Regulation of the Skeletal Muscle Ryanodine Receptor by Calsequestrin.

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P. 37, 1st paragraph, lines 9-10:	correction
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P. 47, 2nd paragraph, line 1:	correction
P. 50, 3rd paragraph, line 1:	correction
P. 58, 2nd paragraph, line 5:	correction
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P. 64, 3rd paragraph, lines2, 6:	correction
P. 68:	page replacement
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Statement

This thesis describes the results of research undertaken in the Muscle Research Group, Division of Molecular Bioscience, John Curtin School of Medical Research, Australian National University, Canberra. This research was supported by an Australian Postgraduate Research Scholarship.

The results and analyses presented in this thesis are my own original work, accomplished under the supervision of Professor Angela Dulhunty and Doctor Derek Laver (School of Biomedical Science, University of Newcastle, Newcastle), except where otherwise acknowledged.

Nicole Beard

Division of Molecular Bioscience

John Curtin School of Medical Research

Australian National University

Sunday, 5 January 2003

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Abstract

Contraction in skeletal and cardiac muscle is initiated by a cascade of events collectively known as excitation-contraction coupling, in which depolarisation of the sarcolemma triggers Ca^{2+} release through ryanodine receptor Ca^{2+} release channels in the sarcoplasmic reticulum. Releasable Ca^{2+} is loosely bound to, and stored on, a high capacity moderate affinity Ca^{2+} binding glycoprotein – calsequestrin – which is located within the sarcoplasmic reticulum lumen. Calsequestrin is tethered to the ryanodine receptor by two anchoring proteins, triadin and junctin, although calsequestrin has been shown to bind to and regulate ryanodine receptors in the absence of these anchors. Calsequestrin has recently been implicated to have a role in cardiac hypertrophy and resultant heart disease, although relatively little is known about the role of calsequestrin in ryanodine receptor function *in vivo*.

The regulation of the skeletal muscle ryanodine receptor by calsequestrin was studied using the lipid bilayer technique. This technique allows monitoring of channel modulation by measuring current passing through the membrane in response to an electrochemical gradient. Currents were recorded using Cs^{+} as the conducting ion. Once incorporated, sarcoplasmic reticulum vesicles orientate in such a way that the cytoplasm of the sarcoplasmic reticulum faces the *cis* chamber, whilst the sarcoplasmic reticulum lumen faces the *trans* chamber. As calsequestrin is found solely in the sarcoplasmic reticulum lumen, it may be isolated to the *trans* chamber.

This study provides novel evidence that calsequestrin inhibits native ryanodine receptor calcium release channel activity. Calsequestrin dissociation from junctional face membrane was achieved by increasing luminal (*trans*) ionic strength from 250 to 500 mM with CsCl, or by exposing the luminal side of ryanodine receptors to high (13 mM) $[Ca^{2+}]$, and dissociation was confirmed with SDS-Page and Western blotting. Calsequestrin dissociation caused a 10-fold increase in the duration of ryanodine receptor channel opening in lipid bilayers. Adding calsequestrin back to the luminal side of the channel after dissociation reversed this activity increase. In addition, a polyclonal anti-calsequestrin antibody added to the luminal solution reduced ryanodine receptor activity before, but not after, calsequestrin dissociation.

A population of ryanodine receptors (~35%) may have initially lacked calsequestrin, since their activity was high and was unaffected by increasing ionic strength, altering luminal [Ca^{2+}], or by anti-calsequestrin antibody: their activity fell when purified calsequestrin was added and they then responded to antibody.

The effects of phosphorylation status on calsequestrin regulation in native ryanodine receptors was shown to affect channel regulation quite differently in native ryanodine receptors, compared to the effects observed in purified ryanodine receptors. Szegedi *et al. (*1999) and Herzog *et al.* (2000) have shown that only dephosphorylated calsequestrin activates the ryanodine receptor, and upon phosphorylation, calsequestrin is unable to alter ryanodine receptor activity. In native calsequestrin-depleted ryanodine receptors, both phosphorylated and dephosphorylated calsequestrin induced significant channel inhibition. Therefore, unlike calsequestrin's effect on purified ryanodine receptors, channel regulation of native ryanodine receptors (with triadin and junctin intact) by calsequestrin is not phosphorylation-dependant.

In contrast to native ryanodine receptors, purified channels (deplete of triadin, junctin and calsequestrin), were not inhibited by calsequestrin. In fact, calsequestrin had a tendency to activate purified ryanodine receptors. This shows that calsequestrin regulates the ryanodine receptor by two distinct mechanisms. Firstly, calsequestrin can bind directly to the ryanodine receptor and activate the channel in a phosphorylation-dependant manner (as reported by [Szegedi *et al,* 1999), Secondly, calsequestrin can inhibit native ryanodine receptor activity, presumably by associating with triadin and junctin and influencing ryanodine receptor activity through this interaction. From these results, it is apparent that calsequestrin associates with the ryanodine receptor via two binding mechanisms, firstly through anchoring proteins triadin and junctin and secondly, by direct association with the luminal domain of the ryanodine receptor. The regulation imposed on ryanodine receptors by calsequestrin is strikingly different, dependent on the presence or absence of triadin or junctin, or the phosphorylation status of calsequestrin.

This is the only detailed study considering the role of calsequestrin on luminal Ca^{2+} regulation of ryanodine receptors. Luminal Ca^{2+} was originally thought to modulate ryanodine receptors solely by binding to Ca^{2+} activation and inhibition sites on the channel. For the first time, luminal [Ca^{2+}]-induced ryanodine receptor activation is

shown to be a result of two independent mechanisms. Raising Ca^{2+} to 5 mM and lowering Ca^{2+} to 100 nM is shown to induce activity due to an initial fast phase, as a result of Ca^{2+} activation, and a slower, secondary phase, due to calsequestrin dissociation. As well, results suggest that the presence of calsequestrin alters the ryanodine receptors response to luminal Ca^{2+}. In the presence of calsequestrin, ryanodine receptors had a tendency to be more active at 1.5 and 2.0 mM *trans* Ca^{2+}, compared with calsequestrin-deplete ryanodine receptors. Calsequestrin may also be involved in the communication of small (and probably physiological) Ca^{2+} fluxes though the channel into the cytoplasm.

These data suggest that calsequestrin reduces ryanodine receptor activity by binding to a co-protein, possibly to the luminal domain of triadin and it is possible that triadin and/or junctin forms part of the signalling pathway by which calsequestrin communicates the luminal Ca^{2+} to the ryanodine receptor, an essential step in sarcoplasmic reticulum Ca^{2+} release and muscle contraction.

Publications

During the course of this study, the following papers and abstracts were published.

Refereed publications:

Beard, N.A.., Dulhunty. and A.F Laver, D.R. (2002). Calsequestrin is an inhibitor of skeletal muscle ryanodine receptor calcium release channel.' *Biophys. J.* 82. 310-320.

Abstracts:

Beard, N.A., Dulhunty, A.F. and Laver, A.F.(2000). The effect of increasing luminal calcium on skeletal muscle calsequestrin. *Proc. Aust. Physiol. Pharm. Soc.* 31(2). 22p.

Sakowska, M.M., Beard, N.A., Fryer, M.W., Dulhunty, A.F. and Laver, D.R. (2001). Regulation of skeletal muscle calcium release channels by inorganic phosphate and calsequestrin. *Proc. Aust. Physiol. Pharm. Soc.* 31(2). 101p.

Beard, N.A., Laver, D.R., and Dulhunty, A.F.(2001). Regulation of skeletal muscle ryanodine receptors by calsequestrin. *Proc. Aust. Physiol. Pharm. Soc.* 30(2). 43p.

Commonly used abbreviations

ATP	adenosine 5' triphosphate
ATR	atractolyside
BAPTA	1,2-bis(2-aminophenoxy)ethane-N, N, N', N'-tetra-acetic acid
BSA	bovine serum albumin
CaM	calmodulin
CHAPS	3-[3-Cholamidopropyl)dimethylammino]-1-propane-sulfonate DTT dithiothreiotol
CK	casein kinase
CSQ	calsequestrin
deP CSQ	dephosphorylated CSQ
ECC	excitation contraction coupling
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether) N, N, N', N'-tetraacetic acid
F_o	open frequency
HRC	histidine rich calcium binding protien
I'	mean current
I'_F	fractional mean current
I'_f/I'_c	relative fractional mean current
JFM	junctional face membrane
Kda	kilodalton
MES	2-[N-Morpholino]ethanesulfolic acid
MOPS	3-[N-Morpholino]propanesulfolic acid
MQ	milli Q
MS	methane sulfonate
NMR	nuclear magnetic resonance
OD	optical density
PC	phosphatatidyl choline
P-CSQ	phosphorylated CSQ
PE	phosphatidylethanolamine
PIPES	Piperazine-N,N'-bis[2-ethanesulfonic acid];1,4-Piperazinediethanesulfonic acid
PMSF	phenylmethylsulfonyl fluoride
P_o	open probability

PS	phosphatidylserine
PVDF	polyvinylidene difluoride
RyR	ryanodine receptor
SDS	sodium dodecyl sulfate
SDS-Page	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SML	sarcolumenin
SR	sarcoplasmic reticulum
T_c	closed time
Temed	N,N,N',N'-Tetramethylethylenediamine
TES	N-tris[Hydroxymethyl]methyl-2-aminoehtanesulfonic acid; 2-([2-Hydroxy-1,1-bis(hydroxymethyl)-ethyl}amino)ethanesulfonic acid
TBS	tris-buffered saline
T_o	open time
TRIS	Tris-[hydroxymethyl]aminomethane
TTBS	TBS with tween
V	voltage

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Chapter 3. Dissociation of CSQ by high ionic strength

Chapter 1

GENERAL INTRODUCTION

1.1 Muscle and excitation-contraction coupling

Both voluntary movement and respiration rely on the contraction of skeletal muscle. Structurally, three distinct membranes found within the muscle cell are involved in the regulation of muscle contraction (Fig. 1.1). (1) The sarcolemma, a specialized plasmalemma membrane, separates the extracellular space from the intracellular myoplasm. (2) The transverse tubules (t-tubules) are invaginations of the sarcolemma, which embed deep within the muscle cell. They allow the internal transmission of the membrane depolarisation induced by an action potential, that is essential in skeletal muscle contraction. Embedded within this membrane, are the dihydropyradine receptors (DHPRs), an L-type Ca^{2+} channel. (3) The sarcoplasmic reticulum (SR), a modified endoplasmic reticulum and large intracellular Ca^{2+} store, which forms an internal membrane system, and consists of a network of tubules aligned longitudinally. The part of the SR membrane facing the t-tubules is known as the terminal cisternae, in which the ryanodine receptor (RyR) – a ligand gated ion channel – is embedded. Ca^{2+} is stored in the lumen of the SR. These three membrane systems and the proteins contained within them are paramount for muscle contraction.

The process of muscle contraction begins with an action potential within a motor neuron, if the summation of both excitatory and inhibitory synapses induces a threshold depolarisation with the soma. The action potential leads to release of acetylcholine into the neuromuscular junction, and acetylcholine binding to acetylcholine receptor cation channels generates an endplate potential. This in turn, elicits an action potential in all muscle cells within the motor unit. A motor unit is defined as an α-motor neuron, its axon and the muscle fibres that it innervates.

This muscle action potential signals the start of a process known as excitation contraction coupling (ECC; Fig 1.1a). In mammalian skeletal muscle, depolarisation spreads along the sarcolemma and deep into the muscle fibre (via the t-tubule invaginations). The DHPR in the t-tubule acts as the voltage sensor for contraction, and in turn induces RyR opening in the SR, across the junctional gap (see Fig 1.1a). In skeletal muscle, this signal is transmitted via a physical coupling between these two

receptors, whereas in cardiac muscle the signal is transmitted via an inward Ca^{2+} current. The activation of RyRs allows Ca^{2+} stored within the SR to diffuse down its electrochemical gradient, into the myoplasm. This Ca^{2+} is then available for interaction with specific sites upon troponin C, enabling actin and myosin filaments to slide past each other (as a result of cross-bridge cycling), and contraction of muscle fibres.

Ca^{2+} available for release through the RyR is stored intraluminally in the SR on a Ca^{2+} binding protein, calsequestrin (CSQ). CSQ is tethered to the junctional face membrane (JFM; the portion of the SR membrane facing the junction of the triad and the SR) by two membrane spanning proteins, triadin and junctin (Fig 1.1b), allowing localization of Ca^{2+} close to the RyR. CSQ, the RyR, triadin and junctin form what is referred to here as the RyR/T/J/CSQ complex.

1.2 Ryanodine receptor

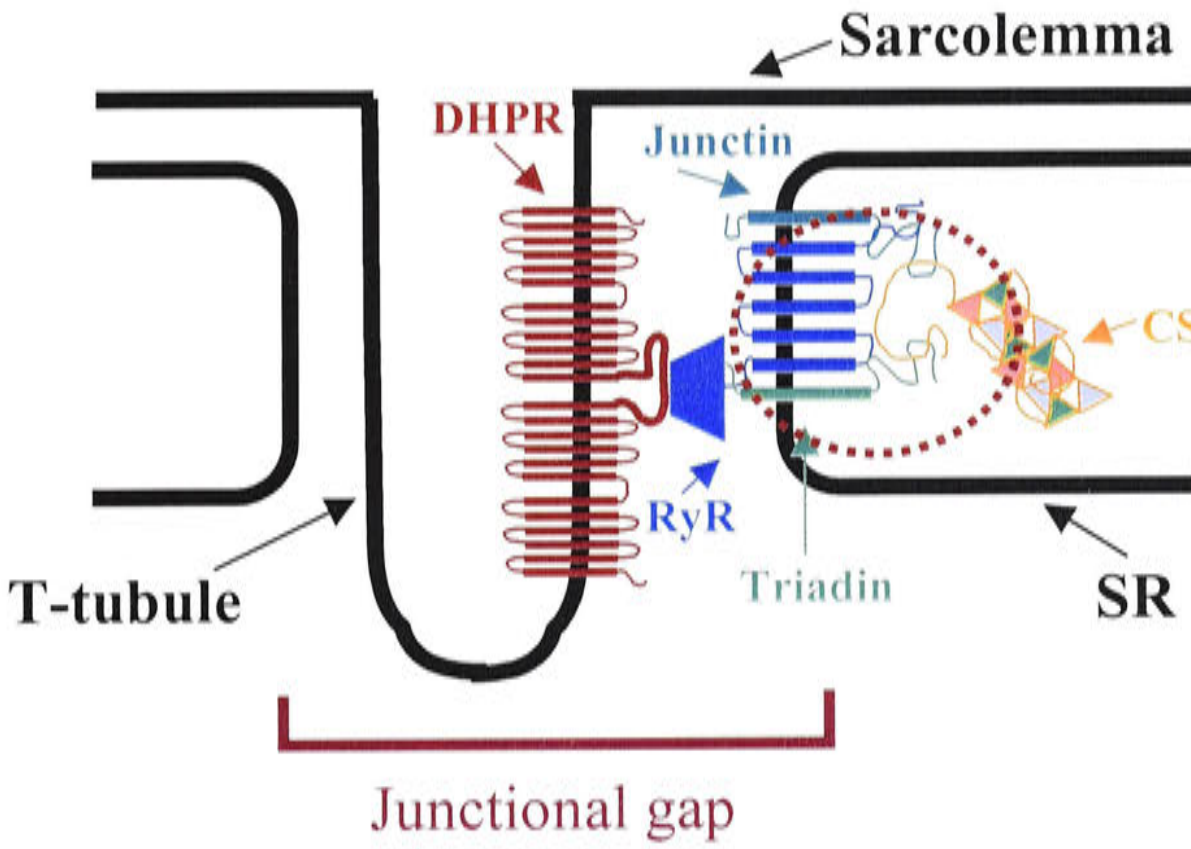
RyRs are ligand-gated Ca^{2+} release channels, so named because they bind the neutral plant alkaloid ryanodine, localized in the root and stem of *Ryania speciosa*. The planar – or black – lipid bilayer technique for observing current flow through ion channels helped identify RyRs in skeletal muscle. Smith *et al.* (1985) were the first to report the presence of a large conductance ion channel, regulated by both adenine nucleotides and ruthenium red (refer to Sections 1.2.6.4 and 1.2.6.6), and thought to be the Ca^{2+} release channel within the SR of skeletal muscle.

1.2.1 Gene/isoforms

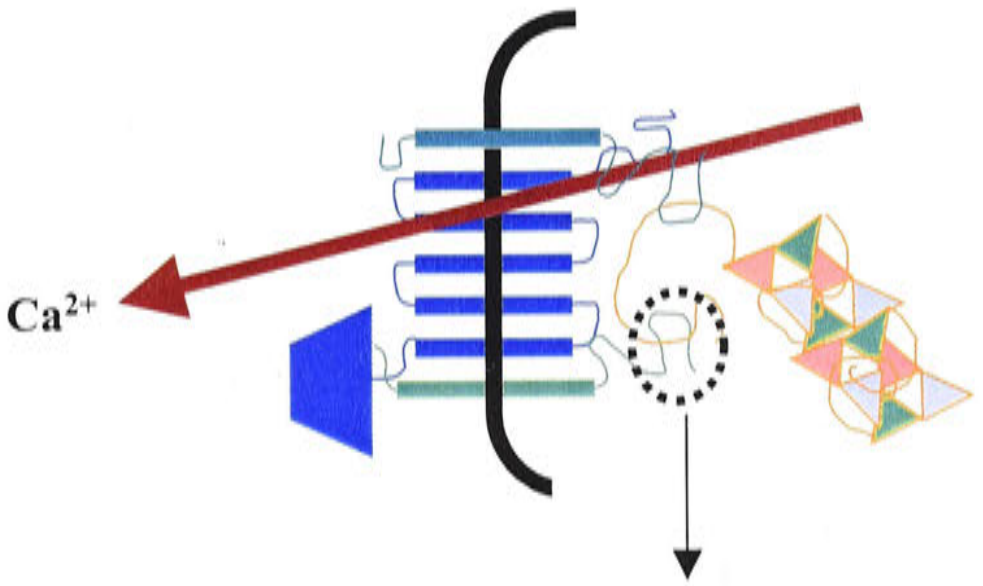
RyRs have been found in species as diverse as mammals, amphibians, avian and insects, but it is the mammalian isoforms that have been extensively studied. Three mammalian genes for the RyR have been sequenced, RyR1, RyR2 and RyR3 (Takeshima *et al.*, 1989; McPherson and Campbell, 1993; Sutko and Airey, 1996). In adult skeletal muscle, RyR1 is the predominant isoform. RyR1 encodes for between 5032-5037 amino acids, dependent on species. The presence of RyR1 is essential for skeletal muscle ECC; in transgenic (dyspedic) mice where the RyR gene is not expressed, ECC is totally lost and animals die at or before birth (Takekura *et al.*, 1994). There are many

Fig. 1.I. (**A**). Schematic representation of the interactions of essential proteins involved in excitation contraction coupling. The SR junction is is circled in red (**B**). The RyR/triadin/junctin/CSQ protein complex , circled in **A** - showing the proposed binding between CSQ, the RyR, triadin and junctin. The interactions of these proteins are in part, responsible for modulating RyR Ca^{2+} release. Residues involved in the interaction between CSQ and Triadin (circled in black) are listed below the diagram. Adapted from Wang et al, 1998 and Sakowska, 2000.

A



B



Triadin ^{210}Arg-Thr-Lys-Glx-Lys-Iso-Glx-Glx-Lys-Thr-Lys-Lys-Glx-Val-Lys^{224}

CSQ ^{354}Glu–Asp–Asp–Asp–Asp–Glu-Asp-Asp-Asp-Asp- Asp-Asp-Asp-Asp^{367}

conserved regions throughout all three isoforms, with an overall homology of 66 – 70%. The inositol 1,4,5-triphosphate (IP_3) receptor is a receptor for IP_3, a second messenger found in muscle sarcolemmal membranes (and other cell types). The IP_3 receptor is the only protein known to be homologous with the RyR, the carboxyl terminal (C-terminal) region being a prime example (Furuichi *et al.*, 1989; Berridge, 1993).

RyR2 is prevalent in cardiac muscle. RyR3, the so-called 'brain isoform', is found only sparingly in brain and is in fact more extensively expressed in smooth muscle and non-muscle tissue (Ogawa *et al.*, 2000). Interestingly in neonates, up to 30% of RyRs found in skeletal muscle are RyR3, these levels fall to below 1% after birth (Bertocchini *et al.*, 1997). The presence of RyR3 is seemingly non-essential for adult skeletal muscle ECC, and is unable to restore ECC in dyspedic mice which lack RyR1 (Flucher *et al.*, 1999). RyR3 is thought to play a role in normal morphogenesis of skeletal muscle (Ikemoto *et al.*, 1997).

Non-mammalian RyRs have been sequenced; α-RyR and β-RyR have been identified in avian, amphibian and fish muscle (Rubtsov and Batrukova, 1997; and references therein), with birds also expressing a mammalian cardiac isoform. The α and β RyRs differ both biochemically and immunologically from their mammalian counterparts. Takeshima *et al.* (1994) first isolated and characterized a RyR gene in *Drosophila melanogaster*, which has a 45% amino acid homology with the skeletal-type RyR.

1.2.2 Alternative splicing

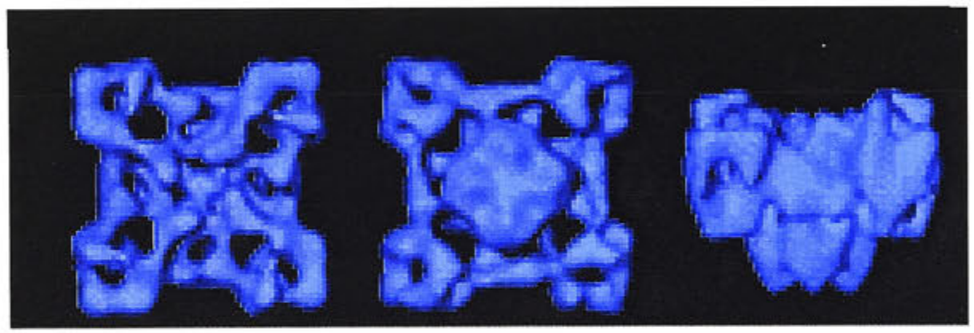
Futatsugi *et al.* (1995) first described an alternate splicing of the skeletal muscle RyR mRNA. Two alternate spliced regions, encompassing several modulatory sequences were localized, characterized by the presence or absence of 15 bp and 18 bp exons, know as ASI and ASII respectively. The absence of ASI resulted in the exclusion of Ala ^{3481} – Gln ^{3485}, whilst the lack of ASII resulted in the omission of Val ^{3865} – Asn ^{3870} (Futatsugi *et al.*, 1995). Both these regions are located within the modulatory region of the RyR and are situated downstream of the proposed Ca^{2+} and ATP binding sites, and upstream of the phosphorylated residue, Ser ^{2843} (Zorzato *et al.*, 1990; Chen *et al.*, 1992; Suko *et al.*, 1993). Distribution and abundance of each splice variant in skeletal muscle is dependant on the stage of muscle development; the expression of the variant lacking ASII increases between embryonic day 18 and birth. This is followed by a gradual

increase in the isoforms containing both ASI and ASII, which are thought to be essential for muscle development (Futatsugi *et al.*, 1995). Whilst the appearance of splice variants may account for observed RyR channel variability, no functional significance has been determined (Sutko and Airey, 1996).

1.2.3 Structural

In skeletal muscle, RyRs form hetrotetramers, each subunit having a molecular weight of ~ 560 kDa (Takeshima *et al.*, 1989; Zorzato *et al.*, 1990). Hydropathy profiles suggest that the majority of the protein (~80%) exists in the cytoplasm (which forms 'foot-like' projections extending from the SR junctional membrane to the triadic-SR junction, close to where the DHPR is located). The remaining 20% forms a compact membrane-spanning structure (Radermacher *et al.*, 1994; Samso and Wagenknecht, 1998). The number of transmembrane helices and residues in the transmembrane domain remains controversial. Reports suggest the existence of four, ten or twelve putative membrane-spanning regions (Takeshima *et al.*, 1989; Zorzato *et al.*, 1990; Radermacher *et al.*, 1994). Reconstructions from cryoelectronmicroscopy (resolution of 25 - 30 A^{o}) gives a picture of the overall profile of the protein, but does not allow identification of residues. The cytoplasmic domain is relatively unstructured, with up to 50% of the volume being unoccupied by protein material (Radermacher *et al.*, 1994). Four electron dense columns appear to provide the scaffolding for this domain, leaving a central channel of 2-3 nm in diameter (See Fig 1.2). Radermacher *et al.* (1994) show ten cytoplasmic domains in each of the four RyR subunits, which form a prism-like structure. Domains 5-6, 7-8 and 9-10 are referred to as clamp structures, thought to form the prism corners. Domain 3 forms one side of the prism, whilst domain 1 provides a connection to the transmembrane segment.

Cardiac and skeletal muscle RyRs are strikingly similar in sequence, although three regions of divergence exist - namely D1, D2 and D3. The largest area of low sequence homology, D1, is found between residues Pro^{4254} - Phe^{4631} in the skeletal RyR, and encompasses residues Ser^{4210} - Val^{4562} in the cardiac isoform (Sorrentino and Volpe, 1993). This region includes the predicted intraluminal loop between transmembrane domain three (M3) and transmembrane domain four (M4) and is thought to include part of the pore forming region (based on the RyR transmembrane segment model proposed by Zorzato *et al.* (1990). Region D2, from amino acids Try^{1342} - Pro^{1403} in skeletal



T-tubule view **SR View** **side view**

Fig. 1.II. ***3D reconstruction of skeletal muscle ryanodine receptor (RyR) from cryoelectronmicroscopy.*** The four-fold symmetry and tetrametric structure are clearly seen in both the t-tubule view and the SR view, whilst both cytoplasmic and transmembrane domains are visible from the SR and side views (taken from Samso *et al.*, 1999).

(cardiac: Asp^{1353} - Tyr^{1397}) and D3, from residues Thr^{1872} - Glu^{1923} (skeletal) which corresponds to amino acids Lys^{1852} - Lys^{1890} in the cardiac isoform, may contain different regulatory domains, responsible for the different properties of cardiac and skeletal RyRs (Sorrentino and Volpe, 1993). In particular, skeletal D3 is much more acidic than its cardiac equivalent and is thought to form the low affinity divalent binding site (Zorzato *et al.*, 1990; see Section 1.2.6.1), responsible for inhibition by Ca^{2+} and Mg^{2+}.

1.2.4 Pore-forming segment

In K^+ channels of *Streptomyces lividans* (one of only two cation channels whose crystal structure has been determined with high resolution (2.3A°)), a region between two membrane-spanning domains, extends into the membrane and forms part of the ion conduction pathway (Doyle *et al.*, 1998). A potential pore forming segment of the RyR (located in the M3/M4 loop) is thought to be analogous to that in voltage-dependent K^+ channels. A Gly-Gly-Iso-Gly motif in RyRs is similar to a motif, found in the K^+ channel (Val-Gly- Try-Gly), which forms part of the selectivity filter (Gao *et al.*, 2000 and references therein).

Sequence analysis of IP_3 receptors suggests that a similar region (Gly-X-Arg-X-Gly-Gly-Gly-X-Gly-Asp), is the pore-forming segment of the IP_3 receptor (Michikawa *et al.*, 1994). A corresponding region is found in rabbit skeletal RyRs, between ^{4890}Gly - Glu ^{4900}. Mutations of amino acids located in the highly conserved Gly^{4822} - Glu ^{4831} region in rabbit cardiac RyRs resulted a loss of channel conductivity and ion selectivity (Du *et al.*, 2001). Site directed mutagenesis of the skeletal muscle RyR of an isoleucine to a threonine (I4898T) resulted in undetectable Ca^{2+} release induced by caffeine and in ^{3}HRy binding (an assay which indicates the open state of a RyR channel; Lynch *et al.*, 1999). In fact, this occurs in human central core disease, which results in hypotonia and muscle weakness (Lynch *et al.*, 1999). Mutations G4894A and D4899N resulted in a reduction in both K^+ and Ca^{2+} conductance (Gao *et al.*, 2000). In mouse cardiac muscle, a point mutation (G4824A) within this stretch of conserved residues (Gly^{4822} - Glu ^{4831}) resulted in a significant decrease in caffeine-induced Ca^{2+} release, and a greatly reduced ^{3}HRy binding (Zhao *et al.*, 1999). These data led to the hypothesis that the residues found within the luminal

loop between M3/M4 contribute to the RyR pore, particularly because single mutations in the region had such an extreme effect on conductance (Gao *et al.*, 2000).

1.2.5 Ion conductance

RyRs are cation selective ion channels, exhibiting an unusually high conductance for monovalent and divalent ions. For example, in 250 mM symmetric K^+, conductance is 770 pS, whilst in symmetric 250 mM Na^+, conductance is 580 pS. Unitary Ca^{2+} conductance (50 mM *trans* Ca^{2+}) is 120 ± 30 pS and Cs^+ conductance (250 mM symmetrical Cs^+) is ~ 525pS; Smith *et al.*, 1986; Smith *et al.*, 1988b; Tinker and Williams, 1992). The ability of the RyR to conduct both choline and TRIS (26 and 22 pS respectively) illustrates that the ion conduction pathway has a selectivity filter which has a known radius of 3.5Å and a length of 10.4 Å (Smith *et al.*, 1988b; Tinker and Williams, 1993; Tinker and Williams, 1995)

With Ca^{2+} as the current carrier, four conductance states have been shown, occurring at equal intervals of ¼ of the maximal conductance state. These substates are also promoted by FKBP12 removal (Ahern *et al.*, 1997a) or pH inhibition, (the later occurring when RyRs are ryanodine modified; Ma and Zhao, 1994). Whether these are due to some form of interaction between each of the four monomers within the RyR, or whether each subunit contains its own ion selective pore is unknown (Meissner, 1994).

1.2.6 RyR regulation

The RyR is regulated by a wide variety of agents, both endogenous and exogenous. The majority of modulation is on the cytoplasmic side, with adenine nucleotides, caffeine, histidine-containing dipeptides, ions (such as cytoplasmic Ca^{2+} and Mg^{2+}), ionic strength changes, lipids and polycationic metabolites, local anaesthetics, oxidizing agents, polyamines, pH, ruthenium red, ryanodine and sphingosine all able to modulate RyR activity (Meissner, 1994; Rubtsov and Batrukova, 1997 and reference therein). On the luminal side, annexin VI (Diaz-Munoz *et al.*, 1990), Ca^{2+} (Sitsapesan and Williams, 1995; Tripathy and Meissner, 1996), ruthenium red (Ma, 1993), sulmazol, themerazol and other oxidizing agents (Eager, 1997; Haarmann *et al.*, 1999) have been reported to affect RyR activity. In addition, on the cytoplasmic side proteins such a calmodulin (CaM; Fuentes *et al.*, 1994), CSQ (see Chapter 3-5 and Kawasaki and Kasai, 1994;

Ohkura *et al.*, 1994; Ohkura *et al.*, 1995; Ohkura *et al.*, 1998; Szegedi *et al.*, 1999; Beard *et al.*, 2002; Herzog *et al.*, 2000; Wang *et al.*, 2001 and Beard *et al.*, 2002), DHPR, triadin (Ohkura *et al.*, 1998), the histidine-rich Ca^{2+} binding protein (HRC) and sarcalumenin (SLM; Hadad *et al.*, 1999), have been shown to interact with, and alter the channel activity of, the RyR. Only those agents and proteins with some significance to this thesis will be considered in greater detail below.

1.2.6.1 Cytoplasmic Ca^{2+}

Cytoplasmic Ca^{2+} is an important modulator of the RyR. SR vesicle Ca^{2+} efflux shows biphasic regulation by Ca^{2+}. At low and high [Ca^{2+}] (nM and mM), RyRs are inhibited, whilst at 10 – 100 µM Ca^{2+}, channel activation occurs (Kirino *et al.*, 1983; Nagasaki and Kasai, 1983; Smith *et al.*, 1986; Meissner, 1986b; Ma *et al.*, 1988; Moutin and Dupont, 1988; Smith *et al.*, 1988a; Fill *et al.*, 1990; Knudson *et al.*, 1993b; Coronado *et al.*, 1994; Laver *et al.*, 1997). Cardiac RyRs are less sensitive to Ca^{2+}-induced inhibition, with channel inhibition occurring at < 10 nM and > 1 mM, compared with 10 - 100 uM in skeletal RyRs (Rousseau and Meissner, 1989; Laver *et al.*, 1997). *In vivo*, skeletal RyR activation by cytoplasmic Ca^{2+} is thought by some authors to be responsible for triggering SR Ca^{2+} release from RyRs that are not coupled to DHPRs. Every second RyR facing the triadic-SR junction does not form an association with DHPRs; these RyRs are thought to be activated by Ca^{2+}-induced Ca^{2+} release, similar to the signal transduction pathway observed between these two receptors in cardiac muscle. However, this is controversial, since Ca^{2+} activation should not occur in the presence of Mg^{2+} (Lamb, 2000).

Chen *et al.* (1992) first showed in a polyclonal antibody study of Ca^{2+} release from SR vesicles, that the region from Gly^{4478} - Pro^{4512} (predicted to be a modulatory region of RyR1 by Takeshima *et al.*, 1989) was involved in Ca^{2+}-induced Ca^{2+} release in skeletal muscle. Antibody techniques were employed again in single channel experiments, to narrow down the region involved in Ca^{2+} activation (Chen *et al.*, 1993). A series of Pro-Glu repeats, found within the sequence Pro^{4489} - Glu^{4499} are thought to contribute to Ca^{2+}-activation; a polyclonal antibody to this sequence was able to inhibit Ca^{2+}-activation of RyRs, without altering modulation by ATP, Mg^{2+}, ruthenium red or ryanodine. These data suggests that this Pro/Glu rich region has an important role in Ca^{2+}-dependant activation. More recently, a single substitution of E3885A (located in

transmembrane domain two (M2) of RyR3) produced a decrease in Ca^{2+} sensitivity without changing modulation of the channel by either ATP or ryanodine (Chen *et al.*, 1998). Thought to be a high affinity Ca^{2+} binding site and termed the Ca^{2+} sensor, it is believed that Glu^{3885} of each monomer (located in close proximity) would form a coordinated region responsible for Ca^{2+} binding and activation of the RyR.

The role of the D3 region (Thr^{1872} – Glu^{1923}) of the RyR is controversial. D3 was first proposed to be the stretch of residues responsible for low affinity Ca^{2+} binding (Ca^{2+}-dependant inactivation; Zorzato *et al.*, 1990). An ΔD3 – RyR mutant, lacking the D3 region, showed an increased sensitivity to Ca^{2+} activation and a decreased sensitivity to Ca^{2+} inhibition (Bhat *et al.*, 1997). These mutant RyRs did not show alterations in ^{3}HRy binding and have similar pharmacology and single channel behaviour to their wild type counterparts, with greater resistance to inhibition by Mg^{2+} (at activating Ca^{2+}). However, studies using a RyR1/RyR2 chimera, (skeletal RyR1 with the cardiac D3 region), failed to show any change in Ca^{2+}-inactivation of ^{3}HRy binding compared with wild type RyRs (Du and MacLennan, 1999). Ca^{2+}-dependent inactivation was reduced in chimeras where the skeletal C-terminus was exchanged for the corresponding cardiac region. With these studies in mind, Du and MacLennan (1999) suggest that Ca^{2+}-dependent inactivation is multifaceted, with inactivation site(s) located within residues Ala^{3726} - Ser^{5037} (encompassing both D3 and C-terminal regions) and decreased sensitivity to inhibition by Ca^{2+} and Mg^{2+} (Owen *et al.*, 1997).

The importance of Ca^{2+} regulation in RyR function is highlighted by the myopathies arising from improper regulation of the RyR. In particular, malignant hyperthermia, which is an autosomally inherited skeletal muscle disorder, characterized by uncontrolled skeletal muscle hypercontraction upon anaesthetic exposure as a result of abnormal Ca^{2+} handling, and is due to a defect in the RyR1 (McCarthy *et al.*, 1990; MacLennan and Phillips, 1992; MacLennan *et al.*, 1992). A mutation of an A614C in the skeletal muscle RyR increased sensitivity of the RyR to Ca^{2+} (Fujii *et al.*, 1991; Laver *et al.*, 1997) and Mg^{2+} (Laver *et al.*, 1997) shifted to the right.

1.2.6.2 Luminal Ca^{2+}

The level of Ca^{2+} loading of the SR is important in regulating Ca^{2+} release from the SR. Increasing or decreasing SR Ca^{2+} load increases or decreases (respectively) the Ca^{2+} release in response to depolarisation (Bassani *et al.*, 1995; Lamb *et al.*, 2001a), cytoplasmic Ca^{2+} (Copello *et al.*, 1997), caffeine (Lamb *et al.*, 2001a), ATP (Donoso *et al.*, 1995) and polylysine (Saiki *et al.*, 1999). Moreover, Lamb *et al.* (2001a) found that endogenous levels of SR Ca^{2+} loading in fast (EDL) and slow twitch (soleus) muscle is the primary factor determining the difference in caffeine sensitivities in the two fibre types. However, the mechanism by which Ca^{2+} load in the SR alters Ca^{2+} release is not clear.

In terms of RyR regulation, the effects of varying luminal [Ca^{2+}] are controversial. Luminal Ca^{2+} regulates RyRs: some studies show that increasing luminal [Ca^{2+}] increases channel activity, whilst other studies report a reduction in channel activity after exposure to increasing luminal [Ca^{2+}]. Luminal Ca^{2+} regulation was first thought to act as a single mechanism; Tripathy and Meissner (1996) suggested that luminal Ca^{2+} activated RyRs by passing through the channel and binding to cytoplasmic sites only, whilst Sitsapesan and Williams (1995) suggested that luminal Ca^{2+} activated RyR by binding to luminal sites only. Recent evidence suggests that luminal Ca^{2+} regulates the cardiac RyR by binding to both activation and inhibition sites on the luminal face of the RyR, as well as flowing through the open channel and interacting with Ca^{2+} activation sites located on the cytoplasmic side of the RyR (Ching *et al.*, 2000; *personal communication*, Rebecca Sitsapesan, National Heart and Lung Institute, Imperial College of Science, Technology and Medicine, London, UK). However, the specific luminal binding region has not been located, in fact studies completed thus far do not rule out the possibility that luminal Ca^{2+} may be activating the RyR by binding to a luminal accessory protein, such as CSQ.

Whether or not the differences observed in RyR regulation by luminal Ca^{2+} are due to using different RyR preparations, or activating cytoplasmic ATP and Ca^{2+} vs no cytoplasmic ATP or Ca^{2+} are unknown, and the exact effect of luminal Ca^{2+} upon RyR regulation is controversial. Some difference may be due to the use of SR vesicles or native RyRs (receptors with triadin, junctin and CSQ attached) by some investigators,

whilst others use purified RyRs, which presumably no longer have these proteins attached.

1.2.6.3 Magnesium

SR vesicle studies showed that Ca^{2+} efflux from skeletal SR was inhibited by mM Mg^{2+} (with mM Ca^{2+}), and Ca^{2+} activation was prevented by mM Mg^{2+} (at μM Ca^{2+}: Kirino *et al.*, 1983; Nagasaki and Kasai, 1983; Meissner *et al.*, 1986a), suggesting that two distinct mechanisms were responsible for this inhibition. Single channel studies also revealed Mg^{2+} inhibition of RyRs (Smith *et al.*, 1986; Meissner *et al.*, 1986a; Laver *et al.*, 1997).

Meissner *et al.* (1986a) and Laver *et al.* (1997) also proposed (in both cardiac and skeletal muscle), a dual mechanism model for inhibition by Mg^{2+}. Mg^{2+} can compete with Ca^{2+} for the Ca^{2+} activation site, preventing Ca^{2+} from inducing RyR opening. Mg^{2+} may also bind to low affinity binding sites (thought to be the Ca^{2+} inhibition site found within the D3 region, see Section 1.2.6.1), which bind divalent cations non-selectively and result in channel inhibition. This second inhibitory mechanism is responsible for inhibition by both Ca^{2+} and Mg^{2+}. The high and low affinity Mg^{2+} inhibition mechanisms operate independently.

1.2.6.4 Adenine nucleotides

Physiologically, ATP enhances Ca^{2+}-induced Ca^{2+} release in both skeletal and cardiac muscle (Kermode *et al.*, 1998; Laver *et al.*, 2001). It is more likely that Mg^{2+} -ATP is the physiological regulator of the RyR, as most of the ATP found intracellularly is complexed by Mg^{2+} (Meissner *et al.*, 1986a; Rubtsov and Batrukova, 1997). ATP has been shown to increase Ca^{2+} release from SR vesicles (Meissner, 1984) and activate single RyRs, in the presence (Meissner, 1986b; Moutin and Dupont, 1988) or absence (Smith *et al.*, 1986) of μM Ca^{2+}. Other adenine nucleotides, such as ADP, adenine AMP-PCP, AMP-PNP and cAMP, have been reported to have similar effects in skeletal muscle (Morii and Tonomura, 1983; Meissner, 1986b; Laver *et al.*, 2001). These compounds are not as effective as ATP at activating the channel. Since RyR activation is not specific for ATP, the mechanism by which adenine nucleotides act is not phosphorylation, but by ligand binding of adenosine to sites on the RyR. The consensus

sequence for the ATP binding site on the skeletal RyR, which is thought to encompasses residues Gly^{4449} - Gly^{4454} and Gly^{4452} - Gly^{4457} (Zorzato *et al.*, 1990), remains to be confirmed by mutagenesis or binding studies.

1.2.6.5 Ryanodine

Ryanodine regulates RyRs by binding to the channel preferentially when it is in the open state (Meissner, 1986b). Channel modulation is biphasic, with activation occurring at nM concentrations, whilst the channel is inhibited by concentrations greater than 100 µM (Lai and Meissner, 1989; McGrew *et al.*, 1989; Carroll *et al.*, 1991). Callaway *et al.* (1994) suggested that both the high and low affinity binding sites, which are associated by either allosteric or steric coupling, are located in the C-terminal region, downstream of Arg^{4475}, and near or within the membrane spanning region.

1.2.6.6 Ruthenium red

Ruthenium red, first shown to block mitochondrial Ca^{2+} transport (Moore, 1971), is a widely used RyR channel blocker. Ca^{2+} efflux from SR vesicles has been shown to be all but blocked in the presence of >20 µM cytoplasmic ruthenium red (Ikemoto *et al.*, 1985; Meissner, 1986b), whilst Ma (1993) demonstrated that ruthenium red inhibits the single channel RyR at sub-µM concentrations (cytoplasmic).

Initially, a scheme explaining ruthenium red binding predicted that two ruthenium red molecules were required to keep the channel in a closed state (Ma, 1993), similar to an acetylcholine receptor (Katz and Thesleff, 1957). Binding to the first site would induce initial closure, whilst the second molecule binding is responsible for long term inhibition (Ma, 1993). More recently, Xu *et al.* (1999) suggested that more than one mechanism existed for ruthenium red regulation of the RyR. The premise is that ruthenium red can inhibit RyRs (activated by Ca^{2+}) by a non-competitive interaction between ruthenium red and the Ca^{2+}. Secondly, ruthenium red (both cytoplasmic and luminal) can induce substate activity, via a voltage dependant mechanism. This model dismisses any suggestion that ruthenium red may occlude the pore.

1.2.6.7 Phosphorylation

RyR activity can be altered by phosphorylation of the RyR complex (Wang and Best, 1992; Herrmann-Frank and Varsanyi, 1993; Hain *et al.*, 1994; Sonnleitner *et al.*, 1998). In skeletal RyRs, Ser ^{2843} has been reported to be phosphorylated *in vivo* (Suko *et al.*, 1993; Varsanyi and Meyer, 1995). Dulhunty *et al.* (2001) report that the enhancement of RyR single channel activity upon application of ATP is partially due to channel phosphorylation by calmodulin kinase 2 (CaMKII). Besides Ser ^{2843}, many other residues are theoretically able to undergo phosphorylation (Ser, Thr, Tyr). Triadin and the histidine rich Ca^{2+} binding protein (HRC) contain numerous Ser, Thr, Tyr residues, are likely candidates for phosphorylation by a membrane bound CaM protein kinase (CaM-Pki; refer to Section 1.5.2 and 1.7.2.1). Whilst the presence of this particular kinase has not been established, phosphorylation of these associated proteins cannot be ruled out as a possible cause of RyR activation by ATP-induced phosphorylation.

1.3 DHPR

The DHPR, named for its affinity for a class of compounds known as dihydropyridines, is found embedded in the t-tubule membrane of muscle. As previously mentioned, the DHPR is an L-type Ca^{2+} channel (Tanabe *et al.*, 1987), which is a voltage dependent cation channel. Only a small percentage of DHPRs in skeletal muscle actually function as Ca^{2+} channels (Dulhunty, 1992 and references therein), and as voltage sensors.

1.3.1 Structural

The skeletal muscle DHPR, having a molecular mass of ~ 390 kDa, consists of four subunits, α_1, α_2-δ, γ and β (Dulhunty, 1992 and references therein). Functionally, $\alpha 1$ is most important, containing the Ca^{2+} binding motif, the voltage sensor and the central ion channel (Tanabe *et al.*, 1987; Tanabe *et al.*, 1988; Adams *et al.*, 1990; Tanabe *et al.*, 1990). There is significant sequence homology between the α_1 subunit and the voltage dependent sodium channel (Takahashi *et al.*, 1987). Containing extensive hydrophobic domains, α_1 also houses the sites for dihydropyridine binding, and a consensus sequence for the phosphorylation site for cAMP-dependent protein kinase (Takahashi *et*

al., 1987). The α_1 subunit contains approximately 2000 residues that are organized into four domains, each containing six transmembrane segments (S_1-S_6; Catterall, 2000 and references therein). The α_2 subunit, which has a limited membrane domain, binds lectins and forms a disulfide-linked dimer with the δ subunit (whose role in DHPR assembly or channel function is not well understood; Takahashi *et al.*, 1987). The β subunit contains another cAMP-dependant protein kinase consensus sequence and is thought not to be embedded in the membrane, but linked intracellularly to α_1. The β subunit, along with the α_1, contributes to a functional coupling between the DHPR and the RyR (Beurg *et al.*, 1999). The γ subunit, which also interacts with α_1, contains four transmembrane domains (Takahashi *et al.*, 1987).

1.3.2 Essential regions of DHPR for skeletal ECC

S_5 and S_6, the fifth and sixth α_1 intramembrane segments of each of the four repeats of the membrane spanning domain, and the membrane-associated loop formed between them, line the pore of the DHPR Ca^{2+} channel, whilst the voltage sensor is contained within S4 (the fourth intramembrane segment; Catterall, 2000 and references therein). The cytoplasmic loop between the second and third domain, termed the II-III loop (encompassing Lys^{720} - Glu^{765}), has been reported to be essential for both skeletal-type ECC and responsible for retrograde signal transduction - whereby an interaction between the RyR and the DHPR increases the Ca^{2+} current through the DHPR (Nakai *et al.*, 1998; Grabner *et al.*, 1999). The application of synthetic peptides in single channel and ^{3}HRy binding studies has led to hypotheses about the interactions between the RyR and the DHPR, particularly the II – III loop. Peptide A – DHPR residues Thr ^{671} - Leu ^{690} – has been shown to both activate and inhibit RyR activity (dependent on voltage), with channel activation thought to represent a physiological binding of the II-III loop (el Hayek *et al.*, 1995; Dulhunty *et al.*, 1999). Further downstream, peptide C (Glu ^{724} – Pro ^{760}) has been reported to inhibit peptide A-induced RyR activity (Saiki *et al.*, 1999; Haarman *et al.*, 2000), and inhibit depolarisation –induced tension in skinned fibres (Lamb *et al.*, 2001b). Applied alone, peptide C was able to activate and inhibit channel activity at low and high concentrations respectively (Haarman *et al.*, 2000).

Physiologically, interactions of DHPR II-III loop regions corresponding to these two peptide sequences may regulate the RyR Ca^{2+} channel. Initially, it was proposed that at

rest, the II-III loop site corresponding to peptide C is bound, rendering the RyR relatively inactive (Saiki *et al.*, 1999). Upon depolarisation, this interaction is removed, allowing the residues of the II-III loop corresponding to peptide A to interact, resulting in SR Ca^{2+} release. More recently, the Ca^{2+} concentration-dependant activation and inhibition of the RyR by peptide C (Haarman *et al.*, 2000) has led to the proposal of two alternate hypotheses (Yamamoto *et al.*, 2002). Firstly, peptide A has no functional role in ECC *in vivo*, and upon depolarisation (at low Ca^{2+}) peptide C activates the RyR, and inhibits ECC at higher [Ca^{2+}]. Alternatively, areas of the II-III loop corresponding to both peptides A and C contribute to activation at low [Ca^{2+}] (rest), and hence trigger ECC. The inhibitory action elicited by II-III loop peptide C region as [Ca^{2+}] rises is enough to inhibit ECC (Mouton *et al.*, 2001).

1.4 CSQ

CSQ is a Ca^{2+} binding protein, located wholly within the lumen of the SR (Yano and Zarain-Herzberg., 1994). CSQ has both a moderate affinity (K_d = 1 mM) and a high capacity for Ca^{2+} (50 mmol/mmol CSQ; MacLennan and Wong, 1971; MacLennan *et al.*, 1983). The main role of CSQ is to sequester Ca^{2+} and to provide a large pool of Ca^{2+} for release via the RyR, from the SR into the myoplasm.

1.4.1 CSQ genes

CSQ is found in all types of muscle; cardiac, skeletal and smooth (MacLennan and Wong, 1971; Ikemoto *et al.*, 1972; Volpe *et al.*, 1994), and has also been isolated in the cerebellum (Volpe *et al.*, 1990) and in plant cells (Krause *et al.*, 1989). Limited genetic information has been obtained about the smooth muscle and cerebellum isoforms of CSQ, but CSQ from both cardiac and fast twitch skeletal muscle (denoted as skeletal CSQ from here on) have been isolated and characterized, and have been found to be coded by two different genes. The slow twitch muscle isoform is identical to cardiac CSQ (Scott *et al.*, 1988; Fliegel *et al.*, 1989a).

1.4.2 Isoform and species homology

The cardiac isoform of CSQ has been fully sequenced in six species; rabbit, human, mouse, chicken, rat and dog (Slupsky *et al.*, 1987; Scott *et al.*, 1988; Park *et al.*, 1998; Rodriguez *et al.*, 1999), and the skeletal isoform has also been sequenced in six species; rabbit, human, mouse, chicken, rat and frog, (Fliegel *et al.*, 1987; Yazaki *et al.*, 1990a; Yazaki *et al.*, 1990b; Treves *et al.*, 1992; Park *et al.*, 1998). Table 1.1 shows identity between each sequence, presented as % homology. Of particular interest are the most extensively studied isoforms (in terms of RyR regulation); rabbit skeletal and canine cardiac, and their comparison with the human isoforms (Fig. 1.3). Amino acid sequence alignment reveals a 95% identity between rabbit and human skeletal CSQ, whilst dog cardiac and the human cardiac isoforms are not so highly conserved (83% identity; see Fig 1.3). Rabbit skeletal and dog cardiac also CSQ share an 83% identity (Fig 1.3). Such homology allows function inference between species and isoforms.

1.4.3 Crystal structure

The crystal structure of CSQ was solved for rabbit skeletal muscle (Wang *et al.*, 1998). Monomeric CSQ has three almost identical domains – domains I, II and III – with each domain containing four α–helices, two bordering either side of the core β-sheet (Wang *et al.*, 1998). It is proposed that folding and compaction of CSQ is promoted by physiological [Ca^{2+}] and [K^{+}] (Wang *et al.*, 1998). He *et al.* (1993) first reported the requirement of a compact, polymerised CSQ for high capacity Ca^{2+} binding; polymerisation is achieved by two interactions, the back-to-back dimer and the front-to-front coupling (Wang *et al.*, 1998). The first interaction (back-to-back) forms a dimer, thought to be initiated by a small increase in [Ca^{2+}] (the levels of which have not been identified). After formation of the back-to-back dimer, it is proposed that another small increase in [Ca^{2+}] induces the second interaction (front-to-front). The front-to-front interaction involves the coupling of back-to-back dimers, more specifically, an interaction between one CSQ molecule from one dimer, with a CSQ molecule from another dimer, which results in CSQ polymerisation (Figs. 1.4 and 1.5).

Several interactions help stabilize and maintain both dimer and polymer formation. The back-to-back interaction is stabilized by intermolecular interaction between domain II (α-helix 4) and domain I (α-helix 3). Salt bridges between Glu^{215} and Lys^{86}, Glu^{215} and

	Rat cardiac	*Rat skeletal*	*Chicken skeletal/ cardiac**	*Dog cardiac*	*Frog skeletal*	*Mouse cardiac*	*Mouse skeletal*	*Human cardiac*	*Human skeletal*	*Rabbit cardiac*
Rabbit skeletal	75	82	85	83	86	82	98	86	98	83
Rabbit cardiac	89	97	94	98	85	96	84	96	82	
Human skeletal	74	80	83	82	85	80	97	84		
Human cardiac	87	94	93	83	86	84	87			
Mouse skeletal	76	82	86	86	87	83				
Mouse cardiac	91	98	93	97	86					
Frog skeletal	79	85	86	86						
Dog cardiac	90	97	94							
Chicken skeletal/cardiac*	85	92								
Rat skeletal	91									

Table 1.1 Amino acid homology (%) between all reported full CSQ sequences (excluding the signal peptide). Homology was calculated using BioEdit sequence alignment editor (version 5.0.6), using the following published protein sequences (accession number in italics); Rabbit skeletal *A2587*; Rabbit cardiac *JQ1396*; Human skeletal *XP001278*, Human cardiac *XP010544*; Mouse skeletal *NP033943*; Frog skeletal *S22418*; Dog cardiac *A28071*, Chicken skeletal/cardiac *A31050*; Rat skeletal *NP058827*, Rat cardiac *AAB58746*.

*Chicken skeletal and cardiac CSQ share identical mRNA and protein sequence.

```
Dog cardiac          eeglnfptyd gkdrvvslte knfkqvlkky dvlclyyhes vssdkvaqkq fqlkeivlel vaqvlehkdi gfvmvdakke aklakklgfd eegslyvlkg
Rabbit skeletal      eegldfpeyd gvdrvinvna knyknvfkky evlallyhep peddkasqrq femeelilel aaqvledkgv gfglvdsekd aavakklglt eedsiyvfke
Rabbit cardiac       eeglnfptyd gkdrvvslse knfkqilkky dllclyyhap vsadkvaqkq fqlkeivlel vaqvlehkei gfvmvdakke aklakklgfd eegslyilkg
Human skeletal       qegldfpeyd gvdrvinvna knyknvfkky evlallyhep peddkasqrq femeelilel aaqvledkgv gfglvdsekd aavakklglt evdsmyvfkg
Human cardiac        eeglnfptyd gkdrvvslse knfkqvlkky dllclyyhep vssdkvtpkq fqlkeivlel vaqvlehkai gfvmvdakke aklakklgfd eegslyilkg
                             10         20         30         40         50         60         70         80         90        100
                     drtiefdgef aadvlvefll dliedpveii nsklevqafe riedqiklig ffkseeseyy kafeeaaehf qpyikffatf dkgvakklsl kmnevdfyep
                     devieydgef sadtlvefll dvledpveli egerelqafe niedeiklig yfknkdsehy kafkeaaeef hpyipffatf dskvakkltl klneidfyea
                     drtiefdgef aadvlvefll dliedpveii nsklevqafe riedhiklig ffksadseyy kafeeaaehf qpyikffatf dkgvakklsl kmnevdfyep
                     devieydgef sadtivefll dvledpveli egerelqafe niedeiklig yfkskdsehy kafedaaeef hpyipffatf dskvakkltl klneidfyea
                     drtiefdgef aadvlvefll dliedpveii ssklevqafe riedyiklig ffksedseyy kafeeaaehf qpyikffatf dkgvakklsl kmnevdfyep
                            110        120        130        140        150        160        170        180        190        200
                     fmdepiaipd kpyteeelve fvkehqrptl rrlrpedmfe tweddlngih ivafaersdp dgyefleilk qvardntdnp dlsivwidpd dfpllvaywe
                     fmeepvtipd kpnseeeivn fveehrrstl rklkpesmye tweddmdgih ivafaeeadp dgyefleilk svaqdntdnp dlsiiwidpd dfpllvpywe
                     fmdeptpipn kpyteeelve fvkehqrptl rrlrpedmfe tweddlngih ivpfaeksdp dgyefleilk qvardntdnp dlsivwidpd dfpllvaywe
                     fmeepvtipd kpnseeeivn fveehrrstl rklkpesmye tweddmdgih ivafaeeadp dgfefletlk avaqdntenp dlsiiwidpd dfpllvpywe
                     fmdepiaipn kpyteeelve fvkehqrptl rrlrpeemfe tweddlngih ivafaeksdp dgyefleilk qvardntdnp dlsilwidpd dfpllvaywe
                            210        220        230        240        250        260        270        280        290        300

                     ktfkidlfkp qigvvnvtda dsvwmeipdd ddlptaeele dwiedvlsgk intedddnee gddgdddedd ddddgnnsde esnddsdddd e
                     ktfdidlsap qigvvnvtda dsvwmemdde edlpsaeele dwledvlege int------- -------e-- -ddd----de d--dd-dddd d
                     ktfkidlfkp qigvvnvtda dsvwmeipdd ddlptaeele dwiedvlsgk intedddnee edd-dddndd ddddngnsde ednddsdedd e
                     ktfdidlsap qigvvnvtda dsvwmemdde edlpsaeele dwledvlege int------- -------e-- --------d- ---dd-dddd d
                     ktfkidlfrp qigvvnvtda dsvwmeipdd ddlptaeele dwiedvlsgk int------e ----dddedd dddd--nsde ednddsdddd de
                            310        320        330        340        350        360        370        380        390
```

Fig. 1.3. ***Comparison of CSQ amino acid sequence from rabbit skeletal, canine cardiac and human skeletal and cardiac isoforms (excluding the signal peptide).*** Sequence alignment was prepared using Bioedit graphical biological sequence editor. Conserved residues are listed purple, whilst resides that are unconserved between species and isoform are in blue. Identical amino acids are in black. Amino acid position is listed below the sequences.

Lys^{24}, and Glu^{169} and Lys^{85} help stabilize this formation (Fig 1.4). In addition, there is an interaction between the dihydrobasic sequence (a sequence found in other Ca^{2+} binding proteins; Blumenthal *et al.*, 1985), and a binding site for the antipsychotic drug, trifluroperazine. The interaction was first thought to be involved in intramolecular binding and folding. It now is thought to occur between two CSQ molecules. This interaction involves salt bridge formation between Lys^{85} and Glu^{223} and Lys^{86} and Glu^{215} (Fig 1.4). Salt bridges, formed between Glu^{55} and Lys^{49} stabilize the front-to-front interaction and involve residues from two α-helices, both from domain I. In conjunction with various interaction between domains I and III from different CSQ molecules, the final polymer is formed (Fig 1.5; Wang *et al.*, 1998).

The front-to-front interaction occurs with the insertion of the N-terminus of CSQ of one dimer into the hydrophobic cleft of the second dimer. Wang *et al.* (1998) propose that CSQs N-terminus is responsible for anchoring CSQ to triadin and junctin, so when CSQ is polymerised (considered its physiological form), no interaction with the anchoring proteins would be possible. Recent evidence suggests that it is the C-terminus (Glu^{354} - Asp^{367}) and not the N-terminus that couples CSQ and triadin, so even if CSQs N-terminus has been buried upon polymerisation, a CSQ/triadin interaction may still occur (Shin *et al.*, 2001 and see section 1.8.6).

1.4.4 Conformational changes in CSQ

Ikemoto *et al.* (1972) first observed a conformational change in CSQ induced by Ca^{2+} binding, which has been confirmed by others (MacLennan and Wong, 1971; Ikemoto *et al.*, 1972; Cala and Jones, 1983; MacLennan *et al.*, 1983; Cozens and Reithmeier, 1984; Slupsky *et al.*, 1987; Wang *et al.*, 1998). When devoid of Ca^{2+}, CSQ structure is mostly random coil, with approx 11% α-helix. Upon Ca^{2+} binding, asymmetry of the protein decreases (Cozens and Reithmeier, 1984), whilst α-helical content increases (Ikemoto *et al.*, 1972; Ikemoto *et al.*, 1974; Ostwald *et al.*, 1974). Besides Ca^{2+}, other cations are able to bind to and induce conformational changes in CSQ. These include Mg^{2+}, K^{+} and Zn^{+} (Ikemoto *et al.*, 1972; Ikemoto *et al.*, 1974; Ostwald *et al.*, 1974; Baksh *et al.*, 1995). Exposure to 1 M KCl induces a radical change in α-helical content, with a shift of up to 19% (Ikemoto *et al.*, 1974). Both Mg^{2+} and Zn^{2+} induce minor changes in CSQs conformation (Ostwald *et al.*, 1974; Baksh *et al.*, 1995).

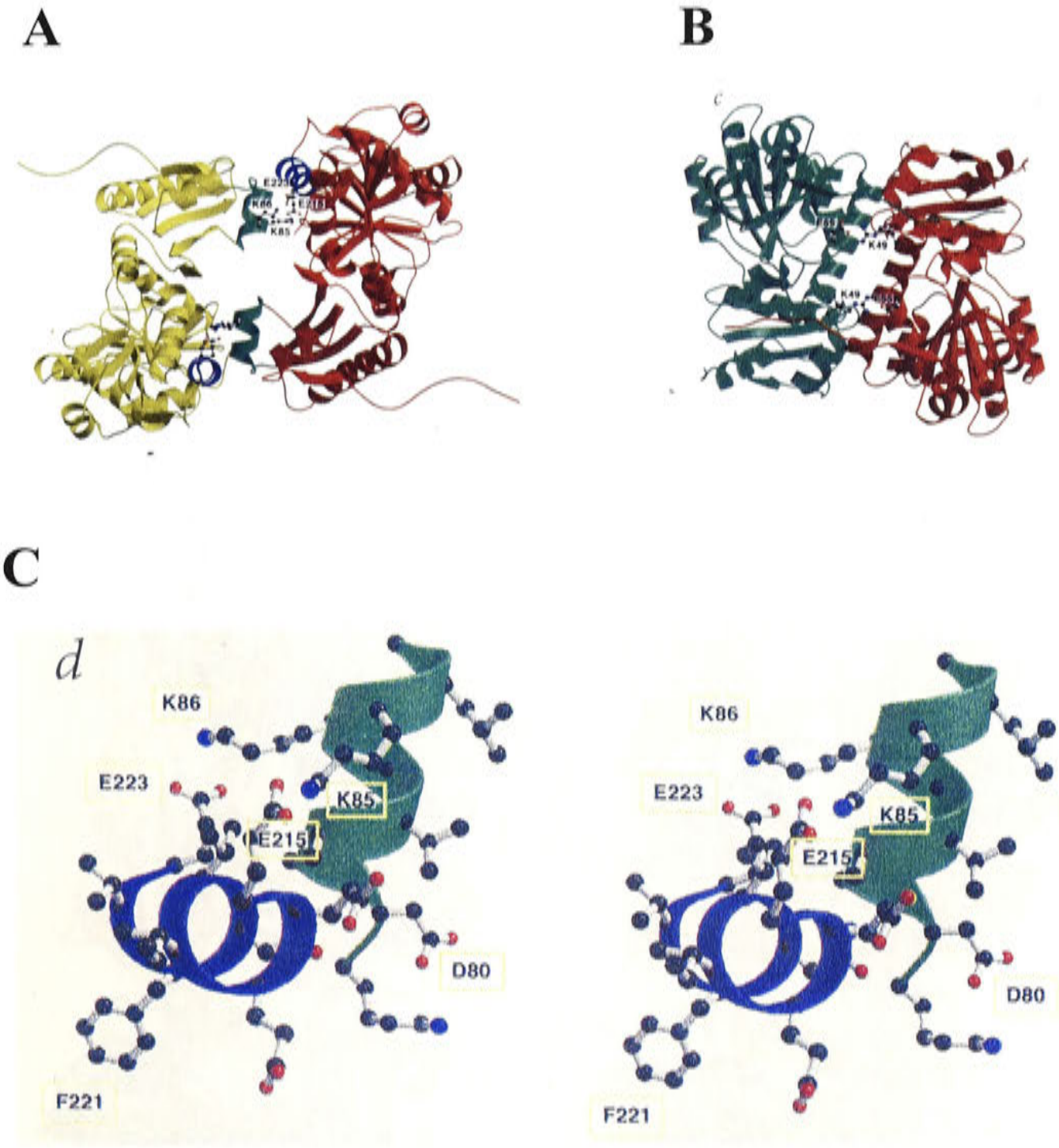


Fig. 1.IV. ***Formation of the CSQ aggregate.*** (**A**). Dimer formation showing the first dimer formed, a back-to-back association and (**B**) the subsequent polymer formation, which induces CSQ aggregation, the front-to-front interaction. (**C**) Ribbon representation of the interaction of the dihydrobasic sequence from monomer one (blue) with the trifluroperazine binding site for monomer two (green). These interactions, stabilized by salt bridge formation , between Lys ^{85} and Glu ^{223} and Lys ^{86} and Glu ^{215} which contribute to the stability of the back-to-back dimer (from Wang *et al.*, 1998).

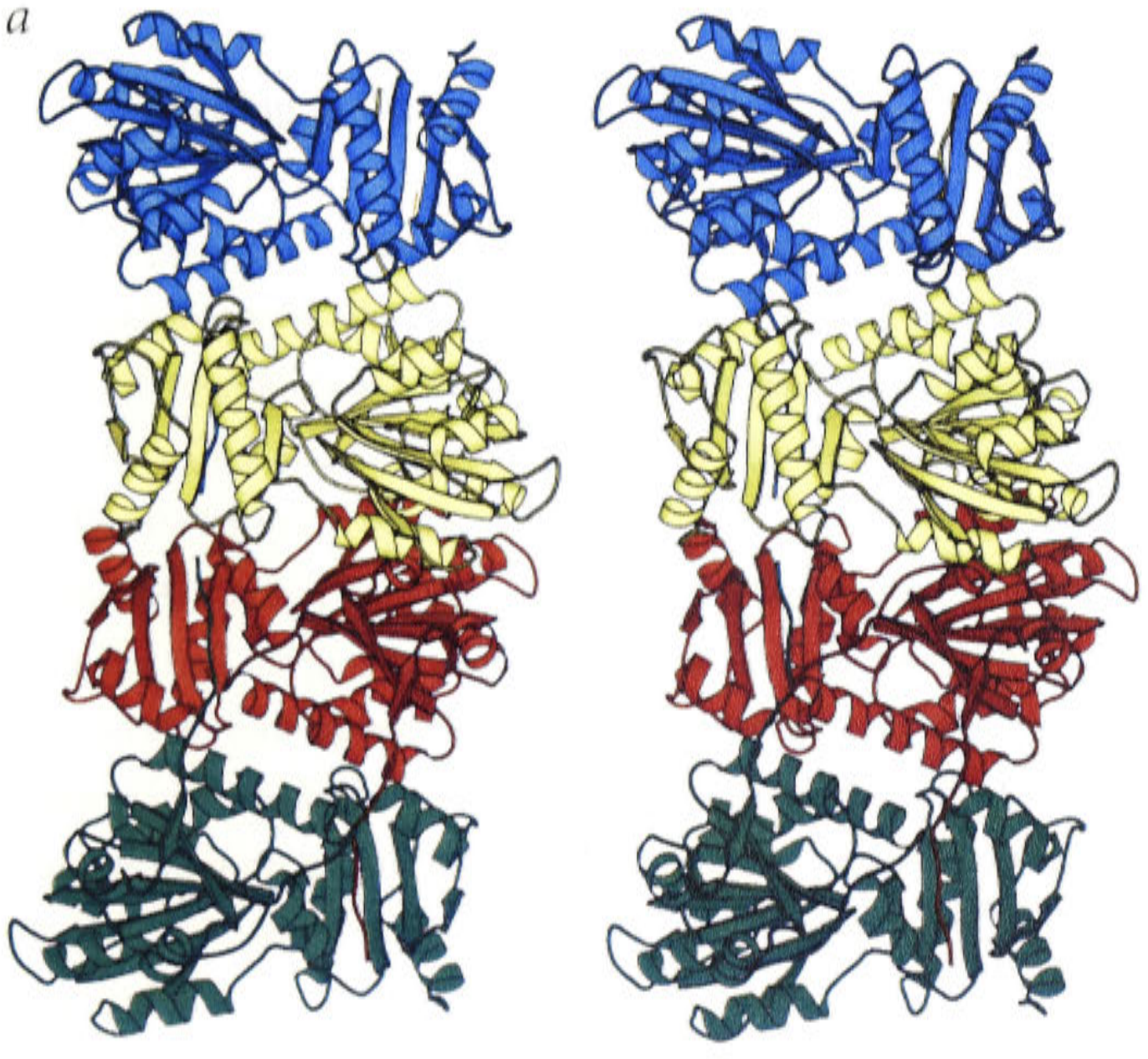


Fig. 1.V. ***Formation of the CSQ aggregate.*** The formation of front-to-front interaction, between two CSQ molecules, already in back-to-back dimer formation, induces the CSQ polymer. The interaction between the CSQ molecules in yellow and red are back-to-back, whilst front-to-front interactions are depicted between molecules in blue and yellow, and red and green (from Wang *et al.*, 1998)

Ca^{2+} binding to cardiac CSQ induced a 2-fold increase in sedimentation coefficient (indicative of a more compact protein structure) and a loss of hydrophobicity (Mitchell *et al.*, 1988). Coupled with the inability of several proteases (including trypsin) to digest the Ca^{2+}-bound protein, these data suggest a Ca^{2+}-induced compaction, allowing both the sites that are susceptible to proteolysis, as well as the hydrophobic sequence to be buried (Mitchell *et al.*, 1988). Presumably, protection of arginine and lysine residues (which trypsin cleaves) occurs when these residues are buried, as CSQ becomes more α-helical (Cozens and Reithmeier, 1984; Ohnishi and Reithmeier, 1987).

1.4.5 Binding of Ca^{2+} and other ions

The Ca^{2+} binding properties of rabbit skeletal CSQ have been reported by various laboratories. MacLennan and Wong (1971) reported Ca^{2+} binding to be 970 nmol/mg CSQ, with a K_d of 40 μM, whilst Meissner *et al.* (1973) reported a similar binding (900-1000 nmol/mg), but a K_d of approximately 4 μM. Extreme differences in K_d in the presence of 100 mM KCl have been reported; Ca^{2+} binding remained constant, whilst the K_d was 800 μM (MacLennan, 1974; Ostwald *et al.*, 1974) and 1.3 μM (Ikemoto *et al.*, 1972). Similar Ca^{2+} binding is reported in dog cardiac CSQ (Mitchell *et al.*, 1988) – between 800 and 900 nmol/mg protein. Ca^{2+} specificity is quite low, with a number of other ions able to compete for the Ca^{2+} binding site (Ikemoto *et al.*, 1974). Ikemoto *et al.* (1974) reports the order of affinity for various cations binding to CSQ as $La^{3+} > Zn^{2+} > Cd^{2+} > Mn^{2+} > Ca^{2+} > Mg^{2+}/Sr^{2+} > K^{+}$, with association constant (M^{-1}) ranging from $3.3 * 10^5$ (La^{3+}) to 4 (K^{+}).

Ca^{2+} binding by CSQ is thought to require only a pair of acidic residues and is driven by a gain in entropy (Krause *et al.*, 1991). Between 18 and 50 sites for Ca^{2+} binding per CSQ monomer have been predicted (MacLennan and Wong, 1971; Ikemoto *et al.*, 1974; Meissner, 1974; Slupsky *et al.*, 1987), but current opinion is that there are 31 Ca^{2+} binding sites for rabbit skeletal muscle CSQ (in the case of rabbit skeletal muscle, Ca^{2+} binding would involve 62 of the protein's 367 amino acids; Krause *et al.*, 1991). Rabbit skeletal CSQ is a highly acidic glycoprotein – having between 33 and 46% acidic residues (depending on both isoform and species), and forming no less than 21 clusters of doublets or triplets (Yano and Zarain-Herzberg., 1994 and references therein). The acidic C-terminus
(^{354}Glu-Asp-Asp-Asp-Asp-Glu-Asp-Asp-Asp-Asp-Asp-Asp-Asp-Asp^{367}) is purported

to be responsible for 26% of the Ca^{2+} binding and to be the site for triadin binding in rabbit skeletal muscle (Shin *et al*, 2001). The C-terminus differs in length between isoforms. Both the rabbit and human skeletal isoforms have a shorter stretch of acidic residues (14 and 9 respectively), compared to dog (38) and human cardiac (26), although these differences are not reflected by significant changes in CSQs Ca^{2+} binding capacity. Therefore, the C-terminus may not be the major Ca^{2+} binding site.

A peptide corresponding to the C-terminus of rabbit skeletal CSQ (Thr^{302}-Glu^{359}), showed an increased Ca^{2+} binding capacity (2 μmol/mg peptide), when compared with native CSQ (~1 μmol/mg CSQ; Ohnishi and Reithmeier, 1987). Ca^{2+} binding sites were presumably exposed in this Asp/Glu-rich peptide that were not accessible, or were hidden in the intact protein. To account for the reduced Ca^{2+} binding capacity of intact CSQ (when compared to that of the C-terminal peptide), the authors suggest that Ca^{2+} binding to the C-terminus signals the conformational change seen in intact CSQ (from Thr^{302}-Glu^{359}), which then buries Ca^{2+} binding sites. These buried sites remain exposed in the truncated C-terminal peptide (Ohnishi and Reithmeier, 1987), and therefore more Ca^{2+} can bind to the peptide.

CSQ binds Mg^{2+} with a lower affinity than Ca^{2+} (Ikemoto *et al.*, 1974). In the presence of mM Mg^{2+}, CSQ binds only 23 Ca^{2+} (compared with 31 when Mg^{2+} is absent). The shift in heat production obtained experimentally after Ca^{2+} binding in the presence of 3 mM Mg^{2+} ($\ddot{A}H°_{Ca^{2+}(+Mg^{2+})}$) is quite different to the theoretical value calculated assuming a direct competition between Ca^{2+} and Mg^{2+} at a single binding site. The measured $\ddot{A}H_{Ca}$ - $\ddot{A}H_{Mg}$ was–15kJ/protein, while the theoretical value was +11.1 kJ/protein (Krause *et al.*, 1991). The difference between the theoretical and experimentally obtained $\ddot{A}H°_{Ca^{2+}}$ in the presence of Mg^{2+} suggests two distinct binding sites for Ca^{2+} and Mg^{2+} although this does not account for the loss of Ca^{2+} binding upon Mg^{2+} addition. It may be that there is a direct competition for some binding sites (so the site is capable of binding both Ca^{2+} and Mg^{2+}) and separate binding at other sites.

