The actions of thiol specific oxidising agents on single channel activity of the ryanodine receptor calcium release channel in cardiac muscle

by

Kevin Richard Eager

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Statement

All experiments and data analysis that are presented in this thesis were performed by the candidate. Sarcoplasmic reticulum vesicles and proteoliposomes containing the purified ryanodine receptor were prepared by Lin Roden.

Kevin R Eager
Division of Neuroscience
John Curtin School of Medical Research
Australian National University
30 May, 1997
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Abstract

Ryanodine receptor (RyR) Ca\(^{2+}\) release channels from sheep ventricular myocardium were incorporated into artificial lipid bilayers. Single channel activity from native and purified RyRs was recorded. The sulphydryl specific oxidising agents, 2,2'-dithiodipyridine and 4,4'-dithiodipyridine, were used to examine the effects of thiol modification on RyR channel activity.

Currents recorded following the incorporation of native sarcoplasmic reticulum vesicles, or proteoliposomes containing the CHAPS-solubilised RyR, had the same properties of the cardiac RyR described by others. The channel recorded has a large cation conductance (~500 pS) and a linear current-voltage relationship in symmetrical 250 mM CsCl. Conductance decreased with high (mM) concentrations of cytoplasmic and luminal Ca\(^{2+}\). The channel is characteristically dependent on cytoplasmic Ca\(^{2+}\) for activation and is sensitive to ryanodine, ATP, Mg\(^{2+}\), caffeine and ruthenium red.

4,4'-dithiodipyridine (4,4'-DTDP) was added to RyRs with a cytoplasmic (cis) Ca\(^{2+}\) concentration of 10\(^{-7}\) M. Channel activity of native and purified RyRs increased within ~1 min and was irreversibly lost ≥5 min after adding 1 mM 4,4'-DTDP (cis). When lower concentrations of 4,4'-DTDP activated the RyR, channel activity was not always lost within the lifetime of the bilayer. Increasing the cis [Ca\(^{2+}\)] from 10\(^{-7}\) to 10\(^{-5}\) M reduced the minimum concentration of 4,4'-DTDP required to activate the RyR from 50 µM to 10 µM. This increase in the sensitivity of the RyR to activation by 4,4'-DTDP may have been due to the presence of Ca\(^{2+}\), or due to the open state of the channel. Adding 1 mM 4,4'-DTDP to native RyRs in 10\(^{-7}\) M Ca\(^{2+}\) increased open probability (P\(_o\)) from ~0.0005 to ~0.065. This was due to a ~15-fold increase in mean open time (T\(_o\)) and a ~20-fold increase in event frequency (F\(_o\)). The increase in T\(_o\) occurred because of the induction of open events of longer duration.

Similar qualitative effects on channel activity occurred after the trans addition of 4,4'-DTDP and the cis and trans additions of the lipophobic oxidising agent thimerosal. 4,4'-DTDP did not affect channel conductance or alter the sensitivity of the channel to Ca\(^{2+}\). The effects of 4,4'-DTDP were prevented by the presence of the thiol reducing agent dithiothreitol (DTT, 2 mM). When the RyR was activated by 4,4'-DTDP, the addition of DTT rapidly reversed the activation and prevented the loss of activity. However, the addition of DTT, after 4,4'-DTDP had abolished channel activity, did not restore channel function. The results indicate that the biphasic effect of 4,4'-DTDP is due to the oxidation of separate cysteine residues on the RyR, or on a protein closely associated with the RyR. Activation of the RyR by 4,4'-DTDP is likely to be due to the oxidation of a highly reactive free sulphydryl (SH) group. The loss of channel activity is likely to occur in a separate reaction, due to the oxidation of a less reactive SH group.
Activation of the RyR by 4,4'-DTDP did not alter usual modification of channel activity by ryanodine or ruthenium red. The actions of 4,4'-DTDP were examined when RyR activity was altered by physiological and pharmacological agents. Properties of channel activation by 4,4'-DTDP were influenced by most reagents tested, but no reagent had any influence on the ability of 1 mM 4,4'-DTDP to induce the loss of channel activity after 5-10 min.

4,4'-DTDP was added to RyRs over a wide range of cis Ca\(^{2+}\) concentrations between \(10^{-9}\) and \(2 \times 10^{-2}\) M. 4,4'-DTDP increased open probability by introducing long components into the open time distributions, increasing mean open time by up to 50-fold. There was no consistent effect of 4,4'-DTDP on channel closed times. 4,4'-DTDP (\(10^{-7}\) to \(10^{-3}\) M) activated the RyR at lower concentrations in \(10^{-3}\) M cis Ca\(^{2+}\). 2,2'-DTDP modified Ca\(^{2+}\)-activated RyR activity in the same way as it was by 4,4'-DTDP. Both reactive disulfides are likely to act at the same site to modify RyR activity. RyRs inhibited by 1 mM Mg\(^{2+}\) (in \(10^{-7}\) M cis Ca\(^{2+}\)) or 10 mM Mg\(^{2+}\) (in \(10^{-2}\) M cis Ca\(^{2+}\)) were activated by 4,4'-DTDP. However, open probability and mean open time, of the 4,4'-DTDP-activated RyRs, were lower than usual because of the presence of Mg\(^{2+}\). The number of openings within the long open time components, of the 4,4'-DTDP-activated RyR, was reduced by Mg\(^{2+}\).

Channels inhibited by pH 6.5 were activated by 1 mM 4,4'-DTDP due to an increase in mean open time and the addition of extra components to the open time distribution. However, the low pH prevented the usual increases in \(T_o\), and the occurrence of open events in the longest time constant component, that were seen following 4,4'-DTDP-activation in pH 7.4. RyRs in \(10^{-7}\) M Ca\(^{2+}\) were activated by ATP (4 mM) and caffeine (2 mM). The addition of 1 mM 4,4'-DTDP to ATP or caffeine-activated RyRs further increased channel activity. The presence of caffeine increased the degree of activation by 4,4'-DTDP, increased mean open time and influenced the distribution of open times. In contrast, the presence of ATP had no effect on most measured parameters of the 4,4'-DTDP-activated RyR including \(P_o\), \(T_o\) and \(F_o\) and the average open and closed time constants. The rate of channel activation by 4,4'-DTDP was increased from \(~40\) s in \(10^{-7}\) M Ca\(^{2+}\) to \(~2 - 3\) s when the channel was activated by either \(10^{-5}\) and \(10^{-4}\) M Ca\(^{2+}\) or by 4 mM ATP. However, the rates of 4,4'-DTDP-induced activation were slow for caffeine-activated and Ca\(^{2+}\)-inhibited RyRs (>35 s). The rate of RyR activation by 4,4'-DTDP appears to be influenced by a complex function of ligand binding and channel open probability. The degree of 4,4'-DTDP-induced activation is also modified by ligand binding and channel open probability. It is argued that 4,4'-DTDP activates the RyR by oxidising a cysteine residue(s) in close proximity to the binding site for ATP. A model is shown to explain the actions of SH reagents on the RyR.
Publications

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

Refereed publications:


Abstracts:


Commonly used abbreviations

2,2'-DTDP  2,2'-dithiodipyridine
4,4'-DTDP  4,4'-dithiodipyridine
ATP      adenosine 5'-triphosphate
BAPTA   1,2-bis(2-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid
CHAPS   3-([3-cholamidopropyl]dimethylammonio)-1-propanesulphonate
CICR    Ca$^{2+}$-induced Ca$^{2+}$ release
DHP      dihydropyridine
DTT      dithiothreitol
EGTA    ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid
E$_{rev}$  reversal potential
FKBP    FK506 binding protein
F$_o$    event frequency
GSH     reduced glutathione
HSR     heavy sarcoplasmic reticulum
kDa     kilodalton
LSR     light sarcoplasmic reticulum
MES     2-[N-Morpholino]ethanesulfonic acid
PC      palmitoyl-oleoyl-phosphatidylcholine
PE      palmitoyl-oleoyl-phosphatidylethanolamine
PMSF   phenylmethylsulfonyl fluoride
P$_o$   open probability
PS      palmitoyl-oleoyl-phosphatidylserine
RDS     reactive disulfide
ROS     reactive oxygen species
RyR     ryanodine receptor
SDS-PAGE sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SH      sulfhydryl
SR      sarcoplasmic reticulum
TES     N-tris[hyroxymethyl]methyl-2-aminoethanesulfonic acid
T$_o$   mean open time
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6.1 Concluding remarks

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Chapter 1

GENERAL INTRODUCTION

The L-type Ca\(^{2+}\) receptor (L-Ca\(^{2+}\)R) is a membrane protein that is specialized for high-affinity, low-affinity Ca\(^{2+}\) ions from the sarcoplasmic (and endoplasmic) reticulum into the cell (Catterall et al., 1984). It is found in many cell types, from muscle cells to neurons and also in non-excitable cells (Sakai and Arimura, 1990). The L-Ca\(^{2+}\)R is a large, 2.5 membrane-spanning ligand-gated ion channel that has a single-channel conductance for Ca\(^{2+}\) of 90-150 pS (Williams, 1972), and in vivo in the free membrane structure, the L-Ca\(^{2+}\)R has been shown to be responsive to specific conditions (Frattini, 1976). It is expressed in various tissues including Ca\(^{2+}\), ATP, Mg\(^{2+}\), IL, and somatostatin (Meissner, 1980). In vivo, the L-Ca\(^{2+}\)R activity is influenced by regulatory proteins that control Ca\(^{2+}\) influx and efflux, including regulatory subunits and Ca\(^{2+}\) buffering proteins. The activity of the L-Ca\(^{2+}\)R is also regulated by Ca\(^{2+}\)-binding proteins and the local environment, including pH and temperature. The L-Ca\(^{2+}\)R activity is also influenced by various cellular factors, including Ca\(^{2+}\)-dependent enzymes and Ca\(^{2+}\)-binding proteins.

1.1 Calcium: Its Involvement in Muscle Contraction

1.1.1 Ca\(^{2+}\) Regulation

Ca\(^{2+}\) plays a crucial role in the regulation of cellular processes, including muscle contraction. The resting cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_{cyt}\)]) is maintained at ~100 nm (Pappas et al., 1984). This is quite remarkable considering that cytosolic Ca\(^{2+}\) gradients across the cell membrane (~1.2 mM Ca\(^{2+}\) in the extracellular fluid) are very steep. The cell membrane itself is impermeable to Ca\(^{2+}\) ions. Ca\(^{2+}\) will only cross the membrane through specialized channels, pumps, and carriers. Within cells, Ca\(^{2+}\) is stored primarily in endoplasmic reticulum (ER) or sarcoplasmic reticulum (SR), a network of specialized organelles (ER). Upon receiving the appropriate signal, the store releases their Ca\(^{2+}\) into the cytoplasm, triggering changes in their function, by modulating a range of physiological effects. The release of Ca\(^{2+}\) activates contraction of muscle fibers.
GENERAL INTRODUCTION

The ryanodine receptor (RyR) is a membrane protein that is specialised to allow the flow of Ca\(^{2+}\) ions from the sarcoplasmic (and endoplasmic) reticulum into the cell (Coronado et al., 1994). It is found in many cell types, from muscle cells to neurones and also in non-excitable cells (Sutko and Airey, 1996). The RyR is a large 2.2 megadalton ligand-gated homotetramer that has a single channel conductance for Ca\(^{2+}\) of \(-90\) to \(-100\) pS (Williams, 1992), and is one of the few membrane structures large enough to be seen under electron microscope (Franzini-Armstrong, 1970). Channel activity of the RyR is regulated by numerous ligands including Ca\(^{2+}\), ATP, Mg\(^{2+}\), H\(^+\) and calmodulin (Meissner, 1994). In the 1980s, investigations revealed that channel activity of the RyR was also affected by reagents that interact with sulphydryl groups. Initial studies showed that heavy metals interact with a sarcoplasmic reticulum protein to cause Ca\(^{2+}\) efflux (Abramson et al., 1983; Bindoli and Fleischer, 1983). Subsequently, the activity of single RyR channels was shown to be increased by sulphydryl oxidising agents including reactive oxygen species (Ondrias et al., 1990; Holmberg et al., 1991). Reactive oxygen species (ROS) and other oxidising agents are potentially very damaging to muscle. Cardiac muscle exposure to oxidising agents during the reperfusion of ischaemic tissue is associated with intracellular Ca\(^{2+}\) overload and cell death (Kaneko et al., 1994). The activation of RyRs by oxidising agents may contribute to the Ca\(^{2+}\) overload that occurs during cardiac reperfusion injury (Holmberg et al., 1991; Boraso and Williams, 1994). A knowledge of the general interactions between RyRs and SH reagents is an important step to understanding the in vivo actions of such reagents on the RyR. The effects of sulphydryl reagents on the single channel activity of cardiac RyRs has not been investigated in depth and is the focus of the present study.

1.1 Calcium: its involvement in muscle contraction

1.1.1 Ca\(^{2+}\) regulation

In most cells, the resting cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) is maintained at ~100 nM (Petersen et al., 1994). This is quite remarkable considering the enormous Ca\(^{2+}\) gradient across the cell membrane (~1.2 mM Ca\(^{2+}\) in the interstitial fluid). The cell membrane itself is impermeable to Ca\(^{2+}\) ions. Ca\(^{2+}\) will only cross the membrane through specialised channels, pumps and carriers. Within cells, Ca\(^{2+}\) is stored primarily in endoplasmic reticulum (ER) or sarcoplasmic reticulum (SR; a muscle specialisation of ER). Upon receiving the appropriate stimuli the stores release their Ca\(^{2+}\) into the cytoplasm, through channels in their membrane, to initiate a range of physiological effects. The increase in [Ca\(^{2+}\)], activates mechanisms to
lower the [Ca$^{2+}$], back to resting levels. These mechanisms extrude Ca$^{2+}$ from the cytosol using sarcolemmal pumps and ion exchangers, or sequester Ca$^{2+}$ back into the intracellular stores via active pumps.

1.1.2 Ca$^{2+}$ and muscle contraction

Rapid changes in [Ca$^{2+}$], enable the contraction and relaxation of muscle. During relaxation [Ca$^{2+}$], is low (~10$^{-7}$ M). The [Ca$^{2+}$] must rapidly rise 1 - 3 orders of magnitude (Meissner, 1986a) to initiate contraction, which occurs when the Ca$^{2+}$ binds to specific Ca$^{2+}$-binding sites on the contractile proteins. The primary source of this Ca$^{2+}$ is the SR. There are two known SR/ER Ca$^{2+}$ channel proteins involved in Ca$^{2+}$ efflux, the RyR and the inositol 1,4,5-triphosphate (IP$_3$) receptor (Petersen et al., 1994). The IP$_3$ receptor is predominantly involved in Ca$^{2+}$ release from smooth muscle and in non-muscle cells (Ogawa, 1994), while the RyR is the functionally important channel in striated muscle contraction.

The process whereby depolarisation of muscle fibres causes muscle contraction is called excitation-contraction coupling (ECC). A model of ECC in striated muscle involves the translation of an electrical signal in the fibre surface to a mechanical action of the contractile proteins utilising chemical mediators. A wave of depolarisation travels along the sarcolemma and deep into the muscle fibres down the transverse tubules. The depolarisation is sensed by the dihydropyridine (DHP) receptor L-type Ca$^{2+}$ channel (voltage sensor). As a result, a signal (either a direct mechanical coupling as hypothesised for skeletal muscle or an inward Ca$^{2+}$ current (I$_{Ca}$) in cardiac muscle) triggers the release of Ca$^{2+}$ from the SR via the RyR-Ca$^{2+}$-release channel. The released Ca$^{2+}$ triggers a series of events that results in muscle contraction: firstly, Ca$^{2+}$ binds to troponin C. This weakens the binding of troponin I to actin, which allows tropomyosin to move laterally. The movement of tropomyosin uncovers the binding site on the actin filament for the myosin head. This enables the actin and myosin filaments to slide past each other, due to the ATP-dependent formation and cycling of cross bridges between the myosin heads and actin filaments, causing contraction of the fibres. The basis for this process was proposed in 1954 in two independent reports (Huxley and Hanson, 1954; Huxley and Niedergerke, 1954) and was termed the sliding filament theory of muscle contraction. This conceptual understanding of ECC represented a giant progression in knowledge and followed the recognition, only a little over half a century earlier, of the importance of external Ca$^{2+}$ in the maintenance of the beating heart (Ringer, 1883).

1.1.3 An historical perspective

By the 1940s, Ca$^{2+}$ was known to have a direct effect on muscle contraction: Heilbrunn’s classical theory of muscle contraction (Heilbrunn and Wiercinski, 1947) suggested that, upon electrical stimulation, Ca$^{2+}$ enters the cell
and contraction occurs. This suggestion was made after the observation that Ca\(^{2+}\) solutions (but not solutions with other mono- or divalent cations) injected into frog skeletal muscle fibres resulted in fibre shortening. However, the proposal that a chemical agent of extracellular origin could enter the muscle fibre and diffuse with sufficient speed into the interior of the muscle fibre to enable simultaneous contraction of the whole fibre was refuted by Hill (1948). His calculations demonstrated that the period between electrical stimulation and contraction was too short for diffusion.

With the opinion that an activator substance was unlikely to result in contraction if released at the sarcolemma upon depolarisation, interest turned to the complex network of internal membrane structures being imaged by electron microscopy. The electron microscope was widely used during the 1950s to study the structure-function aspects of muscle physiology. By the late 1950s investigators were able to describe the complex but highly ordered system of internal membranes. The concept that such a membrane network could carry the excitatory impulses into the muscle was not new (Retzius, 1881), but electron microscopy provided the physical basis for the concept. Porter and Palade (1957) described the regularly spaced “triad” formation, comprising a central transverse tubule (T-tubule) flanked by the lateral sacs of the SR. These triads were periodically localised to regular elements of the sarcomere (Porter and Palade, 1957; Huxley, 1964). Huxley and Taylor (1958) elegantly demonstrated that contractions were only generated at certain restricted regions of the sarcomere, using highly localised membrane depolarisations applied with a glass micropipette. These regions coincided with the location of the triad. This was common in both the frog, where the conducting path and triad position existed in the plane of the Z disk, and the crab where the sensitivity to localised depolarisation focused at the boundary between the A-I bands. Thus, it was reasonable to assume that the triads were important sites at which membrane excitation could result in contraction, and provided an alternative to the diffusion model negated by Hill (1948).

By the mid 1960s the role of the SR and its involvement in ECC became clearer. The Ca\(^{2+}\) responsible for muscle contraction originated from the SR (Hasselbach, 1964) and was stored in its lateral sacs (Costantin et al., 1965). This promoted the suggestion by Costantin et al. (1965) that the lateral sacs of the SR may be the regions “from which Ca\(^{2+}\) is released to trigger contraction”; a proposal that was soon confirmed by Winegrad (1965; 1970) whose initial studies using autoradiographic techniques suggested that Ca\(^{2+}\) was released from the terminal cisternae of the SR. Thus, a picture was emerging that electrical stimulation of the internal membranes of the muscle caused Ca\(^{2+}\) release from the SR, and it was this Ca\(^{2+}\) which was responsible for the eventual contraction of the muscle.

Relaxation of muscle was found to be connected with the removal of Ca\(^{2+}\) from the myoplasm. Relaxation was defined by Hill (1949) as the process
"whereby, after contraction, the muscle returns to its initial length or tension". The proposal that relaxation was due to depletion of ATP from the myoplasm (Engelhardt, 1946) was superseded following the isolation of a "relaxation factor" - a protein with ATPase activity (Marsh, 1951; Marsh, 1952; Bendall, 1952; Bendall, 1953). Ebashi (1960; 1961) and Hasselbach & Makinose (1961) demonstrated the Ca\(^{2+}\) binding properties of the "relaxation factor" upon the formation of native SR membrane into vesicles. These vesicles were capable of accumulating Ca\(^{2+}\) against extremely high electrochemical gradients in the presence of ATP. Soon after, Weber et al. (1963) demonstrated that the microsomal fraction of muscle with the ATPase activity caused relaxation by removing myoplasmic Ca\(^{2+}\), as well as the Ca\(^{2+}\) bound to the contractile proteins.

1.2 Identification and isolation of the RyR

By the mid 1960s there was strong evidence suggesting the SR was the store for the Ca\(^{2+}\) which, when released, caused muscle contraction. However, a connection between the SR and membrane depolarisation had not been established.

Since initial descriptions of the close proximity of the T-tubule to the lateral sacs of the SR (Porter and Palade, 1957), and the recognition of this region as a site for the generation of muscle contraction, over two decades of extensive research passed before a definite link between the two membrane systems was shown. Much of the preliminary work came from two laboratories: Fleischer’s laboratory at Vanderbilt University, and Meissner’s laboratory at the University of North Carolina. This work concentrated, initially, on the mechanism of Ca\(^{2+}\) release. An important study in 1975 (Meissner, 1975) showed that the SR could be fractionated into two populations: a light fraction thought to comprise the longitudinal SR, and a heavy fraction thought to be terminal cisternae SR. Isolation of the heavy fraction showed that the vesicles released Ca\(^{2+}\) when exposed to micromolar concentrations of Ca\(^{2+}\). This observation supported the report of Winegrad (1970) proposing that the longitudinal SR contained relaxation sites while the terminal cisternae contained the release sites. Meissner (1984) showed that the Ca\(^{2+}\) efflux from "heavy" SR was potentiated by adenine nucleotides and inhibited by Mg\(^{2+}\), and suggested that a "putative Ca\(^{2+}\) channel may be active during Ca\(^{2+}\) release". The absence of Ca\(^{2+}\) release from the population of "light" SR vesicles lent further support for the concept that a specific membrane component in terminal cisternae SR was the conduit for Ca\(^{2+}\) efflux.

An increasing number of reagents were found to be capable of altering the Ca\(^{2+}\) efflux across the membrane of the SR. These agents included Ca\(^{2+}\), adenine nucleotides, Mg\(^{2+}\), heavy metals, ruthenium red, ryanodine and local anaesthetics. Consequently, a number of hypotheses were proposed to explain T-tubule activation of Ca\(^{2+}\) release: 1) charge displacement within SR or T-tubule membrane
(Schneider and Chandler, 1973); 2) Ca$^{2+}$ induced Ca$^{2+}$ release (CICR) (Fabiato and Fabiato, 1975); 3) depolarisation of SR membrane (Kasai and Miyamoto, 1973); 4) increase in cytoplasmic pH (Shoshan et al., 1981); 5) IP$_3$-induced Ca$^{2+}$ release (Vergara et al., 1985; Volpe et al., 1985) and 6) sulfhydryl oxidation-induced Ca$^{2+}$ release (Trimm et al., 1986). Most reports by the early to mid 1980s strongly suggested that the release of Ca$^{2+}$ from SR was through a putative "Ca$^{2+}$ channel" (Bindoli and Fleischer, 1983; Chamberlain et al., 1984a; Meissner, 1984).

