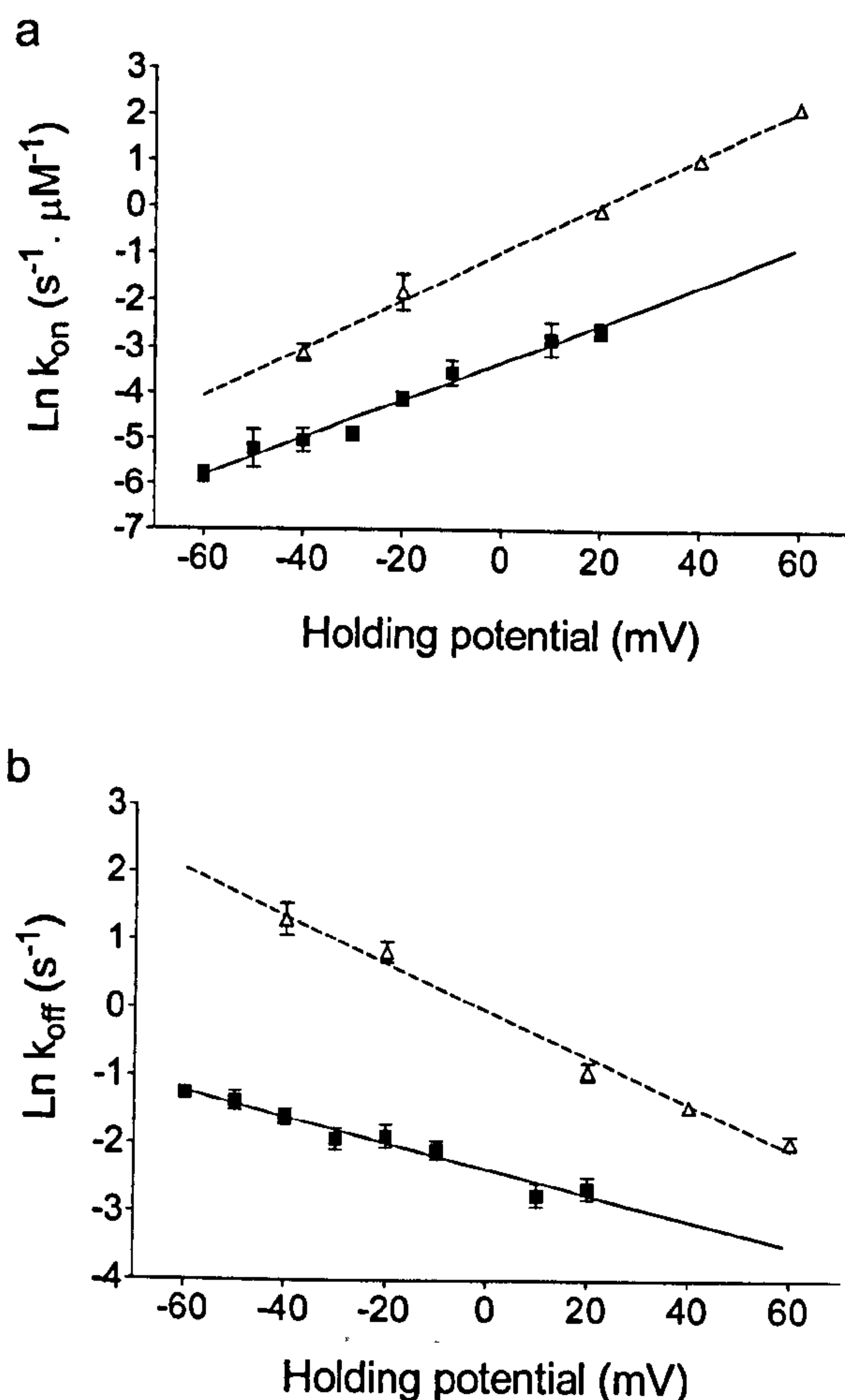


FIGURE 4. (a) The dependence of rates of association of ryanodol (■) and 21-amino-9 α -hydroxyryanodine (Δ) on holding potential. 20 μ M ryanodol was present in the solution at the cytosolic face of the channel. Each point is the mean $\pm$ SEM of 4–10 experiments. 21-amino-9 α -hydroxyryanodine data is from Tanna et al. (1998), with all values of k_{on} normalized for a P_o of 1.0. (b) The dependence of rates of dissociation of ryanodol (■) and 21-amino-9 α -hydroxyryanodine (Δ) on holding potential. 20 μ M ryanodol was present in the solution at the cytosolic face of the channel. Each point is the mean $\pm$ SEM of 4–10 experiments. 21-amino-9 α -hydroxyryanodine data is from Tanna et al. (1998). The solid lines in both a and b were obtained by linear regression with the parameters quoted in the text.