Addition of Ca^{2+} induced an increase in intrinsic fluorescence in CSQ at neutral pH (inferring a Ca^{2+}-dependant conformational change in the protein), which was not observed at the more acidic pH of 6.0 (Hidalgo *et al.*, 1996). Varying pH from 8 to 6 resulted in a decreased heat production, and as binding of Ca^{2+} to CSQ is mainly entropy driven (Krause *et al.*, 1991), these results suggest that a slightly acidic pH

abolishes the Ca^{2+}-induced conformational changes in the protein. A physiological role of pH-induced changes in CSQ conformation has not yet been established, although it has been proposed that a counterflow of protons (compensating for a loss of Ca^{2+} during SR Ca^{2+}release during muscle contraction), may lower luminal pH and prevent CSQ from binding Ca^{2+}, resulting in CSQ unfolding (Hidalgo *et al.*, 1996 and references therein)

1.4.6 Effects of Ca^{2+} dependent conformational changes in CSQ

The regulation of the RyR by luminal Ca^{2+} concentration (see Section 1.2.6.2) is likely to be a combination of activation/inhibition of the RyR by luminal Ca^{2+} binding directly to the RyR complex and luminal Ca^{2+}- dependent changes in CSQ, which alter the influences of CSQ on RyR activity.(Kawasaki and Kasai, 1994; Donoso *et al.*, 1995; Ohkura *et al.*, 1995). Ikemoto *et al.* (1989) reported conformational changes within JFM proteins (not CSQ) when Ca^{2+} was raised from <μM to 10 mM. This conformational change was not observed in JFM preparations where CSQ was absent.

The hypothesis that Ca^{2+} binding to CSQ alters CSQ protein conformation, activating RyRs and thus inducing RyR Ca^{2+} release (in contrast to a direct effect of luminal Ca^{2+} on the RyR) was proposed by Donoso *et al.* (1995). SR Ca^{2+} release is most sensitive to luminal Ca^{2+} over the same range of Ca^{2+} (0.025-2 mM) that induces substantial and reversible conformational changes in CSQ (Ikemoto *et al.*, 1989; He *et al.*, 1993; Donoso *et al.*, 1995). The precise nature of the transmission of luminal [Ca^{2+}] to the RyR by CSQ is yet to be determined

1.4.7 CSQ phosphorylation

Whether or not skeletal CSQ exists *in vivo* in its phosphorylated form is still debated. On one hand, Varsanyi and Heilmeyer (1980) reported the autophosphorylation of CSQ and Campbell and Shamoo (1980) found that CSQ was phosphorylated in SR preparations, suggesting that it is phosphorylated *in vivo*. Alternatively, Cala and Jones (1991) found that isolated cardiac CSQ contained no significant endogenous inorganic phosphate (Pi), indicating that it is not phosphorylated.

The phosphorylation state of CSQ is important in the regulation of purified RyRs by CSQ. There was one report that suggests that dephosphorylation of CSQ by as little as 1% can increase purified channel activity (Szegedi *et al.*, 1999). This effect was [CSQ] dependant; the highest activity observed was after addition of 20 μM dephosphorylated CSQ (the highest concentration studied).

Meggio *et al.* (1981) first showed that casein kinase II (CK II) promoted CSQ phosphorylation. CK II, a protein kinase present in all eukaryotic cells (Pinna, 1997), consists of two β subunits (which are tightly connected) and two α/α′ subunits. This acidophilic hetrotetramer specifically targets and phosphorylates serine/threonines (but preferentially acts on serine; Marin *et al.*, 1986; Kuenzel *et al.*, 1987), although recent reports have shown it can also target tyrosines (Marin *et al.*, 1999b). A higher rate of phosphorylation is reported for residues that are flanked by three acidic amino acids on either side, compared to those that have such residues located on the C-terminal tail (Kuenzel *et al.*, 1987). CKII phosphorylation of the target protein requires a minimal sequence of Ser/Thr-X-X-Asp/Glu, with the negatively charged asparagine/glutamates (at position n+3, where n is the Ser/Thr) being critical for targeting (Meggio *et al.*, 1994). The presence of at least two acidic residues flanking the serine/threonines results in a higher rate of phosphorylation (Meggio *et al.*, 1994).

Whether CK II is found within the SR lumen is not established (Varsanyi and Heilmeyer, 1980; Cala and Jones, 1991; Shoshan-Barmatz *et al.*, 1996b), although Shoshan-Barmatz *et al.* (1996b) provide some evidence that CK II might be located on the luminal side of the SR. CK II and other kinases are thought to exist in the cytoplasm, so that phosphorylation of CSQ, SLM and HRC may occur before targeting to the SR. The location of CK II or other kinases in the SR, would support the hypothesis that RyR activity may be altered by phosphorylation of luminal co-proteins, such as CSQ, HRC and sarcalumenin (SLM).

ATP is essential for CSQ phosphorylation by CK II or any other kinase. At least two possible pathways for ATP transport into the SR have been reported. Shoshan-Barmatz *et al.* (1996a) illustrated that an SR membrane voltage-dependant anion channel (VDAC) may transport ATP into the SR, hence providing the P_i required for CK II phosphorylation (if in fact CK II exists in the SR) of the three Ca^{2+}-sequestering

proteins CSQ, HRC and SLM; see also Section 1.7.2). Alternatively, (Laver, 2001) propose that a small chloride channel (SCl) channel may transport ATP into the SR.

Varsanyi and Heilmeyer (1979) first suggested that rabbit skeletal CSQ has protein kinase properties. Furthermore, CSQ has the ability to autophosphorylate (Varsanyi and Heilmeyer, 1980). This was demonstrated by pre-incubating CSQ with protein kinase, which resulted in an approximately 10-fold increase in phosphorylation, raising V_{max} from 3.7 to 37 pmol.min^{-1}.mg^{-1}. The phosphorylation sites on CSQ have not been unambiguously identified.

After sequencing fast-twitch skeletal rabbit CSQ, Fliegel *et al.* (1987) proposed two potential sites for phosphorylation, Thr^{189} and Thr^{229}. Neither of these residues has the required acidic amino acid at n +3, and therefore does not contain the minimal consensus sequence for phosphorylation by CK II (Fliegel *et al.*, 1987; Meggio *et al.*, 1994). It may be that *in vivo*, these two residues are phosphorylated by some other kinase, such as casein kinase I (CK I) or ε protein kinase C1, for which CSQ is also a substrate *in vitro* (Salvatori *et al.*, 1994; Rodriguez *et al.*, 1999) or are involved in autocatalytic phosphorylation. Neither CK I nor ε protein kinase C1 have been shown to exist within the SR and the role that they may play in CSQ phosphorylation is not understood, although it is possible that CSQ may be phosphorylated by CK I and/or ε protein kinase C1 whilst in the ribosomal form (before targeting to the SR lumen; Rodriguez *et al.*, 1999 and references therein). The first major study to identify which CSQ residues were phosphorylated was by Cala and Jones (1991). Both cardiac (canine) and skeletal (rabbit) CSQ contain several repeats of the minimal structure required for CK II phophorylation in their C-terminal region. It was found that Ser^{378}, Ser^{382}, Ser ^{386} (cardiac) and Thr^{353} (skeletal) could be phosphorylated by CK II. Cardiac CSQ was phosphorylated more rapidly than the skeletal isoform, (V_{max} 240 nmol.min^{-1}.mg^{-1} in cardiac, compared with 0.17 nmol.min^{-1}.mg^{-1} in skeletal), which has been attributed to CK IIs preference for serine over threonine, and the position of three acidic residues in relation to the phosphorylated residue. In comparison, the calculated V_{max} of rabbit skeletal CSQ is 0.4 nmol.min^{-1}.mg^{-1} CSQ (CK II) and 0.9 nmol.min^{-1}.mg^{-1} CSQ (CK I).

1.4.8 Targeting of CSQ to lumen

The luminal retention of endoplasmic reticulum proteins, such as calreticulin and protein disulfide isomerase, are dependent on the presence of a C-terminal sequence Lys-Asx-Glu-Leu, which allows recycling from pre-Golgi to Golgi apparatus (Munro and Pelham, 1987; Nori *et al.*, 1999). This motif is absent from all isoforms of CSQ, so that the mechanism for CSQ segregation in the lumen is a mystery. Originally, it seemed likely that JSR proteins triadin and junctin may be responsible for the segregation of CSQ to the lumen. Both triadin and junctin bind to CSQ and are integral luminal proteins. Deletion of the acidic C-terminus, (the probable binding site on CSQ for triadin), did not alter CSQ segregation or expression in rabbit skeletal myoblast deletion mutants, thus negating the hypothesis that triadin is responsible for luminal retention of CSQ (Nori *et al.*, 1999; Nori *et al.*, 2001a).

Recognition sites within the protein, involving post-translational changes such as phosphorylation and glycosylation, are not responsible for CSQ targeting to the lumen. Nori *et al.* (2000) and Nori *et al.* (2001b) have shown that deletion of the glycosylation site and site-directed mutagenesis of the known phosphorylation site and potential sites indicated by Fliegel *et al.*, 1989b of CSQ, did not alter targeting to the SR lumen.

1.4.9 Overexpression of CSQ

The development of a cardiac CSQ overexpression mouse model was prompted by the observed association between cardiac hypertrophy and an impairment of SR Ca^{2+} handling. It was thought that since CSQ is the major Ca^{2+} storage protein found in SR (MacLennan *et al.*, 1983; Yano and Zarain-Herzberg., 1994), alterations in CSQ expression may be associated with certain cardiomyopathies. Hypertrophy indicates an increase in internal workload (which could occur with inefficient Ca^{2+} handling) and which if sustained, may induce heart failure (Sato *et al.*, 1998).

Mouse hearts overexpressing canine cardiac CSQ developed severe hypertrophy and heart failure (Jones *et al.*, 1998; Wang *et al.*, 2000). The RyR, triadin and junctin were all down-regulated and CSQ overexpression resulted in an impairment of Ca^{2+}-induced Ca^{2+} release, and decreased Ca^{2+} spark frequency (Jones *et al.*, 1998; Wang *et al.*, 2000). In contrast, mice overexpressing murine CSQ showed no down regulation of the

RyR, triadin or junctin expression, but similar to the model of canine cardiac CSQ overexpression, showed both hypertrophy and impaired Ca^{2+}-induced Ca^{2+} release (Sato *et al.*, 1998). The relevance of altered expression of other proteins with canine CSQ over-expression is not known, but it is likely to contribute to changes in Ca^{2+} signalling. These studies illustrates CSQs integral role in establishing Ca^{2+} homeostasis by modulating Ca^{2+} load and release by the SR. Whilst in human heart failure, no alteration of CSQ expression has been detected, this model may be a valuable tool in the study of heart failure prevention by improving Ca^{2+} homeostasis (due to an increase in Ca^{2+} storage capacity) and metabolism (Knollmann *et al.*, 2000).

1.5 Triadin

Brandt *et al.* (1990) first described a unique 95 kDa glycoprotein, later named triadin, found embedded in the SR membrane. This was later characterized as an intrinsic membrane protein located within the terminal cisternae, capable of forming a disulfide linked complex, between triadin monomers (Caswell *et al.*, 1991). A 94 kDa terminal cisternae glycoprotein, also able to form polymer disulfide bonds has also been described (Knudson *et al.*, 1993a). Both triadin and the 94 kDa protein are almost absent in the t-tubule and non-junctional SR, prompting the authors to suggest that both these proteins were identical.

Skeletal muscle triadin contains 706 amino acids (Knudson *et al.*, 1993a; Knudson *et al.*, 1993b). The first 47 N-terminal amino acids are cytoplasmic, with the bulk of the protein existing within the SR lumen. A single transmembrane α-helix consists of 20 residues (Knudson *et al.*, 1993a; Knudson *et al.*, 1993b; Marty *et al.*, 1995). Two cysteine residues, Cys ^{270} and Cys ^{671}, are proposed to form a disulfide-linked oligimer, whilst two potential glycosylation sites are located within triadins luminal domain (Knudson *et al.*, 1993b).

1.5.1 Isoforms

Two cardiac triadin isoforms have been identified in canine cardiac muscle by SDS Page, one migrating at 92 kDa (triadin-3), and an isoforms that forms doublets of 35 kDa and a doublet of 40 kDa (triadin-1; Kobayashi and Jones, 1999) Triadin-2,

proposed by Guo *at al* 1995, was not detected in canine cardiac tissue. Triadin-2 was originally proposed to be a form of triadin-1, resolving at 40 kDa, but recently studies shown the expression of both a glycosylated and de-glycosylated triadin (with mobilities of 40 and 35 kDa respectively), which arise from the same isoform (triadin-3) Only one skeletal isoform has been fully sequenced. The three cardiac isoforms arise from alternate splicing of one gene. All cardiac triadins are identical for the first 264 amino acids, so only part of the luminal sequence of the proteins differs. For the first 663 amino acids, triadin-3 is almost identical to the skeletal muscle isoform (besides missing Ala^{331}). The major differences occur in the C-terminus, with triadin-3 missing the final 39 amino acids (Guo and Campbell, 1995). Cardiac triadin-3 is also missing Cys^{671} and does not forms a disulfide complex like cardiac triadin-1 and triadin-2 (Guo and Campbell, 1995), suggesting both disulfide linkages are required for oligimerization.

The most abundant isoform in cardiac muscle is triadin-1, accounting for at least 95 % of triadin isoforms (Kobayashi and Jones, 1999). The reason for the formation of a doublet (visible on gel electrophoresis as molecular masses of 35 and 40 kDa) is the partial glycosylation status of the protein, and the resultant alteration in mobility of the protein. When glycosylated, the 35 kDa protein is converted into the 40kDa protein, suggesting that only the 40 kDa protein is glycosylated (Kobayashi and Jones, 1999). In mammalian cardiac tissue, triadin-3 is minimally expressed ($\leq$5 %), whilst triadin-2 was undetectable (Kobayashi and Jones, 1999). Like canine cardiac triadin, in mouse cardiac tissue three isoforms (splice variants of the same gene), have been determined, namely mouse triadin 1-3, having molecular weights of 35, 35.5 and 40 kDa, respectively (Hong *et al.*, 2001). Mouse triadin-3 appears to be the glycosylated form of mouse triadin-2.

1.5.2 Phosphorylation

Triadin has been shown to undergo phosphorylation by CaM-Pki (Damiani *et al.*, 1995). This enzyme was originally thought to phosphorylate the cytoplasmic side of the RyR, and RyR activity is altered by phosphorylation (Wang and Best, 1992; Herrmann-Frank and Varsanyi, 1993; Hain *et al.*, 1994; Sonnleitner *et al.*, 1997; Dulhunty *et al.*, 2001). It may be that CaM–Pki alters RyR activity via triadin phosphorylation, rather than, or in conjunction with, direct RyR phosphorylation.

1.5.3 Triadin overexpression

A cardiac mouse model overexpressing triadin was developed to shed some light on the importance of triadin in ECC. Triadin overexpression results in hypertrophy, impaired relaxation and reduced contractility at low (i.e. below normal resting heart rate) frequency, and the reduced expression of junctin and the RyR (Kirchhefer *et al.*, 2001). The authors note that whilst RyR protein expression was significantly reduced, ^{3}HRy binding did not alter from wild type, suggesting that each RyR is activated more easily to compensate the reduced expression (Kirchhefer *et al.*, 2001). Unlike the CSQ overexpression model, triadin overexpression did not lead to heart failure or premature death, possibly due to the down regulation of junctin and the RyR or other compensatory mechanisms. The physiological role of triadin is hard to define from an overexpression model, due to the altered expression of other proteins.

1.6 Junctin

Junctin is an integral membrane protein, found in junctional SR membrane, and is proposed to provide a similar role to triadin as a membrane anchor for CSQ. Although only characterized in cardiac muscle (Mitchell *et al.*, 1988; Jones *et al.*, 1995; Zhang *et al.*, 1997; Wetzel *et al.*, 2000), junctin has been identified in skeletal muscle (Mitchell *et al.*, 1988).

Unlike triadin, junctin is not glycosylated and does not form a disulfide linked complex; cystine residues are absent in both the canine and human isoforms (Jones *et al.*, 1998; Wetzel *et al.*, 2000). Junctin is a shorter protein than triadin, but has a similar membrane topology, with a single membrane spaning domain, a short 21 amino acid cytoplasmic terminus and a long, highly charged luminal tail (Jones *et al.*, 1995). The N-terminus, membrane-spanning region and first 58 luminal residues are highly conserved between species (Jones *et al.*, 1995; Zhang *et al.*, 1997; Dinchuk *et al.*, 2000; Wetzel *et al.*, 2000). The N-terminus has been hypothesized to contain the sites of interaction with triadin, CSQ and the RyR (Zhang *et al.*, 2001). Junctin associates with the RyR, triadin and CSQ within the lumen of the SR. No studies thus far have determined a regulatory action of junctin on any of these proteins (Zhang *et al.*, 1997).

1.6.1 Junctin overexpression

Although functional effects of junctin on the RyR, triadin and CSQ have not been reported, overexpression of cardiac junctin results in abnormalities in muscle contraction, an overall narrowing of the junctional SR, compaction of CSQ and excess t-tubule-SR interactions, observed using electron microscopy (Zhang *et al.*, 2001). These abnormalities might reflect junctins role as an anchor protein for CSQ, and the possibility that the short C-terminus has a role in facilitating a SR - t-tubules association is currently being evaluated (see Zhang *et al.*, 2001). However, they may also be compensatory changes in response to the lack of junctin (for example, changes in expression levels of other proteins).

1.7 Other Ca^{2+} binding proteins

1.7.1 Calmodulin

CaM's role as a Ca^{2+} senor in most eukaryotic cells has been widely characterized. CaM, a 17 kDa protein associating with the RyR, in both a Ca^{2+} bound (Ca^{2+}CaM) and Ca^{2+} free (apoCaM) form (Yang *et al.*, 1994; Tripathy *et al.*, 1995), belongs to a family of Ca^{2+} detectors that contain EF hand sequences (Lewit-Bentley and Rety, 2000 and references therein). An EF hand binds Ca^{2+} within a loop structure, created by a helix-loop-helix (Lewit-Bentley and Rety, 2000 and references therein). Troponin C is an example of another Ca^{2+} binding protein containing an EF hand structure. Up to nine CaM binding sites on the RyR have been predicted (Takeshima *et al.*, 1989; Zorzato *et al.*, 1990; Menegazzi *et al.*, 1994; Wagenknecht *et al.*, 1994), although current consensus is that there is one CaM binding site per RyR subunit (involving residues 3630-3637; Moore *et al.*, 1999). Exogenous CaM can activate and inhibit RyR Ca^{2+} release in a concentration-dependant manner; at nM Ca^{2+} CaM increases Ca^{2+} release, whilst CaM inhibits Ca^{2+} release at mM Ca^{2+} (Tripathy *et al.*, 1995).

Single channel studies imply that RyR Ca^{2+} release is inhibited by a direct interaction with Ca^{2+} CaM (Fuentes *et al.*, 1994). Apart from directly regulating RyR Ca^{2+} release, CaM also stimulates Ca^{2+} uptake. In cardiac muscle, the Ca^{2+}-bound CaM activates

phosphorylation of phospholamban, thus increasing the rate of Ca-ATPase activity and Ca^{2+} uptake (Tada and Inui, 1983).

1.7.2 Histidine-rich Ca^{2+} binding protein and sarcalumenin

Other luminal Ca^{2+}-sequestering proteins have been shown to be substrates for phosphorylases. The histidine-rich Ca^{2+} binding protein (HRC), which is a 165 kDa transmembrane protein that shares many similarities with CSQ, and can be phosphorylated both on the cytoplasmic and luminal sides (Damiani *et al.*, 1995; Orr and Shoshan-Barmatz, 1996). Sarcalumenin (SLM), is a 150 kDa protein, that is also involved in the luminal Ca^{2+} buffering system (Orr and Shoshan-Barmatz, 1996; Shoshan-Barmatz *et al.*, 1996b). Unlike CSQ, which is localized in junctional SR, HRC and SLM are distributed throughout the SR (Seiler *et al.*, 1984; Leberer *et al.*, 1990). Functionally, the roles of HRC and SLM are not well understood, but it is suggested that they may be responsible for non-junctional Ca^{2+} buffering (Cala *et al.*, 1990). Both HRC and SLM are thought to modulate RyRs, in a phosphorylation-dependant manner (see Section 1.7.2.1), although no evidence of direct interaction with the RyR exists. The 53 kDa protein characterized by Leberer *et al.* (1989) appears to be a splice variant of SLM RNA. This variant is missing the second glycosylation site found in SLM and is capable of binding Ca^{2+}.

The CK II-induced phosphorylation of both SLM and HRC (which is a Ca^{2+}-dependant process) inhibits both skeletal and cardiac RyR channel activity and the binding affinity for Ca^{2+} of the RyR (Orr *et al.*, 1991; Shoshan-Barmatz *et al.*, 1996b; Hadad *et al.*, 1999). Like triadin, the HRC can also be phosphorylated by CaM-Pki on the cytoplasmic side of the protein (see Section 1.5.2 and Damiani *et al.*, 1995).

1.7.3 Calreticulin

Calreticulin is a member of the reticuloplasm family. It is considered to be a homolog of CSQ and is present in the endoplasmic reticulum of many proteins (Fliegel *et al.*, 1989a). Like CSQ, calreticulin binds Ca^{2+} with a high capacity; binding 20 – 50 mol Ca^{2+}/mol protein (CSQ: 50 mol/mol), with a kDa of 1-4 mM (CSQ: 1 mM; Pozzan *et al.*, 1994 and references therein). Calreticulin has a similar molecular mass to CSQ (46 kDa compared with 43-45 kDa, respectively) and originally the tissue distribution of

these two Ca^{2+} sequestering proteins was difficult to distinguish. Calreticulin's highly acidic C-terminus contains multiple doublet and triplets of negatively charged residues (Fliegel *et al.*, 1989b), and presumably is a region of multiple Ca^{2+} binding. Calreticulin was first sequenced in liver endoplasmic reticulum (Cala *et al.*, 1990 and references therein) and is only found in trace amounts in skeletal muscle (Ostwald *et al.*, 1974). It therefore probably plays only a minor role in luminal SR Ca^{2+} sequestering.

1.7.4 90 and 100 kDa proteins

The appearance of both a 90 and 100 kDa protein in cardiac muscle, identified as probable Ca^{2+} binding proteins by Stains-ALL (a stain specific for Ca^{2+} binding proteins), illustrates the potential of other Ca^{2+} buffers to playing a part in luminal SR Ca^{2+} homeostasis (Jones *et al.*, 1979b; Jones and Cala, 1981; Cala *et al.*, 1990). Any role played by these uncharacterised proteins is yet to be determined.

1.8 Protein-Protein interactions

1.8.1 RyR and DHPR

The RyR and the DHPR are two proteins known to interact in ECC and there is particular emphasis at present in determining which residues of the RyR and DHPR interact with each other. In 1997, Yamazawa *et al.* (1997) showed that the presence of the RyR D2 region (in particular Thr^{1357}-Pro^{1406}), was essential for ECC, but not essential for eliciting RyR Ca^{2+} release (using electrically evoked and caffeine-induced $[Ca^{2+}]_i$ measurements), although the substitution of the skeletal D2 region for the cardiac D2 sequence did not alter channel function. Although D2 is a region of high divergence between cardiac and skeletal RyRs, a stretch of four alternate negatively charged residues (skeletal Glu^{1327}-Glu^{1333}) is conserved in the cardiac D2 domain (Yamazawa *et al.*, 1997) This suggests that the D2 region may not be responsible for functional differences between cardiac and skeletal ECC, as cardiac ECC requires Ca^{2+} influx through the DHPR, rather than a physical DHPR/RyR interaction. RyR3 does not contain the D2 region and is unable to mediate skeletal ECC, and does not contain the D3 region (Yamazawa *et al.*, 1997).

Chimeric constructs of cardiac RyR with various inserts of the corresponding skeletal sequence showed two particular areas of the RyR responsible for normal RyR-DHPR coupling. The presence of Thr^{1635}-Phe^{2636} (found between regions D1 and D2) in an otherwise cardiac sequence restored, and enhanced the retrograde signal (Nakai *et al.*, 1998; see Section 1.3.2 for the region of DHPR required for retrograde signalling). The replacement of cardiac region (Thr^{2659}-Tyr^{3720}) with the corresponding skeletal region enhanced an already present retrograde signal (Nakai *et al.*, 1998). In addition, Proenza *et al.* (2002) have shown that the sequence containing residues Gln^{1837}-Asn^{2168} of RyR1 help contribute to skeletal-type ECC (although are not essential). These studies illustrate that regions of the RyR that interact with the DHPR are different in retrograde and orthograde (DHPR signalling of RyR Ca^{2+} release) signalling. Seemingly, various regions of the RyR are involved in functional coupling with the DHPR.

1.8.2 RyR and triadin

First shown to interact with the RyR by protein overlay (Brandt *et al.*, 1990; Kim and Caswell, 1990), triadin's luminal domain was identified as the critical region required for triadin/RyR association (Guo and Campbell, 1995). Since this was published, triadin has also been reported to bind to the RyR in a Ca^{2+}-regulated fashion; in the cytoplasm the interaction is optimal at $\leq 10\mu M$ and abolished at higher [Ca^{2+}] (Groh *et al.*, 1999). Residues Asp^{18}-Ser^{46} are essential for binding of triadin to the RyR in the cytoplasm (Groh *et al.*, 1999), although the specific binding sequence is not yet known.

Single channel studies illustrate the role of triadin in RyR activity. Addition of triadin to the cytoplasmic side of bilayer-incorporated purified RyRs resulted in an almost total abolition of channel activity, indicating triadins ability to strongly inhibit RyRs by binding to the cytoplasmic domain (Ohkura *et al.*, 1998). Triadin had no such affect on RyR activity when added to the luminal chamber (Ohkura *et al.*, 1998), suggesting that any interluminal interactions do not regulate the RyR. It seems likely that triadin is bound to the RyR on both the cytoplasmic and luminal sides, although how this affects overall activity of the RyR is still unclear.

1.8.3 RyR and junctin

A peptide corresponding to the luminal domain of junctin was found to bind to the purified RyR, but no interaction was observed with the small cytoplasmic terminus of junctin (Zhang *et al.*, 1997). RyRs interaction with the luminal domain of junctin does not require the presence of Ca^{2+}. Similarly, junctin and triadin can also bind to one another in a Ca^{2+}-independant manner (Zhang *et al.*, 1997).

1.8.4 RyR and SLM/HRC

No physical interaction between the RyR and either SLM or HRC has been demonstrated. As both the SLM and the HRC are believed to modulate the RyR, this raising the possibility that their effects might be exerted through CSQ, triadin or junctin (the only other known luminal SR RyR-binding proteins), although such interactions have not been shown (Hadad *et al.*, 1999).

1.8.5 DHPR and Triadin

Triadin was originally believed to provide a pathway for signal transduction between the DHPR and the RyR, since triadin was shown to associate with the DHPR, using an overlay assay (Brandt *et al.*, 1990), whilst an antibody to triadin was reported to inhibit the slow phase of depolarisation-induced Ca^{2+} release through the RyR (Brandt *et al.*, 1990).

Current opinion on membrane topology of triadin is that the relatively small number of residues existing in the cytoplasm are not substantial enough to mechanically couple the RyR and the DHPR (Knudson *et al.*, 1993b; Marty *et al.*, 1995). Knudson *et al.* (1993a) and Knudson *et al.* (1993b) suggest that the interaction reported by Brandt *et al.* (1990) was non-specific, and Guo and Campbell (1995) were unable to associate peptides corresponding to either the cytoplasmic or luminal triadin domains with the DHPR. Any hypothesis involving an interaction between the DHPR and triadin is still controversial and relies heavily on the existence of an extended cytoplasmic domain (Fan *et al.*, 1995), which is in conflict with the accepted membrane topology of triadin (which places only a short segment on the cytoplasmic side of the RyR).

1.8.6 CSQ and triadin

The luminal region of skeletal triadin contains 46 basic residues, making it a prime target for anchoring the highly acidic CSQ to the SR membrane (Knudson *et al.*, 1993b; Guo and Campbell, 1995; Zhang *et al.*, 1997). Indeed, skeletal CSQ associates with triadin in affinity chromatography experiments (Guo and Campbell, 1995), as does cardiac CSQ (Zhang *et al.*, 1997). In cardiac triadin-1, the CSQ binding domain has been localized to a 25 amino acid Lys-Glu-Lys-Glu, known as the KEKE motif; this interaction being most stable at low [Ca^{2+}] (Kobayashi *et al.*, 2000). Binding to any of the abundant charged residues of CSQ would create a so-called 'polar zipper', associating these two proteins and, since triadin binds to the RyR, CSQ would be tethered close to the channel. The association of skeletal CSQ with triadin is thought to be identical to that in cardiac muscle, since skeletal and cardiac triadin have similar binding motifs (Arg^{210} – Lys^{224}).

1.8.7 CSQ and junctin

CSQ binding to junctin is Ca^{2+}-dependant to some degree; Zhang *et al.* (1997) found optimal interaction in the absence of Ca^{2+}, although a significant interaction occurs even at mM Ca^{2+}. It remains to be seen whether or not small fluxes in luminal Ca^{2+} occurring in the contraction/relaxation cycle may alter CSQ-junctin interaction. Although junctin has numerous KEKE motifs (the residues of triadin that bind to CSQ), no definitive site for CSQ binding has been identified (Kobayashi *et al.*, 2000), and therefore the way in which junctin anchors CSQ remains unclear.

1.8.8 Triadin and HRC

The HRC (see Section 1.7.2) forms a Ca^{2+}-dependant association with rabbit skeletal triadin (Kim and Caswell, 1990). HRC immunoprecipitation studies show that the HRC/triadin interaction is greatest in the absence of Ca^{2+} and is inhibited by [Ca^{2+}] > 1 mM (Lee *et al.*, 2001). Lee *et al.* (2001) have identified nine tandem repeats in the HRC, consisting of histidine-rich sequences, capable of aiding an association with triadin, whilst HRCs binding site is found within residues Glu^{205}-Lys^{260} of triadin. This stretch of amino acids also contains the proposed CSQ binding KEKE motif.

1.9 RyR regulation by CSQ and CSQs role in ECC

The regulation of RyRs by CSQ is somewhat controversial and unresolved. An initial study by Kawasaki and Kasai (1994) showed that addition of CSQ to native RyRs (incorporated into lipid bilayers), previously exposed to high [EGTA], caused a marginal increase in channel activity. Ohkura *et al.* (1995) and Ohkura *et al.* (1998) reported that CSQ significantly increased single channel P_o. In contrast to other reports, Beard *et al.* (2000), Wang *et al.* (2001) and Beard *et al.* (2002) showed that increasing luminal [Ca^2] to levels believed to disrupt CSQ-triadin-junctin interactions (Zhang *et al.*, 1997; Beard *et al.*, 2002), activated RyRs two-fold, suggesting that CSQ is a RyR inhibitor. Ohkura *et al.* (1995) conducted native RyR experiments seemingly in the absence of any luminal Ca^{2+} (luminal [Ca^{2+}] was not clear in the report). It is thought that for CSQ to be attached to the RyR (via junctin and triadin) [Ca^{2+}] should be between 0.01 – 1 mM (Zhang *et al.*, 1997; Wang *et al.*, 1998). This suggests that the reported increase in channel activity seen by Ohkura *et al.* (1995) after the addition of CSQ, may not be due to the tethering of CSQ to the RyR by triadin and junctin. The effect may have been due to a direct RyR-CSQ coupling, shown to activate ryanodine receptors (Herzog *et al.*, 2000; Szegedi *et al.*, 1999). There had been no systematic study of the interaction between CSQ and native RyRs at the time the work for this thesis commenced.

Chapter 2

MATERIALS AND METHODS

2.1 Materials

The polyclonal anti-CSQ antibody was obtained from Swant Chemicals (Switzerland), whilst the monoclonal anti-triadin antibody was kindly donated by Professor Kevin Campbell, Howard Hughes Medical Institute, University of Iowa College of Medicine, Iowa City, Iowa, USA. The anti-sheep, anti-rabbit HRP, and alkaline phosphatase antibodies were purchased from Proscitech (Thuringowa, Australia) and Amersham (Baulkam Hills, Australia). All Nunc Immunoplates and PVDF membrane were obtained from Millipore (Lane Cove, Australia). The high and low molecular weight markers, Silver Stain Plus kit, Novex dry ease mini cellophane and Bio-Rad $^{D}{}_{C}{}^{©}$ protein assay kit were from Bio-Rad (North Ryde, Australia) The spectra/por cellulose ester membrane was from Edwards Instrument Company (Narellan, Australia), HYPER ECL film and ECL reagents from Amersham (Baulkham Hills, Australia), Gelcode© phosphoprotein staining kit from Selby-Biolab (Clayton, Australia), centricon centrifugal concentrators from Amicon (Fawkner, Australia), chromophores D-16 and F-2181 were from Molecular probes (Oregon, USA), and all lipids from Avanti Polar Lipids (Alabama, USA). All other chemicals were from Sigma-Aldrich (Castle Hill, Australia).

2.2 Methods

A number of membrane preparations were isolated throughout this study. Two methods were employed to isolate SR vesicles. RyR enriched SR vesicles (termed terminal cisternae) were isolated from muscle homogenates, and used in single channel bilayer experiments. These vesicles are considered more pure than a heavy SR preparation, which would also contain vesicles enriched with t-tubules and longitudinal SR. In brief, chopped muscle underwent a low g-force centrifugation step, removing cell debris, followed by homogenisation, designed to disrupt the SR structure. The microsomal pellet (heavy or crude SR) was collected after centrifugation. The resultant crude SR pellet was further purified by sucrose gradient centrifugation (27-45% sucrose), which

separated longitudinal SR from terminal cisternae; the latter being the part of the SR that faces the t-tubule and contains the RyR. The first fraction (occurring at the 27-32% interface) contains only longitudinal SR, whilst the fraction at the 32/34% interface is mainly longitudinal SR, with a small amount of terminal cisternae. The fraction found at the 34/38% interface contains terminal cisternae, with some longitudinal SR. The final fraction, at the 38-45% interface is highly enriched in terminal cisternae.

Either SR vesicles or heavy SR were used as a starting preparation in the CSQ isolation procedure, depending on the method used for isolation. The terminal cisternae of SR contains two membrane components, the JFM and the calcium-pump (Ca^{2+}-ATPase) membrane. Two methods were used to isolate CSQ, using either high [NaCl] or high and low [Ca^{2+}] to dissociate CSQ from JFM. Exposure of the terminal cisternae of SR to Triton-X 100 (in the presence of Ca^{2+}), results in the formation of an insoluble pellet of JFM, leaving Ca^{2+}-ATPase in the supernatant (Costello *et al.*, 1986). Addition of 500 mM NaCl to the JFM disrupts ionic interactions (Ikemoto *et al.*, 1989), tethering CSQ to the RyR/triadin/junctin (RyR/T/J) complex, allowing select dissociation of CSQ from this complex. In the first method (high [NaCl] exposure), CSQ's ionic binding properties have been exploited to successfully dissociate this protein from the JFM. In the second method, increasing luminal Ca^{2+} to >10 mM or addition of Ca^{2+} chelators such as EGTA (which subsequently lowers Ca^{2+}_{free}), promotes CSQ dissociation from the RyR/T/J complex (Zhang *et al.*, 1997; Wang *et al.*, 1998), again allowing CSQ's removal. Using Ca^{2+} as a dissociation agent provided a higher yield of CSQ, but sometimes required extra purification steps, whereas NaCl dissociation although not providing such a high yield, yielded CSQ with few contaminating proteins.

To determine direct effect of exogenous CSQ on RyR activity, without co-proteins such as, triadin and junctin present, RyRs were purified from solubilized RyR enriched SR. SR components were separated by fractionation after sucrose gradient centrifugation and RyRs isolated. Purified RyRs do not contain any contaminant triadin, junctin or CSQ (see Ch. 3, Fig. 3. V and Ch. 5, Fig. 5.I) and this preparation was used in CSQ's effects on the channel protein itself, rather than effects via interactions with associated co-proteins.

Procedures 2.3.1 – 2.5.2 were carried out at 4 °C unless otherwise stated.

2.3 SR vesicle preparation

2.3.1 RyR enriched SR vesicle preparation

SR vesicles were prepared according to Chu *et al.* (1988), with minor modifications (Ahern *et al.*, 1994; Ahern *et al.*, 1997b). Mrs S. Pace and Mrs J. Stivala, John Curtin School of Medical Research, Australian National University, Canberra, Australia undertook this preparation. New Zealand male white rabbit back and leg muscle was removed and rinsed in phosphate buffered saline, containing (mM): 137 NaCl, 7 Na_2HPO4, 2.5 $NaH_2PO_4.H_2O$. Once excess fat was removed, the tissue was cut into small pieces and 'snap' frozen in liquid nitrogen and then stored at -70 °C. Fifty gram aliquots of tissue was homogenised in a Waring Blendor (Waring, Connecticut, USA) - 2 x 30 sec on high, 2 x 30 s on low - in *SR buffer1*, containing (mM): 20 imidazole, 300 sucrose, pH 7.4. In all cases, pH was adjusted using a TPS digital pH meter (Bacto Laboratories; Lane Cove, Australia). The homogenate was centrifuged at 11 000 g in a Beckman JA-14 rotor (Beckman Instruments; Gladesville, Australia) for 20 min. The resultant pellet was resuspended in *SR buffer 1* and then homogenised, washed and centrifuged as above. After filtering the supernatant through several layers of cotton gauze, the sample was centrifuged at 100 000 g in a Beckman Ti-45 rotor (Beckman Instruments; Gladesville, Australia) for 1 – 2 h. The heavy SR pellet was resuspended in 3 – 4 ml of *SR buffer 1* and homogenised in a dounce teflon homogeniser (Edwards Instrument Company; Narellan, Australia) with 40 ml of *SR buffer 1*. The homogenate was added to a sucrose density gradient, containing 45%, 38%, 34%, 32% and 27% (w/v) sucrose layers (suspended in 20 mM imidazole, pH 7.4, plus a *standard protease inhibitor solution*, (mM): 1 benzamadine, 0.5 PMSF and (μg/ml) 1 leuopeptin, pepstatin-A), allowing sub-fractionation of vesicles, after centrifugation at 40 000 g in a Beckman SW-28 rotor (Beckman Instruments, Gladesville, Australia) for 16 h. The band appearing at the 38 – 45% interface (RyR enriched fraction) was collected and diluted with 2 volumes of 20 mM imidazole (pH 7.4). Following centrifugation of the diluent at 100 000 g in a Beckman Ti-45 rotor for 1 h, the final pellet was resuspended in 1 ml of *SR buffer 1* with 2 mM DTT (included in a small number of preparations) and *standard protease inhibitor solution*. Aliquots of 15 μl were stored at -70 °C.

2.3.2 Heavy SR preparation

Heavy SR was isolated according to Kim *et al.* (1983). Briefly, 100 - 200 g of muscle tissue was homogenised in a Waring Blendor (4 x 30 s on high speed) in 4 volumes of 2.5 mM NaOH, pH 6.8. The muscle homogenate was centrifuged at 10 000 g in a Sorval GSA rotor (Kendro Laboratory Products; Lane Cove, Australia) for 3 min (to remove residual fat and cell debris) and after a pH adjustment to 6.8, the supernatant was filtered through 8 cotton gauze swabs, followed by a final filter through filter paper (Whatmans no. 2). The filtrate was centrifuged at 17 000 g in a Sorval GSA rotor for 30 min. After homogenising the pellet in 4 volumes of a solution containing (mM): 150 KCl and 20 MES (pH 6.8), the suspension was then centrifuged as above, removing muscle contaminants. The above homogenisation and centrifugation step was repeated and heavy SR collected. Heavy SR was either stored in <5 ml aliquots at –70 °C, or kept on ice briefly (if further purification was to take place the same day).

2.4 RyR purification

RyRs were purified from SR vesicles using methods of Lai *et al.* (1988). SR vesicles were incubated at room temperature for 2 hr in a solubilization medium containing: 25 mM Pipes, 1 M NaCl, 0.5% (w/v) CHAPS/0.25% (w/v) PC, with *RyR specific protease inhibitors* (mM: 0.8 Benzamadine, 0.1 PMSF and µg/ml: 1 leuopeptin, 0.6 pepstatin A). After centrifugation for 15 min at 100 000 g using a Beckman TLA 100.3 rotor (Beckman Instruments; Gladesville, Australia), the solubilized muscle proteins were applied to a sucrose gradient of between 5 – 25% (containing: 5-25% (w/v) sucrose, 25 mM PIPES, 1M NaCl, 0.5% (w/v) CHAPS/ 0.25% (w/v) PC and *RyR specific protease inhibitors*) and ultracentrifuged in a Beckman TLA 100.3 rotor at 80 000 g for 16 hr. Fractions (2 ml) were collected and RyRs visualised after SDS-Page and Western blotting. Those fractions enriched with RyR were concentrated by centrifugation at 5 000 g for 30 min in a Sorval SS-34 rotor (Kendro Laboratory Products; Lane Cove, Australia) using a centricon-30 centrifugal concentrator. The concentrate was then dialyzed overnight against (mM): 500 NaCl, 10 NaPipes, 1 DTT, (µM): 100 EGTA, 200 $CaCl_2$, and 1.5 PMSF, pH 7.4 at 4 °C. Small aliquots (~ 100 µl) were stored frozen at -70 °C.

2.5 CSQ preparation

2.5.1 Dissociation of CSQ from heavy SR

CSQ was purified as described by Ikemoto *et al.* (1989). Heavy SR vesicles were suspended in a solution of (mM): 20 MES, 300 sucrose, plus *standard protease inhibitor solution* (see section 2.3.1) and solubilized using 0.5% (w/v)Triton X-100 (at 0 °C). After incubation for 20 min on ice (vortexing every 5 min), the suspension was then centrifuged at 330 000 g in a Beckman TLA 100.3 rotor for 15 min. The insoluble fraction (containing CSQ attached to JFM) was homogenized in a solution containing (mM): 20 MES, 500 NaCl plus *standard protease inhibitor solution* (pH 6.8), to dissociate CSQ. JFM was then removed by centrifugation at 330 000 g using a Beckman TLA 100.3 rotor for 15 min, leaving CSQ in the supernatant, which was then dialysed twice overnight (see section 2.5.3) against *CSQ storage buffer*, containing (mM): 1 $CaCl_2$, 100 KCl, 10 TES (pH 7.4), to remove residualTriton X-100. The final pellet was suspended at an approximate concentration of 1 mg/ml in *CSQ storage buffer* and stored in <100 µl aliquots at –70 °C.

2.5.2 Purification of CSQ from RyR enriched SR.

CSQ was purified from RyR enriched SR, using the methodology published by Costello *et al.* (1986), with minor changes (*personal communication*, Professor Cecilia Hidalgo, Instituto de Ciencias Biomedicas, Facultad de Medicina, Universidad de Chile, Santiago, Chile). SR vesicles were diluted to 3.3 mg/ml with *CSQ isolation buffer* (mM: 300 sucrose, 0.4 benzamadine, 20 MOPS-Tris, (pH 6.8) and 1 µg/ml leupeptin), with a final [Ca^{2+}] of 1 mM, and incubated on ice for 10 min. Triton X-100 was added to 0.5% (w/v), and the resultant suspension incubated on ice for 20 min, vortexing every 5 min. The suspension was centrifuged at 110 000 g for 15 min or 48 000 g for 1 hr, using a Beckman TLA 100.3 rotor. After resuspending the pellet at 1mg/ml in *CSQ isolation buffer*, the insoluble JFM was homogenized in a dounce glass homogeniser. To remove CSQ from the JFM, EDTA was added to a final concentration of 2 mM and incubated on ice for 20 min. The homogenate was again centrifuged at 110 000 g for 15 min and the supernatant, containing CSQ, was collected. Dependent on the level of purity obtained after EDTA incubation, in some cases CSQ was exposed to 500 mM NaCl,

centrifuged at 100 000 g for 15 min and the supernatant collected, to further purify CSQ.

Following two overnight dialysis steps (see section 2.5.3), the final pellet was suspended at an approximate concentration of 0.4 mg/ml in *CSQ storage buffer* (see section 2.5.1), in <100 µl aliquots at -70 °C. In a number of preparations, 20 mM $CaCl_2$ (in 10 mM TES, pH 7.4) was substituted for 2 mM EDTA (all other procedures remained the same).

2.5.3 Dialysis

To remove residual Triton X-100 and EDTA from CSQ, all preparations underwent overnight dialysis. Dialysis tubing (MW cut-off: 14 - 15 kDa) was soaked in 10% acetic acid (both inside and out) and following a MQ water rinse, was boiled for 10 – 15 min in 2% (w/v) $NaHCO_3$ and 0.02% (w/v) EDTA. Tubing was stored in 10 % (w/v) acetic acid until required. Dialysis was against *CSQ storage buffer* (see section 2.5.1), or on occasion, 10 mM TES and 1 mM $CaCl_2$. Less frequently, dialysis was undertaken using spectra/por cellulose ester membrane pre-prepared dialysis tubing (MW cut-off: 500 Da). In the chromophore study (see section 2.8.2), dialysis tubing with a MW cut-off of 10 kDa was used.

2.6 Electrophoresis and Western Blot

2.6.1 SDS-Page

Electrophoresis of SR vesicles, heavy SR and CSQ isolates on either 3-12%, 5-17% or 12% SDS polyacrylamide gels, was performed following methods of Laemmli (1970). Running gels were prepared, containing (%, w/v): 12, 3-12 or 5-17 bis-acrylamide, 0.025 ammonium persulphate, 0.1 SDS, 0.2 temed and 0.375 M Tris-HCl (pH 8.8). Once set, 2 ml of a stacking gel, containing (%, w/v): 3 or 4 acrylamide, 0.05 ammonium persulphate, 0.1 SDS, 0.2 temed and 0.125 M Tris-HCl (pH 6.8), was placed above the running gel. After diluting high and low molecular weight markers, SR vesicles, heavy SR and CSQ (protein diluted to ~ 1 mg/ml, equi volume with a buffer containing (%, w/v): 2.5 SDS, 10 glycerol, 5 mercaptoethanol, 0.02 bromophenol

blue and 62.5 mM Tris-HCL, pH 6.8), standards and protein were loaded onto the gel. Typically 10 µl of standard and between 5 – 25 µl of protein were loaded into each well. High molecular weight standards contained; Myosin (200 kDa), B-galactosidase (116.25 kDa), Phosphorylase B (97.4 kDa), Serum albumin (66.2 kDa) and Ovalbumin (45 kDa), whilst low molecular weight standards contained; Phosphorylase B (97.4 kDa), Serum albumin (66.2 kDa), Ovalbumin (45 kDa), Trypsin inhibitor (21.5 kDa) and Lysozyme (14.4 kDa). The gel underwent electrophoresis at 35 mA for ~ 1 h, in a buffer containing (mM): 25 Tris, 191 glycine and 0.1% (w/v) SDS.

2.6.1.1 Protein stain

Gels were stained in a Coomassie stain, containing (%, v/w): 0.1 Coomassie Brilliant Blue, 40 ethanol and 10 glacial acetic acid, for at least 30 min with gentle agitation. Excess stain was removed with a destaining solution, containing (%, w/v): 5 acetic acid and 20 ethanol (usually overnight, with one solution change).

2.6.1.2 Silver stain

Silver staining was carried out in accordance with manufacturers of the Silver Stain Plus kit (Bio-Rad; North Ryde, Australia). A silver stain is approximately 50 times more sensitive than a Coomassie stain, and was used when only small quantities of (or dilute) samples were available, or to determine the presence or absence of contaminant protein(s) in a protein isolate – for example, the presence of junctin in a purified CSQ sample (see Chapter 3, section 3.2.3 and Fig. 3.V). In brief, after washing in MQ water for at least 20 min, the gels were placed in a fixative enhancer solution containing 50 % (w/v) ethanol, 10 % (v/v) acetic acid and 10 % (v/v) fixative enhancer concentrate for 20 min, with gentle agitation. The gel was fixed to minimize diffusions of molecules into the gel and remove substances that may interfere with the oxidation/reduction step. The fixed gel was washed twice in MQ water (10 min each wash with gentle agitation) and placed in staining solution, containing (%, v/v): 5 silver complex solution, 5 reduction moderator solution, 5 image development reagent, and immediately before use, 2.5 % (w/v) development accelerator solution. In acidic conditions, silver nitrate reacts with proteins. Upon addition of formaldehyde (in alkaline conditions) and its subsequent oxidization, silver ions are reduced to metallic silver. The reduction of silver ions was continued (aided by the buffering of formic acid produced by

formaldehyde oxidization by sodium carbonate) until sufficient band visualization was achieved. Amino acids lysine, arginine, methionine and cystine are strongly complexed with silver ions. Typically, gels were developed for 10 – 15 min (with gentle agitation) before desired protein band visualization. The reaction was stopped by immersing the gels immediately in a 5% (w/v) acetic acid solution, for at least 15 min. Gels were washed three times in MQ water and then dried (see section 2.6.1.3). Gels were processed in a tank using ~ 50 - 100 ml of solution.

2.6.1.3 Gel drying

After several washes in MQ water, the gels were dehydrated in ~50 ml of *dehydrating buffer*, containing (%, w/v) 5 glycerol, 10 ethanol, with gentle agitation for at least 30 min. The gel was then sandwiched between two sheets of Novex dry ease mini cellophane, which had been exposed to *dehydrating buffer* for ~ 1 min, before pressing in a Novex mini gel drying apparatus (Bio-Rad; North Ryde, Australia). After 24 h pressing, gels were stored in plastic sleeves, protected from light and moisture.

2.6.2 Western blot

As reported by Towbin *et al.* (1992). All steps were completed at room temperature, except where indicated otherwise. Upon completion of electrophoresis, the gel was transferred to a transblot cassette (Bio-Rad; North Ryde, Australia) and a PVDF membrane was placed over the gel. The cassette was clamped in place in a transblot semi-dry cell (containing an ice pack; Bio-Rad; North Ryde, Australia) and electrophoretically transferred at 100 V for 1 h at 4 °C, to ensure sufficient protein transfer, in a transfer buffer containing (mM): 25 Tris, 192 glycine (pH 8.3) and 20% (w/v) ethanol.

Once transferred, the membrane was blocked for 1 h in a 5% (w/v) skim milk solution in *TTBS* (containing (mM): 20 Tris, 500 NaCl and 0.5 % (v/v) Tween-20, pH 7.4), by rotation on a Glas-Col rotator (Glas-Col; Indiana, USA). Following a standard washing step (once for 15 min, and 3 times for 5 min, using fresh *TTBS* each wash), the blot was then exposed to a primary antibody solution, containing 25 µg/ml anti-CSQ, 25 –50 µg/ml anti-RyR or 1.75 µg/ml anti-triadin, in *primary antibody buffer*, containing 1% (w/v) BSA in *TBS* (20 mM Tris, 500 mM NaCl), for 1 h. After the standard washing

step was performed, the blot was incubated overnight at 4 °C (with rotation) in a *secondary antibody buffer* of *TTBS*, with 1% (w/v) bovine serum albumin, and 1: 1000 - 1:4000 (v/v) of either anti-sheep or anti-rabbit horse radish peroxidase (HRP) labelled or alkaline phosphatase labelled antibody. After two standard washing steps, the blot was ready for visualization. For Western blot of the RyR receptor, the following alterations to both the electrophoresis and Western blot procedures were made, (1) a 3-12% or 5-17 % gradient gel was used and (2) Western blot was at transferred at 150 V for the first h, and then decreased to 100 V for the next 2 h.

Occasionally, the blot was incubated in *primary antibody buffer* overnight at 4 °C, and following standard the washing step, was incubated in *secondary antibody buffer* for 1 h at room temperature (with all other steps as above). Typically, each membrane was washed or incubated in between 5 and 10 ml of appropriate solution.

2.6.3 Visualisation

Two methods were used to visualise Western blots, dependent on the type of secondary antibody available. For all blots published in this thesis, HRP labelled antibodies and the ECL development system was used (see section 2.6.3.1), due to its enhanced sensitivity over the alkaline phosphatase antibody development system.

2.6.3.1 HRP labelled antibody visualization

After Western blot, the PVDF membrane blot was placed in an ECL Western blotting detection reagent for 1 min. After exposure to autoradiographic HYPER ECL film in an x-ray film cassette (Kodak, North Ryde, Australia) for between 10 s to 3 min (to achieve visualization of immunoprobed protein bands), HYPER ECL film was developed in a Kodak X-Omat M20 film processor (Kodak, North Ryde, Australia).

2.6.3.2 Alkaline phosphatase antibody visualization

PDVF membrane blots (after Western blot), were washed a further 2 times in *TBS*, then equilibrated for 15 min in a *developer buffer* containing (mM): 100 NaCl, 100 Tris-HCL, pH 9.6. The blot was then rocked, protected from light in *developer buffer* plus (mM): 0.04 mM MgCl2, 300 µM nitrotetrazolium blue and 1.15 nM

5-Bromo-4-Chloro-3-indoxyl phosphate in dimethylsulphonoxide, until immunoprobed bands were distinctly visible (taking between 5 -15 min).

2.7 Protein Determination

All protein determinations (besides that determining the concentration of CSQ when chromophore was attached) were undertaken using the Lowry technique (Lowry *et al.*, 1951), using a Bio-Rad $^{D}{}_{C}$ protein Assay kit. As chromophore absorbs light at 655 nm, the Bradford method of CSQ protein determination was used (595 nm; Bradford, 1976).

2.7.1 Lowry method

Briefly, 5 µl of protein standards (BSA, 0 – 1.44 mg/ml), protein sample or sample buffer (minus protein) were added to a Nunc-Immunoplate 96 well plate. To each well, 20 µl of Reagent A (w/v: 95% alkaline copper tartrate, 5% surfactant) was added, with the plate shaken for ~20 sec on a titretek plate shaker (Flow laboratories; North Ryde, Australia). Two hundred ml of Reagent B (dilute Folins reagent) was then added and the assay was left to develop for at least 15 min. The protein assay was measured using a Bio-Rad micro-titre plate reader (Model 450; Bio-Rad, North Ryde, Australia) detecting light absorbance at 655 nm (reference cell: 405 nm). Protein concentrations were determined from a standard curve generated by Microplate Manager softwear.

2.7.2 Bradford method of protein determination for chromophore-attached CSQ

The *Coomassie Brilliant Blue Reagent* was prepared, according to Bradford (1976). One hundred mg of Coomassie Brilliant Blue was dissolved in 50 ml of 95% (w/v) ethanol and diluted to 1 l with MQ water. To this mixture, 100 ml of 85% (w/v) phosphoric acid was added. The mixture was stirred during the entire procedure. The reagent was kept for no more than two weeks at room temperature.

A standard curve for BSA, (0 - 1.44 mg/ml) was prepared by adding MQ water (where necessary) to a total volume of 0.1 ml. Chromophore -attached CSQ was diluted as appropriate in *CSQ storage buffer* (see section 2.5.1). Five ml of *Coomassie Brilliant*

Blue Reagent was added to the samples, *CSQ storage buffer* (sample blank) and the standards. The mixtures were vortexed and left to incubate at room temperature for 15 min. Absorbance of the samples and the standards were determined at 595 nm using a Cary 3 spectrophotometer (Varian; Frenchs Forest, Australia), against a protein blank (containing no BSA).

2.8 CSQ phosphorylation/dephosphorylation

2.8.1 Phosphorylation status determination.

Two methods were used to determine the phosphorylation status of CSQ. First, the Gelcode® Phosphoprotein staining kit was used. There was some uncertainty as to the sensitivity of this method, as the levels of detection achieved *in house* by the supplier (Pierce laboratories) were not obtained, and the positive control was labelled for phosphorous only very weakly. Secondly, ^{31}P NMR (section 2.8.1.2) was used preferentially because it has high enough sensitivity to determine whether CSQ was fully, partially, or not phosphorylated.

2.8.1.1 Gelcode© Phosphoprotein staining kit

Phosphoprotiens were detected using the GelCode® Phosphoprotein staining kit, following manufacturers instruction (Pierce; Illinois, USA). This kit stains phosphorylated proteins directly on SDS-Page gels, specifically serine and threonine residues within the protein, as the stain targets these phosphoproteins phosphoester linkages. Briefly, after electrophoresis (see section 2.6), the gel was placed in a solution of sulfosalicylic acid for 15 min, after which it was transferred to a sulfosalicylic acid and $CaCl_2$ solution, and agitated for 30 min. After a 20 min incubation at 65 °C in 0.5 M NaOH (and hydrolysis of any phosphoprotein phosphoester linkages and subsequent formation of an insoluble Ca^{2+}-P complex), the gel was treated with an ammonium molybdate solution for 10 min. Following a 20 min agitation in an ammonium molybdate and nitric acid solution, the resultant insoluble nitrophospho-molybdate complex (which binds to phosphorylated serines and threonines) stained green after incubation in methylene green for 15 min. Excess stain was removed by

bathing the gel in sulfosalicylic acid for 15 min. All incubations were done at room temperature in ~ 25 ml of appropriate solution.

2.8.1.2 ^{31}P NMR spectroscopy

This procedure was performed by Dr Marco Casarotto, John Curtin School of Medical Research, Australian National University, Canberra, Australia. CSQ phosphorylation status was determined using ^{31}P NMR spectroscopy. All spectra were acquired on Varian-Inova 500 spectrometer (Varian; Frenchs Forest, Australia) using a spectral width of 15000 Hz, a pulse width of ~15 μs, a spectral frequency of 202.421 MHz, and an acquisition time of 0.33 s. Samples were kept at a constant temperature of 5 °C. NMR samples were ~0.17 mM in an H_2O solution containing 10% D_2O/90% H_2O.

2.8.1.3 Dephosphorylation of CSQ

Acid phosphatase treatment was undertaken as described by Cala and Jones (1991). CSQ was diluted to 50 μg/ml in *CSQ storage buffer* (see section 2.5.1), and 1 mg was dephosphorylated at 30 °C for 30 min in (mM): 20 MES (pH 5.8), 3 EGTA, 0.1% (w/v) triton X-100 and 1 unit of acid phosphatase derived from white potato was used with 100 μg of CSQ . The sample was subject to ^{31}P NMR to detect phosphorylation status of the protein. Small aliquots were frozen in liquid N_2 and stored in *CSQ storage buffer* at –70 °C.

2.9 Fluorescent Labelling of CSQ

Chromophore attachment was undertaken by Professor David Jans and the Nuclear Signalling Laboratory; John Curtin School of Medical Research, Australian National University, Canberra, Australia. Pooled fractions of purified CSQ were concentrated in (mM): 10 TRIS pH 7.4, 100 KCl, and 1 $CaCl_2$, using a centricon-10 centrifugal concentrator. Concentrated CSQ was labelled with either fluorescent dye 5-[4,6-dichlorotriazinyl]aminofluorescein (known as D16) or 6- fluorescein-5-(and-6)-carboxamido)hexanoic acid, succinimidyl ester (5 (6)-SFX), known as F2181. D-16 is a fluoresine dichloratriazine, whilst F-2181 is a succinimidyl ester of fluorescein. D-16 preferentially attaches to serines and threonines, and F-2181 preferentially binds to lysines.

CSQ (0.3 - 1 mg) was incubated with D-16 in 45 mM borate buffer (pH 9.5, with NaOH) at room temperature for 2 h, protected from light. Chromophore attached CSQ was dialysed (see section 2.5.3) in *CSQ storage buffer* (see section 2.5.1) overnight, to remove unbound D-16. The sample was then concentrated using a centricon-10 centrifugal concentrator, and small aliquots stored on ice for immediate use, or frozen at – 70 °C. In the case of F-2181 attachment to CSQ, the following alterations to the above protocol were implemented; the pH of the incubation solution was adjusted to 8.2 with NaOH and the incubation time was reduced to 50 min. The concentration of chromophore labelled protein was determined using the Bradford assay (see section 2.7.2). To detect whether fluorescent labelling altered the RyR regulatory properties of CSQ, a standard re-association assay was performed in the bilayer experiments system. Refer to Fig. 2.I for probable attachment sites of chromophores. It was presumed that all residues targeted by chromophores were labelled by D-16 and F-2181, however this was not determined experimentally.

2.10 Antibody preparation

A polyclonal antibody to skeletal (predominantly) fast twitch fibre (from rabbit back and leg muscle) CSQ was raised in New Zealand male white rabbits. Rabbits were inoculated at four-week intervals, and in each inoculation approximately 1 mg of purified CSQ was used (which was suspended in an equi volume of adjuvant). Up to 200 µl of this suspension was administered to each inoculation site. In the first inoculation step, CSQ was mixed with complete adjuvant, whilst all following inoculants were suspended in incomplete adjuvant. Rabbits were bled from the portal vein in the ear, 9 – 13 days after inoculation, with up to 10 ml collected per rabbit per bleed.

Anti-serum from innoculated rabbits was separated from red blood cells by centrifugation for 10 min in a MSE bench top centrifuge at top speed (unknown, RPM, RCF or origin) at room temperature, and titrated against CSQ to detect levels of antibody present, using an Elissa assay. In brief, a 96 well plate (Nunc Immunoplate) was coated with 100 ng of purified CSQ by incubation (with gentle agitation via a titretek plate shaker) at room temperature. Following a 1 h incubation in 5% skim milk in *TTBS* (see section 2.6.2) which blocks any non-specific protein binding, all wells

were washed three times in *TTBS* and incubated for at least 1 h in anti-serum. Anti-sera dilutions (*in primary antibody buffer*: see section 2.6.2) from $^1/_2$ to $^1/_{2048}$ were used. Wells were washed as above, and anti-rabbit IgG (1/1000 dilution in *secondary antibody buffer*; see section 2.6.2) was added to each well, for 1 h. Wells were rinsed four times in *TTBS* and incubated, protected from light, in (mM): 100 Na_2HPO_4, 490 citric acid, pH 5.0 and 0.01% H_2O_2 (w/v) for 15 min before quantification. The reaction was stopped by the addition of 500 mM H_2SO_4, and the titre quantified spectrophotometrically using a Bio-Rad micro-titre plate reader (Model 450) at 495nm (reference 655 nm). On comparison of pre-immune and bleed titres, a change in OD of 0.5 was considered a significant antibody titre.

2.11 Planar lipid bilayers

2.11.1 Lipid bilayers

In early experiments, bilayer were formed from a lipid mixture containing PE, PS and PC (5:3:2, v/v). In later experiments, a mixture containing only PE and PC (8:2 v/v) were used. It should be noted that the presence of PS was not required for bilayer formation, neither did it appear to alter the integrity of the membrane. In addition, the properties of RyRs and their activation or inhibition by any agents used in this study were not altered by the presence or absence of this lipid (N. Beard, unpublished observation). Once prepared, the chloroform storage medium was evaporated using a stream of N_2, and the lipid mixture was resuspended in *n*-decane, at a concentration of 45-50 mg/ml. The lipid was 'painted' or applied across an aperture of 150 – 250 μm in the wall of a delrin cup, with a glass rod. The lipid thinned spontaneously or via gentle manual manipulation, forming a bilayer (Fig. 2.II A).

The thinning of the lipid film was monitored visually though 20 x magnification using a binocular microscope (Hd Scientific Supplies; Kings Park, Australia), and electrically monitored using the current response to a ramp of 1V/s to measure capacitance. The bilayer separated two chambers, denoted *cis* (cytoplasmic) and *trans* (luminal; see Fig. 2.II B).

1	eegldfpeyd	gvdrvinvna	knyknvfkky	evlallyhep	peddkasqrq	femeelilel
61	aaqvledkgv	gfglvdsekd	aavakklglt	eedsiyvfke	devieydgef	sadtlvefll
121	dvledpveli	egerelqafe	niedeiklig	yfknkdsehy	kafkeaaeef	hpyipffatf
181	dskvakkltl	klneidfyea	fmeepvtipd	kpnseeeivn	fveehrrstl	rklkpesmye
241	tweddmdgih	ivafaeeadp	dgyefleilk	svaqdntdnp	dlsiiwidpd	dfpllvpywe
301	ktfdidlsap	qigvvnvtda	dsvwmemdde	edlpsaeele	dwledvlege	intedddded
361	ddddddd					

Fig. 2.I. ***Position of chromophore attachment sites on CSQ.*** Amino acid sequence from Fliegel *et al*, 1987, accession number A25887. The probable attachment sites for D-16 (preferentially attaching to serine/threonine) are highlighted in blue, whilst the probable attachment sites for F-2181 (a chromophore preferentially attaching to lysine) are shown in red.

SR vesicles were added at a concentration of ~ 33 – 100 µg/ml to the *cis* chamber and stirred until vesicles were incorporated into the bilayer (Fig. 2.III). Vesicles fusion was promoted by the addition of mannitol (300 mM, *cis*) and increasing *cis* CsMS to 250 mM. Vesicle fusion is also promoted by the high osmotic strength (Niles and Cohen, 1987) and increasing *cis* $CaCl_2$ to 4 mM. More specifically, increasing *cis* $CaCl_2$ promotes the binding of vesicles to the membrane. Vesicle incorporation was indicated by the appearance of step changes in current that indicated channel activity.

Cis Cs^+ was 250 mM in all cases (230 mM CsMS and 20 mM CsCl) whilst 300 mM mannitol and 4mM $CaCl_2$ were present during vesicle fusion only in some instances. Mannitol was perfused from the *cis* chamber prior to experimental recording (as the effects of mannitol on RyR activity are undocumented) by exchange with at least 10 bath volumes of mannitol free *cis* solution.

Once RyRs had incorporated, either *cis* Ca^{2+} concentration was lowered to 100 nM, or the *cis* chamber was perfused with ~10 volumes fresh *cis* solution, to prevent further vesicle incorporation. One hundred nM [Ca^{2+}] is insufficient to activate RyRs (Chu *et al.*, 1993; Laver *et al.*, 1995), therefore 2 mM *cis* ATP was added to induce channel opening.

Miller and Racker (1976) show that in more than 99% of all incorporations, the vesicle incorporates such that the cytoplasm of the SR faces the *cis* chamber, whilst the SR lumen faces the *trans* chamber (Fig. 2.III). In this study, >99.5 % of channels responded in a way that indicated that the cytoplasmic surface faced the *cis* chamber. To test whether the RyR had orientated in this manner, channel response to activating agents such as *cis* Ca^{2+} and ATP were tested. These agents have very different affects on the cytoplasmic and luminal sides of RyRs, and can be used to determine channel orientation. Only channels responding in a way that indicated that the cytoplasmic side faced the *cis* chamber were analyzed.

2.11.2 Solutions

2.11.2.1 Standard solution

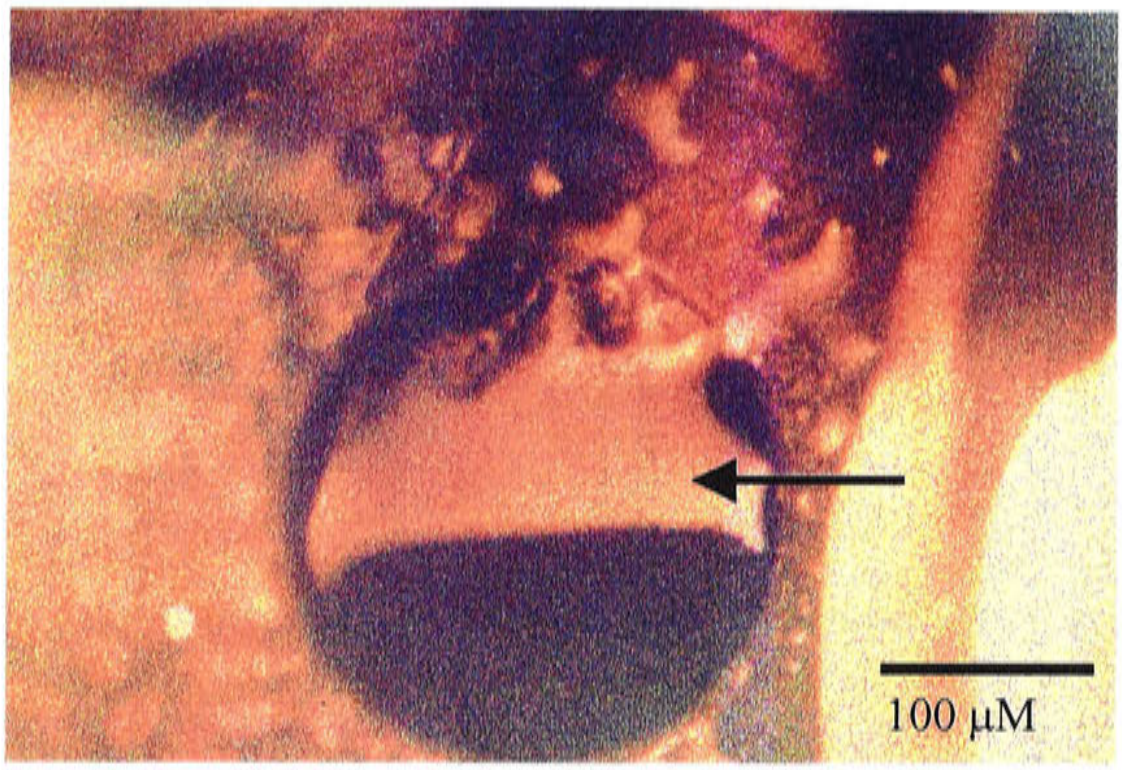
All solutions were made from analytic grade reagents using MQ water and were pH adjusted to 7.4 with CsOH, using a TPS digital pH meter. Standard incorporating solutions which were added to the *cis* of the chamber contained (mM): 230 CsMS, 20 CsCl, 1 $CaCl_2$ and 10 TES (final chamber volume of 1 ml, final pH 7.4) whilst the *trans* chamber contained (mM): 30 CsMS, 20 CsCl, 1 $CaCl_2$ and 10 TES (final volume of 1 ml, final
pH 7.4). Free [Ca^{2+}] was measured using an Ion83 ion meter (Radiometer; Copenhagen, Denmark). When lowering *cis* Ca^{2+} to ~100 nM, 4.2 mM BAPTA (a Ca^{2+} chelator) was added to the *cis* chamber.

Cs^+ was the chosen current carrier because firstly, Cs^+ has a higher conductance (525 pS in symmetric 250 mM Cs^+ (Laver *et al.*, 1995) than Ca^{2+} through the channel, therefore increasing signal-to-noise ratio. Secondly, Cs^+ has also been reported to block SR K^+ currents (Coronado *et al.*, 1992), ensuring that K^+ channels (abundant in muscle), were not recorded. CsMS was chosen to minimize the current through Cl^- channels. Twenty mM CsCl (*cis/trans*) was used because the RyR channel is sensitive to anion composition (Meissner, 1994). In addition the presence of chloride in the solution reduced junction potentials between the solution and the AgCl electrodes.

2.11.2.2 Drug addition

Drugs, ions, antibodies and proteins were added to either the *cis* or *trans* chamber from stock solutions, the compositions of which are listed in Table 2.I. All drugs were stored at -20 °C, except for CsMS, ryanodine and ruthenium red, which were all stored at 4 °C. Antibodies were stored in a working solution at 4 °C for daily use. SR vesicles and CSQ were stored at –70 °C and thawed immediately before use. As pH changes can alter RyR activity (Laver *et al.*, 2000), additions of non-buffered drugs were added to bilayer solutions in a test tube, and pH measured. Variations in pH did not exceed 7.4 ± 0.1; changes in this range would not have any direct effects on RyR activity (Laver *et al.*, 2000).

A



B

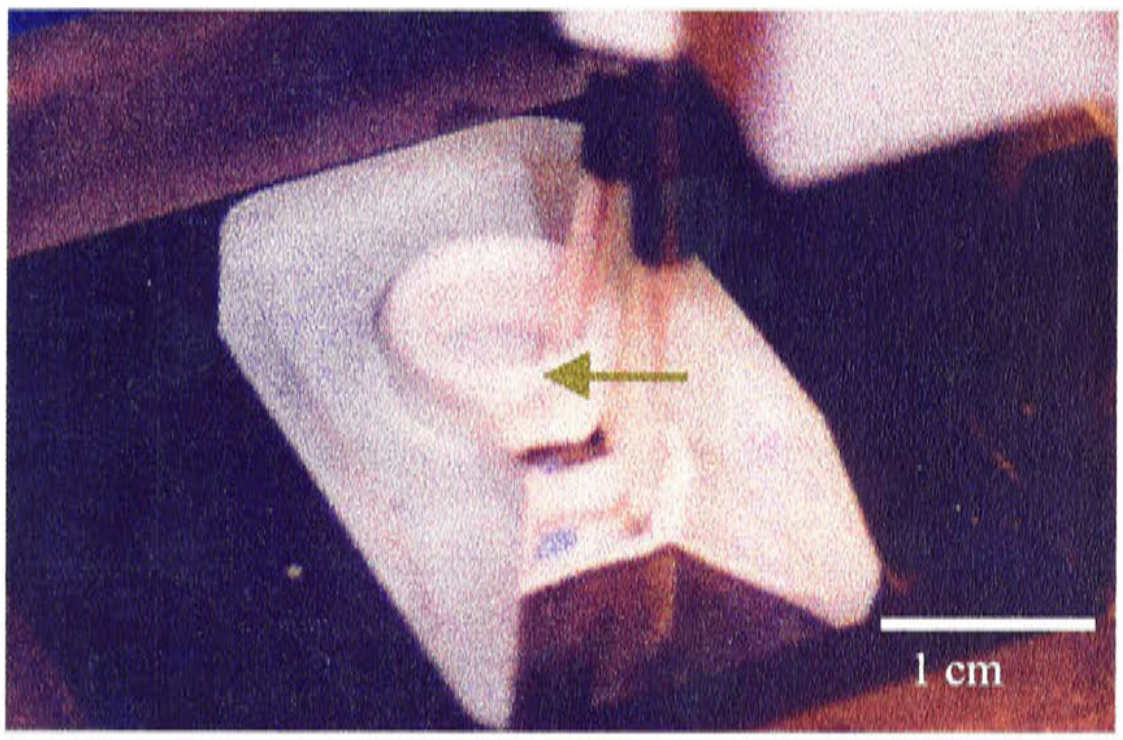


Fig 2.II. A. ***Formation of a black lipid bilayer.*** After lipid mixture is applied across the aperture in a delrin cup, lipid film can be seen (deflecting light) in the upper half circle (arrow). Photograph by D.R.Laver. **B**. Depiction of chamber set up, showing delrin cup (which constitutes *cis* chamber), containing the aperture across which the bilayer is formed, inserted into the chamber holder, forming the *trans* chamber. Note electrodes (arrow), connecting the solution in the chambers with the head stage. Photograph by M.M Sakowska.