1.2.1 Identification of the SR Ca$^{2+}$ release channel

The possibility that a ligand gated channel could be responsible for the flow of Ca$^{2+}$ ions into the myoplasm was strengthened with a report from Meissner’s laboratory in 1985. Ca$^{2+}$ channels in the membrane of terminal cisternae SR (from rabbit skeletal muscle) were discovered (Smith et al., 1985) following the insertion of the SR vesicles into planar lipid bilayers (Miller and Racker, 1976) - bilayers were created using the technique of Mueller et al. (1962). Smith et al. (1985) reported high conductance channels selective for divalent cations that were activated by adenine nucleotides and blocked by ruthenium red. The unusually large conductance of the channel (170 pS in 50 mM Ba$^{2+}$) and its rapid activation kinetics, suggested that the channel was the putative Ca$^{2+}$-release channel in the terminal cisternae membrane.

a) Effect of ryanodine on SR Ca$^{2+}$ release

The highly toxic alkaloid, ryanodine, is isolated from the stem and root of the plant Ryania speciosa Vahl. Ryanodine was widely used for its insecticidal properties but its effects on muscle have been of interest since the 1950s (Jenden and Fairhurst, 1969). Muscle physiologists were interested in the high potency of ryanodine (effects on muscle were observed with concentrations as low as 10$^{-9}$ M) and the varying effects of the drug between species and muscle type (for review see Jenden and Fairhurst, 1969). In vertebrate striated muscle ryanodine produces an irreversible contracture, while in mammalian cardiac muscle ryanodine causes a negative inotropism (Hillyard and Procita, 1959). These findings led to speculation about the actions of ryanodine that ranged from altering Ca$^{2+}$ movement across the SR (Fairhurst and Jenden, 1962) to effects on the sarcolemma (Frank and Sleator, 1975). Sutko et al. (1979) proposed that ryanodine inhibited the release of Ca$^{2+}$ from cardiac SR. Initial studies looking at net Ca$^{2+}$ flux across cardiac and skeletal junctional SR vesicles indicated that ryanodine stimulated Ca$^{2+}$ uptake (Jones and Cala, 1981; Seiler et al., 1984). However, Chamberlain et al. (1984b) confirmed the hypothesis of Sutko et al. (1979) when they showed that ryanodine blocked Ca$^{2+}$ release from canine cardiac SR vesicles, resulting in the rapid Ca$^{2+}$ uptake described in earlier studies.
With the finding that ryanodine blocked SR Ca\(^{2+}\) release, the question arose as to why skeletal muscle would undergo a prolonged state of contracture? This was answered by Fleischer et al. (1985) who demonstrated, using skeletal SR vesicles and low concentrations of ryanodine (100 nM), that the channel could be locked into an open state. The flood of Ca\(^{2+}\) into the myoplasm induced by ryanodine presumably saturated the ability of the SR to reaccumulate the Ca\(^{2+}\), thereby preventing muscle relaxation. This finding was confirmed by Meissner (1986b) who demonstrated, also using skeletal SR vesicles, that ryanodine could indeed stimulate Ca\(^{2+}\) efflux at low concentrations. However, it was also shown that ryanodine could block the Ca\(^{2+}\) release at higher concentrations (>10 µM) (Meissner, 1986b). Finally, Rousseau et al. (1987) incorporated terminal cisternae SR membrane from skeletal and cardiac muscle into lipid bilayers and studied the effects of ryanodine on the activity of single Ca\(^{2+}\) conducting channels. The effect of ryanodine was twofold: it reduced conductance of the channel while increasing, almost to unity, its open probability. The effects of ryanodine were irreversible and were common to both skeletal and cardiac Ca\(^{2+}\) release channels. The ability of ryanodine to block a cloned human cardiac K\(^+\) channel (Bhattacharyya et al., 1995) showed that ryanodine was not specific for the RyR. The apparently conflicting evidence that ryanodine caused contracture in skeletal muscle while producing negative inotropism in cardiac muscle was accounted for by the differences in the Ca\(^{2+}\) handling ability between the tissues. Cardiac muscle has a greater capacity to extrude Ca\(^{2+}\) from the myoplasm via the sarcolemmal Na\(^+\)-Ca\(^{2+}\) exchanger than skeletal muscle (Reuter and Seitz, 1968; Philipson, 1985). Therefore, the ryanodine-induced accumulation of Ca\(^{2+}\), that causes contraction in skeletal muscle may be avoided in cardiac muscle because of efficient Ca\(^{2+}\) removal from the myoplasm. This may cause the depletion of SR Ca\(^{2+}\) stores resulting in a reduction in myocardial contractile strength (Rousseau et al., 1987).

\textbf{b) Isolation of the ryanodine receptor}

The terminal cisternae of SR and the T-tubule are separated by a gap of 10 - 15 nm. Between these two membrane systems are arrays of electron-dense projections, first referred to as SR feet (Franzini-Armstrong, 1970). These “feet” appeared to be attached to the SR membrane, but it was unclear at the time whether they were joined to the T-tubule (Franzini-Armstrong, 1975). However, their localisation between the T-tubule and SR membranes gave reason to speculate that they may provide a mechanism for communication between the two membrane systems. The feet were later shown to be localised to the terminal cisternae, not the longitudinal fraction of SR membrane (Saito et al., 1984).

In 1987, Fleischer and co-workers purified the receptor for ryanodine from the terminal cisternae of skeletal SR (Inui et al., 1987). The purified receptor had a ryanodine binding affinity that was similar to that of SR vesicles. Purification of the RyR showed that the feet structures were the proteins which bind ryanodine,
and that the receptor for ryanodine and the Ca$^{2+}$-release channel were the same “functional unit” (Inui et al., 1987). The high binding affinity of ryanodine to the SR Ca$^{2+}$ release channel proved fundamental in the isolation and purification of the protein.

### 1.3 Isoforms and structure of the RyR

Since the purification of the RyR and its recognition as the “foot structure”, which bridges the gap between the T-tubule and the SR, much effort has focused on the structure-function relationship of the receptor. The number of agents shown to interact with the receptor (see below) is not surprising considering ~90% of the unusually large protein resides in the cytoplasm (Takeshima et al., 1989). Its size and structure reflects the complexity of the job it is required to perform. Not only must it rapidly respond to a signal delivered from the DHP receptor and open its gates to release the Ca$^{2+}$ required for muscle contraction, but there must also be a mechanism to shut the gates to stop the release of Ca$^{2+}$ into the myoplasm and enable the muscle to relax. In addition, the number of regulatory sites for the binding of endogenous agents including Ca$^{2+}$, Mg$^{2+}$ and ATP along with the association of proteins, such as calmodulin, triadin and FK506 binding protein (for review see Coronado et al., 1994), implies that its response to the signal from the DHP receptor is modulated by its environment.

#### 1.3.1 Identification and distribution of three isoforms

There are 3 known isoforms of the mammalian RyR, each encoded for by a separate gene (Takeshima et al., 1995). The primary structures for each of the 3 isoforms have been determined by cloning and sequence analysis of the complementary DNA. The cDNA for the skeletal isoform (RyR1) encodes a protein of 5037 amino acids with a Mr of 565,223 (Takeshima et al., 1989); the cDNA for the cardiac isoform (RyR2) encodes a protein of 4969 amino acids with a Mr of 564,711 (Otsu et al., 1990) and the cDNA for the brain isoform (RyR3) encodes for the smallest of the 3 proteins with 4782 amino acids and a Mr of 551,901 (Hakamata et al., 1992). The 3 isoforms share a 66 - 70% homology in amino acid sequence. The 2.2 megadalton RyR protein is comprised of four identical RyR subunits.

Each of the isoforms has a similar topology based upon hydropathy profiles (Hakamata et al., 1992): the carboxy-terminal tenth of each of the molecules have 4 highly hydrophobic domains, each with at least 20 amino acid residues, designated M1, M2, M3 and M4. These have been suggested as the trans-membrane spanning regions of the protein which may comprise the channel (Takeshima et al., 1989). However, as many as twelve possible membrane spanning regions have been identified (Otsu et al., 1990; Zorzato et al., 1990). The expansive N terminal portions of each of the isoforms have been ascribed to the
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cytoplasm and contain predominantly hydrophilic residues as well as consensus phosphorylation sites and ligand binding sites for nucleotides, calmodulin, Mg$^{2+}$ and Ca$^{2+}$.

Each of the RyR isoforms have a distribution wider than their names suggest. RyR1 is the main isoform in skeletal muscle. However, it has also been found in mouse cerebellum and sea urchin egg, and its mRNA has been found in smooth muscle (McPherson and Campbell, 1993; Coronado et al., 1994; Giannini et al., 1995; Neylon et al., 1995). Low levels of mRNA for RyR1 were also found in heart using sensitive detection techniques (Marks et al., 1989). RyR2 is the predominant isoform in mammalian cardiac muscle. Differences in single channel RyR activity from left ventricular free wall and interventricular septum led to the suggestion that different RyR isoforms were being recorded (Borgatta et al., 1991). However, these variations were not seen in a subsequent study which showed no functional differences in single channel activity or $[^3H]$ryanodine binding characteristics between left ventricular free wall, interventricular septum or atrium (Xu et al., 1993). In addition to its presence in cardiac muscle, RyR2 is also the predominant isoform in the brain and is found in guinea pig endothelium (Otsu et al., 1990; Hakamata et al., 1992; Coronado et al., 1994). The brain isoform (RyR3) has a narrow distribution in the CNS (expressed in highest amounts in the corpus striatum, thalamus, and hippocampus as well as distributions found in the medulla, pons and midbrain). Low levels of RyR3 have also been reported in mammalian skeletal and cardiac muscle (Giannini et al., 1995; Conti et al., 1996), including the skeletal muscle of dyspedic mice (Takeshima et al., 1995), but it is widely expressed in smooth muscle (Hakamata et al., 1992; Coronado et al., 1994). All three RyR isoforms are expressed in the vascular smooth muscle of rat aorta (Neylon et al., 1995).

It is not uncommon for many cell types to express two RyR isoforms. In fish skeletal muscle, 2 RyR isoforms designated α and β have been reported (O'Brien et al., 1995). From the cDNA sequences of frog skeletal muscle, α-RyR has the highest identity (80%) to RyR1 and β-RyR has 86% identity to RyR3 (Ogawa, 1994). The α and β RyR isoforms have also been identified in other non-mammalian vertebrate skeletal muscle (Coronado et al., 1994). It was speculated that muscle types requiring a continuous pattern of activity such as those involved in respiration and posture had a greater RyR3 content. Whether double (or even multiple) isoforms fulfil a functional purpose or are merely an evolutionary ancillary is yet to be determined.

1.3.2 Resolving the structure of the purified RyR

Electron microscopy of the purified mammalian RyR revealed a structure with four-fold symmetry from both skeletal (Lai et al., 1988) and cardiac (Anderson et al., 1989) muscle, as well as the brain (Lai et al., 1992), with
dimensions of \( \sim 30 \times 30 \) nm. These “clover-leaf” or quatrefoil structures are analogous to the feet structures first described by Franzini-Armstrong (1970).

Cryo-electron microscopy and three-dimensional reconstruction have improved the resolution with which the channel can be viewed to \( \sim 3 \) nm. The skeletal RyR is described as a molecule with a large cytoplasmic domain \( (29 \times 29 \times 12 \text{ nm}) \) with a small protrusion into the SR lumen \( (\sim 7 \text{ nm}) \) (Radermacher et al., 1994). The cardiac RyR has a three dimensional architecture which is very similar to that of the skeletal isoform (Sharma et al., 1997). Structurally, the cytoplasmic domain of the RyR has an architecture resembling scaffolding that may be beneficial in withstanding the mechanical forces imposed upon it during fibre contraction and relaxation. A central channel of low density with an apparent diameter of \( 2 - 3 \) nm runs through the transmembrane assembly. The central channel is thought to branch into 4 radially running canals which exit at the junction between the cytoplasmic domain and the transmembrane assembly (Radermacher et al., 1994). Using a slightly different approach to visualise the channel, cryo-electron microscopy and angular reconstruction also provided a three dimensional image of the RyR. With this technique, the channel was viewed in two conformational states: a closed and a “ryanodine modified”, or open, state (Orlova et al., 1996). In the “open” state a hole in the putative transmembrane region was identified. Radermacher et al. (1994) described a “channel plug” (a globular density) in the centre of the putative channel with an apparent diameter of \( 3.0 - 3.5 \text{ nm} \). They speculated that gating involves movement or structural change of this “plug”. The concept of a plug is consistent with the finding of Orlova et al. (1996) who described a “hole” in the open channel which was not visible when the channel was closed. The “canals” and “holes” described in these studies may be the ion conduction pathway of the protein.

1.4 RyR regulation

In the early 1970s, researchers were grappling with the puzzle of linking T-tubule depolarisation with SR \( \text{Ca}^{2+} \) release during ECC. Schneider and Chandler (1973) proposed a model for skeletal muscle involving a direct mechanical coupling between molecules in the membranes of the T-tubule and SR. They found that a voltage dependent charge movement occurred across the T-tubule membrane when ion movement across the membrane was blocked. Based on this observation they speculated that charged molecules in the T-tubule, which spanned the T-tubule/SR gap and were physically blocking SR \( \text{Ca}^{2+} \) release, detected this charge movement. It was this change in charge across the T-tubule membrane that was proposed to cause the movement of the inhibitory molecules thereby relieving the block of \( \text{Ca}^{2+} \) release from the SR. The basis of this model has yet to be disproved. An alternative hypothesis that \( \text{Ca}^{2+} \) stores in the wall of the T-tubule activate the skeletal RyR by CICR has also been proposed (Bianchi, 1968; Bianchi,
1969; Frank, 1980). Unlike skeletal muscle, contraction of cardiac myocytes does not occur in the absence of external Ca$^{2+}$ (Nabauer et al., 1989). The hypothesis of CICR proposed by Fabiato and Fabiato (1975) is widely accepted as the mechanism by which the cardiac RyR is activated. Very few functional studies have been carried out on RyR3 (the brain isoform) but, as for the cardiac channel, it is suspected to be activated by CICR - although, possibly with a higher [Ca$^{2+}$] threshold for activation (Takeshima et al., 1995; Conti et al., 1996).

These hypotheses concentrate on the links between T-tubule potential, RyR activation and intracellular Ca$^{2+}$ transients. However, it has become apparent from numerous in vitro studies that channel activity, after the initial activation, is modulated by numerous endogenous agents such as Mg$^{2+}$, calmodulin, ATP, protein kinases, sulphydryl reagents and even Ca$^{2+}$ itself. The effects of DHP receptor activity, pharmacological reagents and other cytoplasmic or SR membrane proteins on the function of RyR1 and RyR2 are described in this section. The section is intended primarily as a brief introduction into channel function and regulation rather than a comprehensive review of the literature (for recent examples of such reviews see Coronado et al., 1994; Meissner, 1994).

### 1.4.1 Dihydropyridine receptor

The DHP receptor is an L-type Ca$^{2+}$ channel located in the T-tubule of muscle cells as well as in the surface of many other cell types (Schneider, 1994). The protein is comprised of five subunits (α$\text{I}$, α$\text{II}$, β, γ and δ) (Catterall, 1988; Takekura et al., 1995) and carries Ca$^{2+}$ currents that are blocked by dihydropyridines. In skeletal muscle the 212 kDa DHP receptor α$\text{I}$ subunit (Tanabe et al., 1987) serves as the voltage sensor for SR Ca$^{2+}$ release; <5% of the DHP receptors act as functional Ca$^{2+}$ channels (Schwartz et al., 1985; Rios and Brum, 1987). In contrast, the higher molecular weight cardiac DHP receptor α$\text{I}$ subunit (242 kDa), which shares a 66% amino acid sequence homology with the skeletal isoform (Mikami et al., 1989), activates the cardiac RyR by an L-type Ca$^{2+}$ current when the T-tubule membrane is depolarised (Cannell et al., 1987; Nabauer et al., 1989).

#### a) Skeletal muscle

The precise mechanism by which DHP receptor activation results in SR Ca$^{2+}$ release is unknown, but ultrastructural and biophysical evidence supports Schneider and Chandler’s (1973) proposal for a direct mechanical coupling between the DHP receptor and the RyR (Rios and Brum, 1987; Block et al., 1988; Tanabe et al., 1990). Tanabe et al. (1990) showed that the putative cytoplasmic loop between transmembrane segments II and III of the α$\text{I}$ subunit was critical for skeletal type ECC. Subsequently, an interesting study was conducted in which the II - III loop was expressed in Escherichia coli (Lu et al., 1994). Adding the
expressed skeletal or cardiac II-III loop peptide to single channel skeletal RyRs, incorporating into lipid bilayers, increased the open probability of the channel. The open probability of the cardiac RyR was unaffected by addition of either the skeletal or the cardiac II-III loop peptide. This suggested that the variability in channel activation mechanisms between the skeletal and cardiac RyR isoforms is due to differences in the RyR molecule rather than the DHP receptor. This suggestion is supported by the recent study of Nakai et al. (1997) who showed that the reciprocal signalling that occurs between the DHP receptor and the RyR in skeletal muscle is a feature unique to RyR1.

DHP receptors in skeletal T-tubule cluster in groups of four to form a "tetrad" (Block et al., 1988). Morphological (Block et al., 1988) and biochemical (Bers and Stiffel, 1993) studies provide evidence that the ratio of RyRs to the tetrads is close to 2:1. Viewing the junctional T-tubule membrane from freeze-fracture images, the tetrads appear to be located directly opposite alternate RyRs (Block et al., 1988). A simple stoichiometry of this kind does not refute the hypothesis of a direct physical coupling between the DHP receptor and the RyR. However, of interest is the apparently "spare" or tetrad-free RyR: whether this RyR is activated by a CICR mechanism is not known. Another group of RyRs that are not directly associated with tetrads are the extra-junctional RyRs, which may also contribute to Ca\(^{2+}\) entry into the myoplasm during ECC (Dulhunty et al., 1992).

b) Cardiac muscle

The formation of DHP receptors into the tetrad conformation that is seen in skeletal muscle, is not apparent in cardiac T-tubule membrane. The ratio of RyRs to DHP receptors in myocytes is ~3 - 10 (depending on species) compared to 0.52 in skeletal triads (Bers and Stiffel, 1993). Such a stoichiometry suggests that a regenerative signal could be produced by CICR. A direct physical coupling between DHP receptor and the RyR was put forward as an hypothesis for cardiac ECC (Cohen and Lederer, 1988), but has received little support in subsequent studies (Balke and Wier, 1991). However, a functional coupling exists between the DHP and ryanodine receptors in cardiac myocytes (Sham et al., 1995; Adachi-Akahane et al., 1996). Ca\(^{2+}\) entering the myocyte in response to T-tubule depolarisation, i.e. through the DHP receptor, is 1 - 2 orders of magnitude more effective in gating Ca\(^{2+}\) efflux from the SR than Ca\(^{2+}\) entry through the Na\(^{+}\)/Ca\(^{2+}\) exchanger (Sham et al., 1995). This suggests a close association between the DHP receptor and the RyR. Interestingly, RyRs in cardiac myocytes are not just located junctionally or extra-junctionally on the terminal cisternae of SR. Cardiac myocytes contain corbular SR which is not in physical contact with membrane of the T-tubule or the sarcolemma, and contains RyRs which participate in CICR during ECC (Jorgensen et al., 1993).
1.4.2 Endogenous modulators of RyR activity

Many pharmacological studies of the RyR have been performed using either SR vesicles or single RyRs incorporated into an artificial lipid bilayer. In bilayer studies, the cytoplasmic face of the channel is accessible to the cis chamber, the luminal face of the channel is accessible to the trans chamber (Miller, 1978; Smith et al., 1985). Such a system has the advantages of experimental access to either face of the channel and the capacity to exchange either solution, thus providing the ideal environment for application and removal of drugs. Most RyR modulating agents affect the channel from its myoplasmic side, as may be expected from its large cytoplasmic N terminal portion. Only Ca$^{2+}$ (Sitsapesan and Williams, 1994a; Sitsapesan and Williams, 1995a), annexin VI (a Ca$^{2+}$/phospholipid binding protein of unknown function that is found in SR lumen) (Diaz Munoz et al., 1990), rose bengal (Xiong et al., 1992), ruthenium red (Ma, 1993), sulmazole (Williams and Ashley, 1989) and thimerosal (Eager et al., 1995) have been shown to affect RyR activity when added to its luminal face.

a) Calcium

Ca$^{2+}$ is the primary activator of both RyR1 and RyR2 (Sitsapesan et al., 1995), having the ability to activate the channel in the absence of other ligands. There are few ligands that activate the RyR in the absence of Ca$^{2+}$. While ATP (for RyR1) (Smith et al., 1986) and thiol oxidising agents (Xiong et al., 1992; Boraso and Williams, 1994; Eager et al., 1997) do activate the channel in the absence of Ca$^{2+}$, they are unable to activate the channel fully. Interestingly, Ca$^{2+}$ by itself does not fully activate the channel; rarely does the channel open probability increase to greater than 0.4 in the absence of secondary activating ligands such as ATP (Williams, 1992). In the absence of other ligands, the concentration of Ca$^{2+}$ for half maximal activation ($K_A$) of the RyR is 0.7 - 44 µM (Rousseau and Meissner, 1989; Chu et al., 1993; Sitsapesan and Williams, 1994b; Laver et al., 1995). Maximum Ca$^{2+}$-activation occurs between ~1 - 100 µM (Chu et al., 1993; Laver et al., 1995). Higher concentrations inhibit the skeletal RyR (Chu et al., 1993; Laver et al., 1995), but the cardiac RyR is not inhibited until the [Ca$^{2+}$]$_{ei}$ rises above ~1 mM (Laver et al., 1995). The bell-shaped Ca$^{2+}$-activation curve suggests that the RyR has two (cytoplasmic) Ca$^{2+}$ binding sites: a high affinity Ca$^{2+}$-activation site ($K_A$ of 0.7 - 44 µM) and a low affinity Ca$^{2+}$-inhibition site. The half maximal inhibition ($K_I$) differs between skeletal and cardiac RyRs: $K_I$ for RyR1 varies from 0.4 - 0.7 mM (Chu et al., 1993; Laver et al., 1995) and RyR2 from 5 - 15 mM (Fruen et al., 1994b; Laver et al., 1995).

The effects of luminal Ca$^{2+}$ on RyR activity are currently being evaluated. Estimates of the free Ca$^{2+}$ concentration of skeletal SR (3.6 - 3.9 mM; Volpe and Simon, 1991; Fryer and Stephenson, 1996) are considerably higher than cardiac (100 - 150 µM; Varro et al., 1993; Bassani and Bers, 1995). In cardiac myocytes,
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approximately 43% depletion of SR Ca\(^{2+}\) occurs during a normal twitch, but this can vary from 10 - 60% depending on I\(_{Ca}\) and species - possibly reflecting different physiological requirements (Bassani et al., 1995).