interaction with the receptor on RyR would then involve a voltage-dependent equilibrium between receptor states of different affinity (Tanna et al., 1998). In such a scheme (Fig. 6), the transmembrane potential would provide an energy source for the movement of a charged residue, or domain, of RyR within the electric field that would produce a conformational change and result in altered receptor affinity. The difference in voltage dependence of k_{on} and k_{off} for ryanodol indicates a degree of asymmetry in the energy profile for the transition between the different affinity states of the receptor; the electrical distance covered by the charged residue in

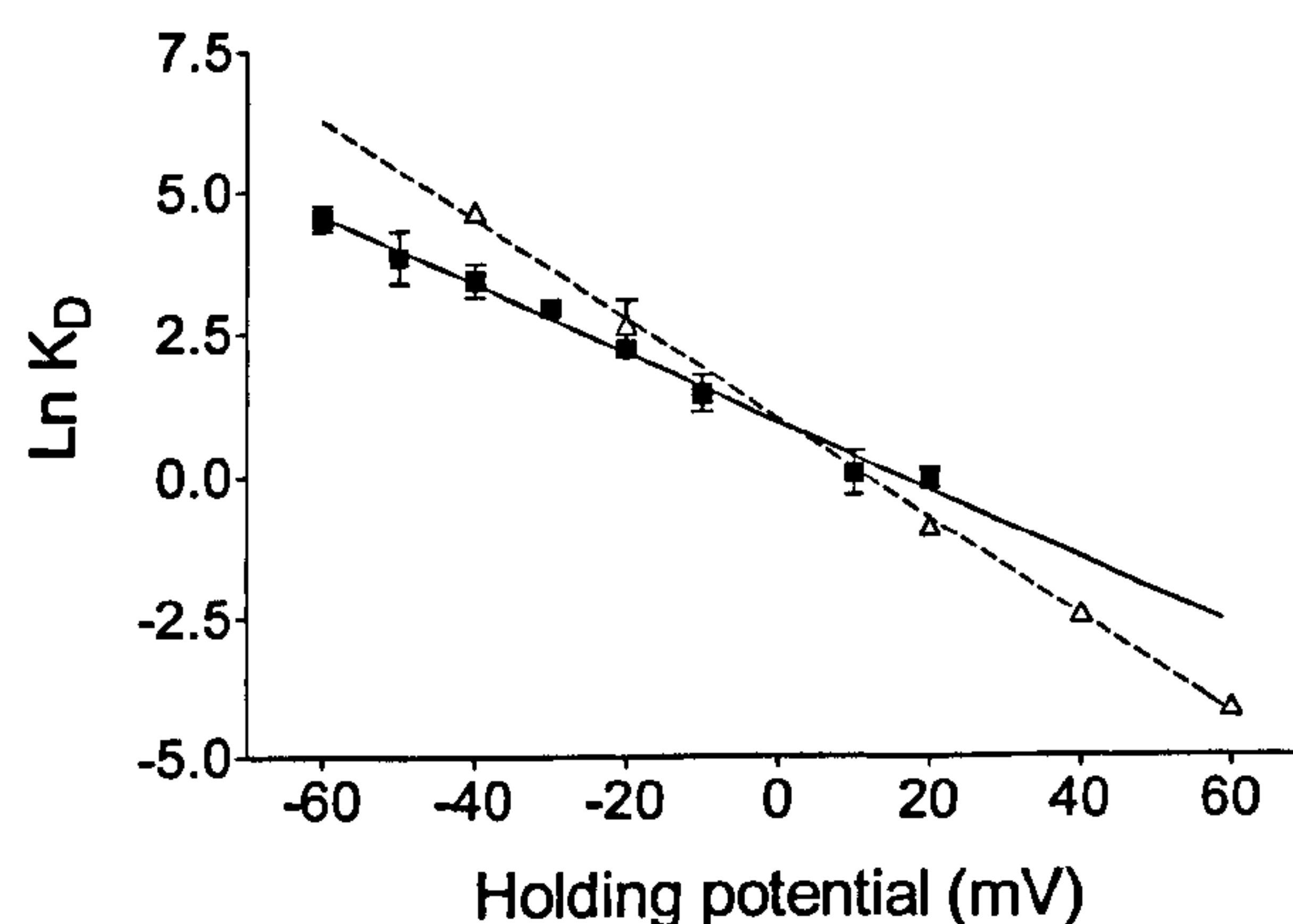


FIGURE 5. The relationship of the dissociation constant [$K_D = k_{off} (s^{-1})/k_{on} (\mu M^{-1} s^{-1})$] of ryanodol (■) and 21-amino-9 α -hydroxyryanodine (Δ) to the transmembrane holding potential.

the forward and reverse reactions may be different, or the amount of charge moved in the forward and reverse reactions may be different due to screening (Moczydlowski, 1986).