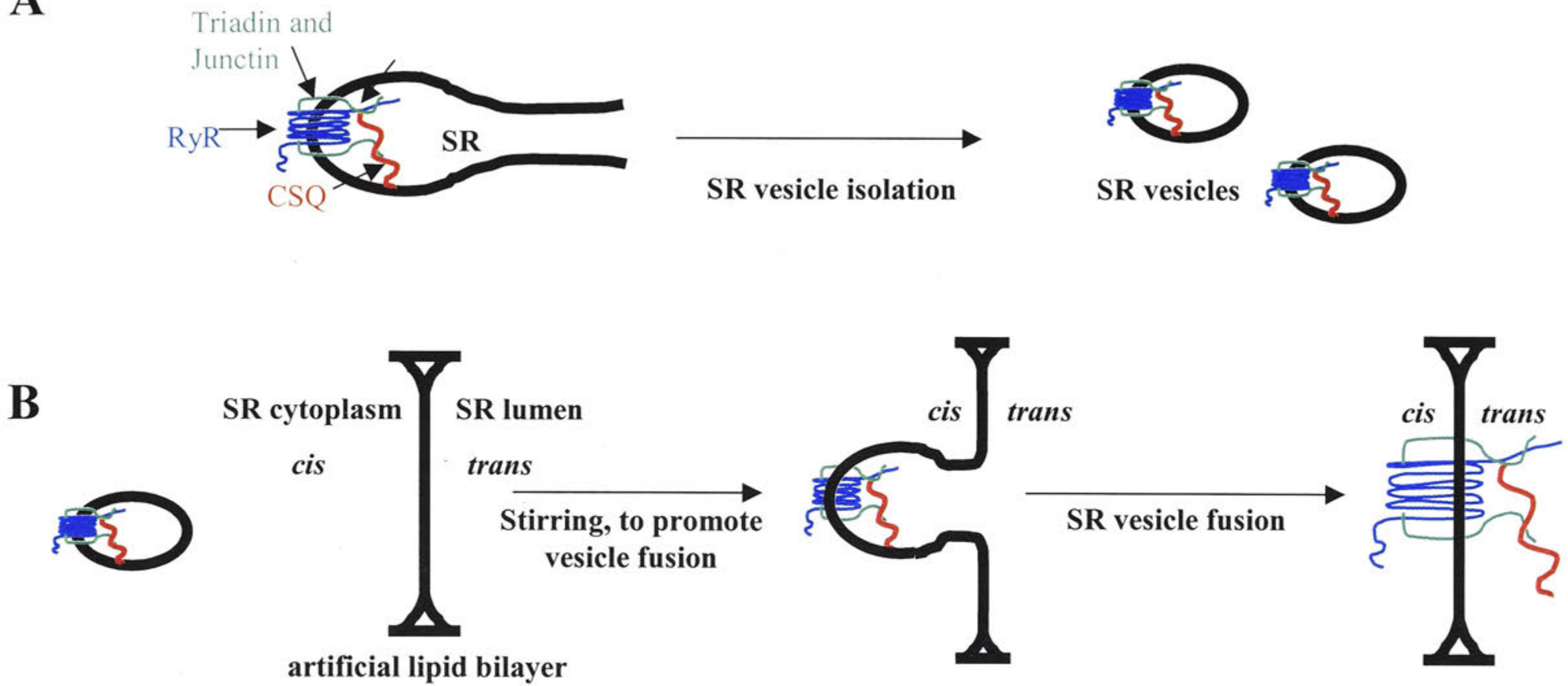


Fig. 2.III. Diagram of ***RyR orientation.*** **A**.After vesicle isolation (section 2.10.2), the SR lumen (where CSQ is located) is enclosed within the vesicle. **B.** Upon fusion of the vesicle with the artificial lipid bilayer, the cytoplasmic side of the RyR always faced the *cis* chamber, whilst the SR lumen faced the *trans* chamber. Adapted from Sakowska, 2000

Solution	Composition
Anti-CSQ	25 μg/ml in *1° antibody buffer* with 0.02% (w/v) NaN_3
Anti-triadin	14 μg/ml in *1° antibody buffer* with 0.02% (w/v) NaN_3
ATP	200 mM in 10 mM TES, pH 7.4
BAPTA	100 mM
$CaCl_2$	200 mM, in 10 mM TES, pH 7.4
CsMS	1.5/2 M, pH 7.4
CSQ	0.2 - 1mg/ml, in 1 mM $CaCl_2$, 100 mM KCL, 10 mM TES, pH 7.4
$MgCl_2$	200 mM, in 10 mM TES, pH 7.4
NaN_3	1%, w/v
Ruthenium red	1 mM
Ryanodine	1 mM

Table 2.1. ***Components of stock solutions used.*** All solutions added to the *trans* or *cis* chamber were done from the above stock solutions. In all cases, pH was adjusted with CsOH at room temperature. BAPTA and ruthenium red were stored protected from light, due to their light sensitivity.

After addition of each drug, ion, antibody or protein, the bath was stirred for at least 10 s, to ensure even dispersion through the solution. In the case of CSQ and anti-CSQ antibody additions, if it appeared that binding had not occurred (i.e. no change in channel activity was observed), stirring was initiated for 15 s every 1 or 2 min, to minimize the protein or antibody settling at the bottom of the chamber.

2.11.2.3 Solution exchange

A back-to-back perfusion set up allowed solution exchange from either chamber, without altering chamber volume. Old solution was simultaneously removed from the chamber while fresh solution was added to the chamber. Perfusion of 10 bath volumes (~ 10 ml) results in a dilution of 1000 (C. Haarmann, unpublished observation). In the case of experiments examining Ca^{2+} regulation of RyRs, the $[Ca^{2+}]$ is of upmost importance. In the most extreme case, where *trans* solution containing 13 mM Ca^{2+} was replaced with standard *trans* solution (containing 1 mM Ca^{2+}), a 10 volume perfusion of the *trans* chamber would result in a $[Ca^{2+}]$ of ~ 1.013 mM.

2.11.3 Channel recording

During vesicle addition and incorporation, prior to channel recording, standard *cis* and *trans* solutions contained (mM): 20 $CsCl_2$, 10 TES, 1 $CaCl_2$, 230/30 CsMS (*cis*/*trans*). After channel incorporation, control channel activity was recorded, after the addition of 2 mM ATP and occasionally 3 mM $MgCl_2$ to the *cis* chamber and 200mM CsMS added to the *trans* chamber to give symmetrical solutions. All other agents were added to the *trans* chamber, except for ruthenium red, ryanodine and in a small number of channels, CSQ.

To confirm that the recorded channel activity was from RyRs and not from another type of ion channel (such as a K^+ or Cl^- channel), in addition to selecting Cs^+ as the conducting ion and MS as the major anion, specific compounds ruthenium red (*cis*, 10 μM; Ma, 1993), or ryanodine (*cis*, 10 μM; Carroll *et al.*, 1991) were routinely added at the end of the experiment.

The *trans* chamber was held at virtual ground, and voltage applied to the *cis* chamber. However, in accordance with physiological convention, bilayer potential is expressed as:

$$V = V_{cytoplasmic} - V_{lu\ min\ al} \qquad \text{[Eq. 2.10-1]}$$

During recording, the voltage was changed from +40 to – 40 mV (and vice versa) at each 30 s time interval to determine the possible voltage-dependence of effects on channel activity. All recordings were done in symmetrical solutions (with respect to Cs^+, Cl^- and MS), except for ionic dissociation experiments, where *trans* ionic strength was increased to 500 mM. To account for these differences in ionic conditions, $V_{holding}$ was always +/- 40 mV from the reversal potential for Cs^+.

Electrical connections with the chambers were made via silver chloride coated silver wire electrodes, connected to an amplifier head stage. In some cases, electrodes were immersed in salt bridges (2% (w/v) agar in, 1 mM TES, 1 mM $CaCl_2$ and 250/50 CsCl (*cis*/*trans*)), which minimized changes in liquid junction potentials arising from a change in the composition of the solution. Electrodes were connected to an amplifier head stage. Currents were recorded and the voltage was controlled by an Axopatch 200A amplifier (Axon Instruments; California, USA).

Data were recorded at a bandwidth of 5 kHz on videotape, using pulse code modulation (SDR Clinical Technology; Middle Cove Australia). Prior to analysis, the current signal was replayed through a 1 kHz filter, and then sampled at 2 kHz. On fewer occasions, data were filtered at 5 kHz, then sampled at 10 kHz and stored directly on computer (refer to Fig. 2.IV).

2.12 Data analysis

Channel events were detected using a program developed *in house*, Channel 2 (developed by Professor Peter W Gage and Mr Michael Smith, JCSMR). Channel openings and closures were detected on a 30 s recording and used to calculate the following:

$$\text{Open probability } (P_o) = \frac{T_{open}}{T_{time}} \qquad \text{[Eq. 2.11-1]}$$

$$\text{Mean open time } (T_o; \text{ ms}) = \frac{T_{open}}{n} \qquad \text{[Eq. 2.11-2]}$$

$$\text{Mean closed time } (T_c; \text{ ms}) = \frac{T_{closed}}{n} \qquad \text{[Eq. 2.11-3]}$$

$$\text{Open frequency } (F_o; \text{ events/s}) = \frac{n}{T_t} \qquad \text{[Eq. 2.11-4]}$$

In the above equations, T_{open} is the total channel open time, T_{closed} is the total channel closed time, T_{time} is total time (s) of analysed record, and n is the number of events. The threshold detector for events detection was set at either 25 or occasionally 50 % of the maximum single channel conductance, with a closed threshold of 12.5 or 25% (respectively). Discriminators of 25/12.5% allow detection of subconductance levels observed in RyRs. In some cases where indicated, T_c was analysed within bursts. A burst is defined as a group of events terminated by ≥1s of closure. Mean current (I, pA), was also measured and is the average of all data points in a 30 s recording.

Channel activity was assessed either from P_o or fractional mean current (I'_F; I'/maximal current) and in all cases was measured from ≥ 30 s of continuous data. It is common for more than one RyR to incorporate into a bilayer and T_o, T_c and F_o were not analysed for multichannel recordings.

2.13 Statistics

Average data are presented as mean ± SEM when n>3, or mean ± SD when n=3. Individual data are presented where n<3. A paired Students *t*-test was used to detect significance of the difference between control and test values from a single set of channels. Where appropriate, the non-parametric sign test was used to assess significance. To test for the difference of means of two independent samples, either the Mann-Whitney or Students *t*-test for unpaired data was used. A p value of <0.05 was considered significant.

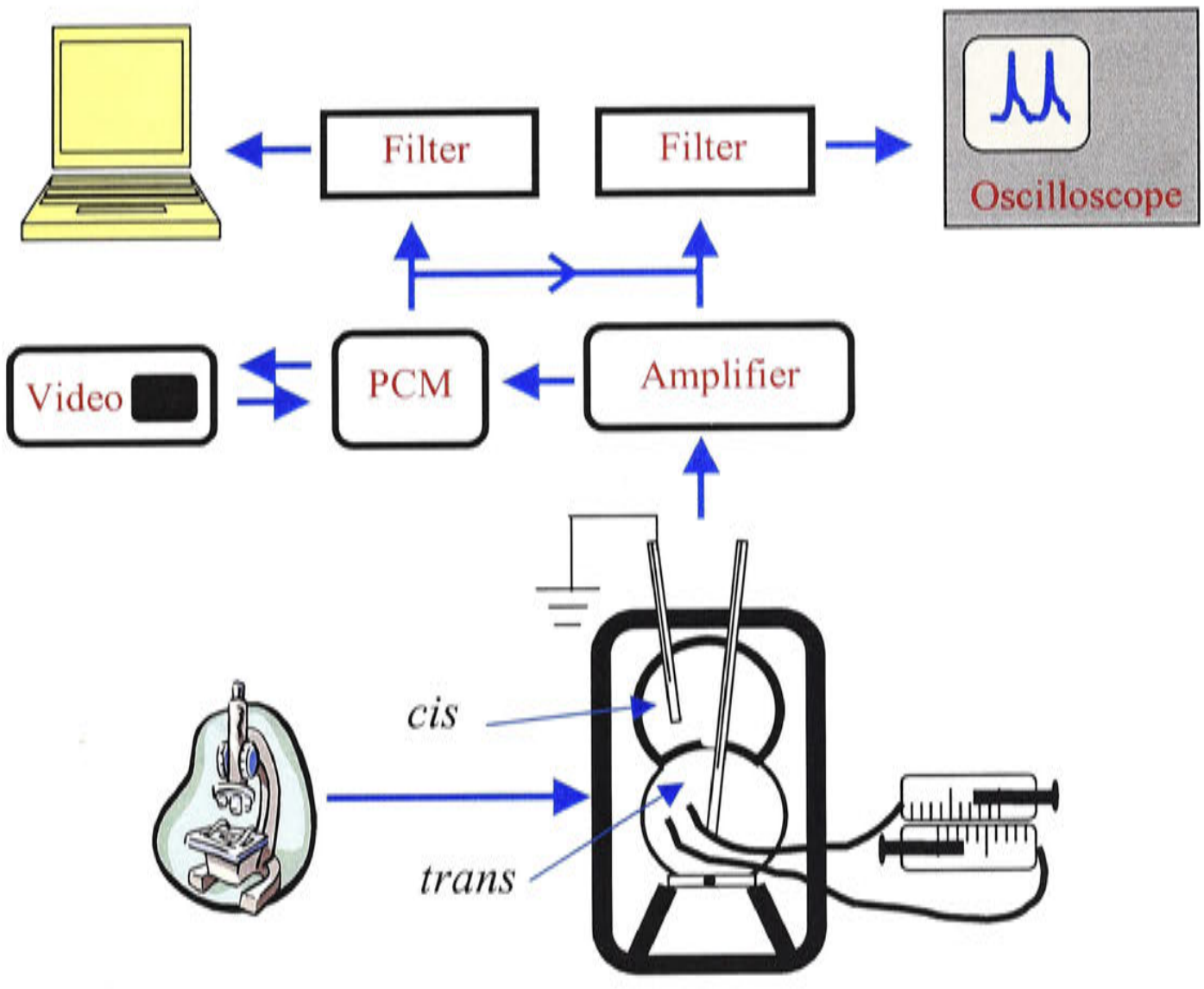


Fig 2.IV. ***Cartoon diagram of the bilayer setup.*** Lipids were formed across a 150 – 250 μm aperture of a delrin cup, which was viewed through a binocular microscope. Silver chloride coated silver wires provided the electrical connection to the solution and currents were then amplified and recorded onto videotape using PCM, whilst simultaneously filtered and displayed on an oscilloscope. Data recorded on videotape was filtered and digitized for computer analysis. Back to back syringes allow solution exchange from either chamber, without altering chamber volume

DISSOCIATION OF CSQ USING HIGH IONIC STRENGTH SOLUTIONS.

3.1 INTRODUCTION

Miller and Racker (1976) were the first to show that fusing SR vesicles isolated from skeletal muscle with artificial (or black) lipid bilayer membranes allowed incorporation of SR ion channels. This technique allows monitoring of channel activity by measuring current passing through the membrane in response to an electrochemical gradient. This is a powerful tool for studying alterations in both ion channel conductance and gating, in response to the addition and removal of ions, pharmacological agents and proteins. Of particular interest and importance, is the study of the SR Ca^{2+} release channel, the RyR.

Using the bilayer technique, the skeletal muscle RyR has been characterized as a cation selective ion channel, with rapid gating kinetics and high conductance (770 pS in 250 mM symmetric K^+ and approximately 500 pS in 250 mM symmetric Cs^+; Smith *et al.*, 1986; Tinker and Williams, 1992). In addition to RyRs, the SR also contains K^+, Cl^- and voltage gated anion channels. By using Cs^+ as the current carrier, K^+ currents are blocked (Cukierman *et al.*, 1985), and by conducting experiments at the reversal potential of Cl^-or by using other impermeant anions, the contribution of currents from non-RyR channels can be minimized. Abundant investigation has shown that the RyR is activated by μM cytoplasmic Ca^{2+} (Coronado *et al.,* 1994 and references therein) and mM ATP (Meissner *et al.*, 1986a; Moutin and Dupont, 1988), and inhibited by mM Mg^{2+} (Smith *et al.*, 1986; Meissner *et al.*, 1986a; Laver *et al.*, 1997) and by 10 μM ryanodine and ruthenium red (Meissner, 1986b; Ma, 1993).

Both the Ca^{2+} binding properties and kinetics of CSQ, the major Ca^{2+} binding protein found within the SR, have been studied extensively. The role that this protein plays in regulation of the RyR, and hence the regulation of SR Ca^{2+} release, is still not well understood. Conflicting evidence shows CSQ to both activate (Kawasaki and Kasai, 1994; Ohkura *et al.*, 1994; Hidalgo and Donoso, 1995; Ohkura *et al.*, 1995; Szegedi *et*

study and Wang *et al.*, 2001). CSQ was reported to be localized near the RyR, through interactions with anchoring proteins, triadin and junctin, but not to be directly associated with the RyR (Zhang *et al.*, 1997). More recent evidence shows that a close interaction between the RyR and CSQ can occur *in vitro*, suggesting a direct physical coupling between these two proteins (Murray and Ohlendieck, 1998). In addition, Szegedi *et al.* (1999) and Herzog *et al.* (2000) report that in purified RyRs, CSQ induces an increase in channel activity and Ca^{2+} release that is dependent on the phosphorylation state of CSQ. No detailed study of the interaction between CSQ and the RyR, or any functional or regulatory implications of such an interaction, has been reported thus far.

In single channel studies using the lipid bilayer technique, the luminal side of the SR faces the *trans* chamber (Laver *et al*, 1995). As CSQ has no cytoplasmic or membrane-spanning domain, it is located wholly in the *trans* chamber and can be dissociated and re-associated by simply altering *trans* bath conditions, or adding exogenous CSQ. An increase in ionic strength (to 500 mM, with NaCl) has been reported to dissociate CSQ from junctional face membrane (JFM) in biochemical studies (Ikemoto *et al.*, 1989). Exposure of solubilized JFM to high [NaCl] disrupts charge-charge interactions, thought to be responsible for tethering CSQ to the JFM), allowing the selective removal of this protein. Such an ionic strength change can be utilized while using the lipid bilayer technique. This allows channel activity to be investigated in detail (in real time), with alterations in channel gating and conductance induced by addition or removal of drugs, ligands and proteins, observed in a controlled environment.

The aims of experiments in this chapter were to firstly, confirm the Cs^+ current recorded in lipid bilayer experiments arose from RyR channel activity, secondly, to selectively dissociate CSQ from native RyRs and determine any regulatory effects this protein may have on channel activity. Thirdly, to re-associate exogenous CSQ with the RyR, to reveal whether any RyR activity changes induced by CSQs removal could be reversed by its re-introduction. Finally, to apply anti-CSQ antibody to the *trans* chamber, to determine whether any effects this antibody had on CSQ conformation were able to alter RyR regulation by CSQ.

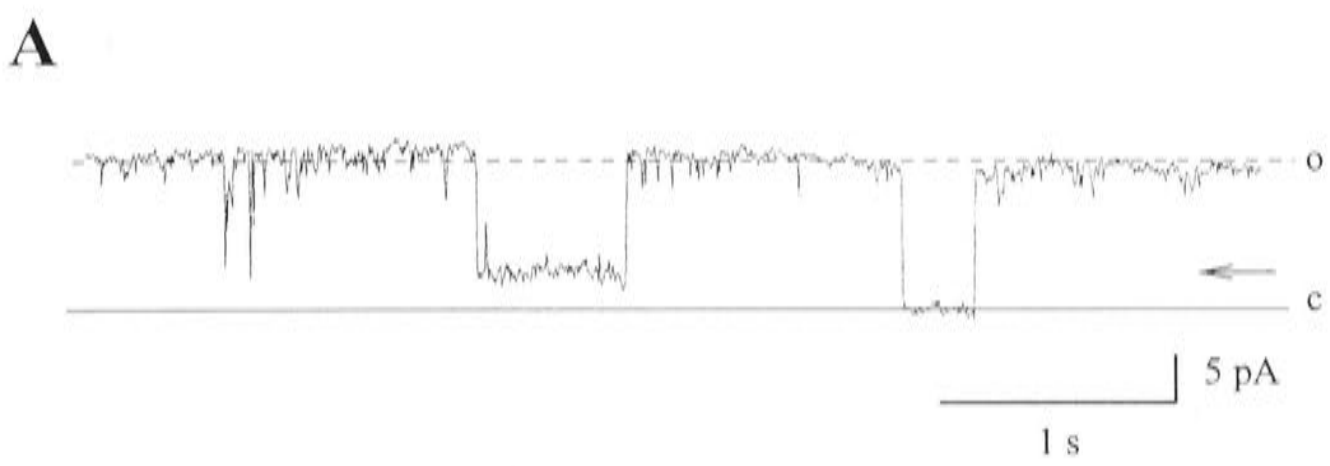
3.2 Results

3.2.1 Bilayers – general observations

As a general rule, after formation of a lipid bilayer and addition of SR vesicles, RyR incorporation occurred within 15 min, and was usually indicated by a baseline shift in the current signal. If incorporation of a RyR had not occurred after 20 min, bilayers were broken and reformed in the presence of SR vesicles.

Due to the sensitivity of the RyR to the isolation procedure and to ensure that the protein isolated with the SR was a fully functioning RyR (responding pharmacologically in a typical manner), channels from each batch of SR were carefully tested after isolation and purification. Responses to Mg^{2+}, ATP, ruthenium red (or ryanodine) and both cytoplasmic and luminal Ca^{2+} were examined. Any preparation not showing typical RyR behaviour was not used. Any experiments involving channels that did not show an increase in activity after the addition of 2 mM ATP and an activity decrease after lowering Ca^{2+} to 100 nM with BAPTA (and in the case of channels with 2 mM Mg^{2+} added, channel inhibition) were terminated, because channels were not functioning as normal RyRs, or were incorporated in the incorrect orientation into the bilayer.

On some occasions, single channel multiple subconductance levels were observed (Fig. 3.I A). As a general rule, subconductance states of 25%, 50% or 75% were seen. Subconductance levels induced by a particular condition or addition of any protein, ion or drug used in this study were never observed. Subconductance levels can be further seen in the all points histogram in Fig. 3.I B, with conductances of 90.25 pS (corresponding to a 25% conductance state) and 361.5 pS (maximal channel opening; which although low, is within the range observed from RyRs). A multitude of subconductance levels induced by FKBP12 removal has been reported by Ahern *et al.* (1997a) and Ahern *et al.* (1997b). Substate activity is more commonly observed in purified RyRs (Lai and Meissner, 1989), and might be due to the removal of FKBP12. In all bilayer data presented in this chapter, the composition of *cis* and *trans* solutions



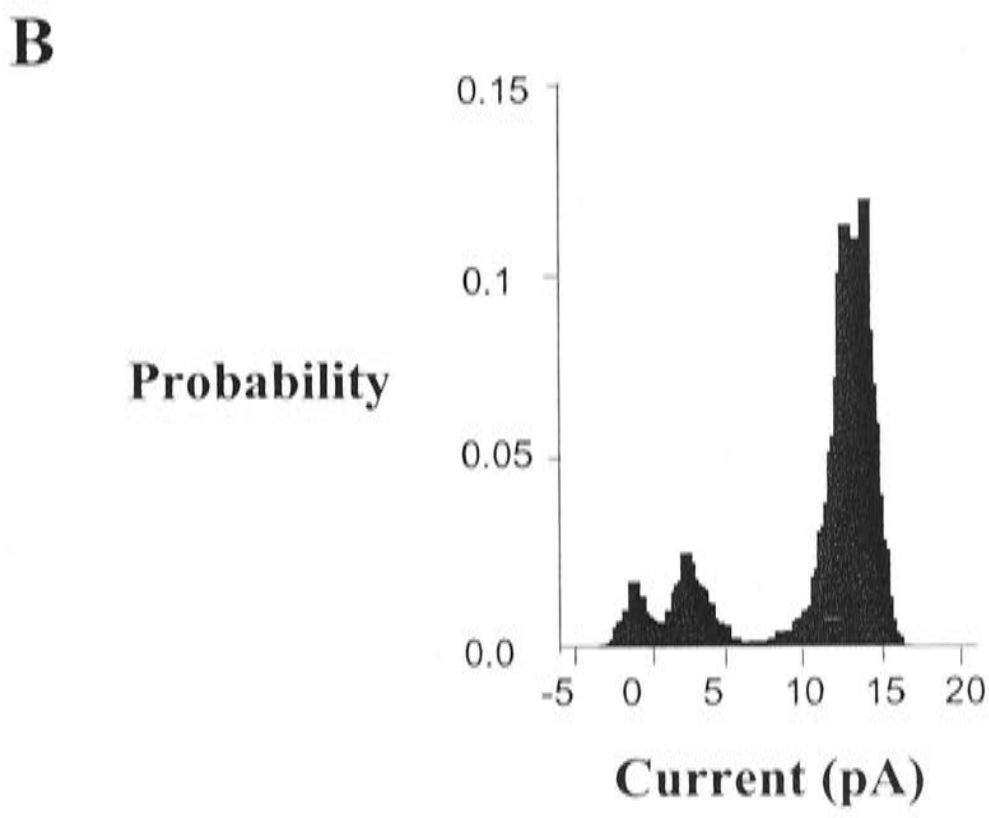


Fig. 3.I. ***Substate activity observed in native RyRs.*** (**A**) Example of substate activity in single RyR. The subconductance level (arrow in A) was observed at approximately 25 % of the maximum conductance, in addition to maximal opening (broken line) and closed (closed line) levels. (**B**) All points amplitude histogram of activity at +40 mV, showing channel closed stated (0 pA), open state (14.46 pA, 361.5 pS) and substate (3.61 pA, 90.25 pS). Data was analyzed over a 1 min recording.

were as follows, unless otherwise specified: *cis* 100 nM free Ca^{2+} (1mM $CaCl_2$ plus 4.5 mM BAPTA), (in mM) 2 ATP, and 250 Cs^+ (230 mM CsMS, 20 mM $CsCl_2$; *trans* (mM) 250 or 500 Cs^+ and 1 Ca^{2+} (as $CaCl_2$). In many instances (57%), more than one channel was observed in the bilayer. Up to six channels per bilayer were observed in the course of this study (eg. Fig. 3.II), although it was uncommon to observe more than two. For purposes of analysis, bilayers with more than two active channels were not used. As an interesting aside; when n >2 channels were observed, they were almost always (84%) from so-called 'high activity' channels (refer to Chapter 4, Section 4.2.1). However, since multiple channel activity was identified by summed openings to conductance levels that were multiples of the maximum single channel conductance, it was possible that multiple channels were not observed if the probability of channel opening was low.

3.2.2 Effect of characterized agents on native channel activity

3.2.2.1 ATP

ATP is a physiological enhancer of Ca^{2+}-induced Ca^{2+} release and has been shown to increase Ca^{2+} release from RyRs (Smith *et al.*, 1986). As well as being the physiological activator in muscle, ATP has also been proposed to increase RyR activity by phosphorylating the RyR or an associated co-protein in lipid bilayers (Dulhunty *et al.*, 2001). Two mM ATP was added to the *cis* chamber, evoking a 2.2-fold increase in open probability (P_o; n=5; Figs. 3.IIA & Table 3.I). Open time (T_o) showed a significant increase, whilst closed time (T_c) decreased to 10% of control activity (Table 3.I). Despite increasing in all but one channel, open frequency (F_o) did not increase significantly overall, because of the large variability in frequency between channels (Table 3.I). Fig. 3.IIA and Table 3.I contains results obtained with channels at positive potentials; it should be noted that at a negative potential, similar effects were observed. ATP (2mM) was used as part of the standard *cis* solution as an activator in all experiments, as standard *cis* [Ca^{2+}] of 100 nM is sub-activating, and thus control channel activity is difficult to examine quantitatively. Two mM ATP has about the same activating effect on bilayer-incorporated RyRs as the 8 mM concentration found *in vivo*, due to some of the ATP being complexed with $MgCl_2$ (*personal communication*, Dr Derek Laver, University of Newcastle, Newcastle, Australia).

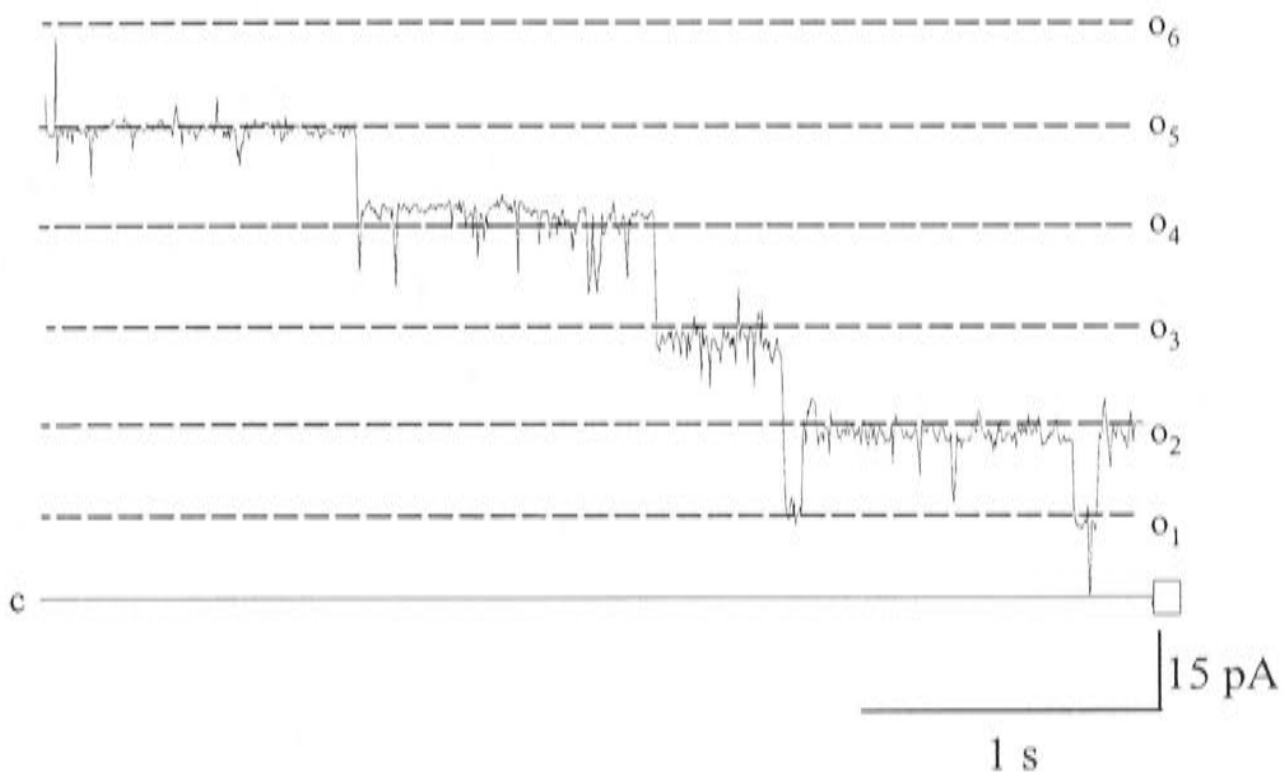


Fig. 3.II. ***Currents observed after incorporation of multiple RyRs into the lipid bilayer***. Simultaneous activity of at least six RyRs at level o_6. Activity was recorded at +40 mV. Channel openings are indicated by upward deflections from the closed line (c), indicating the closed state of channels. O_n represents the open state of *n* channels.

Table 3.I ***Single channel parameters of RyR response to addition of 2 mM cis ATP.***

	Pre-ATP addition	Post-ATP addition	
P_o	0.20 ± 0.10	0.44 ± 0.66	*
T_o (ms)	2.32 ± 0.48	5.60 ± 1.53	*
T_c (ms)	34.67 ± 21.36	3.34 ± 1.67	*
F_o (events/s)	85.82 ± 49.44	90.06 ± 15.26	

The addition of 2 mM ATP caused significant (*, $p<0.05$, t-test) increase in P_o, T_o, and a significant shortening of T_c. No significant change in F_o was observed.

3.2.2.2 Ca^{2+}

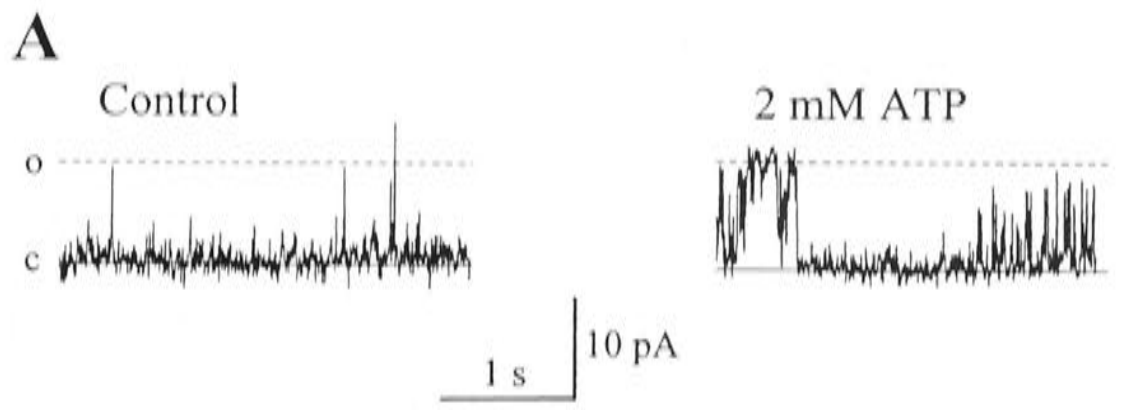
RyR response to both *cis* Mg^{2+} and Ca^{2+} have been well documented and can be used to indicate whether the channel has incorporated in the correct orientation (refer to Chapter 1, Section 1.2.6.1 & 1.2.6.3). Both Mg^{2+} and Ca^{2+} are reported to bind at the same inhibition and activation sites (Meissner, 1986b; Laver *et al.*, 1997). Fig. 3.III B illustrates the biphasic nature of the effect of *cis* Ca^{2+} on RyR activity; with channel inhibition in the presence of nM and mM $[Ca^{2+}]$, and activation occurring in the presence of μM Ca^{2+}. Each point is the average of between 3 - 10 channels, presented as mean ± SEM (Fig. 3.III B). Each channel was exposed to no more than three different *cis* $[Ca^{2+}]$. The solid line (Fig. 3.III D) is the least square fit of the data to the Hill equation;

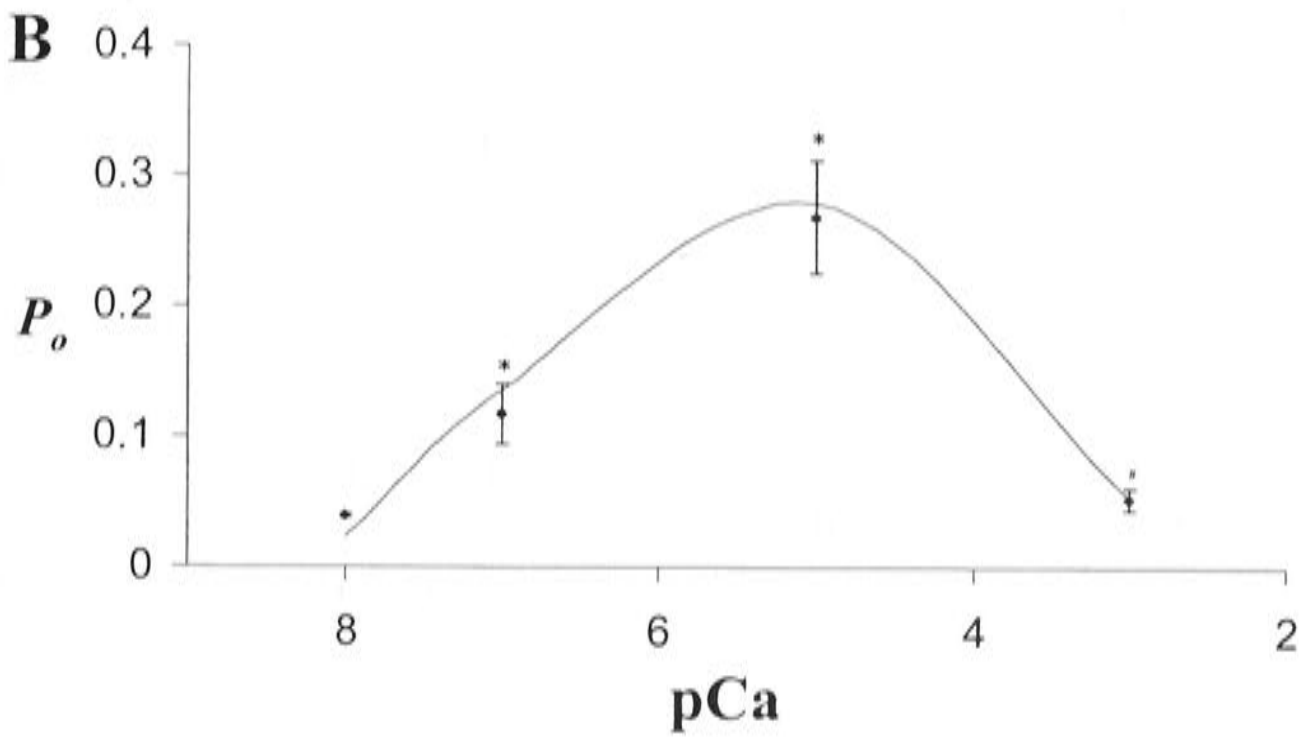
$$P_o = \left(P_{\max(Ca)}\right)\left\{\frac{1}{\left(1+\left(K_{a(Ca)}/\left[Ca^{2+}\right]\right)^{n_{a(Ca)}}\right)}\right\}\left\{\frac{1}{\left(1+\left(\left[Ca^{2+}\right]/K_{i(Ca)}\right)^{n_{i(Ca)}}\right)}\right\} \qquad \text{[Eq. 3.2-1]}$$

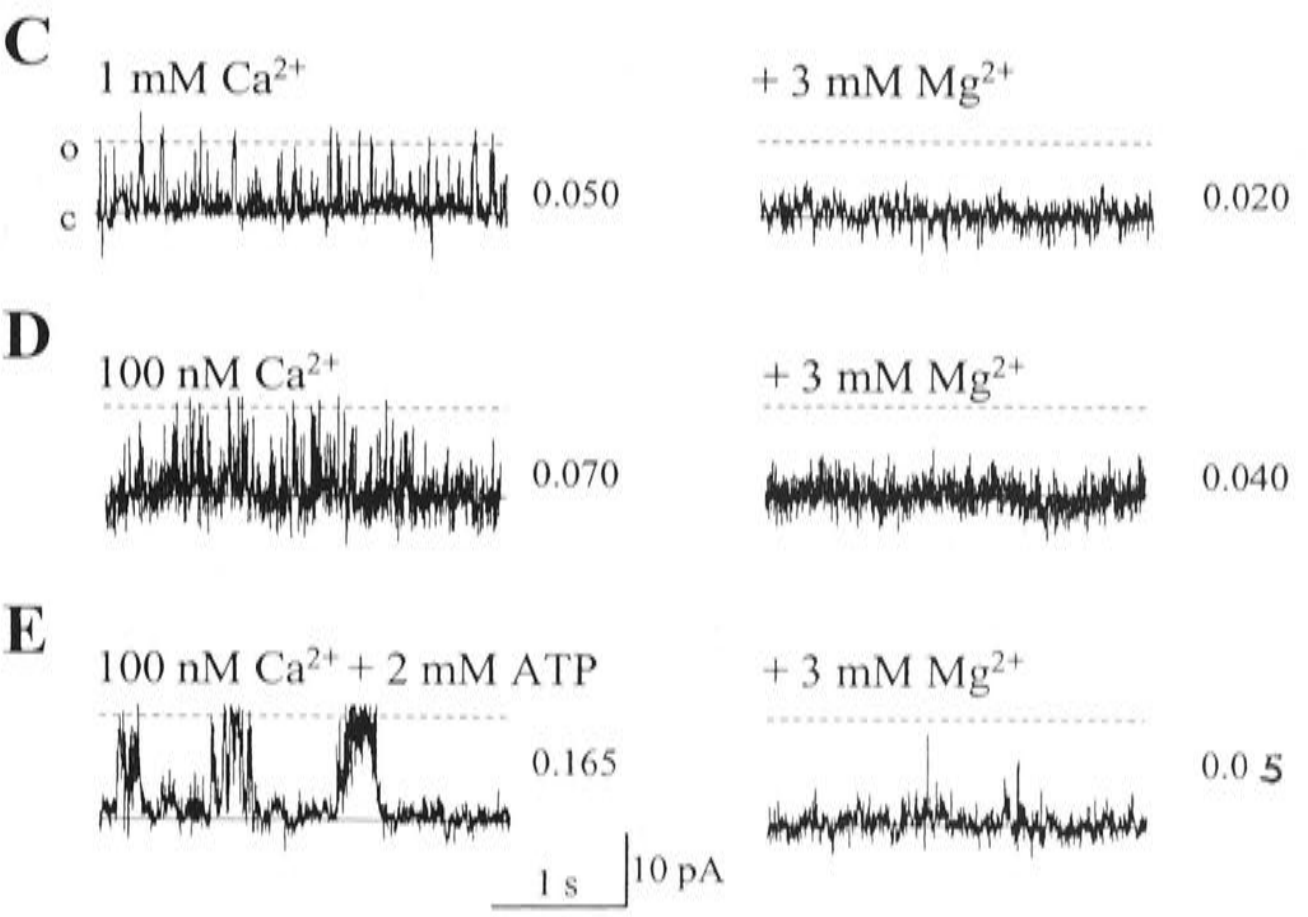
where $P_{max(Ca)}$ is the open probability at maximally activating Ca^{2+} in the absence of other ligands, $n_{a(Ca)}$ and $n_{i(Ca)}$ are the Hill co-efficients for activation (a) and inhibition (i) respectively, and $K_{a(Ca)}$ and $K_{i(Ca)}$ correspond to the apparent dissociation for activation and inhibition.

Channel P_o increased from 0.039 ± 0.004 to 0.117 ± 0.023 ($p < 0.05$, t-test) upon increasing *cis* Ca^{2+} from 10 nM to 100 nM, further increasing to 0.269 ± 0.043 ($p < 0.05$, t-test) when channels were exposed to 10 μM Ca^{2+}. Channel activity dropped dramatically (to 0.052 ± 0.009; $p < 0.05$, t-test) when *cis* Ca^{2+} was subsequently increased to 1 mM. These results compare favourably with those reported by Fill *et al.* (1990), although higher activation is reported here (Fig. 3.III) at low *cis* Ca^{2+} (pCa 7). Because the response to Ca^{2+} was immediate; channels were either analysed immediately after aliquot addition and stirring had ceased, or 30 s after this (depending whether at positive or negative potential, as activity was observed for 30 s at each potential). After the initial rise or fall in activity, no significant change in the activation or inhibition induced at a particular *cis* Ca^{2+} was observed up to 5 min after application, indicating the P_o rise was not due to transient RyR activation.

Fig 3.III. ***Typical RyR response to cis ATP, Ca^{2+} and Mg^{2+}.*** V_m-E_{Cs+} was +40mV under all conditions. (**A**) control activity with 250 mM *trans* $Cs^+$and 1 mM Ca^{2+}(left panel), and after addition of 2 mM ATP (right panel); (**B**) *cis* Ca^{2+} dependence of native RyRs in the absence of ATP. Each point is the mean P_o for 4-10 channels. The solid line is the best least squares fit of the the Hill equation to the data (Eq. 3.2-1), with a $K_{a(Ca)}$ of 1.19 * 10^{-7} and a $K_{i(Ca)}$ of 2.25 * 10^{-4}. An * indicates average values significantly different from those recorded under control conditions and a # indicates a significant difference from the previous condition ($p<0.05$, t-test); (**C-E**) the response of channels to 3 mM $MgCl_2$ (right panels), in the presence of (left panel) 1 mM Ca^{2+} (**C**), 1 nM Ca^{2+} (**D**) and 1 mM Ca^{2+} and 2 mM ATP (**E**). Channel I'_F is shown to the right of traces **C-E**. Single channel opening is upward from the zero current level (continuous line) to the maximum open conductance (broken line).


A
Control
2 mM ATP
o
c
1 s
10 pA


B
0.4
0.3
P_o
0.2
0.1
0
8
6
4
2
pCa


C
1 mM Ca^{2+}
+ 3 mM Mg^{2+}
o
c
0.050
0.020
D
100 nM Ca^{2+}
+ 3 mM Mg^{2+}
0.070
0.040
E
100 nM Ca^{2+} + 2 mM ATP
+ 3 mM Mg^{2+}
0.165
0.0 5
1 s
10 pA

3.2.2.3 Mg^{2+}

In earlier experiments, Mg^{2+} was added to the control *cis* solution in an attempt to keep solutions as 'physiological' as possible. Due to Mg^{2+} inhibitory actions, its addition often reduced channel P_o to close to zero. Therefore Mg^{2+} was omitted in the majority of later experiments in order to obtain meaningful measurements of control activity. Fig. 3.III C-E shows that at different *cis* Ca^{2+} and ATP concentrations, 3 mM Mg^{2+}_{total} (1.26 mM Mg^{2+}_{free}) was able to decrease fractional mean current (I'_F), and induce Mg^{2+} inhibition typical of RyR channels. In the presence of 1 mM Ca^{2+}, Mg^{2+}induced a 2.5-fold decrease in channel activity (Fig. 3.III C). At 1 mM *cis* Ca^{2+}, Mg^{2+}reduced channel I'_F in the absence and presence of ATP by 43 and 56%, respectively (Fig. 3.III D-E). At 1 nM Ca^{2+}, Mg^{2+}_{free} was 2.82 mM in the absence of ATP and 1.2 mM in the presence of ATP, due to Mg^{2+} and Ca^{2+} complexing with ATP. It is interesting that in the presence of 1 nM Ca^{2+} and 2 mM ATP, Mg^{2+} could reduce channel activity by a greater amount than when ATP was absent (even though ATP significantly lowers Mg^{2+}_{free}). Mg^{2+}-ATP has been reported to regulate RyRs (Meissner, 1994), and may be inhibiting RyR activity at this very low cis Ca^{2+} concentration. We cannot rule out that these unexpected results may also be due to channel variation, since a detailed study of Mg^{2+} inhibition was not performed. The aim of this limited Mg^{2+} study was simply to determine whether the native RyRs used throughout this study exhibited typical RyR behaviour.

3.2.2.4 Ruthenium red

Ruthenium red has been shown to block Ca^{2+} transport in both mitochondria (Moore, 1971) and RyRs (Ma, 1993). Addition of 10 μM *cis* ruthenium red reduced native channel activity to virtually zero (P_o = 0.007 at + 40 mV and 0.003 at – 40 mV; Fig. 3.IV A-D). The channel was blocked within 15 s of addition with only very brief and infrequent openings observed. Routinely, ruthenium red was added at the end of an experiment, to confirm the channel was a RyR.

3.2.2.5 Ryanodine

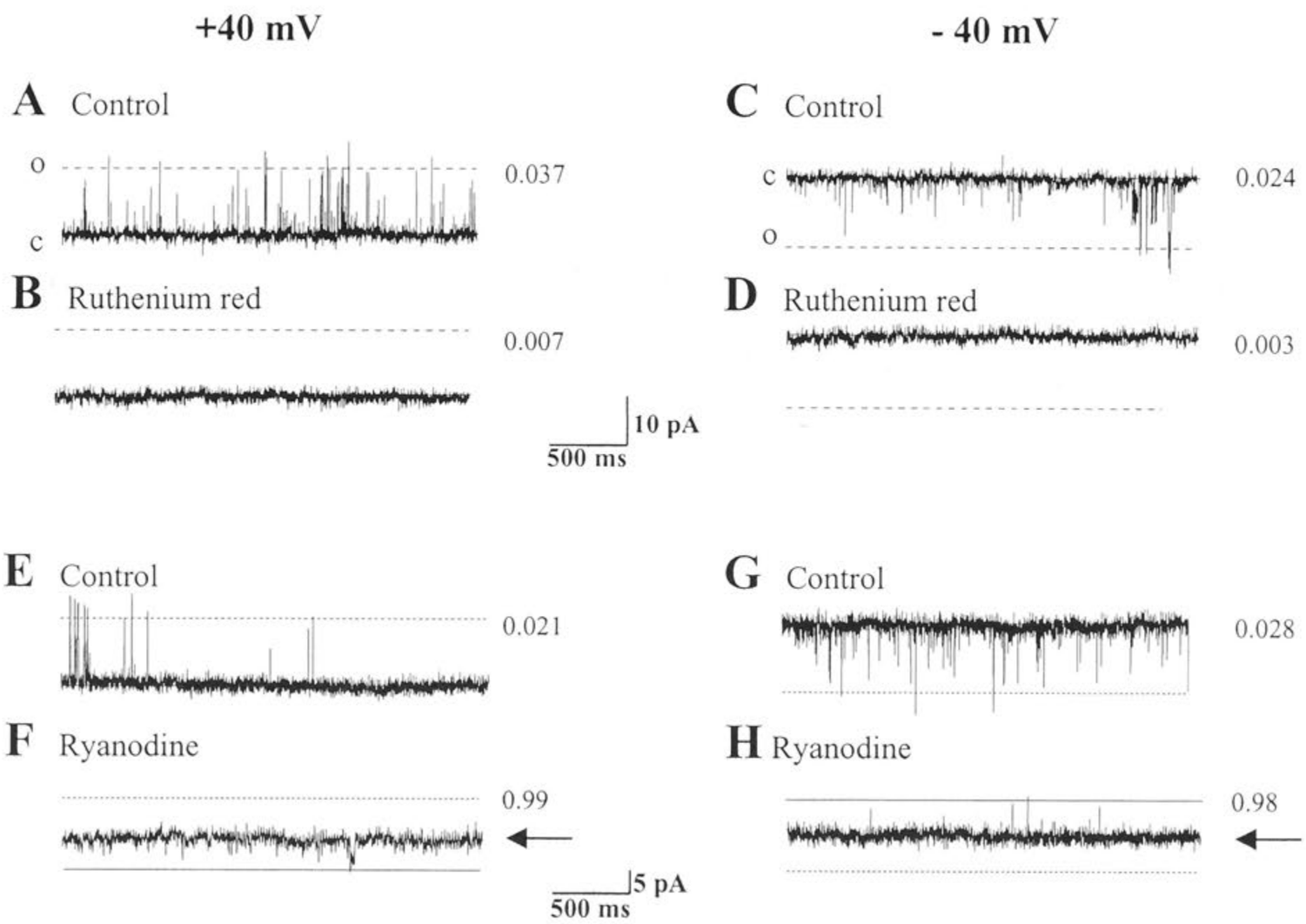
Plant alkaloid ryanodine is able to reduce RyR channel to a low (approximately 50%) conductance level, and 'locks' the channel into this state (Rousseau and Meissner, 1989). Ryanodine (^{3}HRy) binding is dependent on the RyR being in a partially or fully open state. The use of ryanodine was limited in this study, as in many experiments, ligands and proteins (for example exogenous CSQ) induced substantial channel closure, with very brief and infrequent openings. In these cases, it was preferential to add ruthenium red (which does not require the RyR to be active for binding) to confirm that the channel was a RyR.

Fig. 3.IV E-H shows a channel whose activity upon addition of *cis* 10 μM ryanodine, was reduced to an approximately 50% conductance state, indicated by an arrow to the left of traces F&H. No further openings above a 50% conductance level were observed within 5 min of addition of ryanodine. The 50% block occurred within 20 s of ryanodine addition.

3.2.3 CSQ purification

The SR terminal cisternae contains two membrane components, the JFM (containing the RyR, CSQ, triadin and junctin) and the extra junctional membrane containing the calcium-pump (Ca^{2+}-ATPase). Exposure of the terminal cisternae of the SR to Triton-X 100 (in the presence of Ca^{2+}), leads to Ca^{2+}-ATPase extraction and results in the formation of an insoluble JFM pellet, leaving Ca^{2+}-ATPase in the supernatant (Costello *et al.*, 1986). Addition of 500 mM NaCl to the JFM disrupts the ionic interactions, tethering CSQ to the RyR/triadin/junctin (RyR/T/J) complex, allowing selective dissociation of CSQ from this complex (Fig. 3.V). Thus, CSQ's ionic binding properties may be exploited to successfully dissociate this protein from solubilized JFM. Such treatment allowed partial purification of CSQ, which was contaminated with a protein, seen as a faint band at approximately 30 kDa. The 30 kDa protein was removed following a second overnight dialysis. The contaminating protein has not been positively identified in this study, but it may be the 30 kDa luminal protein reported by Yamaguchi *et al.* (1995). The presence of the 30 kDa protein had no effect on RyR

Fig. 3.IV. ***Response of native RyRs to cis modulators ryanodine and ruthenium red.*** (**A**) Control activity with 250/250 mM (*cis/trans*) Cs^+; (**B**) after addition of 10 μM ruthenium red; (**C&D**) repeat of **A&B**, at – 40 mV; (**E**) control activity with 250/250 mM (*cis/trans*) Cs^+; (**F**) after addition of 10 μM ryanodine; (**G&H**) repeat of **E&F**, at – 40 mV. V_m-E_{Cs+} was +40mV under conditions **A**,**B**,**E**,**F** and –40mV under conditions **C**,**D**,**G**,**H**. Single channel opening is upward from the zero current level (+40 mV) and downward from the zero current level (-40 mV; continuous line) to the maximum open conductance (broken line). Open probability (P_o) of the channel is shown to the right of the current traces. The arrow in **F&H** indicates an ~ 50% reduction in maximum conductance of the channels upon exposure to ryanodine. Channel traces in the presence of ryanodine and ruthenium red were recorded within 90 s of drug addition.


+40 mV
- 40 mV
A Control
o
c
0.037
B Ruthenium red
0.007
10 pA
500 ms
C Control
c
o
0.024
D Ruthenium red
0.003
E Control
0.021
F Ryanodine
0.99
5 pA
500 ms
G Control
0.028
H Ryanodine
0.98

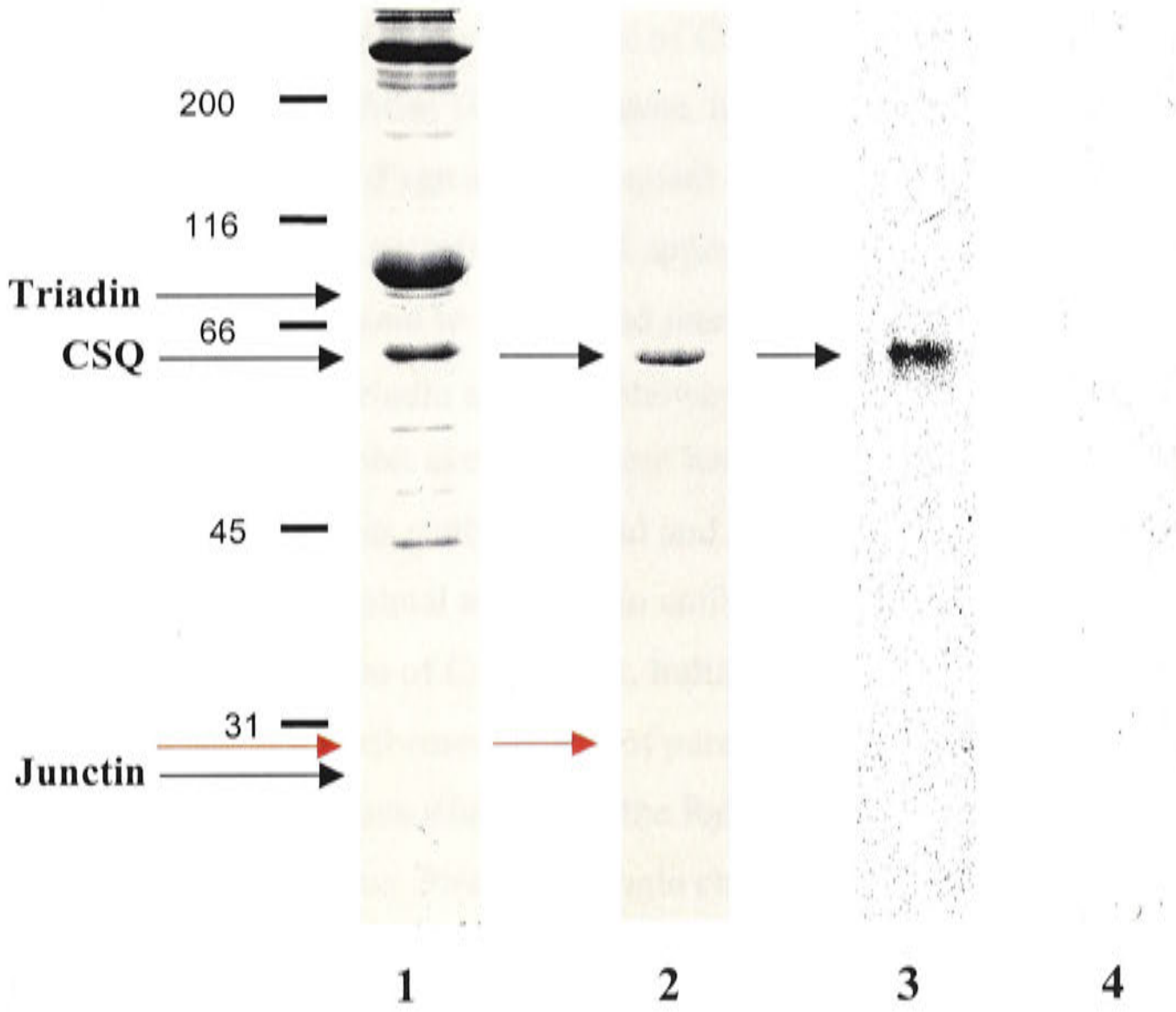


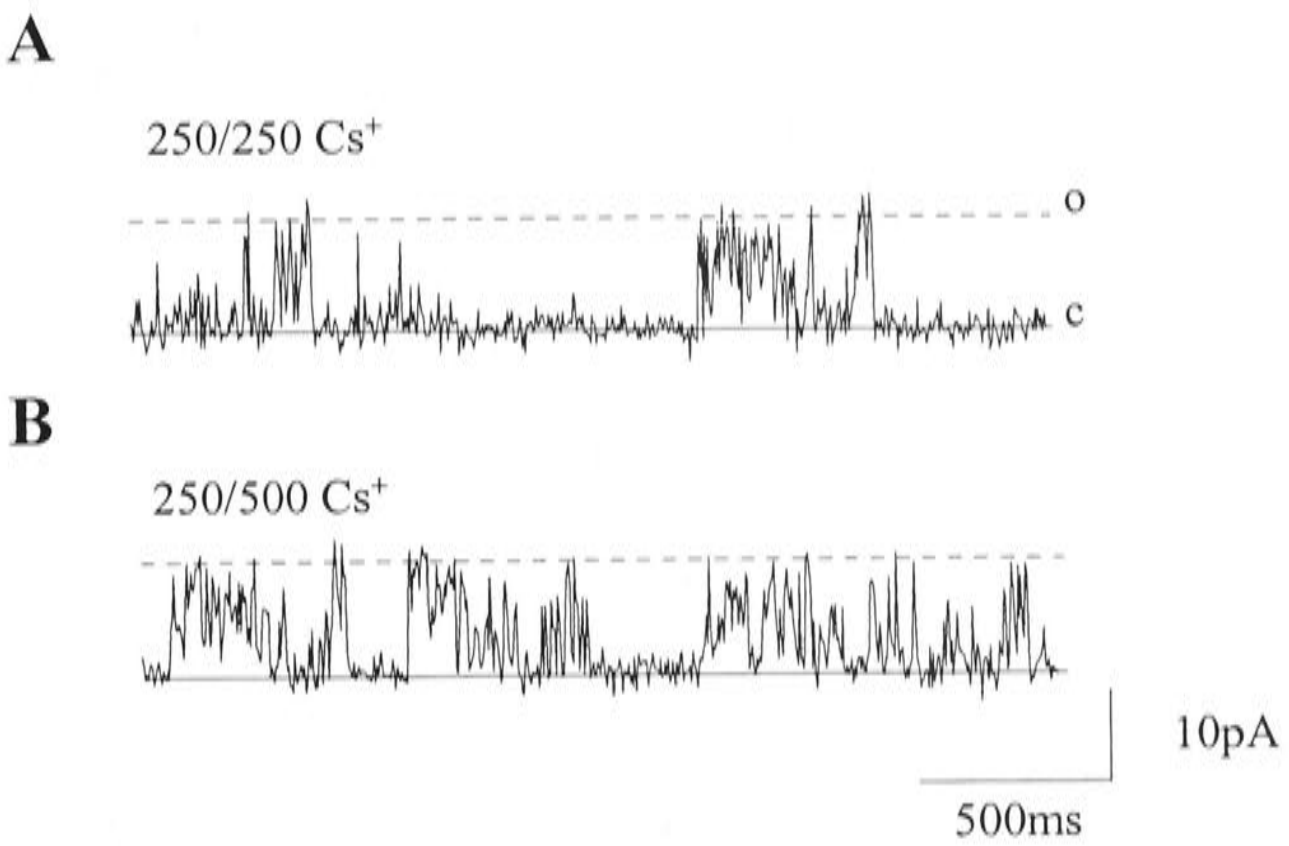
Fig. 3.V. ***Purification of CSQ.*** SDS-PAGE (12%) showing a 55 kDa band in heavy SR (lane 1) and in supernatant after exposure of JFM to 500 mM NaCl (lane 2). The band was identified as CSQ by anti-CSQ antibody (lane 3), whilst no triadin was detected by anti-triadin antibody in the isolated CSQ sample (lane 4) . The position of molecular weight markers is shown to the left of lane 1. Proteins were transferred onto Immobillon-P PVDF membranes, prior to probing with anti-CSQ and anti-triadin antibodies. There was no band observed at 26 kDa in the isolated CSQ sample (lane 2) that would correspond to junctin (one of the major CSQ binding protein in the lumen). Note the appearance of a very faint band (indicated by a red arrow), thought to be the 30 kDa protein.

activity and did not alter the RyR response to CSQ (refer to Figs. 3.XI & 3.XII and Section 3.2.5.3).

This method of CSQ isolation allows removal of CSQ from solubilized JFM, without removing triadin or junctin. From 100 g of tissue, up to 5 mg of CSQ was isolated (see Chapter 2, Section 2.5). SDS-Page and subsequent Coomassie stain showed that isolated CSQ did not contain protein bands at approximately 95 or 26 kDa (Fig. 3.V, lane 2), which would correspond to triadin and junctin respectively. Furthermore, immunodetection with anti-triadin antibody showed no visible levels of triadin in the isolate (Fig. 3.V lane D). Rabbit skeletal junctin has not been sequenced and antibodies to sequenced isoforms (such as canine skeletal and canine cardiac junctin) do not cross-react with the rabbit skeletal anti-junctin antibody (*personal communication*, Prof. Larry Jones, Krannert Institute of Cardiology, Indianapolis, IN, USA), therefore junctin immunodetection was not performed. It was of paramount importance in this study that triadin and junctin remained associated with the RyR and did not contaminate the isolated CSQ, for three reasons. Firstly, in single channel studies, luminal (or *trans*) ionic strength was raised to dissociate CSQ from the RyR/T/J complex, and exogenous CSQ was re-associated with the channel. Thus, triadin and junctin needed to remain associated with the RyR to bind the added CSQ. Secondly, to show any regulation of the native RyR by CSQ, it was important that all three proteins proposed to bind CSQ (the RyR, triadin and junctin) were present. Finally, to be certain that any modulation of the RyR by exogenous CSQ was CSQ-specific, it was important that triadin and junctin did not contaminate the CSQ preparation, particularly as triadin has been shown to inhibit RyR activity (Ohkura *et al.*, 1998).

3.2.4 Increasing ionic strength – dissociation of CSQ from SR vesicle in lipid bilayers

The high ionic strength CSQ purification method (see above and Chapter 2, Section 2.5.2) was modified to dissociate CSQ from SR vesicles in lipid bilayers. Channel activity was analysed at potentials 40 mV positive and negative to the Cs^+ reversal potential (see Chapter 2, Section 2.11.4). In all the bilayer experiments presented in this chapter from here on the composition of *cis* and *trans* solutions is as follows, unless otherwise specified: *cis* 100 nM free Ca^{2+}(1 mM $CaCl_2$ plus 4.5 mM BAPTA), (in mM)



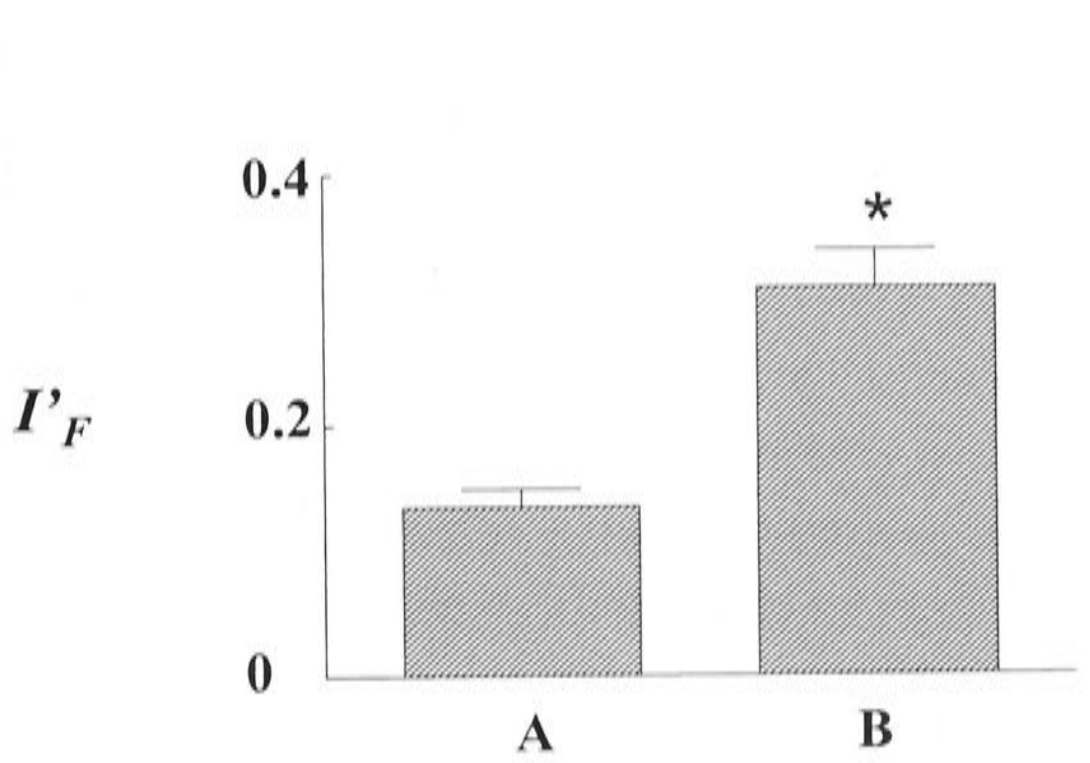


Fig 3.VI. ***Increasing trans ionic strength activates RyRs.*** (**A**) Control activity with 250/250 mM Cs^+; (**B**) after raising *trans* ionic strength to 500 mM Cs^+; (**C**) fractional mean current (I'_F) of average data (n=59 channels), under conditions shown in **A**&**B**. *Cis*/*trans* [Cs^+] are shown above the records in **A**&**B**. $V_m - E_{Cs+}$ was +40mV under all conditions. Single channel opening is upwards from zero current level (continuous line) to maximum conductance (broken line). Asterisks (*) indicate average values significantly different from control ($p<0.05$, t-test).

2 ATP, and 250 Cs^+; *trans* (mM) 250 or 500 Cs^+ and 1 Ca^{2+}. In the case of average data presented in this chapter, channels were analyzed for 30 s, unless stated otherwise. After incorporating native SR into lipid bilayers, the observed RyR openings were both infrequent and brief (Fig. 3.VI A). Addition of 250 mM Cs^+ to the *trans* chamber (which raises *trans* Cs^+ to 500 mM) induced a 2.3-fold increase in I'_F (Fig. 3.VI C). In three channels, RyRs were exposed to high *trans* Cs^+ for up to 10 min. In these channels, after the initial increase (occurring at 89 s ± 15 s), no further significant change in activity was observed, indicating that the activation was a sustainable change in channel gating and probably not due to random variations in channel gating (see Section 3.2.4.1.2).

3.2.4.1 Irreversible increase in channel activity

3.2.4.1.1 500mM Cs^+-induced activity

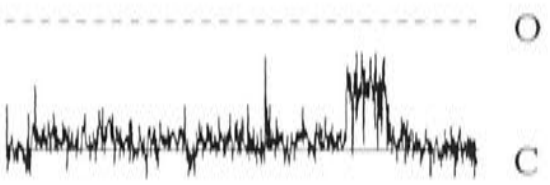
To determine whether the increase in activity seen after addition of *trans* Cs^+ was due to ionic strength increase *per se*, or whether some irreversible event (such as protein dissociation) had occurred, the *trans* chamber was perfused and the solution replaced with 250 mM Cs^+ (the solution used for control recording). In these channels, control *cis* solution also contained 3 mM $MgCl_2$. As previously shown in Figs. 3.VI, channel activity increased upon Cs^+ increase to 500 mM, with both P_o and T_o showing significant rises (2.3 and 13-fold, respectively). Activity then remained unchanged when Cs^+ was restored to control levels (via perfusion) with 250 mM Cs^+ (Figs. 3.VII B-C & 3.VIII). In fact, P_o and T_o did not change further after subsequent Cs^+ additions and further perfusion. Although burst T_c fell in 5 out of 8 RyRs with 500 mM Cs^+, average burst T_c did not change significantly (Fig. 3.VIII), due to channel variability. An irreversible increase in channel activity is consistent with CSQ removal. Once dissociated, CSQ did not re-associate, because it was diluted enormously in the *trans* chamber solution, and was further diluted following perfusion of the *trans* bath (perfusion of 10 chamber volume results in a dilution of 1000).

The activation induced by raising [Cs^+] was not so pronounced at negative potentials (Figs. 3.VII & 3.VIII). An increased in I'_F in all channels was seen, from 0.137 ± 0.05 to 0.441 ± 0.175 ($p < 0.05$), a 3.2-fold increase. Following perfusion, there was a small, but insignificant drop in I'_F, which was still 2.2-fold higher ($p < 0.05$, sign test) than

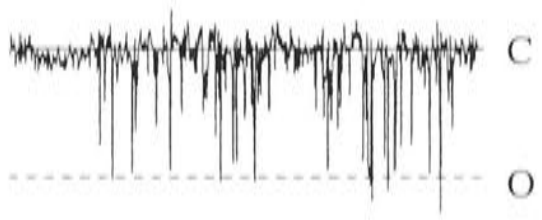
Fig 3.VII. ***Increasing trans ionic strength to 500 mM causes an irreversible increase in single RyR channel activity.*** (**A**) Control activity with 250 mM *trans* Cs^+in the presence of 3 mM $MgCl_2$; (**B**) after increasing *trans* Cs^+ to 500 mM with CsCl; (**C**) perfusing the *trans* chamber with 250 mM Cs^+; (**D&E**) repeat of **B&C**. **F-J** repeat of **A-E**, but at –40mV. V_m-E_{Cs+} was +40mV for **A-E** and –40mV for **F-J**. Single channel opening is upward from the zero current level (+40 mV) and downward from the zero current level (-40 mM; continuous line) to the maximum open conductance (broken line). Cis/*trans* [Cs^+] is shown above each record.