With Ca\(^{2+}\) as the sole cis agonist, luminal Ca\(^{2+}\) does not affect the gating of skeletal or cardiac RyRs (Sitsapesan and Williams, 1994a; Sitsapesan and Williams, 1995a), although millimolar concentrations of luminal Ca\(^{2+}\) reduce RyR current amplitude in both RyR isoforms (Tinker et al., 1992; Ahern et al., 1994). Interestingly, the open probability of skeletal and cardiac RyRs is increased by high micromolar to millimolar concentrations of luminal Ca\(^{2+}\) when the channel is activated by cytoplasmic sulmazole (Sitsapesan and Williams, 1994a) or ATP (Sitsapesan and Williams, 1995a; Lukyanenko et al., 1996; Tripathy and Meissner, 1996) in the absence of cytoplasmic Ca\(^{2+}\). The physiological significance of channel regulation by luminal Ca\(^{2+}\) is unclear, but Sitsapesan and Williams (1994a, 1995a) speculate that the influence of Ca\(^{2+}\) is due to a luminal Ca\(^{2+}\) -binding site which is exposed when conformational changes occur in the molecule after the binding of either sulmazole or ATP in the absence of cytoplasmic Ca\(^{2+}\). In contrast, Tripathy and Meissner (1996) suggest that the luminal Ca\(^{2+}\) flowing through the channel has access to Ca\(^{2+}\) binding sites on the cytoplasmic side of the RyR.

b) ATP

ATP was first shown to activate single skeletal RyRs both in the presence (Smith et al., 1985) and absence (Smith et al., 1986) of cytoplasmic Ca\(^{2+}\). ATP was later shown to have similar effects on the cardiac channel, although only in the presence of Ca\(^{2+}\) (Rousseau et al., 1986; Williams and Ashley, 1989). Other adenine nucleotides (AMP, ADP, adenine, adenosine and the non-hydrolysable ATP analogue AMP-PCP), but not the trinucleotides (CTP, GTP, ITP or UTP) (Meissner, 1984; Meissner, 1994), also induce Ca\(^{2+}\) release from skeletal and cardiac SR vesicles and activate single RyR channels in lipid bilayers (Meissner, 1984; Meissner et al., 1986; Meissner and Henderson, 1987; McGarry and Williams, 1994a). Consensus sequences for adenine nucleotide binding (GXGXXG) were identified in both skeletal and cardiac RyRs (Takeshima et al., 1989; Otsu et al., 1990). The consensus sequence was found at several sites in RyR1, including the sections between amino acid residues 4449 - 4454 and 4452 - 4457. These two sites were in the region containing other putative modulator binding sites and were also close to the putative transmembrane segment M1 (see section 1.3.1) (Takeshima et al., 1989). For RyR2, the most likely nucleotide binding region lay between amino acid residues 2619 - 2652 (Otsu et al., 1990). A photoaffinity analogue of ATP (3'-O-(4-benzoyl-ATP) identified a likely ATP binding site(s) for the skeletal RyR on the 27 kDa and 13 kDa fragments of the RyR protein (Zarka and Shoshan-Barmatz, 1993). It was
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suggested that each RyR monomer may contain two or more different nucleotide binding sites.

The skeletal and cardiac RyRs are optimally active in the presence of micromolar Ca\(^{2+}\) and millimolar ATP (Meissner et al., 1986). However, RyRs are also partially activated by millimolar ATP at resting \([Ca^{2+}]\), (Smith et al., 1985; Rousseau et al., 1986; Smith et al., 1986). Because ATP is in both skeletal and cardiac myoplasm at millimolar concentrations (Smith et al., 1985; Murphy et al., 1989; Zarka and Shoshan-Barmatz, 1993), inhibitors of RyR activity in the myoplasm, such as Mg\(^{2+}\) and calmodulin (discussed in section 1.4.5), must also be important in the modulation of the channel if the RyR is closed under resting conditions (Smith et al., 1985).

c) Mg\(^{2+}\)

Mg\(^{2+}\) inhibits Ca\(^{2+}\) release from SR vesicles (Nagasaki and Kasai, 1983; Meissner, 1984; Smith et al., 1985; Meissner et al., 1986) and current passed by single RyRs in lipid bilayers (Rousseau et al., 1986; Smith et al., 1986; Ashley and Williams, 1990). The inhibition was thought to occur by Mg\(^{2+}\) competitively binding to the Ca\(^{2+}\)-activation site on the RyR (Nagasaki and Kasai, 1983). Meissner et al. (1986) subsequently suggested that high Mg\(^{2+}\) concentrations inhibited \(^{45}\)Ca\(^{2+}\) efflux from skeletal SR vesicles by binding to additional low affinity sites on the RyR. Lamb and Stephenson (1991) showed that activation of DHP receptors could relieve the Mg\(^{2+}\)-inhibition of RyRs in 1 mM, but not 10 mM, Mg\(^{2+}\). They proposed that, during skeletal muscle contraction, activation of the DHP receptor lowers the affinity of the RyR for Mg\(^{2+}\), thereby relieving the Mg\(^{2+}\)-inhibition and opening the Ca\(^{2+}\) release channel. Laver et al. (1997) showed that Mg\(^{2+}\) inhibits single skeletal and cardiac RyR channels by two independent mechanisms. Firstly, Mg\(^{2+}\) competitively binds to the Ca\(^{2+}\)-activation site to inhibit the channel at resting \([Ca^{2+}]\), as previously proposed (Nagasaki and Kasai, 1983). Secondly, Mg\(^{2+}\) binds to the low affinity Ca\(^{2+}\)-inhibition site (Meissner et al., 1986), that doesn’t discriminate between Ca\(^{2+}\) and Mg\(^{2+}\). Free Mg\(^{2+}\) in the myoplasm is at concentrations that fall within the binding affinities for both of the RyR Mg\(^{2+}\)-binding sites. While total [Mg\(^{2+}\)], is \(\sim 14\) mM in skeletal muscle, most of this is compartmentalised into the mitochondria and the SR. Of the \(\sim 6\) mM that is diffusible in the cytoplasm, most is bound to ATP, forming the Mg\(^{2+}\)-ATP complex essential for muscle contraction and Ca\(^{2+}\)-ATPase activity, with smaller percentages bound to creatine phosphate and parvalbumin (Lamb and Stephenson, 1992). Early estimates for free [Mg\(^{2+}\)], of up to 3.2 mM (Fabio, 1983) were too high, partially due to poor ion selectivity of Mg\(^{2+}\) ionophores (Hall et al., 1991; Silverman et al., 1991). Present estimates range from 0.8 - 1.3 mM in skeletal (Lamb and Stephenson, 1992) and 0.5 - 1.6 mM in cardiac muscle (Murphy et al., 1989; Agus and Morad, 1991; Hall et al., 1991; Silverman et al., 1991; Dulhunty et al., 1996). However, the [Mg\(^{2+}\)], can potentially increase under
changing physiological conditions e.g. during sustained muscle contraction, ATP levels can be depleted (Zarka and Shoshan-Barmatz, 1993) causing the dissociation of the Mg\(^{2+}\)-ATP complex with a subsequent increase in free [Mg\(^{2+}\)], (Murphy et al., 1989; Dennis et al., 1991; Kawai et al., 1996).

d) \(pH\)

RyR channels from striated SR are sensitive to physiologically relevant changes in intracellular pH (pHi) (Rousseau and Pinkos, 1990). The pHi in skeletal and cardiac muscle is generally maintained between 7.2 and 7.4 (Gevers, 1977; Lazdunski et al., 1985; Dennis et al., 1991). A decrease in pHi of \(\geq 0.2\) pH units inhibits SR Ca\(^{2+}\) release, and single channel activity, of both the skeletal and the cardiac RyR (Meissner and Henderson, 1987; Rousseau and Pinkos, 1990; Favero et al., 1995b). Interestingly, increases in pHi, (of \(\sim 0.2\) pH units) cause the development of muscle tension (Shoshan et al., 1981). This observation led to the proposal that changes in pH gradients across skeletal SR may be the physiological mechanism by which Ca\(^{2+}\) is released from the SR (Shoshan et al., 1981). However, this hypothesis has not received widespread acceptance. Because of the intense metabolic activity that can potentially occur in the highly worked or fatiguing muscle cell (Favero et al., 1995b) and the resulting production of protons, primarily through ATP hydrolysis and CO\(_2\) retention (Gevers, 1977; Dennis et al., 1991), a highly efficient proton extrusion and buffering system is essential. Sometimes, however, this system is inadequate to deal with the demands placed upon it, resulting in intracellular acidification. This situation occurs upon reperfusion of the ischaemic heart, where pHi can drop to \(< 7.0\) (Poole-Wilson, 1978). The precise cause of this acidification is unknown. However, the increase in [Ca\(^{2+}\)], that occurs after reperfusion (Shen and Jennings, 1972; Hearse, 1977) could be involved in the intracellular acidification. Increases in [Ca\(^{2+}\)] have been shown to decrease pHi, (Vaughan Jones et al., 1983), possibly through competition for common intracellular binding sites (Vaughan Jones et al., 1983; Dennis et al., 1991).

e) \(Phosphorylation\)

Protein kinases and phosphatases are often used as intracellular signalling agents to modulate cellular activity (Hain et al., 1995). Many ion channels, including the RyR (Meissner, 1994), are modulated by phosphorylation (Levitan, 1994). Seiler et al. (1984) reported that high molecular weight proteins in skeletal and cardiac SR vesicles, analogous to the SR foot structures, were phosphorylated by the endogenous Ca\(^{2+}\)-calmodulin-dependent protein kinase and by an exogenous catalytic subunit of cAMP-dependent protein kinase. The phosphorylated proteins were later identified as RyRs. Subsequent reports have shown both the skeletal RyR (at serine 2843; Sukk et al., 1993) and the cardiac RyR (at serine 2809; Witcher et al., 1991) to be phosphorylated by several
Introduction

different protein kinases including Ca\(^{2+}\)-calmodulin-dependent protein kinase, Ca\(^{2+}\)-calmodulin-dependent protein kinase II, cAMP-dependent protein kinase and cGMP-dependent protein kinase (Hohenegger and Suko, 1993; Strand et al., 1993; Suko et al., 1993; Hain et al., 1994; Hain et al., 1995; Valdivia et al., 1995). While reports on both skeletal and cardiac RyRs show single channel activation by phosphorylation (Witcher et al., 1991; Herrmann-Frank and Varsányi, 1993; Valdivia et al., 1995), possibly through increases in the sensitivity of the channel to Ca\(^{2+}\) and ATP (Herrmann-Frank and Varsányi, 1993; Valdivia et al., 1995), another shows inhibition of the skeletal RyR (Wang and Best, 1992). The functional studies have yielded inconsistent results; the physiological significance of RyR phosphorylation is yet to be determined (Coronado et al., 1994; Meissner, 1994).

1.4.3 *Pharmacological characterisation of RyRs*

*a) Ryanodine and ruthenium red*

Ryanodine and ruthenium red are widely used for identification of the RyR. The skeletal and cardiac RyRs bind ryanodine with 4 possible sites, each with different affinity: 1 - 4 nM for the high affinity ryanodine binding site, and 30 - 50 nM, 500 - 800 nM and 2 - 4 µM for the other three sites (Pessah and Zimanyi, 1991). This finding corroborates the report of Lai et al. (1989) who suggested the presence of both high and low affinity \[^{3}H\]ryanodine binding sites with a ratio of 1:3. The Hill coefficient is close to 1 for the high affinity site (Lai et al., 1989; Pessah and Zimanyi, 1991) and progressively decreases to ~0.25 for the lowest affinity site (Pessah and Zimanyi, 1991). This suggests that ryanodine binds to 4 identical sites with decreasing affinity, and that each interact allosterically in a negatively cooperative manner (Pessah and Zimanyi, 1991). Evidence for negative cooperativity was demonstrated by a reduction in the rate of the association kinetics for \[^{3}H\]ryanodine as the unlabelled ryanodine concentration was increased (Buck et al., 1992). However, other groups have suggested positive cooperativity (an unusual situation for ligand binding) in the allosteric interactions between high and low affinity ryanodine binding, since binding at the low affinity site decreased the dissociation rate of \[^{3}H\]ryanodine from its high affinity site (Lai et al., 1989; McGrew et al., 1989). The strong binding of ryanodine to its receptor with slow dissociation rates (McGrew et al., 1989), and the availability of tritiated ryanodine, has enabled the purification, subsequent cloning and identification of the RyR (Imagawa et al., 1987; Inui et al., 1987; Takeshima et al., 1989; Meissner, 1994).

Ryanodine binds preferentially to the open channel (Michalak et al., 1988). This fact enabled \[^{3}H\]ryanodine to be used widely as an indicator of channel gating (Coronado et al., 1994), since the fraction bound provided a measure of the channel’s open probability. In single channel studies, where RyRs are incorporated into lipid bilayers, ryanodine is commonly used to identify the channel. The
addition of low concentrations of ryanodine (<10 µM) reduces conductance of the channel to ~50% of its maximum, while open probability increases almost to unity irrespective of cytoplasmic Ca²⁺ (Rousseau et al., 1987; Laver et al., 1995). Under these conditions ryanodine is presumably bound to the high affinity site (Lai et al., 1989). Higher concentrations of ryanodine irreversibly block the channel by binding to the low affinity sites (Lai et al., 1989).

Ruthenium red is a strong inhibitor of RyR activity. At nanomolar concentrations it inhibits Ca²⁺ efflux from skeletal and cardiac SR vesicles (Chamberlain et al., 1984b; Meissner, 1984), and inhibits the binding of [³H]ryanodine to its receptor (Pessah et al., 1985). Before the RyR was identified as the SR Ca²⁺ release channel, the actions of ruthenium red were thought to occur via a specific ionic interaction with some component in the SR Ca²⁺ efflux pathway (Chamberlain et al., 1984b). This suspicion was later confirmed when ruthenium red (micromolar) rapidly and irreversibly blocked single RyRs incorporated into lipid bilayers from both skeletal and cardiac muscle (Smith et al., 1985; Rousseau et al., 1986). Ma (1993) investigated the mechanism for this block and presented evidence that ruthenium red is an open channel blocker that has multiple binding sites located in the conduction pore of the channel. Because of its high affinity for the RyR, as well as its rapid action, ruthenium red is a widely used tool for identification of the RyR. However, due to its relative lack of specificity (Moore, 1971), it is often used in conjunction with other RyR markers such as ryanodine, caffeine or ATP.

b) Caffeine

Caffeine induces Ca²⁺ release from skinned frog skeletal muscle and SR vesicles (Meissner, 1984) due to the direct activation of RyRs (Rousseau et al., 1988; Rousseau and Meissner, 1989). Because of this it is often used as a probe of RyR channel function. However, caffeine also affects L-type Ca²⁺ currents in cardiac muscle, therefore its actions are not specific for the RyR (Zahradnik and Palade, 1993). The increase in Ca²⁺ current across the SR caused by caffeine is due to an increase in RyR open probability, not a change in channel conductance (Sitsapesan and Williams, 1990). Two different mechanisms for caffeine activation have been described for the cardiac RyR: a Ca²⁺-dependent mechanism whereby 0.5 - 2 mM caffeine appears to sensitize the channel to Ca²⁺, and a second Ca²⁺-independent mechanism where higher caffeine concentrations (>5 mM) activate the channel in the absence of Ca²⁺ (Sitsapesan and Williams, 1990). The channel kinetics after activation by caffeine, in the absence of Ca²⁺, were different to the kinetics of channels activated by a combination of caffeine and Ca²⁺, or by Ca²⁺ alone. This suggested that caffeine was not increasing the sensitivity of the RyR to Ca²⁺ in the Ca²⁺-independent mechanism (Sitsapesan and Williams, 1990). While caffeine, and its related methylxanthines, shares a similar molecular structure
to adenine (Meissner, 1984; Melzer et al., 1995), caffeine and adenine nucleotides bind to different sites on the cardiac RyR (McGarry and Williams, 1994a).

1.4.4 Other RyR modulators

Other ligands that modulate RyR activity are numerous and include the anthraquinones (Abramson et al., 1988a), aryldisulfides (Zaidi et al., 1989), cocaine (Tsushima et al., 1996), dantrolene (Nelson et al., 1996), endogenous polyamines (e.g. spermine) (Uehara et al., 1996), heavy metals (Abramson et al., 1983), milrinone (Holmberg and Williams, 1991), procaine and other local anaesthetics (Shoshan-Barmatz and Zchut, 1993; Tinker and Williams, 1993; Zahradniková and Palade, 1993), reactive oxygen species (Holmberg et al., 1991), scorpion toxins (Valdivia et al., 1992) and sulmazole (Williams and Holmberg, 1990). See Coronado et al. (1994) for a more comprehensive list of the agents that affect RyRs. Some of these reagents can be classified under the heading of thiol or sulfhydryl reagents. Interactions between such reagents and the RyR may be important under physiological or pathological conditions and are discussed more completely in section 1.5.

Most of the ligands mentioned above, as well as Ca²⁺, Mg²⁺, adenine nucleotides and calmodulin (Meissner and Henderson, 1987), affect the channel activity of skeletal and cardiac RyRs in a qualitatively similar manner. However, quantitative differences in their effects on the isoforms do exist, such as the differences in sensitivity between RyR1 and RyR2 to >10⁻⁴ M Ca²⁺ (Zimanyi and Pessah, 1991; Laver et al., 1995). Differences in ligand sensitivity also extend to “secondary” activating or inhibitory ligands such as ATP and Mg²⁺. These differences may be due to: a) slight variations in the ligand binding sites between RyR isoforms; b) differences in Ca²⁺ sensitivity of the isoforms if the ligand interacts with the receptor via a Ca²⁺-dependent mechanism (Sitsapesan et al., 1995) or c) the influence of co-proteins such as triadin or FK binding proteins.

Isoform specific ligands also exist: perchlorate ions affect only the skeletal isoform (Ma et al., 1993; Fruen et al., 1994a) whereas the cardiac glycosides e.g. digoxin (McGarry and Williams, 1993) affect only the cardiac RyR. Cyclic ADP-ribose was also suspected to be isoform specific, activating only the non-skeletal RyR isoforms (Morrisette et al., 1993). It was shown to stimulate Ca²⁺ release from sea urchin eggs through an inositol 1,4,5-triphosphate-insensitive mechanism (Galianone et al., 1991) and activate the cardiac (Sitsapesan et al., 1994) but not the skeletal RyR (Morrisette et al., 1993). In contrast, Sitsapesan and Williams (1995b) showed that 1 - 10 µM cyclic ADP-ribose activated sheep skeletal RyRs in solutions containing ~10 µM cis Ca²⁺ and millimolar trans Ca²⁺. Interestingly, cyclic ADP-ribose did not affect the open probability of the skeletal RyR when the trans [Ca²⁺] was lowered to <40 µM. They suggested that Morrisette et al. (1993) failed to see an effect on open probability after adding
cyclic ADP-ribose because of the low \textit{trans} \( \text{Ca}^{2+} \) used in their experiments \((-1 - 3 \, \mu\text{M})\) (Sitsapesan and Williams, 1995b).

1.4.5 \textbf{Proteins associated with the RyR}

The SR is a highly specialised internal membrane structure designed to store \( \text{Ca}^{2+} \). In addition to simply storing \( \text{Ca}^{2+} \), it must also be able to release \( \text{Ca}^{2+} \) into the myoplasm and reaccumulate it upon demand. Each of the three functions are carried out by separate proteins: 1) calsequestrin - a \( \text{Ca}^{2+} \) binding protein located in the lumen of the SR; 2) RyRs - \( \text{Ca}^{2+} \) release channels which are packed primarily into the junctional face of the terminal cisternae and 3) \( \text{Ca}^{2+},\text{Mg}^{2+} \)-ATPase - a pump located primarily in the longitudinal SR and non-junctional terminal cisternae, designed to transport myoplasmic \( \text{Ca}^{2+} \) into the SR lumen. In addition to these SR proteins, other membrane or cytosolic proteins may directly or indirectly affect \( \text{Ca}^{2+} \) transport across the SR. This may occur through interactions with the RyR. When the characteristics of the RyR were investigated after it was removed from its native SR membrane by CHAPS -solubilisation (i.e. a purified RyR), or after the RyR was cloned and expressed, certain properties were sometimes seen that were not commonly observed in the native channel. Firstly, purification of the RyR resulted in a channel with more substate activity than seen in the native channel (Lai and Meissner, 1989; Laver et al., 1995). However, Sitsapesan and Williams (1994b) found that channel gating of cardiac RyRs was not modified by purification. Secondly, RyR1 cloned from cDNA and expressed in COS-1 cells also displayed a high degree of substate activity as well as having an extremely large conductance in some channels, while still displaying the pharmacological properties of the native channel (Chen et al., 1993). The differences suggest that other membrane or cytosolic proteins, removed during solubilisation or not expressed with the RyR, may be important in usual RyR function.

\textit{a) Calmodulin}

Calmodulin inhibits the \( \text{Ca}^{2+} \)-activated skeletal and cardiac RyR at concentrations of \(-1 - 2 \, \mu\text{M}\) (Meissner and Henderson, 1987; Smith et al., 1989). Calmodulin is a ubiquitous 16.8 kDa \( \text{Ca}^{2+} \)-binding protein that, in the presence of \( \text{Ca}^{2+} \), regulates many enzymic reactions (Klee et al., 1980; Chiesi and Carafoli, 1983). Its concentration in skeletal and cardiac muscle of \(-2 - 6 \, \mu\text{M}\) (Fabriato, 1983; Ogawa, 1994) is relatively low compared to the concentration in the brain (10 - 50 \( \mu\text{M}\); Klee et al., 1980; Ogawa, 1994). Using electron microscopy, a binding site for calmodulin on the cytoplasmic surface of each of the four 565 kDa RyR subunits has been revealed (Wagenknecht et al., 1994; Samso et al., 1997). Because this calmodulin binding site is at least 10 nm from the transmembrane assembly it was speculated that the binding of calmodulin to this site must induce long range conformational changes in the protein to alter channel gating properties.
(Wagenknecht et al., 1994). In addition to the ability of calmodulin to inhibit the RyR in activating $\text{Ca}^{2+}$ concentrations, it has recently been shown that calmodulin can activate the skeletal RyR in the absence of $\text{Ca}^{2+}$ ($\leq 0.1 \mu \text{M}$) (Tripathy et al., 1995). Under these (low [$\text{Ca}^{2+}$]) conditions, up to 16 calmodulin molecules bind to each RyR tetramer and, presumably, keep the channel in a state of low activation. The physiological implications for this observation are not known. The effects of calmodulin on the RyR are likely to be due to a direct interaction between calmodulin and the RyR since they are seen in the absence of ATP and hence are unlikely to be caused by a $\text{Ca}^{2+}$/calmodulin-dependent kinase (Meissner, 1986a; Coronado et al., 1994; Melzer et al., 1995).

b) Triadin

Triadin is a 95-kDa SR membrane glycoprotein originally identified in skeletal muscle (Brandt et al., 1990; Knudson et al., 1993a; Knudson et al., 1993b). Its localisation to the junctional SR and high abundance led to the proposal that it may play a role in the $\text{Ca}^{2+}$ homeostasis of skeletal muscle (Knudson et al., 1993a). The presence of triadin in cardiac SR was an issue of some controversy in the early 1990s: Brandt et al. (1993) isolated a 95-kDa protein from rat ventricular muscle that they identified as triadin. Conversely, Knudson et al. (1993b) could not find evidence for such a protein in rabbit cardiac or brain tissue using Northern blot analysis of mRNA or Western blot analysis. However, Knudson et al. (1993b) did not dismiss the possibility that the techniques they used may not have detected low levels of expression, or that the protein may not be recognised by the antibodies used. Using polyclonal antibodies to skeletal triadin, Guo et al. (1996) identified and characterised three cardiac triadin isoforms including a 92-kDa protein. This confirmed the presence of triadin in rabbit heart and, in doing so, indicated that triadin was not involved in a task specific for skeletal muscle. The molar content of triadin and the RyR in the skeletal muscle are similar (Caswell et al., 1991). In cardiac muscle, estimates of triadin content from rat ventricle were considerably lower than that seen in skeletal muscle (Brandt et al., 1993). Similarly, there are also $\sim 2$-$3$-fold fewer RyRs and $\sim 10$-fold fewer DHP receptors in cardiac than in skeletal muscle (Brandt et al., 1993).