While the experiments reported here provide an unequivocal demonstration that modulation of ryanoid binding by voltage is not dependent on the ryanoid carrying a positive charge, they do suggest that the net charge of the ryanoid may produce small quantitative differences in the influence of transmembrane voltage. The total voltage dependence, monitored from variations in k_{on} and k_{off} , with voltage, of 21-amino-9 α -hydroxyryanodine is slightly greater than that of ryanodol, and this produces a significant difference in the influence of voltage on the resulting K_D s for the two ryanoids. How could the net charge of the ryanoid alter the influence of voltage on the interaction of the ligand with its receptor? The binding of 21-amino-9 α -hydroxyryanodine may induce a different conformer of the ligand–RyR complex diagrammed in Fig. 6 than that induced by ryanodol. The difference in fractional conductance of the ryanodol–RyR and 21-amino-9 α -hydroxyryanodine–RyR complexes is evidence of such a difference. The binding of the two ryanoids may induce different distributions of charge in the ryanoid–RyR complex, either directly by addition of another ionic charge or indirectly through alteration of one or more of the RyR acid dissociation constants by interaction with the electric charge of 21-amino-9 α -hydroxyryanodine. The small difference in voltage dependence of the neutral and cationic ryanoid indicates that movement of charge is involved in the voltage-induced transition; however, the small magnitude of the effect makes interpretation difficult. While not eliminating the possibility of allosteric interactions between the ryanodine binding site and the ion conduction pathway, a mechanism where the ryanoid binding site is placed within the voltage drop

TABLE I
The Influence of holding Potential on the Interaction of Ryanodol and 21-Amino-9 α -Hydroxyryanodine with Sheep RYR2

	Ryanodol	21-Amino-9 α -Hydroxyryanodine
Slope ($z_{\text{on}}F/RT$)	0.041 ± 0.003 ($r = 1.0$)	0.051 ± 0.002 ($r = 1.0$)
z_{on}	1.03	1.29
Slope ($-z_{\text{off}}F/RT$)	-0.019 ± 0.002 ($r = 1.0$)	-0.0344 ± 0.002 ($r = 1.0$)
z_{off}	0.48	0.87
z_{total}	1.51	2.16
k_{on} at 0 mV ($\mu\text{M}^{-1} \text{s}^{-1}$)	0.035	0.365
k_{off} at 0 mV (s^{-1})	0.095	0.990
K_d at 0 mV (μM)	2.81	2.79

Data for 21-amino-9 α -hydroxyryanodine are from Tanna et al. (1998) with values of k_{on} normalized for a P_o of 1.0. Values of k_{on} , k_{off} , and K_d at 0 mV are calculated from the regression lines fitted to data in Figs. 4 and 5.

across the channel is appealing because of its simplicity. Under these circumstances, the small increase in voltage dependence arises from the movement of a charged ryanoid into and/or out of the electric field. A location of the ryanoid binding site within the voltage drop of RyR would be consistent with the proposal that the receptor site is within the conduction pathway of the channel. The conduction pathway of the RyR channel is almost certainly formed from residues within the carboxyl terminal domains of the four RyR monomers that together make up the functional homotetramer (Bhat et al., 1997; Zhao et al., 1999) and investigations involving the proteolytic digestion of channels labeled with [^3H]ryanodine or a [^3H]photoactivated derivative of ryanodine have established that the high affinity ryanodine binding site on the skeletal muscle isoform of RyR

(RyR1) is located at the carboxyl terminus of the molecule (Callaway et al., 1994; Witcher et al., 1994). Very recent experiments indicate that mutations around the probable pore-forming region of the RyR channel produce reductions or abolition of the binding of [^3H]ryanodine and could be interpreted as indicating this region as a component of the ryanoid binding site (Zhao et al., 1999).