+40 mV **-40 mV**

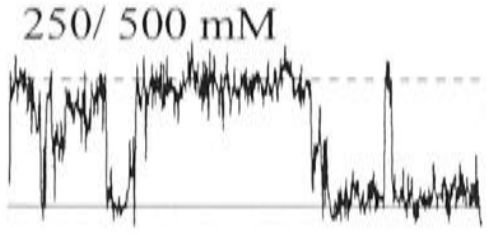
A 250/250 mM Cs^+



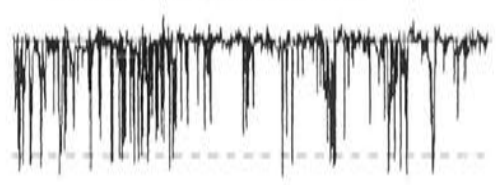
F 250/ 250 mM Cs^+



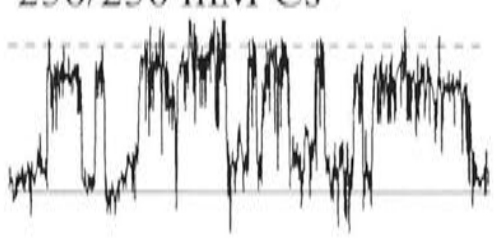
B 250/ 500 mM



G 250/ 500 mM Cs^+

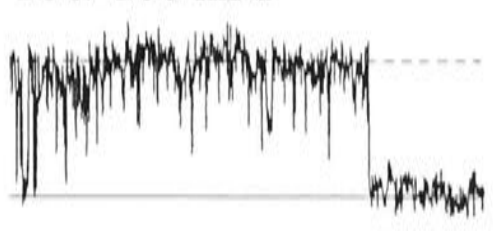


C 250/250 mM Cs^+

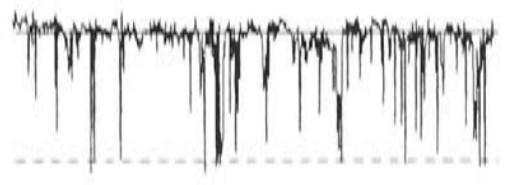


H 250/ 250 mM Cs^+

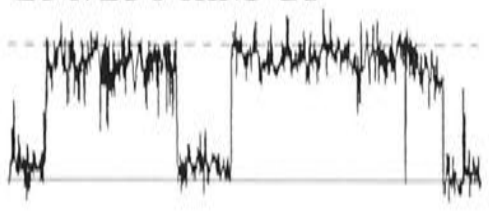
D 250/ 500 mM



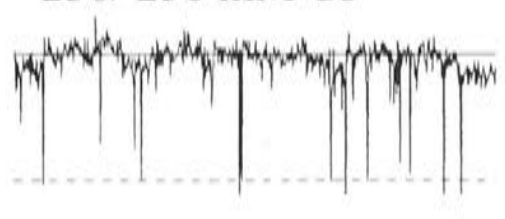
I 250/ 500 mM Cs^+



E 250/250 mM Cs^+

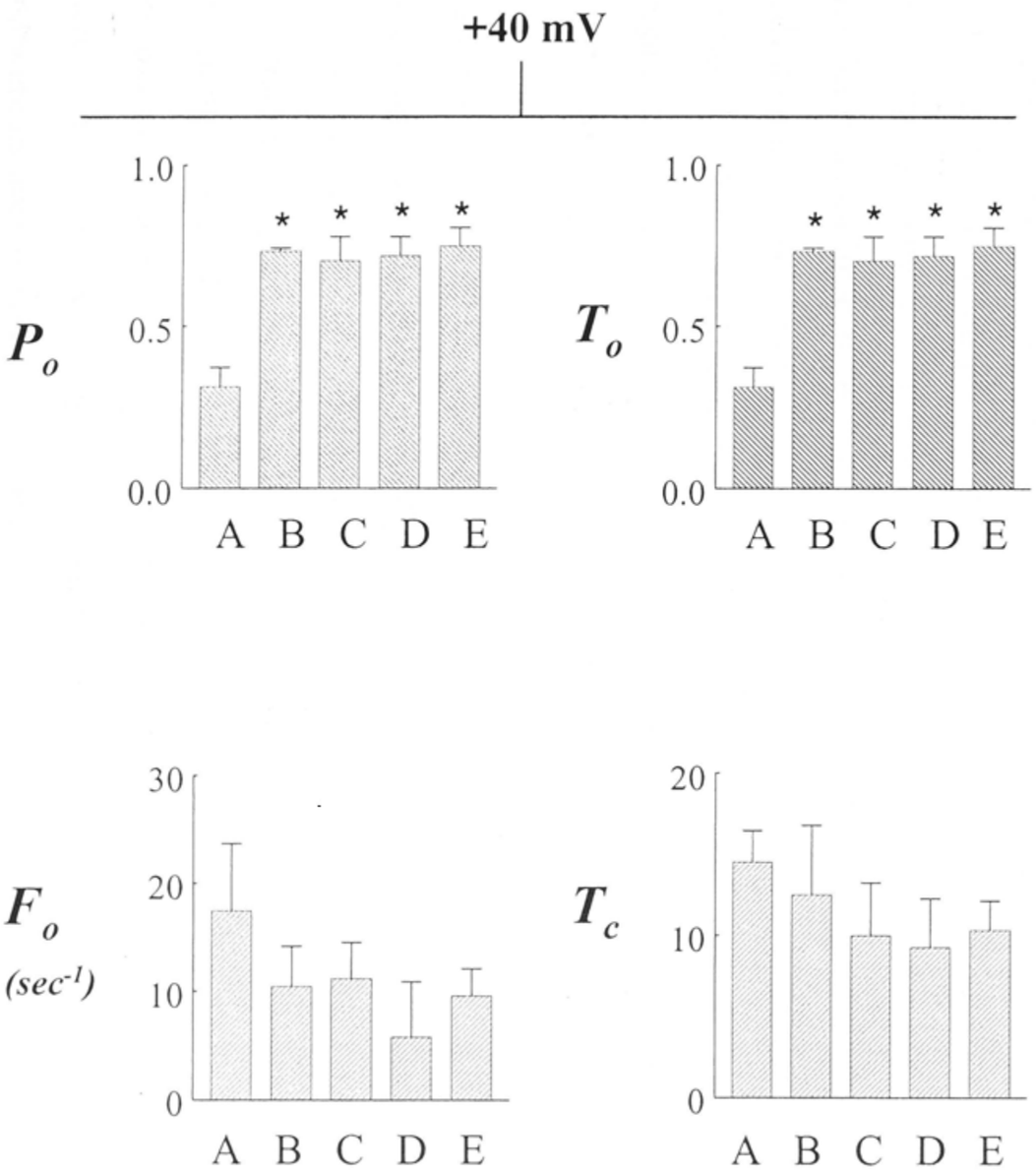


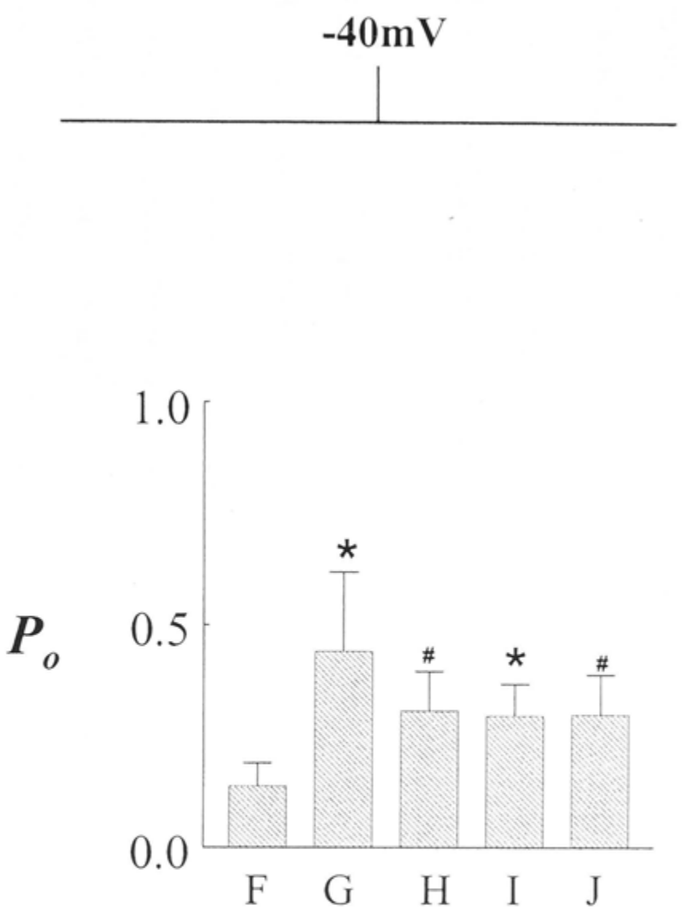
J 250/ 250 mM Cs^+



1 s 6 pA

Fig. 3.VIII. ***Single channel parameters, increasing trans ionic strength to 500 mM causes an irreversible increase in single RyR channel activity.*** Average data (n = 8 at +40 mV; n=5 at –40 mV) for P_o,T_o, F_o and burst T_c, at +40 mV, as shown in Fig. 3.VII. (where **A**: 250/250 Cs^+ (*cis*/*trans*), **B**: raising *trans* $Cs^+$to 500 mM; **C**: after perfusions of the *trans* chamber with 250 Cs^+, **D&E**: same as **B&C**). Average data for P_o, for conditions **F-J** (which are a repeat of **A-E**, but at –40mV), as shown in Fig. 3.VII. Asterisks (*) indicate average values significantly different from control ($p<0.05$, t-test) and cross hatch (#) indicate average values significantly different from control (sign test, $p<0.05$) .


+40 mV
P_o
1.0
0.5
0.0
A B C D E
T_o
1.0
0.5
0.0
A B C D E
F_o
(sec-1)
30
20
10
0
A B C D E
T_c
20
10
0
A B C D E


-40mV
P_o
1.0
0.5
0.0
F G H I J

activity recorded pre-Cs^+ increase (Fig. 3.VIII). Due to the number of multiple channel recordings in this data set, T_o, T_c and F_o were not analysed. In two bilayers with single channels, the Cs^+-induced T_o increase was 9.6 and 4.0-fold. Neither T_c nor F_o changed significantly (the Cs^+-induced F_o increase was ≤1.2-fold from control, whilst T_c decreased ≤1.4-fold from control).

3.2.4.1.2 Time-dependence of RyR activation

Both Sakowska (2000) and Gallant *et al.* (2001) reported a time-dependent RyR activation in channels recorded for 10 min – 1 h. There is no apparent experimental basis for this; recordings were done without the addition of activators. Even without standard *cis* activators μM Ca^{2+} and mM ATP, distinct activity increases were observed in some cases (Sakowska, 2000), but not always (Gallant *et al.*, 2001). In light of these observations, all efforts were made to show reversal of activation by either removal of the ligands inducing activity rise, or replacement of an inhibitory protein (e.g. CSQ). However, it should be noted that the average time a channel was recorded was 15 min, and spontaneous P_o changes over this time frame when reported were ~10% (Sakowska, 2000). In all experiments presented here, increasing *trans* ionic strength resulted in a P_o or I'_F increase to two to four times higher than control, so even if part of this increase was due to an intrinsic increase in channel activity, a substantial proportion of RyR activation is a direct result of the application of high Cs^+. It will be shown later (see Section 3.2.5) that subsequent addition of CSQ reduced activity to within 95% of the original control activity. This indicates that intrinsic activation was less than 6% in the present experiments.

3.2.4.1.3 ATP phosphorylation

To discount possible effects of ATP phosphorylation, bilayers were formed in both the presence and absence of 2 mM ATP. Dulhunty *et al.* (2001) have shown that ATP can irreversibly phosphorylate approximately 60% of RyRs and this results in activation becoming irreversible after 5 and 10 min exposure to ATP. This time frame is the approximate time after ATP exposure that a rise in activity was observed following an increase in Cs^+ concentration to 500 mM. Five channels were incorporated into bilayers and were incubated with ATP for at least 15 min before channel incorporation, which was at least 20 min before activity was recorded in the presence of 500 mM *trans* Cs^+ (Fig 3.IX). These channels responded to 500 mM Cs^+ in a similar fashion to channels

Table 3.II. ***Comparison of I'_f/I'_c of activity after exposure to 500 mM Cs^+. The effects of phosphorylation of RyRs by ATP addition on observed activity.***

	I'_f/I'_c
ATP added ~ 5 min before exposure to 500 mM Cs^+	2.54 ± 0.242
ATP added >20 min before exposure to 500 mM Cs^+	2.34 ± 0.25 *1

RyR phosphorylation in the presence of ATP does not account for the increase in RyR activity observed after addition of 500 mM Cs^+. A marginally smaller (*1, $p < 0.15$) relative mean current, I'_f/I'_c was observed 20 min after ATP addition, when ATP had presumably phosphorylated the channel before the experiment commenced (phosphorylation of the RyR has been shown to occur within 10 min of addition of ATP; Dulhunty *et al*, 2001).

which were exposed to 2 mM ATP approximately 2 min before increasing [Cs^+] (and therefore may have undergone ATP phosphorylation during the CSQ dissociation period; compare Figs. 3.VIII & 3.IX). Fig. 3.IX shows a typical increase in relative mean current, test relative to control (I'_t/I'_c, representing a change in I'_F from 0.054 ± 0.01 to 0.135 ± 0.04) observed after exposure to 500 mM Cs^+ shown at least 22 min after addition of ATP to the *cis* chamber. Presumably if ATP phosphorylation were occurring, phosphorylation-dependent effects would have induced a rise in RyR activity well before this stage.

The activity of channels after prolonged exposure to ATP (Fig. 3.IX) did not look very different from that after brief exposure to ATP (Fig 3.VIII above). This was not surprising because ATP-induced phosphorylation simultaneously causes an irreversible activation of RyRs and reduces the activation of the channel by ligand binding of ATP. Therefore phosphorylation is not necessarily reflected in an increase in activity during response to ATP; it is seen as a failure to reverse the activation by ATP removal (Dulhunty *et al.*, 2001).

Table 3.II illustrates that the change in I'_t/I'_c for those channels thought to have undergone ATP phosphorylation well before Cs^+ was increased to 500 mM, was not different from channels exposed to ATP approximately 5 min before [Cs^+] increase (and the subsequent channel activity rise induced by CSQ dissociation). These data suggest that ATP phosphorylation is not responsible for the activation induced when *trans* Cs^+ is increased to 500 mM.

3.2.5 Re-association of CSQ

3.2.5.1 The effect of *trans* CSQ on the single channel parameters of channels previously exposed to 500 mM Cs^+

At +40 mV, I'_t/I'_c (which initially increased 3-fold from control levels upon [Cs^+] increase, and was not altered by subsequent reduction of Cs^+ from 500 to 250 mM) was reduced 2.3-fold by the addition of 20 μg CSQ (Fig. 3.X). Whilst marginally higher than control levels, I'_t/I'_c recorded upon re-association of CSQ did not differ significantly from control (Fig. 3.X E). At –40 mV, the drop induced by CSQ addition

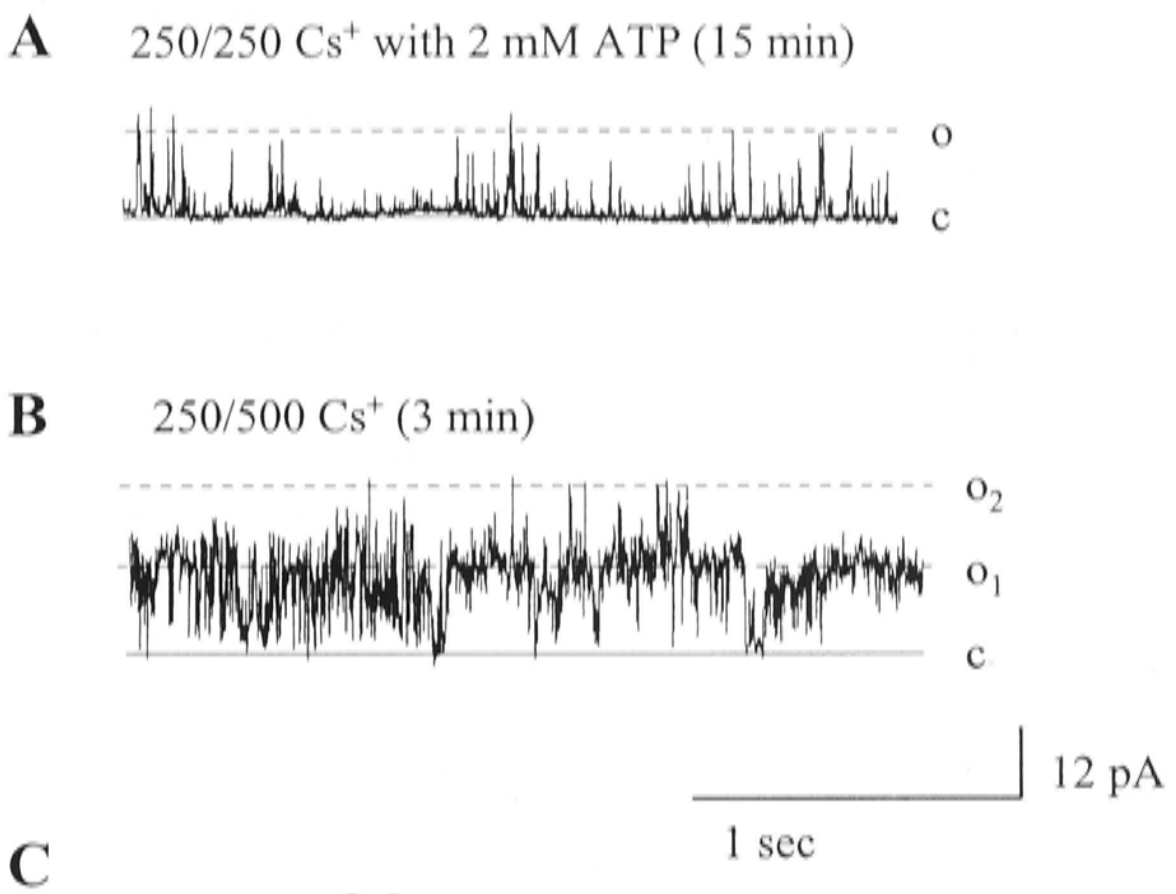


Fig. 3.IX. ***Response of potentially phosphorylated RyRs to an increase in [Cs^+] (250 to 500 mM trans Cs^+).*** (**A**) Control activity, 15 min after the addition of ATP to the chamber (approximately 20 mins before channel activity rise was observed); (**B**) at least 3 min after *trans* increase to 500 mM Cs^+, *cis/trans* Cs^+ is shown above **A&B**; (**C**) average data (n = 5) for relative mean current (test relative to control; I'/I'_c) under conditions shown in **A&B**. V_m-E_{Cs+} was +40mV under all conditions. Single channel opening is upward from the zero current level (continuous line) to the maximum open conductance (o, o_1 and o_2; broken line). Asterisks (*) indicate average values significantly different from control ($p<0.05$, t-test).

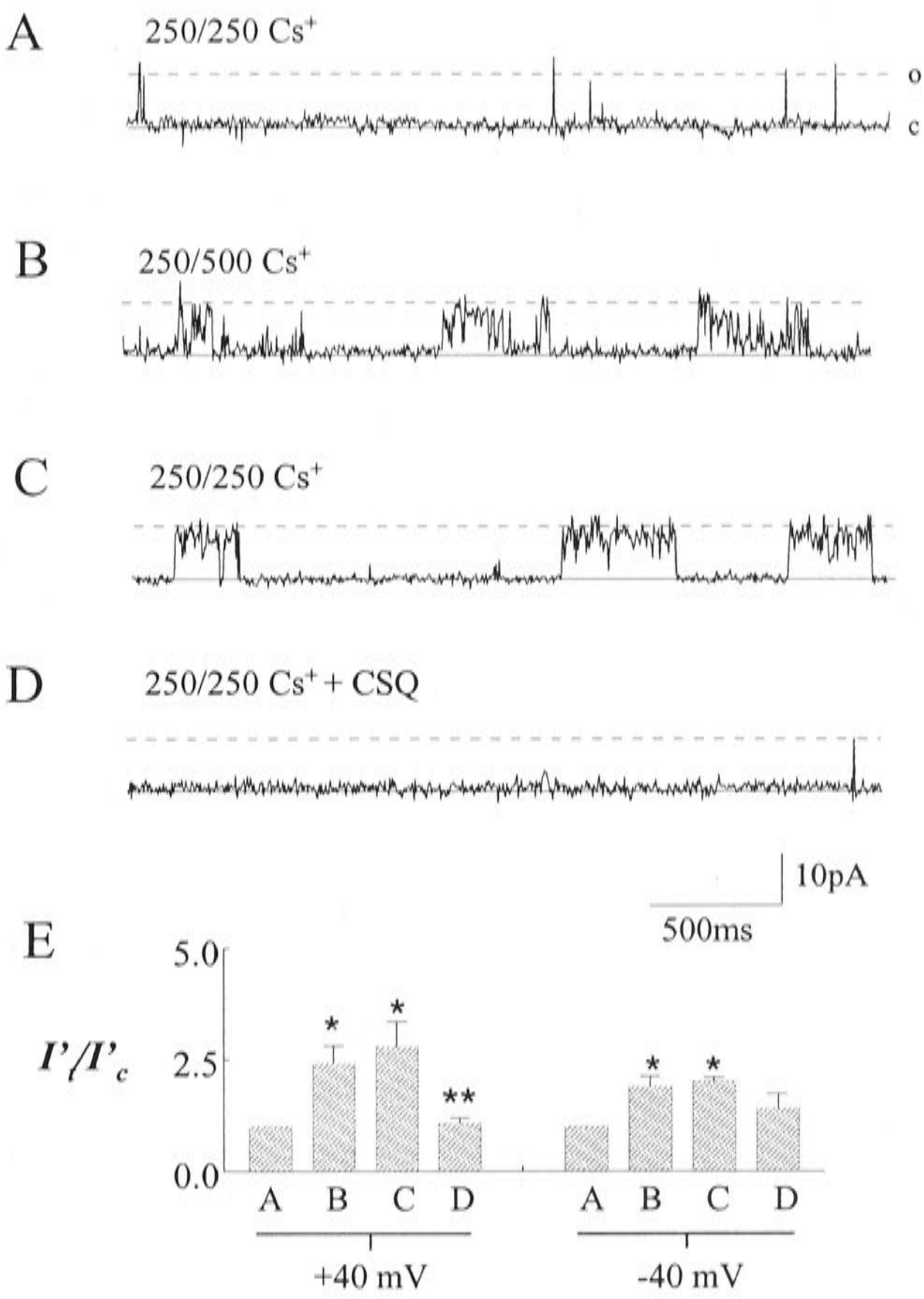


Fig. 3.X. ***The effect of increasing trans ionic strength to 500 mM can be reversed by the addition of 20 μg CSQ.*** (**A**) Control activity with 250 mM *trans* Cs^+; (**B**) after increasing *trans* Cs^+ to 500 mM with CsCl; (**C**) perfusing the *trans* chamber with 250 mM Cs^+; (**D**) addition of 20 μg *trans* CSQ. *Cis/trans* Cs^+ is shown above records **A-D;** (**E**) Average data (n = 5) showing relative mean current - test relative to control (I'_t/I'_c) under conditions shown in **A-D** at both +40 and –40 mV. V_m-E_{Cs+} was +40mV under all conditions shown in **A-D**. Single channel opening is upward from the zero current level (continuous line) to the maximum open conductance (broken line). Asterisks (*) indicate average values significantly different from control, ** indicates activity significantly different from the previous conditions ($p<0.05$, t-test).

was less pronounced. The initial 1.9-fold increase in I'_i/I'_c due to ionic strength increase was only reversed 1.4-fold by CSQ addition. Despite this, channel activity was still not significantly different from control levels ($P > 0.06$, t-test; Fig. 3.X E). It may be that phosphorylation upon ATP addition, thought to be responsible for a small proportion of RyR activation (see Section 3.2.4.1.3), could be responsible for an irreversible component of the activation of RyRs during exposure to high ionic strength, and may explain CSQs inability to fully restore control activity of RyRs. Intrinsic RyR activation (discussed above) may also contribute to this irreversible increase in activity.

As it appears that CSQ-induced changes in RyR activity are not voltage-dependent, data obtained only at positive potentials is reported in subsequent experiments. The only exception to this is when a new RyR or CSQ preparation was used, and in Chapter 5, when investigation the role of luminal Ca^{2+} on RyR activity.

3.2.5.2 Channel-CSQ dose response curve

The irreversible increase in channel activity resulting from the increase in $[Cs^+]$ was reversed by the addition of 20 μg CSQ to the *trans* chamber. Fig 3.XI is a dose response curve, illustrating the response of CSQ-depleted RyRs (after exposure to 500 mM Cs^+ and subsequent perfusion with 250 mM Cs^+, resulting in a 2.6-fold increase in P_o) to various [CSQ], over a 10 min time period.

There was no significant change in channel activity of RyRs exposed to 0, 1, 2 or 5 μg CSQ, but channel P_o was significantly lowered upon addition of 20 or 50 μg CSQ. In CSQ-responsive channels (those exposed to ≥ 20μg CSQ), activity was recorded for 15 min after CSQ addition, with no detectable alteration in channel activity observed after the initial drop. Presumably, CSQ binds to the RyR/T/J complex with a high affinity, as it is able to sustain inhibition for 10 min. Either CSQ associates and remains associated, or if CSQ does dissociate, it re-associates quickly, so that the overall inhibition on RyR activity is maintained. The latter is unlikely because CSQ remains associated with the native RyR channel until dissociation with high ionic strength or other manipulations (see Chapter 5), when the [CSQ] in the *trans* chamber is infinitely low. As there was no observable difference in the inhibition shown at 20 μg or 50 μg,

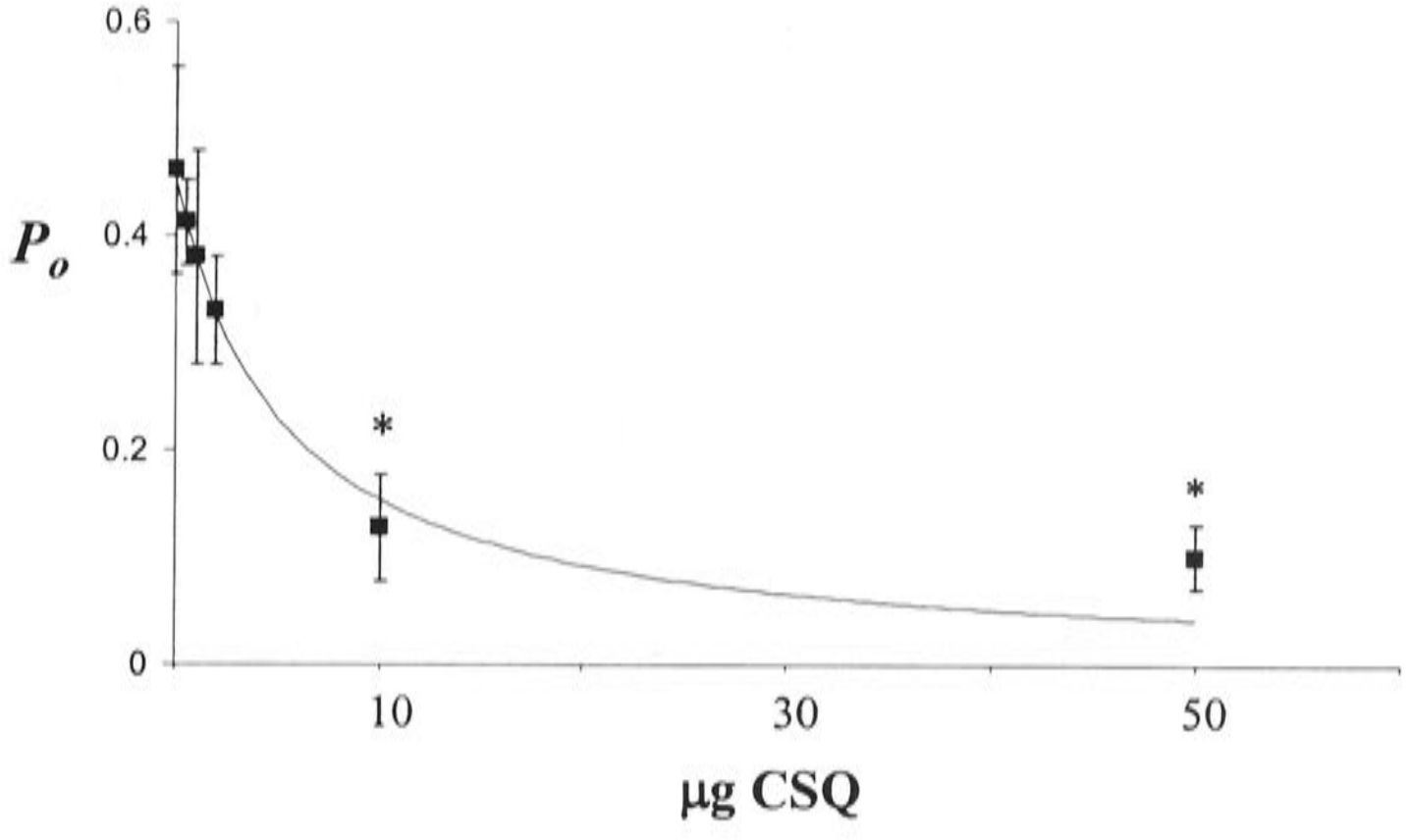


Fig. 3.XI. ***Concentration dependent inhibition of native RyRs by exogenous CSQ.*** Data is presented as open probability (P_o) at various [CSQ]. Error bars are SEM, except for data point at 2 µg CSQ, where n = 3 and SD is presented. V_m-E_{Cs+} was +40mV. Asterisks * indicates a significantly lower P_o than at control (0 µg CSQ; $P<0.05$, t-test). Each point is the mean P_o for 3-7 channels.

the lower concentration was chosen for further experiments (Figs. 3.X, 3.XII-III). The dose response curve is shown for activity obtained at +40 mV.

3.2.5.3 Effect of the 30 kDa protein on channel activity

In another set of experiments, the effect of CSQ containing the contaminant 30 kDa protein was tested on CSQ-dissociated RyRs. After Cs^+ increase to 500 mM and subsequent *trans* perfusion (with expected sustained increase in channel activity), 20 μg CSQ + the 30 kDa protein essentially reversed the increase in I'_t/I'_c (Fig. 3.XII). A second overnight dialysis of the CSQ preparation containing the 30 kDa protein (refer to Chapter 2, Section 2.5.3 for details) was sufficient to remove this protein from the CSQ preparation, (Fig. 3.V). In both the absence and presence of the 30 kDa contaminating protein, CSQ was able to reverse the ionic strength-induced increase in channel activity to levels close to control (CSQ with the 30 kDa protein: $I'_t/I'_c = 1.07$ and 0.102 (n=2); CSQ without the 30 kDa protein: $I'_t/I'_c = 1.378 \pm 0.31$ (n=5), showing that CSQ and not the 30 kDa protein, induced channel inhibition.

3.2.5.4 Effect of heat-treated CSQ and CSQ storage buffer

To ensure that RyR inhibition was CSQ-induced, rather than a non-specific effect of the protein or a component of the buffer in which it was stored, both heat-treated CSQ in storage buffer and CSQ storage buffer (without CSQ) were added to the *trans* chamber. A volume of 100 μl CSQ buffer minus CSQ was added to the *trans* chamber, which was in excess (by 15 – 50%) of the volume of buffer added to channels, as part of the CSQ re-association experiments. It was assumed that boiling CSQ for 10 min would denature the protein. Fig. 3.XIII A-C shows that addition of storage buffer minus CSQ, after the ionic strength increase to 500 mM (resulting in an 2.1-fold increase in I'_t/I'_c, which was maintained after subsequent perfusion with 250 mM Cs^+ (data not shown)), did not alter. Neither storage buffer minus CSQ, nor the addition of 20 μg heat-treated CSQ in storage buffer (Fig. 3.XIII D-F) reduced channel activity from the high [Cs^+]-induced increase. In 6 channels, I'_F was 0.54 ± 0.143 after exposure to high [Cs^+] and 0.57 ± 0.14 after 100 μl CSQ buffer minus CSQ, whilst in another 4 channels, I'_F was 0.35 ± 0.07 after dissociation (500 *trans* Cs^+ and subsequent perfusion with 250 mM Cs^+) and 0.33 ± 0.06 after addition of *trans* heat-treated CSQ. Thus, channel inhibition

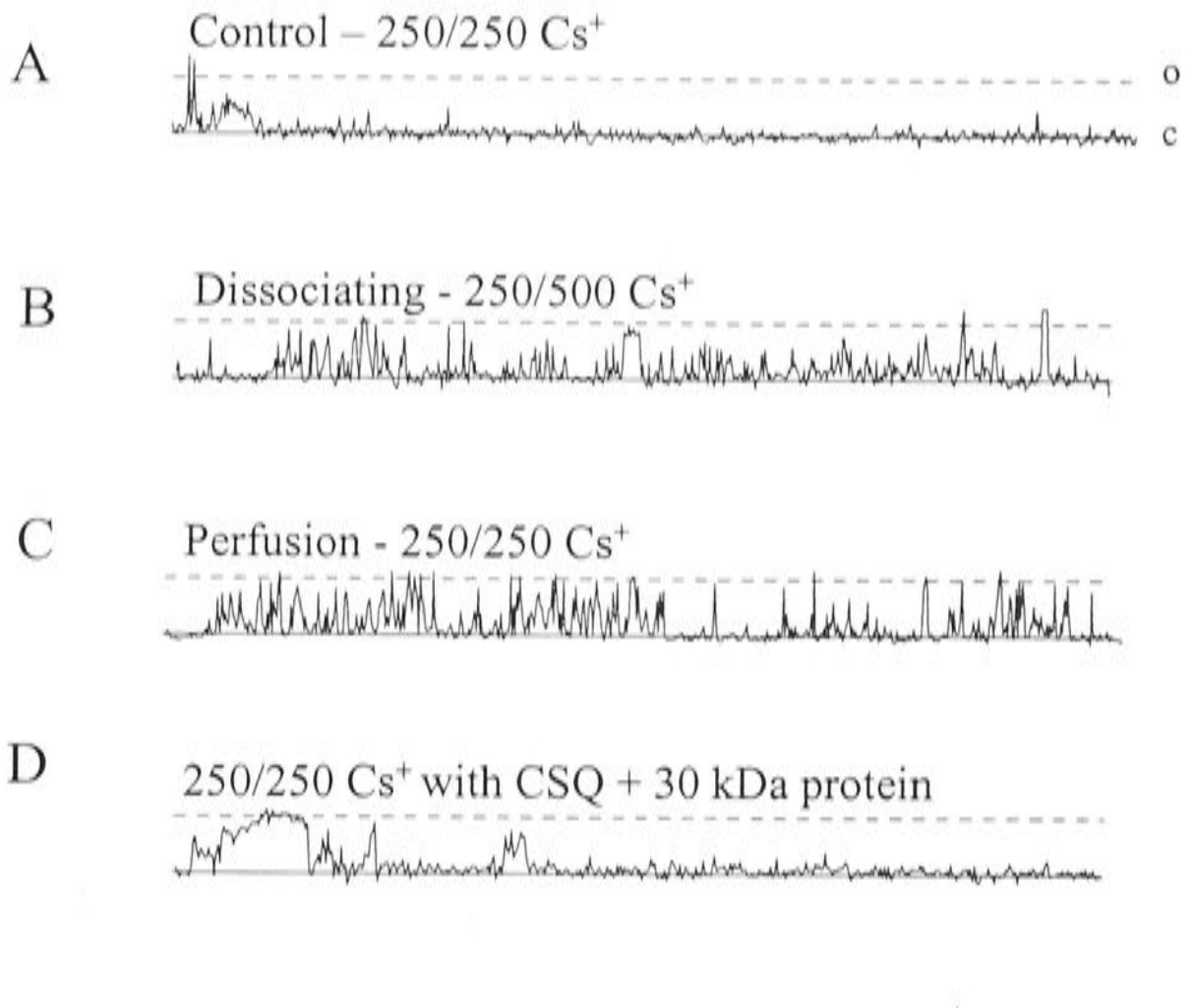


Fig. 3.XII. ***Increasing trans ionic strength to 500 mM can be reversed by the addition of 20 μg CSQ + 30 kDa protein.*** (**A**) Control activity with 250 mM *trans* Cs^+; (**B**) after increasing *trans* Cs^+ to 500 mM with CsCl; (**C**) perfusing the *trans* chamber with 250 mM Cs^+; (**D**) Addition of 20 μg CSQ + contaminating 30 kDa protein. *Cis/trans* Cs^+ is shown above **A**-**D**; (**E**) Average data (bar graph, n = 2) showing fractional mean current (I'_F) under conditions shown in **A**-**D**. Nested line graph shows data from each of the two individual channels. V_m-E_{Cs+} was +40mV under all conditions. Single channel opening is upward from the zero current level (continuous line) to the maximum open conductance (broken line).

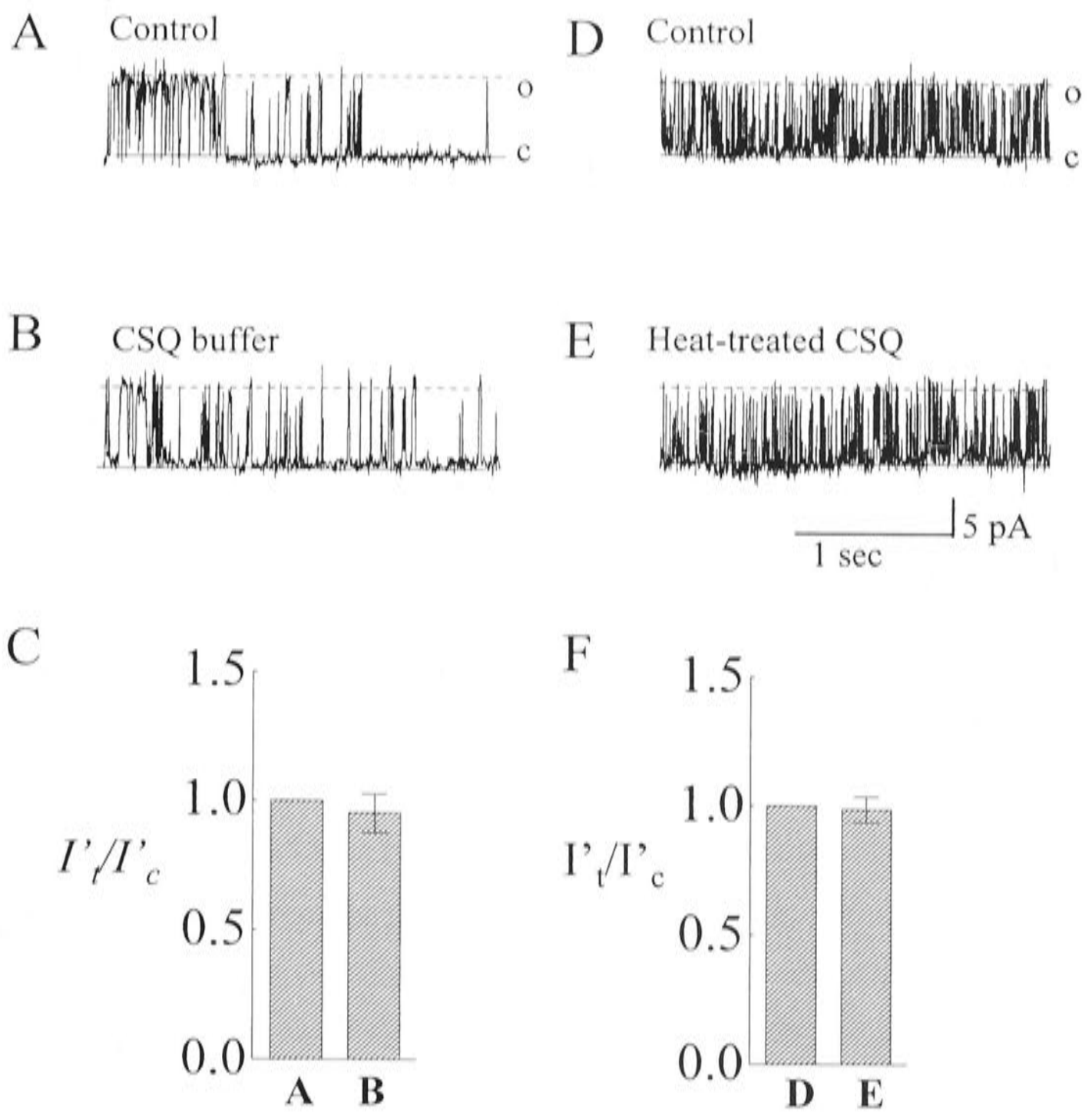


Fig 3.XIII. ***Neither CSQ storage buffer (without CSQ) nor heat treated CSQ in storage buffer reversed activity induced by CSQ dissociation in single channel RyRs.*** (**A**) Control activity with 250 mM *trans* Cs^+, after increasing Cs^+ to 500 mM (to dissociate CSQ) and *trans* perfusion with 250 mM Cs^+; (**B**) after addition of 100 µl CSQ storage buffer (without CSQ): (mM) 1 $CaCl_2$, 10 TES, 100 KCl; (**C**) average data (n = 6) for relative mean current, I'_f/I'_c (test relative to control conditions shown in A) for **A&B**; (**D**) control activity with 250 mM *trans* Cs^+, after increasing Cs^+ to 500 mM (to dissociate CSQ) and *trans* perfusion with 250 mM Cs^+; (**E**) after addition of 20 µg heat treated CSQ. (**F**) average data (n = 4) for, I'_f/I'_c (test relative to control conditions shown in D) for **C&D**. No significant differences in activity from control was observed for either heat-treated CSQ or CSQ buffer. V_m-E_{Cs+} was +40mV under all conditions. Single channel opening is upward from the zero current level (continuous line) to the maximum open conductance (broken line).

by buffered CSQ can be attributed to CSQ alone, and not a non-specific effect due to the presence of protein or a component of the buffering system. Ms Magda Sakowska undertook the study involving the addition of heat-treated CSQ under my supervision.

3.2.6 Anti-CSQ antibody inhibits RyR single channel activity

3.2.6.1 Specificity of anti-CSQ antibody to CSQ

Polyclonal antibodies display a variety of affinities and activity to the antigen in question. Provided that there is no cross-reactivity with other antigens, polyclonal antibodies are a more effective way of identifying antigens than monoclonal antibodies.

A polyclonal anti-CSQ antibody was tested for specificity to CSQ using SDS-Page and Western blot. The potential for non-specific antibody interactions were examined. Fig. 3.XIV (lane 2) shows anti-CSQ antibody binding specifically to a protein resolving at approximately 55 kDa (CSQ) in a SR sample, with no other bands visible in the Western blot. Anti-CSQ antibody was used at a concentration of ten times that normally used in this laboratory, to optimise the chance of non-specific antibody binding. These results indicate that any effects of anti-CSQ antibody on RyR channel behaviour were likely to be induced by anti-CSQ antibody binding to CSQ alone.

The effects of non-specific interaction of IgG with CSQ were also examined in a second control experiment. IgG was added to the *trans* chamber at 0.15 – 1.5 μg and did not alter RyR I'_F, despite incubation for 10 min, and frequent stirring (every 30-45 s; Fig. 3.XV). This confirms the specificity of anti-CSQ antibody for CSQ and shows that the effects induced by anti-CSQ antibody addition (below) were not simply due to the binding of non-specific IgG. The channels presented as part of Fig. 3.XV were undertaken (in part) by Ms Magda Sakowska under my supervision.

Brandt *et al.* (1992) first showed that the application of an antibody specific for triadin could inhibit the slow phase of depolarisation-induced SR Ca^{2+} release, but not the fast component, demonstrating that an antibody to a luminal RyR regulating protein may

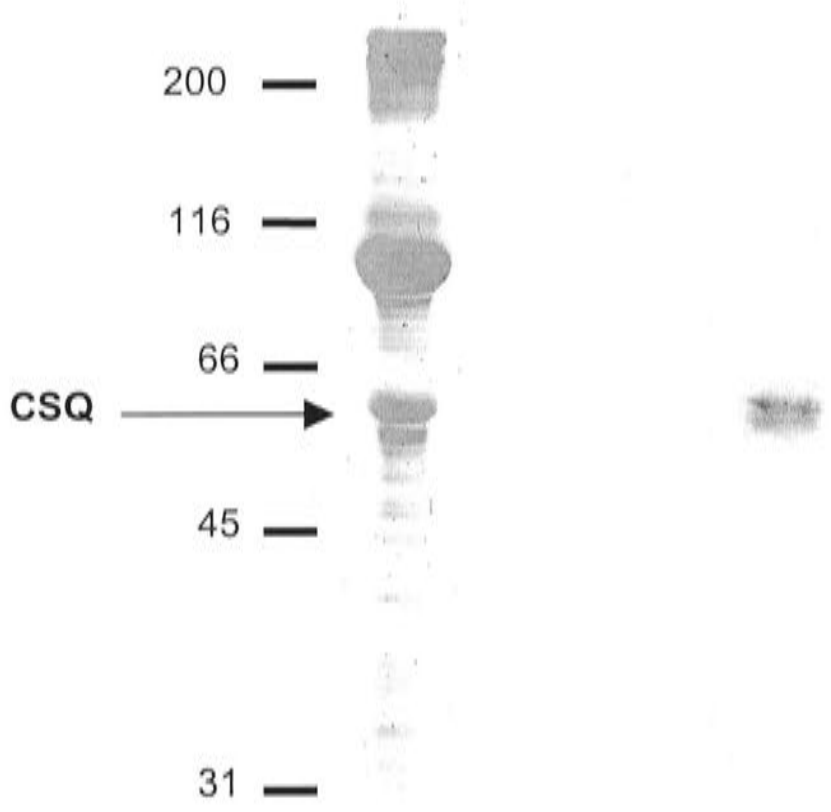


Fig. 3.XIV. ***SDS-Page (12%) and Western blot, showing the specificity of the anti-CSQ antibody for CSQ in native SR.*** The band resolving at ~55 kDa in the SR sample (lane 1) was identified as CSQ by anti-CSQ antibody (lane 2). Note that anti-CSQ antibody does not detect the presence of any other proteins within the SR isolate. The position of molecular weight markers is shown to the left of lane 1. Proteins were transferred onto Immobillon-P PVDF membranes, prior to probing with anti-CSQ antibody.

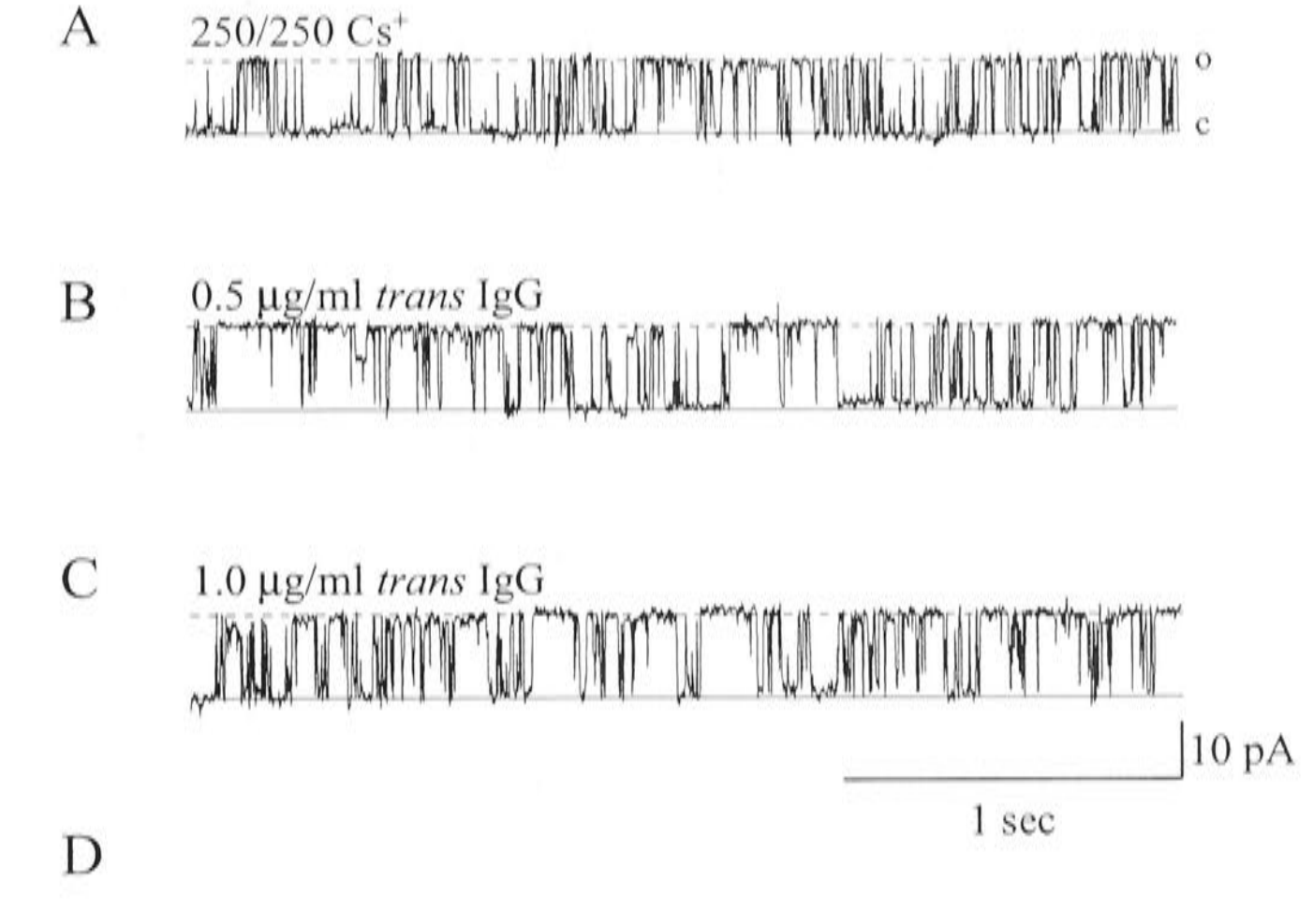


Fig 3.XV. ***IgG does not affect single RyR channel activity.*** (**A**) Single channel activity under control conditions; (**B**) after adding 0.5 µg/ml IgG to the *trans* solution; (**C**) after adding 1.5 µg/ml IgG to the *trans* solution, *cis*/*trans* Cs^+ is shown above record **A**; (**D**), dose response curve for for average data (n=4) for relative mean current (I'_f/I'_c). All I'_f/I'_c were insignificantly different from control (0 µg IgG). V_m-E_{Cs} was at +40 mV, single channel opening is upward from the zero current (continuous line) to the maximum open conductance (broken line). Instead of 2 mM cis ATP, 2 mM AMP-PCP was used to activate the channels.

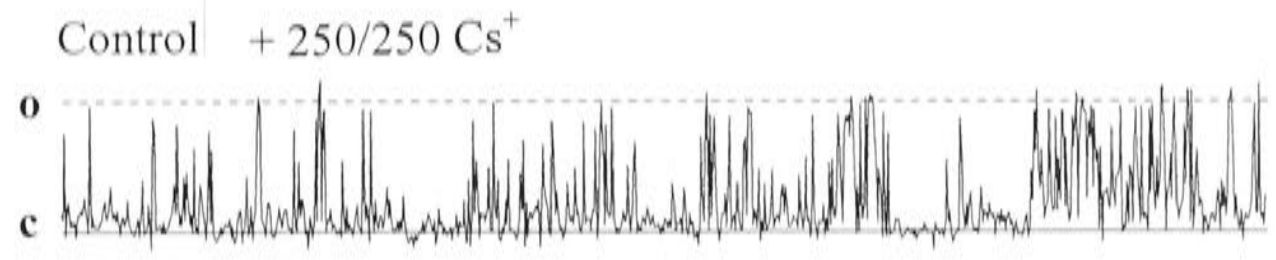
induce regulatory changes on the RyR. Following on from this idea (and to further investigate the interaction between the RyR and CSQ), the effects of a polyclonal anti-CSQ antibody on RyR activity were investigated. Addition of 0.5 μg anti-CSQ antibody (stored in 0.02% NaN_3) to the *trans* chamber significantly inhibited RyRs (Fig. 3.XVI), at +40 mV (in these channels, control *cis* solution also contained 3 mM $MgCl_2$). Inhibition occurred within 30 s of the antibody being added to the *trans* chamber (and subsequent stirring). Anti-CSQ antibody addition resulted in a 5.9-fold reduction in P_o (Fig. 3.XVI C), due to a 5.1-fold lengthening of burst T_c (Fig. 3.XVI E) causing a 5.7-fold reduction in channel F_o (Fig. 3.XVI F). No significant change in T_o was observed. At negative potentials, 0.5 μg anti-CSQ antibody induced a 3.6-fold fall in P_o, due to a 2.8-fold reduction in F_o (Fig. 3.XVII).

Anti-CSQ antibody did not alter activity in 10 of 10 RyRs when added to the *trans* solution following treatment with 500 mM *trans* Cs^+, which increased I'_f/I'_c 2.3-fold (Fig. 3.XVIII). Conversely, RyR activity fell in each of 3 experiments in which RyRs were exposed to antibody (0.5 μg/ml) after they had been exposed to 500 mM *trans* Cs^+, and then to 20 μg purified CSQ. I'_F fell from 0.05 ± 0.02 (after re-association of CSQ) to 0.016 ± 0.007 (addition of anti-CSQ antibody; $p<0.05$, t-test). Together these results suggest that (a) the antibody inhibited RyRs by binding to CSQ and (b) confirms that the high ionic strength solution dissociated CSQ from the RyR complex. Two of ten channel presented in this set of experiments contained 3 mM *cis* $MgCl_2$.

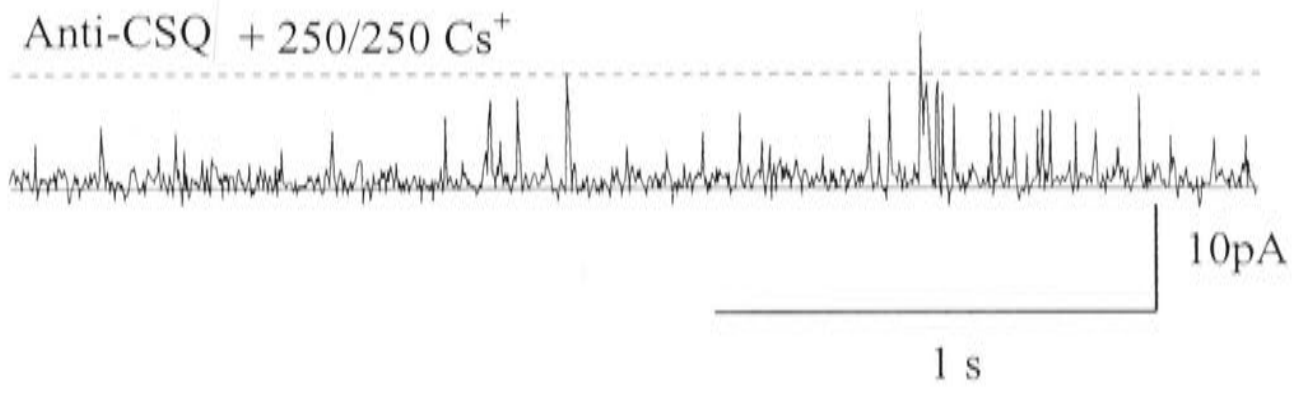
NaN_3, a commonly used laboratory preservative, was used at 0.02% in the anti-CSQ antibody solution. In muscle isolation, NaN_3 is commonly added to inhibit H^+-ATPase activity and block mitochondrial ATP-dependent calcium transport. To be certain that anti-CSQ antibody and not NaN_3 was inhibiting RyR activity, NaN_3 was added to the *trans* chamber at the same concentration as that added as part of the anti-CSQ antibody solution (60 nM which is approximately $4x10^{-6}$ % (w/v)). In 3 channels, no change in I'_F was observed upon NaN_3 addition (Fig. 3.XIX). In a representative channel, [NaN_3] was increased to 60 μM, with no alteration in channel activity observed. Hence, it is the anti-CSQ antibody, and not NaN_3, that inhibits the channel. Two of the three channels presented in this section were obtained by Ms Magda Sakowska (under my supervision).

Fig. 3.XVI. ***Anti-CSQ antibody reduces single RyR channel activity at +40 mV.*** (**A**) Single channel activity under control conditions; (**B**) after adding 0.5 μg/ml anti-CSQ antibody to the *trans chamber*. In **A&B**, [Cs^+]s *cis/trans* are shown above each record; (**C-F**) average data for P_o (**C**), T_o (**D**), F_o (**E**) and burst T_c (**F**), under control conditions (**A**) and after adding anti-CSQ antibody (**B**). Average data represent each of 6 individual channels, which are means, with SE error bars. Nested line graph shows data from each of 6 channels. V_m-E_{Cs} was at +40 mV, single channel opening is upward from the zero current (continuous line) to the maximum open conductance (broken line). An * indicates average values significantly different from control ($p<0.05$, t-test).

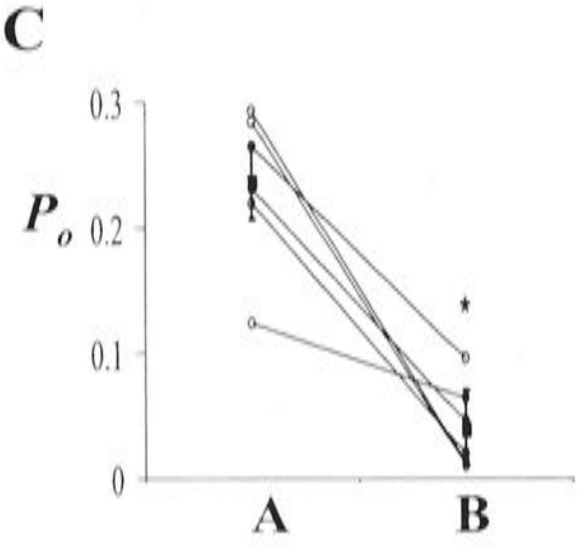
A


Control
+ 250/250 Cs+
o
c

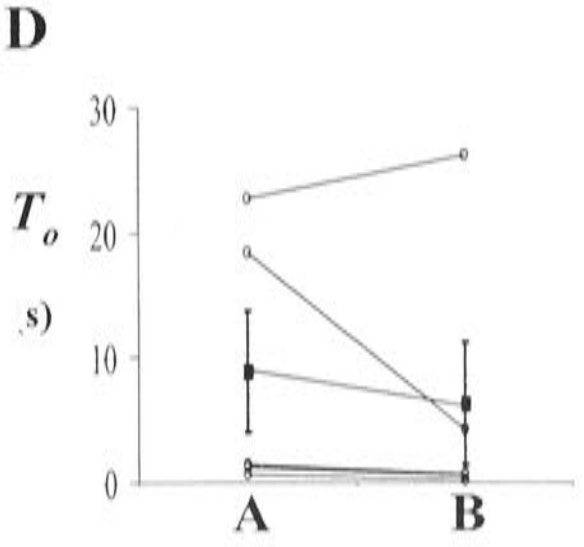
B


Anti-CSQ
+ 250/250 Cs+
10pA
1 s

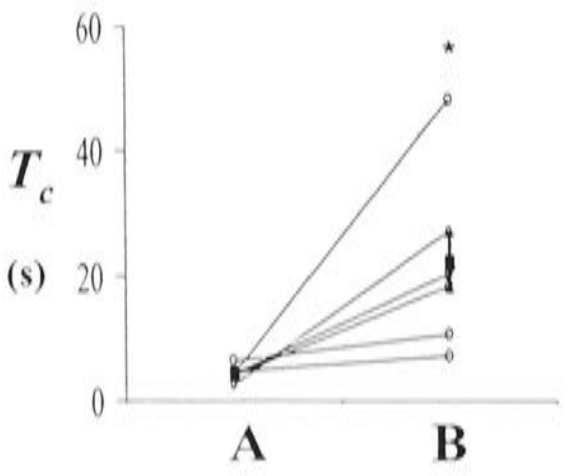
C


Po
0.3
0.2
0.1
0
A
B

D


To
(s)
30
20
10
0
A
B

E


Tc
(s)
60
40
20
0
A
B

F

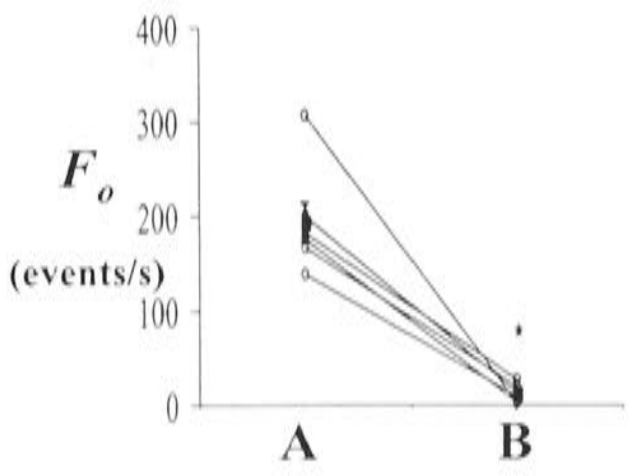
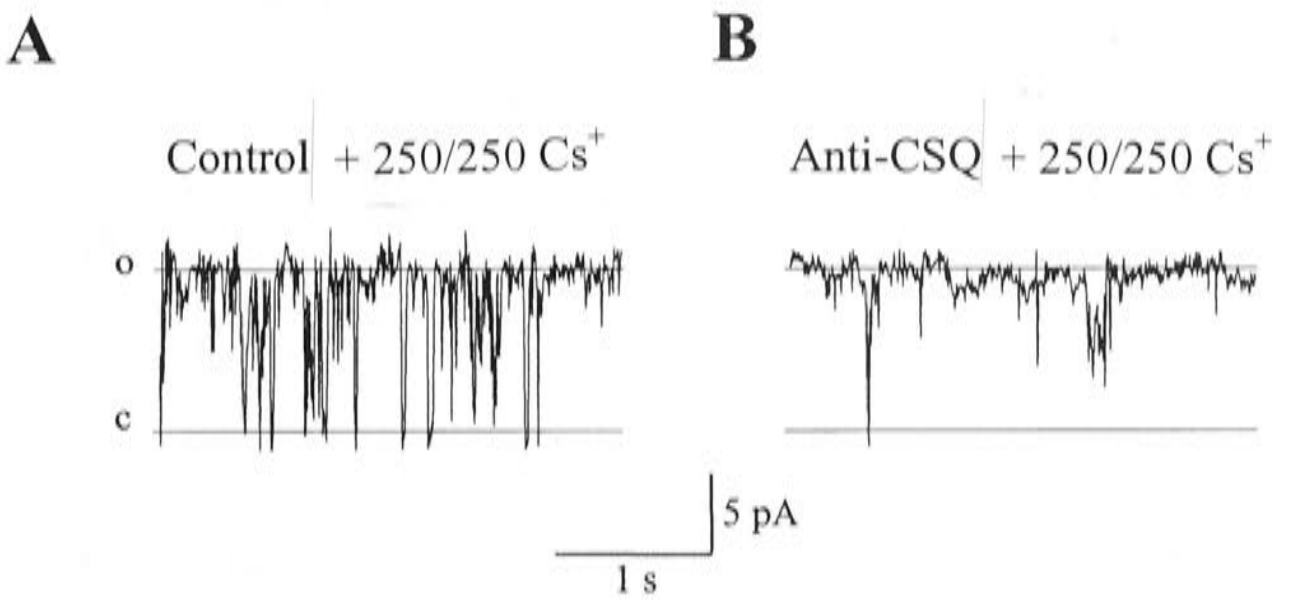
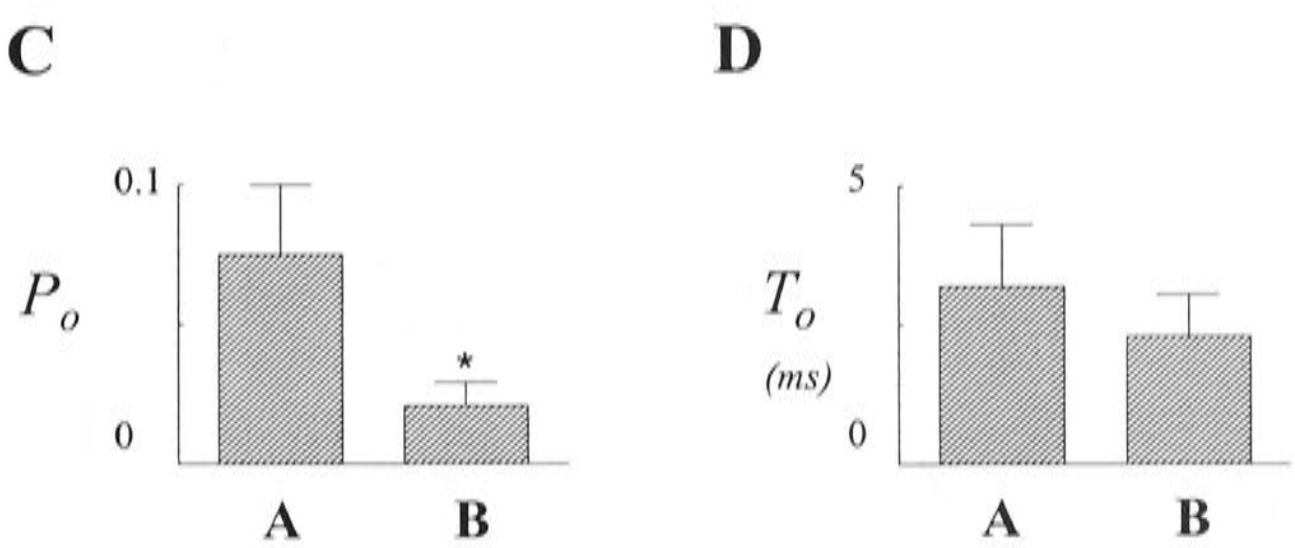
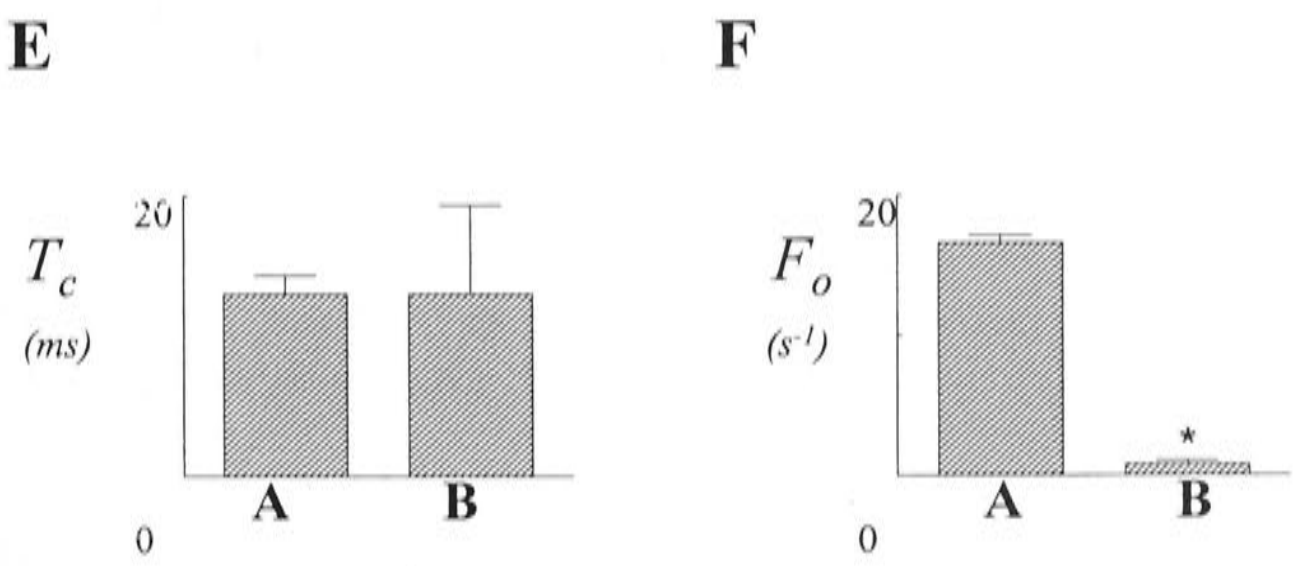

Fo
(events/s)
400
300
200
100
0
A
B

Fig. 3.XVII. ***At –40 mV, anti-CSQ antibody reduces single RyR channel activity.*** (**A**) Single channel activity under control conditions; (**B**) after adding 0.5 μg/ml anti-CSQ antibody to the *trans* solution. In **A**&**B**, *cis/trans* [Cs^+] are shown above each record; (**C-F**) average data (n = 6) for P_o (**C**), T_o (**D**), F_o (**E**) and burst T_c (**F**), under control conditions (**A**) and after adding anti-CSQ antibody (**B**). V_m-E_{Cs} was at -40 mV, single channel opening is upward from the zero current (continuous line) to the maximum open conductance (broken line). An * indicates average values significantly different from control conditions ($p<0.05$, t-test).


A
B
Control + 250/250 Cs+
Anti-CSQ + 250/250 Cs+
o
c
5 pA
1 s


C
D
0.1
Po
0
*
A
B
5
To
(ms)
0
A
B


E
F
20
Tc
(ms)
0
A
B
20
Fo
(s-1)
0
*
A
B

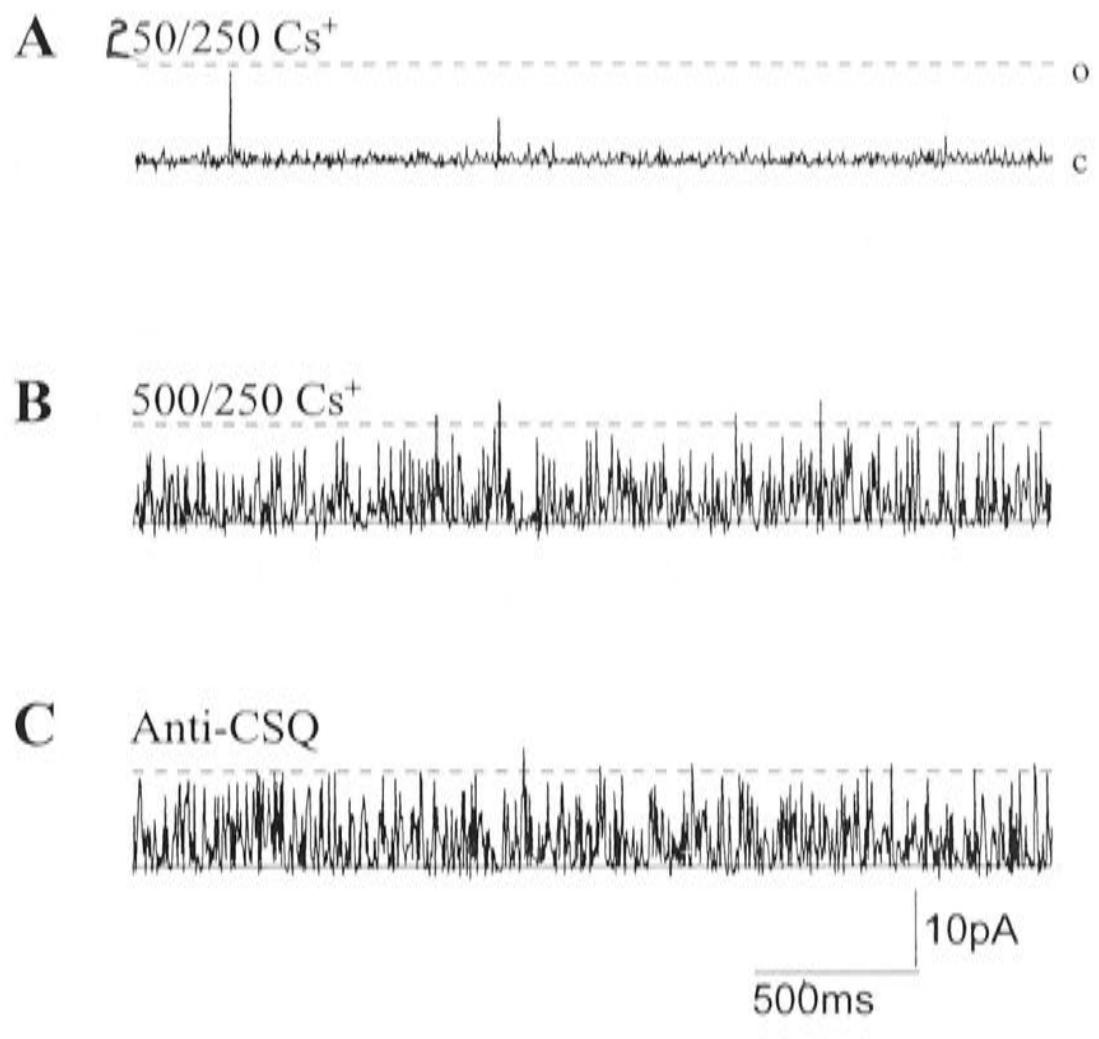


Fig. 3.XVIII. ***Anti-CSQ antibody does not alter activity in CSQ-depleted single RyRs***. (**A**) Control channel activity; (**B**) after increasing *trans* Cs^+ to 500 mM; (**C**) after adding 0.5 µg/ml anti-CSQ antibody to the *trans* chamber. *Cis/trans* Cs^+ are shown above traces **A-B**;(**D**) Average mean current (test relative to control; I'/I'_c; n = 9) under control conditions control (**A**), after increasing *trans* Cs^+ to 500 mM (**B**) and after addition of anti-CSQ (**C**). V_m-E_{Cs} was +40 mV, single channel opening is upward from zero current (continuous line) to the maximum open conductance (broken line). In 2 of the 10 channels, 3 mM $MgCl_2$ *cis* was used. The * indicates that there is a significant increase in I'/I'_c above after increasing *trans* ionic strength to 500 mM ($p<0.05$, t-test).

3.2.6.2 Response of RyRs to an *in-house* anti-CSQ antibody

The results in the previous section show that anti-CSQ antibody can be used as a tool in determining whether or not RyR channels are associated with CSQ, i.e. whether the RyR/T/J complex has CSQ attached (see Chapter 4, Section 4.2.1.). The predominantly fast twitch rabbit CSQ anti-CSQ antibody used in this study was purchased from Swant chemicals (Switzerland), and the epitope of CSQ this antibody is raised against was unknown. An *in-house* polyclonal anti-CSQ antibody was generated (see Chapter 2, Section 2.10), because the commercial preparation of anti-CSQ antibody was no longer available. The *in-house* antibody was made against the whole CSQ protein. To determine whether the *in-house* anti-CSQ antibody induced similar RyR activity responses to commercially available anti-CSQ antibody, both the serum containing anti-CSQ antibody and the first bleed serum (rabbit serum obtained before CSQ inoculation) were added to RyRs incorporated in bilayers.

3.2.6.2.1 Single channels response to pre-inoculation bleed serum

Addition of up to 0.43 mg/ml pre-inoculation bleed serum was added to the *trans* chamber in 3 channels, stirring every min for 10 min, and as expected, the pre-inoculation bleed serum did not alter channel activity from levels recorded under standard control conditions (see Section 3.2.1 for control conditions; Fig. 3.XX A-C).

3.2.6.2.2 Single channels response to final bleed serum

Between 1 and 800 μg final bleed serum was added to the *trans* chamber, and similar to the results observed upon addition of the pre-inoculation bleed serum to the *trans* chamber, *in-house* anti-CSQ antibody did not alter RyR activity. I'_F was 0.066 ± 0.014 under control conditions and 0.062 ± 0.015 after addition of serum containing anti-CSQ antibody (Fig. 3.XX D-E). After addition, the channels were recorded for up to 10 min, stirring every minute, to optimise anti-CSQ antibody binding, with no change in I'_F. Unlike the commercially available anti-CSQ antibody, the *in-house* anti-CSQ antibody did not induce any observable alterations in RyR activity.