It was predicted that the amino terminus of the skeletal muscle triadin isoform, consisting of 47 amino acid residues, is cytoplasmic, and that most of the remainder of the highly basic molecule is luminal (Knudson et al., 1993b). The cytoplasmic amino terminus for skeletal and cardiac triadin are identical, whereas the luminal portions of the skeletal and cardiac molecule show little homology (Peng et al., 1994; Guo et al., 1996). The difference is reflected in the slightly lower molecular mass of the cardiac isoform (between 92- and 95-kDa, compared to 95-kDa in skeletal muscle) (Brandt et al., 1993; Guo et al., 1996). It is interesting that triadin from both muscle types share a common topology: analysis
of hydrophobicity profiles shows a single hydrophobic stretch of ~20 amino acid residues - a sufficient length to span the membrane (Knudson et al., 1993b; Guo et al., 1996). While triadin does not bind to the DHP receptor (Guo and Campbell, 1995), the luminal portion of triadin from skeletal muscle was shown to bind the RyR and interact with calsequestrin (Guo and Campbell, 1995). This led to the suggestion that triadin may anchor calsequestrin to the junctional face of the SR and provide a functional contact with the RyR. This concept was supported after the fluorogenic coumarin maleimide, 7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin, was shown to form an adduct with a small percentage of "hyperreactive" thiols on both the skeletal RyR and triadin under conditions which favoured the closed conformation of the channel. This suggested a communication between the two proteins (Liu et al., 1994; Liu and Pessah, 1994). The similar size, localisation and structural topology of the two triadin isoforms, along with their close association with the RyR, suggest that triadin may perform a common functional or structural role in regulation of Ca\(^{2+}\) efflux from the SR in both skeletal and cardiac muscle (Knudson et al., 1993a; Guo et al., 1996).

c) FK binding proteins

The immunosuppressive drugs FK-506 and cyclosporin A, used for treating graft rejection and other autoimmune disorders (Lam et al., 1995), bind to the ubiquitous cytosolic immunophilins FK binding protein (FKBP) and cyclophilin respectively. Interestingly, little is known of the physiological role of the immunophilins (Timerman et al., 1993). However, it has recently been shown that the 11.8-kDa FK-506 binding protein (FKBP12) is tightly associated with the purified skeletal RyR (Jayaraman et al., 1992), and the 12.6-kDa FKBP with the cardiac RyR (Sewell et al., 1994; Lam et al., 1995). The molar ratio of approximately four FKBPs per RyR suggests that one FKBP is associated with each RyR monomer (Timerman et al., 1993; Timerman et al., 1996). This is supported by evidence which shows that FKBP12 binds to four symmetrically located regions on the periphery of the cytoplasmic domain of the RyR (Samso et al., 1997). Amongst the various mammalian FKBPs (including FKBP12, FKBP12.6, FKBP13, FKBP25, FKBP52 and FKBP54) FKBP12 and FKBP12.6 have an 85% amino acid sequence homology and are the most closely related (Sewell et al., 1994). To investigate the effects of FKBP12 on single channel activity, Brillantes et al. (1994) expressed skeletal RyRs without the FKBP12 protein. This resulted in channel activity with multiple subconductance states. Coexpression of the RyR with FKBP12, or adding FKBP12 to the expressed RyR in bilayers, restored channel activity with a stable full conductance (Brillantes et al., 1994). When FK-506 was added to skeletal RyRs in lipid bilayers the degree of substate activity, along with open probability and mean open time increased (Ahern et al., 1994; Brillantes et al., 1994). These effects were attributed to dissociation of the binding protein and/or inhibition of the isomerase activity of the binding
proteins. Interestingly, the removal of FKBP from cardiac RyRs in lipid bilayers with FK-506 or rapamyacin increased the amount of substate activity and increased P0 in one study (Kaftan et al., 1996), but did not affect single channel activity in another (Timerman et al., 1996). Further evidence in support of a role for the binding proteins in channel modulation was gained when the depressed Ca\(^{2+}\) uptake rates of FKBP -deficient terminal cisternae vesicles were restored to control levels in the FKBP -reconstituted vesicles (Timerman et al., 1993). Hence, FK binding proteins are bound to, and essential for, the normally functional skeletal RyR (Brillantes et al., 1994), and may also functionally associate with the cardiac RyR (Kaftan et al., 1996).

d) Others

A number of other proteins that are localised to the triad junction have also been shown to modulate, or are associated with, the RyR. Calsequestrin is the major luminal Ca\(^{2+}\)-binding protein. It may sense the Ca\(^{2+}\) release “trigger” through an interaction with the RyR itself, or through an indirect interaction with RyR -associated proteins, to cause the dissociation of bound Ca\(^{2+}\) (Campbell et al., 1983; Aaron et al., 1984; Ikemoto et al., 1991). A 106 -kDa protein has been purified from skeletal SR vesicles by biotin-avidin chromatography. When incorporated into lipid bilayers it conducts cations and shows properties similar to the native and purified RyR (Hilkert et al., 1992; Mirzabekov et al., 1993). Another protein belongs to the annexins - a family of Ca\(^{2+}\)/phospholipid binding proteins. The 67 -kDa SR luminal protein, annexin VI, has been shown at nanomolar concentrations to modify gating of single skeletal RyRs from the luminal side of the channel (Diaz Munoz et al., 1990) - one of the few agents known to modulate the channel from the luminal side. Calpain II is an endogenous cytosolic protease that, in vitro, degrades the RyR into 315- and 150 -kDa proteolytic fragments. When the proteolysed fragments are incorporated into lipid bilayers, cationic currents resembling those of the native RyR are observed, except for a high open probability and the loss of usual channel gating (Rardon et al., 1990). Finally, two glycolytic enzymes, glyceraldehyde 3-phosphate dehydrogenase (GADP) and aldolase, are associated with the RyR and possibly other junctional proteins. GADP has been proposed as a mechanical link between the RyR and the DHP receptor in skeletal muscle (Caswell and Brandt, 1989). The function of aldolase is unknown (Meissner, 1994) but an association with the RyR is suggested (Caswell and Brandt, 1989).

1.5 Interactions between thiol reagents and the RyR

Sulphhydryl (SH) reagents influence Ca\(^{2+}\) flux across the SR, possibly through the modification of cysteine residues on the RyR (Abramson and Salama, 1988). The involvement of SH groups in SR Ca\(^{2+}\) release and, more specifically,
on the activity of the RyR is addressed in the remainder of this introduction. Thiol groups on proteins are, chemically, extremely reactive (van Iwaarden et al., 1992) and hence are prime candidates for modulation. Critical SH groups are thought to be involved in the gating processes of the RyR and have been studied for over a decade. SH oxidising agents increase the permeability of terminal cisternae SR vesicles to $\text{Ca}^{2+}$ through a direct interaction with the RyR $\text{Ca}^{2+}$ release channel. This section outlines the history that led to these conclusions, from the early work on muscle contraction to single channel studies, but first describes the type of reactions involved in these processes.

1.5.1 Biological thiol interactions

Each monomer of the cardiac RyR contains 89 cysteine residues (Otsu et al., 1990), but the number of free SH groups (i.e. reactive thiols) on the RyR is unknown. Because of the high reactivity of cysteine residues, some are likely to be in the cystine, or disulfide, form while many more are likely to be inaccessible to SH reagents due to shielding by the tertiary structure of the protein. In addition, the reactivity of an individual thiol can vary considerably depending on factors such as its basicity and polarisation as well as the electrostatic interactions between the thiol and its neighbours (van Iwaarden et al., 1992). In essence, the reaction between reagent and protein thiol, generally the thiolate ion ($RS^-$) (van Iwaarden et al., 1992), depends on the intrinsic reactivity of the thiolate ion (nucleophile) to an electrophilic reagent (Brocklehurst, 1979).

The number of compounds that can interact with protein thiols is enormous. The various reagents can be grouped into categories, such as the reagent class (e.g. metal ions, reactive oxygen species, organomercurials, quinone compounds or arsenic compounds) or the type of reaction in which they are involved (e.g. oxidation, alkylation, arylation or thiol-disulfide exchange) (for review see van Iwaarden et al., 1992). Few of the reagents have an absolute specificity for cysteine residues. Only the reactive disulfides, 2,2'-dithiodipyridine or 4,4'-dithiodipyridine, or other disulfides such as 5,5'-dithiobis-(2-nitrobenzoic acid) interact solely with cysteine residues in proteins (Brocklehurst, 1979; van Iwaarden et al., 1992). The organomercurials and arsenoxides react preferentially with thiols, but binding to other groups in proteins does occur (Brocklehurst, 1979; van Iwaarden et al., 1992). Metal ions generally have a low thiol specificity (Stadtman, 1990).

The thiol reactions of particular interest to studies on the RyR are shown in equations 1.1 - 1.4 (below). They include interactions between protein SH groups and metal ions (1.1), disulfides (1.2 and 1.3) and other oxidation reactions in which inter- or intramolecular disulfide bonds are formed (1.4). However, other types of interactions between SH groups and SH reagents occur, such as additions to activated double bonds (e.g. $N$-ethylmaleimide), as well as reactions between thiols
and the quinones or arsenic compounds (van Iwaarden et al., 1992). The first of the three reactions relevant to investigations into the RyR, is the reversible reaction between a protein thiol (RSH) and a metal ion or an organomercurial which results in the production of a mercaptide (van Iwaarden et al., 1992):

\[
RSH + M^+_c \leftrightarrow RSM_c + H^+ \tag{1.1}
\]

Secondly, in the highly specific thiol-disulfide exchange reaction between a protein thiol and a disulfide (R₂-S-S-R₂), the complete reaction consists of two reversible steps (Brocklehurst, 1979):

\[
R_1SH + R_2S-S-R_2 \leftrightarrow R_1S-S-R_2 + R_2SH \tag{1.2}
\]

\[
R_1SH + R_1S-S-R_2 \leftrightarrow R_1S-S-R_1 + R_2SH \tag{1.3}
\]

Due to the specificity of disulfides they are often used as labelling agents, although their relatively large size can be disadvantageous if structural information is desired (van Iwaarden et al., 1992). Finally, the oxidation of SH groups by reagents such as H₂O₂, iodine and ferricyanide takes the following format:

\[
2RSH \leftrightarrow R-S-S-R + 2H^+ + 2e^- \tag{1.4}
\]

This oxidation reaction is also reversible but it is not specific, since other residues such as tyrosine, tryptophan and methionine can be oxidised by these reagents (van Iwaarden et al., 1992). SH groups can also be spontaneously oxidised by atmospheric oxygen (air- or auto-oxidation) (van Iwaarden et al., 1992), in a reaction that may be catalysed by small concentrations of the transition metal ions such as copper and iron (Cavallini et al., 1968; De Marco et al., 1971; Cheeseman and Slater, 1993).

### 1.5.2 Thiol reagents affect muscle contraction: an historical perspective

Publications on the effects of SH reagents on skeletal muscle (Liu et al., 1948) and neuromuscular transmission (Ellis and Beckett, 1954) date back many decades. Okamoto and Kuperman (1966) speculated that SH groups could play a key role in the biphasic contractile response of frog sartorius muscle to the SH interacting organomercurials, \( p \)-chloromercuribenzoate (pCMB) and \( p \)-chloromercuribenzenesulfonate (pCMBS), along with \( N \)-ethylmaleimide (NEM) which is also known to react with SH groups (van Iwaarden et al., 1992). In the absence of external stimuli, repetitive low-amplitude contractions were observed upon addition of pCMB and pCMBS, while NEM produced a large but short lived contraction. Both pCMB and pCMBS caused an eventual loss of contractile ability, while the effect of NEM was incompletely reversed with repeated washings in Ringers. Muscle exposed to NEM in the presence of the reducing agent \( l \)-cysteine showed no changes in tension or stimulus evoked contractions (Okamoto and Kuperman, 1966). This indicated that free SH groups were required for the physiological response to occur.
Chang et al. (1970) examined the physiological role of SH groups in neuromuscular transmission. Using oxidative, alkylating and mercaptide forming agents they showed conduction blockade of the motor axon, depolarisation of the muscle membrane and sustained contracture in frog and rat nerve-muscle preparations. However, the SH reagents had complex and varying effects on neuromuscular transmission and few conclusions were drawn.

New techniques enabled the SR membrane to be isolated, and Ca\(^{2+}\) flux across the membrane investigated (Fairhurst and Jenden, 1966; Fuchs et al., 1968). In the first report to show a direct effect of an agent on SR Ca\(^{2+}\) efflux, Fairhurst and Hasselbach (1970) found that 5 mM Zn\(^{2+}\) produced a marked stimulation of Ca\(^{2+}\) efflux from heavy SR. Because Zn\(^{2+}\) was known to inhibit Ca\(^{2+}\) uptake from skeletal and cardiac muscle (Chimoskey and Gergely, 1968), experiments were conducted in the absence of an energy supply to block Ca\(^{2+}\) uptake. Since the Zn\(^{2+}\)-induced Ca\(^{2+}\) efflux was independent of Ca\(^{2+}\) uptake they concluded that Zn\(^{2+}\) had somehow increased the permeability of the SR membrane to Ca\(^{2+}\).

The mechanism by which Zn\(^{2+}\) induced the Ca\(^{2+}\) efflux was unclear. A report that another heavy metal, Hg\(^{2+}\), also stimulated Ca\(^{2+}\) efflux from rabbit skeletal SR vesicles (Chiesi and Inesi, 1979) suggested a possible link with the actions of Zn\(^{2+}\). In 1983 further evidence showed that heavy metals and SH reagents caused Ca\(^{2+}\) efflux from the SR. Abramson et al. (1983) found that heavy metals induced SR Ca\(^{2+}\) efflux with the following order of potency: Cu\(^{2+}\) > Hg\(^{2+}\) > Ag\(^{+}\) > Cd\(^{2+}\) ≥ Zn\(^{2+}\) > CH\(_3\)Hg > NEM. Because the order of potency paralleled their strength of reaction with SH groups, it was suggested that the reagents induced the Ca\(^{2+}\) efflux through interactions with SH groups. In addition, Bindoli and Fleischer (1983) found that dithiothreitol partially reversed a pCMBS-induced Ca\(^{2+}\) release, strongly suggestive of a pCMBS-thiol interaction. Dithiothreitol (DTT) is a powerful reducing agent that is widely used because of its high water solubility and low probability of air oxidation (Cleland, 1964). It is used for maintaining thiols in their reduced state or reducing disulfide bridges in proteins and peptides.

These and associated studies provided the ground work for the subsequent investigations into the effects of SH reagents on Ca\(^{2+}\) flux across the SR.

1.5.3 Oxidation-induced Ca\(^{2+}\) release

Subsequent studies have focused on determining the target protein of the reagents and the type of reaction that causes the effects. Various techniques have been used to address these questions including intact and skinned muscle fibres, \([^{1}\text{H}]\)ryanodine binding studies, Ca\(^{2+}\) flux studies from SR vesicles and single channel studies. The number of SH reagents found to effect Ca\(^{2+}\) efflux from SR vesicles quickly grew, along with the picture as to how the reagents were altering SR membrane permeability to Ca\(^{2+}\). The classes of reagent that are of particular
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relevance to oxidation-induced changes in SR Ca\textsuperscript{2+}-permeability fall under three broad headings corresponding to equations 1.1 - 1.4 (section 1.5.1): a) the binding of heavy metals; b) mixed disulfide reactions in which an endogenous SH group on the RyR forms a mixed disulfide with an exogenously added thiol or disulfide containing agent and c) oxidation reactions in which an externally added agent catalyses the oxidation of two endogenous thiols in close proximity (termed vicinal thiols) to form a disulfide bridge.

\textbf{a) Heavy metals}

Heavy metals were proposed, in the 1950s, to cause sustained muscular contraction by blocking the SR Ca\textsuperscript{2+} uptake (Hasselbach and Weber, 1955). It was subsequently shown that high concentrations of heavy metals inhibited the Ca\textsuperscript{2+},Mg\textsuperscript{2+}-ATPase thereby blocking Ca\textsuperscript{2+} uptake (Chimoskey and Gergely, 1968; Brunder et al., 1988; Oba et al., 1989) and preventing muscle relaxation. Salama and Abramson (1984) found that Ca\textsuperscript{2+} efflux from heavy SR vesicles was induced by micromolar concentrations of Ag\textsuperscript{+}. They proposed that the Ag\textsuperscript{+}-induced Ca\textsuperscript{2+} efflux was due to Ca\textsuperscript{2+} release from a protein other than the Ca\textsuperscript{2+},Mg\textsuperscript{2+}-ATPase, since it was blocked by ruthenium red and local anaesthetics (agents known to block Ca\textsuperscript{2+} efflux in muscle fibres and heavy SR). Because of the low concentrations used, Ag\textsuperscript{+} was unlikely to be blocking Ca\textsuperscript{2+} uptake, therefore they suggested it was acting at the physiological Ca\textsuperscript{2+} release site.

By the end of the 1980s it was clear that heavy metals induced Ca\textsuperscript{2+} efflux from the SR, through an interaction with the RyR or a protein(s) closely associated with the RyR. Supporting evidence from both skeletal and cardiac preparations includes the following. 1) Heavy metals were significantly more potent in inducing rapid Ca\textsuperscript{2+} release from vesicles of heavy SR (HSR) than from light SR (LSR) (Fairhurst and Hasselbach, 1970; Salama and Abramson, 1984; Tatsumi et al., 1988). 2) In skeletal SR vesicles, known inhibitors of the RyR such as ruthenium red (Salama and Abramson, 1984; Brunder et al., 1988; Moutin et al., 1989; Oba et al., 1989) and Mg\textsuperscript{2+} (Salama and Abramson, 1984; Tatsumi et al., 1988) blocked the Ag\textsuperscript{+}-induced Ca\textsuperscript{2+} efflux. 3) Ruthenium red also inhibited the Ag\textsuperscript{+}-induced Ca\textsuperscript{2+} release in cardiac SR vesicles (Prabhu and Salama, 1990a). 4) Physiological pH maximised Ag\textsuperscript{+}-induced Ca\textsuperscript{2+} release (Salama and Abramson, 1984). 5) In skinned muscle fibres, a high [Mg\textsuperscript{2+}] inhibited a transient, but not a tonic, Ag\textsuperscript{+}-induced contracture (Oba et al., 1989). 6) Procaine (a known inhibitor of the CICR mechanism) reduced the duration of the Ag\textsuperscript{+}-induced contraction in skinned frog skeletal fibres (Oba et al., 1995).

There is no suggestion that heavy metals act as physiological activators of the RyR or modulators of Ca\textsuperscript{2+} flux across the SR (Abramson and Salama, 1989). However, it is of biophysical and pathological interest that they do bind to the RyR to activate the channel, possibly through interactions with a SH group(s)
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(Abramson et al., 1983). In addition, they have proved to be a useful tool for investigation of RyR structure. The heavy metals were used to show that the thiols on proteins in the SR, to which metals bind and induce Ca$^{2+}$ efflux, are located in a relatively hydrophilic environment. The more hydrophobic SH reagents were less effective in releasing Ca$^{2+}$ from the vesicles (Abramson et al., 1983; Bindoli and Fleischer, 1983). In order to find out more about the connection between thiol oxidation and channel activation, other more specific SH reagents were used to investigate their effects on Ca$^{2+}$ efflux from the SR.

b) Mixed disulfide reactions

The SH containing molecules cysteine, cysteamine and homocysteine are biologically common mercaptans. By themselves they have no ability to induce Ca$^{2+}$ efflux from SR vesicles; in fact, cysteine is a mildly effective reducing agent with a redox potential of -210 mV at pH 7.0 (Cleland, 1964). However, the addition of cysteine (25 - 100 μM), in the presence of catalytic concentrations of Cu$^{2+}$ or Hg$^{2+}$ (2 - 5 μM), produced Ca$^{2+}$ efflux from actively loaded skeletal SR vesicles (Trimm et al., 1986) and rapid twitches in rabbit skinned skeletal fibres (Salama et al., 1992). At the concentrations used, neither cysteine nor the heavy metals alone had any effect on Ca$^{2+}$ flux from vesicles (Trimm et al., 1986). Interestingly, in rabbit skinned fibres, 10 μM cysteine induced rapid phasic contractions in 25% of fibres tested (Salama et al., 1992). This may have been due to an endogenous catalyst. The metals appeared to be catalysing the formation of a mixed disulfide between the SH group of the mercaptan and an endogenous SR thiol (Trimm et al., 1986). This oxidative mechanism is supported with the evidence that: 1) glutathione (GSH; but not its oxidised form - GSSG) inhibits muscle contraction induced by heavy metals (Salama et al., 1992); 2) excess DTT prevents the Cu$^{2+}$/cysteine -induced Ca$^{2+}$ efflux from SR vesicles (Trimm et al., 1986); 3) the rate of Cu$^{2+}$ -catalysed mercaptan -induced Ca$^{2+}$ efflux from SR vesicles parallels the relative rate of autooxidation of the mercaptans in the presence of Cu$^{2+}$ (Trimm et al., 1986) and 4) using $^{35}$S-Cys + Cu$^{2+}$, covalent labelling of several SR proteins was observed using SDS-gel electrophoresis techniques (no labelling in the absence of Cu$^{2+}$) (Abramson and Salama, 1989).

The aryldisulfides, 2,2'-dithiodipyridine (2,2'-DTDP) and 4,4'-dithiodipyridine (4,4'-DTDP), referred to as reactive disulfides (RDS), also induce a rapid Ca$^{2+}$ efflux from the SR by oxidising a critical SR SH group(s) (Zaidi et al., 1989). These agents are absolutely specific for free SH groups which they oxidise with the accompanying production of the highly light absorbing thiopyridone. This can be measured with quantitative spectrophotometry (Kenyon and Bruice, 1977; Zaidi et al., 1989). 2,2'- and 4,4'-DTDP each contain two pyridyl rings which are separated by a disulfide bridge. Where 2,2'-DTDP has the disulfide bond at the two position on the pyridyl ring, 4,4'-DTDP has it on the four position. These compounds induce Ca$^{2+}$ efflux, from actively and passively loaded
SR vesicles (Zaidi et al., 1989), when SH groups on an SR protein reacts with the RDS. This reaction results in a mixed disulfide, linking the SR protein and a pyridyl ring. Interestingly, RDSs inhibit Ca\(^{2+}\) efflux when concentrations used are higher than those required to stimulate Ca\(^{2+}\) efflux. Zaidi et al. (1989) showed that while low concentrations of RDS (3 × 10\(^{-6}\) M) stimulated the binding of \(^{3}H\)ryanodine to the RyR, binding was inhibited at concentrations above 3 × 10\(^{-6}\) M (4,4'-DTDP) or ~5 × 10\(^{-5}\) M (2,2'-DTDP). Since the binding of \(^{3}H\)ryanodine to the receptor provides a measure of channel open probability, these studies suggest that higher concentrations of RDSs close the RyR and may reduce the Ca\(^{2+}\) permeability across the SR.

Both mercaptans and arylsulfides increase Ca\(^{2+}\) efflux from the SR through the RyR. This was shown by: 1) the ability of a high [Mg\(^{2+}\)] to inhibit RDS -induced Ca\(^{2+}\) efflux from actively and passively loaded SR vesicles (Zaidi et al., 1989); 2) the inability of RDS -induced contraction to occur in the presence of ruthenium red (Zaidi et al., 1989); 3) significantly higher mercaptan -induced Ca\(^{2+}\) release from HSR than LSR (Trimm et al., 1986); 4) the block of mercaptan -induced Ca\(^{2+}\) release from SR vesicles by ruthenium red (Trimm et al., 1986) and 5) 4,4'-DTDP increasing current through a bilayer containing multiple native skeletal RyRs (Nagura et al., 1988).

c) Oxidation of thiols

Reactions which involve the oxidation of two vicinal thiols, resulting in the formation of a disulfide bond, are described in this section. This type of reaction is of particular physiological interest because of the possibility of spontaneous autooxidation (see section 1.5.1). Other reagents capable of oxidising endogenous thiols to form disulfides include iodine and ferricyanide, along with the potentially reactive molecules classified as reactive oxygen species (ROS). These include hydrogen peroxide (H\(_2\)O\(_2\)), singlet oxygen (O\(_2^*\)), hypochlorous acid (HOCl) and free radicals such as the hydroxyl radical (\(^{1}O\(_2^*\)) and nitric oxide (NO). Oxidation reactions with these reagents are not specific to SH groups, since such reagents can also oxidise other amino acid residues such as tyrosine, tryptophan, methionine, histidine, arginine, lysine and proline (van Iwaarden et al., 1992; Castilho et al., 1996).