In addition, several features of the interaction of ryanoids with the RyR channel are consistent with the proposal that the high-affinity binding site for these ligands is located within the conduction pathway of the channel. The magnitude of equilibrium [^3H]ryanodine binding is altered by interventions that modify RyR channel P_o . Activating ligands such as Ca^{2+} , caffeine, and ATP increase binding while ligands that lower P_o , such as Mg^{2+} and ruthenium red, reduce binding (Chu et al., 1990; Holmberg and Williams, 1990; Hawkes et al., 1992; Meissner and El-Hashem, 1992). These observations have given rise to the proposal that the high affinity binding site for [^3H]ryanodine is only accessible when the channel is open and, as a consequence, equilibrium [^3H]ryanodine binding is used routinely as a method of assessing the P_o of RyR channels. A direct demonstration that the high-affinity binding of a ryanoid to the RyR channel requires the channel to be open was provided in the studies of Tanna et al. (1998), in which a linear dependence of the rate of association of 21-amino-9 α -hydroxyryanodine on RyR channel P_o was demonstrated.

The dissociation constants monitored in these single-channel experiments are in the same range as those determined for RyR in intact cardiac sarcoplasmic reticulum membrane vesicles in competition binding studies with [^3H]ryanodine [$2 \mu\text{M}$ 21-amino-9 α -hydroxyryanodine (Welch, W., unpublished results) and $1 \mu\text{M}$ ryanodol (Welch et al., 1997)]. The good agreement of the dissociation constants determined by two very different methods indicates that the parameters determined for individual RyRs in planar bilayers are relevant to the receptor in the native SR membrane. Using the values in