It is of interest to compare the vastly different action of the commercially available and the *in-house* anti-CSQ antibodies, and their abilities to modulate RyR activity. The commercially available anti-CSQ antibody was able to significantly reduce RyR

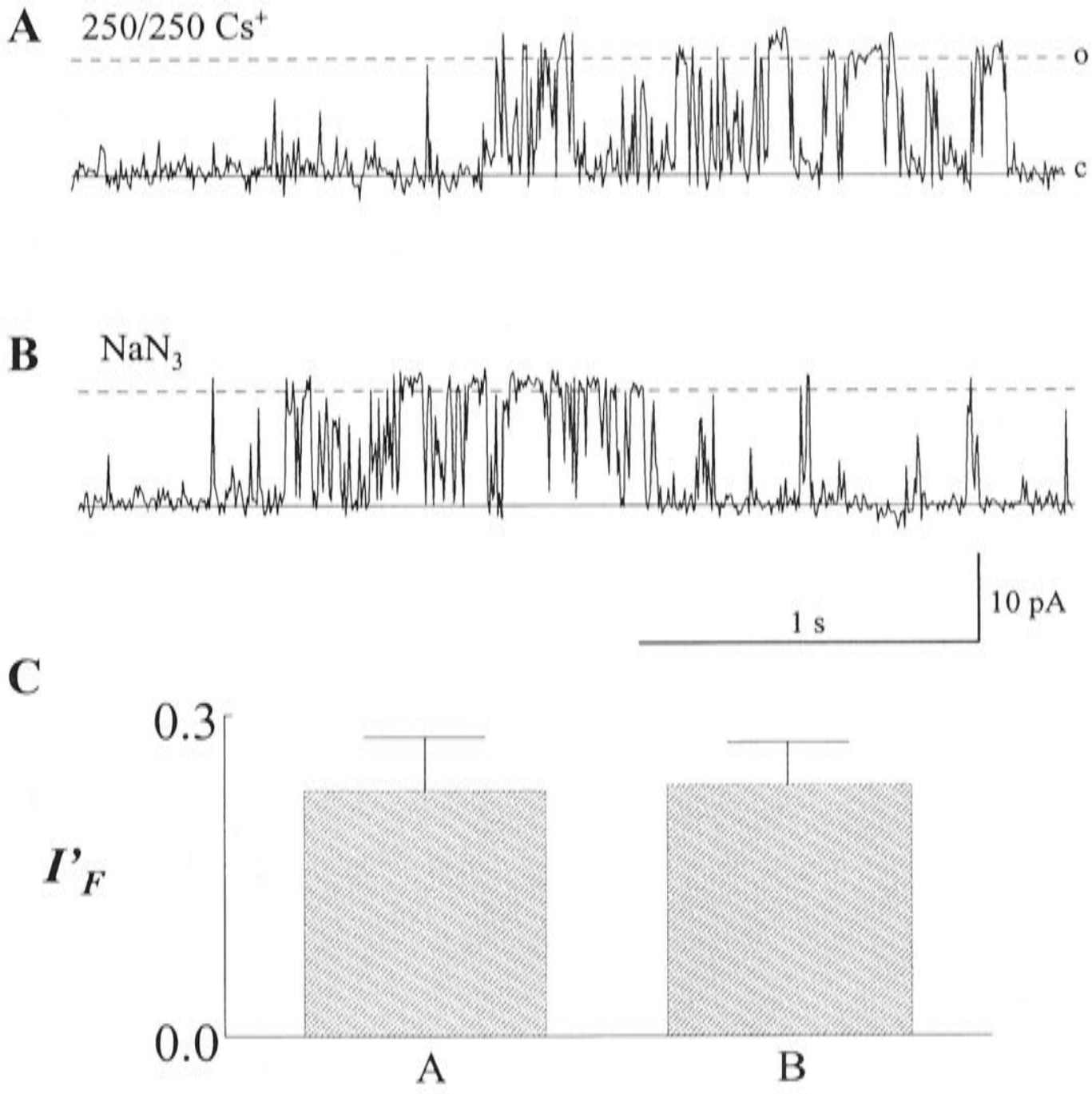


Fig. 3.XIX. ***NaN_3 does not inhibit single RyR channel activity.*** (**A**) Single channel activity under control conditions; (**B**) after adding 60 nmol NaN_3 to the *trans* solution. *Cis/trans* Cs^+ is shown above record **A**; (**C**) average data (n=3) for fractional mean current (I'_F), for control (**A**) and after addition of NaN_3 (**B**). There is no significant difference in I'_F between control and NaN_3. V_m-E_{Cs} was at +40 mV, single channel opening is upward from the zero current (continuous line) to the maximum open conductance (broken line).

channel activity, whilst the *in-house* anti-CSQ antibody was unable to modulate RyR activity. The *in-house* anti-CSQ antibody was generated against the whole CSQ protein, but it is not known whether the commercially available anti-CSQ antibody was generated against the whole protein, or a small CSQ peptide. Immunodetection data illustrates that the *in-house* anti-CSQ antibody binds to CSQ (see Fig.3.XIX). It is possible that both the commercially available and the *in-house* anti-CSQ antibodies bind to different epitopes, with the commercially available binding influencing CSQ conformation, in a manner that allowed it to alter RyR activity. The *in-house* antibody either did not alter CSQ conformation upon binding, or altered it in a manner that did not influence RyR activity.

Fig. 3.XX. ***Neither pre-immune serum nor final bleed serum (after CSQ inoculation) inhibit single RyR channel activity.*** (**A**) Single channel activity under control conditions; (**B**) after adding 100 nmol pre-immune bleed to the *trans* chamber; (**C**) fractional mean current (I'_F) plot, of two channels, under conditions **A**&**B**; (**D**) repeat of **A**; (**E**) after adding 100 nmol final bleed serum to the *trans* solution; (**F**) average data (n=4) for I'_F, for conditions **C**&**D**. There is no significant difference in I'_F between control and either the pre-inoculation or the final bleed serum. V_m-E_{Cs} was at +40 mV, single channel opening is upward from the zero current (continuous line) to the maximum open conductance (broken line, o, o_1 & o_2).

A Control

o

c

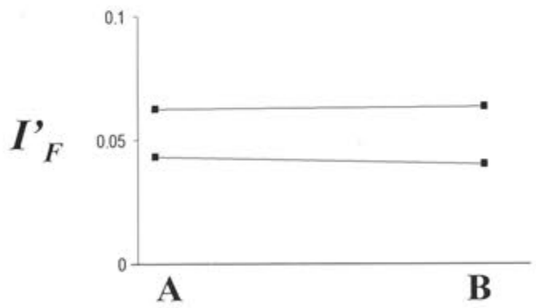
B

Pre-inoculation serum

1 s

10 pA

C



D Control

o_2

o_1

c

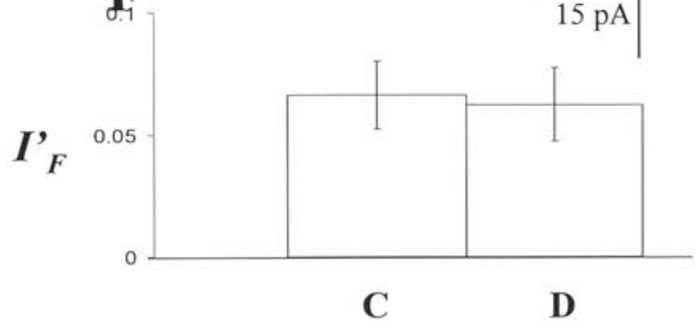
E

Final bleed serum

15 pA

1 s

F



3.3 DISCUSSION

The results in this section illustrate that associations of CSQ with the native RyR/T/J complex in lipid bilayers decreases channel activity in the presence of the cytoplasmic Ca^{2+} and ATP concentrations, and luminal Ca^{2+} concentrations which are present in resting muscle. High luminal ionic strength (500 mM) used to dissociate CSQ from the JFM, caused an increase in channel activity which was reversed by the re-association of exogenous CSQ. In addition, changes in CSQ induced by the binding of commercially available anti-CSQ antibody, further inhibit RyR activity. Finally, one anti-CSQ antibody emerged as a useful tool in detecting whether CSQ is associated with RyR/T/J complex.

3.3.1 RyR identification and characterization

As previously mentioned, RyRs are not the only ion channels found in SR (and in fact make up the minority of all channels found within the SR); occasionally small conductance ion channels were observed which were not responsive to ryanodine. Having said this, in 97% of all channel incorporations under the conditions used here, the ion channel was identified as a RyR by its response to various activators and inhibitors. For example, incorporated channels exhibited RyR-typical *cis* Ca^{2+} activation (at μM Ca^{2+}) and inhibition (at mM and nM Ca^{2+}). In addition, ATP induced channel activation, whilst Mg^{2+} inhibition was observed in channels under varying Ca^{2+} and ATP concentrations. Also, channel responses to ryanodine and ruthenium red were characteristic of RyRs. Ryanodine is a specific modulator of RyRs, while ruthenium red has been shown to also affect Ca^{2+} channels in mitochondria, although mitochondrial ion channels are highly unlikely to be present in a SR isolate. All channels analysed displayed a conductance of approximately 250 - 525 pS in symmetric Cs^{+} (250 mM), which falls well within the ranges reported for skeletal muscle RyRs.

3.3.1.1 Multichannel bilayers and substates

It is worth noting that although channel activity with an amplitude of approximately15 pA indicated single RyR channel activity, this does not mean that only one channel was present in the bilayer. It was more likely (due to the frequent

occurrence of two or more active channels under activating conditions), that more than one channel has incorporated into the bilayer, with only one of these channels active. Given the geometry of RyRs the JFM (two parallel rows of 30 nm RyRs) and the size of the vesicles (100 nm diameter), it would be surprising if vesicles did not contain several RyR, with only one or two active.

Substate activity is a property of native RyRs. Subconductance levels may be due to channel gating events that are improperly resolved by the recording apparatus, or to the channel opening to several discrete conductance levels. These conductance levels are present in native channel activity and can be enhanced by the removal of a RyR associated protein (such as FKBP12), or addition of ryanodine, both of which results in functional alterations of RyRs. In the absence of the binding protein, three maintained subconductance levels are observed (25, 50 & 75% of maximal conductance; Ahern *et al.*, 1997a; Brillantes *et al.*, 1994), although brief openings to some levels were observed in the presence of FKBP12. Studies of FKBP depleted RyRs suggest that RyRs possess four coupled conduction pathways (Liu and Pessah, 1994). The presence of subconductance levels in the present data could be due to FKBP12 removal, but this is unlikely. There is no evidence to suggest that channels used in this study were lacking FKBP12. Indeed, Western blots reveal the presence of FKBP12 in all native SR preparations examined by other members of the group.

Subconductance levels of 25, 50, and 75% were observed in native RyRs (Fig. 3.I and Ahern *et al.*, 1997a). The presence of substates corresponding to multiples of ¼ of maximal activity suggests that at times, the four ion conduction pathways become uncoupled. The reason for spontaneous uncoupling remains unclear, but subconductance levels are observed in many types of ion channels.

Subconductance states might be due to the presence of another small conductance channel in the bilayer, although this was unlikely, due to the relatively fast switch observed between a substate and maximal open state (see Fig. 3.I).

3.3.2 Dissociation and Re-association of CSQ from the RyR

It was first shown by Ikemoto *et al.* (1989) that CSQ could be dissociated from solubilized JFM, simply by increasing surrounding ionic strength. As in the detailed lipid bilayer study presented here, a significant proportion of CSQ was selectively removed by exposure to 500 mM NaCl. Presumably, ionic bonds attaching CSQ to triadin and junctin are broken by the high ionic strength, leaving the protein free and easy to isolate.

3.3.2.1 Re-association of CSQ

In contrast to the findings of Ikemoto *et al.* (1989), who report CSQ-enhanced SR Ca^{2+} release, several lines of evidence have been presented here which show that CSQ may be an inhibitor of the RyR. Firstly, increasing ionic strength dissociated CSQ and irreversibly raised channel activity, suggesting that the RyR was inhibted by CSQ. Secondly, addition of exogenous CSQ returned RyR activity close to control levels. Finally, after increasing Cs^+ to 500 mM (dissociating conditions), the RyR could no longer respond to the commercially produced anti-CSQ antibody, again suggesting that the increase in activity was due to dissociation of CSQ by the increase in ionic strength.

Re-association of exogenous CSQ did not return channel P_o exactly to control levels, although CSQ addition did result in a significant fall from dissociation-induced activation. The failure of CSQ to fully reverse the increase in activity may be explained by two factors: first, spontaneous long-term RyR channel activation, which is thought to contribute to a small rise in I'_F and P_o (see Section 3.2.4.1.2) and second, phosphorylation of the RyR by ATP (see Section 3.2.4.1.3). Both these phenomena may account for a small rise in channel activity from control levels, which could not be reversed by CSQ re-association. It seems unlikely that insufficient CSQ was re-associated with the RyR/T/J complex to fill all available binding sites, and hence be responsible for the inability of exogenous CSQ to totally reverse dissociation-induced RyR activation. Although an abundance of CSQ exists in the terminal cisternae (approximately 100 mg/ml SR; Williams and Beeler, 1986), the percentage of CSQ localized within the terminal cisternae and associated with the RyR/T/J complex is not precisely known. The addition of 20 µg CSQ to a bilayer bath with 1 or 2 RyRs

incorporated is well in excess of the amount of CSQ that is capable of binding to the RyR/T/J complex (as a rule, 33 – 100 μg of SR vesicles were added to the *cis* chamber in lipid bilayer recordings, even if it is assumed that all of this protein incorporated into the bilayer, CSQ only represents 7% of vesicle protein, approximately 2.3 – 7 μg). The addition of 50 μg CSQ did not alter channel activity any more than 20 μg, again suggesting that excess CSQ was available for binding to the RyR/T/J complex. Alternatively, lack of complete reversibility could suggest that high ionic strength permanently alters the ability of CSQ to interact with triadin and junctin and to alter RyR function. As CSQ can still bind to the RyR/T/J complex (Fig. 3.X-XII), believed to occur through triadin and junctin (Zhang *et al.*, 1997), 500 mM Cs^+ apparently does not dissociate triadin or junctin, or substantially alter the binding sites on these proteins for CSQ. Since triadin is thought to be linked to the RyR via a disulfide bridge, it is unlikely that it would be dissociated by high ionic strength.

3.3.2.2 CSQ activation vs. inhibition of the RyR

Several laboratories have reported that CSQ enhances SR Ca^{2+} release (Ikemoto *et al.*, 1989; Hidalgo and Donoso, 1995) and raises RyR activity (Kawasaki and Kasai, 1994; Ohkura *et al.*, 1994; Ohkura *et al.*, 1995). However, a preliminary report by Wang *et al.* (2001) suggests an inhibition of RyRs by CSQ, similar to that presented here (Figs 3.III, IV & VII). It is interesting to ponder the reasons for the opposite effects of CSQ on RyR regulation. Several possibilities come to mind. Firstly, the study presented here used 2 mM ATP to activate the RyR, added to mimic *in vivo* conditions. No other study of CSQ regulation of RyRs employing the lipid bilayer techniques used this adenine nucleotide as an activator. Could it be that the inhibitory nature of CSQ is potentiated by the presence of ATP? The effects of luminal Ca^{2+} are vastly different in both the presence and absence of this ligand (Sitsapesan and Williams, 1995; Tripathy and Meissner, 1996). Secondly, it may be that the binding of ATP to the channel has long-range effects on the ability of CSQ to regulate Ca^{2+} release, and therefore alter RyR activity. Finally, it may not be the binding of ATP to the channel *per se*, but rather ATP-induced phosphorylation of the RyR that facilitates CSQ inhibition. Protein phosphorylation may induce a small conformational shift in the RyR, exposing CSQ inhibitory sites, or limiting access to CSQ, triadin or junctin activation sites. Whilst these last two proposals are highly speculative, it is possible that

differences in laboratory techniques (such as the absence or presence of ATP) might be responsible for the observed difference in CSQs ability to regulate the RyR.

A second difference in technique is seen in experiments by Kawasaki and Kasai (1994), who used RyRs that had been depeleted of CSQ prior to incorporation into the bilayer, to study CSQ regulation of single channels. Kawasaki and Kasai (1994) show a minimal increase in RyR activity upon addition of CSQ (immediately after addition of 1 mM *trans* Ca^{2+}) , a rise that might not be significant (neither average data nor statistical inference was reported). It was not until bath stirring (which occurred after *both* Ca^{2+} and CSQ had been added), that activity increased dramatically. Ca^{2+} was added because it has been reported that CSQ both associates with the RyR/T/J complex, and forms a polymer (required for high capacity Ca^{2+} binding) at the physiological Ca^{2+} concentration (1 mM; He *et al.*, 1993). Possibly, RyR activity presumed to be induced by CSQ was in fact Ca^{2+}-induced. Increases in *trans* Ca^{2+} (from approximately 0 to 10 mM) are well known to activate native RyRs (Sitsapesan and Williams, 1995; Tripathy and Meissner, 1996). Similar effects of luminal Ca^{2+} on RyR activity are also described in Chapter 5 (see Section 5.3 for further discussion).

The conflicting reports of the action of CSQ on RyR activity are not due simply to the incorporation of the RyR/T/J complex into an artificial membrane. This study and Wang *et al.* (2001), which report CSQ as an inhibitor of RyRs, and Kawasaki and Kasai (1994), Ohkura *et al.* (1994) and Ohkura *et al.* (1995), who report CSQs ability to activate RyRs, all use the lipid bilayer technique. Another possible reason for the difference in CSQ regulation of RyRs is that an essential co-protein may remain associated with RyRs after the relatively mild dissociating procedures used here and by Wang *et al.* (2001), but not in other studies. Exposure to 500 mM *trans* Cs^{+} (with 1 mM *trans* Ca^{2+}) can disrupt charge-charge interactions between CSQ and junctin/triadin (Ikemoto *et al.*, 1989), and thus may dissociate CSQ, while leaving other RyR co-proteins in place (Fig. 3.V; Wang *et al.*, 2001 and Chapter 5, Section 5.2.3 and Figs. 5.I) use $\geq$10 mM *trans* Ca^{2+} to promote CSQ unfolding and possibly its reversion to the monomeric form, rendering CSQ unable to bind to the RyR/T/J complex (Wang *et al.*, 1998). These two (high ionic strength or high Ca^{2+}) mild dissociating procedures are unlikely to result in the removal of triadin and junctin from the RyR, as this association

is covalent and therefore not ionic strength or Ca^{2+}-dependent (see Chapter 5, Section 5.2.1).

On the other hand, it is clear that many proteins in addition to CSQ (including triadin and junctin), are dissociated from the SR by incubation with EGTA and CHAPS (Kawasaki and Kasai, 1994; Ohkura *et al.*, 1994; Ohkura *et al.*, 1995). In fact, Costello *et al.* (1986), after using EGTA to dissociate CSQ from other JFM proteins, showed a contaminant protein resolving around 97 kDa (which may have been triadin – the protein was not identified). Dissociation of essential proteins could account for the failure of CSQ to inhibit channel activity after incubation with EGTA or solubilization. The procedure could remove or modify triadin, a protein proposed here to be essential in the signalling pathway for CSQ inhibition of the RyR. As both Kawasaki and Kasai (1994) and Ohkura *et al.* (1998) use EGTA to prepare CSQ-depleted SR vesicles, could the activation observed by them be due to a direct CSQ-RyR interaction, as seen by Szegedi *et al.* (1999) and Herzog *et al.* (2000; see Chapter 4, Section 4.2.2.3 & 4.3.2 for further investigation of this hypothesis)?

Whilst the possible reasons for the reported differences observed in RyR regulation by CSQ are many, it should be noted that this study is the first to show the actions of both the removal and re-addition of CSQ from native RyRs incorporated into lipid bilayers. Re-association of CSQ restores the RyR activity close to pre-dissociation levels, providing the strongest evidence to date for CSQs role as a RyR regulator.

3.3.2.3 Re-association of CSQ in preparations also containing the 30 kDa protein

CSQ was able to restore activity to close to pre-dissociation levels whether or not a contaminant 30 kDa protein was present. If the 30 kDa protein can be partially isolated by increasing [NaCl] in a JFM preparation, it may also either fully or partially dissociate after $[Cs^+]$ increase to 500 mM Cs^+ in the lipid bilayer setup. The identity of the 30 kDa protein is unknown, but a protein binding to CSQ, termed the 30 kDa protein (and thought to be an ADP/ATP translocase) has been reported recently and is postulated to have a role in RyR regulation (Yamaguchi and Kasai, 1998). It is interesting to note that this protein co-purified with CSQ, whilst other CSQ-binding

proteins, triadin and junctin, did not. Possibly, the addition of 500 mM NaCl did not raise the ionic strength to a high enough level to release CSQ from the 30 kDa protein, assuming that they are associated as suggested by Yamaguchi and Kasai (1998). Although the preliminary experiments presented here do not suggest a role for the 30 kDa protein in RyR regulation, Yamaguchi *et al.* (1999) reported that atractyloside (ATR), an inhibitor of the ATP/ADP translocase (thought to be the 30 kDa protein, see above) reduced choline flux through RyRs in a Ca^{2+}-dependent fashion. At activating *cis* Ca^{2+} (10^{-5} M), the number of RyRs inhibited by ATR was approximately half, whilst choline influx rate through the RyR (and hence RyR activation), was maximized at lower *cis* [Ca^{2+}] ($10^{-5} - 10^{-7}$ M). According to these findings the 30 kDa protein should activate RyRs in the presence of 100 nM *cis* Ca^{2+}. This did not occur (Fig. 3.VI). The single channel studies reported by Yamaguchi *et al.* (1999) were performed in the absence of *trans* Ca^{2+} and it was probable that CSQ had dissociated from the RyR before the addition of ATR, (see Chapter 5, Section 5.2.3 for further evidence of low Ca^{2+}-induced CSQ dissociation). Therefore, these experiments would only show regulation by the 30 kDa protein, and not by CSQ. In light of current findings (see Fig. 3.XII), it seems probable that although the 30 kDa protein may be a RyR activator, its modulatory role is suppressed by the presence of CSQ. The mechanism for this is unknown, but CSQ may limit access to RyR binding site(s) on the 30 kDa protein, (presuming the 30 kDa protein binds to CSQ), and it may no longer be able to associate with the RyR.

3.3.3 Anti-CSQ antibody

There are several possible explanations for the different actions of the two anti-CSQ antibodies. The *in-house* anti-CSQ antibody may have not been at a high enough concentration to bind to CSQ within 10 min of exposure. Up to 800 µg of serum protein was added to the *trans* chamber (corresponding to up to 500 µl of serum). It was not feasible to purify the antibody from the final bleed serum, as only enough anti-CSQ antibody for up to six RyR channel experiments (and one specificity characterization experiment) would be purified, taking into account that at least 10% of antibody would be lost in the purification process. As neither pre-inoculation bleed serum, nor the serum containing antibody had any effect on RyR activity, antibody purification was not completed. The reasons for anti-CSQ antibody's inability to modulate RyR activity

could not be due to an interference of serum proteins with the antibody, since the pre-inoculation bleed serum would contain all potential contaminant proteins that could be found in the antibody containing serum.

It is possible that the commercially available anti-CSQ antibody was generated to a small peptide fragment of CSQ and not the whole protein, as was the case with the *in-house* antibody. It is conceivable that binding of anti-CSQ antibody to only part of the CSQ molecule (which would be the case with an antibody generated against a fragment of CSQ), induced a conformational shift that resulted in inhibition. The *in-house* anti-CSQ antibody that recognized and bound to epitopes over the whole CSQ molecule may not (a) bind to the same epitope as the commercially available antibody or (b) only a very small fraction of the *in-house* polyclonal antibody may bind to the epitope that allows it to influence RyR activity. The usefulness of the commercially produced anti-CSQ antibody lay in its ability to induce substantial RyR inhibition only when CSQ was present. The commercial anti-CSQ antibody was therefore used to determine whether or not CSQ was tethered to the RyR, in experiments presented in Chapter 4 (see Section 4.2.1.1).

3.3.4 Physiological implications of CSQ regulation of RyRs

Whilst gross changes in ionic strength (enough to dissociate CSQ) within the lumen are highly unlikely to occur *in vivo*, these data provide insight into the mechanisms of CSQ regulation of the RyR. Do small changes in the concentration of polyvalent ions occur *in vivo*, which result in CSQ dissociation? This hypothesis is explored further in Chapter 5.

Dissociation of CSQ is not the only way that CSQ can inhibit ryanodine receptors, as a conformational change (assumed to occur after binding of anti-CSQ antibody to CSQ) is enough to elicit RyR inhibition. It is certainly possible that such a change may be induced *in vivo*, by either alterations in total Ca^{2+} (the levels of which alter dramatically in muscle), K^+ or H^+ during the SR Ca^{2+} release/uptake cycle, or induced by structural changes in associated proteins upon RyR Ca^{2+} release. It is important to note that regulation of RyRs within the lumen may well rely on an interplay of proteins such as triadin, junctin, CSQ and the 30 kDa protein, with the summation of modulations

imposed by these proteins contributing to a highly controlled RyR Ca^{2+} release. Whatever the physiological mechanism may be, the data presented in this chapter are the first to systematically study the effect of CSQ dissociation/re-association on RyR regulation and the first to show CSQ as an inhibitor of SR Ca^{2+} release.

Chapter 4

MODULATORS OF CSQ ACTION ON RYRS: MODULATORS OF THE MODULATOR.

4.1 Introduction

Several different observations (listed below) suggest that the effect of CSQ on RyR activity can vary depending on the specific experimental conditions, and indicate that the effect of CSQ is subject to modulation by factors such as whether or not CSQ is already associated with the RyR, phosphorylation (of CSQ) and the presence of co-proteins. Data presented in Chapter 3 shows that only 68% of RyRs responded to manipulation intended to alter CSQ association with the RyR/T/J complex. 32 % of RyRs did not respond characteristically to increasing [Cs^+] or exposure to anti-CSQ antibody (those responding were described in Chapter 3). This observation raised the possibility that two populations of RyRs existed, one having CSQ associated and one lacking this association. This possibility is examined in this chapter.

CSQ may be phosphorylated *in vivo* Varsanyi and Heilmeyer, 1980; activation of purified RyRs by CSQ has been reported to be phosphorylation dependent – only dephosphorylated exogenous CSQ modulated purified RyR activity Szegedi *et al.*, 1999; Herzog *et al.*, 2000. These data were the first to illustrate (a) a direct CSQ-RyR interaction and (b) that the phosphorylation status of CSQ is important in RyR regulation by CSQ. It is unknown whether the interaction of CSQ with native RyRs (those containing RyR co-proteins, such as triadin and junctin), requires CSQ dephosphorylation in the same way as the interactions with purified RyRs. Therefore, the effect of CSQ phosphorylation is also considered in this chapter.

As discussed previously, CSQ has been reported to either activate or inhibit RyRs Kawasaki and Kasai, 1994; Ohkura *et al.*, 1995; Ohkura *et al.*, 1998; Beard *et al.*, 1999; Szegedi *et al.*, 1999; Beard *et al.*, 2000; Wang *et al.*, 2001; Beard *et al.*, 2002. As reported in Chapter 3, CSQ dissociation (by increasing *trans* Cs^+ to 500 mM) activated native RyRs in the presence of *cis* 100 nM Ca^{2+} and 2 mM ATP. Re-association of exogenous CSQ reduced channel activity to pre-dissociation levels, illustrating CSQs ability to inhibit RyRs. Wang *et al.* (2001) reported preliminary data also showing CSQ as an inhibitor of cardiac RyRs. In the only other comprehensive lipid bilayer study of CSQ modulation of the RyR, Szegedi *et al.* (1999) reported that purified RyRs were

either unaffected or activated by CSQ, depending on the phosphorylation state of CSQ. The possibility that the different actions of CSQ depend on the association of co-proteins with the RyR is examined here.

Changes in channel activity thought to reflect CSQ dissociation from the RyR, reported in Chapters 3 (above) and 5 (below), were slow taking several minutes to complete. It should be possible to independently measure the rate of CSQ dissociation from the RyR using fluorescence techniques. Therefore the final question addressed in this chapter is the rate of CSQ dissociation from the RyR/T/J complex. In an attempt to examine this, two chromophores were attached to CSQ to enable CSQ dissociation to be monitored in stopped flow experiments.

The aims of the experiments in this chapter were as follows;
(1) to explore the possibility that two discrete populations of RyRs exist.
(2) to study the effects of dephosphorylated CSQ on the RyR/T/J complex and compare with effects of phosphorylated CSQ (data presented in Chapter 3),
(3) to investigate the possibility that CSQ can regulate RyR activity by either binding directly to the RyR, or indirectly, by binding to triadin and junctin,
(4) to determine accurately the dissociation time of CSQ from native RyRs, using stopped flow techniques.

4.2 RESULTS

4.2.1 Two populations of RyRs

4.2.1.1 Non-responsive native RyRs

In 22 of the 69 native RyRs exposed to 500 mM *trans* Cs^+, activity did not increase significantly from control following ionic strength increase (after 5 min exposure to 500 mM Cs^+; Chapter 3). In all the bilayer experiments presented in this chapter the composition of *cis* and *trans* solutions are as follows, unless otherwise specified: *cis* 100 nM free Ca^{2+}(1 mM $CaCl_2$ plus 4.5 mM BAPTA), (in mM) 2 ATP, and 250 Cs^+; *trans* (mM) 250 or 500 Cs^+ and 1 Ca^{2+}. In the case of average data presented in this chapter, channels were analyzed for 30 s, unless stated otherwise.

Ten of the 22 non-responsive RyRs were exposed to 500 mM *trans* Cs^+ for up to 8 min, with no increase in channel activity evident. The control activity in these channels was unusually high. The channels did however, respond to addition of 20 μg exogenous CSQ to the *trans* chamber (following *trans* perfusion with 250 mM Cs^+ in 5 out of 5 channels tested). The control activity in these channels was unusually high. Addition of exogenous CSQ resulted in a significant drop in channel activity, at both positive and negative potentials (data shown at + 40mV; Fig. 4.I), and there was a further drop in the relative mean current (test relative to control, I'_t/I'_c) following RyR incubation with 5 μg anti-CSQ antibody (n=3, Fig. 4.I). In this set of experiments, 3 mM $MgCl_2$ was included as part of the *cis* solution.

In addition, in 2 of 9 channels (not previously exposed to high ionic strength), control activity in the presence of Mg^{2+} was also unusually high, and exposure to *trans* 0.5 μg anti-CSQ antibody did not alter fractional mean current (I'_F) significantly from control (Fig. 4.II). RyRs were incubated in anti-CSQ for up to 8 min, and stirred every min, to ensure antibody association. It should be noted that commercial anti-CSQ was used in the results presented in Section 4.2.1. In this set of experiments, 3 mM $MgCl_2$ was included as part of the *cis* solution.

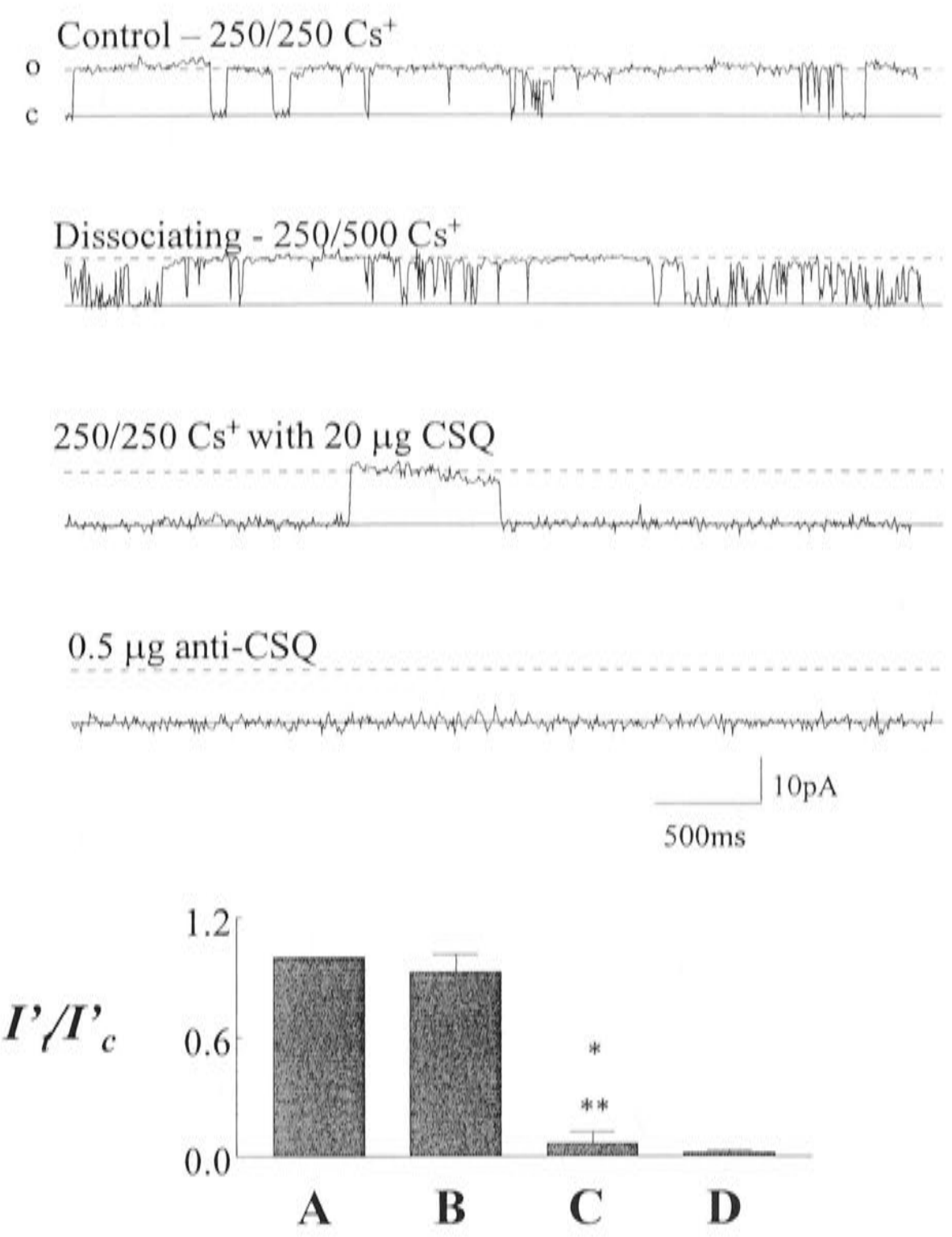


Fig. 4.I. ***Exogenous CSQ inhibited channels which did not respond to an increase in trans*** Cs^+. (**A**) Control activity with 250 mM *trans* Cs^+, in the presence of 3 mM *cis* $MgCl_2$; (**B**) after increasing *trans* Cs^+ to 500 mM with CsCl; (**C**) perfusing the *trans* chamber with 250 mM Cs^+ and subsequent addition of 20 µg CSQ; (**D**) 0.5 µg commercial anti-CSQ antibody; (**E**) Average data (n = 5-10) showing relative mean current (test relative to control, I'_t/I'_c) under conditions shown in **A**-**D**. V_m-E_{Cs+} was +40mV under all conditions. Single channel opening is upward from the zero current level (continuous line) to the maximum open conductance (broken line). Error bars are SEM, except in the case of CSQ and anti-CSQ antibody (n=3), where SD is presented. Asterisks (*) indicate average values significantly different from control, ** indicates activity significantly different from the previous condition ($p<0.05$, t-test)

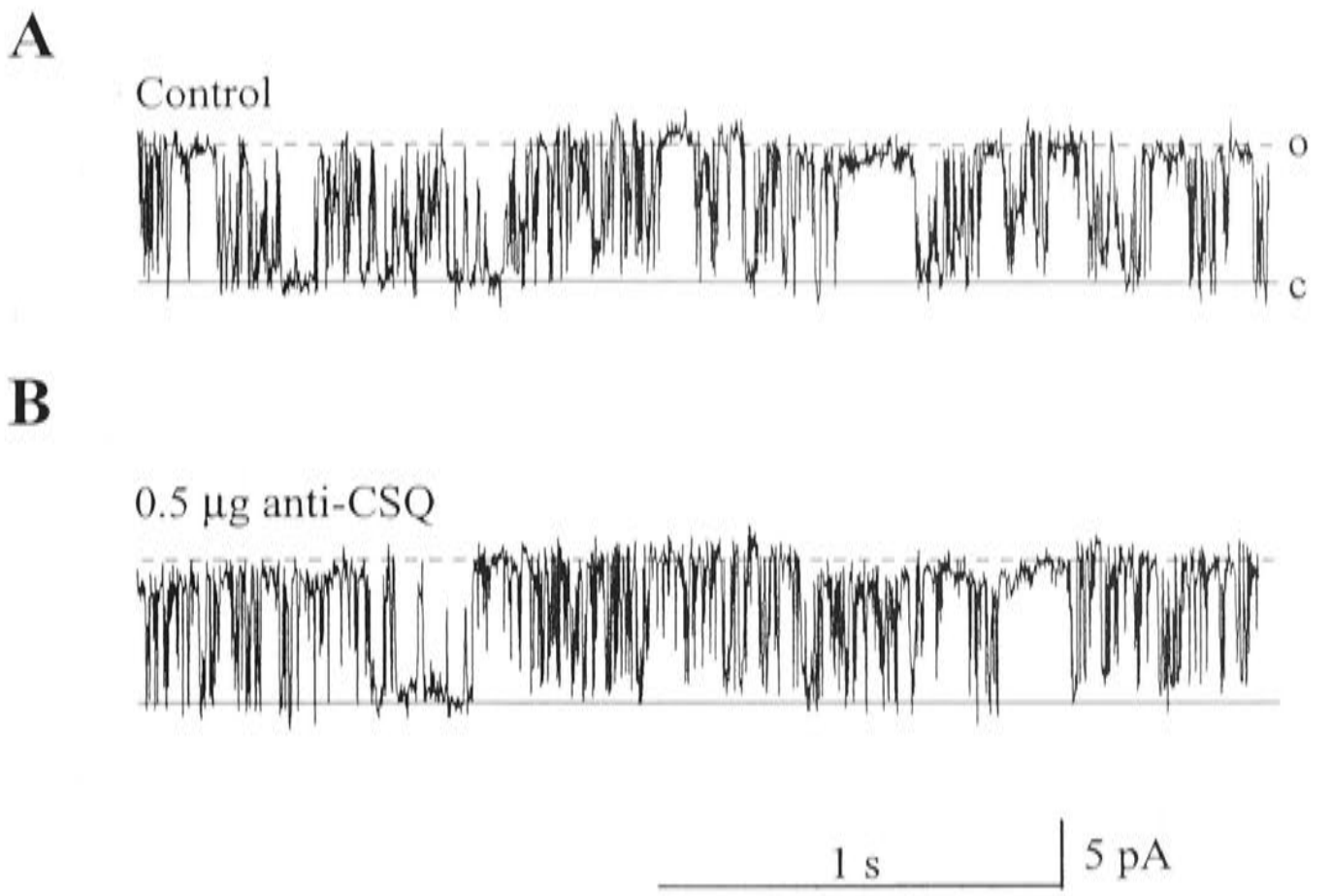


Fig. 4.II. ***High activity RyRs did not respond to trans anti-CSQ antibody.*** (**A**) Control activity with 250 mM *trans* Cs^+ in the presence of 3 mM *cis* $MgCl_2$; (**B**) after adding 0.5 µg *trans* commercial anti-CSQ antibody; (**C**) Individual data (n = 2) showing fractional mean current (I'_F) under conditions shown in **A**-**B**. V_m-E_{Cs+} was +40mV under all conditions. Single channel opening is upward from the zero current level (continuous line) to the maximum open conductance (broken line).

The unresponsive channels (to high ionic strength or anti-CSQ antibody) had a significantly higher control activity (I'_F), than channels that responded to 500 mM Cs^+ (in the presence of *cis* 2mM ATP and 10^{-7} M Ca^{2+}; Fig. 4.III). Fig 4.III shows a frequency distribution of 69 native channels, of which, 22 did not respond to increased [Cs^+], whilst 47 showed the expected increase in activity. The plot shows that the vast majority of unresponsive channels had a higher control activity than their responsive counterparts. Average data shows that those unresponsive to Cs^+ had a significantly higher control I'_F ($p<10^{-13}$, t-test) than responsive RyRs, indicating that there were two populations of RyRs. Conceivably, these high activity channels did not have CSQ bound to them originally. The possibility that fractions of RyRs may not have CSQ associated with them was first reported by Yamaguchi *et al.* (1997). If this explanation is correct (see Figs. 4.I-III), it might be that RyRs without tethered CSQ would have a higher control activity. Presumably, the inhibition imposed by CSQ had been removed, but the channels would respond to CSQ addition, and channel activity should fall. This hypothesis is consistent with the initial activity increase upon exposure to 500 mM Cs^+ (in 47 channels) being due to dissociation of CSQ, as CSQ removal results in RyRs with a higher activity (due to the removal of CSQs inhibition).

4.2.1.2 Addition of exogenous CSQ to low and high activity channels

Low activity RyRs (those thought to have CSQ tethered to them), did not show any alteration in channel activity after the addition of 20 µg exogenous CSQ (Fig. 4.IV A-C), suggesting that these channels already had CSQ associated with them. Channels were observed for 10 min, with no evident (or indeed significant) alteration in RyR I'_F. In contrast, high activity channels responded to the addition of 20 µg CSQ, with I'_i/I'_c falling 2.3-fold (Fig. 4.IV D-F) in 6 channels. These results support the theory that two populations of RyRs are distinguished by their association with CSQ.

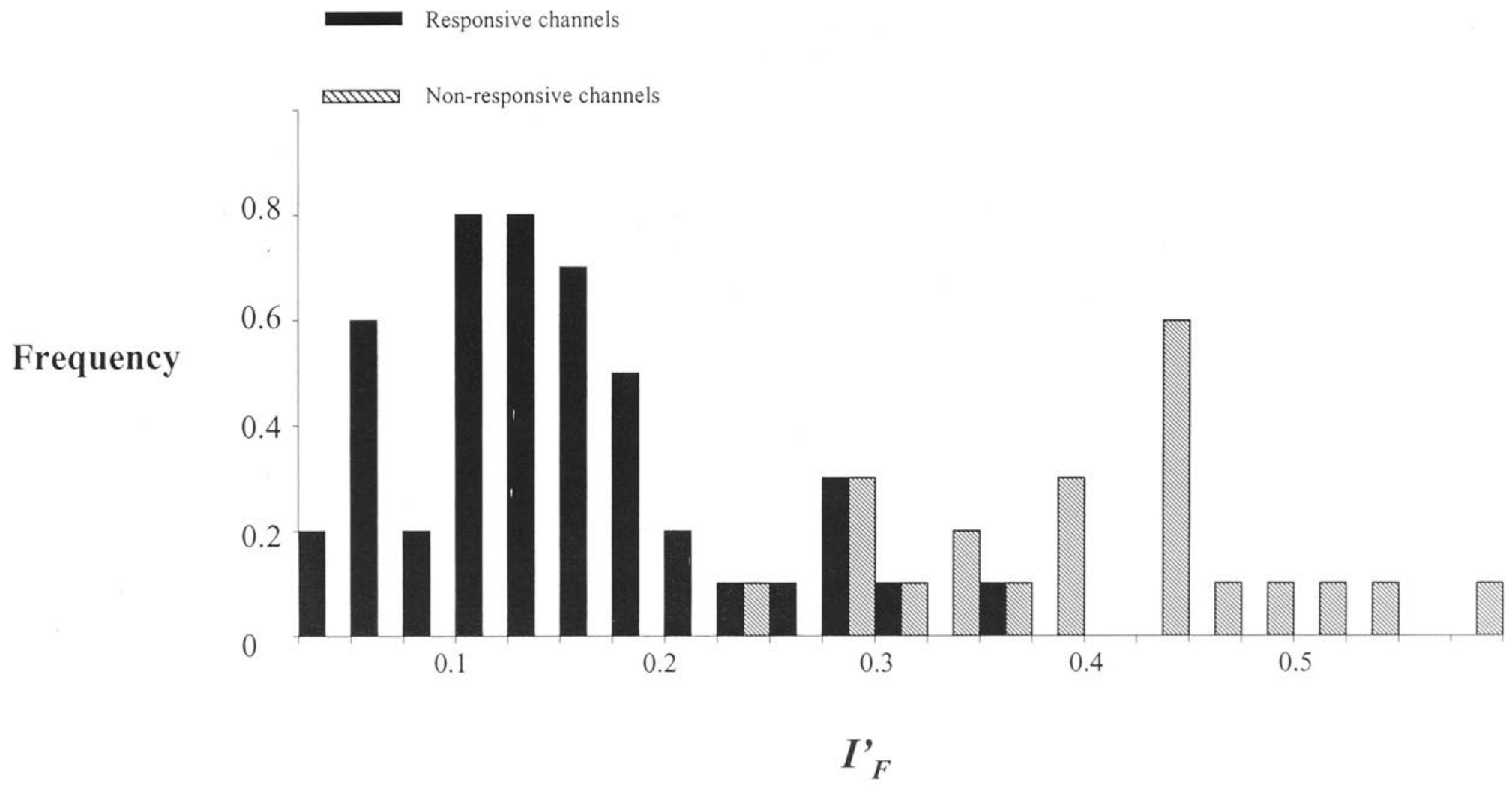
4.2.1.3 The cause of CSQ depleted RyRs

The existence of a population of RyRs lacking CSQ may have been due to higher than normal ionic strength in control solutions. *In vivo*, the ionic strength within the lumen is in equilibrium with the cytoplasm, and is approximately 150 mM (Berne and Levy, 1990). It may be that increasing ionic strength from 50 mM (which is the *trans* [Cs^+] used to incorporate RyRs, pre-control levels) to 250 mM Cs^+ (control levels),

dissociated CSQ from a number of RyRs, while the whole RyR population was stripped of CSQ after exposure to 500 mM Cs^+. To test this possibility, ionic strength was increased from 50 mM to 250 mM (Fig 4.V). Average I'_t/I'_c (in 29 channels) did not increase (Fig. 4.V). It is interesting to note that one channel showed a ~2.4-fold rise in I'_t/I'_c, as a result of raising [Cs^+] to 250 mM. It seems that the increase in channel activity in this case may have been due to CSQ dissociation, as this channel did not respond to subsequent addition of anti-CSQ antibody. This dissociation may have been due to uneven stirring, resulting in a local high [Cs^+], close to the CSQ-RyR/T/J interaction site(s); high enough to dissociate CSQ (i.e. close to 500 mM Cs^+, as additions were made from a 1.5M stock). None-the-less, the results in Fig. 4.V suggest that it is unlikely that the dissociation of CSQ occurs with the pre-control Cs^+ increase (from 50 – 250 mM Cs^+). If CSQ removal were the cause of channel unresponsiveness, then the dissociation must have occurred sometime before lipid bilayer channel incorporation of RyRs.

It appeared that some batches of SR contained channels that exhibited low activity, and therefore may have contained RyRs with tethered CSQ (Table 4.I). In particular, in batch 11a/98, 8 channels were incorporated into lipid bilayers, all of which had low control activity (in the presence of *cis* 2mM ATP and 10^{-7} M Ca^{2+}). Conversely in batch 07/99, all 16 channels incorporated had high control channel activity, whilst in batch number 03a/01, 41/46 incorporations exhibited high activity. A pattern emerged, showing that certain batches of vesicles had either a high proportion of RyRs with high activity (channels without CSQ attached), whilst other batches contained channels with low control activity. Of course, the number of channels used from certain batches was low (less than 10 channels were recorded from 5 of the 20 batches) and a few batches showed both RyR populations. Taken together, these data indicate that CSQ-depleted RyRs existed prior to SR vesicle isolation (or were depleted during SR vesicle isolation) and prior to reconstitution with bilayers. The mechanism of CSQ depletion warrants further investigation. Indeed RyRs without tethered CSQ may occur *in vivo* as suggested by Yamaguchi *et al.* (1997), or CSQ may have been phosphorylated in some cases and not others (see Section 4.2.2 for further investigation of this possibility).

Fig 4.III. ***Control activity of single RyR channels that respond to procedures that influence CSQ was lower than control activity of responsive channels.*** Control mean current as a fraction of maximum current (I'_F) is shown. Channels whose activity increased with 500 mM *trans* Cs^+ (CSQ dissociation) or decreased with *trans* anti-CSQ antibody are indicated by the black bars; channels which showed no consistent change in I'_F after either exposure to 500 mM *trans* Cs^+ or 0.5 μg/ml *trans* anti-CSQ are indicated by the shaded bars. The Student's t-test indicates that there are two populations of RyR channel (see Results).


Responsive channels
Non-responsive channels
Frequency
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0.6
0.4
0.2
0
0.1
0.2
0.3
0.4
0.5
I'_F

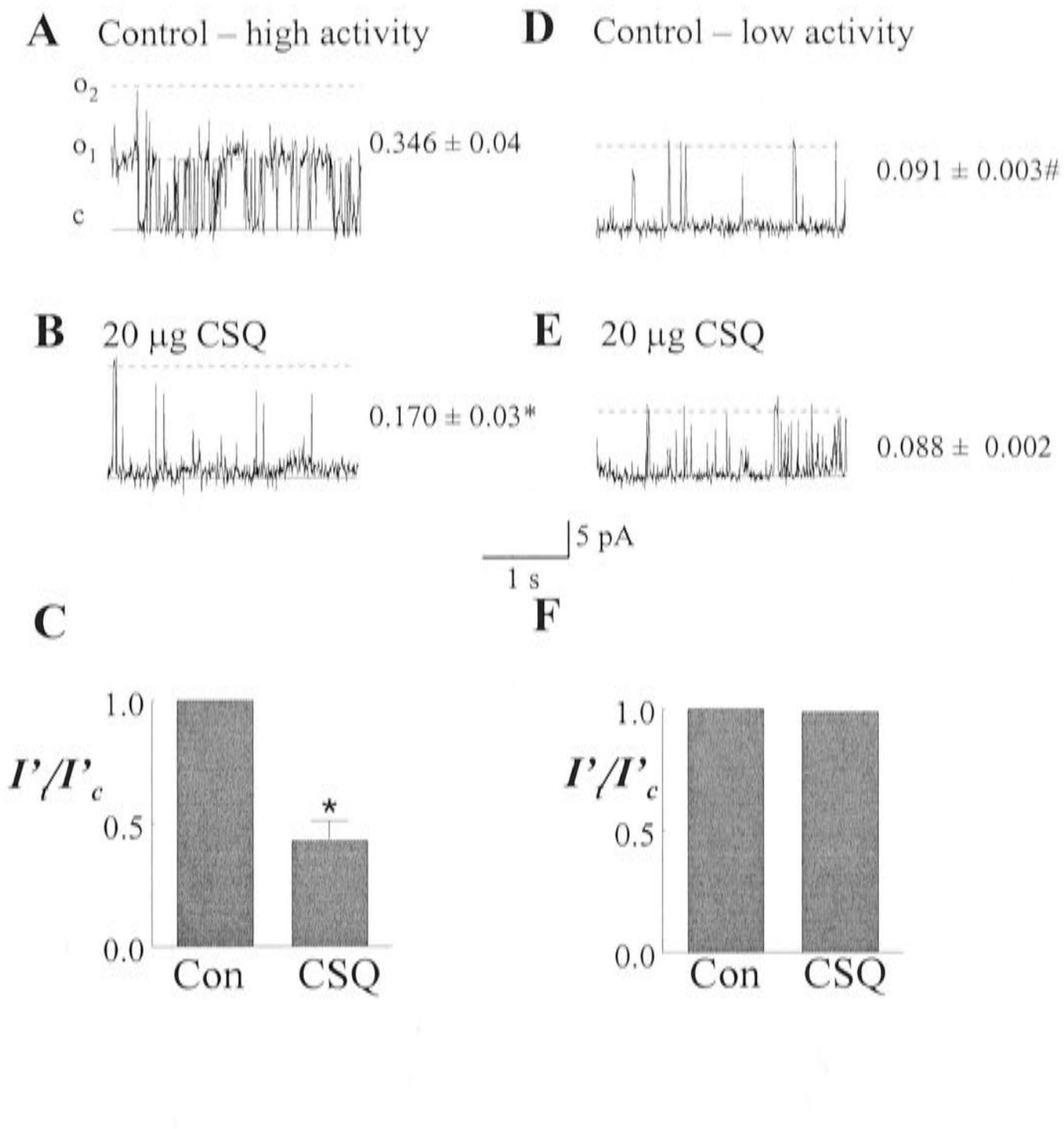


Fig. 4.IV. ***CSQ was able to reduce channel activity only in RyRs with high initial activity.*** (**A**) Control activity of high activity channel; (**B**) after addition of 20 µg CSQ; (**C**) average data (n=4) for relative mean current; I_f/I_c under conditions shown in **A&B;** (**D**) control activity of low activity channel; (**E**) after addition of 20 µg CSQ; (**F**) average data I'_f/I'_c (n=6) for under conditions shown in **D&E**. I'_F is shown to the right of **A**,**B**,**D&E**. In **E**, up to 100 µg CSQ was added to the *trans* chamber, with no alteration in channel activity observed (data not shown). V_m-E_{Cs+} was +40mV under all conditions. Channel opening is upward from the zero current level (continuous line) to the maximum open conductance (broken line, o_1 & o_2). Asterisks (*) indicate average values significantly different from control ($p<0.05$, t-test).

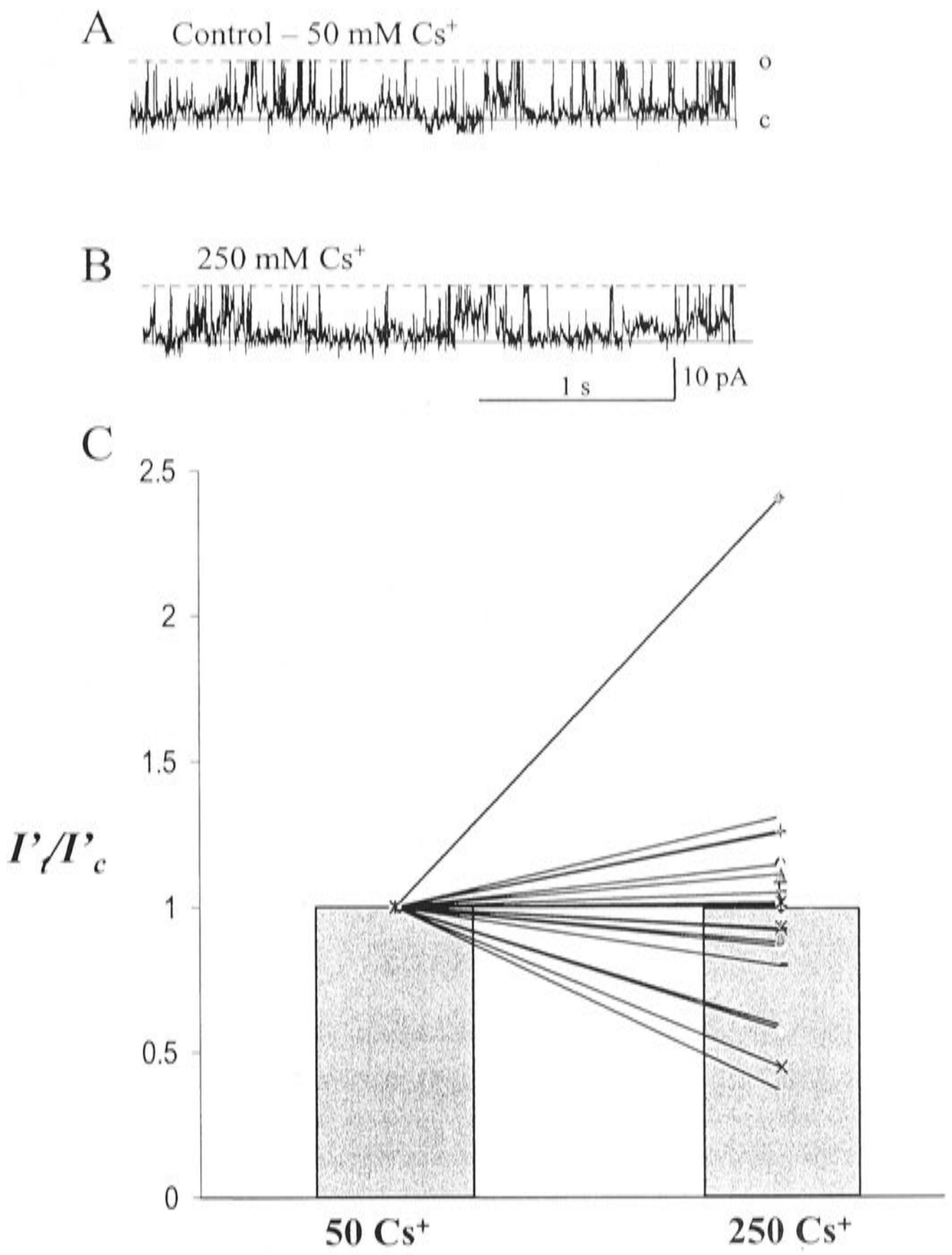


Fig. 4.V. ***Increasing Cs^+ from 50 to 250 mM did not dissociate CSQ.*** (**A**) Activity with 50 mM *trans* Cs^+; (**B**) after increasing *trans* Cs^+ to 250 mM with CsCl; *trans* Cs^+ is above **A&B**; (**C**) Bar graph represents relative mean current, I'_t/I'_c (test relative to control), showing average data (n = 21) of **A&B**, nested line graph represents each of 21 single channel I'_t/I'_c. V_m-E_{Cs+} was +40mV under all conditions. Single channel opening is upward from the zero current level (continuous line) to the maximum open conductance (broken line).

Table 4.I. Batch specificity of responsive and non-responsive (to 500 mM Cs^+ or anti-CSQ antibody) RyR channel behaviour

Batch Identifier	Total channels used from batch	Number with high activity (%)	Number with low activity (%)
05/98	4	4 (100)	0
06/98	1	1 (100)	0
11a/98	8	0	8 (100)
11b/98	6	5 (83)	1 (17)
12/98	2	2 (100)	0
07/99	16	16 (100)	0
08/99	1	1 (100)	0
09/99	2	2 (100)	0
10/99	3	3 (100)	0
12/99	8	4 (50)	4 (50)
12/99	15	11 (73)	4 (27)
05/00	2	2 (100)	0
12/00	2	2 (100)	0
03a/01	46	41 (89)	5 (11)
03b/01	3	3 (100)	0
04/01	3	3 (100)	0
06/01	8	6 (75)	2 (25)

Channels were either exposed to 500 Cs^+, or 0.5 μg anti-CSQ. Channels were termed unresponsive if activity did not increase or decrease (as expected) after 5 min of exposure. Percentages of channels exhibiting responsive or non- responsive are given in parentheses.

4.2.2 Phosphorylation and dephosphorylation of CSQ

To determine what effects the phosphorylation status of CSQ had upon CSQ regulation of native RyRs, the phosphorylation status of the CSQ isolated for use in the present experiments was determined by ^{31}P nuclear magnetic resonance (^{31}P NMR) analysis.

4.2.2.1 Determination of the degree of phosphorylation of purified CSQ

Fig 4.VI compares ^{31}P NMR spectra indicating phosphorous levels in the CSQ sample, before and after dephosphorylation. The horizontal axis (ppm) corresponds to a chemical shift, reflecting the environment the phosphorous atoms are in. The original CSQ sample displays a resonance peak at approximately 0.2 ppm, (expected for phosphorous), indicating that CSQ is at least in part, phosphorylated. The appearance of a broad resonance peak, at between -1.5 to -2.8 (red arrow, top trace) observed in the phosphorylated sample, may indicate phosphorous atoms in aggregated protein (see Section 4.2.2.2). Protein precipitation was observed after NMR analysis, suggesting that some of the CSQ aggregated during the 24 hr testing period. From this NMR data, it was not possible to determine how many residues were phosphorylated.

4.2.2.2 Dephosphorylation of CSQ

CSQ was dephosphorylated according to the methods of Cala and Jones (1991; see also Chapter 2, Section 2.8.1.3). Once dephosphorylated, 2-4 mg of dephosphorylated CSQ was subjected to ^{31}P NMR, to determine that dephosphorylation had in fact occurred. The peak observed in the phosphorylated sample of CSQ at approximately 0.2 ppm (Fig. 4.VI, red arrow, top trace) disappeared after dephosphorylation. The appearance of a small peak (approximately -1.2 ppm, red arrow, bottom trace), close to the broad peak observed in the phosphorylated sample, may again be explained by some phosphorus atoms in aggregated protein. It may be that the phosphatase cannot access bound phosphate (which may be buried within aggregated protein), and hence dephosphorylate residues, when CSQ is aggregated. This would account for the small amount of aggregated CSQ still in a phosphorylated form, resulting in the appearance of a small resonance at –1 ppm. In terms of phosphorylation levels, the dephosphorylated CSQ sample contained at least three times less phosphorus than the phosphorylated CSQ sample (as indicated by the area under each graph). It is important to note that only

non-aggregated CSQ was used in the lipid bilayer study using dephosphorylated CSQ (see Section 4.2.2.3), to try and optimise the yield of dephosphorylated CSQ.

4.2.2.3 Regulation of native RyRs by dephosphorylated CSQ

The ^{31}P NMR data indicates that the exogenous CSQ added to CSQ-depleted RyRs (Figs. 3.X-XII & 4.XII) was phosphorylated. The action of dephosphorylated CSQ on RyRs was examined. After control recording, native RyRs were exposed to 500 mM Cs^+ to dissociate endogenous CSQ, which resulted in a 2.2 fold increase in I'_F (Fig. 4. VII). After subsequent perfusion of the *trans* chamber with 250 mM Cs^+, 20-50 µg of dephosphorylated CSQ was added to the *trans* chamber. Dephosphorylated CSQ reduced I'_F 1.5-fold at +40 mV, and 1.6-fold at –40 mV (Fig. 4.VII, data not shown at –40 mV) in much the same way as the native phosphorylated CSQ (see Chapter 3, Fig 3.X-3.XII). In additional experiments, up to 100 µg of dephosphorylated CSQ (*trans*) was added to high activity RyRs. Addition of dephosphorylated CSQ induced a 2.1-fold reduction in I'_f/I'_c at +40 mV, and a 2.0-fold decrease at –40 mV (Fig. 4.VIII). These channels had a control I'_F of >0.4; a similarly high control I'_F was observed in high activity channels not responding to 500 Cs^+ or anti-CSQ antibody addition (see Figs. 4.III-IV), therefore it is likely that these channels did not have CSQ attached.

It appears that the phosphorylation status of CSQ had no influence on CSQs ability to inhibit native RyRs. Table 4.II compares the inhibition of native RyRs, induced by both phosphorylated CSQ and dephosphorylated CSQ. There was no significant difference in the % inhibition (I'_F upon CSQ re-association as a % of dissociation) induced by either phosphorylated or dephosphorylated CSQ, on native CSQ-dissociated or high activity RyR I'_F. Apparently, altering the phosphorylation status of exogenous CSQ did not alter the functional interaction of CSQ with the native RyR/T/J complex.

4.2.3 The response of purified RyRs to CSQ

In 1998, the first evidence that CSQ may directly regulate the RyR was published by Szegedi *et al.* (1999). Purified RyRs were incorporated into lipid bilayers and *trans* addition of dephosphorylated CSQ enhanced open probability, while phosphorylated CSQ had no effect. Presumably, these purified RyRs did not contain either triadin or junctin, although evidence for this was not shown. It was therefore important to

Original CSQ sample

dePCSQ

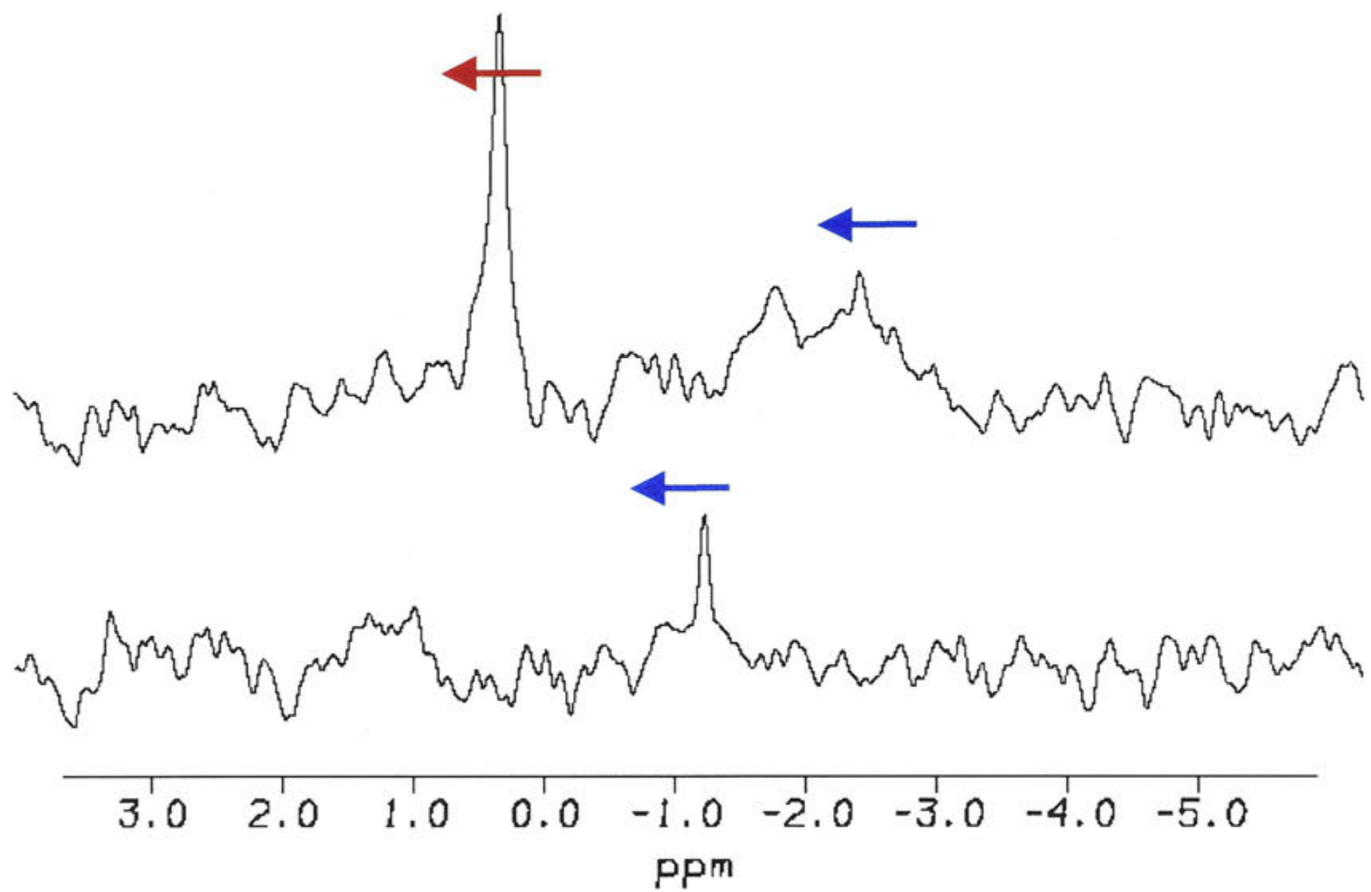


Fig. 4.VI. ***^{31}P NMR spectrum of skeletal muscle CSQ.*** The spectra were obtained using a spectral width of 15000 Hz, a pulse width of ~15 μs, a spectral frequency of 202.421 MHz, and an acquisition time of 0.33 s. The resonances in the upper trace (from the original CSQ sample) and lower trace (deP-CSQ) are indicated with red and blue arrows.

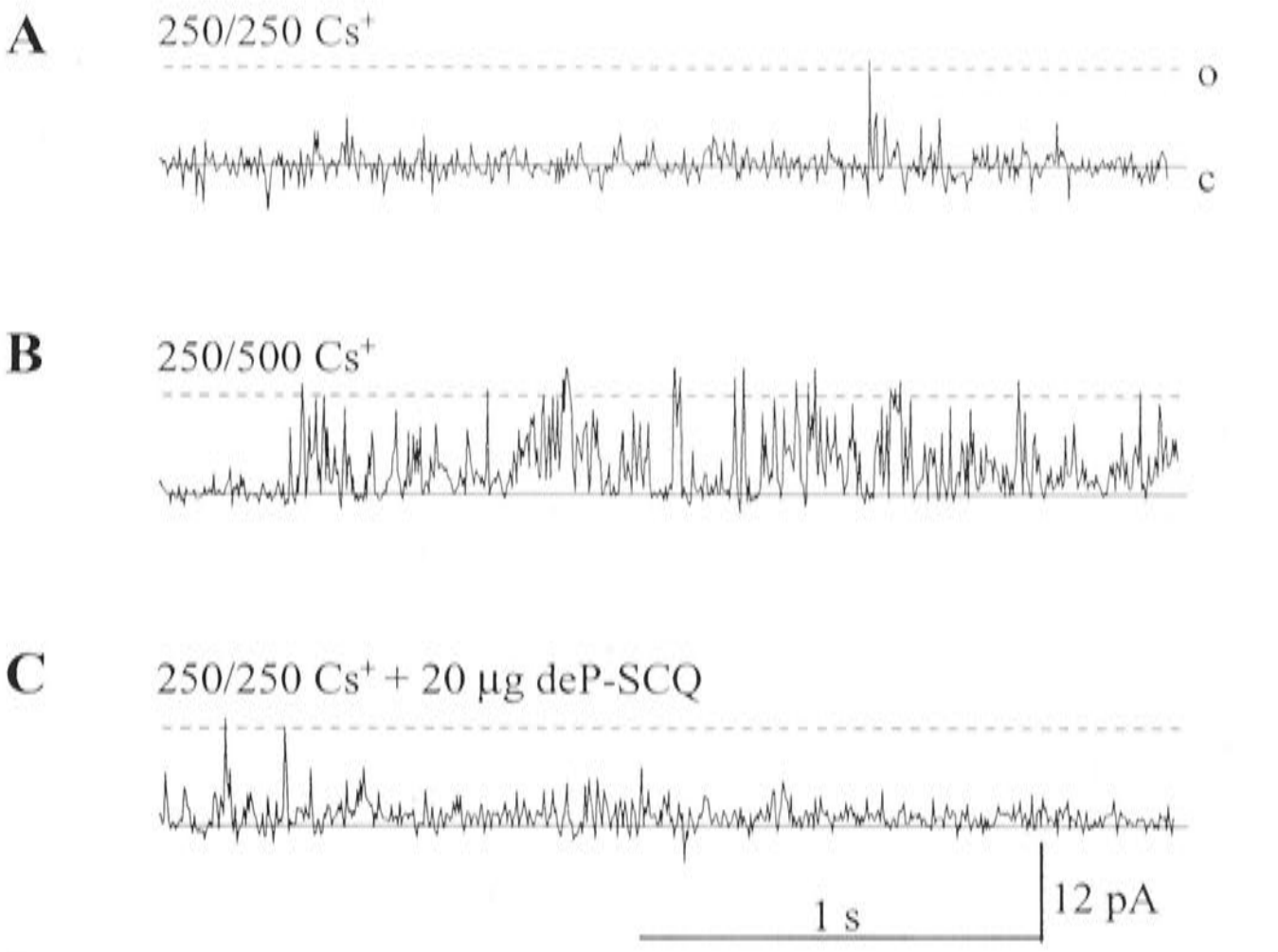


Fig. 4.VII. Exogenous dephosphorylated-CSQ inhibited CSQ-dissociated RyRs. (**A**) Control activity with 250 mM *trans* Cs^+; (**C**) Increasing trans Cs^+ to 500 mM with $CsCl_2$; (**C**) Following *trans* perfusion with 250 mM Cs^+, addition of 20 µg dephosphorylated CSQ (deP-CSQ), *cis*/*trans* Cs^+ is shown above **A-C**; (**D**) Average data (n = 4) showing relative mean current (I'/I'_c, test relative to control) under conditions shown in **A-C**. V_m-E_{Cs+} was +40mV in all cases Single channel opening is upward from the zero current level (continuous line) to the maximum open conductance (broken line). Asterisks (*) indicate average values significantly different from control and ** indicates value significantly different from the previous condition. ($p<0.05$, t-test).

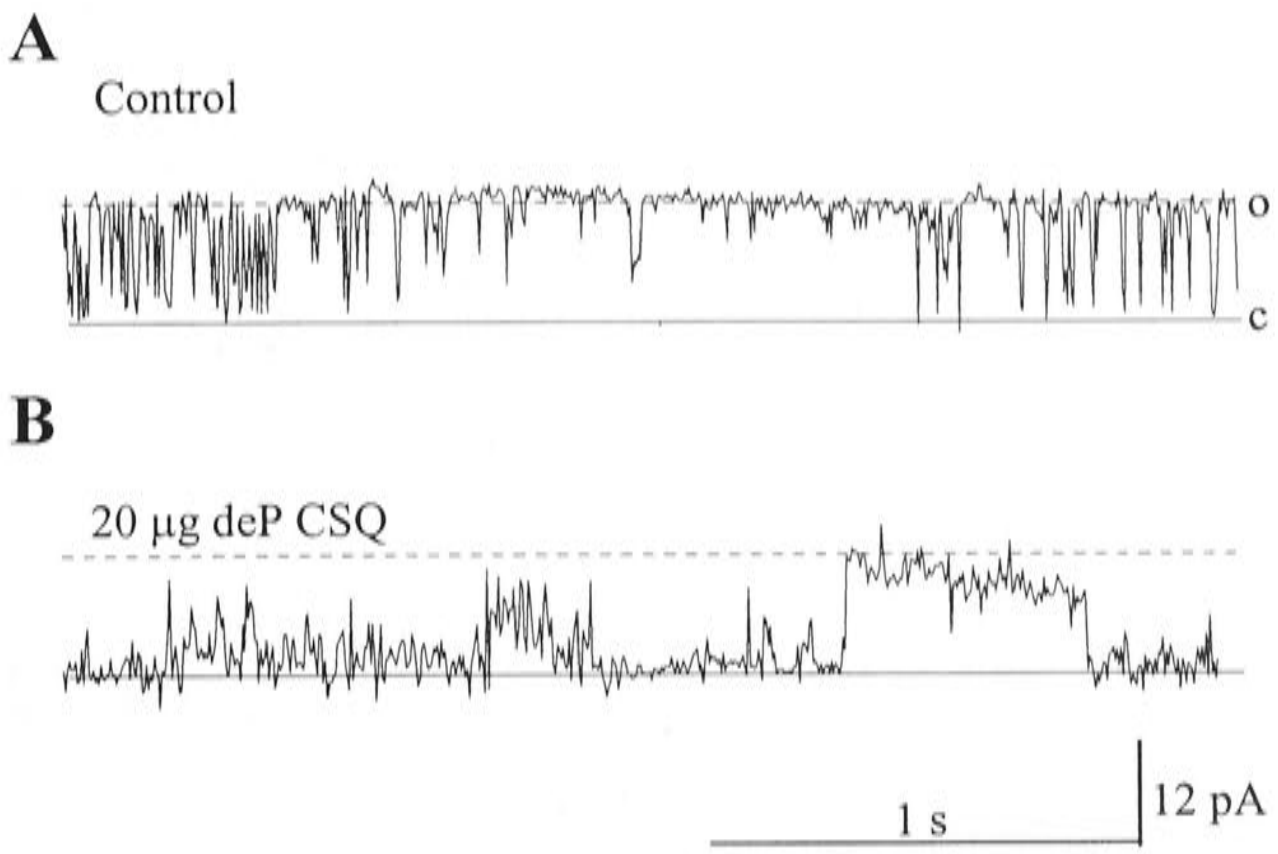


Fig. 4.VIII. Exogenous dephosphorylated-CSQ inhibited high activity channels. (**A**) Control activity with 250 mM *trans* Cs^+; (**B**) addition of 20 µg dephosphorylated CSQ. $Vm-E_{Cs+}$ was +40mV in **A** (con) & **B** (deP-CSQ). (**C**) Average data (n = 4) showing relative mean current (I'/I'_c, test relative to control) under conditions shown in **A&B**, at both + and – 40 mV. V_m-E_{Cs+} was +40mV in **A-C** and –40mV in **C**. Single channel opening is upward from the zero current level (continuous line) to the maximum open conductance (broken line). Asterisks (*) indicate average values that are significantly different from control.