ROS have been shown to alter SR Ca\(^{2+}\) permeability and are known to react freely with protein thiols. They are generated at low concentrations during the normal course of cell metabolism (Jackson and O'Farrell, 1993) but the endogenous radical scavengers, such as GSH, control their intracellular levels (Ambrosio et al., 1995). ROS are also generated by our biological defence system. An immune response often involves the release by phagocytes of cytotoxic products to combat pathogens. These products can include lysozyme and peroxidases, as well as ROS such as superoxide, hydrogen peroxide and the
The effects of ROS on cardiac muscle are of particular interest since, upon reperfusion of the ischaemic heart, the invasion of polymorphonuclear leukocytes at the previously ischaemic tissue results in the release of a variety of powerful oxidant species including HOCl and 'OH (Favero et al., 1995a; Steare and Yellon, 1995). HOCl reacts particularly strongly with the sulfur containing amino acids and appears to be one of the most toxic and physiologically significant of the ROS generated during cardiac reperfusion injury (Fukui et al., 1994), although the hydroxyl radical is considered equally deleterious under such conditions (Wu et al., 1996). Fukui et al. (1994) demonstrated that (patho)physiologically relevant concentrations of HOCl (100 µM) increased the [Ca\(^{2+}\)], in single rat ventricular myocytes. This rise was significantly suppressed by reducing agents, but only those permeable to membrane such as DTT or the esterified cysteines, not GSH or cysteine. DTT was also able to restore the [Ca\(^{2+}\)], close to basal levels after the levels of Ca\(^{2+}\) were raised by HOCl. This study strongly suggested that HOCl oxidised intracellular SH groups to raise [Ca\(^{2+}\)], but did not show which protein(s) may have contributed to this effect.

Another ROS implicated in thiol oxidation is nitric oxide (NO). The physiological significance of NO was first shown when a factor (termed “endothelium derived relaxation factor” and later shown to be NO) was found to modulate vascular tone (Furchgott and Zawadzki, 1980). It is now implicated in many tasks including intracellular signalling (Ignarro, 1991) and host defence (Gross and Wolin, 1995). NO may also be involved in the free radical -induced damage that occurs after ischaemia-reperfusion. This is possible since it is produced in endocardial and endothelial cells (Schulz et al., 1991) as well as the cardiomyocytes (Schulz et al., 1992). NO synthase is also present in human skeletal muscle; mRNA for this enzyme is more abundant in human skeletal muscle than in human brain (Nakane et al., 1993). Unlike 'OH, NO is not highly reactive (Butler et al., 1995) and is unable to directly oxidise thiols (Upchurch, Jr. et al., 1995). NO must first be oxidised to form N\(_2\)O\(_3\) before interacting with the thiol according to equation 1.5. The S-nitrosothiol (RSNO) that is formed may be unstable, and can revert to a thiol if a disulfide bond is not rapidly formed. However, if two vicinal thiols are oxidised to S-nitrosothiol groups, a disulfide bond plus 2NO may form (Eq. 1.6) (Lei et al., 1992).

\[
\text{N}_2\text{O}_3 + \text{RSH} \rightarrow \text{RSNO} + \text{H}^+ + \text{NO}_2^- \quad (1.5)
\]

\[
2\text{RSNO} \rightarrow \text{R-S-S-R} + 2\text{NO} \quad (1.6)
\]

Effects of NO donors on the activity of single RyRs, and Ca\(^{2+}\) efflux from SR vesicles, have been inconclusive. The NO donor S-nitroso-N-acetylpenicillamine (SNAP) inhibited the activity of individual skeletal and cardiac RyRs in lipid bilayers, and inhibited Ca\(^{2+}\) release from SR vesicles (Mészáros et al., 1996; Zahradniková et al., 1997). In contrast, the activity of single skeletal RyRs and
Ca\textsuperscript{2+} efflux from SR vesicles was stimulated by SNAP and other NO donors (Stoyanovsky et al., 1997). A third study showed that NO donors have no direct effect on single channel activity of skeletal RyRs (Aghdasi et al., 1997a). Willmott et al. (1996) suggested that NO activates the RyR through an indirect pathway. They showed that Ca\textsuperscript{2+} was released from an IP\textsubscript{3}-insensitive intracellular store in sea urchin eggs by a cGMP/cADP-ribose-dependent pathway activated by NO. More work is required to understand the effects of NO on the activity of the RyR.

The suggestion that HOCl and other ROS act on intracellular Ca\textsuperscript{2+} stores is consistent with findings in both skeletal and cardiac tissue. HOCl (100 µM) and H\textsubscript{2}O\textsubscript{2} (2 mM) induce Ca\textsuperscript{2+} efflux from actively loaded rabbit skeletal vesicles (Trimm et al., 1986). Singlet oxygen and superoxide free radicals, generated from the illumination of 50 nM rose bengal (Holmberg and Williams, 1992), along with H\textsubscript{2}O\textsubscript{2} (3 mM) (Boraso and Williams, 1994) cause an increase in the open probability (if only transient) of single sheep RyR incorporated into lipid bilayers. H\textsubscript{2}O\textsubscript{2} is likely to act by an oxidation reaction since 3 mM H\textsubscript{2}O\textsubscript{2} (normally sufficient to activate the channel) had no effect on RyR activity in the presence of 10 mM DTT, while subsequent additions of ATP and caffeine activated the channel (Boraso and Williams, 1994). Studies investigating isolated channels in synthetic lipid bilayers provide strong evidence that these agents act directly on the RyR itself or, at least, on molecules tightly associated with it. While oxygen radicals may have deleterious effects on membrane structure and proteins, the intrinsic function of the RyR does not appear to be affected by ischaemia (Darling et al., 1992) although the number of RyRs may decrease (Zucchi et al., 1994).

Many of the molecules that are classified as ROS are extremely reactive. They react with and modulate the activity of the RyR, but are not specific for the channel. They have also been shown to affect sarcolemmal Na\textsuperscript{+},K\textsuperscript{+}-ATPase and the Na\textsuperscript{+}/H\textsuperscript{+}-exchanger (Kukreja et al., 1990; Xie et al., 1990) along with other intracellular ion regulatory proteins such as the Ca\textsuperscript{2+},Mg\textsuperscript{2+}-ATPase (Eley et al., 1991a; Viner et al., 1996).

d) Phthalocyanine dyes and quinones

The phthalocyanine dyes, such as alcian blue and luxol fast blue MBSN, contain a centrally located Cu\textsuperscript{2+} and have been used as stains, such as for the visualisation of glycosaminoglycans (Hronowski and Anastassiades, 1979). The possibility that these dyes could induce SR Ca\textsuperscript{2+} release was investigated because of their structural similarity to the metalloporphyrins which catalyse many redox reactions (Abramson et al., 1988b). The dyes were found to induce Ca\textsuperscript{2+} efflux from skeletal SR vesicles (Abramson et al., 1988b; Koshita et al., 1993). As for Ag\textsuperscript{+}-induced Ca\textsuperscript{2+} release, alcian blue-induced Ca\textsuperscript{2+} efflux is specific for HSR rather than LSR (Koshita et al., 1993), and is inhibited by ruthenium red (Abramson et al., 1988b; Koshita et al., 1993) and Mg\textsuperscript{2+} (Koshita et al., 1993).
Alcian blue appears to oxidise endogenous SH groups to produce its effect. This is indicated, firstly, by the report that DTT, added after completion of alcian blue-induced Ca\(^{2+}\) efflux, resulted in the reuptake of 80% of the released Ca\(^{2+}\) (Abramson et al., 1988b). Secondly, pre-incubation of the reagent in a reducing environment (i.e. in the presence of DTT, cysteine or GSH) abolished its ability to induce Ca\(^{2+}\) efflux, while subsequent re-oxidation restored its potency (Abramson et al., 1988b; Koshita et al., 1993).

Quinones oxidise SH groups to form disulfides but can also alkylate the SH groups, hence they are not a particularly specific class of reagent (van Iwaarden et al., 1992). Some quinones have been shown to induce Ca\(^{2+}\) release from SR vesicles. Abramson et al. (1988a) found that a particular class of quinone, the anthraquinones, was especially effective in releasing Ca\(^{2+}\) from skeletal SR vesicles. The anthraquinones, such as doxorubicin (DXR) and daunorubicin, are widely used antineoplastic agents that appear, like the phthalocyanine dyes, to activate the skeletal and cardiac RyR by oxidising endogenous SH groups (Abramson et al., 1988a; Abramson and Salama, 1989; Ondrias et al., 1990). DXR is a positive inotrope as well as being one of the most effective of the anthraquinones available for the treatment of human cancers (Doroshow, 1983). However, its use is limited due to its cardiotoxic side effects. The ability of DXR to generate superoxide anion and \(\text{H}_2\text{O}_2\) in specific cardiac compartments, including the SR (Doroshow, 1983), has led to the hypothesis that the cardiotoxicity of DXR may result from membrane peroxidation and altered membrane permeability to Ca\(^{2+}\) caused by the ROS (Doroshow, 1983).

The activation of the single cardiac RyR by DXR was followed by channel inhibition after 8 min (Ondrias et al., 1990). This biphasic effect of DXR on the RyR is not a unique property of DXR, or the quinones. Ag\(^{+}\)-induced Ca\(^{2+}\) release from skeletal SR vesicles showed a bell shaped activation curve: concentrations of Ag\(^{+}\) above \(\sim 30 \mu\text{M}\) had a progressively depressive effect on peak Ag\(^{+}\)-induced Ca\(^{2+}\) efflux (Moutin et al., 1989). In addition, ROS (Holmberg and Williams, 1992) and RDSs (Eager et al., 1997) initially increased the open probability of single RyRs incorporated into lipid bilayers but, subsequently, caused the irreversible loss of channel activity. The biphasic effects on RyR activity, induced by DXR as well as the metals, disulfides and ROS, suggests that these different classes of SH reagent all interact with the RyR via a common mechanism. The delay before the irreversible loss of channel activity that was seen in single channel studies (Ondrias et al., 1990; Holmberg and Williams, 1992; Eager et al., 1997) could be a time-dependent consequence of the modulation of a critical SH group on the RyR. Alternatively, separate SH groups with different reagent affinity or accessibility may mediate the effects on the RyR, as seen with the SR Ca\(^{2+},\text{Mg}^{2+}\)-ATPase (Murphy, 1976; Thorley-Lawson and Green, 1977).
1.5.4 Physiological relevance of RyR oxidation

Mammalian cells contain high concentrations (0.5 - 10 mM) of GSH (Meister and Anderson, 1983). The reduced form of GSH, a tripeptide (γ-Glu-Cys-Gly), is converted to its oxidised form (glutathione disulfide, GSSG) by GSH peroxidase, or during contact with free radicals or other oxidants. GSSG is then reconverted to GSH using the enzyme GSSG reductase and NADPH. The ratio of GSH:GSSG in the cytoplasm is extremely high (Sies et al., 1972; Meister and Anderson, 1983). Hence, the intracellular environment is conducive to the maintenance of thiols. If this is the case, how then can intracellular thiols be oxidised? Under conditions of marked toxicity or oxidative stress, [GSH], is decreased while [GSSG], is substantially increased (Sies et al., 1972, Curella et al., 1985). Curello et al. (1985) showed that the ratio of GSH:GSSG fell from 45 in the perfused rabbit heart, to 8 in the reperfused heart following 90 min of ischaemia. This reduced ratio may then leave the cell vulnerable to oxidative damage (Curella et al., 1985; Steare and Yellon, 1995; Sirsjo et al., 1996).

The failure of skeletal muscle contraction (Brunder et al., 1988) and the non-activation of single cardiac RyRs (Boraso and Williams, 1994; Eager et al., 1997), after the addition of oxidising agents in the presence of millimolar concentrations of reducing agents, suggested that reducing agents could prevent oxidation -induced RyR activation. However, the addition of alcian blue or plumbagin (a phthalocyanine dye and a naphtholquinone respectively), in the presence of 5 mM GSH or DTT, induced Ca\(^{2+}\) release from skeletal SR vesicles through an oxidation reaction (Koshita et al., 1993). The authors suggested that the oxidising agents reacted more quickly with the protein thiols than they did with the reducing reagent. Because thiol oxidation could occur even in the presence of physiological concentrations of GSH, it has been suggested that redox reactions may be involved in physiological SR Ca\(^{2+}\) release (Koshita et al., 1993). However, it is more likely that oxidation, and subsequent activation, of RyRs occurs only under extreme conditions or pathological states when the cell is exposed to oxidative stress such as during cardiac reperfusion injury (Holmberg et al., 1991), ischaemia of skeletal muscle (Sirsjo et al., 1996), muscle fatigue (Favero et al., 1995b) or aging (Stadtman, 1992). Cells are more vulnerable to oxidation at these times when the ratio of GSH:GSSG is lowered.

1.5.5 SH reagents and single RyR activity

SH reagents have been applied to single RyRs incorporated into lipid bilayers. These included doxorubicin (Ondrias et al., 1990), RDSs (Eager et al., 1997), ROS such as H\(_2\)O\(_2\) and the ROS -generating compound rose bengal (Holmberg et al., 1991; Holmberg and Williams, 1992; Xiong et al., 1992; Ma et al., 1993; Boraso and Williams, 1994; Favero et al., 1995a), ascorbate/iron (Stoyanovsky et al., 1994), glutathione disulfide (Zable et al., 1997), fluorescent
maleimides (Liu et al., 1994), thimerosal (Abramson et al., 1995), N-ethylmaleimide and diamide (Aghdasi et al., 1997b). In all cases, the channels were activated, although sometimes only transiently. Eight of the thirteen studies also reported a loss of RyR activity, subsequent to activation, within minutes of exposure to the reagents (Ondrias et al., 1990; Holmberg et al., 1991; Holmberg and Williams, 1992; Liu et al., 1994; Abramson et al., 1995; Favero et al., 1995a; Aghdasi et al., 1997b; Eager et al., 1997). This biphasic effect explained earlier observations from [3H]ryanodine binding studies where low concentrations of SH reagent stimulated the binding of [3H]ryanodine to its receptor, while higher concentrations actually depressed binding (Zaidi et al., 1989). Similar observations were made in Ca\(^{2+}\) flux studies from SR vesicles where high concentrations of a SH reagent depressed peak oxidation-induced effects (Moutin et al., 1989; Prabhu and Salama, 1990b). In contrast to channel activation with thiol oxidising agents, millimolar concentrations of the reducing agents, DTT and GSH, depress channel activity (Boraso and Williams, 1994; Zable et al., 1997). These experiments on single RyRs strongly suggest that the RyR is the target for SH reagents. However, it is possible that proteins tightly associated with the RyR contain the thiols that are modulated by SH reagents, and that the oxidation of these proteins causes the indirect activation of the RyR.

Prior to the publication of some of the work from the present study (Eager et al., 1997), there were no reports on the effects of SH specific reagents on single RyRs. There was also a lack of analysis of single channel activity of RyRs modulated by thiol reagents. However, Boraso and Williams (1994) showed that high concentrations of H\(_2\)O\(_2\) increased the open probability of RyRs in 10\(^{-5}\) and <10\(^{-9}\) M Ca\(^{2+}\) through a direct effect on the gating mechanism of the channel. Understanding the mechanisms by which specific SH reagents influence the activity of RyRs, i.e. the role of cysteine residues in RyR gating processes, is important in order to gain an insight into the actions of oxidising agents on the RyR under physiological and pathological conditions. In order to adequately assess the role of SH groups in RyR activity, it is necessary to use thiol specific reagents to avoid the potential effects that non-specific reagents may have on groups other than thiols. The RDSs, 2,2'- and 4,4'-DTDP, are absolutely specific for free SH groups (Brocklehurst, 1979), and have been shown to act on the RyR to stimulate the binding of [3H]ryanodine to its receptor (Zaidi et al., 1989), induce Ca\(^{2+}\) efflux from SR vesicles (Zaidi et al., 1989; Prabhu and Salama, 1990b) and increase current through a bilayer containing multiple skeletal RyRs (Nagura et al., 1988).

In the present study, single cardiac RyR channels were incorporated into lipid bilayers and were exposed to RDSs, and other SH reagents, to examine the following issues: 1) how single channel activity is affected in the presence of RDSs; 2) the mechanism(s) by which RDSs affect RyR activity; 3) how RDSs affect activity of the RyR that is modified by endogenous modulators of the channel and
4) whether more than one thiol group on the RyR is involved in the modification of channel activity following thiol oxidation.
MATERIALS AND METHODS

Chapter 2

2.1 Preparation of native SR vesicles

Native SR vesicles were prepared using methods based on those of Chen et al. (1980) and Blaustein et al. (1981). Sheep hearts were removed from sublethally stunned (5% pentobarbitone IV) followed by exsanguination) and rinsed in ice-cold buffer A (in mM: 20 imidazole, 330 sucrose, 5 N6N, 0.5 DTT, pH 7.0) at room temperature. The hearts were removed, stripped of visible fat, and comminuted using a metal chopper. The whole was homogenized in fresh buffer A using a Waring blender (4 x 15 sec bursts at high speed) within 15 min of excision.

The homogenate was centrifuged at 9,000 rpm in a Beckman JA-14 rotor for 20 min. The supernatant was filtered through several layers of gauze then ultracentrifuged at 30,000 rpm in a Beckman Ti-50 rotor for 2 hr. The crude vesicles pellet was resuspended in buffer A plus 2 mM DTT and 10 μg/ml leupeptin, snap frozen in liquid nitrogen and stored in either liquid nitrogen (volumes of ~1 ml) or at –70°C (volumes of 20 ml).

Some preparations were subfractionated on a discontinuous sucrose density gradient consisting of 25%, 18%, 14%, and 20% sucrose layers (w/v) in buffer B (in mM: 400 KCl, 0.5 MgCl₂, 0.5 CaCl₂, 0.5 EGTA and 20 imidazole, pH 7.0). The crude pellet was resuspended in buffer A using a glass Dounce homogenizer (10-30 strokes), homogenized until the sucrose density gradient and centrifuged at 20,000 rpm in a Beckman SW-28 rotor for 1 hr. The pellet collected from the 18-20% interface was diluted 1:4 in buffer B then centrifuged at 20,000 rpm in the Ti-50 rotor for 1 hr. The pellet was resuspended in buffer A plus 2 mM DTT, snap frozen and stored in the same manner as the crude vesicles (see above).

All preparations were carried out at 4°C. Buffers A and B contained the protease inhibitors leupeptin (1 μg/ml), pepstatin A (1 μM), benzamidine (1 mM) and PMSF (0.7 mM). Protein concentrations were assayed according to Lowry et al. (1951).

2.2 Purification of the ryanodine receptor

The cationic fraction was depleted using the cationic detergent, CHAPS, and assessed on the protocol of Kao et al. (1988) and Lindsay and Williams (1992). CRD vesicles (protein concentration of 10-20 mg/ml) removed from storage in liquid nitrogen were thawed. Vesicles were resuspended in buffer C (in mM: 15000 NaCl, 0.1 EGTA, 0.15 CaCl₂, 2 DTT and 25 imidazole, pH 7.2) and subfractionation
MATERIALS AND METHODS

2.1 Preparation of native SR vesicles

Native SR vesicles were prepared using methods based on those of Chu et al. (1988) and Sitsapesan et al. (1991). Sheep hearts were removed from anaesthetised ewes (5% pentobarbitone (IV) followed by oxygen/halothane) and rinsed in ice cold buffer A (in mM: 20 imidazole, 300 sucrose, 3 NaN_3, 0.5 DTT, pH 7.4 at room temperature with HCl). Ventricular tissue was removed, stripped of visible fat and connective tissue and diced. The tissue was homogenised in fresh buffer A using a Waring blender (4 x 15 sec bursts at high speed) within 15 min of excision.

The homogenate was centrifuged at 9,000 rpm in a Beckman JA-14 rotor for 20 min. The supernatant was filtered through several layers of gauze then ultracentrifuged at 30,000 rpm in a Beckman Ti-45 rotor for 2 hrs. The crude vesicle pellet was resuspended in buffer A plus 2 mM DTT and 10 µg/ml leupeptin, snap frozen in liquid nitrogen and stored in either liquid nitrogen (volumes of ~1 ml) or at -70°C (volumes of 20 µl).

Some preparations were subfractionated on a discontinuous sucrose density gradient consisting 45%, 38%, 34% and 28% sucrose layers (w/v) in buffer B (in mM: 400 KCl, 0.5 MgCl_2, 0.5 CaCl_2, 0.5 EGTA and 20 imidazole, pH 7.4). The crude pellet was resuspended in buffer A using a glass Dounce homogeniser (6 - 8 strokes), loaded onto the sucrose density gradient and centrifuged at 20,000 rpm in a Beckman SW-28 rotor for 16 hr. The band collected from the 38 - 45% interface was diluted 3 -fold in buffer B then centrifuged at 32,000 rpm in the Ti-45 rotor for 1 hr. The pellet was resuspended in buffer A plus 2 mM DTT, snap frozen and stored in the same manner as the crude fraction (see above).

All procedures were carried out at 4°C. Buffers A and B contained the protease inhibitors leupeptin (1 µg/ml), pepstatin A (1 µM), benzamidine (1 mM) and PMSF (0.7 mM). Protein concentrations were assayed according to Lowry et al. (1951).

2.2 Purification of the ryanodine receptor

The cardiac RyR was solubilised using the zwitterionic detergent, CHAPS, based on the methods of Lai et al. (1988) and Lindsay and Williams (1991). Crude vesicles (protein concentration of 10 - 20 mg/ml) removed from storage in liquid nitrogen were thawed. Vesicles were resuspended in buffer C (in mM: 1,000 NaCl, 0.1 EGTA, 0.15 CaCl_2, 2 DTT and 25 imidazole, pH 7.2) and solubilisation
initiated with the addition of CHAPS (0.5 or 1.0% w/v) and L-α-phosphatidylcholine (2.5 mg/ml). The vesicles (diluted to a protein concentration of 2 mg/ml) were homogenised in a glass Dounce homogeniser and placed on ice for 1 hr. The preparation was then centrifuged at 36,000 rpm in a Ti-75 rotor for 45 min to remove the insoluble material. The supernatant in buffer C plus CHAPS (0.5 or 1.0% w/v) and PC (2.5 mg/ml) was layered on a 5 - 25% continuous sucrose gradient to isolate the RyR from other solubilised membrane components. This was centrifuged at 28,000 rpm in an SW-28 rotor for 16 hours. From these centrifuge tubes 2 ml fractions were collected and the aliquots subjected to electrophoresis on 3 - 12% SDS-polyacrylamide gels which were silver stained to identify the fractions containing the RyR.

The solubilised RyR was subsequently reconstituted into proteoliposomes of PC by dialysis. The RyR containing fractions were dialysed overnight at 4°C against a buffer containing (in mM) 100 NaCl, 0.1 EGTA, 0.15 CaCl$_2$ and 25 imidazole at pH 7.2 with at least three changes of buffer. Sucrose was added to the proteoliposomes to give a final sucrose concentration of 200 mM before snap freezing in liquid nitrogen and storage in -70°C.