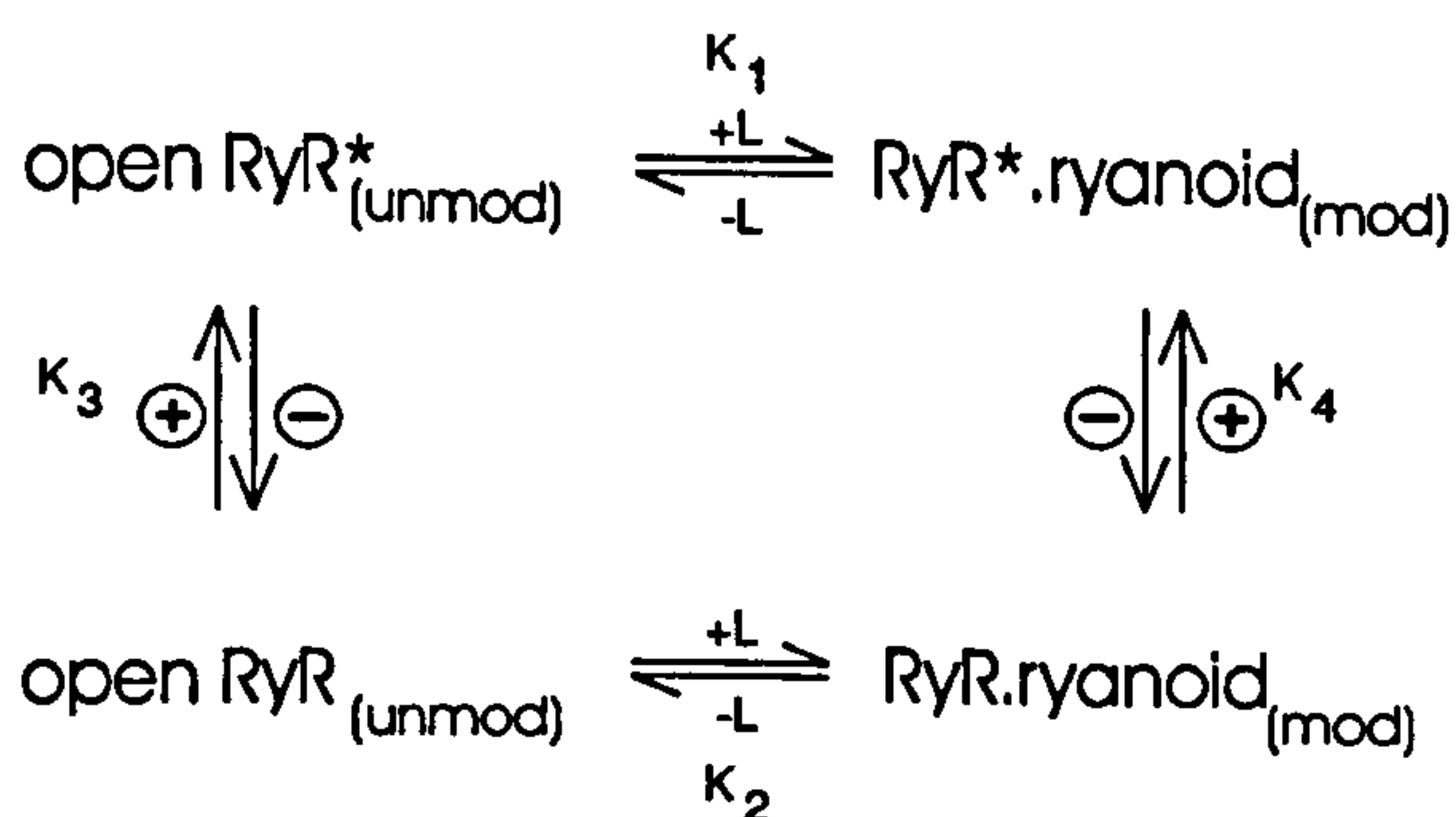


FIGURE 6. Scheme summarizing the influence of transmembrane voltage on the interaction of ryanoids with the high affinity binding site of RyR (based on information presented in this communication and Tanna et al., 1998). The ryanoid binding site is only available when the channel is open and only accessible from the cytosolic side of the channel. L represents the ligand (any ryanoid), and + and - indicate the application of positive and negative potential to the cytosolic side of the channel. A minimum of two forms of the vacant receptor exist in this model (low affinity, open RyR; high affinity, open RyR*). Increasing positive potential shifts the equilibrium toward open RyR* by increasing the value of the equilibrium constants K_3 and K_4 . If the ryanoid binding site is located within the voltage drop across the channel, then charged ryanoids such as 21-amino-9 α -hydroxyryanodine will experience an additional influence of voltage.

Table I, the difference in binding energy of the two ryanoids is only 4 kcal/mol. The small difference in binding energy demonstrates that the differences in channel function observed with 21-amino-9 α -hydroxyryanodine and ryanodol are more likely to result from the detailed differences in electrical and steric interactions between the receptor and ligand rather than from differences in the global ryanoid-RyR interaction energy. Note that compared with ryanodine ($K_d = 2$ nM; Welch et al., 1997), the high dissociation constant of 21-amino-9 α -hydroxyryanodine arises from a 4 kcal/mol unfavorable interaction between the ligand and receptor (either electrostatic repulsion, poor solvation, or both). Steric interactions can be discounted since placing the bulky BODIPY derivative at the 21 position caused only a small perturbation in binding (Welch et al., 1994). In contrast, the 4 kcal/mol reduction in binding energy of ryanodol compared with ryanodine is due to the loss of the pyrrole carbonyl. That is, while relative to ryanodine, both ryanodol and 21-amino-9 α -hydroxyryanodine have 4 kcal/mol less binding energy; this results in one case from the omission of an important interaction, while in the other case it is due to the addition of an unfavorable interaction.

Independent of voltage-induced changes, there are very marked differences in both the rates of association and dissociation for ryanodol and 21-amino-9 α -hydroxyryanodine with RyR. For example, at a holding potential of 0 mV, ryanodol is $\sim 10\times$ less likely to associate ($\Delta\Delta G = 1.4$ kcal/mol) with the ryanodine binding site on RyR than 21-amino-9 α -hydroxyryanodine; once bound, ryanodol leaves this site $\sim 10\times$ slower ($\Delta\Delta G = 1.4$ kcal/mol) than 21-amino-9 α -hydroxyryanodine. Therefore, the difference in energy barriers limiting association with and dissociation from the ryanoid binding site is considerably greater than the difference in total binding energy of the two ryanoids.

In summary, the experiments described in this report were designed to examine the mechanisms involved in the striking influence of transmembrane holding potential on the interaction of ryanoids with the high affinity binding site on the RyR channel. Our earlier observation of a strong influence of voltage on the interaction of a positively charged ryanoid with RyR could be explained either by the movement of the charged ligand into the voltage drop of the channel to reach its binding site and/or a voltage-driven conformational alteration in RyR leading to altered affinity of the receptor. Here we demonstrate that the apparent dissociation constant for the neutral ryanoid, ryanodol, decreases as the potential at the cytosolic face of the channel is made increasingly positive, and that this results from alterations to both the rates of association of ryanodol with its receptor and dissociation of ryanodol from its receptor. This observation provides very strong

evidence in support of a voltage-driven alteration in ryanoid receptor affinity as the major determining factor in the influence of transmembrane holding potential on the interactions of ryanoids with RyR (Fig. 6).

While this mechanism underlies the influence of voltage on the interaction of both neutral and positively charged ryanoids, our experiments indicate that a small additional voltage-dependent effect can be observed with the positively charged 21-amino-9 α -hydroxyryanodine. This may reflect the movement of the charged moiety from the cytosolic bulk solution to a binding site within the voltage drop across the channel, presumably in the conduction pathway of the channel.

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