Table 4.II. ***Comparison of RyR inhibition by phosphorylated CSQ and dephosphorylated CSQ.***

CSQ-induced inhibition of high activity native RyRs

	% Inhibition
Addition of phosphorylated CSQ	57 ± 7
Addition of dephosphorylated CSQ	51 ± 16

CSQ-induced inhibition of low activity native RyRs

	Inhibition
Addition of phosphorylated CSQ	59 ± 7
Addition of dephosphorylated CSQ	48 ± 8

Inhibition is shown as a percentage of I'_F measured after initial CSQ dissociation. Data is presented as mean ± SEM. There was no significant difference in inhibition induced by either phosphorylated CSQ or dephosphorylated CSQ in either low activity channels (after CSQ dissociation by Cs^+) or high activity channels.

determine whether the decrease in native RyR activity seen upon CSQ re-association reported in Chapter 3 (Figs. 3.X-XII), was due to a direct interaction between CSQ and the RyR, or to CSQ affecting RyR activity after associating with triadin and junctin (inducing a regulatory effect through either one of these proteins). To ascertain the role of triadin and junctin in CSQ inhibition of RyR activity, both purified skeletal RyRs and RyRs expressed in a CHO cell line were studied using single channel experiments.

4.2.3.1 Purification of the RyR

The purification of RyRs from SR vesicles was achieved after solubilized SR had been fractionated on a discontinuous sucrose gradient. SDS-Page (and subsequent silver stain) of native SR showed strong bands at approximately 400 kDa (presumably the RyR; Fig.4.IX A, lane 1) and at approximately 110 kDa, (presumably Ca^{2+}-ATPase), and proteins resolving at approximately 97, 55 and 31 kDa (which presumably are triadin, CSQ and junctin, respectively). CHAPS solubilized SR (purified RyRs) showed a strong band at approximately 400 kDa, and a weak band at approximately 110 kDa (Fig. 4.IX A, lane 2), with no CSQ, triadin or junctin, indicating that the solubilized fraction contained mostly RyR, with minimal contaminant Ca^{2+}-ATPase.

Immunoprobing the gels with anti-CSQ and anti-triadin antibodies showed that CSQ (Fig. 4.IX A, lane 3,) and triadin (Fig. 4. IX A, lane 5) were present in native SR, but were removed by RyR purification (Fig. 4.IX A, lanes 4&6 respectively). A faint band at 26 kD (which corresponds to junctin) can be seen in the native SR (Fig. 4. IX A, lane 1), but was not present in the solubilized fraction (Fig. 4.IX A, lane 2). It was assumed that Ca^{2+}-ATPase does not regulate RyR activity or normally bind to triadin, junctin or CSQ, since there was no published evidence suggesting that Ca^{2+}-ATPase has a role in modulation of the RyR.

4.2.3.2 Characteristics of CHAPS solubilized RyR and RyRs expressed in CHO cell to ATP, Ca^{2+} and ruthenium red.

Fig. 4.X shows that purified RyRs respond to *cis* Ca^{2+} and ATP in a similar manner to native channels. A reduction of *cis* Ca^{2+} from 1 mM to 100 nM (by the addition of 4.5 mM BAPTA) reduced channel activity 9-fold (Fig. 4.X A, panel 2). *Trans* Ca^{2+} was held at 1 mM throughout the experiments. As with native RyRs, purified channels that were partially inhibited by 1 mM Ca^{2+} had a higher activity than channels at sub-

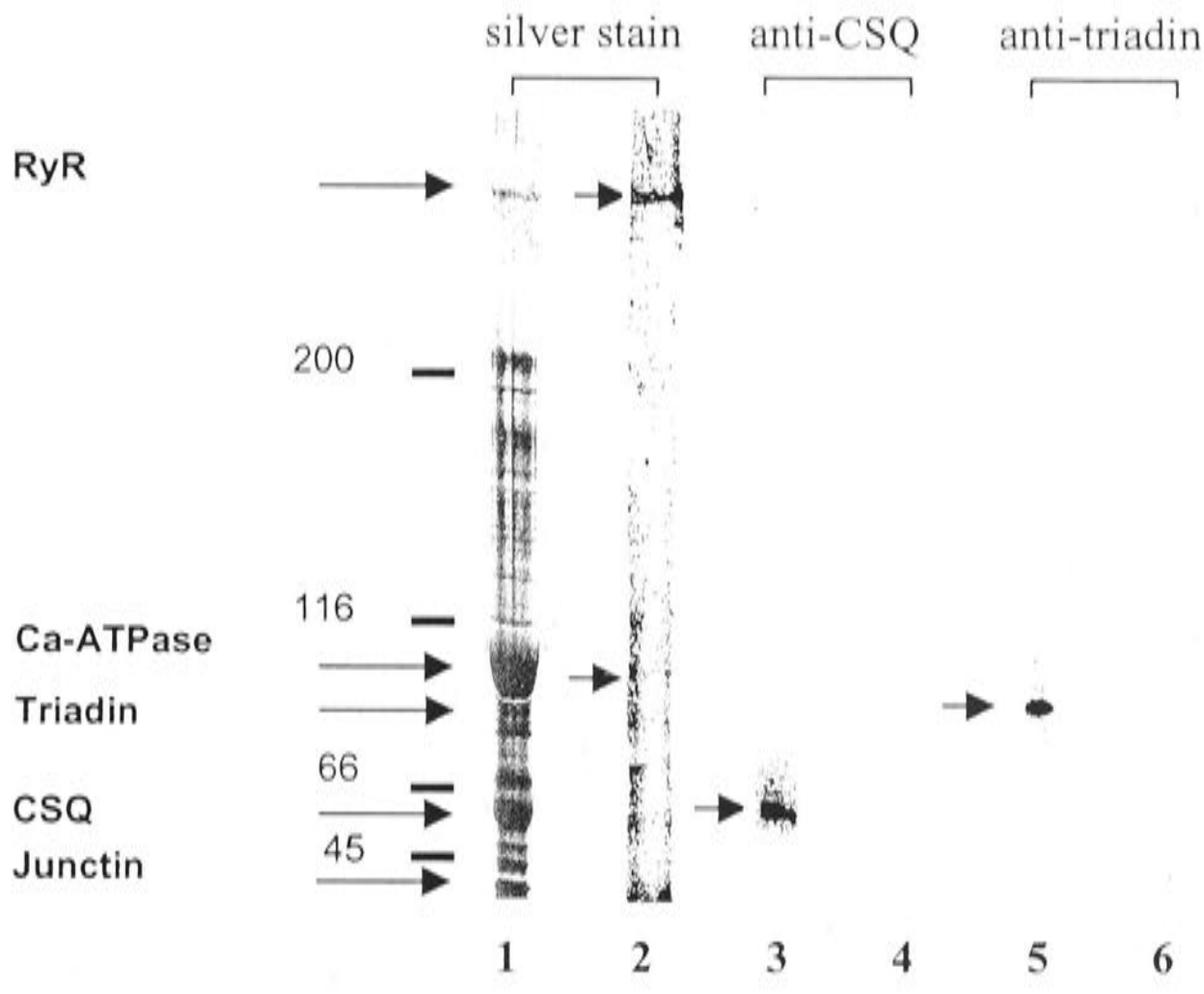


Fig. 4.IX. ***Purification of the RyR.*** Silver stain of SDS-PAGE (5%-17%) showing a ~110 kDa and ~400 kDa protein in native SR (lane 1), after purification of RyRs (lane 2). The resolution of the silver stain in lane 2 has been enhanced to illustrate the removal of all SR proteins (besides RyR and Ca^{2+}-ATPase) following the purification procedure. CSQ (lanes 3& 4) and triadin (lanes 5 & 6) were detected in native SR membranes (lanes 3 & 5), but not in purified RyR fractions (lanes 4 & 6), after immunoprobing with anti-CSQ or anti-triadin antibodies. The protein located at ~ 110 kDa is presumed to be Ca^{2+}-ATPase, and the 400 kDa protein to be the RyR. The position of molecular weight markers is shown next to lane 1. There was no band at 26 kDa in the purified RyR sample (lane 2) that would correspond to junctin. Proteins were transferred onto Immobilon-P PVDF membranes, prior to probing with antibody.

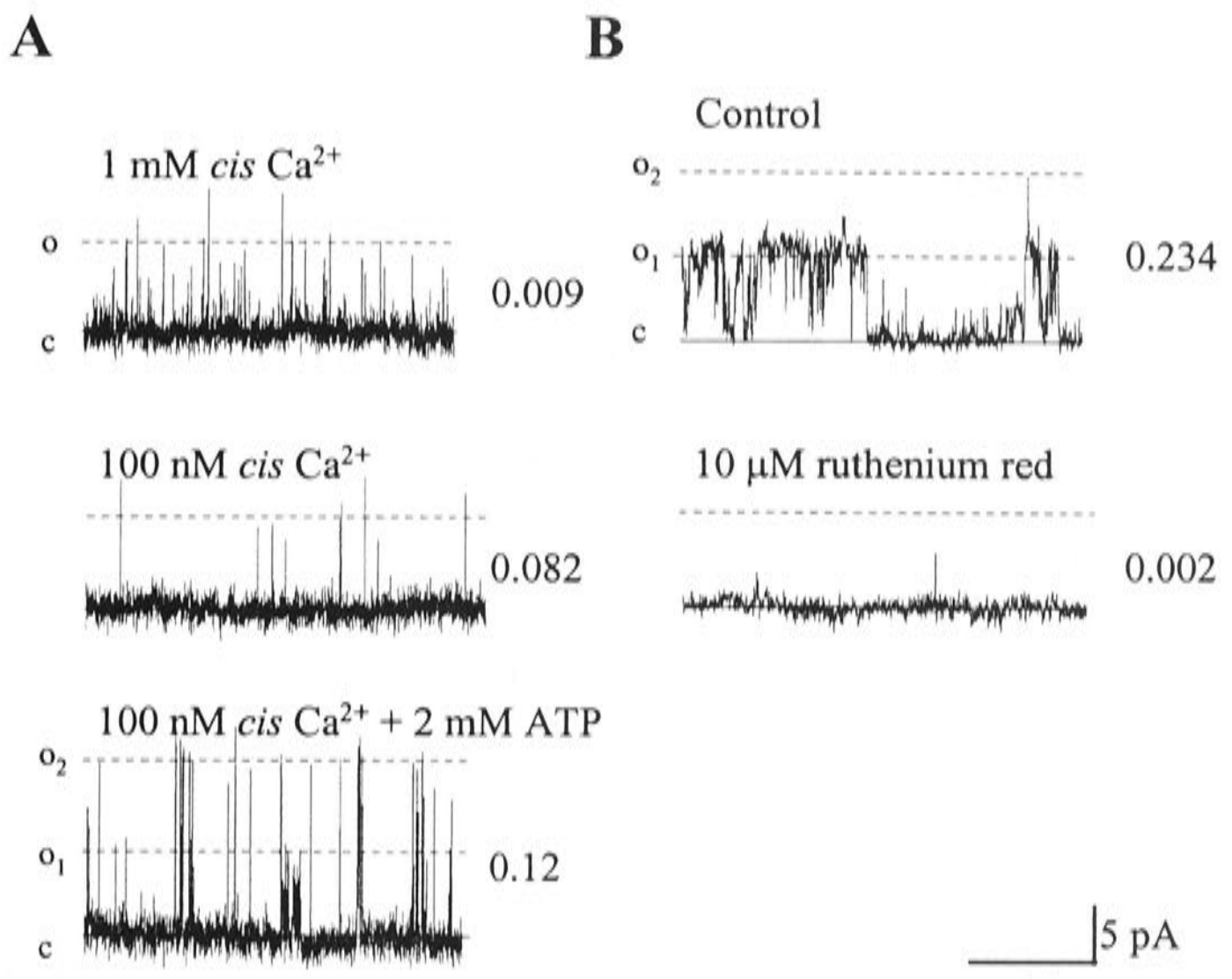


Fig. 4.X. ***Activation and inhibition of purified RyRs by cis Ca^{2+}, ATP and ruthenium red.*** (**A**) Control activity with 250/250 Cs^+ (*cis/trans*), 1 mM *cis* Ca^{2+} (panel 1), after lowering *cis* Ca^{2+} to 100 nM with 4.5 mM BAPTA (panel 2), and after addition of 2 mM ATP. (**B**) control activity with 250/250 Cs^+ (*cis/trans*), 100 nM *cis* Ca^{2+} (panel 1), and channel inhibition upon addition of 10 μM *cis* ruthenium red (panel 2). Individual channel fractional mean current (I'_F) is shown to the right of channel traces in **A-B**. V_m-E_{Cs+} was +40mV under all conditions. Single channel opening is upward from the zero current level (continuous line) to the maximum open conductance (broken line, o_1 & o_2).

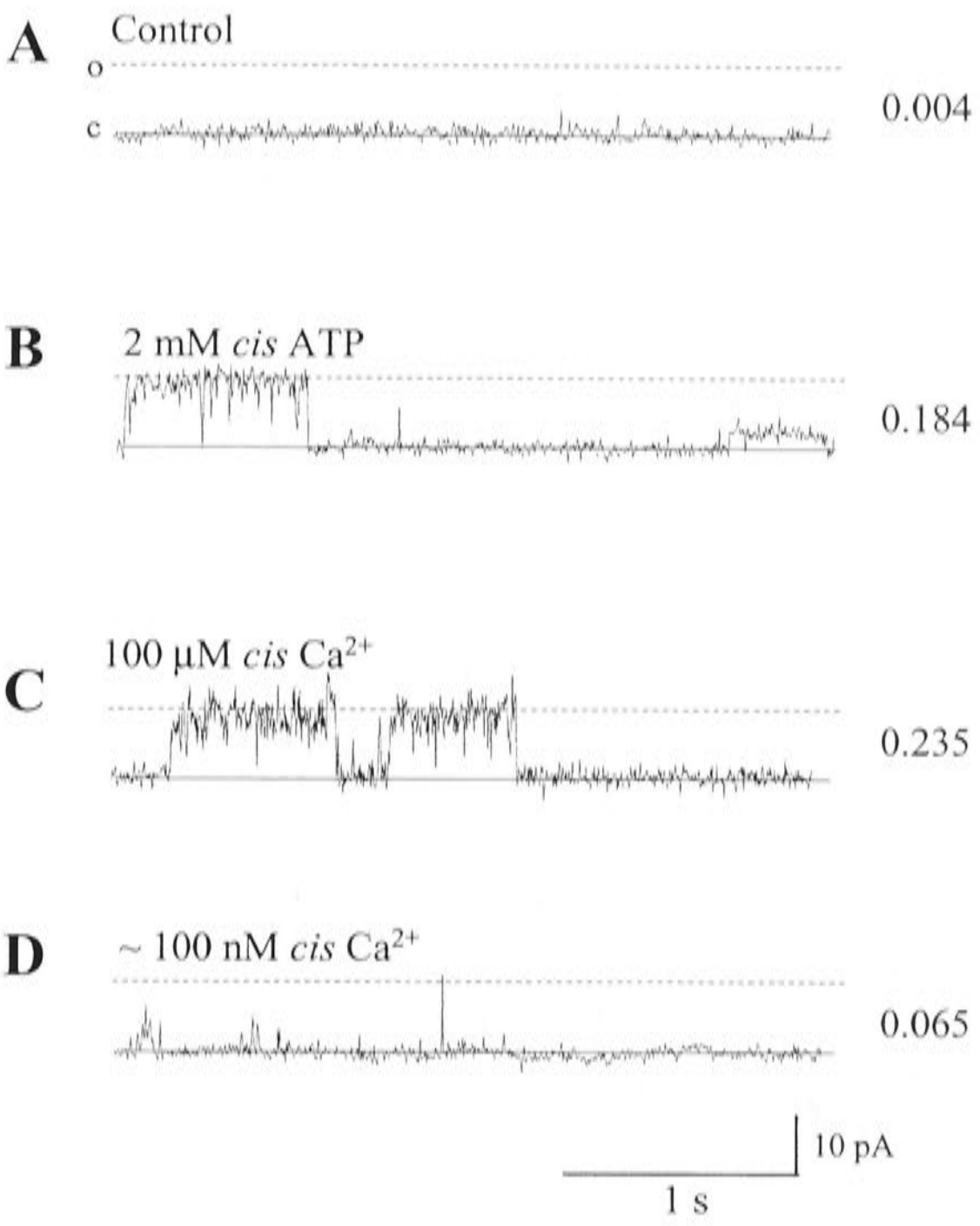


Fig. 4.XI. ***Activation and inhibition of RyRs expressed in CHO cells by cis modulators ATP and Ca^{2+}.*** (**A**) Control activity with 250/250 mM (*cis/trans*) Cs^{+}, 1 mM *cis* Ca^{2+}, (**B**) after addition of 2 mM ATP; (**C**) lowering *cis* Ca^{2+} to 100 µM (by adding 0.59 mM BAPTA); (**D**) lowering *cis* Ca^{2+} to ~ 100 nM (by adding a further 3.61 mM BAPTA). Fractional mean current (I'_F) of channel is shown to the right of each current trace, measured from 30 s of continuous channel recording. V_m-E_{Cs+} was +40mV under conditions. Single channel opening is upward from the zero current level (continuous line) to the maximum open conductance (broken line).

activating 100 nM cis Ca^{2+}. Addition of 2 mM *cis* ATP (using the same bilayer with two open channels) induced a 1.5-fold rise in channel I'_F (Fig. 4.X A, panel 3). Like native RyRs, the calculated Cs^+ conductance (250/250 mM) for purified RyRs was close to 525 pS, comparable with purified RyR conductance cited in literature (see Chapter 1, Section 1.2.5).

In another bilayer containing two active purified RyRs, the application of 10 μM ruthenium red induced channel inhibition; I'_F dropped from 0.234 (± 0.09) to 0.002 (±0.006; Fig. 4.X B). This drop was almost instantaneous (and occurred within the 15 s stirring period) and was sustained for over 5 min.

The application of 2 mM *cis* ATP (in the presence of 1 mM *cis/trans* Ca^{2+}) also induced an increase in the activity of RyRs expressed in CHO cells, increasing I'_F from close to zero (0.004) to 0.184 (Fig. 4.XI A-B). A reduction in *cis* free Ca^{2+} from the partially inhibiting concentration of 1 mM to 100 μM (activating Ca^{2+} concentration), by addition of 0.59 mM BAPTA, induced a further 1.3-fold rise in channel activity (Fig. 4.XI C), whilst subsequent addition of 3.61 mM BAPTA (reducing *cis* free Ca^{2+} to ~ 100 nM), resulted in significant channel inhibition (Fig. 4.XI D).

These data (Figs. 4.X-XI) indicate that both purified RyRs and RyRs expressed in a CHO cell line display similar ligand-induced modulation to that of native RyRs (Figs. 3.I-IV) and that cited in literature Coronado *et al.*, 1994; Meissner, 1994.

4.2.3.3 Effect of CSQ on purified RyRs

To test whether the CSQ-induced RyR inhibition was mediated by triadin and junctin, purified RyRs were incorporated into bilayers and exposed to either 20 or 50 μg *trans* CSQ (Fig. 4.XII). Fig. 4.XII illustrates that CSQ addition either increased activity (n=3: $I'_t/I'_c = 3.34 \pm 1.65$, Fig. 4.XII A-C) or caused no significant change in RyR activity (n = 2: $I'_t/I'_c = 0.96 \pm 0.012$, Fig. 4.XII, D-F. The lack of CSQ inhibition of purified RyRs is consistent with CSQ requiring anchoring protein(s) to mediate an inhibitory effect, since the removal of the only two proteins known to bind to CSQ and the RyR (triadin and junctin) prevented inhibition of RyRs by CSQ.

4.2.3.4 Effect of CSQ on RyRs expressed in CHO cells

The addition of 20 µg *trans* CSQ did not induce any change in the activity of RyRs expressed in CHO cell (Fig. 4.XIII). CSQ was incubated in the *trans* chamber for up to 8 min, with no apparent effect upon RyR activity. Up to 150 µg of CSQ was added to the *trans* chamber (with frequent stirring), a high enough [CSQ] to ensure interaction with the native RyR (see Ch. 3, section 3.3.2.1 for an estimate of CSQ localized in the terminal cisternae and that required for physiological addition in lipid bilayer experiments). This particular CHO cell line expresses only RyRs, without muscle co-proteins (such as triadin, junctin or CSQ; Bhat *et al.*, 1997).Due to the small sample size (n=2; because of a small amount of protein available for these experiments), this data are insufficient to be conclusive. However, the results are consistent with the effects of CSQ on purified RyRs presented here (Fig. 4.XIII) and with those of Szegedi *et al.* (1999) and Herzog *et al.* (2000), and show that phosphorylated CSQ does not inhibit RyR activity when co-proteins are absent. Certainly, CSQ regulation of RyRs without co-proteins such as triadin and junctin present, is far different from regulatory effects on the native channel. These data provide further evidence that the anchoring protein(s) are necessary for CSQ inhibition of the ryanodine receptor, and this is the first evidence from one laboratory showing that CSQ can regulate RyRs by two distinctly different mechanisms.

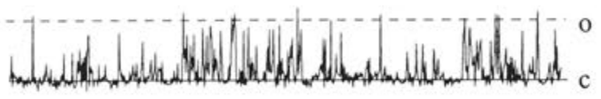
4.2.4 Attachment of chromophores to CSQ

As reported in Chapter 3 (Section 3.2.4 and Figs. 3.VI-VIII), CSQ was dissociated from RyRs upon increasing Cs^+ to 500 mM, after 89 ± 15s. This does not give an exact measurement of the rate of CSQ dissociation, because time measurements are not accurate due to the slow mixing times. To determine the exact time taken to dissociate CSQ from the RyR/T/J complex, it was proposed to employ stopped flow experiments, to determine the rate of CSQ dissociation more accurately. To achieve this, CSQ had to be dissociated from solubilized JFM, and exogenous CSQ (with an attached chromophore) re-associated. Once associated, chromophore-attached CSQ could be dissociated by either increasing [Cs^+] to 500 mM, or raising Ca^{2+} to 13 mM (see Chapter 5, Section 5.2.2.2 for data showing that high Ca^{2+} dissociation of CSQ). It was planned to calculate dissociation time by observing the alterations in fluorescence which occur upon CSQ dissociation from the JFM. It was assumed that CSQ would undergo a

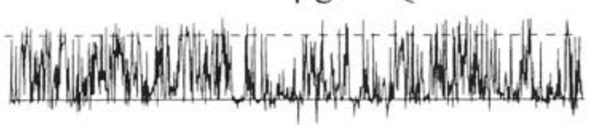
Fig. 4.XII. ***Association of 20 μg CSQ either activated or did not alter purified RyR activity.*** **A-C**, channels inhibited by CSQ (n = 3), **D-F**, channels non-responsive to CSQ (n=2). *Cis/trans* Cs^+ are shown above traces **A-B** & **D-E**. (**A**) Control activity with 250 mM *trans* Cs^+; (**B**) after addition of 20 μg CSQ; (**C**) bar graph represents average data, fractional mean current (I'_F) of 3 channels under conditions shown for **A** (con) & **B** (CSQ), nested line graph represents each of 3 single channel I'_F; (**D&E**) repeat of **B&C**; (**F**) bar graph represents average data I'_F of 2 channels under conditions shown in **D** (con) & **E** (buffer), nested line graph represents each of 2 single channel I'_F. V_m-E_{Cs+} was +40mV under all conditions. Single channel opening is upward from the zero current level (continuous line) to the maximum open conductance (broken line). Asterisks (*) indicate average values significantly different from control ($p<0.05$, t-test).

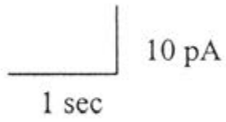
CSQ-responsive purified RyRs

A 250/250 Cs^+

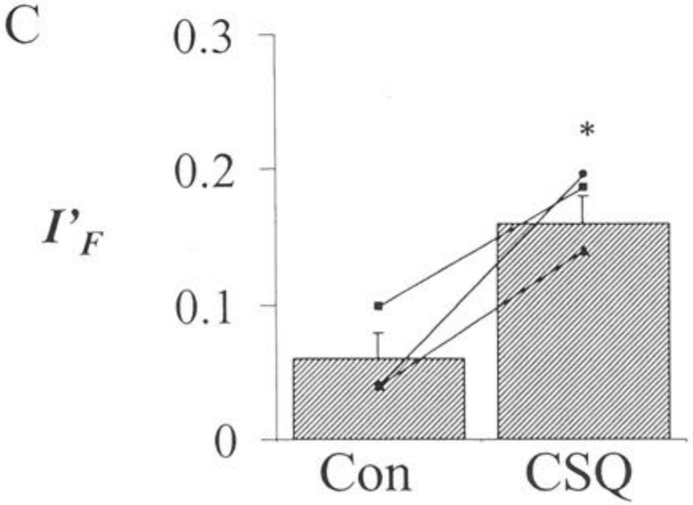


B 250/250 Cs^+ + 20 μg CSQ



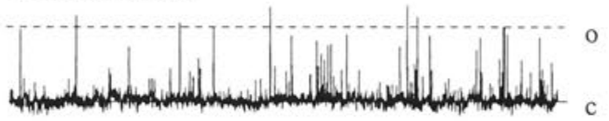


C

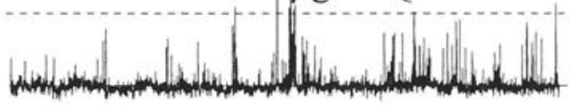


Non CSQ-responsive purified RyRs

D 250/250 Cs^+



E 250/250 Cs^++ 20 μg CSQ



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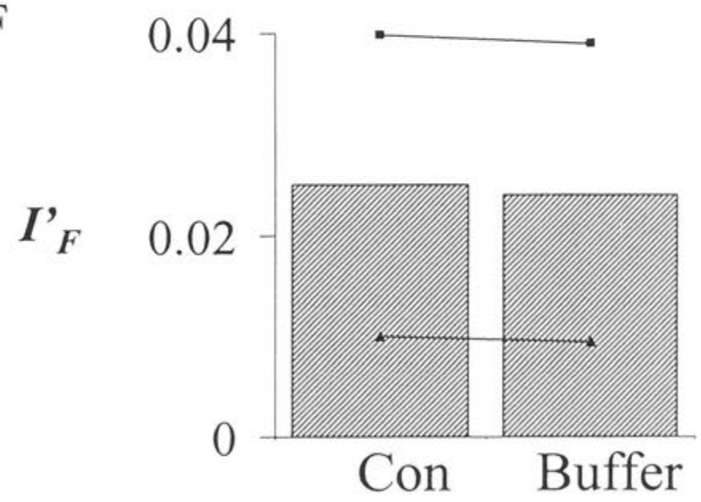


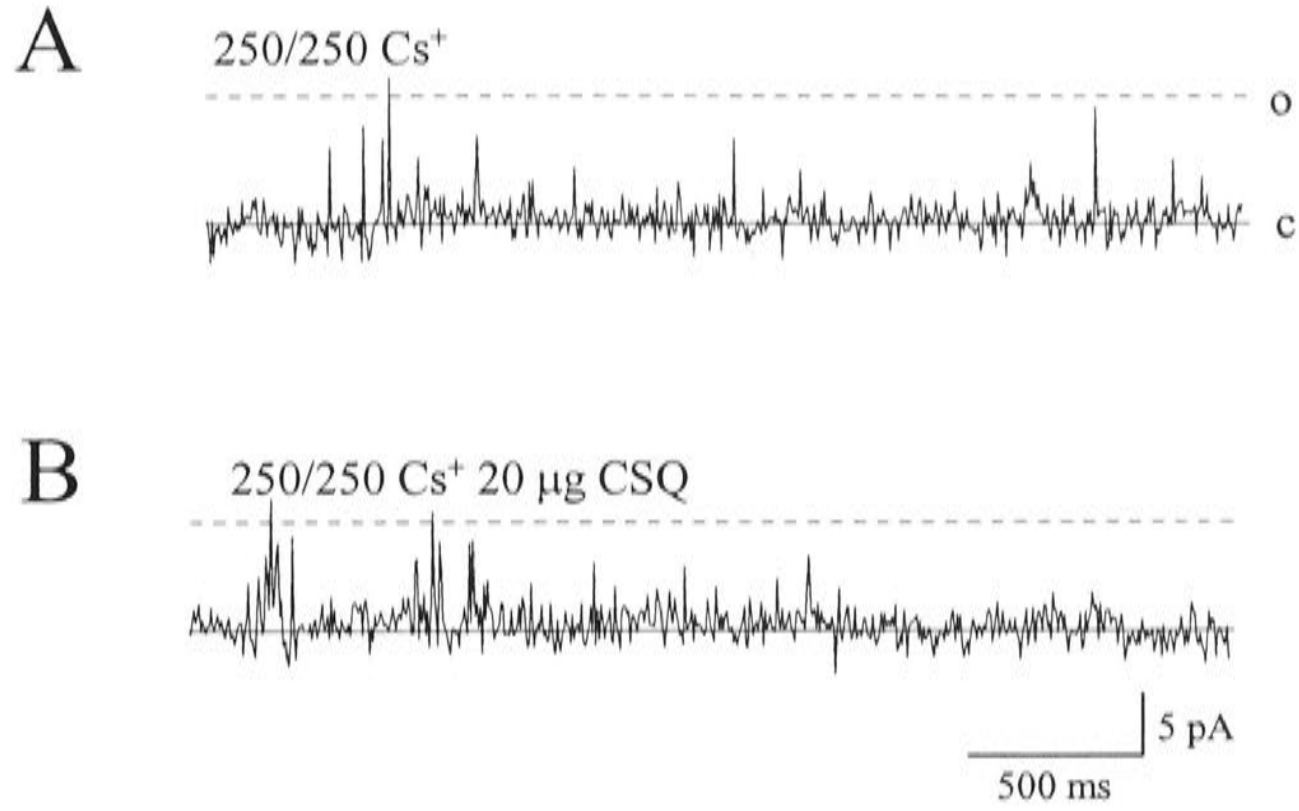


Fig 4.XIII. ***Addition of CSQ to RyRs expressed in CHO cells had no effect on single channel activity.*** (**A**) Control activity with 250 mM *trans* Cs^+; (**B**) after addition of 20 µg *trans* CSQ; (**C**) Bar graph represents average data, showing fractional mean current (I'_F) of 2 channels under conditions shown in **A&B,** with the nested line graph representing each of two single channel I'_F. V_m-E_{Cs+} was +40mV under all conditions. Single channel opening is upward from the zero current level (continuous line) to the maximum open conductance (broken line). *Cis/trans* Cs^+ is shown above each trace.

conformational change upon dissociation, which would be reflected by an alteration in fluorescence. Two chromophores were chosen, the first which binds to threonines and serines preferentially (called D-16) and the second, which attaches to lysines preferentially (F-2181). The skeletal muscle CSQ sequence contains 24 lysines, 11 threonines and 14 serines, so that several chromophores should attach to each CSQ molecule. Once the chromophores were attached (see Chapter 2, Section 2.8.2 for methodology of attachment), it was important to test whether this attachment had some how altered the way in which CSQ regulated, or associated with, the RyR/T/J complex.

4.2.4.1 CSQ-D16 and CSQ-F2181 do not alter RyR activity

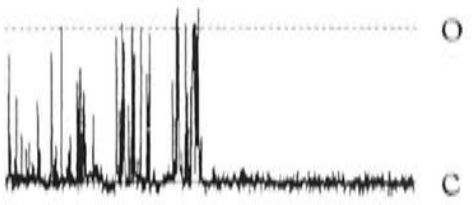
A rise in channel activity was induced by increasing Cs^+ to 500 mM, and as expected upon perfusion with 250 mM Cs^+, activity did not alter further (Figs. 4.XIV & XV A&B). Addition of 20 µg CSQ-D16 or CSQ-F2181 did not then change channel activity, despite stirring every 30-45 s for 10 mins, to facilitate binding (Fig. 4.XIV). Following perfusion of the *trans* chamber with 250 mM Cs^+, unmodified CSQ (20 µg) was able to reduce channel activity to pre-Cs^+ I'_F (Figs. 4.XIV C-E). In one channel, after dissociation and perfusion, 100 µg CSQ-D16 was added to the *trans* chamber, with no alteration in I'_F observed. In another experiment, a high activity channel that did not respond to 500 mM Cs^+, channel activity was not altered by incubation with 50 µg CSQ-D16 (Fig. 4.XIV E-G).

Furthermore, in two channels that did not respond to additions of either CSQ-D16 or CSQ-F2181, 20µg of unmodified CSQ was added to the *trans* chamber (following perfusion with 250 mM Cs^+, to remove the chromophore-conjugated CSQ). Channel activity was reduced to close to control levels. Somehow, chromophore attachment alters CSQs ability to bind to the RyR/T/J complex, and hence inhibit CSQs regulation of the RyR.

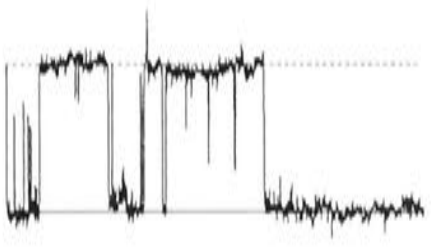
To ensure that the process of chromophore attachment (i.e. temporary high pH and exposure to room temperature at various stages in the process) did not destabilize CSQ, and cause its apparent inability to bind to the RyR/T/J complex and inhibit RyRs, unmodified CSQ was treated in the same way as CSQ during chromophore attachment. Fig 4.XV shows the effect of addition of CSQ, which had been temporarily exposed to both the high pH and temperature used in chromophore attachment, i.e. pH 9.5 and 25

Fig. 4.XIV. ***Addition of CSQ with attached chromophores CSQ-D16 and CSQ-F2181 did not inhibit channel activity.*** (**A**) Low activity channel; control activity with 250 mM *trans* Cs^+; (**B**) after increasing *trans* Cs^+ to 500 mM with CsCl, and an increase in channel activity was observed; (**C**) after perfusing the *trans* chamber with 250 mM Cs^+ (not shown) and subsequent addition of 50 μg CSQ-D16; to control solution) under conditions shown in **A-C**, **(D)** average data (n=2) showing fractional mean current (I'_F) of channel response to CSQ-D16 (n=3) and CSQ-F2181 (n=1), under conditions shown in **A-C** and **E-G**. (**E-G**) Repeat of **A-C**, in high activity channel, not responsive to 500 Cs^+. V_m-E_{Cs+} was +40mV under all conditions. Single channel opening is upward from the zero current level (continuous line) to the maximum open conductance (broken line). Individual channel I'_F is shown next to records **E-G**.

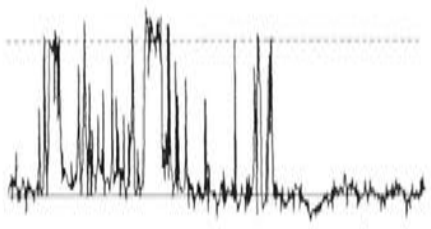
A Control



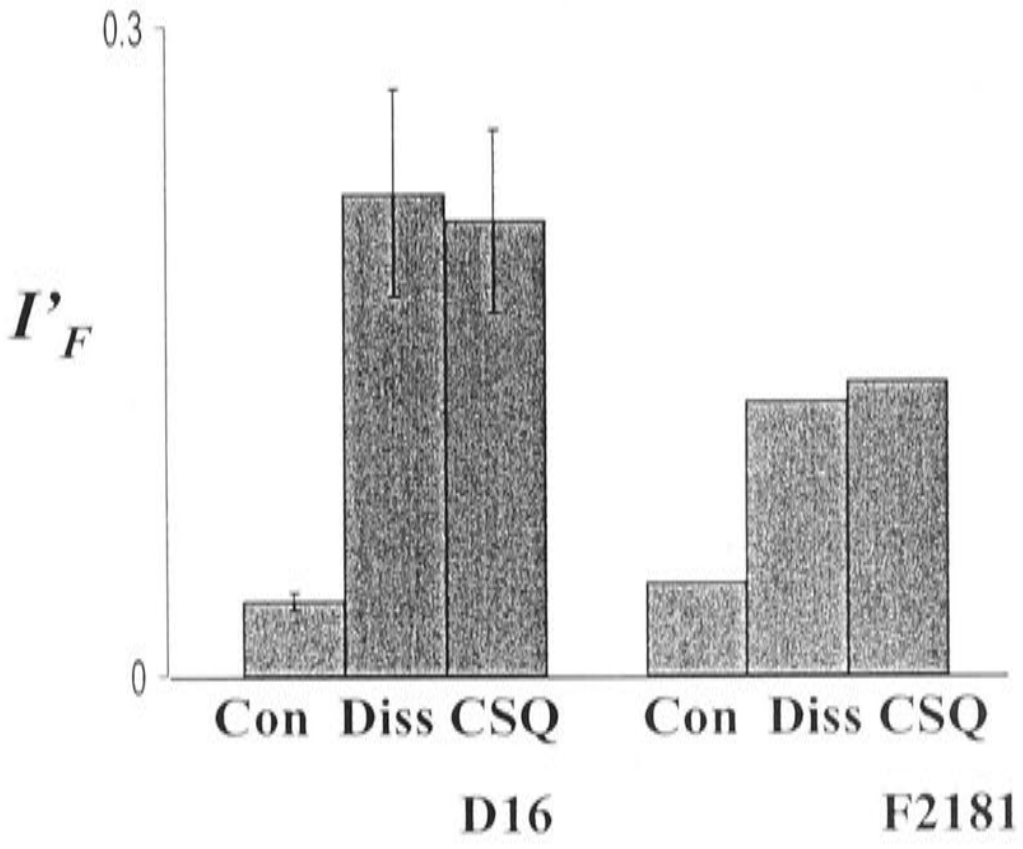
B Dissociate CSQ



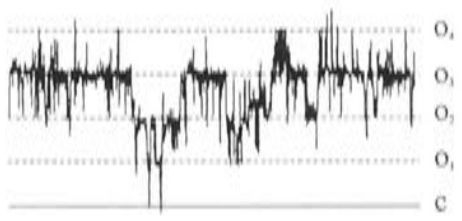
C 50 µg CSQ-D16



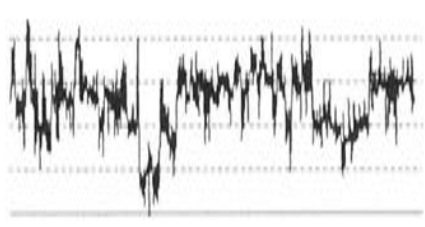
D



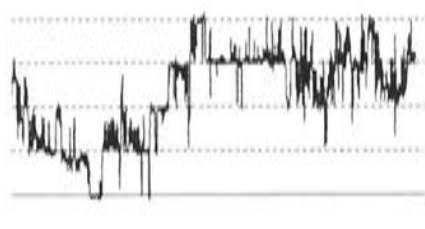
E Control

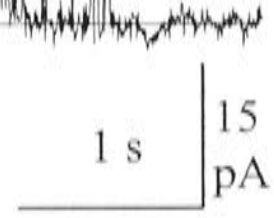


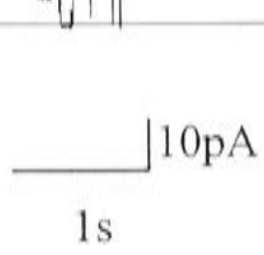
F Dissociate CSQ



G 50 µg CSQ-F2181







°C for 3 h, before the pH was restored to 7.4 and the CSQ sample was placed on ice. After the expected rise in RyR activity induced by 500 mM Cs^+ (and subsequent *trans* perfusion with 250 mM Cs^+), addition of 20 µg of the treated CSQ reduced channel activity to close to control levels, in the same way as the re-association study presented in Chapter 3 (Figs. 3.X-XII and Section 3.2.5). It is apparent that the attachment of D-16 and F-2181 (with a resultant functional/structural modification) prevented CSQ from inhibiting RyR activity. The failure to inhibit was not a result of extended exposure to high temperature or high pH.

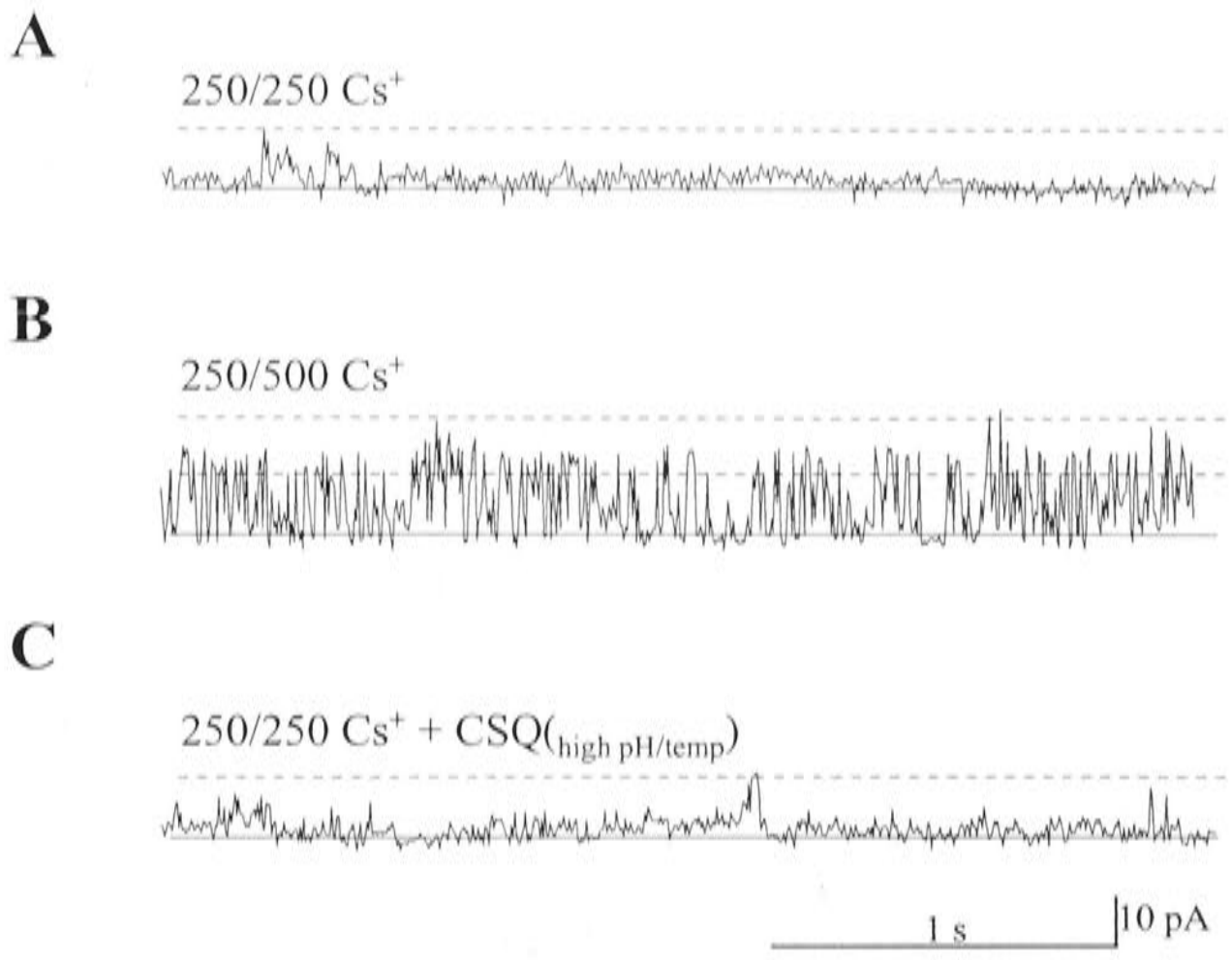


Fig. 4.XV. ***Exposure of CSQ to high pH and temperature (conditions used in chromophore attachment) did not alter the ability of CSQ to inhibit RyRs.*** (**A**) Control activity with 250 mM *trans* Cs^+; (**B**) after increasing *trans* Cs^+ to 500 mM with CsCl; (**C**) after perfusing the *trans* chamber with 250 mM Cs^+ (not shown) and subsequent addition of 20 µg CSQ, previously exposed to high pH and temperature, *cis/trans* Cs^+ are shown above **A-C**; (**D**) average data (n = 4) showing mean current (I'_F) under conditions shown in **A-C**. V_m-E_{Cs+} was +40mV under all conditions. Single channel opening is upward from the zero current level (continuous line) to the maximum open conductance (broken line). Asterisk (*) indicate values significant from control, crosshatch (#) indicates value significant from the previous condition($p<0.05$, t-test)

4.3 Discussion

The primary findings discussed in this section are that:

(1) There are two populations of RyRs, those with CSQ associated, as well as a CSQ-depleted population.

(2) The phosphorylation status of CSQ did not influence CSQs ability to inhibit native RyR activity; in fact both phosphorylated CSQ and dephosphorylated CSQ produced an approximately 2.0-fold reduction in I'_F in native RyRs. In contrast, only dephosphorylated CSQ activated purified RyRs Herzog *et al.*, 2000.

(3) CSQ modulation was strikingly different in purified and native RyRs. Re-association of CSQ inhibited native RyRs, whilst CSQ tended to activate purified RyRs.

(4) Attachment of chromophores D-16 and F-2181, which target lysine, serine and threonine residues, abolished CSQs ability to inhibit native RyRs.

4.3.1 Two populations of RyRs

Firstly, 68% of RyRs observed initially with low control activity, were activated after exposure to high ionic strength. The return to control ionic strength did not restore activity to pre-dissociation levels, in fact RyR activity fell only upon addition of CSQ. The other 32% of channels observed, termed 'high activity' channels (with high initial channel activity), did not respond to ionic strength increase. However, these high activity channels responded to exogenous CSQ addition, with a significant lowering of channel activity. Therefore at the start of the experiments, one population of RyRs has CSQ associated, and a second population did not have associated CSQ.

The absence of CSQ was not due to a lack of the necessary accessory protein(s) (likely to be junctin/triadin), because channel activity fell after excess CSQ was added to high activity channels, showing that CSQ could bind to and regulate channels. If triadin and junctin were absent, it is expected that CSQ re-association would not result in channel

inhibition, as was the case in CSQ regulated purified RyRs (where no triadin or junctin is present).

That high, and not low, activity channels responded to CSQ addition (by inhibiting RyR activity), firstly supports the proposal of the existence of two populations of RyRs, and secondly, supports the notion that a significant number of CSQ binding sites on triadin and junctin are occupied by CSQ in low activity channels. If a significant proportion of CSQ binding sites on accessory proteins were available, it would be expected that I'_F would fall in low activity channels due to CSQ eliciting an inhibitory effect.

These results do not show whether some RyRs lacked CSQ *in vivo* (suggested by Yamaguchi *et al.*, 1997), or whether CSQ was dissociated during preparation of SR vesicles. Wang *et al.* (2000), showed that cardiac myocytes which overexpress CSQ have impaired Ca^{2+} release and a reduction of spontaneous Ca^{2+} sparks (i.e. reduced RyR activity). If RyRs are partially depleted of CSQ *in vivo* (Yamaguchi *et al.*, 1997), then the findings of Wang *et al.* (2000) may be explained. Some RyRs in cardiac myocytes that originally lacked CSQ may have been inhibited when CSQ was over expressed, because we only observed CSQ-induced inhibition in RyRs that lacked CSQ. Of course protein overexpression may also induce other unrelated and non-specific changes.

The role that the degree of CSQ association with RyRs *in vivo* may play in regulating SR Ca^{2+} release would depend on the role CSQ plays in RyR regulation. In native RyRs, evidence presented here suggests that CSQ is a powerful RyR inhibitory protein. Other accessory proteins, such as triadin, FKBP12, DHPR, HRC and possibly the 30 kDa protein, have also been shown to modulate RyR activity. Presumably, CSQ forms part of a signalling pathway with triadin and possibly other proteins. If CSQ plays a major role in RyR regulation by inhibiting SR Ca^{2+} release, the fact that approximately 68% of RyRs contain associated CSQ may mean that the degree of CSQ association with the RyR can be modulated *in vivo*, and this modulation may influence RyR activity.

The absence of CSQ in some cases provides additional insight into the marked heterogeneity commonly observed in RyR activity (Laver *et al.*, 1995; Copello *et al.*, 1997). The observed differences in activity within the CSQ-depleted and CSQ

associated groups was less than the variability of the total RyR population, indicating that differences in CSQ status may be an important factor in RyR variation. The modulatory differences observed within the two RyR groups may stem from RyRs that are differently coupled to other accessory proteins known to affect RyR function, such as DHPR Melzer *et al.*, 1995, FK506-binding Timerman *et al.*, 1993; Ahern *et al.*, 1994 and calmodulin Tripathy *et al.*, 1995, or RyRs in different oxidation or phosphorylation states.

4.3.2 CSQ regulation of purified RyRs

CSQ decreased the activity of native RyRs incorporated into bilayers (see Chapter 3, Figs. 3.X-XII), but had no effect on (or tended to increase) the activity of RyRs that had been purified prior to bilayer incorporation (Fig.4.XII). These data indicate that CSQs regulatory effect on RyRs is dependent on the composition of the native RyR complex. Since triadin and junctin bind to both the RyR and CSQ Zhang *et al.*, 1997, and these proteins were absent in the CHAPS purified RyRs (Fig.4.X), it is possible that one or both of triadin and junctin mediate the CSQ effect. It has already been shown that triadin antagonises CSQ-induced changes in RyR activity Ohkura *et al.*, 1998, which provides further evidence that triadin forms part of the CSQ signalling pathway for RyR regulation. It has also been shown that CSQ binds triadin and junctin (Guo and Campbell, 1995, Zhang *et al.*, 1997). Therefore it is most likely that the RyR/T/J complex is required for CSQ inhibition of RyRs.

Although functional data (see Szegedi *et al.* (1999) and Herzog *et al.* (2000)) suggest that CSQ binds directly to the RyR when triadin and junctin are absent, there is no binding data to support such an interaction. The binding sites for CSQ on the RyR, or for the RyR on CSQ have not yet been identified. CSQ was unable to associate with peptides to the proposed RyR luminal loops, M1/2 (4580-4640) and M3/4(4859-4917; Herzog *et al.*, 2000); based on the RyR1 membrane topology model proposed by Zorzato *et al.* (1990). As the number and location of the RyR luminal loops are still controversial: (Zorzato *et al.* (1990) proposed twelve luminal loops, while Takeshima *et al.* (1989) proposed four luminal loops), it may be that the amino acids corresponding to the M1/2 or M3/4 regions do not exist in the lumen.

Although there is no binding data, Kawamoto *et al.* (1986) showed that CSQ is one of four proteins that can associate with the RyR (using immunoelectron microscopy), and Murray *et al.* (1998; using chemical crosslinking and immunoblot analysis), reported a close interaction between CSQ and the ryanodine receptor suggesting a physical coupling. On the other hand, binding of CSQ to triadin has been shown. Recently it has been reported that the Asp-rich region of skeletal CSQ, (Glx^{354}-Asx^{367}), is the site for triadin association (Shin *et al.*, 2001).

The *in vivo* action of triadin/junctin on the RyR is not known, although the activity of purified RyRs was decreased by triadin (Ohkura *et al.*, 1998; Groh *et al.*, 1999). Peptides corresponding to the cytoplasmic domain of triadin inhibited purified RyR activity, indicating that the functional interaction between triadin and the RyR may occur on the cytoplasmic side of the SR membrane (Groh *et al.*, 1999). CSQ binding to triadin might somehow influence the interaction between triadins cytoplasmic domain (the proposed site of triadins functional regulation of the RyR) and the RyR, enabling triadin to further inhibit RyR activity. On the other hand, Caswell *et al.* (1999) have shown an interaction between the purified skeletal RyR and bacterially expressed peptides corresponding to the luminal domain of triadin (Leu^{110}-Lys^{280}), but reported no interaction with a triadin peptide (Thr^{2}-Pro^{49}) encompassing the cytoplasmic domain of triadin (residues 1-47). Therefore some contradiction concerning the location of the triadin binding site exists.

4.3.3 Phosphorylation status of CSQ

CSQs ability to inhibit native RyRs was independent of CSQs phosphorylation status, in contrast to CSQ-induced activation of purified RyR activity, which depended so heavily on CSQ being dephosphorylated (Szegedi *et al.*, 1999; Herzog *et al.*, 2000). These different findings support the suggestion that CSQ modulates RyR activity via two mechanisms; (1) by binding directly to the RyR, in a phosphorylation-dependant manner, as shown by Szegedi *et al.* (1999) and Herzog *et al.* (2000), and (2) by inducing RyR inhibition by interactions with triadin and junctin in a phosphorylation-independent manner. Whether or not both mechanisms of CSQ regulation are operating in native RyRs is not known. If so, the reported CSQ activation induced by a direct RyR-CSQ interaction is overshadowed by the triadin/junctin mediated CSQ regulation, seen as an overall RyR inhibition of the RyR by CSQ.

Whether or not phosphorylation and dephosphorylation of CSQ regulates RyRs *in vivo* would depend on whether CSQ is phosphorylated before luminal segregation, or after targeting to the lumen. This in turn, would depend on the location of specific kinases responsible for CSQ phosphorylation, and whether or not ATP could be transported into the lumen of the SR. It is not known which kinase specifically phosphorylates CSQ *in vivo*, or whether such kinases are present in the SR lumen (or indeed whether CSQ is phosphorylated inside the lumen). CK II has been shown to phosphorylate CSQ; it can phosphorylate Thr^{353} (Cala and Jones, 1991). To date, the presence of CK II within the SR lumen has been intimated, but not proven Shoshan-Barmatz *et al.*, 1996. It is of course possible, that either Thr^{353} or another phosphorylatable residue may be phosphorylated by another kinase, which could be present in the lumen. CSQ has also been identified as a potentially good substrate for CK I and ε protein kinase I, but current evidence suggests that within muscle, these kinases exist only in the cytoplasm and not in the lumen (Salvatori *et al.*, 1994; Rodriguez *et al.*, 1999). The phosphorylation status of isolated CSQ (Fig. 4.VI) suggests that CSQ in the SR lumen is phosphorylated, therefore dephosphorylation-dependent CSQ activation of the RyR, or indeed regulation of the RyR due to phosphorylation changes in CSQ, may not be physiologically relevant.

4.3.4 Attachment of chromophores to CSQ

It is obvious that chromophore attachment alters CSQ's ability to bind to the RyR/T/J complex, possibly by binding to a residue/residues important in the functional coupling of CSQ to the RyR/T/J complex, or to Ca^{2+} sequestration, or to self-compaction. This might somehow alter CSQ's conformation, or inhibit residues involved in CSQ binding to the RyR, triadin or junctin. Whilst stopped flow experiments could not be used to examine CSQ dissociation rates, this study has provided insight into the apparent importance of threonines/serines, and/or lysines in CSQ's structural and/or functional capacity.

4.3.5 Physiological Significance

These results (see Section 4.2.3), coupled with those of Szegedi *et al.* (1999) and Herzog *et al.* (2000) are intriguing. CSQ can activate the RyR (dependant on phosphorylation status), when triadin and junctin are absent, but not when the anchoring

proteins are present; native RyRs were inhibited by CSQ (regardless of phosphorylation status). One must question the role of both interactions *in vivo*; certainly the existence of two mechanisms adds a new dimension to the role that CSQ may play in RyR modulation. CSQ may bind to the RyR both directly and via triadin and junctin, with the inhibition induced by these two anchor proteins overriding RyR regulation by any direct coupling of CSQ to the channel. If this were so, it would explain the overall inhibitory effect observed in the native RyR/T/J complex used in this study. The possibility that other proteins, such as the 30 kDa protein, binds to and modulates either CSQ or the RyR, would only add to the complex nature of RyR regulation by luminal proteins. On the other hand, CSQ might have a much higher affinity for triadin than the RyR. When triadin is present, CSQ may bind to triadin, causing RyR inhibition, whilst when triadin is absent, CSQ may interact with the RyR directly (as no triadin is available to compete for CSQ binding), eliciting activation. Another possibility is that upon binding, triadin blocks access to RyR binding sites on CSQ.

Whether or not phosphorylation-dependant changes in CSQ play a role in regulating RyR activity depends on two things; (1) whether a CSQ-RyR interaction *in vivo* occurs, and (2) whether or not CSQ can be phosphorylated *in vivo*. Further investigation in this area would help define the role of CSQ in SR Ca^{2+} release regulation.

What has emerged from the work in this chapter is that the regulation of RyR activity by luminal factors may be as complex as RyR regulation by cytoplasmic factors. Almost nothing is known about the effects of RyR regulation by proteins such as junctin, the 30 kDa protein, HRC and SLM, and if and how these proteins interact with each other, the RyR, and triadin and CSQ (the later two proteins being the only two known to regulate the RyR that have been investigated at any length). To understand the nature of luminal regulation of the RyR, (and indeed SR Ca^{2+} release), further investigation into the interaction of such proteins, and their effects on the RyR is needed.

ROLE OF CSQ IN THE EFFECTS OF LUMINAL Ca^{2+} ON RYR ACTIVITY

5.1 INTRODUCTION

Maintenance of luminal Ca^{2+} at physiological concentrations is important in stabilizing the structural formation of the complex formed between RyR, triadin, junctin and CSQ (Wang *et al.*, 1998). In fact, CSQs associations with junctin and triadin are inhibited in the presence of >10 mM Ca^{2+} (Zhang *et al.*, 1997). Low [Ca^{2+}]s are thought to uncouple the interactions that help stabilize CSQ in a polymer formation, and possibly revert CSQ to a random coil structure (Wang *et al.*, 1998). Furthermore, increases in [Ca^{2+}] from 0-3 mM have been reported to induce substantial, reversible changes in CSQs conformation, leading to the proposal that CSQ is a regulator of SR Ca^{2+} release (Ikemoto *et al.*, 1989b; Ikemoto *et al.*, 1991; Gilchrist *et al.*, 1992; He *et al.*, 1993). It is therefore possible that gross alterations in luminal Ca^{2+} may result in reduced RyR regulation by CSQ, either by the removal of CSQ, or even by changing CSQ conformation.

The effects of changes in luminal [Ca^{2+}] on RyR activity have been studied extensively, although with somewhat conflicting results. Increasing luminal [Ca^{2+}] above physiological levels has been reported to either increase (Sitsapesan and Williams, 1995; Herrmann-Frank and Lehmann-Horn, 1996; Tripathy and Meissner, 1996), or decrease RyR activity (Ma *et al.*, 1988; Fill *et al.*, 1990; Szegedi *et al.*, 1999). Luminal Ca^{2+} probably binds to luminal Ca^{2+} activation sites (located on the RyR or another associated luminal proteins), resulting in Ca^{2+} activation, although regulation via diffusion through the pore and interaction with cytoplasmic Ca^{2+} activation sites has also been proposed (Tripathy and Meissner, 1996). No study thus far has fully investigated the possible effects of luminal Ca^{2+} on RyR regulation by CSQ.

In this chapter, the effects of high and low luminal Ca^{2+} on RyR activity in the presence and absence of CSQ, are presented. The premise that Ca^{2+}-induced conformational changes in CSQ effect RyR activity was explored over a physiological luminal Ca^{2+} range of 1-3 mM. In addition, the Ca^{2+}concentrations tested lie within the range of Ca^{2+} (0.05 – 10 mM) that SR Ca^{2+} release is known to be most sensitive to luminal Ca^{2+} concentrations (Donoso *et al.*, 1995).

5.2 Results

5.2.1 Ca^{2+} dissociation of CSQ

CSQ binding to triadin and junctin, as well as the polymerisation of CSQ are Ca^{2+}-dependent processes (He *et al.*, 1993; Zhang *et al.*, 1997; Wang *et al.*, 1998). Free luminal Ca^{2+} of 1 mM is required to ensure CSQ/triadin/junctin interactions, and to stabilize CSQs polymer structure. As mentioned previously, Zhang *et al.* (1997) have shown that luminal Ca^{2+} of $\geq$10 mM disrupts CSQs interaction with the RyR/triadin/junctin (RyR/T/J) complex. The present results show that incubating JFM in 13 mM Ca^{2+} can also dissociate CSQ (Fig. 5.I). A high [Ca^{2+}] wash used during purification resulted in a greater yield of CSQ (when followed by an exposure to 500 mM NaCl, to maximize protein purity), 7-8 mg CSQ/100 g muscle (13 mM Ca^{2+} plus high ionic strength dissociation) vs 5 mg CSQ/100 g muscle (ionic strength dissociation alone). All isolated protein samples were subjected to SDS-Page, silver staining and immunodetection, using anti-CSQ antibody. Exposing SR vesicles (Fig. 5.I, lane 1) to the high Ca^{2+} CSQ purification protocol (see Chapter 2, Section 2.5.2) successfully removed CSQ (Fig. 5.I, lane 2) from the solubilized JFM (Fig. 5.I, lane 3), as shown by silver stain and by immunoprobing with anti-CSQ antibody (Fig. 5.I, lanes 4-6). No detectable levels of triadin were found in the CSQ isolated by high or low Ca^{2+}, and the band at 26 kDa (presumably junctin), seen in the JFM sample (Fig. 5.I, lane 1) has also been removed by CSQ purification (Fig 5.I, lane 2). It is unlikely that a significant fraction of the CSQ was lost due to proteolytic degradation, since proteolic inhibitors were present throughout the isolation procedure (see Chapter 2, Section 2.5).

5.2.2 Response of RyR to increasing *trans* [Ca^{2+}]

5.2.2.1 Luminal Ca^{2+} dose response curve

Due to the disparity in the literature regarding the exact nature of luminal Ca^{2+} regulation of RyRs (see Chapter 1, Section 1.2.6.2), luminal Ca^{2+} was raised from 1 to 20 mM to determine the effects of raising luminal Ca^{2+} under standard *cis* conditions - activating *cis* ATP (2 mM) and sub-activating *cis* Ca^{2+} (100 nM), in the presence of

CSQ. In all bilayer data, the composition of *cis* and *trans* solutions is as follows, unless otherwise specified: *cis* 100 nM free Ca^{2+} (1mM $CaCl_2$ plus 4.5 mM BAPTA), (in mM) 2 ATP, and 250 Cs^+; *trans* (mM) 250 or 500 Cs^+ and 1 Ca^{2+}. In the case of average data presented in this chapter, channels were analyzed for 30 s, unless stated otherwise.

Fig. 5.II illustrates the biphasic response of RyRs to luminal Ca^{2+}, whereby increasing Ca^{2+} from 1 mM to ~ 4.5 mM induces an increase in channel open probability (P_o) - whilst further increases in *trans* Ca^{2+} to 20 mM resulted in channel inhibition with a P_o close to that recorded in the presence of 1 mM *trans* Ca^{2+}. The curve was fitted with a least squares fit to the Hill equation (see Chapter 3. Eq. 3.2-1 for Hill equation), giving a P_{max} of 0.43, Ca^{2+} binding affinities of $K_{a(ca)}$ = 1.7 mM and $K_{i(ca)}$ = 13.1 mM, with Hill co-efficients for activation ($n_{a(ca)}$) and inhibition ($n_{i(ca)}$) of 3.35 and 2.91, respectively. Channel activity was analysed within 1 min of changing [Ca^{2+}], to determine effects of luminal Ca^{2+} on RyR activity, presumably when CSQ was present (as CSQ dissociation is thought to take longer than 1 min; see Section 5.2.4). Thirteen mM Ca^{2+} was chosen as the concentration used to attempt CSQ dissociation experiments because this [Ca^{2+}] results in a significantly higher P_o than 1 mM *trans* Ca^{2+} (see Fig 5.II) and > 10 mM *trans* Ca^{2+} was previously reported to prevent the interaction between CSQ and triadin and junctin (Zhang *et al.*, 1997).

5.2.2.2 Time dependence and reversibility of raising luminal [Ca^{2+}].

5.2.2.2.1 13 mM trans Ca^{2+}

The effects of high luminal [Ca^{2+}] on the RyR/T/J/CSQ complex and RyR activity was examined by exposing the *trans* solution bathing bilayer-incorporated RyRs to 13 mM *trans* Ca^{2+}. As illustrated in Fig. 5.III at positive potentials, RyR P_o increased significantly after exposure to 13 mM *trans* Ca^{2+}. The increase was rapid and occurred within 30 s. In addition to this immediate increase in channel activity, the time course of the response of RyRs showed another distinct slower phase; where P_o increased over several minutes (Fig. 5.III C). The secondary increase in activity was characterized by significant increases in average P_o and open frequency (F_o), and a decline in closed time (T_c; Fig. 5.III F,H,I). Open time (T_o) decreased slightly (but not significantly) from the primary increase, although this increase was still significantly higher than control (Fig. 5.III G). When *trans* Ca^{2+} was returned to 1 mM by perfusion of the *trans* chamber, the channel activity fell significantly but did not return to its original (control) level.

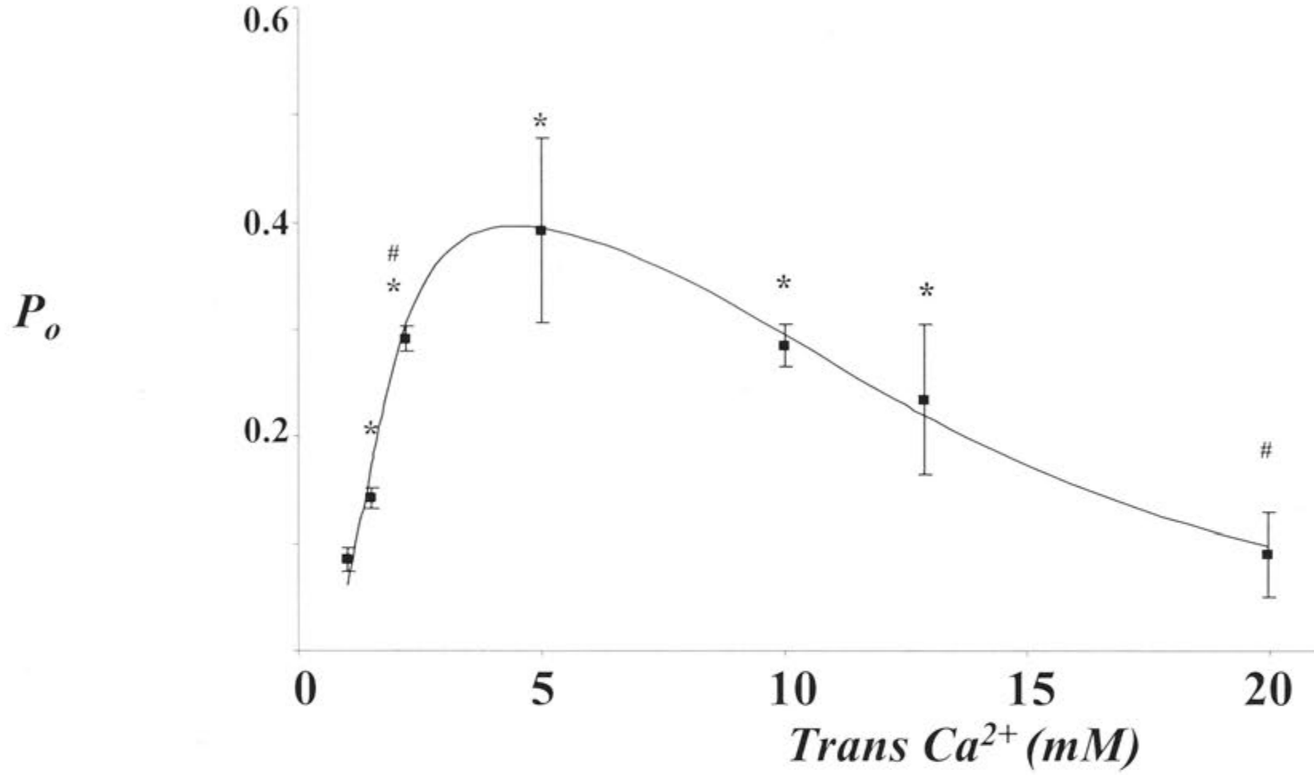


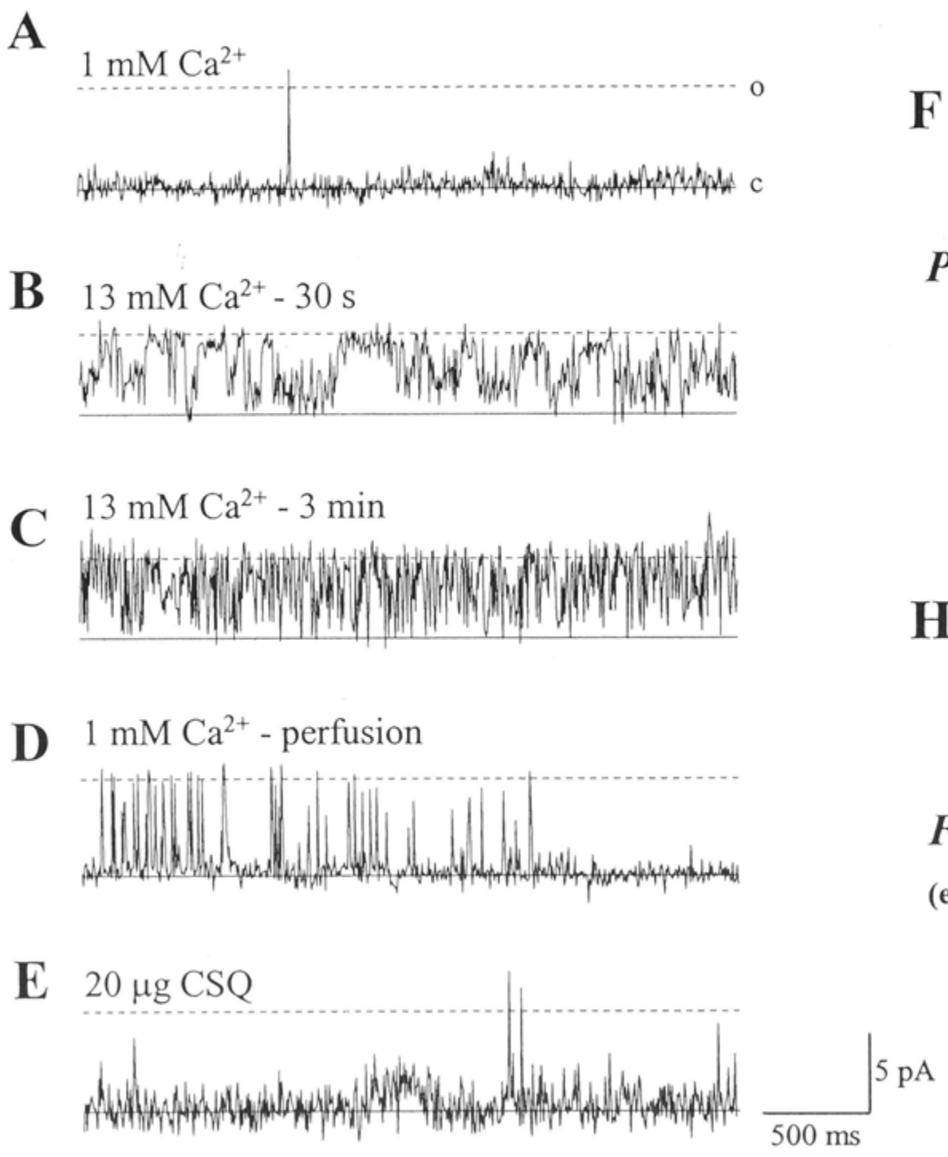
Fig 5.II. ***Biphasic response of RyRs to increasing trans Ca^{2+} - dose response curve, with ATP as the sole cytoplasmic activator.*** Experimental conditions: *cis* (mM) 250 Cs^+, 2 ATP (activating) and 1 nmol Ca^{2+}(sub-activating); *trans* (mM) 250 Cs^+, 1-20 Ca^{2+}. *Trans* $Ca^{2+}$was altered by aliquot additions of 200 mM stock Ca^{2+}(Ca^{2+} concentration was determined by a calcium electrode). Each data point is the mean open probability (P_o), error bars are SEM for n=3-16. Data points show the average results of analysis of P_o starting 1 min after addition of Ca^{2+}. The solid line is the least squares fit to the Hill equation (Eq. 3.2-1). Asterisks (*) indicates a significant change from control, crosshatch (#) indicates a significant change from the previous condition (1 mM Ca^{2+}).