### 2.3 Planar lipid bilayer technique

Lipids were prepared from a mixture of PE, PS and PC. The lipids, suspended in chloroform, were purchased from Avanti Polar Lipids (Alabama, USA) and stored in original containers at -70°C or in glass vessels under nitrogen, for daily use, at -20°C. The lipids (prepared fresh each day) were brought to room temperature, mixed in a 5:3:2 ratio (PE:PS:PC), dried of chloroform under a stream of nitrogen then resuspended in n-decane (Aldrich, 99%+) at a concentration of 50 mg/ml. The bilayers were formed in solution across an aperture of ~150 - 250 μm in the wall of a Delrin cup (Cadillac Plastics, Australia). Lipid was transferred to the surface of the cup and spread over the aperture using a flame polished glass rod. The lipid thinned either spontaneously, or after gentle mechanical assistance with the glass rod, to form a bilayer. The thinning of the lipid was monitored electrically by measuring its capacitance; bilayers had a capacitance of between 150 - 320 pF. The measured capacitance was slightly greater than the calculated values of ~90 - 250 pF (based on a bilayer capacitance of 0.5 μF/cm$^2$; Benz et al., 1975). The difference between measured and theoretical values is due to the “background” capacitance comprising the wall of the cup itself (~20 pF) and the lipid surrounding the bilayer.

The standard solutions in each of the chambers (volume for each = 1 ml) for channel recording were (in mM) 250 CsCl, 1 CaCl$_2$, 10 TES (cis), and 50 CsCl, 1 CaCl$_2$, 10 TES (trans), both titrated to pH 7.4 with CsOH. In some experiments, one of the following was added to the cis chamber to aid vesicle
incorporation: a) 250 mM CsCl (increasing overall [CsCl] to 500 mM); b) 4 mM CaCl$_2$ or c) 500 mM mannitol. Increasing the [Ca$^{2+}$]$_{cis}$ promotes the binding of vesicles to the bilayer, and increasing osmotic strength in the cis chamber induces vesicle rupture (Niles and Cohen, 1987) and, hence, channel incorporation. Interestingly, by increasing the [CsCl] to 500 mM, the Ca$^{2+}$-inhibition mechanism in cardiac RyRs is disrupted (Laver et al., 1995). Consequently, this method of facilitating incorporation was not used when the Ca$^{2+}$-inhibition mechanism was investigated in the subsequent experiment.

Native SR membrane vesicles or the CHAPS-solubilised RyR in proteoliposomes were added to the cis chamber to a final protein concentration of 10 - 20 µg/ml and mechanically stirred (Miller and Racker, 1976). Incorporation was monitored on an oscilloscope and usually occurred within seconds to minutes of protein addition with the above solutions, in which Cs$^+$ and Cl$^-$ were the primary current carriers. Incorporation was evident as a deflection from baseline which indicated transfer of current between the cis and trans chambers. The orientation of the RyR in the vesicles was such that, upon incorporation of the vesicles with the bilayer, the cis chamber corresponded to the cytoplasmic side of the channel and the trans chamber to the luminal side (Miller and Racker, 1976). This orientation, for each channel incorporated into the bilayer, was confirmed by testing the sensitivity of the channel to cis Ca$^{2+}$ or ATP, or by adding the toxins ryanodine or ruthenium red to the cis chamber at the end of the experiment. The addition of these agents to the cytoplasmic face of the RyR causes a known response in the channel activity which is not seen when added to the luminal face of the channel. This showed that >99% of all RyRs incorporated with their cytoplasmic face accessible to the cis chamber. Experiments were terminated for the few RyRs that did not respond to cis addition of these reagents.

To prevent additional fusions, stirring was stopped and the cis chamber was perfused with 4.5 - 6 volumes (4.5 - 6 ml) using a back to back syringe system. This removed a sufficient proportion of vesicles to prevent further incorporation and set up the cis chamber with the desired experimental solution. The perfusion system enabled a change of solution from either the cis or the trans chambers by simultaneously adding a fresh solution and removing the resident solution. A 4.5-volume perfusion produces a solution change of >95% (Laver et al., 1995), and was used to adjust [CsCl] and also the Ca$^{2+}$ at concentrations between 10$^{-3}$ and 10$^{-9}$ M. It was important to consistently obtain an accurate [Ca$^{2+}$] following perfusion: in the worst instance, a 95% solution change when lowering [Ca$^{2+}$] from 1 mM to either 1 µM or 0.1 µM will give a final [Ca$^{2+}$] of 1.41 µM and 0.12 µM respectively. These calculations account for the presence of 2 mM BAPTA in the solution and were performed using a software package, Buffta (developed by R.G. Ryall, Dept of Haematology, Flinders Medical Centre, South Australia), to calculate the theoretical value for free ions in solution. Perfusion was not
detrimental to bilayer stability. Consequently, numerous exchanges of solution could be conducted during the course of an experiment.

The cis chamber was held at virtual ground while the trans chamber was voltage clamped at set potentials, relative to ground, using an Axopatch 200A amplifier (Axon Instruments). To conform to standard conventions, the bilayer potential was expressed as:

\[ V_{\text{applied}} = V_{\text{cis}} - V_{\text{trans}} = V_{\text{cytoplasm}} - V_{\text{lumen}} \]

Thus, at a holding potential of +40 mV (cis), positive current flowed from the cis to the trans chamber.

All experiments were carried out at room temperature (21-24°C), with solutions brought to the equivalent temperature before perfusion, and at a holding potential of +40 mV (the Cl⁻ reversal potential (ECl⁻)) unless otherwise specified.

### 2.4 Chemicals and solutions

All solutions were made from salts of “analytical reagent” grade or better and, along with the water soluble drugs, were prepared using MilliQ deionised water. All solutions were titrated to pH 7.4 (unless otherwise specified) with CsOH, using a digital pH meter (TPS Pty Ltd, Australia). Salts were obtained from Aldrich (CsCl and CsOH), Ajax (CaCl₂, KCl and NaCl) and Sigma (MgCl₂). The drugs ATP, DTT, GSH, ruthenium red and thimerosal were purchased from Sigma, ryanodine from Calbiochem and caffeine from Ajax. BAPTA, EGTA, TES, MES, leupeptin, benzamidine, pepstatin A, PMSF and CHAPS were purchased from Sigma. Ethyl alcohol-d (ethanol) was purchased from Aldrich and mannitol from Ajax.

Under experimental conditions the cis and trans chambers contained 250/50 mM CsCl respectively, 10 mM TES and 1 mM CaCl₂ titrated to pH 7.4 with CsOH (unless otherwise stated). Cs⁺ was used as the charge carrier instead of Ca²⁺. Cs⁺ has a number of advantages over Ca²⁺ including: 1) a high conductance (525 pS in symmetrical 250 mM CsCl; Laver et al., 1995) compared to that of Ca²⁺ (~90 pS; Ashley and Williams, 1990; Sitsapesan et al., 1991), resulting in a greater signal to noise ratio; 2) no known effect by itself on the activity of the RyR (at ≤250 mM), compared to Ca²⁺ which has binding sites of high and low affinity which modulate channel activity (Laver et al., 1995) and 3) a very low conductance (Cukierman et al., 1985) through, or the block of currents (Coronado et al., 1992) carried by, SR K⁺ channels. Solutions of low Ca²⁺ (≤ 10⁻⁵ M) contained 250 mM CsCl, 10 mM TES and 2 mM BAPTA, titrated to the desired [Ca²⁺] with CaCl₂. Free [Ca²⁺] was measured during titration with a Ca²⁺-selective electrode (Radiometer ION83). Calibration of this electrode was monitored by
checking its estimates of free Ca\(^{2+}\) against theoretical estimates (using Buffa) with standard reaction constants (Tsien, 1980). The cis chamber was perfused with at least 4.5 volumes of low (<10\(^{-3}\) M) Ca\(^{2+}\) solutions. To increase the [Ca\(^{2+}\)]\(_{cis}\) above 1 mM, small volumes of a 1 M CaCl\(_2\) stock solution were added. pH in the cis chamber was lowered (to 6.5) by perfusing at least 4.5 volumes of the low pH solution (containing 250 mM CsCl, 10 mM MES (pKa 6.1) and 1 mM CaCl\(_2\), titrated to pH 6.5 with CsOH). At the end of the experiment the desired pH in the cis chamber was confirmed with a pH meter.

The following reagents were all added from stock solutions to the cis or trans chambers while stirring for 5 - 10 sec (maximum stock concentration; mM): 2,2\,'-DTDP (100), 4,4\,'-DTDP (100), ATP (500), caffeine (100), DTT (1000), GSH (200), ruthenium red (1), ryanodine (2) and thimerosal (100). The pH of the ATP and GSH stock solutions were titrated to 7.4 using CsOH, with 10 mM TES as a buffer. Addition of drugs at the concentrations used (from 100 nM to 10 mM) had no effect on pH. All drugs were dissolved in H\(_2\)O, except 2,2\,'- and 4,4\,'-DTDP which were dissolved in ethanol. Ryanodine and ruthenium red were stored at 4°C; the remainder were stored in daily use aliquots at -20°C, and thawed as required. The ethanol added with 2,2\,'- and 4,4\,'-DTDP never exceeded 1% volume of either chamber. 1% ethanol in the cis chamber did not affect RyR activity (see section 4.2.2d and Fig. 4.4) (Oba et al., 1997; Eager et al., 1997).

### 2.5 Single channel recording

Silver chloride coated silver wire in salt bridges with 2% agar (containing 250/50 mM CsCl (cis/trans), 1 mM CaCl\(_2\) and 10 mM TES) provided the electrical contact between the bath and the amplifier headstage. The salt bridges were used to minimise junction potentials arising during solution changes. Voltage was controlled and currents recorded using an Axopatch 200A amplifier (Axon Instruments). These currents were displayed in real time on a Tektronix 5223 digitising oscilloscope (Tektronix).

Data were captured using two methods. In the first and most frequently used (77.2% of all RyRs recorded), data were recorded at a bandwidth of 5 kHz (Axopatch 200A) on videotape (JVC VCR, model HR-J400U) using pulse code modulation (Vetter PCM Recorder, model 200). The signal was later replayed and passed through a 1 kHz (10-pole lowpass Bessel, -3 dB) filter and sampled at 2 kHz (Axotape 12.01, Axon Instruments) using a Labmaster 125 MHz Interface (Axon Instruments). The second method was to filter data at a bandwidth of 1 kHz with the Axopatch 200A amplifier (4-pole lowpass Bessel), sample at 2 kHz (Axotape) using the Labmaster 125 MHz Interface and store directly on computer (486DX2).
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Sampling data at rates that are greater than double the filter frequency is considered by some (Colquhoun and Sigworth, 1983) to be necessary for an accurate reproduction of channel activity. To ensure that the sampling rate used in the present study did not significantly affect data interpretation, identical sections of channel recordings were resampled at 5 kHz (with a 1 kHz filter). Data were analysed and compared with that produced at the standard 2 kHz sampling rate (see section 3.6 and Fig. 3.8).

2.6 Data analysis

Channel events were detected using an in-house analysis program Channel2, developed by PW Gage and M Smith. A 50% threshold discriminator used to detect channel openings and closures was used to calculate channel open probability (P_o; channel open time/total time), mean open time (T_o, ms; channel open time/total number events) and event frequency (F_e, events s^-1; total number of events/length of record). The 50% discriminator was valid for the cardiac RyR since activity to sub-maximal conductance levels was seldom observed (see section 3.13). Open and close times, also captured by Channel2, were transferred to a spreadsheet program (Quattro Pro, version 5.00, Borland International) where frequency distributions from sections of channel records of at least 2 min were collated. Open and close times were sorted into bins of set duration or bins of variable width with equal separation (7 bins/decade) on a logarithmic scale (Sigworth and Sine, 1987). A multiple exponential (Eq. 2.1) was applied to yield the time constant(s) of decay.

\[ (a_1 \exp(-x/a_2) + a_n \exp(-x/a_n)) \]  

(2.1)

For bins of variable width, the graph of the square root of the frequency plotted against the log binned duration showed peaks corresponding to exponential components in the distribution. Exponential components within the distribution were fitted to the distribution of log-binned data using a least squares analysis. For display of average trends in the distributions, open time constants were allocated to: \( \tau_1, <3 \text{ ms} \); \( \tau_2, 3 - 12 \text{ ms} \); \( \tau_3, 12 - 50 \text{ ms} \); \( \tau_4, 50 - 500 \text{ ms} \) and \( \tau_5, >500 \text{ ms} \) and closed time constants allocated to: \( \tau_1, <3 \text{ ms} \); \( \tau_2, 3 - 20 \text{ ms} \); \( \tau_3, 20 - 100 \text{ ms} \); \( \tau_4, 100 - 500 \text{ ms} \) and \( \tau_5, >500 \text{ ms} \) (Eager et al., 1997).

2.6.1 Statistics

To test the significance of the difference in the mean values from a single group of channels before and after a treatment, the student’s paired 2-tailed t-test was used. Because proportional changes in the measured parameters were of interest, the difference between mean of the logarithms of the variables was tested (Statistical Consulting Unit, ANU) (Eager et al., 1997). When the difference in the mean values between two different groups of channels was being tested, the
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independent 2-tailed t-test (using the f-test for homogeneity of variance) was used. A value of \(<0.05\) was considered significant. Data are presented as mean ± 1 SEM.
CHARACTERISATION OF THE CARDIAC RYANODINE RECEPTOR

The RyR has been extensively studied using the technique developed by Harker and Rios et al. (1990), which enables the protein to be incorporated into a lipid bilayer and electrically stimulated. Properties of the single cardiac RyR were first described by Rios et al. et al. (1989). It is a cation-selective channel with a higher probability to conduct than unclamped cardiac in a mixed salt solution (150 mM KCl, 10 mM NaCl) (Williams et al., 1992; Corrando et al., 1994). The conductance of the channel is voltage-dependent, showing a bell-shaped curve that increases from a low level at depolarizations of 15 mV to a maximum of about 20 mV. At a 150 mM KCl membrane potential of 90 mV, the channel conductance was found to be approximately 1500 pS, and its self-maximal conductance was reached at a [K+] of 200 mM, when the conductance-voltage relationship was fitted to a Michaelis-Menten type saturation curve (Lindsey et al., 1991).

The aim of this chapter is to show that the Ca2+ current that was recorded and described as cardiac RyR current does, in fact, pass through the RyR protein. To do this the current-voltage relationship and pharmacological responses of the native and the purified channel are examined and compared to similar measurements of the RyR reported in the literature.
3.1 INTRODUCTION

The RyR has been extensively studied using the technique developed by Miller and Racker (1976) which enables the protein to be incorporated in a lipid bilayer and electrically visualised. Properties of the single cardiac RyR were first described by Rousseau et al. (1986). It is a cation-selective channel with a higher permeability to divalent than monovalent cations in a mixed salt solution \( P_{Ca^{2+}}/P_{K^+} = 6.6 \) (Williams, 1992; Coronado et al., 1994). The conductance of the channel to divalent cations is considerably less than its conductance of monovalent cations in single salt solutions: the RyR has a \( Ca^{2+} \) conductance of \(-90 \) pS (Ashley and Williams, 1990; Sitsapesan et al., 1991) compared to 462 pS for \( Cs^+ \) (symmetrical 250 mM \( Cs^+ \), 10 \( \mu \)M \( Ca^{2+} \)) (Sitsapesan and Williams, 1994a). The RyR has an extremely large unit conductance: maximum \( K^+ \) conductance of the purified channel was found to saturate at \(-900 \) pS, and its half-maximal conductance was reached at a \([K^+]_o\) of 20 mM, when the conductance-activity relationship was fitted to a Michaelis-Menten type saturation curve (Lindsay et al., 1991).

The aim of this chapter is to show that the \( Cs^+ \) current that was recorded and described as cardiac RyR current does, in fact, pass through the RyR protein. To do this the current-voltage relationships and pharmacological responses of the native and the purified channel are examined and compared to similar parameters of the RyR reported in the literature.
RESULTS

3.2 Bilayer characteristics and stability

Bilayers into which SR vesicles and proteoliposomes were incorporated were made from a mixture of phospholipids dissolved in n-decane (see Methods). Decane was used since it allows bilayers with a stable capacitance to be produced (Coronado et al., 1992). Maximum capacitance of the bilayers was ~320 pF. Lipid bilayers maintained a constant capacitance for >2 hrs in the solutions used (Laver et al., 1995) and, after vesicle incorporation, had a peak to peak noise no greater than ~4 pA (at a 1 kHz bandwidth). Several noise sources are introduced into the system by inadequate grounding and small changes in bilayer capacitance from vibration (Coronado et al., 1992). Added noise can result from mechanical and microphonic disturbances and was kept to a minimum by placing the experimental apparatus on an air table to reduce vibrations.

3.3 Currents observed upon vesicle incorporation

The presence of Cl⁻ in the cis chamber is beneficial for vesicle incorporation (Coronado et al., 1992), hence the use of CsCl salts in the solutions. Single Cl⁻ conducting channels were described in cardiac SR by Rousseau (1989) and are thought to act as counter-ion channels, in conjunction with various cation channels, to offset the electrogenic effects of Ca²⁺ flux across the SR membrane (Meissner, 1983; Fink and Veigel, 1996). In order to observe the Cs⁺ currents through the RyR, without interference from the Cl⁻ currents, all recordings (unless otherwise mentioned) were made at +40 mV (E_Cl⁻, see below) as suggested by Coronado et al. (1992).

Incorporation of 61% of native SR vesicles into the bilayer occurred within 10 min of protein addition (n = 1530) but only 33% of proteoliposomes (into which the CHAPS -solubilised, or purified, RyR was inserted) incorporated within the same time (n = 84). Vesicle incorporation was detected on the oscilloscope as an increase in current level, i.e. an increase in bilayer conductance. Three types of current were observed upon incorporation of the vesicles or proteoliposomes. 1) A Cs⁺ current, with an observed reversal potential (E_reversal) close to -18 mV and a chord conductance at +40 mV for the native channel of 259 ± 1 pS (n = 3) (Fig. 3.1A) and 266 ± 7 pS (n = 7) for the purified channel (Fig. 3.1B). The rectification, or non-linearity, of the I/V relationship was due to the asymmetrical salt solutions. With symmetrical salt solutions, a linear I/V relationship was obtained (see section 3.5). The E_reversal of -18 mV was different to the theoretical E_{Cs+} of -40 mV in 250/50 mM CsCl (calculated using the Nernst equation). This was
Fig. 3.1 Current-voltage relationships of the native and the CHAPS-solubilised ryanodine receptor. Channels were recorded after incorporation of native SR membrane (A) or proteoliposomes containing the purified RyR (B) in 250/50 mM CsCl, and symmetrical 1 mM CaCl$_2$ and 10 mM TES. Each point in the I/V relationship is the mean single channel current obtained in 3 (A) or 7 (B) experiments. The lines are third order polynomials which were used to obtain the $E_{rev}$ ± SEM for the ordinate fall within the symbols. Channel traces show examples of channel activity at different holding potentials, with no net current being passed by the channel at -20 mV (the potential close to $E_{rev}$). Dotted lines indicate the channel closed level.
because the presence of symmetrical Ca\textsuperscript{2+}, which competes with Cs\textsuperscript{+} for a binding site within the RyR pore (Tinker et al., 1992), reduces Cs\textsuperscript{+} conductance (see section 3.5) and moves the \( E_{rev} \) towards \( E_{Ca^{2+}} \) (0 mV). Records of single channels at different holding potentials show the similarities in channel activity and maximum conductance between the native and purified RyRs (Fig. 3.1). These currents were observed following the incorporation of 74% of native vesicles \((n = 1530)\) and 36% of proteoliposomes \((n = 84)\). 2) A Cl\textsuperscript{-} current, similar to that described by Rousseau (1989), that had an observed \( E_{rev} \) of \( \pm 40 \) mV (the theoretical \( E_{rev} \) for Cl\textsuperscript{-} in 250/50 mM CsCl) and a conductance of \( \pm 70 \) pS (Fig. 3.2A). This Cl\textsuperscript{-} current was seen in 61% of native vesicle incorporations \((n = 1530)\), but was not seen after proteoliposome incorporation. 3) A channel with properties similar to the VDAC\textsuperscript{+} channels described from outer mitochondrial membrane (Mirzabekov et al., 1993). This channel displayed multiple conductance levels (substates - see section 3.4.3) as \( V_m \) was increased to \( \pm 30 \) mV and a large unitary conductance \( (\pm 500 \) pS in 250/50 CsCl, 1 mM CaCl\textsubscript{2}). Such activity was seen in only 18 of 1530 native SR incorporations. These channels were more frequently observed upon incorporation of proteoliposomes; properties were similar to those seen from native vesicles and occurred in 82% of incorporations \((n = 84)\; ;\; see \ Fig. \ 3.2B)\.

The vast majority of experiments were conducted on RyRs incorporated from native SR vesicles. Because 48% of these incorporations included Cl\textsuperscript{-} channels, all experiments (except those used to obtain I/V curves, in which bilayers containing only a single Cs\textsuperscript{+} conducting channel were selected) were conducted at \( E_{Ca^{2+}} \) (see above) to prevent Cl\textsuperscript{-} flux interfering with the cation current. Analysis of RyR activity was carried out only when a single Cs\textsuperscript{+} conducting channel was active in the bilayer (e.g. Fig. 3.2Ci). Occasionally two (Fig. 3.2Cii) or more (Fig. 3.2Ciii) RyR channels were simultaneously active in the bilayer. In such cases, the current from one channel was superimposed upon that of another. Such large currents were unlikely to be a single large conductance channel because a single event from the maximum current to the baseline (or closed state) was rarely, if ever, seen, and because the maximum current was a multiple of the maximum single cation current.