However, addition of 20 μg of purified CSQ to the *trans* chamber did cause a further reduction in activity back to control levels. Since addition of exogenous CSQ does not alter the activity of channels that are already associated with CSQ (see Ch. 4. Fig. 4.IV), the CSQ-induced reduction in activity seen here provides additional evidence that high luminal Ca^{2+} had dissociated CSQ from the RyR complex. At negative potentials (in 4 out of 7 channels), RyR activity rose immediately upon incubation with 13 mM Ca^{2+}, whilst in 3 out of 7 channels, relative mean current (I'_t/I'_c) initially decreased in response to the increase in [Ca^{2+}] (Fig. 5.IV). Tripathy and Meissner (1996) have reported a voltage dependence of luminal Ca^{2+} regulation of purified RyRs, with a significant decrease in channel P_o observed upon increasing *trans* Ca^{2+} from 1 to 10 mM, in the presence of 2 mM ATP and 45 nM Ca^{2+}_{free} (*cis*) at –40mV. In the present experiments, overall average channel activity rose significantly within 30 s of 13 mM Ca^{2+} application, and after 3 min all channels showed an increase in I'_t/I'_c, consistent with CSQ dissociation (Fig. 5.IV). Similar to changes observed at +40 mV (see previous paragraph and Fig. 5.III), when reducing [Ca^{2+}] from 13 mM to 1 mM, channel activity was reduced at -40 mV ($P<0.05$, sign test), but remained significantly higher than control (Fig. 5.IV C&E). Only addition of 20 μg CSQ returned P_o to control levels (Fig. 5.IV D-E).

5.2.2.2.2 The effects of 13 mM Ca^{2+} on CSQ-depleted RyR activity

To investigate the role CSQ plays in Ca^{2+}-dependent activation of RyRs, the effect of high luminal [Ca^{2+}] was measured in RyRs after CSQ had been removed by exposure to 500 mM ionic strength. Exposure to high luminal ionic strength produced a 1.8-fold increase in RyR activity, and presumably dissociated CSQ from the RyR/T/J complex (See Chapter 3, Section 3.2.4). When *trans* [Ca^{2+}] was subsequently raised to 13 mM, RyRs activity increased during stirring time (Fig. 5.V C). However, in contrast to the RyRs with associated CSQ (Figs. 5.III & IV), the time course of activation of CSQ-depleted RyRs after an increase in luminal Ca^{2+} did not show the slow phase (i.e. there was no significant increase in activation over the 3 min exposure to 13 mM Ca^{2+}; Fig. 5.V D). Returning [Ca^{2+}] to 1 mM by either perfusion of the *trans* chamber or the addition of 12 mM BAPTA, restored RyR activity to levels seen prior to the increase in luminal [Ca^{2+}], but not to the low levels seen before high Cs^+ induced dissociation of CSQ (Fig. 5.V E). Only re-association of 20 μg CSQ could return channel activity to control levels (Fig. 5.V F). Similar to results obtained in the Cs^+ dissociation and CSQ

Fig. 5.III. ***At +40 mV, increasing trans Ca^{2+} from 1 mM to 13 mM caused an increase in single RyR channel activity, which could only be fully reversed by addition of exogenous CSQ***. **(A)** Control channel activity, with 1 mM *trans* Ca^{2+}; **(B)** 30 sec after increasing *trans* Ca^{2+} to 13 mM; **(C)** 3 mins after increasing *trans* to 13 mM; **(D)** after perfusing *trans* chamber with 1 mM Ca^{2+}; **(E)** Addition of *trans* 20 µg CSQ. **F-I**, Average data (n=9) for (**F**) P_o (**G**) T_o (**H**) T_c and (**I**) F_o for conditions in **A-E**. V_m- E_{Cs} was +40 mV. Single channel opening is upward from zero current (continuous line) to maximum open conductance (broken line). An asterisk (*) indicates average values significantly different from control ($p<0.05$, t-test), whilst ** and ## indicate average values are significantly different from the previous condition (t-test and sign test respectively).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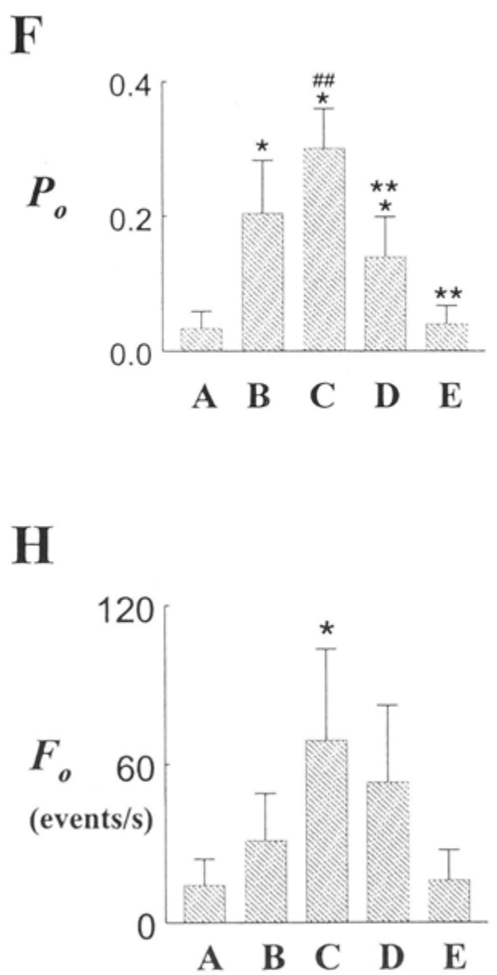
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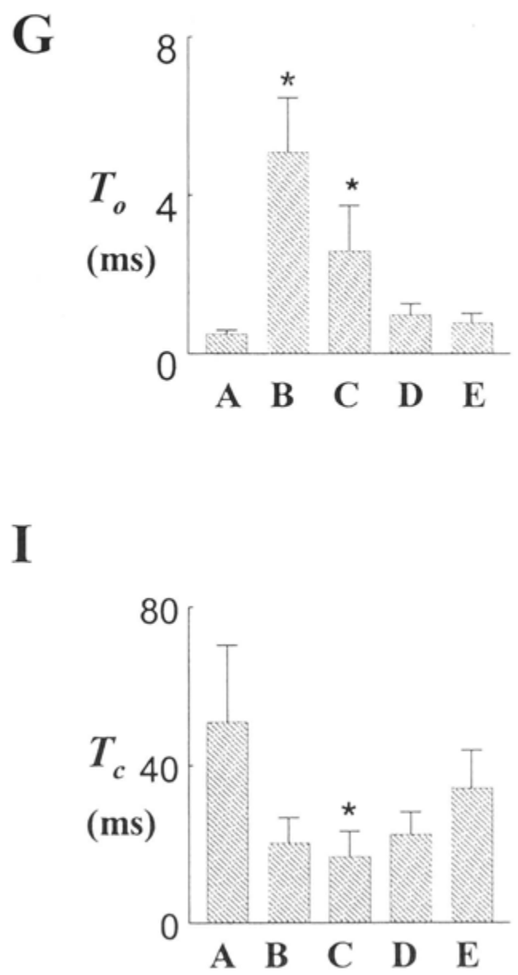
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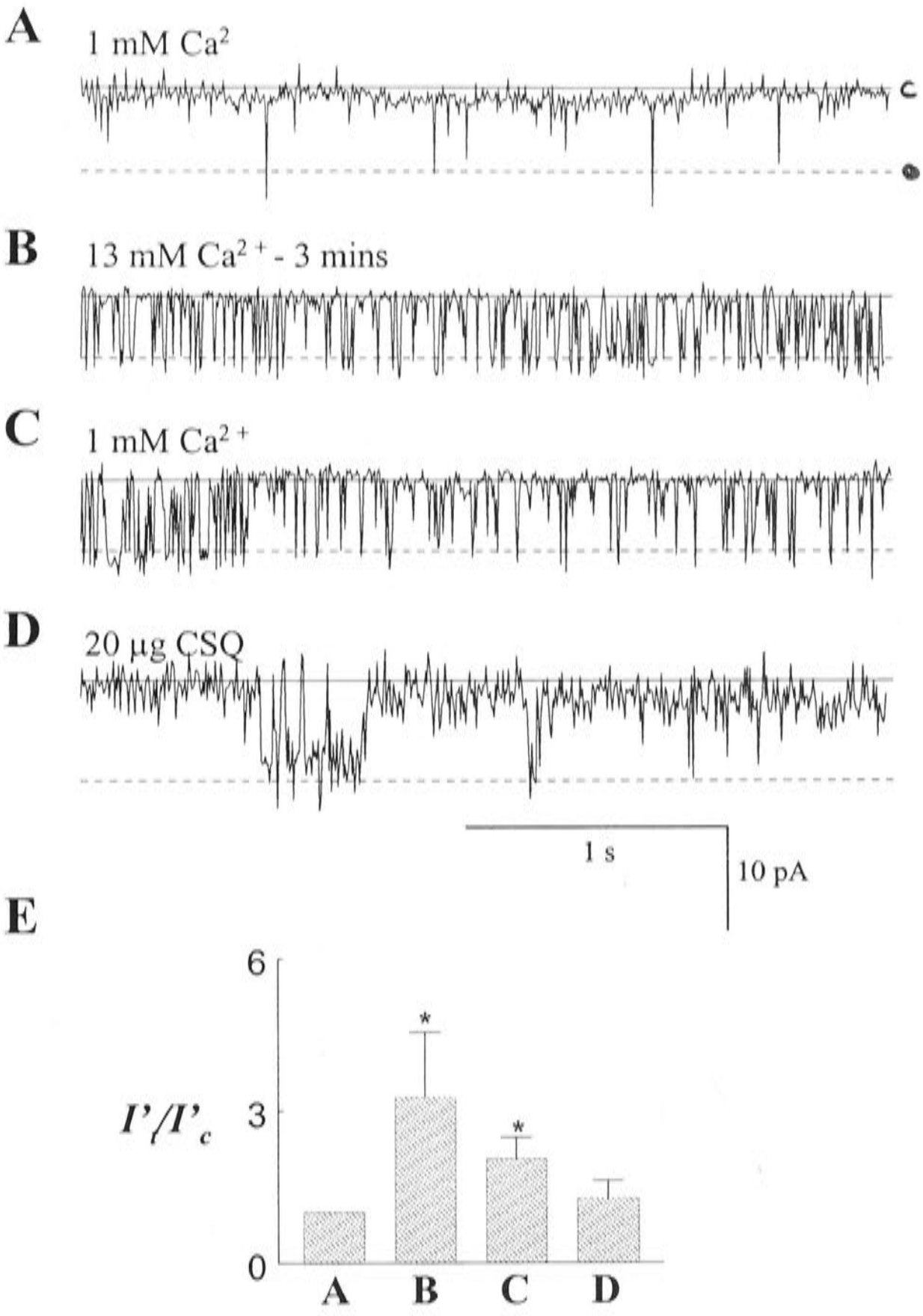


Fig. 5.IV. ***At –40 mV, increasing trans Ca^{2+} from 1 mM to 13 mM caused an increase in single RyR channel activity, which could only be fully reversed by addition of exogenous CSQ.*** **(A)** Control channel activity, with 1 mM *trans* Ca^{2+}; **(B)** 3 mins after increasing *trans* Ca^{2+} to 13 mM; **(C)** after perfusing the *trans* chamber with 1 mM Ca^{2+}; **(D)** Addition of *trans* 20 µg CSQ. **(E)** Average data (n=3-7) for I'_t/I'_c for conditions shown in **A-D**. V_m-E_{Cs} was -40 mV. Single channel opening is downward from zero current (continuous line) to maximum open conductance (broken line). (*) indicates average values significantly different from control ($p<0.05$, t-test).

re-association experiments, (see Fig. 3.XI), 20 µg CSQ was able to reduce the activity of the channels to levels not significantly different from the pre-CSQ dissociation levels (Fig. 5.V E). Thus, the different effects of luminal Ca^{2+} on RyRs with and without CSQ indicates that 1) luminal Ca^{2+} activates RyRs by both CSQ-dependent and independent mechanisms, and 2) that CSQ dissociation underlies the slower component of RyR activation. The relatively slow secondary increase in RyR activity seen with high [Ca^{2+}] was similar to the slow increase in activity observed after an increase in ionic strength, suggesting that in both cases, CSQ dissociation from the RyR complex takes 1.5-3 min.

5.2.2.2.3 5 mM trans Ca^{2+}

Upon increasing *trans* Ca^{2+} from 1 mM to 5 mM, an almost immediate and significant increase in channel fractional mean current (I'_F), from 0.086 ± 0.01 to 0.186 ± 0.04 was observed (Fig. 5.VI). A secondary rise in channel activity was observed after 3 min exposure to 5 mM *trans* Ca^{2+}, with I'_F increasing to 0.291 ± 0.03. Similar to the results observed upon addition of 13 mM *trans* Ca^{2+}, perfusion of the *trans* chamber with 1 mM Ca^{2+} reduced channel activity somewhat, but not to control levels. In 3 out of 3 channels tested, addition of CSQ reduced channel activity to a level that was not significantly different from control (Fig. 5.VI). Furthermore, in 1 out of 1 channels, application of 20 µg *trans* anti-CSQ antibody after CSQ addition further reduced channel activity (data not shown). These results suggest that 5 mM *trans* Ca^{2+} may also dissociate CSQ from the RyR/T/J complex.

5.2.2.2.4 2 mM trans Ca^{2+}

Exposing RyRs to 2 mM *trans* Ca^{2+} (in a similar protocol to that followed in Section 5.2.2.2.3), did not induce CSQ dissociation from RyRs (Fig 5.VII). Despite incubation in 2 mM Ca^{2+} for up to 15 min, channel I'_F was seen to increase almost immediately 1.7-fold from control levels (1 mM *trans* Ca^{2+}), with no secondary increase in channel activity observed (Fig. 5.VII B&C), despite incubation in 2 mM Ca^{2+} for up to 15 min. Reperfusion of the *trans* chamber with 1 mM Ca^{2+} restored RyR I'_F to control levels, illustrating the activity increase upon raising [Ca^{2+}] to 2 mM was reversible and not due to the removal of a RyR-associated protein, such as CSQ.

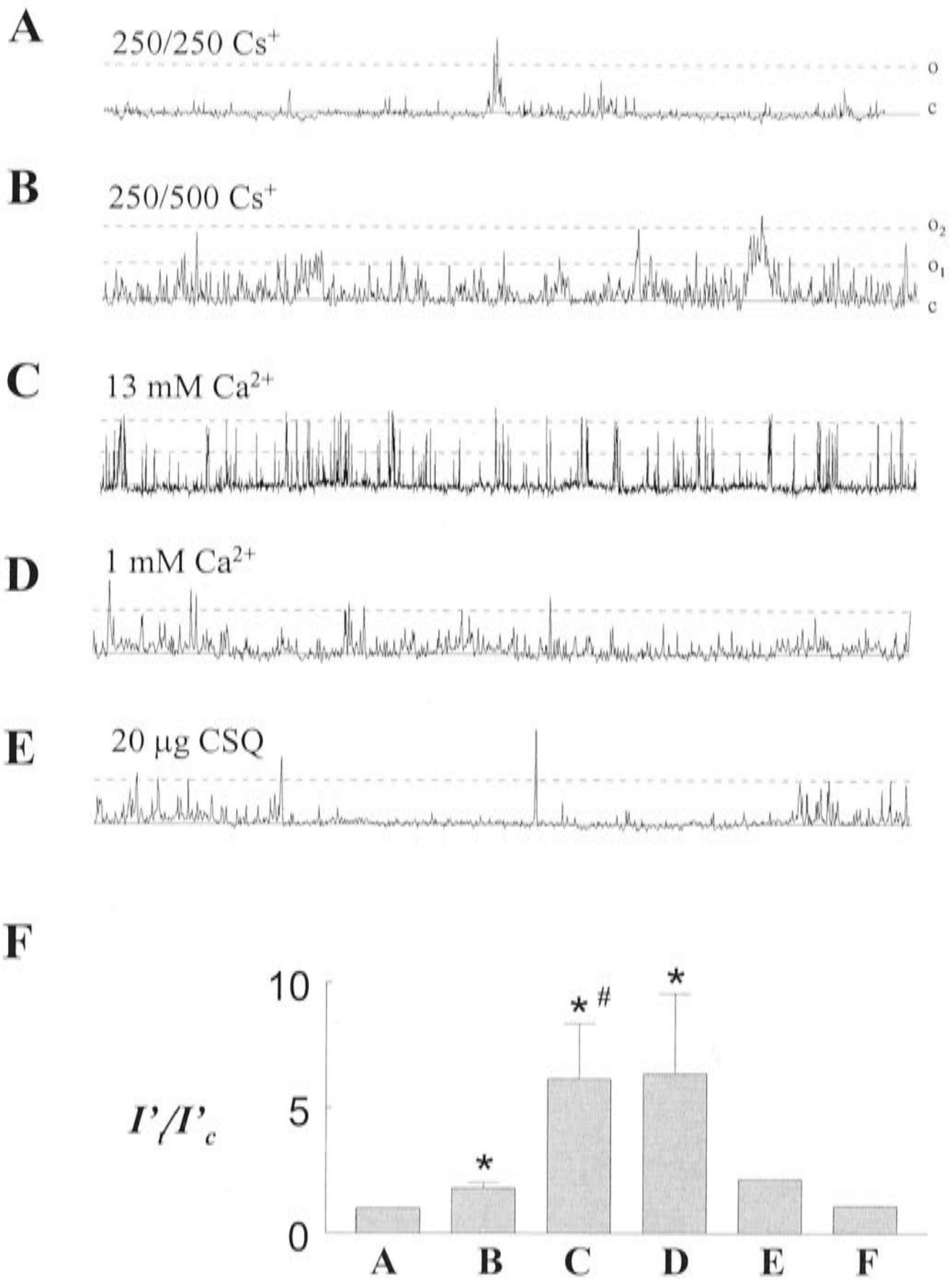


Fig. 5.V. ***Addition of 13 mM Ca$^+$ to the trans bath induced an immediate RyR activation, in the absence of CSQ.*** (**A**) Control activity with 250 mM *trans* Cs^+; (**B**) after increasing *trans* Cs^+ to 500 mM; (**C**) $[Ca^{2+}]$ increase from 1mM to 13 mM; (**D**) reducing $[Ca^{2+}]$ back to 1mM; (**E**) Addition of *trans* 20 μg CSQ; (**F**) Average data ($I'_t/I'_{c)}$ for **A-C** (n=4), **D** (n=3) and **E** (n=1), under conditions shown in **A-E,** plus after the addition of 0.5 μg/ml commercial anti-CSQ (listed as **F**). V_m-E_{Cs+} was +40mV under all conditions. Channel openings are upward from the zero current level (continuous line) to the maximum open conductance (broken line) of each of two channels (O_1 and O_2). Asterisks (*) indicate average values significantly different from control, whilst crosshatch (#) indicates significant different from the previous condition ($p<0.05$, t-test).

5.2.3 Low luminal Ca^{2+} concentrations may also irreversibly increase RyR activity.

Wang *et al.* (1998) illustrated that in the absence of Ca^{2+}, CSQ reverts to an unfolded monomeric form, which is unable to associate with anchoring proteins. CSQ has been shown to associate with junctin and triadin in a Ca^{2+}-dependent manner (Zhang *et al.* (1997), whilst Costello *et al.* (1986) used EDTA (a Ca^{2+} chelator) to isolate CSQ from a permeabilized JFM preparation. Therefore, the possibility that low or minimal Ca^{2+} could dissociate CSQ from the RyR complex in lipid bilayers was investigated.

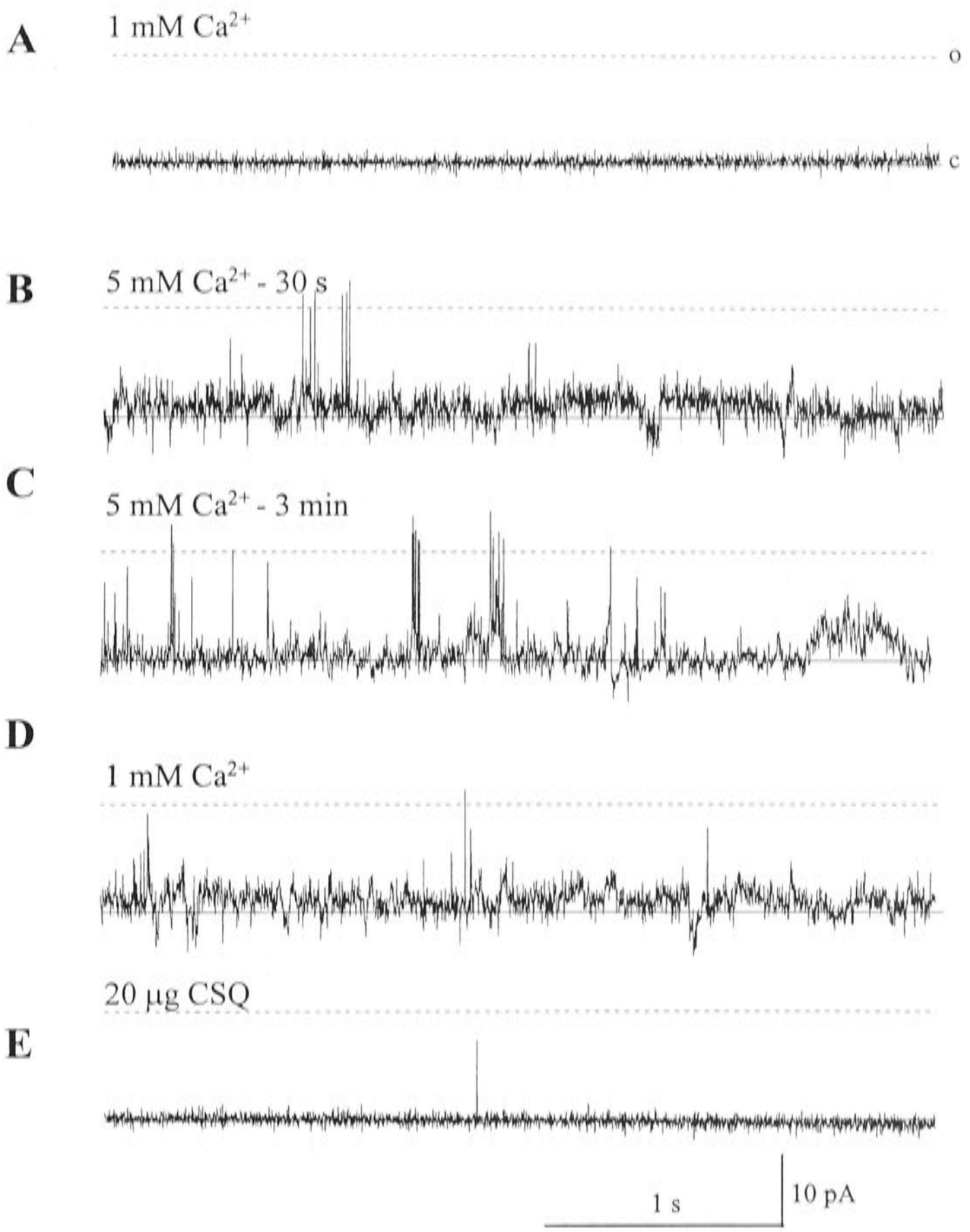
5.2.3.1 100 µM *trans* Ca^{2+}

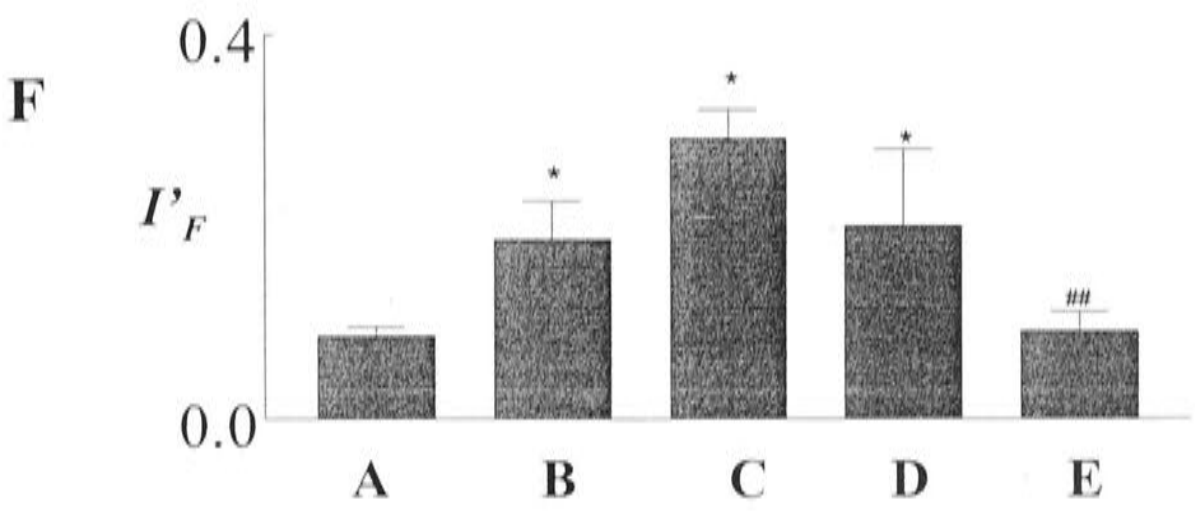
Surprisingly, addition of 900 µM BAPTA, a Ca^{2+} chelator (who's addition resulted in a free [Ca^{2+}] of 100 µM) to the *trans* chamber actually resulted in an almost immediate increase in channel activity, when compared with that recorded at 1 mM. In the two most comprehensive luminal Ca^{2+} studies, µM luminal Ca^{2+} has been shown to reduce channel activity below that observed in the presence of 1 mM luminal Ca^{2+} (Sitsapesan and Williams, 1995; Tripathy and Meissner, 1996). Channel activity increased 2.8-fold from that recorded in the presence of 1 mM *trans* Ca^{2+} (Fig. 5.VIII). No secondary increase in channel activity (which would indicate CSQ dissociation) was observed, despite frequent chamber stirring (every minute) and exposure to 100 µM Ca^{2+} for up to 10 min. Perfusion of the *trans* chamber with control solution (containing 1 mM Ca^{2+}) resulted in a complete reversal of channel activation, similar to that recorded under control conditions. This indicates that the rise in channel activity was not due to the dissociation of CSQ, or of some other protein.

5.2.3.2 100 nM *trans* Ca^{2+}

Reducing *trans* Ca^{2+} from 1 mM to 100 nM (by the addition of 4.5 mM BAPTA) resulted in an immediate 1.7-fold increase in channel I'_F (Fig. 5.IX). A further increase in channel activity (raising I'_F 2.8-fold from control) was observed after 3 mins of incubation with 100 nM Ca^{2+}. Perfusion of the *trans* chamber with control solution (1 mM Ca^{2+}) partially restored control I'_F, and like incubation with both 5 and 13 mM

Fig. 5.VI. ***Increasing trans Ca^{2+} from 1 mM to 5 mM resulted in two distinct stages of RyR activation***. **(A)** Control channel activity, with 1 mM *trans* Ca^{2+}; **(B)** 30 sec after increasing *trans* Ca^{2+} to 5 mM; **(C)** 3 mins after increasing *trans* Ca^{2+} to 5 mM; **(D)** after perfusing *trans* chamber with 1 mM Ca^{2+}; **(E)** addition of 20 μg CSQ; **(F)** average data (n=3-6) for fractional mean current (I'_F) for conditions shown in **A-E**. V_m-E_{Cs} was +40 mV. Single channel opening is upward from zero current (continuous line) to maximum open conductance (broken line). Asterisk(*) indicate average values significantly ($p<0.05$, t-test) different from control) and ## double cross hatch indicate average values significantly ($p<0.05$, sign test) different from the previous condition.


A
1 mM Ca2+
o
c
B
5 mM Ca2+ - 30 s
C
5 mM Ca2+ - 3 min
D
1 mM Ca2+
20 µg CSQ
E
1 s
10 pA


F
0.4
0.0
I'F
*
*
*
###
A
B
C
D
E

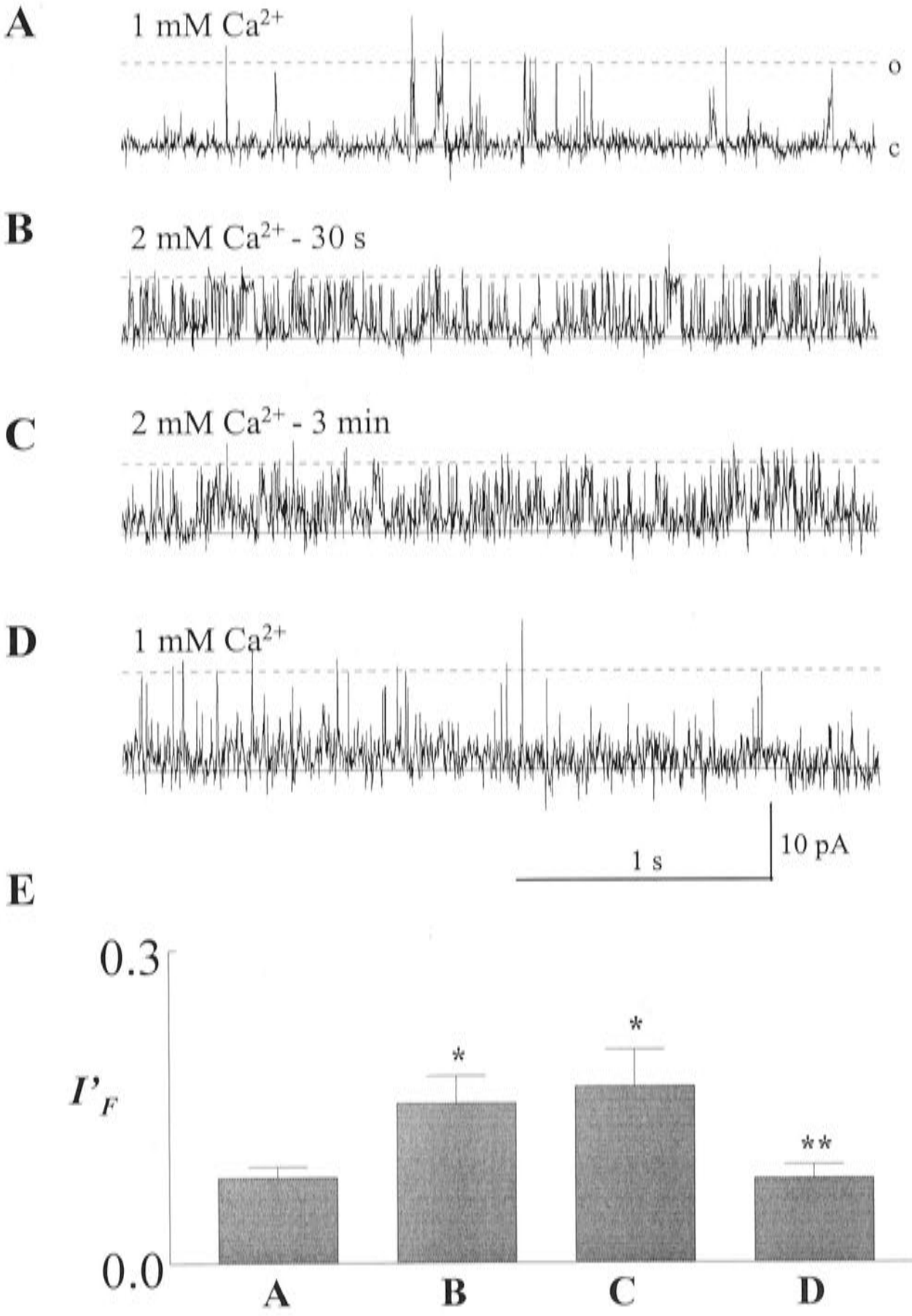


Fig. 5.VII. ***Increasing trans Ca^{2+} from 1 mM to 2 mM did not result in two distinct stages of RyR activation***. **(A)** Control channel activity, with 1 mM *trans* Ca^{2+}; **(B)** 30 sec after increasing trans Ca^{2+} to 2 mM; **(C)** 3 mins after increasing *trans* to 2 mM; **(D)** after perfusing *trans* chamber with 1 mM Ca^{2+}; **(E)** Average data (n=4-8) for I'_F for conditions shown in **A-D**. V_m-E_{Cs} was +40 mV. Single channel opening is upward from zero current (continuous line) to maximum open conductance (broken line). (*) indicate average values significantly different from control and ** indicates values different from the previous condition ($p<0.05$, t-test).

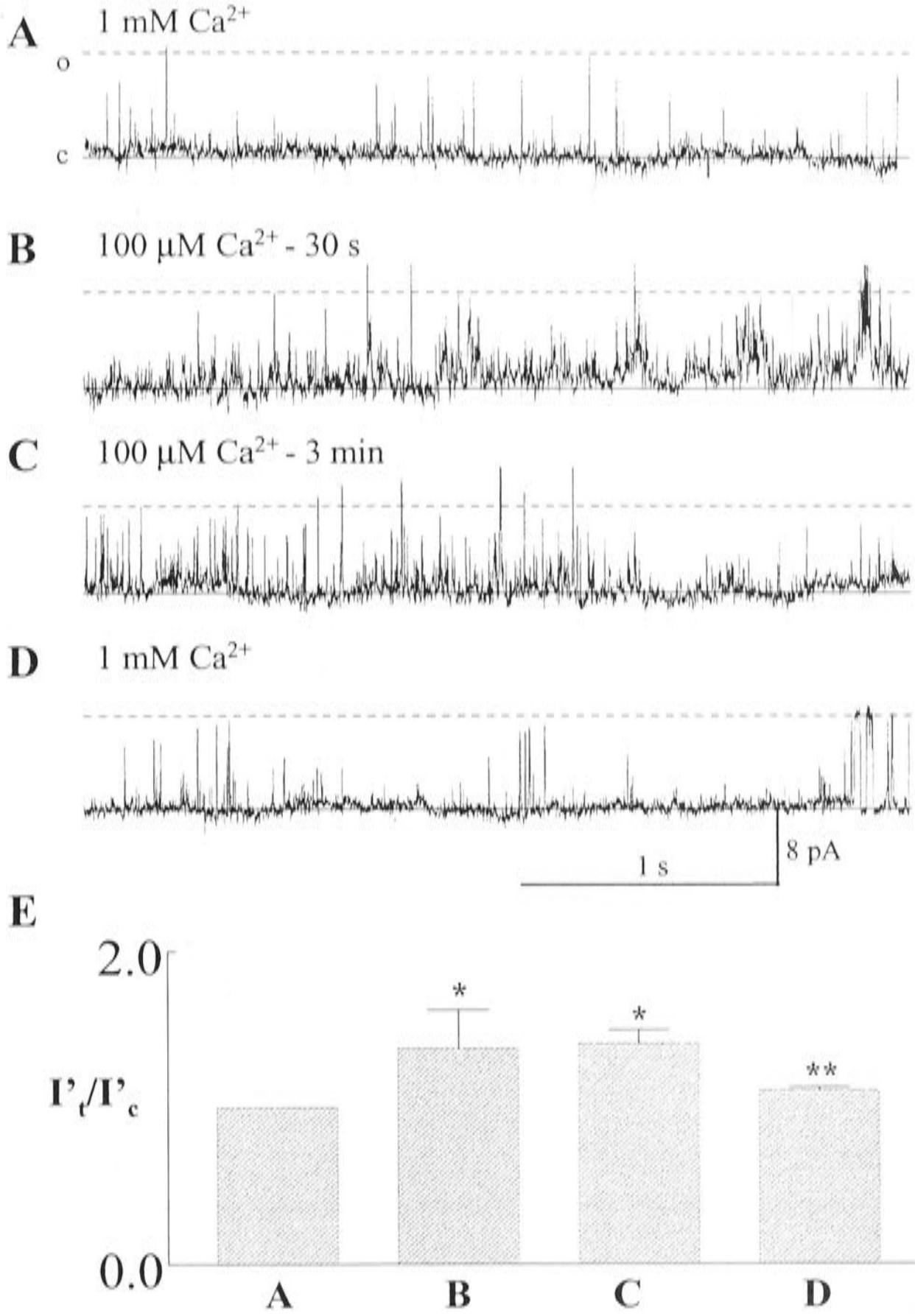
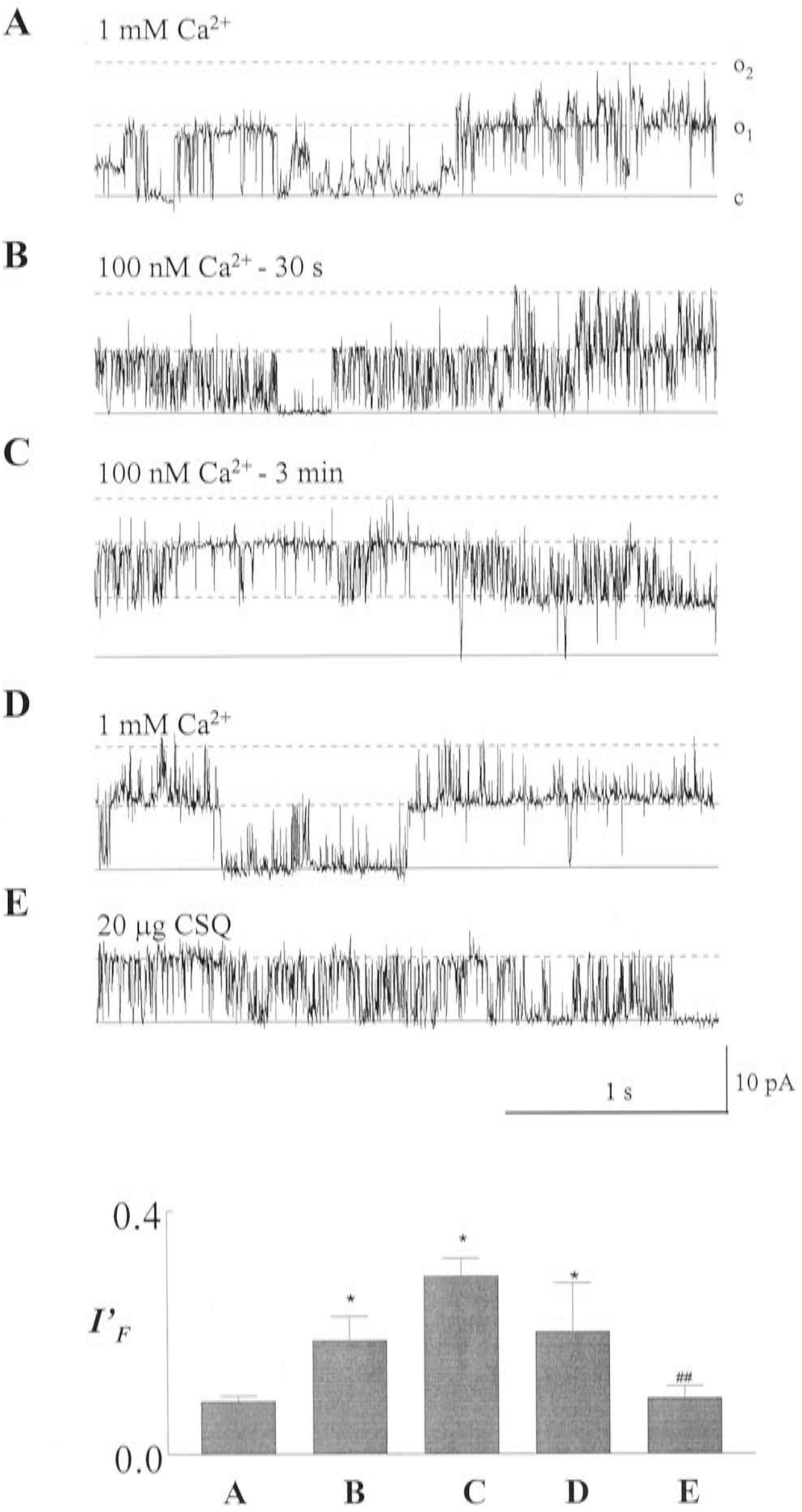


Fig. 5.VIII. ***Decreasing trans Ca^{2+} from 1 mM to 100 µM did not activate RyRs in two stages.*** **(A)** Control channel activity, with 1 mM *trans* Ca^{2+}; **(B)** 30 sec after decreasing *trans* Ca^{2+} to 100 µM by addition of 900 µM BAPTA; **(C)** 3 min after decreasing *trans* Ca^{2+} to 100 µM ; **(D)** after perfusing *trans* chamber with 1 mM Ca^{2+}; **(E)** Average data (n=3-6) for fractional mean current (I'_t/I'_c) for conditions shown in **A-D**. V_m- E_{Cs} was +40 mV. Single channel opening is upward from zero current (continuous line) to maximum open conductance (broken line). Asterisk (*) indicate average values significantly different from control and ** indicates average values different from the previous condition ($p<0.05$, t-test).

Fig. 5.IX. ***Decreasing trans Ca^{2+} from 1 mM to 100 nM resulted in activation of RyR in two distinct stages.*** V_m-E_{Cs} was +40 mV. Single channel opening is upward from zero current (continuous line) to maximum open conductance (broken line). Solutions contained: *cis*, 100 nM Ca^{2+}, 2 mM ATP and 250 mM Cs^+; *trans*, 1 mM or 100 nM Ca^{2+} and 250 mM Cs^+. **(A)** Control channel activity, with 1 mM *trans* Ca^{2+}; **(B)** 30 sec after decreasing *trans* Ca^{2+} to 100 nM; **(C)** 3 mins after decreasing *trans* Ca^{2+} to 100 nM; **(D)** after perfusing *trans* chamber with 1 mM Ca^{2+}; **(E)** Addition of 20 μg CSQ; **(F)** Average data (n=3-12) for fractional mean current (I'_F) for conditions shown in **A**-**E**. Asterisk(*) indicates average values significantly ($p<0.05$, t-test and sign test respectively) different from control and ## indicates average values significantly ($p<0.05$, sign-test) different from the previous condition.


A
1 mM Ca2+
o2
o1
c
B
100 nM Ca2+ - 30 s
C
100 nM Ca2+ - 3 min
D
1 mM Ca2+
E
20 μg CSQ
1 s
10 pA
0.4
0.0
I'F
*
*
*
##
A
B
C
D
E

Ca^{2+}, channel activity was restored upon re-association of 20 μg CSQ (Fig 5.IX). Thus, lowering luminal Ca^{2+} to 100 nM appeared to dissociate CSQ from the RyR/T/J complex.

5.2.4 Comparison of single channel parameters and dissociation times induced by 500 mM Cs^+, 13 mM and 100 nM Ca^{2+}.

The application of 13 mM *trans* Ca^{2+} caused a smaller increase in P_o due to CSQ dissociation, than did 500 mM Cs^+ (Table 5.I). The increase due solely to CSQ dissociation was calculated from the difference between activities recorded 30 s after Ca^{2+} was increased to 13 mM Ca^{2+} (which is activity due to Ca^{2+} binding to Ca^{2+} activation sites), and the secondary increase observed after 3 min exposure to high Ca^{2+} (see Fig 5.III C). Five hundred mM Cs^+ increased P_o by increasing the channel T_o 8-fold, with no alteration in either T_c or F_o. On the other hand, CSQ dissociation by high luminal Ca^{2+} induced a P_o rise by increasing the F_o of channels (Table 5.I). This difference could be due to either Cs^+ or Ca^{2+} regulating the RyR by mechanisms after CSQ-dissociation (CSQ-independent mechanisms). Alternatively, high Cs^+ dissociation might result in more CSQ removal (removing more of the inhibitory effect) than that induced by raising Ca^{2+} to 20 mM. However, this is unlikely, as the purification procedure using high ionic strength to dissociate calsequestrin from soluabilized JFM resulted in a lower yield of CSQ than the protocol using high Ca^{2+} (see Chapter 2, section 2.2).

A comparison of dissociation times for the three dissociation agents - 500 mM Cs^+, 13 mM Ca^{2+} and 100 nM Ca^{2+} - revealed that the high [Cs^+] may induce CSQ dissociation more rapidly than either high or low Ca^{2+} (Table 5.II). Dissociation time was calculated from the second of application of 500 mM Cs^+, Ca^{2+} or BAPTA, until reversible effects could be seen. Dissociation took at least 89 s with high [Cs^+], supporting the assumption that any increase in channel activity immediately after application of 5 mM, 13 mM or 100 nM Ca^{2+} was not due to CSQ dissociation.

Table 5.I *Changes in single channel parameters occurring with the three methods of CSQ dissociation, 500 mM Cs^+, 13 mM and 100 nM Ca^{2+} at +40 mV).*

	500 mM Cs^+ (Change relative to control (250 mM Cs^+))	**13 mM Ca^{2+}** (Change relative to control (1 mM Ca^{2+}))	**100 nM Ca^{2+}** (Change relative to control (1 mM Ca^{2+}))
I'_F	2.54*	3.01*	2.15*
P_o	2.81*	2.89*	na
T_o	8.54*	2.19*	na
T_c	0.86	0.07	na
F_o	1.01	2.67*	na

* Represents a significant change from control ($p \leq 0.05$). The channel parameter increase due solely to CSQ dissociation was calculated from the difference between activity recorded initially (30 s after Ca^{2+} was increased to 13 mM or decrease to100 nM Ca^{2+}), and the secondary increase in activity which occurred between 30 sec and 3 mins after exposure to high or low Ca^{2+}. Na denotes parameter not analysed.

Table 5.II *Comparison of time taken in seconds (s) to dissociate CSQ, using 500 mM Cs^+, 13 mM and 100 nMmM Ca^{2+} at +40 mV.*

	Dissociation time (s) 500 Cs^+	Dissociation time (s) 13 mM Ca^{2+}	Dissociation time (s) 100 nM Ca^{2+}
Mean	89	110	123
SEM	15	11	18
N	17	7	5

5.2.5 Effects of the presence and absence of CSQ on changes in RyR activity induced by luminal Ca^{2+} between 1-3 mM

The response of RyRs either with or without CSQ to variations in luminal [Ca^{2+}] of between 1-3 mM Ca^{2+} (encompassing the range of luminal [Ca^{2+}] over which SR Ca^{2+} release was most sensitive to luminal Ca^{2+} concentrations; Donoso *et al.*, 1995), was investigated. Channels were incorporated in the presence of 1 mM luminal Ca^{2+}, and Ca^{2+} was increased to 1.5, 2, 2.5 and 3 mM in a step-wise fashion, and activity observed at both positive and negative potentials.

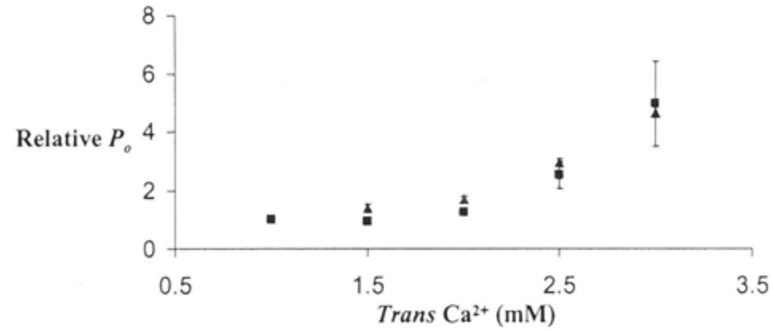
5.2.5.1 Response of RyRs with attached CSQ to small increases in luminal Ca^{2+}

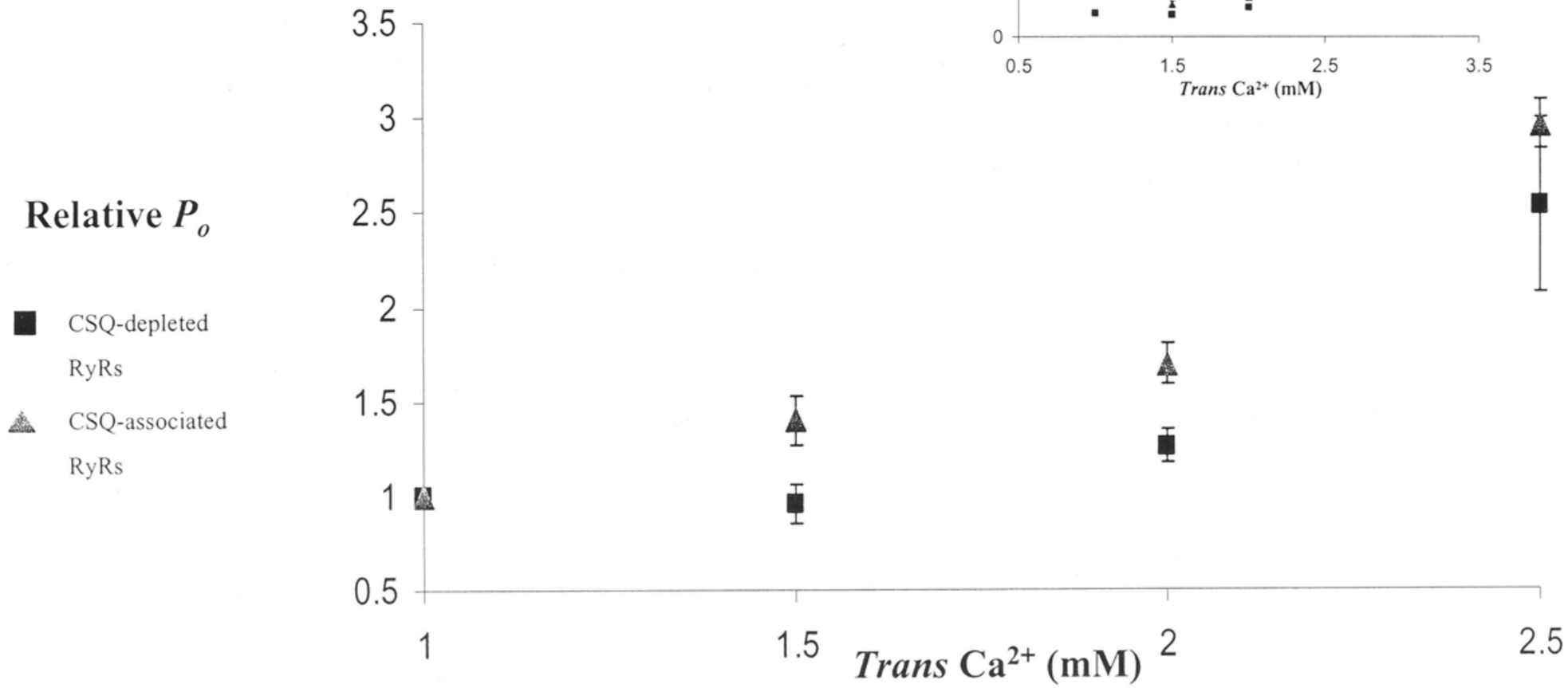
Channels that were not pre-exposed to 500 mM Cs^+ (i.e. channels with CSQ attached) showed a small increase in I'_l/I'_c upon incubation with 1.5 and 2 mM Ca^{2+} (1.2- and 1.3-fold increase respectively), compared with control levels in 1mM Ca^{2+}. Upon exposure to 2.5 and then 3 mM Ca^{2+}, channel I'_l/I'_c increased 2.9- and 4.6-fold (respectively), from control (Fig 5.X). Channels were analysed within 30 s of exposure to the increased [Ca^{2+}]. Increases in [Ca^{2+}] at this level were presumed not to dissociate CSQ (see Section 5.2.2). In fact, two representative channels that were left exposed to 3 mM *trans* Ca^{2+} for up to 8 min, did not show a secondary increase in channel activity, suggesting that 3 mM luminal Ca^{2+} could not dissociate CSQ.

5.2.5.2 Response of CSQ-depleted RyRs to small increases in luminal Ca^{2+}

To dissociate CSQ, RyRs were exposed to 500 mM Cs^+ until an increase in channel activity was observed, indicating CSQ dissociation (see Chapter 3, Section 3.2.4). Once the increase in activity occurred and remained stable (for 30s), the *trans* chamber was perfused with control solution (containing 250 mM Cs^+). As seen previously, no alteration in channel activity was detected after perfusion.

Fig 5.X. ***Response of RyRs to 1-3 mM Ca^{2+} in the absence and presence of CSQ.*** Experimental conditions: *cis* (mM) 250 Cs^{+}, 2 ATP (activating) and 1 nmol Ca^{2+}(sub-activating); *trans* (mM) 250 Cs^{+}, 1-3 Ca^{2+}. *Trans* $Ca^{2+}$was altered by aliquot additions of 200 mM stock Ca^{2+}(Ca^{2+} concentration was determined by a calcium electrode). CSQ was dissociated from RyRs prior to increasing [Ca^{2+}] by exposure to 500 mM Cs^{+}. Each data point is the mean open probability (P_o) relative to control (1 mM Ca^{2+}), error bars are SE for n=7-8 in the absence and presence of CSQ. CSQ was dissociated from the RyR/T/J complex by pre-treatment with 500 mM Cs^{+}. The inset shows relative Po in response to a wider range of *trans* Ca^{2+} (1.0 – 3.5 mM) than the main figure, in the same conditions as listed above.


8
6
4
2
0
Relative P_o
0.5
1.5
2.5
3.5
Trans Ca^{2+} (mM)


Relative P_o
CSQ-depleted RyRs
CSQ-associated RyRs
3.5
3
2.5
2
1.5
1
0.5
1
1.5
2
2.5
Trans Ca^{2+} (mM)

The activity of CSQ-depleted channels increased upon exposure to increasing luminal Ca^{2+} from 1-3 mM (Fig. 5.X). At 2.5 and 3 mM luminal Ca^{2+}, the removal of CSQ did not significantly alter the RyRs response to Ca^{2+} activation. At 2.5 mM Ca^{2+}, RyRs without CSQ increased 1.2-fold from control (compared with a 1.3-fold rise in CSQ associated channels), whilst at 3 mM, a 4.1-fold rise in channel current was recorded (CSQ associated RyRs: 4.9-fold). The presence of CSQ tended to enhance the activation of RyRs by the two lower Ca^{2+} concentrations. CSQ-depleted RyRs tended to be less responsive to alterations in luminal Ca^{2+}; at 1.5 and 2 mM Ca^{2+}, RyR activity rose 1.2- and 1.3-fold from control (compared with an almost 1.5-fold increase at 1.5 mM and a 1.7-fold rise at 2 mM, from control activity recorded in CSQ associated RyRs). Whilst, neither of these increases are significant (P= 0.092 at 1.5 mM and 0.07 at 2 mM), it appears that a conformational change induced by Ca^{2+} exposure might influence the way in which CSQ may regulates the RyR .

5.3 Discussion

5.3.1 Regulation of RyRs by high luminal [Ca^{2+}]

The level of Ca^{2+} loading of the SR is important in regulating Ca^{2+} release. Increasing or decreasing SR Ca^{2+} load, increases or decreases (respectively) the sensitivity of Ca^{2+} release to depolarisation (Bassani *et al.*, 1995; Lamb *et al.*, 2001a), cytoplasmic Ca^{2+} (Copello *et al.*, 1997), caffeine (Lamb *et al.*, 2001a), ATP (Donoso *et al.*, 1995) and polylysine (Saiki and Ikemoto, 1997). Moreover, Lamb *et al.* (2001a) found that different endogenous levels of SR Ca^{2+} loading in fast (EDL) and slow twitch (soleus) muscle was the primary factor determining the difference in caffeine sensitivities of the two fibre types. However, it has not been clear how the Ca^{2+} load in the SR alters Ca^{2+} release.

These effects of SR Ca^{2+} load on Ca^{2+} release are also reflected in single RyR channel studies. Increasing luminal Ca^{2+} increases channel activity in both purified (Tripathy and Meissner, 1996) and native RyRs (Sitsapesan and Williams, 1995). This increase in activity has been attributed to Ca^{2+} flowing from the luminal solution, through the channel, and binding to Ca^{2+} activation sites on the cytoplasmic side of the RyR (Tripathy and Meissner, 1996). An alternative hypothesis is that luminal Ca^{2+} is sensed by regulatory sites on the luminal side of the RyR (Sitsapesan and Williams, 1995; Ching *et al.*, 2000). Since these bilayer studies were performed under conditions where CSQ (and possibly triadin and junctin) were not likely to have been present, regulation by CSQ bound to triadin/junctin can be discounted. Triadin and junctin were not present when RyRs were purified using CHAPS solubilization (Tripathy and Meissner, 1996). It was also likely that CSQ was not present when native RyR were incorporated into bilayers in the absence of luminal Ca^{2+} (Sitsapesan and Williams, 1995; Ching *et al.*, 2000).

It has been proposed in other studies that CSQ is involved in RyR regulation by luminal Ca^{2+} (see review by Donoso *et al.*, 1995). SR Ca^{2+} release is most sensitive to luminal Ca^{2+} over the same range of Ca^{2+} (0-2 mM) that induces substantial, reversible conformational changes in CSQ (Ikemoto *et al.*, 1989b; He *et al.*, 1993). Evidence is

presented, which suggests that high levels of luminal [Ca^{2+}] can activate RyRs by at least two mechanisms; one that is independent of CSQ, and another that involves CSQ dissociation from the RyR/T/J complex. Raising Ca^{2+} from 1 mM to 13 mM dissociates CSQ from the JFM (Figs. 5.III-V), inducing a 2.4–fold rise in RyR activity, which could be fully reversed only by replacing the CSQ (Fig. 5.V). Wang *et al.* (2001) also found that 10 mM Ca^{2+} induces a 2.1-fold increase in P_o of cardiac RyRs in bilayers. Both studies are consistent with previous findings that high luminal [Ca^{2+}] disrupts the charge-charge interactions binding triadin and junctin to CSQ (Mitchell *et al.*, 1988; Zhang *et al.*, 1997). Presumably the CSQ-independent mechanism is due to a direct activation of the RyR by luminal Ca^{2+} seen in single channel studies in the absence of CSQ (see above).

5.3.2 Regulation of RyRs at low luminal Ca^{2+}

Both Tripathy and Meissner (1996) and Sitsapesan and Williams (1995) report that RyRs respond to luminal [Ca^{2+}] increases over the range of nM to mM by increasing channel P_o. The most comparable investigation (in terms of the RyR preparation used, muscle type and standard *cis* conditions) to that reported here (see Fig. 5.II), is that of Sitsapesan and Williams (1995), who report an increase in channel P_o when increasing luminal Ca^{2+} from 5 μM to 2 mM. For example, Sitsapesan and Williams (1995) show that in ATP activated channels (in the presence of sub-activating *cis* Ca^{2+}), P_o was reduced by 100 μM *trans* Ca^{2+} when compared with activity recorded at 1 mM (*trans*), whereas Fig. 5.XI illustrates an increase in channel activity upon lowering luminal Ca^{2+} to this concentration. In both this study and that of Sitsapesan and Williams (1995) ATP was the only cytoplasmic activating ligand, native skeletal RyRs were used and Cs^{+} was the current carrier. The two differences between the studies are that Sitsapesan and Williams (1995) incorporated the RyR in relatively low luminal Ca^{2+} (5 μM), and the RyRs were isolated from sheep, not rabbit. It is possible that 5 μM *trans* Ca^{2+} may induce CSQ dissociation or a conformational change in CSQ. Thus, the effect of luminal Ca^{2+} on CSQ and RyR activity might be quite different after exposure to 5 μM *trans* Ca^{2+}. Such vastly different RyR regulation by any ligand has not previously been reported between species and is not likely to be responsible for the differences between the studies.

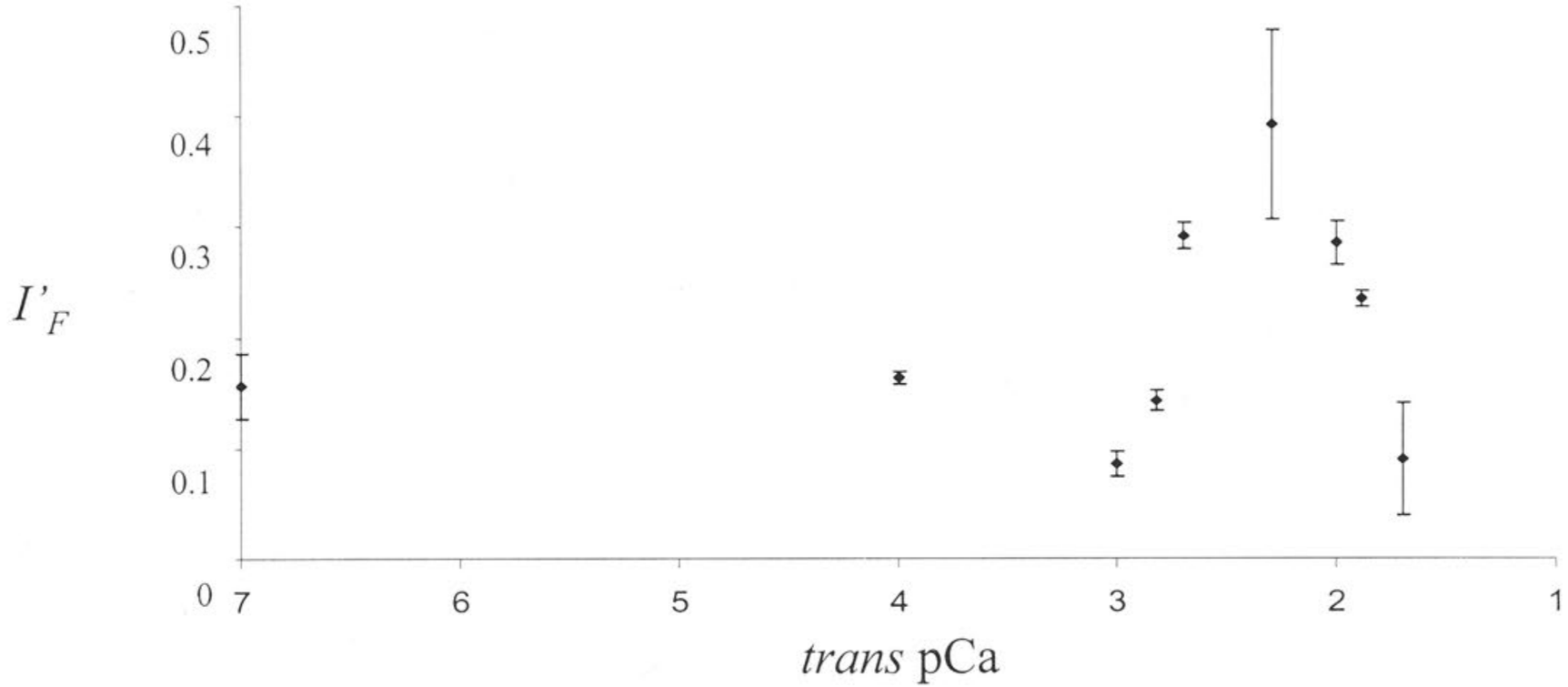


Fig 5.XI. ***The response of native RyRs to increasing trans Ca^{2+} from pCa 7 (100 nM) to pCa 1.7 (20 mM).*** Experimental conditions: *cis* (mM) 250 Cs^+, 2 ATP (activating) and 1 nmol Ca^{2+}(sub-activating); *trans* 250 mM Cs^+, 100 nM-20 mM Ca^{2+}. *Trans* $Ca^{2+}$was altered by aliquot additions of 200 mM stock Ca^{2+}(Ca^{2+} concentration was determined by a calcium electrode) and 100 mM stock BAPTA. Data points are means, error bars are SEM; n=3-16. Data points show the average results of analysis of fractional mean current (I'_F) 1 min after addition of Ca^{2+}.

In fact, no other study of the effects luminal Ca^{2+} reports both lowering and raising luminal Ca^{2+} from 1 mM results in an increase in channel activity. Whilst both Herrmann-Frank and Lehmann-Horn (1996) and Fill *et al.* (1990) report maximal activation occurring at μM Ca^{2+} levels, and a reduction upon increasing luminal Ca^{2+} to 1 mM, both report channel P_o decreasing as $[Ca^{2+}]$ is increased from μM to mM. These results (see Fig 5.XI for dose response curve) warrant further investigation. Herrmann-Frank and Lehmann-Horn (1996) incorporate vesicles in the presence of 50 μM *trans* Ca^{2+}, whilst Fill *et al.* (1990) use 10 μM. Is it possible that at such a low Ca^{2+}, CSQ conformation is altered, CSQ is dissociated, or that other RyR regulatory proteins are compromised in such a way that the response to luminal $[Ca^{2+}]$ is altered, when compared to the response shown when CSQ is attached, or in a physiological conformation?

Potentially, regulation by luminal Ca^{2+} might be due to the effects of Ca^{2+} on another co-protein, such as triadin (Ching *et al.*, 2000). Another possibility is that the RyRs response to CSQ may be altered at different $[Ca^{2+}]$, like calmodulin which modifies the RyR in a *cis* Ca^{2+}-dependent manner (Tripathy *et al.*, 1995). The CSQ status of the RyRs used was not determined in any of the previous luminal Ca^{2+} investigations. CSQ association with the RyR may explain the increases in RyR activity with decreasing *trans* Ca^{2+} shown in Figs. 5.VIII-IX.

Another possibility is that BAPTA affects the RyR or an associated luminal protein directly, resulting in RyR activation when Ca^{2+} was reduced below 1 mM . Costello *et al.* (1986) show that a 97 kDa protein (presumably triadin) is cleaved from solubilized JFM by EDTA (another weaker Ca^{2+}chelator). Since triadin has been shown to inhibit RyRs (Ohkura *et al.*, 1998), its modification or removal may result in enhanced RyR activity.

It is important to note that although the effect of luminal Ca^{2+} at $[Ca^{2+}]$ lower than physiological levels are contradictory, both low and high luminal Ca^{2+} are able to dissociate CSQ from the RyR/T/J complex, and the dissociation further illustrates CSQs role as a RyR inhibitor.

5.3.3 High Cs^+ vs high or low Ca^{2+} as a CSQ dissociation agent

Mechanistically, the differences in CSQ dissociation time between high Cs^+ and high Ca^{2+} exposure (see Section 5.2.4) might be explained by the way in which each agent removes CSQ from the RyR/T/J complex. High ionic strength disrupts CSQs ionic interaction with triadin and junctin, and hence the RyR. High and low Ca^{2+} have been shown to disrupt CSQs polymer formation and monomeric folding (He *et al.*, 1993; Wang *et al.*, 1998). Whether or not the removal of CSQ from the RyR/T/J complex requires both the disruption of the polymer complex and reversion to a random coil structure, is not known. None-the-less, the formation of a CSQ polymer is a two-step process, involving the formation of a front-to-front dimer, followed by a back-to-back interaction. It seems possible that the removal of CSQ from the RyR/T/J complex by high and low Ca^{2+} may be more time consuming, simply due to the fact that depolymerisation may have to occur prior to CSQ dissociation.

It is interesting to note that both high Ca^{2+} and high Cs^+ induced an increase in RyR P_o in very different ways, Ca^{2+} which increased F_o, while Cs^+, which increased T_o. Channel activity in both sets of experiments was returned to control levels upon re-association of CSQ, indicating that neither of these dissociation agents compromised the CSQ binding site on triadin, junctin or the RyR. It is quite possible that high Ca^{2+}, high Cs^+ or both of these agents dissociate luminal RyR regulating proteins in addition to CSQ. A possible contender would be the 30 kDa protein (refer to Chapter 3, Section 3.3.2.3 for further information of the possible role of this protein in RyR regulation and Yamaguchi *et al.*, 1999). A protein resolving at 30 kDa on SDS Page gel appeared after purification of CSQ using high ionic strength, but not after Ca^{2+}-induced dissociation. It might be that the 30 kDa protein was removed along with CSQ in the lipid bilayer experiments, when exposed to 500 mM *trans* Cs^+. If the removal of the 30 kDa protein explains the different RyR gating with high Cs^+-induced CSQ dissociation, removal of the 30 kDa protein may be increasing T_o but not further increasing P_o (although this is unlikely, unless it were due to a compensatory mechanism, where one would expect a drop in F_o or an increase in T_c, which was not observed). Although re-association of exogenous CSQ with the 30 kDa protein restored control channel activity in CSQ dissociation experiments (see Chapter 3, Fig. 3.XII), the effects of the absence and presence of this protein on channel T_o were not investigated (due to the presence of mainly

multi-channel bilayers in this set of experiments). It is also possible that the Ca^{2+} binding proteins sarcolumenin and the histadine rich Ca^{2+} binding protein also undergo some kind of dissociation, or change in structure in response to the conditions used to dissociate CSQ, and that these changes alter the way they regulate the RyR. Both these proteins are proposed to regulate the RyR, and any Ca^{2+}-induced effects on these proteins may alter RyR gating.

5.3.4 CSQs role in regulating RyRs at 1-3 mM luminal Ca^{2+}

Ikemoto *et al.* (1972) and Ostwald *et al.* (1974) reported changes in CSQ conformation occurring over the range of Ca^{2+} concentrations tested here, which have been postulated to be responsible for an increase in SR Ca^{2+} release rate constants observed in the range of 1-3 mM Ca^{2+} (Donoso *et al.*, 1995).

No significant difference in luminal [Ca^{2+}] regulation of RyR activity was observed at 2.5 or 3 mM Ca^{2+}, whether or not CSQ was associated with the RyR. It should be noted however, that at 1.5 and 2 mM Ca^{2+}, RyR activity had a tendency to be higher in RyRs with attached CSQ. These results warrant further exploration. It is possible that changes in CSQ conformation, induced by altering luminal Ca^{2+} from 1-3 mM (as suggested by Ikemoto *et al.,* 1989 and He *et al.,* 1993) play a role in RyR regulation. Another possibility is that direct binding of CSQ to the RyR communicates luminal Ca^{2+} to the RyR. This would rely on a direct CSQ-RyR interaction *in vivo*, which has yet to be established. Given that triadin binds to both the RyR and CSQ, and regulates the RyR (Zhang *et al.*, 1997; Ohkura *et al.*, 1998), it might be that CSQ and triadin form part of a luminal Ca^{2+} signaling pathway. Indeed it has been suggested that triadin might be involved in mediating activation of RyRs by luminal Ca^{2+} (Ching *et al.*, 2000).

A detailed study, comparing RyR regulation by luminal Ca^{2+} (1-3 mM) in both native and purified RyRs would help ascertain the role that CSQ, and in fact all other luminal RyR regulating proteins, play in communication of small (and probably physiological) Ca^{2+} fluxes into the cytoplasm, and further our understanding of the complex role that luminal proteins play in both Ca^{2+} signaling and RyR regulation.

5.3.5 Physiological Implications

Taken together, these results show that luminal Ca^{2+} regulates the RyR via two mechanisms. Firstly, Ca^{2+} can bind to activation sites, found on the RyR or an associated protein, inducing channel activation (when luminal Ca^{2+} is raised from 1 – 13 mM, or reduced to ≤100 μM). Secondly, changes in luminal Ca^{2+} concentrations can dissociate CSQ, inducing a significant rise in RyR activity. Whether or not luminal Ca^{2+} reaches levels that might induce CSQ dissociation *in vivo* remains to be determined, although Fryer and Stephenson (1996) have reported that in the case of severe fatigue, Ca^{2+} forms a Ca-P precipitate, thereby lowering free Ca^{2+} below 1 mM. This may result in some CSQ dissociation and altered RyR regulation. It may also be that local changes in $[Ca^{2+}]$, close to CSQ, may be sufficient to induce CSQ dissociation. However, this seems unlikely as there is thought to be free Ca^{2+} diffusion thoughout the terminal cisternae.

The role of CSQ in conveying luminal Ca^{2+} concentrations to the RyR *in vivo* is not clear. Luminal Ca^{2+} might regulate RyRs via dissociation of CSQ. Although, it is not likely that the dissociation of CSQ from the JFM would be caused by normal physiological changes in luminal $[Ca^{2+}]$, and may not be an important mechanism *in vivo*. However, levels of luminal Ca^{2+} of ~10 mM are obtained experimentally when loading the SR to above normal levels (Lamb *et al.*, 2001a), and such loading leads to enhanced Ca^{2+} release. Whether this increase occurs due to CSQ dissociation from the JFM in the SR or another mechanism (or a combination of both), remains to be investigated. Another possibility is that CSQ is an important co-protein that modifies RyR function in response to changes in luminal Ca^{2+} in a similar manner to calmodulin and its association with cytoplasmic Ca^{2+} (Tripathy and Meissner, 1996). Like calmodulin in the cytoplasm, the effect of CSQ on RyR function may depend on the Ca^{2+} binding status of CSQ (and CSQ conformation), which could vary with changes in luminal Ca^{2+}. It is also probable that smaller changes in $[Ca^{2+}]$ might alter CSQ conformation and cause subtle changes in RyR activity. Finally, given that triadin binds to both CSQ and to the cytoplasmic side of the RyRs (Groh *et al.*, 1999), it is possible that CSQ and triadin could form a signalling pathway that communicates the luminal Ca^{2+} concentration to the cytoplasmic side of the RyR.

GENERAL CONCLUSIONS

6.1 Introduction

This study describes the effects that the Ca^{2+} binding protein CSQ has on RyR activity in skeletal muscle. Up until now, little has been know about the role of CSQ in regulating Ca^{2+} release through the RyR, with conflicting reports suggesting CSQ as both an activator (Kawasaki and Kasai, 1994; Ohkura *et al.*, 1995; Ohkura *et al.*, 1998; Szegedi *et al.*, 1999; Herzog *et al.*, 2000 and an inhibitor (Beard *et al.*, 1999a; Beard *et al.*, 2000a; Wang *et al.*, 2001; Beard *et al.*, 2002) of channel activity.

The novel findings of this report were as follows:

1) Exposing the luminal side of the SR to high ionic strength (500 mM Cs^+), resulted in an increase in channel activity, reversible by addition of exogenous CSQ.

2) Application of anti-CSQ antibody to the *trans* chamber also resulted in significant RyR inhibition, presumably by inducing a conformational shift in CSQ, which in turn affected the way in which CSQ regulates the RyR.

3) Two populations of RyRs exist, those with 'high' control activity and those with 'low' control activity. The RyR population with high control activity did not respond to either 500 mM Cs^+ exposure or to *trans* addition of anti-CSQ antibody, suggesting that this population lacked anchored CSQ, a hypothesis first suggested by Yamaguchi *et al.*, 1997.

4) Native RyRs respond in a different manner to CSQ than purified RyRs. Addition of *trans* CSQ had a tendency to activate purified RyRs, suggesting that CSQ regulates RyRs in different ways; a direct CSQ-RyR binding inducing RyR activation, whilst the presence of known anchoring proteins triadin and junctin allowed CSQ to inhibit RyR activity.

5) In native RyRs, the phosphorylation status of CSQ does not influence CSQ's ability to inhibit RyRs. Both phosphorylated and dephosphorylated CSQ induces a significant drop in RyR activity. This is in contrast to the regulation shown by CSQ in purified RyRs, where only the addition of dephosphorylated CSQ could induce significant change in channel activity, an activation of RyRs. Once again, these data illustrate the different regulation imposed by CSQ on the RyR when anchoring proteins triadin and junctin are present.