### 3.4 Identification of the cation current: pharmacology

#### 3.4.1 Ca\textsuperscript{2+} dependence of the native cation channel

The Ca\textsuperscript{2+} dependence of the native Cs\textsuperscript{+} conducting channel was tested to see whether increases in the cis [Ca\textsuperscript{2+}] from nanomolar through to millimolar levels would affect its level of activity in a manner consistent with that previously described for the cardiac RyR. Figure 3.3A shows the average results from the analysis of channels recorded for 1 - 2 min at different Ca\textsuperscript{2+} concentrations. Most
**Fig. 3.2** Currents observed after incorporation of native SR membrane and proteoliposomes containing the purified RyR. A - Activity of a Cl⁻ conducting channel from native SR in 250/50 mM CsCl, 10⁻⁵/10⁻³ M CaCl₂. At a holding potential of -40 mV current flowed from the cis to the trans chamber, therefore openings are downward transitions from the closed level (dotted line). B - VDAC -like channel activity after addition of proteoliposomes to the cis chamber. Recorded in 250/50 mM CsCl, 1 mM CaCl₂ at +40 mV. C - Examples of RyR activity after incorporation of native SR vesicles: (i) a bilayer containing a single active RyR with 250/50 mM CsCl, 1 mM CaCl₂ at a holding potential of +40 mV. (ii) A channel record showing two simultaneously active RyRs in the bilayer. O₁ indicates maximum open level of n channels. Recording conditions identical to C1 but with 10⁻³ M Ca²⁺. (iii) RyR activity from a bilayer containing at least six active channels. Recorded in 500/50 mM CsCl, 1 mM CaCl₂ at +40 mV.
individual channels were exposed to more than one \([Ca^{2+}]\) during an experiment. Hence, the values of open probability \(P_o\), mean open time \(T_o\) and event frequency \(F_o\) (see Methods) from individual channels were included in the data for up to three different \(Ca^{2+}\) concentrations. Open probability was determined for each channel and displayed as the mean ± SEM for \(n\) channels. The solid line is the best least squares fit of the data to the Hill equation (Eq. 3.1):

\[
P_o = P_{\text{max}} \left( \frac{1}{1 + (K_A / [Ca^{2+}])^{H_A}} \right) \left( 1 - \frac{1}{1 + (K_I / [Ca^{2+}])^{H_I}} \right)
\]

(3.1)

where \(P_{\text{max}}\) is the open probability of the fully activated channel, \(K_A\) and \(K_I\) are the binding affinities for activation and inhibition respectively, and \(H_A\) and \(H_I\) are the Hill coefficients for activation and inhibition respectively. The Hill coefficients are equivalent to the number of ions, in this case \(Ca^{2+}\) ions, that bind cooperatively to the protein to produce channel activation or inhibition. Fitting the mean values of \(P_o\) (Fig. 3.3A) with Eq. 3.1 gave: \(P_{\text{max}} = 0.25\), \(K_A = 7.5\ \mu\text{M}\), \(H_A = 1.17\), \(K_I = 3.5\ \text{mM}\) and \(H_I = 2.0\). Overall, the activation and inhibition characteristics obtained are consistent with those of the cardiac RyR: 1) the data follow a typical “bell shaped” \(Ca^{2+}\)-activation curve (Laver et al., 1995); 2) \(P_{\text{max}} (0.25)\) is similar to the value of 0.31 when \(P_o\) was calculated from steady state recordings of ~3 min (Sitsapesan and Williams, 1994b), although lower than 0.55 when \(P_o\) was calculated from within bursts (Laver et al., 1995); 3) \(K_A (7.5\ \mu\text{M})\) falls into the range reported for the cardiac RyR of 0.7 - 44 \(\mu\text{M}\) (Rousseau and Meissner, 1989; Chu et al., 1993; Sitsapesan and Williams, 1994b; Laver et al., 1995); 4) \(H_A (1.17)\) is similar to Hill coefficient values reported for the RyR of 1.0 - 1.1 (Chu et al., 1993; Laver et al., 1995), 5) \(K_I (3.5\ \text{mM})\) was lower than the 15 \(\text{mM}\) reported when fitting mean values of \(P_o\), although it falls within the range of values obtained when data from individual RyRs were fitted by the Hill equation (1.5 - 7 \(\text{mM}\) (Laver et al., 1995) and 6) \(H_I (2.0)\) is comparable to the values reported by Laver et al. (1995) of 1.7 (or 2.0 when Hill coefficients were obtained from individual channels). Comparison of the \(Ca^{2+}\)-inhibition data with published data for the single cardiac RyR is limited to a single report (Laver et al., 1995). Other single channel studies investigating the \(Ca^{2+}\)-dependence of the cardiac RyR failed to detect inhibition with higher concentrations of \(Ca^{2+}\) (Rousseau and Meissner, 1989; Chu et al., 1993): possibly because insufficiently high concentrations of \(Ca^{2+}\) were tested, or possibly because the property of the channel which confers \(Ca^{2+}\)-inhibition, a “fragile” characteristic of the channel, was removed during vesicle preparation (Laver et al., 1995).

Average open probability increases with a \([Ca^{2+}] > 10^{-7}\ \text{M}\), reaches a peak at between 10^{-4} and 10^{-3} \(\text{M}\) and declines as the \([Ca^{2+}]\) rises above 10^{-3} \(\text{M}\). Mean open time and event frequency were obtained from the same channels from which \(P_o\) was measured. Both \(T_o\) and \(F_o\) display a similar dependence on the \([Ca^{2+}]_{\text{cis}}\) as \(P_o\).
Fig. 3.3  \( \text{Ca}^{2+} \)-dependence of the native RyR. Channels were recorded at +40 mV in 250/50 mM CsCl, 1 mM trans \( \text{CaCl}_2 \) and a cis \([\text{Ca}^{2+}]\) as indicated. A - Each point is the mean of \( P_\text{o} \), \( T_\text{o} \) or \( F_\text{o} \) of \( n \) channels. Vertical bars show ± SEM where these exceed the dimensions of the symbol. Data from each channel were recorded over 1 - 2 min. The solid line for \( P_\text{o} \) is the best least squares fit of the data to the Hill equation (Eq. 3.1) which gave: \( P_{\text{max}} = 0.25 \), \( K_A = 7.5 \mu \text{M} \), \( H_A = 1.17 \), \( K_I = 3.5 \text{ mM} \) and \( H_I = 2.0 \) (see text). Lines link data points for \( T_\text{o} \) and \( F_\text{o} \). B - Records from three separate RyRs provide examples of channel activity in subactivating, activating and inhibiting \( \text{Ca}^{2+} \) concentrations. The \( P_\text{o} \) shown was calculated from the 5 s section of channel activity displayed.
(Fig. 3.3A). There are increases in both $T_o$ and $F_o$ as the $[Ca^{2+}]$ rises above $10^{-7}$ M, showing that the changes in $P_o$ are due to $T_o$ as well as $F_o$ (lines connect mean values of $T_o$ and $F_o$ for clarity). It is interesting that the inhibition of the channel, as $[Ca^{2+}]_{cis}$ rises to $\geq 5 \times 10^{-3}$ M, initially occurs due to a rapid decline in $T_o$ to very low values, which is followed by a decrease in $F_o$ as the $[Ca^{2+}]$ is further increased. Records from 3 separate RyRs at subactivating ($10^{-7}$ M), activating ($10^{-5}$ M) and inhibitory ($2 \times 10^{-2}$ M) $Ca^{2+}$ show single channel activity typical of that seen at each $[Ca^{2+}]$ (Fig. 3.3B). The $P_o$ for each of the records are close to the mean values in Figure 3.3A.

3.4.2 Response of the native cation channel to endogenous/exogenous ligands

The $Ca^{2+}$-sensitive $Cs^+$ conducting channel was exposed to drugs and salts, that affect the RyR in a known and reproducible manner, to confirm its identification as the RyR. Figure 3.4 shows the effects on the native $Cs^+$ conducting channel of such reagents added to the cis chamber in the normal experimental solutions. In the top three panels (Fig. 3.4A-C) the channel was activated with $10^{-3}$ M $Ca^{2+}$. In the lower two (Fig. 3.4D-E), control activity was lowered by reducing the cis $Ca^{2+}$ concentration to $10^{-7}$ M to highlight the effects of the potentiating reagents.

a) Ryanodine and Ruthenium Red

Ryanodine ($3 - 30$ µM) reduced the maximum conductance of all channels to a level $\sim 50\%$ of their maximum value within 2 min ($<20$ s in 53% of the channels; $n = 17$; Fig. 3.4A) - no further openings to the maximum conductance were observed. This result is similar to that of Rousseau et al. (1987). Channel closures became brief resulting in a high $P_o$ in the ryanodine-modified state. The high $P_o$ of the ryanodine-modified channel was independent of the cis $[Ca^{2+}]$ (Rousseau and Meissner, 1989; Laver et al., 1995), with the same effects observed at different $[Ca^{2+}]_{cis}$: $10^{-7}$ M ($n = 2$), $10^{-6}$ M ($n = 3$), $10^{-3}$ M ($n = 7$) and $2 \times 10^{-2}$ M ($n = 5$).

Ruthenium red was first found to block mitochondrial $Ca^{2+}$ transport (Moore, 1971) but was subsequently shown to irreversibly block the RyR from striated and smooth muscle with low to sub micromolar concentrations (Smith et al., 1985; Rousseau et al., 1986; Ma, 1993). Figure 3.4B shows the block of the high conductance cation current from SR membrane with 10 µM ruthenium red. Channel block was seen in all channels (91% within 10 s) with 10 µM ruthenium red at $10^{-3}$ M ($n = 10$) and $2 \times 10^{-2}$ M ($n = 1$) $Ca^{2+}$.

b) $Mg^{2+}$

$Mg^{2+}$ inhibits the skeletal and cardiac RyR (Meissner, 1994). It has two proposed binding sites on the channel: one, with a $Mg^{2+}$ affinity of $\sim 1$ mM, where
**Fig 3.4 Characterisation of the native RyR using pharmacological and physiological reagents.** Drugs were added to the *cis* chamber after a control recording in 250/50 mM CsCl, 1 mM *trans* Ca$^{2+}$ and either 10$^{-3}$ or 10$^{-7}$ M *cis* Ca$^{2+}$. Holding potential was +40 mV. Channel traces in the presence of the drug were recorded within 100 s of drug addition.
$Mg^{2+}$ competes with $Ca^{2+}$ for the $Ca^{2+}$-binding site thus preventing $Ca^{2+}$-activation; the other with a lower $Mg^{2+}$ affinity which shares a common site with $Ca^{2+}$ to inhibit the channel (Laver et al., 1997). To test the effects of $Mg^{2+}$ on the high conductance cation channel two sets of experiments were carried out. Firstly, to look at the effects of $Mg^{2+}$ on the $Ca^{2+}$-activation site, the $cis \, [Ca^{2+}]$ was reduced to $10^{-7}$ M (close to the resting $[Ca^{2+}]$). Activity of the channels was typically low: $P_o = 0.0008 \pm 0.0004$, $T_o = 1.19 \pm 0.12 \, ms$ and $F_o = 0.59 \pm 0.3 \, s^{-1}$. Addition of 1 mM $Mg^{2+}$ to the $cis$ chamber significantly reduced all three parameters to $P_o = 1 \times 10^{-5} \pm 7 \times 10^{-6}$, $T_o = 0.51 \pm 0.02 \, ms$ and $F_o = 0.02 \pm 0.01 \, s^{-1}$ ($P < 0.001; n = 5$). BAPTA was used to buffer the $cis \, Ca^{2+}$ concentration to $10^{-7}$ M. While BAPTA has a much lower affinity for $Mg^{2+}$ than for $Ca^{2+}$ (Tsien, 1980), addition of $Mg^{2+}$ could have altered the free $[Ca^{2+}]$ by displacing $Ca^{2+}$ ions from the BAPTA. Consequently, the free $[Ca^{2+}]$ and $[Mg^{2+}]$ were calculated (using Buffta): the $[Ca^{2+}]$ remained essentially unchanged at $1.13 \times 10^{-7}$ M while the free $[Mg^{2+}]$ was close to the 1 mM added to the solution ($9.47 \times 10^{-4}$ M).

The second set of experiments was designed to look at the effects of $Mg^{2+}$ binding to its low affinity site. The channels were activated with 1 mM $Ca^{2+}$ then exposed to 10 mM $Mg^{2+}$ (Fig. 34C). $Mg^{2+}$ altered channel gating: while average $P_o$ was reduced from $0.14 \pm 0.06$ to $0.06 \pm 02$ the difference was not significant since $P_o$ in 3 of 7 channels remained unaltered. However, $T_o$ fell in all channels with a significant average reduction from $5.8 \pm 2.6$ to $0.77 \pm 0.04 \, ms$ ($P < 0.01; n = 7$). $F_o$, like $P_o$, was not significantly altered. The high concentration of $Mg^{2+}$ also reduced the conductance of the channel. The rapid flickering during $Mg^{2+}$-inhibition, evident from the channel record in Figure 3.4C (right), meant that individual open events were not always fully resolved, giving the appearance of a reduction in channel conductance. When channel openings were long enough in duration to be fully resolved, it was found that $Mg^{2+}$ did cause a significant reduction in channel conductance of $22.4 \pm 1.2\%$: from $233 \pm 10 \, pS$ in control conditions to $181 \pm 7 \, pS$ after the addition of 10 mM $Mg^{2+}$ ($P < 0.0001; n = 7$). This was due to $Mg^{2+}$ competing with $Cs^+$ for passage through the channel, which reduces the conductance of the RyR.

For each of the two sets of $Mg^{2+}$-inhibition experiments, the 2 min period immediately after $Mg^{2+}$ addition was compared to a 2 min control recorded directly prior to $Mg^{2+}$. The effects of high concentrations of $Mg^{2+}$ on the $Ca^{2+}$-activated channel were similar to those reported for the skeletal (Smith et al., 1986) and the cardiac (Hymel et al., 1988; Laver et al., 1997) RyR.

c) **Caffeine and ATP**

Finally, two agents known to potentiate RyR activity - caffeine (Meissner and Henderson, 1987; Rousseau and Meissner, 1989; Sitsapesan and Williams, 1990) and the endogenous adenine nucleotide, ATP (Smith et al., 1985; Rousseau...
et al., 1986) - were tested on the cation channel to see if the reported effects of these agents could be reproduced. Experiments were conducted in the normal solutions, with the cis [Ca\(^{2+}\)] reduced to 10\(^{-7}\) M to lower control channel activity. Addition of 2 mM caffeine to the cis chamber produced no change in conductance, but a 10 -fold increase in \(P_o\) from 0.003 ± 0.002 to 0.032 ± 0.01 (\(P < 0.001; n = 6\); Fig. 3.4D), through 3 -fold increases in both \(T_o\) (\(P < 0.0001\)) and \(F_o\) (\(P < 0.01\)). The lack of effect on conductance and an increase in \(P_o\) is consistent with the previously reported effects of caffeine on the single cardiac RyR (Rousseau and Meissner, 1989, Sitsapesan and Williams, 1990). However, while the increase in \(P_o\) resulting from increases in both frequency and duration of open events is in agreement with the findings of Rousseau and Meissner (1989), it is in contrast to a report of Sitsapesan and Williams (1990) who show that caffeine activation occurs through an increase in event frequency only.

The cardiac RyR is also activated by millimolar concentrations of ATP. A putative nucleotide binding consensus sequence in the cardiac RyR at residues 2619 - 2652 lies in the cytoplasmic domain of the channel (Otsu et al., 1990) and is in the proposed “modulatory” region of the channel (McPherson and Campbell, 1993). The effects of ATP on the native SR cation channel were investigated by comparing the channel activity during a 2 min control recording to the channel activity during a 1 - 2 min period immediately after ATP addition. No effects of ATP (2 mM) were observed when it was added to channels in 10\(^{-9}\) M cis Ca\(^{2+}\) (\(n = 4\)) in accordance with Rousseau et al. (1986). However, with a cis Ca\(^{2+}\) concentration of 10\(^{-7}\) M, ATP (4 mM) significantly increased each of \(P_o\), \(T_o\) and \(F_o\) (\(P < 0.0001; n = 12\); Figs. 3.4E&3.5), while having no effect on channel conductance. \(P_o\) was increased >30 -fold from 0.0005 ± 0.0002 to 0.016 ± 0.004 primarily through an increase in \(F_o\) (15 -fold), and also through a 2 -fold increase in \(T_o\). These results are similar to the reported effects of ATP on the cardiac RyR: Tsushima et al. (1996) reported an increase in \(P_o\) from 0 to 0.015 (in 10\(^{-7}\) M Ca\(^{2+}\)) with 2 mM ATP, also through an increase in both frequency and duration of open events.

The effects of ATP on measured single channel properties are shown in Figure 3.5. In each of 12 channels, ATP produced increases in \(P_o\), \(T_o\) and \(F_o\) (Fig. 3.5A) - the mean values for control and ATP are shown (filled circles) to the left and right (respectively) of the single channel values. A quantitative analysis of the channel open and closed durations was used to determine qualitatively the processes involved in gating. Since the distributions of open and closed times from single channels can be dispersed over several orders of magnitude, the method of Sigworth and Sine (Sigworth and Sine, 1987) was used to display the distributions using a logarithmic time scale. This method enables simultaneous comparison of the distribution of short and long events. The effects of ATP on open event durations were determined by comparing the distribution of open times before and
Fig. 3.5 Effects of ATP on activity of the native RyR in subactivating Ca\(^{2+}\).
ATP (4 mM) was added to twelve RyRs in 10\(^{-7}\) M Ca\(^{2+}\). A - Values of P\(_o\), T\(_o\) and F\(_o\) were measured during a 2 min control recording and compared to values in the presence of ATP measured over a 1-2 min period. The data for each channel, before and after ATP, were plotted and joined by a straight line. The filled circles to the left and right of the single channel data are the mean of the control and ATP data, respectively; vertical bars ± SEM. Asterisks indicate significant differences from control: *** P < 0.001. Bi - The distribution of open times before and after ATP for a single channel were compared by plotting the square root of frequency (√f) of open times in bins of logged durations (see section 2.6). Data were compiled from 1-2 min of channel activity. The exponential function, obtained from the best least squares fit to the data, is shown as the solid line through the data. A single exponential fit to both control and ATP data gave time constants of 1.95 ms and 5.45 ms respectively. Bii - The time constants calculated for each of the twelve channels were allocated to groups, averaged and plotted. Horizontal axis - time constant (τ, ms). Vertical axis - proportion of events (area, %). Horizontal bars show ± SEM for τ, and vertical bars ± SEM of the area, where these exceed the dimensions of the symbol.
**A**

Individual channels

- \[ \log P_0 \]
- \[ \log T_0 \]
- \[ \log F_0 \]

Control

ATP

The response of cardiac membranes to Ca-conducting channel in the pharmacological responses produced by ATP is shown.

**B**

- \[ i \]

- \[ ii \]

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<th>[ \sqrt{t} ]</th>
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<th>[ Area(%) ]</th>
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5.4.1 Purified versus the native channel: calcium current

Calcium incorporation is greater than the incorporation of native skeletal Ca channels, indicating that the Ca-conducting channel, following incorporation into the pure peptide in a manner that is more than in native vesicles (ADP). However, the incorporation of Ca channels from either preparation, ATP activated, shows a large relationship for the purified channel. ATP activation caused a substantial increase of 80 ± 7 \% and a control decrease of 12% 

\( \pm 1.5 \% \) compared to 15% \( \pm 2 \% \) and -18.5 \( \% \) \( \pm 3 \% \) respectively for the native channel in skeletal muscle. No analysis of...
after ATP. Open events for each channel were sorted into bins of variable width (7 bins/decade) on a logarithmic scale (see Methods; Sigworth and Sine, 1987). The exponential components, seen as peaks within each distribution, were fitted to the log binned data using a least squares analysis. Figure 3.5Bi shows the distribution of open times for a single channel during a control recording in $10^{-7}$ M Ca$^{2+}$ (circles) and after activation by 4 mM ATP (squares). A single exponential component (solid line) fitted each distribution with time constants of 1.95 ms (control) and 5.45 ms (ATP). The open time distribution for the control data in $10^{-7}$ M Ca$^{2+}$ were all fitted by single exponential components: each of these fell into the shortest time constant group ($\tau_1 < 3$ ms) with an average time constant of $1.6 \pm 0.2$ ms (Fig. 3.5Bii). After ATP addition, the open time distributions for 11 of 12 channels were still fitted by a single exponential component (the 12th requiring a double) but the values for these components fell into two groups: either $\tau_1$ (<3 ms) or $\tau_2$ (3 - 12 ms). The average time constants after ATP activation were: $\tau_1 = 2.4 \pm 0.2$ ms (n = 5) and $\tau_2 = 5.2 \pm 1.1$ ms (n = 8) (Fig. 3.5Bii).

The response of the native SR Cs$^+$-conducting channel to the pharmacological reagents provides strong evidence that the channel is the RyR.

### 3.4.3 Purified verses the native channel - similarities/differences

Incorporation of proteoliposomes took longer than the incorporation of native vesicles, and the probability of observing the Cs$^+$ conducting channel following incorporation was lower in proteoliposomes (~0.36) than in native vesicles (~0.74). However, characteristics of the Cs$^+$ conducting cation channel from either preparation were essentially identical: 1) the current-voltage relationship for the purified channel in 250/50 mM CsCl, 1 mM CaCl$_2$ had a slope conductance of $266 \pm 7$ pS and a $E_{rev}$ of $-17.7$ mV (n = 7) compared to $259 \pm 1$ pS and $-18.5$ mV (n = 3) respectively for the native channel in identical solutions (see Fig. 3.1); 2) the purified channel showed the same dependence on Ca$^{2+}$ for activation as the native channel. At subactivating Ca$^{2+}$ ($10^{-7}$ M) channel activity was low ($P_o = 0.0003 \pm 0.0003$; n = 4); activity increased with $10^{-6}$ M Ca$^{2+}$ ($P_o = 0.025 \pm 0.009$; n = 2) and was optimal in $10^{-3}$ M Ca$^{2+}$ and 3) ryanodine (10 µM) locked the channel into a sub-conductance state at ~50% of the maximum conductance and irreversibly blocked the channel at higher concentrations (Fig. 3.6A). These facts strongly support the conclusion that the Cs$^+$-conducting channel recorded after proteoliposome incorporation is the RyR.

Only one aspect of channel activity between the native and the purified RyR was found to differ in the present study. The purified RyR showed a greater likelihood of substate activity (Fig. 3.6B) (Lai and Meissner, 1989) compared to the native channel (Williams, 1992; Laver et al., 1995), even though conductance and gating characteristics were similar (Lai and Meissner, 1989; Sitsapesan and Williams, 1994b; Zahradniková and Zahradnik, 1995) (Fig. 3.1). No analysis of
Fig. 3.6 The purified RyR is modified by ryanodine and has subconductance levels. A - Effect of ryanodine (Ry) on the CHAPS-solubilised RyR recorded in 250/250 mM CsCl, 10^-6/10^-9 M CaCl2 at +40 mV. Left - Single channel activity. Right - All points histogram of the 2 s trace on the left. B - Single channel activity of the purified RyR showing subconductance levels. Below - All points histogram of the 10 s trace above. Recorded in 250/50 CsCl, 1 mM CaCl2 at a holding potential of +60 mV.
RyR substate activity was performed, since most experiments used the native channel which displayed minimal substate activity (i.e. most events were transitions between the maximum conducting level and the closed level, unless the events were too brief to be fully resolved e.g. in the Ca$^{2+}$- or Mg$^{2+}$-inhibited channels). Laver et al. (1995) showed a further difference between the native and purified channels: the purified RyR did not possess the Ca$^{2+}$-inhibition properties, that were seen in the native RyR (see Fig. 3.3), when the [Ca$^{2+}$] was ≥10 mM. This was attributed to the Ca$^{2+}$-inhibition mechanism, which is a “fragile” property of the channel and is removed during the somewhat harsh purification process.