6) Both high ($\geq$5 mM) and low ($\leq$100 nM) luminal Ca^{2+} can induce CSQ dissociation from the RyR/triadin/junctin complex. Altering luminal Ca^{2+} to these levels resulted in activation via two independent mechanisms. Firstly, an immediate rise in channel activity was seen immediately, due to Ca^{2+} activation of the RyR and was only reversible by replacing control luminal [Ca^{2+}] (1 mM). A secondary phase of channel activity increase was also observed, due to CSQ dissociation, occurring approximately 3 min after the alteration in luminal [Ca^{2+}]. This increase in activity could only be reversed to control levels upon association of exogenous CSQ.

6.2 RyR/T/J/CSQ complex

6.2.1 The role of triadin and junctin

Strong evidence suggests that CSQ is an inhibitor of native RyR activity, with three methods of CSQ dissociation (exposure to high ionic strength, and high and low Ca^{2+}) all resulting in a significant activation of the channel, which was completely reversible by CSQ re-association. It is apparent that luminal proteins are required for CSQ to inhibit RyRs, because when RyRs are purified, CSQ strongly activates the channel Szegedi *et al.*, 1999; Herzog *et al.*, 2000. Only two proteins residing in the SR lumen (triadin and junctin) have been shown to bind both to CSQ and the RyR, although it is possible that other luminal proteins (SLM, HRC, 30 kDa) might be responsible for communicating CSQ-imposed RyR regulation (although no direct interaction with the ryanodine receptor or CSQ has been shown). Whilst triadin has been shown to inhibit RyRs, no effects of junctin on RyR activity have been reported thus far. Although a similar protein to triadin – in terms of number of transmembrane segments, it's role in the RyR/T/J/CSQ complex and it's highly charged luminal domain – in terms of interacting with CSQ, junctin is a little more complex. Unlike the discrete CSQ binding domain, consisting of a KEKE motif, (Arg^{210}-Lys^{224}) found on triadin, seemingly more than one site on junctin is required for interaction with CSQ. Kobayashi *et al.* (2000) showed that deletion of any one of three luminal KEKE motifs resulted in inhibition of the junctin/CSQ interaction. This suggests that there is not a single binding site on junctin for CSQ, that multiple interaction sites exist and that all these sites need to interact for the CSQ/junctin interaction to be stable. Certainly, to fully understand the complex arrangement and regulation imposed by the RyR/T/J/CSQ complex, it is essential to discover if and how junctin influences RyR behaviour. It would be of great interest to study which of these KEKE motifs (or other sites on junctin) are required for stability of the protein, and which may be involved in a functional regulation of the RyR or CSQ.

6.2.2 Direct CSQ-RyR interaction

To help to understand more about the specific interactions between CSQ and the RyR, specific binding sites on CSQ for the RyR, and the RyR and CSQ need to be identified. Herzog *et al* (2000) looked at the binding of CSQ to two segments of the RyR, the M1/2 and M3/4 segments, which correspond to residues 4580-4640 and 4859-4917. Neither of these two loops interacted with CSQ. The number and location of the RyR luminal loops are still controversial - Zorzato *et al.*, (1990) proposed twelve luminal loops, while Takeshima *et al* (1989) proposed four luminal loops. Therefore the amino acid residues thought to correspond to the M1/2 or M3/4 regions may not in fact be on the luminal side of the protein. This aside, identification of a definitive site(s) on the RyR for CSQ association would provide more credence to the proposal of a direct CSQ-RyR interaction. It is also interesting to ponder a physiological basis for CSQ associating with the RyR both directly, and via triadin and junctin. As previously mentioned, these interactions result in activation (direct binding) and inactivation (binding via triadin and junctin) of the RyR. This study has shown in native SR, that re-associating CSQ with the CSQ-deplete RyR/T/J complex resulted in RyR inhibition. Either, the direct CSQ-RyR interaction occurs *in vivo*, but the resultant channel activation is masked by a stronger CSQ-induced inhibition of the RyR/T/J complex. Or, *in vivo*, a direct interaction does not occur.

6.3 RyR regulation by luminal Ca^{2+} binding proteins SLM and HRC?

Making the RyR/T/J/CSQ complex much more intriguing, is the existence of Ca^{2+} binding proteins, the HRC and SLM. Knowing that these are believed to modulate the RyR (see Chapter 1, section 1.7.2), the question arises; does SLM/HRC exert a RyR regulatory effects through its binding to CSQ, which in turn can exerts its effects via a signalling pathway through triadin and junctin (see preliminary reports Beard *et al.*, 1999; Beard *et al.*, 2000; Wang *et al.*, 2001) or on CSQ bound directly to the RyR Szegedi *et al.*, 1999; Herzog *et al.*, 2000? Physiologically, what is the effect of this seemingly complex interplay of several modulatory mechanisms; is an inhibition induced by SLM/HRC overshadowed by CSQ modulation Kawasaki and Kasai, 1994;

Meissner (1996), Gyorke and Gyorke (1998) and Ching *et al.* (2000) also activated the channels with cytoplasmic ATP. It should be noted that Tripathy and Meissner (1996) have shown voltage-dependant activation of RyRs by luminal Ca^{2+} in the absence of ATP. The observed RyR activity increase is consistent with an increased Ca^{2+} release rate from SR observed by Ikemoto *et al.* (1989) and Donoso *et al.* (1995).

6.4 Anti-CSQ

How the polyclonal anti-CSQ antibody specifically regulates the RyR simply by binding with CSQ is still unclear. How CSQs structure is altered by the antibody could quite easily be determined using nuclear magnetic resonance, comparing structural alteration of CSQ in both the presence and absence of the antibody. It would be of interest to determine whether or not anti-CSQ was inducing total channel inhibition by binding to CSQ and thus inducing a conformational shift that either exposes a site that may bind to the RyR (and induce inhibition). Alternatively such a conformational change might alter the way in which CSQ binds to other proteins (such as triadin and junctin) and alter the way these proteins interact and regulate the RyR. Such structural data would uncover important details such as how conformational changes in CSQ may alter RyR activity and possibly give information on changes in the protein conformation upon binding ligands, such as Ca^{2+}.

6.5 Luminal Ca^{2+}

In terms of RyR regulation, the effects of luminal Ca^{2+} still remain controversial. Fill *et al.* (1990; using native RyRs) and Ma *et al.* (1988) and Szegedi *et al.* (1999) - using purified RyRs - showed that increasing luminal Ca^{2+} decreased RyR activity. In contrast, Tripathy and Meissner (1996; using purified RyRs, with ATP to activate) and Herrmann-Frank and Lehmann-Horn (1996; using purified RyRs), reported a biphasic response to increasing luminal [Ca^{2+}], with Tripathy and Meissner (1996) reporting maximal activation occurring at $\geq$ 5 mM Ca^{2+} (at +40 mV, or 0.25 mM at – 40 mV). Sitsapesan and Williams (1995; using native RyRs, with ATP to activate), showed an increase in channel open probability (P_o) as luminal Ca^{2+} was increased, provided cytoplasmic ATP and Ca^{2+} already regulated the channels. Similarly, Györke and Györke (1998) and Ching *et al.* (2000) report a Ca^{2+}-dependence of channel activation in cardiac RyRs. All researchers, except Tripathy and Meissner (1996) use cytoplasmic Ca^{2+} at activating levels. In addition, Sitsapesan and Williams (1995), Tripathy and

Meissner (1996), Györke and Györke (1998) and Ching *et al.* (2000) also activated the channels with cytoplasmic ATP. It should be noted that Tripathy and Meissner (1996) have shown voltage-dependant activation of RyRs by luminal Ca^{2+} in the absence of ATP. The observed RyR activity increase is consistent with an increased Ca^{2+} release rate from SR observed by Ikemoto *et al.* (1989) and Donoso *et al.* (1995).

Increasing luminal Ca^{2+} (from pM to mM) has been reported to activate RyRs Sitsapesan and Williams, 1995; Herrmann-Frank and Lehmann-Horn, 1996; Tripathy and Meissner, 1996; Györke and Györke, 1998; Ching *et al.*, 2000. It appears that probably only one of these studies would have had RyRs with CSQ attached (Györke and Györke, 1998), due to the use of either purified RyRs or exposure of CSQ to <nM Ca^{2+}, which is thought to inhibit CSQ folding, polymerisation and interactions with the RyR complex (Wang *et al.*, 1998 and Chapter 5). Tripathy and Meissner (1996) and Herrmann-Frank and Lehmann-Horn (1996) show RyR activation by luminal Ca^{2+} in purified channels (no CSQ), whilst Ikemoto *et al.* (1989) and Donoso *et al.* (1995) report an enhanced release rate from SR vesicles containing CSQ upon increasing luminal Ca^{2+}. The differences observed between luminal Ca^{2+}-dependence of RyR activity and SR Ca^{2+} release cannot be attributed entirely to the presence or absence of CSQ. Only a detailed study which takes account of the differences between the methods used in the different studies (i.e. presence or absence of ATP, co-proteins and cytoplasmic Ca^{2+}), will be able to resolve the exact effects of luminal Ca^{2+} concentration on RyR activity.

6.6 Physiological relevance of CSQ mutation or altered expression

To date, two physiological conditions have reported alternations in CSQ expression or a CSQ mutation. Firstly, catecholamine-induced polymorphic ventricular tachycardia is a disease that can deteriorate to ventricular fibrillation and induce sudden death upon exercise, stress or catecholamine infusion. The autosomal dominant form of this disease have been shown to be due - in part at least – to mutations in the RyR gene in cardiac muscle (Priori *et al.*, 2002). Recently, a missense mutation in the cardiac CSQ gene has been described, which gives rise to an autosomal recessive form of this disease Lahat *et al.*, 2001. This mutation leads to a single amino acid change from an

aspartic acid to a histidine at residue 307 (d307h). This occurs close to the highly charged Ca^{2+} binding domain (Glu^{354}-Asp^{367}) of CSQ. It is proposed that mutated CSQ may serve to increase the free Ca^{2+} in the SR, resulting in Ca^{2+} overload.

It is very important to discover what effects this single amino acid mutation has on the formation (or lack of it) of the RyR/T/J/CSQ complex, and effects on RyR activation and calcium release, as well as the RyR response to small luminal Ca^{2+} changes (that occur within the contraction/relaxation cycle. For example, does replacing a negatively charged residues (Asp) to a positively charged amino acid (His) alter the structure of CSQ in such a way that either the Ca^{2+} binding domain becomes partially buried and unavailable for Ca^{2+} binding? Or, does a structural change inhibit the interaction of CSQ with its co-proteins (triadin and junctin) or the RyR? How much of a role does the up-regulation of other luminal Ca^{2+} binding proteins play in Ca^{2+} sequestration in the SR lumen? As yet, a CSQ knockout model has not been generated, and hence the importance of CSQ in regular muscle function has not been elucidated. Both the CSQ mutation (Lahat *et al.*, 2001) and the overexpresion studies (Jones *et al*, 1998, Knollman *et al*, 2000, Wang *et al*, 20009) suggest that CSQ (at normal expression levels) is required for fully functioning muscle, but do not really elucidate its role. It would be interesting to see whether the total absence of expressed CSQ would result in non-functioning muscle (particularly in the heart), and death.

Secondly, an increased expression of skeletal muscle CSQ and CSQ-like proteins has been found in mice with streptozotocin-induced diabetes (Howarth *et al*, 2002). These mice had a 2.5 times greater skeletal muscle Ca^{2+} -binding capacity, but showed no such alteration in binding capacity and not alteration in CSQ expression in cardiac muscle. Diabetics present with an elevated cytosolic Ca^{2+} level in muscle, and the authors suggest that up-regulation of the SR Ca^{2+} homeostasis proteins may be a compensatory mechanism, enabling extra luminal Ca^{2+} buffering, thereby reducing the cytosolic Ca^{2+} levels (Howarth *et al*, 2002). This is the first report of altered CSQ expression apparent in a skeletal muscle disorder.

These two disorders illustrate the importance of the understanding of the RyR/T/J/CSQ complex, and in particular, how CSQ regulates Ca^{2+} release through the SR, which eventually leads to muscle contraction. Whilst this thesis presents the first study that has

systematically studied the interactions of CSQ with native RyRs and the resultant channel modulation, the results illustrate the need for more investigation into the mechanisms of RyR/CSQ interaction and SR Ca^{2+} sequestration and release.

Bibliography

Adams, B.A., Tanabe, T., Mikami, A., Numa, S. and Beam, K.G. 1990. Intramembrane charge movement restored in dysgenic skeletal muscle by injection of dihydropyridine receptor cDNAs. *Nature*. 346: 569-72.

Ahern, G.P., Junankar, P.R. and Dulhunty, A.F. 1994. Single channel activity of the ryanodine receptor calcium release channel is modulated by FK-506. *FEBS. Lett.* 352: 369-374.

Ahern, G.P., Junankar, P.R. and Dulhunty, A.F. 1997a. Subconductance states in single-channel activity of skeletal muscle ryanodine receptors after removal of FKBP12. *Biophys. J.* 72: 146-62.

Ahern, G.P., Junankar, P.R. and Dulhunty, A.F. 1997b. Ryanodine receptors from rabbit skeletal muscle are reversibly activated by rapamycin. *Neurosci. Lett.* 225: 81-4.

Baksh, S., Spamer, C., Oikawa, K., McCubbin, W.D., Heilmann, C., Kay, C.M. and Michalak, M. 1995. Zn^{2+} binding to cardiac calsequestrin. *Biochem. Biophys. Res. Commun.* 209: 310-315.

Bassani, J.W., Yuan, W. and Bers, D.M. 1995. Fractional SR Ca release is regulated by trigger Ca and SR Ca content in cardiac myocytes. *Am. J. Physiol.* 268: C1313-C1319.

Beard, N.A., Dulhunty, A.F. and Laver, D.R. 2000. The effect of increasing luminal calcium on skeletal muscle calsequestrin. *Proc. Aust. Physiol. Pharmacol. Soc.* 21: 23p.

Beard, N.A., Laver, D.R. and Dulhunty, A.F. 1999. Regulation of skeletal muscle ryanodine receptors by calsequestrin. *Proc. Aust. Physiol. Pharmacol. Soc.* 30: 43p.

Beard, N.A., Sakowska, M.M., Dulhunty, A.F. and Laver, D.R. 2002. Calsequestrin is an inhibitor of skeletal muscle ryanodine receptor calcium release channels. *Biophys. J.* 82: 310-20.

Berne, R.M. and Levy, M.N. 1990. Principles of Physiology. 1990 C. V. Mosby Company, Missouri. 690 pp.

Berridge, M.J. 1993. Inositol trisphosphate and calcium signalling. *Nature*. 361: 315-25.

Bertocchini, F., Ovitt, C.E., Conti, A., Barone, V., Scholer, H.R., Bottinelli, R., Reggiani, C. and Sorrentino, V. 1997. Requirement for the ryanodine receptor type 3 for efficient contraction in neonatal skeletal muscles. *EMBO. J.* 16: 6956-6963.

Beurg, M., Ahern, C.A., Vallejo, P., Conklin, M.W., Powers, P.A., Gregg, R.G. and Coronado, R. 1999. Involvement of the carboxy-terminus region of the dihydropyridine receptor beta1a subunit in excitation-contraction coupling of skeletal muscle. *Biophys. J.* 77: 2953-2967.

Blumenthal, D.K., Takio, K., Edelman, A.M., Charbonneau, H., Titani, K., Walsh, K.A. and Krebs, E.G. 1985. Identification of the calmodulin-binding domain of skeletal muscle myosin light chain kinase. *Proc. Natl. Acad. Sci. U.S.A.* 82: 3187-91.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248-54.

Brandt, N.R., Caswell, A.H., Brunschwig, J.P., Kang, J.J., Antoniu, B. and Ikemoto, N. 1992. Effects of anti-triadin antibody on Ca^{2+} release from sarcoplasmic reticulum. *FEBS. Lett.* 299: 57-59.

Brandt, N.R., Caswell, A.H., Wen, S.R. and Talvenheimo, J.A. 1990. Molecular interactions of the junctional foot protein and dihydropyridine receptor in skeletal muscle triads. *J. Memb. Biol.* 113: 237-51.

Brillantes, A.M., Bezprozvannaya, S. and Marks, A.R. 1994. Developmental and tissue-specific regulation of rabbit skeletal and cardiac muscle calcium channels involved in excitation- contraction coupling. *Circ. Res.* 75: 503-510.

Cala, S.E. and Jones, L.R. 1983. Rapid purification of calsequestrin from cardiac and skeletal muscle sarcoplasmic reticulum vesicles by Ca^{2+}-dependent elution from phenyl-sepharose. *J. Biol. Chem.* 258: 11932-11936.

Cala, S.E. and Jones, L.R. 1991. Phosphorylation of cardiac and skeletal muscle calsequestrin isoforms by casein kinase II. Demonstration of a cluster of unique rapidly phosphorylated sites in cardiac calsequestrin. *J. Biol. Chem.* 266: 391-398.

Cala, S.E., Scott, B.T. and Jones, L.R. 1990. Intralumenal sarcoplasmic reticulum Ca^{2+}-binding proteins. *Sem. Cell. Biol.* 1: 265-275.

Callaway, C., Seryshev, A., Wang, J.P., Slavik, K.J., Needleman, D.H., Cantu, C., Wu, Y., Jayaraman, T., Marks, A.R. and Hamilton, S.L. 1994. Localization of the high and low affinity [3H]ryanodine binding sites on the skeletal muscle Ca^{2+} release channel. *J. Biol. Chem.* 269: 15876-15884.

Campbell, K.P. and Shamoo, A.E. 1980. Phosphorylation of heavy sarcoplasmic reticulum vesicles: identification and characterization of three phosphorylated proteins. *J. Memb. Biol.* 56: 241-8.

Carroll, S., Skarmeta, J.G., Yu, X., Collins, K.D. and Inesi, G. 1991. Interdependence of ryanodine binding, oligomeric receptor interactions, and Ca^{2+} release regulation in junctional sarcoplasmic reticulum. *Arch .Biochem. Biophys.* 290: 239-47.

Caswell, A.H., Brandt, N.R., Brunschwig, J.P. and Purkerson, S. 1991. Localization and partial characterization of the oligomeric disulfide-linked molecular weight 95,000 protein (triadin) which binds the ryanodine and dihydropyridine receptors in skeletal muscle triadic vesicles. *Biochem.* 30: 7507-7513.

Caswell, A.H., Motoike, H.K., Fan, H. and Brandt, N.R. 1999. Location of ryanodine receptor binding site on skeletal muscle triadin. *Biochem.* 38: 90-97.

Catterall, W.A. 2000. Structure and regulation of voltage-gated Ca^{2+} channels. *Annu .Rev. Cell .Dev. Biol.* 16: 521-55.

Chen, S.R., Ebisawa, K., Li, X. and Zhang, L. 1998. Molecular identification of the ryanodine receptor Ca^{2+} sensor. *J. Biol. Chem.* 273: 14675-14678.

Chen, S.R., Zhang, L. and MacLennan, D.H. 1992. Characterization of a Ca^{2+} binding and regulatory site in the Ca^{2+} release channel (ryanodine receptor) of rabbit skeletal muscle sarcoplasmic reticulum. *J. Biol. Chem.* 267: 23318-26.

Chen, S.R., Zhang, L. and MacLennan, D.H. 1993. Antibodies as probes for Ca^{2+} activation sites in the Ca^{2+} release channel (ryanodine receptor) of rabbit skeletal muscle sarcoplasmic reticulum. *J. Biol. Chem.* 268: 13414-21.

Ching, L.L., Williams, A.J. and Sitsapesan, R. 1999. AMP is a partial agonist at the sheep cardiac ryanodine receptor. *Br. J. Pharmacol.* 127: 161-171.

Ching, L.L., Williams, A.J. and Sitsapesan, R. 2000. Evidence for Ca^{2+} activation and inactivation sites on the luminal side of the cardiac ryanodine receptor complex. *Circ. Res.* 87: 201-206.

Chu, A., Dixon, M.C., Saito, A., Seiler, S. and Fleischer, S. 1988. Isolation of sarcoplasmic reticulum fractions referable to longitudinal tubules and junctional terminal cisternae from rabbit skeletal muscle. *Meth. Enzymol.* 157: 36-46.

Chu, A., Fill, M., Stefani, E. and Entman, M.L. 1993. Cytoplasmic Ca^{2+} does not inhibit the cardiac muscle sarcoplasmic reticulum ryanodine receptor Ca^{2+} channel, although Ca^{2+} induced Ca^{2+} inactivation of Ca^{2+} release is observed in native vesicles. *J. Memb. Biol.* 135: 49-59.

Copello, J.A., Barg, S., Onoue, H. and Fleischer, S. 1997. Heterogeneity of Ca^{2+} gating of skeletal muscle and cardiac ryanodine receptors. *Biophys. J.* 73: 141-156.

Coronado, R., Kawano, S., Lee, C.J., Valdivia, C. and Valdivia, H.H. 1992. Planar bilayer recording of ryanodine receptors of sarcoplasmic reticulum. *Meth. Enzymol.* 207: 699-707.

Coronado, R., Morrissette, J., Sukhareva, M. and Vaughan, D.M. 1994. Structure and function of ryanodine receptors. *Am. J. Physiol.* 266: C1485-C1504.

Costello, B., Chadwick, C., Saito, A., Chu, A., Maurer, A. and Fleischer, S. 1986. Characterization of the junctional face membrane from terminal cisternae of sarcoplasmic reticulum. *J. Cell. Biol.* 103: 741-753.

Cozens, B. and Reithmeier, R.A. 1984. Size and shape of rabbit skeletal muscle calsequestrin. *J. Biol. Chem.* 259: 6248-52.

Cukierman, S., Yellen, G. and Miller, C. 1985. The K^+ channel of sarcoplasmic reticulum. A new look at Cs^+ block. *Biophys J.* 48: 477-84.

Damiani, E., Picello, E., Saggin, L. and Margreth, A. 1995. Identification of triadin and of histidine-rich Ca^{2+}-binding protein as substrates of 60 kDa calmodulin-dependent protein kinase in junctional terminal cisternae of sarcoplasmic reticulum of rabbit fast muscle. *Biochem. Biophys. Res. Commun.* 209: 457-465.

Diaz-Munoz, M., Hamilton, S.L., Kaetzel, M.A., Hazarika, P. and Dedman, J.R. 1990. Modulation of Ca^{2+} release channel activity from sarcoplasmic reticulum by annexin VI (67-kDa calcimedin). *J. Biol. Chem.* 265: 15894-9.

Dinchuk, J.E., Henderson, N.L., Burn, T.C., Huber, R., Ho, S.P., Link, J., O'Neil, K.T., Focht, R.J., Scully, M.S., Hollis, J.M., Hollis, G.F. and Friedman, P.A. 2000. Aspartyl beta -hydroxylase (Asph) and an evolutionarily conserved isoform of Asph missing the catalytic domain share exons with junctin. *J.Biol.Chem.* 275: 39543-54.

Donoso, P., Prieto, H. and Hidalgo, C. 1995. Luminal calcium regulates calcium release in triads isolated from frog and rabbit skeletal muscle. *Biophys. J.* 68: 507-515.

Doyle, D.A., Morais Cabral, J., Pfuetzner, R.A., Kuo, A., Gulbis, J.M., Cohen, S.L., Chait, B.T. and MacKinnon, R. 1998. The structure of the potassium channel: molecular basis of K^+ conduction and selectivity. *Science.* 280: 69-77.

Du, G.G. and MacLennan, D.H. 1999. Ca^{2+} inactivation sites are located in the COOH-terminal quarter of recombinant rabbit skeletal muscle Ca^{2+} release channels (ryanodine receptors). *J. Biol. Chem.* 274: 26120-6.

Du, G.G., Oyamada, H., Khanna, V.K. and MacLennan, D.H. 2001. Mutations to Gly2370, Gly2373 or Gly2375 in malignant hyperthermia domain 2 decrease caffeine and cresol sensitivity of the rabbit skeletal-muscle Ca^{2+}-release channel (ryanodine receptor isoform 1). *Biochem. J.* 360: 97-105.

Dulhunty, A.F. 1992. The voltage-activation of contraction in skeletal muscle. *Prog. Biophys. Mol. Biol.* 57: 181-223.

Dulhunty, A.F., Laver, D., Curtis, S.M., Pace, S., Haarmann, C. and Gallant, E.M. 2001. Characteristics of irreversible ATP activation suggest that native skeletal ryanodine receptors can be phosphorylated via an endogenous CaMKII. *Biophys. J.* 81: 3240-52.

Dulhunty, A.F., Laver, D.R., Gallant, E.M., Casarotto, M.G., Pace, S.M. and Curtis, S. 1999. Activation and inhibition of skeletal RyR channels by a part of the skeletal DHPR II-III loop: effects of DHPR Ser687 and FKBP12. *Biophys. J.* 77: 189-203.

Eager, K.R. 1997. The actions of thiol specific oxidizing agents on single channel activity of the ryanodine receptor calcium release channel in cardiac muscle. *In* John Curtin School of Medical Research. Australian National Univeristy, Canberra. 150.

el Hayek, R., Antoniu, B., Wang, J., Hamilton, S.L. and Ikemoto, N. 1995. Identification of calcium release-triggering and blocking regions of the II-III loop of the skeletal muscle dihydropyridine receptor. *J. Biol. Chem.* 270: 22116-22118.

el Hayek, R. and Ikemoto, N. 1998. Identification of the minimum essential region in the II-III loop of the dihydropyridine receptor alpha 1 subunit required for activation of skeletal muscle-type excitation-contraction coupling. *Biochem.* 37: 7015-7020.

Fan, H., Brandt, N.R. and Caswell, A.H. 1995. Disulfide bonds, N-glycosylation and transmembrane topology of skeletal muscle triadin. *Biochem.* 34: 14902-8.

Fill, M., Coronado, R., Mickelson, J.R., Vilven, J., Ma, J.J., Jacobson, B.A. and Louis, C.F. 1990. Abnormal ryanodine receptor channels in malignant hyperthermia. *Biophys. J.* 57: 471-475.

Fliegel, L., Burns, K., MacLennan, D.H., Reithmeier, R.A. and Michalak, M. 1989b. Molecular cloning of the high affinity calcium-binding protein (calreticulin) of skeletal muscle sarcoplasmic reticulum. *Biophys. J.* 264: 21522-8.

Fliegel, L., Burns, K., Opas, M. and Michalak, M. 1989a. The high-affinity calcium binding protein of sarcoplasmic reticulum. Tissue distribution, and homology with calregulin. *Biochim. Biophys. Acta.* 982: 1-8.

Fliegel, L., Ohnishi, M., Carpenter, M.R., Khanna, V.K., Reithmeier, R.A. and MacLennan, D.H. 1987. Amino acid sequence of rabbit fast-twitch skeletal muscle calsequestrin deduced from cDNA and peptide sequencing. *Proc. Nat. Acad. Sci. USA.* 84: 1167-1171.

Flucher, B.E., Conti, A., Takeshima, H. and Sorrentino, V. 1999. Type 3 and type 1 ryanodine receptors are localized in triads of the same mammalian skeletal muscle fibers. *J. Cell. Biol.* 146: 621-630.

Fryer, M.W. and Stephenson, D.G. 1996. Total and sarcoplasmic reticulum calcium contents of skinned fibres from rat skeletal muscle. *J.Physiol. (Lond).* 493: 357-370.

Fuentes, O., Valdivia, C., Vaughan, D., Coronado, R. and Valdivia, H.H. 1994. Calcium-dependent block of ryanodine receptor channel of swine skeletal muscle by direct binding of calmodulin. *Cell Calcium.* 15: 305-16.

Fujii, J., Otsu, K., Zorzato, F., de Leon, S., Khanna, V.K., Weiler, J.E., O'Brien, P.J. and MacLennan, D.H. 1991. Identification of a mutation in porcine ryanodine receptor associated with malignant hyperthermia. *Science.* 253: 448-51.

Furuichi, T., Yoshikawa, S., Miyawaki, A., Wada, K., Maeda, N. and Mikoshiba, K. 1989. Primary structure and functional expression of the inositol 1,4,5-trisphosphate-binding protein P400. *Nature.* 342: 32-8.

Futatsugi, A., Kuwajima, G. and Mikoshiba, K. 1995. Tissue-specific and developmentally regulated alternative splicing in mouse skeletal muscle ryanodine receptor mRNA. *Biochem. J.* 305: 373-378.

Gallant, E.M., Curtis, S., Pace, S.M. and Dulhunty, A.F. 2001. Arg(615)Cys substitution in pig skeletal ryanodine receptors increases activation of single channels by a segment of the skeletal DHPR II-III loop. *Biophys. J.* 80: 1769-82.

Gao, L., Balshaw, D., Xu, L., Tripathy, A., Xin, C. and Meissner, G. 2000. Evidence for a role of the lumenal M3-M4 loop in skeletal muscle Ca^{2+} release channel (ryanodine receptor) activity and conductance. *Biophys. J.* 79: 828-840.

Gilchrist, J.S., Belcastro, A.N. and Katz, S. 1992. Intraluminal Ca^{2+} dependence of Ca^{2+} and ryanodine-mediated regulation of skeletal muscle sarcoplasmic reticulum Ca^{2+} release. *J. Biol. Chem.* 267: 20850-20856.

Grabner, M., Dirksen, R.T., Suda, N. and Beam, K.G. 1999. The II-III loop of the skeletal muscle dihydropyridine receptor is responsible for the Bi-directional coupling with the ryanodine receptor. *J. Biol. Chem.* 274: 21913-21919.

Groh, S., Marty, I., Ottolia, M., Prestipino, G., Chapel, A., Villaz, M. and Ronjat, M. 1999. Functional interaction of the cytoplasmic domain of triadin with the skeletal ryanodine receptor. *J. Biol. Chem.* 274: 12278-12283.

Guo, W. and Campbell, K.P. 1995. Association of triadin with the ryanodine receptor and calsequestrin in the lumen of the sarcoplasmic reticulum. *J. Biol. Chem.* 270: 9027-9030.

Guo, W., Jorgensen, A.O. and Campbell, K.P. 1994. Characterization and ultrastructural localization of a novel 90- kDa protein unique to skeletal muscle junctional sarcoplasmic reticulum. *J. Biol. Chem.* 269: 28359-28365.

Györke, I. and Györke, S. 1998. Regulation of the cardiac ryanodine receptor channel by luminal Ca^{2+} involves luminal Ca^{2+} sensing sites. *Biophys. J.* 75: 2801-2810.

Haarman, C.S., Laver, D.R. and Dulhunty, A.F. 2000. Effect of two different portions of the II-III loop of the dihydropyridine receptor on single ryanodine receptors. *Proc. Aust. Physiol. Pharmacol. Soc.* 31: 35p.

Haarmann, C.S., Fink, R.H. and Dulhunty, A.F. 1999. Oxidation and reduction of pig skeletal muscle ryanodine receptors. *Biophys. J.* 77: 3010-22.

Hadad, N., Meyer, H.E., Varsanyi, M., Fleischer, S. and Shoshan-Barmatz, V. 1999. Cardiac sarcalumenin: phosphorylation, comparison with the skeletal muscle sarcalumenin and modulation of ryanodine receptor. *J. Memb. Biol.* 170: 39-49.

Hain, J., Nath, S., Mayrleitner, M., Fleischer, S. and Schindler, H. 1994. Phosphorylation modulates the function of the calcium release channel of sarcoplasmic reticulum from skeletal muscle. *Biophys. J.* 67: 1823-33.

He, Z., Dunker, A.K., Wesson, C.R. and Trumble, W.R. 1993. Ca^{2+} -induced folding and aggregation of skeletal muscle sarcoplasmic reticulum calsequestrin. The involvement of the trifluoperazine-binding site. *J. Biol. Chem.* 268: 24635-24641.

Herrmann-Frank, A. and Lehmann-Horn, F. 1996. Regulation of the purified Ca^{2+} release channel/ryanodine receptor complex of skeletal muscle sarcoplasmic reticulum by luminal calcium. *Pflugers Arch.* 432: 155-157.

Herrmann-Frank, A. and Varsanyi, M. 1993. Enhancement of Ca^{2+} release channel activity by phosphorylation of the skeletal muscle ryanodine receptor. *FEBS. Lett.* 332: 237-42.

Herzog, A., Szegedi, C., Jona, I., Herberg, F.W. and Varsanyi, M. 2000. Surface plasmon resonance studies prove the interaction of skeletal muscle sarcoplasmic reticular Ca^{2+} release channel/ryanodine receptor with calsequestrin. *FEBS. Lett.* 472: 73-77.

Hidalgo, C. and Donoso, P. 1995. Luminal calcium regulation of calcium release from sarcoplasmic reticulum. *Biosci. Rep.* 15: 387-397.

Hidalgo, C., Donoso, P. and Rodriguez, P.H. 1996. Protons induce calsequestrin conformational changes. *Biophys. J.* 71: 2130-7.

Hong, C.S., Ji, J.H., Kim, J.P., Jung, D.H. and Kim do, H. 2001. Molecular cloning and characterization of mouse cardiac triadin isoforms. *Gene.* 278: 193-9.

Howarth, F.C., Glover, L., Culligan, K., Qureshi, M.A. and Ohlendieck, K. 2002. Calsequestrin expression and calcium binding is increased in streptozotocin-induced diabetic rat skeletal muscle though not in cardiac muscle. *Pflugers Arch.* 444: 52-8.

Ikemoto, N., Antoniu, B., Kang, J.J., Meszaros, L.G. and Ronjat, M. 1991. Intravesicular calcium transient during calcium release from sarcoplasmic reticulum. *Biochem.* 30: 5230-5237.

Ikemoto, N., Antoniu, B. and Meszaros, L.G. 1985. Rapid flow chemical quench studies of calcium release from isolated sarcoplasmic reticulum. *J. Biol. Chem.* 260: 14096-100.

Ikemoto, N., Bhatnagar, G.M., Nagy, B. and Gergely, J. 1972. Interaction of divalent cations with the 55,000-dalton protein component of the sarcoplasmic reticulum. Studies of fluorescence and circular dichroism. *J. Biol. Chem.* 247: 7835-7837.

Ikemoto, N., Nagy, B., Bhatnagar, G.M. and Gergely, J. 1974. Studies on a metal-binding protein of the sarcoplasmic reticulum. *J. Biol. Chem.* 249: 2357-65.

Ikemoto, N., Ronjat, M., Meszaros, L.G. and Koshita, M. 1989. Postulated role of calsequestrin in the regulation of calcium release from sarcoplasmic reticulum. *Biochem.* 28: 6764-6771.

Ikemoto, T., Komazaki, S., Takeshima, H., Nishi, M., Noda, T., Iino, M. and Endo, M. 1997. Functional and morphological features of skeletal muscle from mutant mice lacking both type 1 and type 3 ryanodine receptors. *J. Physiol.* 501: 305-312.

Jones, L.R., Besch, H.R., Jr., Fleming, J.W., McConnaughey, M.M. and Watanabe, A.M. 1979b. Separation of vesicles of cardiac sarcolemma from vesicles of cardiac sarcoplasmic reticulum. Comparative biochemical analysis of component activities. *J. Biol. Chem.* 254: 530-539.

Jones, L.R. and Cala, S.E. 1981. Biochemical evidence for functional heterogeneity of cardiac sarcoplasmic reticulum vesicles. *J. Biol. Chem.* 256: 11809-11818.

Jones, L.R., Suzuki, Y.J., Wang, W., Kobayashi, Y.M., Ramesh, V., Franzini-Armstrong, C., Cleemann, L. and Morad, M. 1998. Regulation of ca signaling in transgenic mouse cardiac myocytes overexpressing calsequestrin. *J. Clin. Invest.* 101: 1385-1393.

Jones, L.R., Zhang, L., Sanborn, K., Jorgensen, A.O. and Kelley, J. 1995. Purification, primary structure, and immunological characterization of the 26-kDa calsequestrin binding protein (junctin) from cardiac junctional sarcoplasmic reticulum. *J. Biol. Chem.* 270: 30787-30796.

Katz, S. and Thesleff, B. 1957. A study of the 'desentisation' produced by acetylcholine at the motor end-plate. *J. Physiol.* 138: 63-80.

Kawamoto, R.M., Brunschwig, J.P., Kim, K.C. and Caswell, A.H. 1986. Isolation, characterization, and localization of the spanning protein from skeletal muscle triads. *J. Cell. Biol.* 103: 1405-1414.

Kawasaki, T. and Kasai, M. 1994. Regulation of calcium channel in sarcoplasmic reticulum by calsequestrin. *Biochem. Biophys. Res. Commun.* 199: 1120-1127.

Kermode, H., Williams, A.J. and Sitsapesan, R. 1998. The interactions of ATP, ADP, and inorganic phosphate with the sheep cardiac ryanodine receptor. *Biophys J.* 74: 1296-304.

Kim, D.H., Ohnishi, S.T. and Ikemoto, N. 1983. Kinetic studies of calcium release from sarcoplasmic reticulum in vitro. *J. Biol. Chem.* 258: 9662-9668.

Kim, K.C. and Caswell, A.H. 1990. Isolation of a terminal cisternea protein which may link the dihydropyridine receptor to the junctional foot protein in skeletal muscle. *Biochem.* 29: 9281-9289.

Kirchhefer, U., Neumann, J., Baba, H.A., Begrow, F., Kobayashi, Y.M., Reinke, U., Schmitz, W. and Jones, L.R. 2001. Cardiac hypertrophy and impaired relaxation in transgenic mice overexpressing triadin 1. *J. Biol. Chem.* 276: 4142-9.

Kirino, Y., Osakabe, M. and Shimizu, H. 1983. Ca^{2+}-induced Ca^{2+} release from fragmented sarcoplasmic reticulum: Ca^{2+}-dependent passive Ca^{2+} efflux. *J. Biochem. (Tokyo)*. 94: 1111-1118.

Knollmann, B.C., Knollmann-Ritschel, B.E., Weissman, N.J., Jones, L.R. and Morad, M. 2000. Remodelling of ionic currents in hypertrophied and failing hearts of transgenic mice overexpressing calsequestrin. *J. Physiol.* 525: 483-498.

Knudson, C.M., Stang, K.K., Jorgensen, A.O. and Campbell, K.P. 1993a. Biochemical characterization of ultrastructural localization of a major junctional sarcoplasmic reticulum glycoprotein (triadin). *J. Biol. Chem.* 268: 12637-12645.

Knudson, C.M., Stang, K.K., Moomaw, C.R., Slaughter, C.A. and Campbell, K.P. 1993b. Primary structure and topological analysis of a skeletal muscle- specific junctional sarcoplasmic reticulum glycoprotein (triadin). *J. Biol. Chem.* 268: 12646-12654.

Kobayashi, Y.M., Alseikhan, B.A. and Jones, L.R. 2000. Localization and characterization of the calsequestrin-binding domain of triadin 1. Evidence for a charged beta -strand in mediating the protien-protein interaction. *J. Biol. Chem.* 275: 17639-17646.

Kobayashi, Y.M. and Jones, L.R. 1999. Identification of triadin 1 as the predominant triadin isoform expressed in mammalian myocardium. *J. Biol. Chem.* 274: 28660-28668.

Krause, K.H., Chou, M., Thomas, M.A., Sjolund, R.D. and Campbell, K.P. 1989. Plant cells contain calsequestrin. *J. Biol. Chem.* 264: 4269-4272.

Krause, K.H., Milos, M., Luan Rilliet, Y., Lew, D.P. and Cox, J.A. 1991. Thermodynamics of cation binding to rabbit skeletal muscle calsequestrin. Evidence for distinct Ca^{2+}- and Mg^{2+}-binding sites. *J. Biol. Chem.* 266: 9453-9459.

Kuenzel, E.A., Mulligan, J.A., Sommercorn, J. and Krebs, E.G. 1987. Substrate specificity determinants for casein kinase II as deduced from studies with synthetic peptides. *J. Biol. Chem.* 262: 9136-40.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 227: 680-685.

Lahat, H., Pras, E., Olender, T., Avidan, N., Ben-Asher, E., Man, O., Levy-Nissenbaum, E., Khoury, A., Lorber, A., Goldman, B., Lancet, D. and Eldar, M. 2001. A Missense Mutation in a Highly Conserved Region of CASQ2 Is Associated with Autosomal Recessive Catecholamine-Induced Polymorphic Ventricular Tachycardia in Bedouin Families from Israel. *Am J Hum Genet.* 69: 1378-84.

Lai, F.A., Erickson, H.P., Rousseau, E., Liu, Q.Y. and Meissner, G. 1988. Purification and reconstitution of the calcium release channel from skeletal muscle. *Nature.* 331: 315-319.

Lai, F.A. and Meissner, G. 1989. The muscle ryanodine receptor and its intrinsic Ca^{2+} channel activity. *J. Bioenerg. Biomembr.* 21: 227-246.

Lamb, G.D. 2000. Excitation-contraction coupling in skeletal muscle: comparisons with cardiac muscle. *Clin. Exp. Pharmacol. Physiol.* 27: 216-224.

Lamb, G.D., Cellini, M.A. and Stephenson, D.G. 2001a. Different Ca^{2+} relelasing actions of caffeine and depolarization in skeletal muscle fibres of the rat. *J. Physiol.* 531: 715-728.

Lamb, G.D., El-Hayek, R., Ikemoto, N. and Stephenson, D.G. 2001b. Effects of dihydropyridine receptor II-III loop peptides on Ca^{2+} release in skinned skeletal muscle fibers. *Am. J. Physiol.* 279: C891-C905.

Laver, D. 2001. The power of single channel recording and analysis: its application to ryanodine receptors in lipid bilayers. *Clin. Exp. Pharmacol. Physiol.* 28: 675-86.

Laver, D.R., Baynes, T.M. and Dulhunty, A.F. 1997. Magnesium inhibition of ryanodine receptor calcium channels: evidence for two independent mechanisms. *J. Memb. Biol.* 156: 213-229.

Laver, D.R., Eager, K.R., Taoube, L. and Lamb, G.D. 2000. Effects of cytoplasmic and luminal pH on Ca^{2+} release channels from rabbit skeletal muscle. *Biophys. J.* 78: 1835-51.

Laver, D.R., Lenz, G.K. and Lamb, G.D. 2001. Regulation of the calcium release channel from rabbit skeletal muscle by the nucleotides ATP, AMP, IMP and adenosine. *J. Physiol.* 537: 763-778.

Laver, D.R., Roden, L.D., Ahern, G.P., Eager, K.R., Junankar, P.R. and Dulhunty, A.F. 1995. Cytoplasmic Ca^{2+} inhibits the ryanodine receptor from cardiac muscle. *J. Memb. Biol.* 147: 7-22.

Leberer, E., Charuk, J.H., Clarke, D.M., Green, N.M., Zubrzycka Gaarn, E. and MacLennan, D.H. 1989. Molecular cloning and expression of cDNA encoding the 53,000- dalton glycoprotein of rabbit skeletal muscle sarcoplasmic reticulum. *J. Biol. Chem.* 264: 3484-3493.

Leberer, E., Timms, B.G., Campbell, K.P. and MacLennan, D.H. 1990. Purification, calcium binding properties, and ultrastructural localization of the 53,000- and 160,000 (sarcalumenin)-dalton glycoproteins of the sarcoplasmic reticulum. *J. Biol. Chem.* 265: 10118-24.

Lee, H.G., Kang, H., Kim, D.H. and Park, W.J. 2001. Interaction of HRC (histidine-rich Ca^{2+} binding protein) and triadin in the lumen of sarcoplasmic reticulum. *J. Biol. Chem.* 14: 14.

Lewit-Bentley, A. and Rety, S. 2000. EF-hand calcium-binding proteins. *Curr. Opin. Struct. Biol.* 10: 637-43.

Liu, G. and Pessah, I.N. 1994. Molecular interaction between ryanodine receptor and glycoprotein triadin involves redox cycling of functionally important hyperreactive sulfhydryls. *J. Biol. Chem.* 269: 33028-33034.

Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. 1951. Protein Measurement With the Folin Phenol Reagent. *J. Biol. Chem.* 193: 265-275.

Lynch, P.J., Tong, J., Lehane, M., Mallet, A., Giblin, L., Heffron, J.J., Vaughan, P., Zafra, G., MacLennan, D.H. and McCarthy, T.V. 1999. A mutation in the transmembrane/luminal domain of the ryanodine receptor is associated with abnormal Ca^{2+} release channel function and severe central core disease [see comments]. *Proc. Nat. Acad. Sci. USA.* 96: 4164-4169.

Ma, J. 1993. Block by ruthenium red of the ryanodine-activated calcium release channel of skeletal muscle. *J. Gen. Physiol.* 102: 1031-1056.

Ma, J., Fill, M., Knudson, C.M., Campbell, K.P. and Coronado, R. 1988. Ryanodine receptor of skeletal muscle is a gap junction-type channel. *Science.* 242: 99-102.

MacLennan, D.H. 1974. Isolation of a second form of calsequestrin. *J. Biol. Chem.* 249: 980-4.

MacLennan, D.H., Campbell, K.P. and Reithmeier, R.A.F. 1983. Calsequestrin. *In* Calcium and Cell Funciton. Vol. IV. W.Y. Cheung, editor. Academic Press, New York. 152-173.

MacLennan, D.H., Otsu, K., Fujii, J., Zorzato, F., Phillips, M.S., O'Brien, P.J., Archibald, A.L., Britt, B.A., Gillard, E.F. and Worton, R.G. 1992. The role of the

skeletal muscle ryanodine receptor gene in malignant hyperthermia. *Symp. Soc. Exp. Biol.* 46: 189-201.

MacLennan, D.H. and Phillips, M.S. 1992. Malignant hyperthermia. *Science.* 256: 789-94.

MacLennan, D.H. and Wong, P.T. 1971. Isolation of a calcium-sequestering protein from sarcoplasmic reticulum. *Proc. Nat. Acad. Sci. USA.* 68: 1231-1235.

Marin, O., Meggio, F., Marchiori, F., Borin, G. and Pinna, L.A. 1986. Site specificity of casein kinase-2 (TS) from rat liver cytosol. A study with model peptide substrates. *Eur. J. Biochem.* 160: 239-44.

Marin, O., Meggio, F., Sarno, S., Cesaro, L., Pagano, M.A. and Pinna, L.A. 1999. Tyrosine versus serine/threonine phosphorylation by protein kinase casein kinase-2. A study with peptide substrates derived from immunophilin Fpr3. *J. Biol. Chem.* 274: 29260-5.

Marty, I., Robert, M., Ronjat, M., Bally, I., Arlaud, G. and Villaz, M. 1995. Localization of the N-terminal and C-terminal ends of triadin with respect to the sarcoplasmic reticulum membrane of rabbit skeletal muscle. *Biochem. J.* 307: 769-774.

McCarthy, T.V., Healy, J.M., Lehane, M. and Heffron, J.J. 1990. Recent developments in the molecular genetics of malignant hyperthermia: implications for future diagnosis at the DNA level. *Acta. Anaesthesiol. Belg.* 41: 107-12.

McGrew, S.G., Wolleben, C., Siegl, P., Inui, M. and Fleischer, S. 1989. Positive cooperativity of ryanodine binding to the calcium release channel of sarcoplasmic reticulum from heart and skeletal muscle. *Biochem.* 28: 1686-91.

McPherson, P.S. and Campbell, K.P. 1993. Characterization of the major brain form of the ryanodine receptor/Ca^{2+} release channel. *J. Biol. Chem.* 268: 19785-90.

Meggio, F., Deana, A.D. and Pinna, L.A. 1981. Endogenous phosphate acceptor proteins for rat liver cytosolic casein kinases. *J. Biol. Chem.* 256: 11958-61.

Meggio, F., Marin, O. and Pinna, L.A. 1994. Substrate specificity of protein kinase CK2. *Cell Mol Biol Res*. 40: 401-9.

Meissner, G. 1974. Isolation of Sarcoplasmic reticulum from Skeletal Muscle. *In* Methods in Enzymology. Vol. 31. S.P. Colowick and N.O. Kaplan, editors. Academic press, NY. 238-243.

Meissner, G. 1984. Adenine nucleotide stimulation of Ca^{2+}-induced Ca^{2+} release in sarcoplasmic reticulum. *J. Biol. Chem.* 259: 2365-2374.

Meissner, G. 1986. Ryanodine activation and inhibition of the Ca^{2+} release channel of sarcoplasmic reticulum. *J. Biol. Chem.* 261: 6300-6306.

Meissner, G. 1994. Ryanodine receptor/Ca^{2+} release channesl and their regulation by endogenous effectors. *Ann. Rev. Physiol.* 56: 485-508.

Meissner, G., Conner, G.E. and Fleischer, S. 1973. Isolation of sarcoplasmic reticulum by zonal centrifugation and purification of Ca^{2+} -pump and Ca^{2+} -binding proteins. *Biochim. Biophys. Acta.* 298: 246-269.

Meissner, G., Darling, E. and Eveleth, J. 1986a. Kinetics of rapid Ca^{2+} release by sarcoplasmic reticulum. Effects of Ca^{2+}, Mg^{2+}, and adenine nucleotides. *Biochem.* 25: 236-44.

Melzer, W., Herrmann-Frank, A. and Luttgau, H.C. 1995. The role of Ca^{2+} ions in excitation-contraction coupling of skeletal muscle fibres. *Biochim. Biophys. Acta.* 1241: 59-116.

Menegazzi, P., Larini, F., Treves, S., Guerrini, R., Quadroni, M. and Zorzato, F. 1994. Identification and characterization of three calmodulin binding sites of the skeletal muscle ryanodine receptor. *Biochem.* 33: 9078-84.

Michikawa, T., Hamanaka, H., Otsu, H., Yamamoto, A., Miyawaki, A., Furuichi, T., Tashiro, Y. and Mikoshiba, K. 1994. Transmembrane topology and sites of N-glycosylation of inositol 1,4,5-trisphosphate receptor. *J. Biol. Chem.* 269: 9184-9.

Miller, C. and Racker, E. 1976. Ca^{2+}-induced fusion of fragmented sarcoplasmic reticulum with artificial planar bilayers. *J. Memb. Biol.* 30: 283-300.

Mitchell, R.D., Simmerman, H.K. and Jones, L.R. 1988. Ca^{2+} binding effects on protein conformation and protein interactions of canine cardiac calsequestrin. *J. Biol. Chem.* 263: 1376-1381.

Moore, C.L. 1971. Specific inhibition of mitochondrial Ca2+ transport by ruthenium red. *Biochem. Biophys. Res. Commun.* 42: 298-305.

Moore, C.P., Rodney, G., Zhang, J.Z., Santacruz-Toloza, L., Strasburg, G. and Hamilton, S.L. 1999. Apocalmodulin and Ca^{2+} calmodulin bind to the same region on the skeletal muscle Ca^{2+} release channel. *Biochem.* 38: 8532-7.

Morii, H. and Tonomura, Y. 1983. The gating behavior of a channel for Ca^{2+}-induced Ca^{2+} release in fragmented sarcoplasmic reticulum. *J. Biochem. (Tokyo).* 93: 1271-85.

Moutin, M.J. and Dupont, Y. 1988. Rapid filtration studies of Ca^{2+}-induced Ca^{2+} release from skeletal sarcoplasmic reticulum. Role of monovalent ions. *J. Biol. Chem.* 263: 4228-35.

Mouton, J., Marty, I., Villaz, M., Feltz, A. and Maulet, Y. 2001. Molecular interaction of dihydropyridine receptors with type-1 ryanodine receptors in rat brain. *Biochem. J.* 354: 597-603.

Munro, S. and Pelham, H.R. 1987. A C-terminal signal prevents secretion of luminal ER proteins. *Cell.* 48: 899-907.

Murray, B.E., Froemming, G.R., Maguire, P.B. and Ohlendieck, K. 1998. Excitation-contraction-relaxation cycle: role of Ca^{2+}-regulatory membrane proteins in normal, stimulated and pathological skeletal muscle (review). *Int.J Mol.Med.* 1: 677-687.

Murray, B.E. and Ohlendiek,K. 1998. Complex formation between calsequestrin and the ryanodine receptor in fast- and slow-twitch skeletal muscle. *FEBS. Lett.* 429: 317-322.

Nagasaki, K. and Kasai, M. 1983. Fast release of calcium from sarcoplasmic reticulum vesicles monitored by chlortetracycline fluorescence. *J. Biochem. (Tokyo).* 94: 1101-9.

Nakai, J., Sekiguchi, N., Rando, T.A., Allen, P.D. and Beam, K.G. 1998. Two regions of the ryanodine receptor involved in coupling with L-type Ca^{2+} channels. *J. Biol. Chem.* 273: 13403-13406.

Niles, W.D. and Cohen, F.S. 1987. Video fluorescence microscopy studies of phospholipid vesicle fusion with a planar phospholipid membrane. Nature of membrane-membrane interactions and detection of release of contents. *J. Gen. Physiol.* 90: 703-35.

Nori, A., Furlan, S., Patiri, F., Cantini, M. and Volpe, P. 2000. Site-Directed Mutagenesis and Deletion of Three Phosphorylation Sites of Calsequestrin of Skeletal Muscle Sarcoplasmic Reticulum. *Exp. Cell. Res.* 260: 40-49.

Nori, A., Gola, E., Tosato, S., Cantini, M. and Volpe, P. 1999. Targeting of calsequestrin to sarcoplasmic reticulum after deletions of its acidic carboxy terminus. *Am. J. Physiol.* 277: C974-C981.

Nori, A., Valle, G., Furlan, S., Massimino, M.L. and Volpe, P. 2001a. Retention of skeletal muscle calsequestrin in the terminal cisternae upon dleetion of the 2nd or 3rd domain. *Biophys. J. (Annual Meeting Abstracts).* 80: 57a.

Nori, A., Valle, G., Massimino, M.L. and Volpe, P. 2001b. Targeting of Calsequestrin to the Sarcoplasmic Reticulum of Skeletal Muscle upon Deletion of Its Glycosylation Site. *Exp. Cell. Res.* 265: 104-13.

Ogawa, Y., Kurebayashi, N. and Murayama, T. 2000. Putative roles of type 3 ryanodine receptor isoforms (RyR3). *Trends. Cardiovasc. Med.* 10: 65-70.

Ohkura, M., Furukawa, K., Fujimori, H., Kuruma, A., Kawano, S., Hiraoka, M., Kuniyasu, A., Nakayama, H. and Ohizumi, Y. 1998. Dual regulation of the skeletal muscle ryanodine receptor by triadin and calsequestrin. *Biochem.* 37: 12987-12993.

Ohkura, M., Furukawa, K., Tu, A.T. and Ohizumi, Y. 1994. Calsequestrin is a major binding protein of myotoxin alpha and an endogenous Ca^{2+} releaser in sarcoplasmic reticulum. *Eur. J. Pharmacol.* 268: R1-R2.

Ohkura, M., Ide, T., Furukawa, K., Kawasaki, T., Kasai, M. and Ohizumi, Y. 1995. Calsequestrin is essential for the Ca^{2+} release induced by myotoxin alpha in skeletal muscle sarcoplasmic reticulum. *Can. J. Physiol.* 73: 1181-1185.

Ohnishi, M. and Reithmeier, R.A. 1987. Fragmentation of rabbit skeletal muscle calsequestrin: spectral and ion binding properties of the carboxyl-terminal region. *Biochem.* 26: 7458-7465.

Orr, I., Gechtman, Z. and Shoshan-Barmatz, V. 1991. Characterization of Ca^{2+}-dependent endogenous phosphorylation of 160,000- and 150,000-Dalton proteins of sarcoplasmic reticulum. *Biochem. J.* 276: 89-96.

Orr, I. and Shoshan-Barmatz, V. 1996. Modulation of the skeletal muscle ryanodine receptor by endogenous phosphorylation of 160/150-kDa proteins of the sarcoplasmic reticulum. *Biochim. Biophys. Acta.* 1283: 80-8.

Ostwald, T.J., MacLennan, D.H. and Dorrington, K.J. 1974. Effects of cation binding on the conformation of calsequestrin and the high affinity calcium-binding protein of sarcoplasmic reticulum. *J. Biol. Chem.* 249: 5867-71.

Owen, V.J., Taske, N.L. and Lamb, G.D. 1997. Reduced Mg^{2+} inhibition of Ca^{2+} release in muscle fibers of pigs susceptible to malignant hyperthermia. *Am.J.Physiol.* 272: C203-11.

Park, K.W., Goo, J.H., Chung, H.S., Kim, H., Kim, D.H. and Park, W.J. 1998. Cloning of the genes encoding mouse cardiac and skeletal calsequestrins: expression pattern during embryogenesis. *Gene*. 217: 25-30.

Pinna, L.A. 1997. Protein kinase CK2. *Int. J. Biochem. Cell. Biol.* 29: 551-4.

Pozzan, T., Rizzuto, R., Volpe, P. and Meldolesi, J. 1994. Molecular and cellular physiology of intracellular calcium stores. *Physiol. Rev.* 74: 595-636.

Priori, S.G., Napolitano, C., Memmi, M., Colombi, B., Drago, F., Gasparini, M., DeSimone, L., Coltorti, F., Bloise, R., Keegan, R., Cruz Filho, F.E., Vignati, G., Benatar, A. and DeLogu, A. 2002. Clinical and molecular characterization of patients with catecholaminergic polymorphic ventricular tachycardia. *Circ.* 106: 69-74.

Proenza, C., O'Brien, J., Nakai, J., Mukherjee, S., Allen, P.D. and Beam, K.G. 2002. Identification of a region of RyR1 that participates in allosteric coupling with the alpha(1S) (Ca(V)1.1) II-III loop. *J Biol Chem*. 277: 6530-5.

Radermacher, M., Rao, V., Grassucci, R., Frank, J., Timerman, A.P., Fleischer, S. and Wagenknecht, T. 1994. Cryo-electron microscopy and three-dimensional reconstruction of the calcium release channel/ryanodine receptor from skeletal muscle. *J. Cell. Biol.* 127: 411-23.

Rodriguez, M.M., Chen, C.H., Smith, B.L. and Mochly-Rosen, D. 1999. Characterization of the binding and phosphorylation of cardiac calsequestrin by epsilon protein kinase C. *FEBS. Lett.* 454: 240-246.

Rousseau, E. and Meissner, G. 1989. Single cardiac sarcoplasmic reticulum Ca^{2+}-release channel: activation by caffeine. *Am. J. Physiol.* 256: H328-33.

Rubtsov, A.M. and Batrukova, M.A. 1997. Ca-Release Channels (Ryanodine Receptors) of Sarcoplasmic Reticulum: Structure and Properties. A Review. *Biochem.* 62: 933-945.

Saiki, Y., El-Hayek, R. and Ikemoto, N. 1999. Involvement of the Glu724-Pro760 region of the dihydropyridine receptor II-III loop in skeletal muscle-type excitation-contraction coupling. *J. Biol. Chem.* 274: 7825-32.

Saiki, Y. and Ikemoto, N. 1997. Fluorescence probe study of the lumenal Ca^{2+} of the sarcoplasmic reticulum vesicles during Ca^{2+} uptake and Ca^{2+} release. *Biochem Biophys Res Commun.* 241: 181-6.

Sakowska, M.M. 2000. Regulation of Skeletal Muscle Calcium release Channels by Inorganic Phosphate and Calseqestrin. *In* Biochemistry and Molecular Biology. Australian National University, Canberra. 99.

Salvatori, S., Furlan, S. and Meggio, F. 1994. Dual role of calsequestrin as substrate and inhibitor of casein kinase-1 and casein kinase-2.
198: 144-149.

Samso, M. and Wagenknecht, T. 1998. Contributions of electron microscopy and single-particle techniques to the determination of the ryanodine receptor three-dimensional structure. *J. Struct. Biol.* 121: 172-180.

Sato, Y., Ferguson, D.G., Sako, H., Dorn, G.W., 2nd, Kadambi, V.J., Yatani, A., Hoit, B.D., Walsh, R.A. and Kranias, E.G. 1998. Cardiac-specific overexpression of mouse cardiac calsequestrin is associated with depressed cardiovascular function and hypertrophy in transgenic mice. *J. Biol. Chem.* 273: 28470-28477.

Scott, B.T., Simmerman, H.K., Collins, J.H., Nadal Ginard, B. and Jones, L.R. 1988. Complete amino acid sequence of canine cardiac calsequestrin deduced by cDNA cloning. *J. Biol. Chem.* 263: 8958-8964.

Seiler, S., Wegener, A.D., Whang, D.D., Hathaway, D.R. and Jones, L.R. 1984. High molecular weight proteins in cardiac and skeletal muscle junctional sarcoplasmic reticulum vesicles bind calmodulin, are phosphorylated, and are degraded by Ca^{2+}-activated protease. *J. Biol. Chem.* 259: 8550-7.

Shin, D.W., Ma, J. and Kim, D.H. 2001. The asp-rich region at the carboxyl-terminus of calsequestrin binds to Ca^{2+} and interacts with triadin. *FEBS. Lett.* 486: 178-182.

Shoshan-Barmatz, V., Hadad, N., Feng, W., Shafir, I., Orr, I., Varsanyi, M. and Heilmeyer, L.M. 1996a. VDAC/porin is present in sarcoplasmic reticulum from skeletal muscle. *FEBS. Lett.* 386: 205-10.

Shoshan-Barmatz, V., Orr, I., Weil, S., Meyer, H., Varsanyi, M. and Heilmeyer, L.M. 1996b. The identification of the phosphorylated 150/160-kDa proteins of sarcoplasmic reticulum, their kinase and their association with the ryanodine receptor. *Biochim. Biophys. Acta.* 1283: 89-100.

Sitsapesan, R. and Williams, A.J. 1995. The gating of the sheep skeletal sarcoplasmic reticulum Ca^{2+}- release channel is regulated by luminal Ca^{2+}. *J. Memb. Biol.* 146: 133-144.

Slupsky, J.R., Ohnishi, M., Carpenter, M.R. and Reithmeier, R.A. 1987. Characterization of cardiac calsequestrin. *Biochem.* 26: 6539-6544.

Smith, J.S., Coronado, R. and Meissner, G. 1985. Sarcoplasmic reticulum contains adenine nucleotide-activated calcium channels. *Nature.* 316: 446-449.

Smith, J.S., Coronado, R. and Meissner, G. 1986. Single channel measurements of the calcium release channel from skeletal muscle sarcoplasmic reticulum. Activation by Ca^{2+} and ATP and modulation by Mg^{2+}. *J. Gen. Physiol.* 88: 573-588.

Smith, J.S., Coronado, R. and Meissner, G. 1988a. Techniques for observing calcium channels from skeletal muscle sarcoplasmic reticulum in planar lipid bilayers. *Meth. Enzymol.* 157: 480-489.

Smith, J.S., Imagawa, T., Ma, J., Fill, M., Campbell, K.P. and Coronado, R. 1988b. Purified ryanodine receptor from rabbit skeletal muscle is the calcium-release channel of sarcoplasmic reticulum. *J. Gen. Physiol.* 92: 1-26.

Sonnleitner, A., Conti, A., Bertocchini, F., Schindler, H. and Sorrentino, V. 1998. Functional properties of the ryanodine receptor type 3 (RyR3) Ca^{2+} release channel. *EMBO. J.* 17: 2790-2798.

Sonnleitner, A., Fleischer, S. and Schindler, H. 1997. Gating of the skeletal calcium release channel by ATP is inhibited by protein phosphatase 1 but not by Mg^{2+}. *Cell Calcium*. 21: 283-90.

Sorrentino, V. and Volpe, P. 1993. Ryanodine receptors: how many, where and why? *Trends. Pharmacol. Sci.* 14: 98-103.

Stange, M., Tripathy, A. and Meissner, G. 2001. Two domains in dihydropyridine receptor activate the skeletal muscle Ca^{2+} release channel. *Biophys. J.* 81: 1419-29.

Suko, J., Maurer-Fogy, I., Plank, B., Bertel, O., Wyskovsky, W., Hohenegger, M. and Hellmann, G. 1993. Phosphorylation of serine 2843 in ryanodine receptor-calcium release channel of skeletal muscle by cAMP-, cGMP- and CaM-dependent protein kinase. *Biochim. Biophys. Acta.* 1175: 193-206.

Sutko, J.L. and Airey, J.A. 1996. Ryanodine receptor Ca^{2+} release channels: does diversity in form equal diversity in function? *Physiol. Rev.* 76: 1027-71.

Szegedi, C., Sarkozi, S., Herzog, A., Jona, I. and Varsanyi, M. 1999. Calsequestrin: more than 'only' a luminal Ca^{2+} buffer inside the sarcoplasmic reticulum. *Biochem. J.* 337: 19-22.

Tada, M. and Inui, M. 1983. Regulation of calcium transport by the ATPase-phospholamban system. *J. Mol.Cell. Cardiol.* 15: 565-75.

Takahashi, M., Seagar, M.J., Jones, J.F., Reber, B.F. and Catterall, W.A. 1987. Subunit structure of dihydropyridine-sensitive calcium channels from skeletal muscle. *Proc. Nat. Acad. Sci. USA.* 84: 5478-82.

Takekura, H., Sun, X. and Franzini Armstrong, C. 1994. Development of the excitation-contraction coupling apparatus in skeletal muscle: peripheral and internal calcium release units are formed sequentially. *J. Mus. Res. Cell. Mot.* 15: 102-118.

Takeshima, H., Iino, M., Takekura, H., Nishi, M., Kuno, J., Minowa, O., Takano, H. and Noda, T. 1994. Excitation-contraction uncoupling and muscular degeneration in mice lacking functional skeletal muscle ryanodine-receptor gene. *Nature*. 369: 556-9.

Takeshima, H., Nishimura, S., Matsumoto, T., Ishida, H., Kangawa, K., Minamino, N., Matsuo, H., Ueda, M., Hanaoka, M. and Hirose, T. 1989. Primary structure and expression from complementary DNA of skeletal muscle ryanodine receptor. *Nature*. 339: 439-445.

Tanabe, T., Beam, K.G., Adams, B.A., Niidome, T. and Numa, S. 1990. Regions of the skeletal muscle dihydropyridine receptor critical for excitation-contraction coupling. *Nature*. 346: 567-9.

Tanabe, T., Beam, K.G., Powell, J.A. and Numa, S. 1988. Restoration of excitation-contraction coupling and slow calcium current in dysgenic muscle by dihydropyridine receptor complementary DNA. *Nature*. 336: 134-9.

Tanabe, T., Takeshima, H., Mikami, A., Flockerzi, V., Takahashi, H., Kangawa, K., Kojima, M., Matsuo, H., Hirose, T. and Numa, S. 1987. Primary structure of the receptor for calcium channel blockers from skeletal muscle. *Nature*. 328: 313-8.

Timerman, A.P., Ogunbumni, E., Freund, E., Wiederrecht, G., Marks, A.R. and Fleischer, S. 1993. The calcium release channel of sarcoplasmic reticulum is modulated by FK-506-binding protein. Dissociation and reconstitution of FKBP-12 to the calcium release channel of skeletal muscle sarcoplasmic reticulum. *J. Biol. Chem.* 268: 22992-22999.

Tinker, A. and Williams, A.J. 1992. Divalent cation conduction in the ryanodine receptor channel of sheep cardiac muscle sarcoplasmic reticulum. *J. Gen. Physiol.* 100: 479-93.

Tinker, A. and Williams, A.J. 1993. Probing the structure of the conduction pathway of the sheep cardiac sarcoplasmic reticulum calcium-release channel with permeant and impermeant organic cations. *J Gen Physiol.* 102: 1107-29.

Tinker, A. and Williams, A.J. 1995. Measuring the length of the pore of the sheep cardiac sarcoplasmic reticulum calcium-release channel using related trimethylammonium ions as molecular calipers. *Biophys J.* 68: 111-20.

Towbin, H., Staehelin, T. and Gordon, J. 1992. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. 1979. *Biotech.* 24: 145-149.

Treves, S., Vilsen, B., Chiozzi, P., Andersen, J.P. and Zorzato, F. 1992. Molecular cloning, functional expression and tissue distribution of the cDNA encoding frog skeletal muscle calsequestrin. *Biochem J.* 283: 767-72.

Tripathy, A. and Meissner, G. 1996. Sarcoplasmic reticulum lumenal Ca^{2+} has access to cytosolic activation and inactivation sites of skeletal muscle Ca^{2+} release channel. *Biophys. J.* 70: 2600-2615.

Tripathy, A., Xu, L., Mann, G. and Meissner, G. 1995. Calmodulin activation and inhibition of skeletal muscle Ca^{2+} release channel (ryanodine receptor). *Biophys. J.* 69: 106-119.

Varsanyi, M. and Heilmeyer, L.M., Jr. 1979. The protein kinase properties of calsequestrin. *FEBS. Lett.* 103: 85-88.

Varsanyi, M. and Heilmeyer, L.M., Jr. 1980. Autocatalytic phosphorylation of calsequestrin. *FEBS. Lett.* 122: 227-230.

Varsanyi, M. and Meyer, H.E. 1995. Sarcoplasmic reticular Ca^{2+} release channel is phosphorylated at serine 2843 in intact rabbit skeletal muscle. *Biol. Chem. Hoppe. Seyler.* 376: 45-9.

Volpe, P., Furlan, S. and Damiani, E. 1990. Calsequestrin, a component of the Inositol 1,4,5-triphosphate- sensitive Ca^{2+} store of chicken cerebellum. *Neuron*. 5: 713-721.

Volpe, P., Martini, A., Furlan, S. and Meldolesi, J. 1994. Calsequestrin is a component of smooth muscles: the skeletal-and cardiac-muscle isoforms are both present, although in highly variable amounts and ratios. *Biochem. J.* 301: 465-469.

Wang, J. and Best, P.M. 1992. Inactivation of the sarcoplasmic reticulum calcium channel by protein kinase. *Nature*. 359: 739-41.

Wang, J., Maertz, N.A., Lokua, A.J., Kranias, E.G. and Valdivia, H.H. 2001. Regulation of Cardiac Ryanodine Receptors Activity By Calsequestrin. *Biophys. J. (Annual Meeting Abstracts)*. 80: 590a.

Wang, S., Trumble, W.R., Liao, H., Wesson, C.R., Dunker, A.K. and Kang, C.H. 1998. Crystal structure of calsequestrin from rabbit skeletal muscle sarcoplasmic reticulum. *Nature Struct. Biol.* 5: 476-83.

Wang, W., Cleemann, L., Jones, L.R. and Morad, M. 2000. Modulation of focal and global Ca^{2+} release in calsequestrin- overexpressing mouse cardiomyocytes. *J. Physiol.* 524: 399-414.

Wetzel, G.T., Ding, S. and Chen, F. 2000. Molecular Cloning of Junctin from Human and Developing Rabbit Heart. *Mol. Gen. Metab.* 69: 252-258.

Williams, R.W. and Beeler, T.J. 1986. Secondary structure of calsequestrin in solutions and in crystals as determined by Raman spectroscopy. *J. Biol. Chem.* 261: 12408-13.

Xu, L., Tripathy, A., Pasek, D.A. and Meissner, G. 1999. Ruthenium red modifies the cardiac and skeletal muscle Ca^{2+} release channels (ryanodine receptors) by multiple mechanisms. *J. Biol. Chem.* 274: 32680-91.

Yamaguchi, N., Igami, K. and Kasai, M. 1997. Kinetics of depolarization-induced calcium release from skeletal muscle triads in vitro. *J. Biochem. (Tokyo)*. 121: 432-439.

Yamaguchi, N., Kagari, T. and Kasai, M. 1999. Inhibition of the ryanodine receptor calcium channel in the sarcoplasmic reticulum of skeletal muscle by an ADP/ATP translocase inhibitor, atractyloside. *Biochem. Biophys. Res. Commun.* 258: 247-251.

Yamaguchi, N. and Kasai, M. 1998. Identification of 30 kDa calsequestrin-binding protein, which regulates calcium release from sarcoplasmic reticulum of rabbit skeletal muscle. *Biochem. J.* 335: 541-547.

Yamaguchi, N., Kawasaki, T. and Kasai, M. 1995. DIDS binding 30-kDa protein regulates the calcium release channel in the sarcoplasmic reticulum. *Biochem. Biophys. Res. Commun.* 210: 648-653.

Yamamoto, T., El-Heyek, R. and Ikemoto, N. 2000. Postulated Role of Inter-domain Interaction within the Ryanodine Receptor in Ca^{2+} Channel Regulation. *Trends. Cardiovasc. Med.* 10: 310-6.

Yamazawa, T., Takeshima, H., Shimuta, M. and Iino, M. 1997. A region of the ryanodine receptor critical for excitation- contraction coupling in skeletal muscle. *J. Biol. Chem.* 272: 8161-8164.

Yang, H.C., Reedy, M.M., Burke, C.L. and Strasburg, G.M. 1994. Calmodulin interaction with the skeletal muscle sarcoplasmic reticulum calcium channel protein. *Biochem.* 33: 518-25.

Yano, K. and Zarain-Herzberg.A. 1994. Sarcoplasmic reticulum calsequestrins:Structural and functional properties. *Mol. Cell. Biol.* 135: 61-70.

Yazaki, P.J., Salvatori, S. and Dahms, A.S. 1990a. Amino acid sequence of chicken calsequestrin deduced from cDNA: comparison of calsequestrin and aspartactin. *Biochem.Biophys.Res.Commun.* 170: 1089-95.

Yazaki, P.J., Salvatori, S., Sabbadini, R.A. and Dahms, A.S. 1990b. Calsequestrin, an intracellular calcium-binding protein of skeletal muscle sarcoplasmic reticulum, is homologous to aspartactin, a putative laminin-binding protein of the extracellular matrix. *Biochem.Biophys.Res.Commun.*, 166: 898-903.

Zhang, L., Franzini-Armstrong, C., Ramesh and Jones, L.R. 2001. Structural Alterations in Cardiac Calcium Release Units Resulting from Overexpression of Junctin. *J. Mol.Cell. Cardiol.* 33: 233-247.

Zhang, L., Kelley, J., Schmeisser, G., Kobayashi, Y.M. and Jones, L.R. 1997. Complex formation between junctin, triadin, calsequestrin, and the ryanodine receptor. Proteins of the cardiac junctional sarcoplasmic reticulum membrane. *J. Biol. Chem.* 272: 23389-23397.

Zhao, M., Li, P., Li, X., Zhang, L., Winkfein, R.J. and Chen, S.R. 1999. Molecular identification of the ryanodine receptor pore-forming segment. *J. Biol. Chem.* 274: 25971-25974.

Zorzato, F., Fujii, J., Otsu, K., Phillips, M., Green, N.M., Lai, F.A., Meissner, G. and MacLennan, D.H. 1990. Molecular cloning of cDNA encoding human and rabbit forms of the Ca^{2+} release channel (ryanodine receptor) of skeletal muscle sarcoplasmic reticulum. *J. Biol. Chem.* 265: 2244-56.