3.5 Effect of Ca$^{2+}$ on maximum RyR current

Although the Cs$^+$-conducting channel appeared to be the RyR, the I/V relationship showed a non-linearity and a slope conductance (at +40 mV) of ~260 pS in 250/50 mM CsCl (Fig. 3.1). These properties differed from some reports in the literature which showed linear I/V curves and conductances in 250/50 CsCl or symmetrical 250 mM Cs$^+$ of ~500 pS (Smith et al., 1988; Chu et al., 1993). The reason for these discrepancies was due to the use of asymmetrical salt solutions and a high trans [Ca$^{2+}$] (Ahern et al., 1994). Ca$^{2+}$ competes with monovalent cations for a binding site within the channel pore (Tinker et al., 1992), thereby reducing channel conductance. To show that the solutions used were responsible for the reduced conductance and the non-linearity of the I/V relationship, the conductance properties of RyRs in different salt solutions were examined. In control solutions (250/50 mM CsCl, 10$^{-5}$ M Ca$^{2+}$), the purified RyR had a non-linear I/V relationship with a $E_{rev}$ of -17.7 mV and a chord conductance (at +40 mV) of 266 ± 7 pS (n = 7) (Fig. 3.7A - circles). In symmetrical solutions (250 mM CsCl), the I/V relationship was linear with a $E_{rev}$ close to 0 mV (Fig. 3.7A - triangles and squares). Channel conductance was increased, close to the values reported (above), by lowering the [Ca$^{2+}$]: conductance (at +40 mV) increased from 258 ± 4.2 pS in control solutions (250/50 mM CsCl, 1 mM CaCl$_2$) to 535 ± 4.8 pS after the perfusion of solutions containing 250/250 mM CsCl and 10$^{-6}$/10$^{-9}$ M Ca$^{2+}$ (n = 3). Examples of the effects of lowering the [Ca$^{2+}$] on channel conductance are shown. Firstly, by comparing the I/V curves of two separate channels in either 1 mM symmetrical Ca$^{2+}$ (Fig. 3.7A - triangles; 333 pS) or 10$^{-6}$/10$^{-9}$ M Ca$^{2+}$ (Fig. 3.7A - squares; 525 pS). Secondly, by comparing the channel records of a single RyR (Fig. 3.7B) in asymmetrical CsCl with 1 mM Ca$^{2+}$ (left) to the records following the perfusion of both chambers with symmetrical CsCl and a low Ca$^{2+}$ concentration (right). The different gating pattern at the negative holding potential is a factor of the voltage-dependence of open probability; the channel is more open at positive potentials (Rousseau and Meissner, 1989; Laver et al., 1995).
3.6 Data presentation and analysis

Data were collected at room temperature and current-voltage (I/V) relationships were constructed from these recordings in usual control solutions (Fig. 3.7A). Single channel current-voltage (I/V) relationships were constructed from the single channel currents using the state selection algorithm as described in Methods. The single channel currents were then compared across different temperature and 

Fig. 3.7  Effects of symmetrical solutions and [Ca$^{2+}$] on the current-voltage relationship and channel conductance of the purified RyR. A - I/V data of the purified RyR in usual control solutions (●, from Fig. 3.1) were compared to I/V curves of two separate RyR channels in symmetrical 250 mM CsCl solutions: one with 10^{-3} M symmetrical CaCl$_2$ (▲) (E$_{rev}$ = -1.4 mV); the other with 10^{-6}/10^{-9} M CaCl$_2$ (★★) (E$_{rev}$ = +2.2 mV). B - Single channel traces showing the effects of altering the ionic composition of the solutions on maximum conductance: left - activity in usual control solutions; right - activity from the same channel after lowering the [Ca$^{2+}$] and making the concentration of CsCl symmetrical.
3.6 Data acquisition: sampling frequency

Data were sampled at the double the filter frequency. This was considered sufficient to describe the gross changes in channel activity described in this study. However, concerns about sampling at less than 5 times the filter frequency exist (Colquhoun and Sigworth, 1983). Therefore, results were compared from analysis of identical sections of data which were sampled at 2 and 5 kHz (with a 1 kHz filter). This was to see if there were any differences in the results, and to assess whether an increase in the sampling rate was warranted.

Three 30 s sections of activity from separate channels were selected because of the different patterns of channel activity (Fig. 3.8A). Each record was sampled at 2 and 5 times the filter frequency then analysed using Channel2 to determine $P_0$, $T_0$, $F_0$ (Fig. 3.8B) and open times (from which open time constants were subsequently obtained). Both $P_0$ and the number of exponentials required to fit the open time distributions were independent of the sampling rate. Each open time distribution contained 3 exponentials: the time constants, and fraction of events in each, varied <20%. However, there were exceptions: in channel (i) the longest component (~300 ms) was ~25% briefer at 5 kHz than at 2 kHz, because brief closures were resolved at the higher sampling rate. In addition, the shortest time constant (~1 ms) in channels (ii) and (iii) contained ~40% more events (with corresponding decreases in the percentage of events in longer components) at 5 kHz than at 2 kHz. The table (Fig. 3.8B) shows the effects of increasing the sampling rate on $T_o$ and $F_o$. Average open duration decreased between ~9 - 25% with corresponding increases in event frequency.

The primary difference between data analysed at 2 or 5 times the filter frequency was seen in $T_o$ and $F_o$. However, these differences were small in relation to the changes induced with the reagents under investigation in this study. Hence, conclusions would not be influenced by possible “undersampling” (Eager et al., 1997).
Fig. 3.8 **Comparison of RyR activity sampled at 2 and 5 kHz with a 1 kHz filter.** A - Left - 30 s sections of channel recordings were selected on the basis of different patterns of channel activity. Right - Time constants were obtained from the distribution of open times, for the 30 s of channel activity (left), after the data were sampled at 2 and 5 kHz. The time constants were plotted (see legend to Fig. 3.5). Straight lines connect the points. B - Summary of $P_0$, $T_0$, and $F_0$ for the three sections of channel activity (Ai - iii) at the two different sampling rates.
DISCUSSION

3.7 Identification of the RyR

Channel activity, with properties identical to those described by others for the cardiac RyR, was observed after membrane from cardiac SR or proteoliposomes containing the purified RyR were incorporated into a lipid bilayer. Vesicle incorporation inserted channels into the bilayers which were identified as RyRs, Cl⁻ channels and occasionally a VDAC-like channel. Proteoliposome incorporation inserted channels into the bilayer producing RyR activity and VDAC-like currents. RyR activity was observed in 74% of all incorporations from SR vesicles and 36% of incorporations from proteoliposomes. Cs⁺ was the main cation in the cis and trans solutions. With 250 mM CsCl in the cis chamber and 50 mM CsCl in the trans chamber, current passed by RyRs reversed at close to -20 mV and flowed from the cis to the trans chamber at potentials more positive than this. The ryanodine-sensitive Cs⁺ current was shown to pass through the RyR because of the sensitivity of the current to various ligands that are well known to affect RyR activity. These ligands included ryanodine, ruthenium red, Ca²⁺, Mg²⁺, ATP and caffeine. In addition, the maximum conductance of the channel was the same as that reported for the RyR.

3.8 Recording current from single RyR channels

3.8.1 Incorporation of vesicles and proteoliposomes

The total number of RyRs present in a bilayer after vesicle incorporation could not be precisely determined. Occasionally, the activity of more than one channel was observed. This was recognised when two or more channels were simultaneously active and the current from one was superimposed upon that of another. This was unlikely to be a single channel with substate activity because the maximum conductance was a multiple of RyR maximum conductance, and transitions from the maximum to closed levels were rarely observed. In the single RyR, transitions between maximum open and closed levels were common. Why were individual RyRs observed in some bilayers and double or multiple RyRs in others? Heavy SR vesicles are ~100 - 200 nm in diameter and may contain variable numbers of RyRs. If vesicles usually contain zero or only a single functional RyR, then multiple RyRs present in the bilayer may be due to the simultaneous fusion of vesicles that were clustered together. Alternatively, more than one vesicle may have incorporated before any single channel became active. To reduce the possibility of the latter, the solutions contained concentrations of Ca²⁺ that favoured RyR activation and, as soon as RyR activity was observed, the
cis solution was replaced to remove vesicles or proteoliposomes and thus prevent further incorporation.

There is also a possibility that more than one RyR may be present in a bilayer, but only one is active at any point in time. This would give the impression that only a single active RyR is present in the bilayer. Under these circumstances the presence of more than one RyR would be difficult to detect because maximum channel conductance between individual RyRs is very consistent. The possibility of this occurring is reduced with an activating \([Ca^{2+}]\) in the incorporating solution i.e. favouring channel activation. Interestingly, a situation in which two channels with different gating patterns, which were alternately active in the one bilayer, could explain modal gating that is observed with RyR activity. Channel activity has been shown to rapidly switch between slow and rapid gating (Laver et al., 1995; Zahradnikova and Zahradnik, 1995). However, such gating switches have been observed in sections of channel activity when the channel is very active. Unless there is a high degree of cooperativity between two channels, allowing them to alternately open and close, then it is very unlikely that modal gating is due to the activity of more than one channel.

### 3.8.2 Sampling frequency

The data were filtered at 1 kHz and sampled at twice the filter frequency. Increasing the sampling rate allows the detection of shorter events and thus collects data that more closely reflects the original signal. However, it also has the drawback of increasing the amount of data for analysis and storage. While a sampling rate that is five times the filter frequency is recommended (Colquhoun and Sigworth, 1983), the rate used in the present study was shown to be sufficient to describe the data and is used in other studies which also analyse the single channel activity of RyRs (McGarry and Williams, 1994a; Schiefer et al., 1995).

### 3.9 RyR conductance

The maximum RyR conductance of 535 pS in 250 mM symmetrical CsCl and low concentrations of Ca\(^{2+}\) was consistent with the values of ~500 pS reported in the literature (Williams, 1992). However, under normal recording conditions, in asymmetrical CsCl and higher concentrations of Ca\(^{2+}\), the conductance was significantly reduced because channel conductance is sensitive to millimolar concentrations of Ca\(^{2+}\). The \(cis\) \([Ca^{2+}]\) was adjusted according to the requirements of the experiment. The \(trans\) \([Ca^{2+}]\) was maintained at \(10^{-3}\) M. The SR \([Ca^{2+}]\) of skeletal muscle is in the millimolar range, but measurements suggest that the SR \([Ca^{2+}]\) in myocytes is closer to \(100\ \mu M\) (see section 1.4.2a). Millimolar \(trans\) Ca\(^{2+}\) was used in the experiments because earlier estimates of luminal \([Ca^{2+}]\) were higher (Bers, 1991) than present estimates, and many other studies investigating single cardiac RyRs have used millimolar concentrations of \(trans\) Ca\(^{2+}\) (Rousseau and
Meissner, 1989; Smith et al., 1989; Williams and Ashley, 1989). In addition, it is possible that estimates of luminal Ca\(^{2+}\) may be somewhat low because values of the SR [Ca\(^{2+}\)] comprise only the Ca\(^{2+}\) content of the SR that is releasable by caffeine (Bassani et al., 1995). Consequently, the trans [Ca\(^{2+}\)] was maintained at 1 mM in most experiments. Importantly, for channels activated solely by cis Ca\(^{2+}\), the steady state open probability and channel gating are not affected by increasing the luminal [Ca\(^{2+}\)] from nanomolar to millimolar concentrations (Sitsapesan and Williams, 1994a; Sitsapesan and Williams, 1995a).

The I/V relationship of the purified RyR was essentially indistinguishable from that of the native channel. Under usual recording conditions, both exhibited a rectifying I/V curve and a \(E_{\text{rev}}\) close to -18 mV.

### 3.10 Ca\(^{2+}\)-dependence of the cardiac RyR

The primary activating ligand for the cardiac RyR is Ca\(^{2+}\). Unlike its skeletal counterpart, the cardiac RyR has an absolute dependence on extracellular Ca\(^{2+}\) for its activation in vivo. It is inactive at a resting intracellular Ca\(^{2+}\) concentration, but is activated by the influx of Ca\(^{2+}\) through the sarcolemmal L-type Ca\(^{2+}\) channels (Fabiatand Fabiato, 1975; Cannell et al., 1987; Nabauer et al., 1989; Niggli and Lederer, 1990). Although the cardiac RyR is \(\sim 1.5\)-fold more sensitive to Ca\(^{2+}\) activation than the skeletal RyR (Zimanyi and Pessah, 1991) both isoforms are activated by \(\geq 0.3\) μM Ca\(^{2+}\) (Meissner et al., 1986, Chu et al., 1993; Laver et al., 1995). The Ca\(^{2+}\)-dependence of \(P_0\) for the cardiac RyR was fitted to the Hill equation. This produced the characteristic “bell shape” for Ca\(^{2+}\)-dependence of \(P_0\) (Chamberlain et al., 1984a; Meissner, 1984) and provided quantitative information on the activation and inhibition of the RyR by Ca\(^{2+}\). Half activation and inactivation were in the low micromolar and low millimolar range respectively. The values obtained for the Hill coefficients suggest that a single Ca\(^{2+}\) ion binds to the Ca\(^{2+}\)-activation site to activate the channel, but two Ca\(^{2+}\) ions are required to bind cooperatively for channel inhibition. This is consistent with the findings of other studies investigating the Ca\(^{2+}\)-activation of single cardiac RyRs (Chu et al., 1993; Laver et al., 1995). However, higher values (between 2.4 and 4.0) were obtained when the Hill coefficients were calculated from the Ca\(^{2+}\)-activation curves of individual cardiac RyRs (Sitsapesan and Williams, 1994b). The higher Hill coefficients were accounted for by an improvement in resolution of channel activity, due to the higher single channel conductance when the monovalent ion, Cs\(^+\), was used as the conducting ion instead of Ca\(^{2+}\) (Sitsapesan and Williams, 1994b). However, Cs\(^+\) was also used as the conducting ion in the present study and others (Chu et al., 1993; Laver et al., 1995), in which the values obtained for the Hill coefficient were close to one. Laver et al. (1995) suggested that these lower values for Hill coefficients may be due to “averaging of results from several channels with varying Ca\(^{2+}\) sensitivities”.

Ca\(^{2+}\) concentrations >10\(^{-7}\) M activated both the native and the purified RyR. Maximum \(P_o\) was obtained in 10\(^{-3}\) M Ca\(^{2+}\). Analysis of Ca\(^{2+}\)-activation, for the native RyR, showed that Ca\(^{2+}\) increased both \(T_o\) and \(F_o\). \(T_o\) was maximal in 10\(^{-5}\) M Ca\(^{2+}\) and declined with millimolar concentrations of Ca\(^{2+}\), whereas \(F_o\) was highest in low millimolar concentrations of Ca\(^{2+}\) and declined as the concentrations were increased above 5 x 10\(^{-3}\) M. These findings are consistent with the reports from some laboratories (Smith et al., 1988; Schiefer et al., 1995) but differ from the findings of others. For example, Williams and co-workers have shown that Ca\(^{2+}\) activation of the cardiac RyR occurs primarily through an increase in event frequency with little or no change in event duration (Williams and Ashley, 1989; Ashley and Williams, 1990). Smith et al. (1986) also showed that Ca\(^{2+}\) increased \(P_o\) in the skeletal RyR by increasing the total number of events with no effect on the duration of open times. Other differences between laboratories include the Hill coefficients (discussed previously) and the [Ca\(^{2+}\)] for half maximal activation, which varies between ~1 \(\mu\)M and ~44 \(\mu\)M (Rousseau and Meissner, 1989; Chu et al., 1993; Sitsapesan and Williams, 1994b; Laver et al., 1995). The differences in RyRs, between laboratories, that cause variation in the properties of Ca\(^{2+}\)-activation have not been determined. However, a possible cause of the variation may be due to the primary anion used in the \textit{cis} solution. Meissner et al. (1997) showed that anion specific binding sites on the skeletal RyR regulate channel activity by modifying the apparent Ca\(^{2+}\) binding affinity of the receptor. Channels were more sensitive to Ca\(^{2+}\)-activation in the presence of the chloride anion, which was used in the present study, than other anions such as MES or PIPES (piperazine-N\(^{\prime}\)N\(^{\prime}\)-bis-2-ethanesulfonic acid). Interestingly, Fruen et al. (1996) found increased Ca\(^{2+}\)-activation of skeletal, but not cardiac, RyRs when chloride was substituted for propionate. In addition, some variation may arise from protein preparation procedures which, for example, may lead to dissimilar states of phosphorylation or oxidation.

The concentration of Ca\(^{2+}\) required to activate the RyR may also depend on the surface charge on the lipid. Most laboratories use a mixture of phospholipids in the bilayers into which RyRs are incorporated. These often include the negatively charged phosphatidylserine. However, in some laboratories, channels are routinely incorporated into bilayers containing no charged lipids e.g. 100\% phosphatidylethanolamine. The influence of surface charge on a bilayer has previously been shown to affect channel conductance (Bell and Miller, 1984). SR K\(^+\) channels were incorporated into lipid bilayers containing differently charged lipids. Compared to the conductance of the channel in a neutral bilayer, conductance was reduced in a positively charged bilayer and increased in a negatively charged bilayer (Bell and Miller, 1984). This observation was explained by a higher [K\(^+\)] at the mouth of the channel, compared to the bulk [K\(^+\)]. The difference is determined by the surface potential and the bulk [K\(^+\)] according to the Gouy-Chapman theory. Tu et al. (1994) suggested that the [Ca\(^{2+}\)] at the luminal
face of the RyR was higher than that of the bulk solution. This was considered likely because of negative surface charges at the luminal mouth of the channel. It was speculated that the consequence of this local Ca\(^{2+}\) accumulation might be to act as a preselection filter for the conduction pathway. On the myoplasmic surface of the SR membrane, a bilayer with a net negative charge will induce an increase in the [Ca\(^{2+}\)] at the bilayer surface. A higher local [Ca\(^{2+}\)] may be sensed by the RyR, as suggested by Williams and Ashley (1989). If the Ca\(^{2+}\) -activation site on the RyR was sufficiently close to the bilayer, then it may be influenced by the higher local [Ca\(^{2+}\)]. Consequently, RyRs incorporated in bilayers which are negatively charged, may be activated with lower “bulk” concentrations of Ca\(^{2+}\) than required in neutral bilayers. The influence of surface charge, if any, on the [Ca\(^{2+}\)] sensed by the Ca\(^{2+}\) -activation site on the RyR is highly dependent on the distance of the site from the membrane; the influence of surface charge diminishes as the distance increases. The overall effect of surface potential depends on salt concentrations: in a more concentrated electrolyte solution the effect decays over a shorter distance because there is more screening of the surface charge potential (Bell and Miller, 1984). The distance of decay, or the Debye length, is ~1 nm in a mammalian Ringer’s solution (Hille, 1992) and can be calculated for a given salt solution using the expanded Gouy-Chapman theory (Bell and Miller, 1984).

### 3.11 Pharmacology

The RyR is modulated in a precise manner by many physiological and pharmacological reagents. Ryanodine and ruthenium red were added to RyRs in activating concentrations of cis Ca\(^{2+}\). Ruthenium red rapidly blocked the channel, while ryanodine influenced both gating and channel conductance. The purified RyR was similarly affected by low concentrations of ryanodine, with higher concentrations blocking channel activity. The effects of ryanodine are widely recognised to be irreversible. However, Zimanyi et al. (1992) reported that pretreatment of the skeletal RyR with 0.5 mM DTT enabled full restoration of control activity after 10 µM ryanodine had locked the channel into the characteristic ryanodine -induced substate. The perfusion of ryanodine from the cis chamber followed by addition of Ca\(^{2+}\) and 0.5 mM DTT restored control activity. This effect could not be reproduced in the present study using cardiac RyRs with 1 mM DTT (n = 5, results not presented). While this may be due to differences in the RyR isoforms, more thorough investigations would be required to support this possibility.

Mg\(^{2+}\) inhibits both striated muscle RyR isoforms, albeit with different affinities. Because Mg\(^{2+}\) inhibits RyRs by two independent mechanisms (i.e. interacting with the Ca\(^{2+}\) -activation and Ca\(^{2+}\) -inhibition mechanisms), both were investigated. Firstly, Mg\(^{2+}\) -inhibition via the Ca\(^{2+}\) -activation mechanism occurs because Mg\(^{2+}\) binds to the Ca\(^{2+}\) -activation site. This was shown by the termination
of essentially all RyR activity in 10^{-7} \text{ M Ca}^{2+} with 1 \text{ mM Mg}^{2+}. However, when the cis [Ca^{2+}] is increased to >10^{-6} \text{ M, 1 mM Mg}^{2+} no longer inhibits channel activity because Ca^{2+} competitively binds to the Ca^{2+} -activation site in favour of Mg^{2+} (Laver et al., 1997). The involvement of the second Mg^{2+} -inhibition mechanism was demonstrated by adding high concentrations of Mg^{2+} (10 mM) to channels activated by 1 \text{ mM Ca}^{2+}. The higher [Mg^{2+}] was sufficient to bind to the low affinity Ca^{2+}/Mg^{2+} -inhibition site(s), therefore inhibited the channel via the Ca^{2+} -inhibition mechanism. Mg^{2+} -inhibition via the Ca^{2+} -activation mechanism is likely to be important in both skeletal (Lamb and Stephenson, 1991) and cardiac muscle ECC. By binding to the Ca^{2+} -activation site, Mg^{2+} appears to inhibit channel activity until Mg^{2+} -inhibition is relieved (Lamb and Stephenson, 1991). This is likely to occur following the specific ECC signal which arrives at the triad following depolarisation of the surface membrane.

Caffeine (millimolar) activated RyRs in 10^{-7} \text{ M Ca}^{2+} by increasing both \( T_{o} \) and \( F_{o} \). The increased \( P_{o} \) in the present study was comparable with the effects of caffeine reported by Rousseau and Meissner (1989) who described the concentration -dependent effects of caffeine on the activity of the cardiac RyR, but lower than the caffeine -induced increase in \( P_{o} \) reported by Sitsapesan and Williams (1990). Many other studies using caffeine, or caffeine analogues such as sulmazole, also showed activation of the RyR by increasing \( T_{o} \) and \( F_{o} \) (Rousseau and Meissner, 1989; Williams and Holmberg, 1990; McGarry and Williams, 1994b). In contrast to these studies, Sitsapesan and Williams (1990) found that caffeine -induced activation of the native cardiac RyR in 10^{-7} \text{ M Ca}^{2+} was due primarily to an increase in \( F_{o} \) with no appreciable effect on \( T_{o} \), and suggested that caffeine activated the channel by increasing its sensitivity to Ca^{2+}. This was not the case in the present study. The properties of channels activated by caffeine were compared to the properties of a separate group of channels, with a similar \( P_{o} \), that were activated solely by Ca^{2+}. RyRs activated by caffeine (\( P_{o} \sim 0.032 \)) had a 3 -fold higher \( T_{o} \) and a 2 -fold lower \( F_{o} \) than channels activated by 10^{-5} \text{ M Ca}^{2+} (\( P_{o} \sim 0.022 \)). These differences do not support the proposal that caffeine activates RyRs by increasing their Ca^{2+} sensitivity. The differences between laboratories in the effects of caffeine on the RyR may be due to the two different mechanisms (Ca^{2+} -dependent and Ca^{2+} -independent) by which caffeine can activate the RyR (see section 1.4.3b). Sitsapesan and Williams (1990) showed that adding high concentrations of caffeine (>5 mM) to RyRs, in the absence of Ca^{2+}, activated the channels by increasing both \( T_{o} \) and \( F_{o} \). This was similar to the effects of caffeine observed in the present study. It is possible that the differences in the properties of caffeine -induced activation between laboratories is due to different sensitivities of the channels to the ligands.

Cardiac RyRs were activated by millimolar ATP in 10^{-7} \text{ M Ca}^{2+}, but not in 10^{-9} \text{ M Ca}^{2+}. This was in agreement with other reports on the effects of adenosine
nucleotides on the cardiac RyR (Rousseau et al., 1986; McGarry and Williams, 1994a). The activation of the RyR was due to increases in both event frequency and event duration, which is consistent with the activation of skeletal and cardiac RyRs by ATP and adenosine (Smith et al., 1986; McGarry and Williams, 1994a). Unlike the cardiac RyR, the skeletal RyR is activated by ATP in the absence of Ca\(^{2+}\) (Smith et al., 1986; Tripathy and Meissner, 1996). The strict requirement for Ca\(^{2+}\) in the ATP-activation of the cardiac RyR suggests that the cardiac RyR is more Ca\(^{2+}\)-dependent than the skeletal isoform (Williams and Ashley, 1989). This agrees with an absolute Ca\(^{2+}\) requirement for physiological cardiac RyR activation, and a higher Ca\(^{2+}\) sensitivity for activation of the cardiac isoform than the skeletal isoform (Zimanyi and Pessah, 1991). However, Ca\(^{2+}\) also influences the activity of the ATP-activated skeletal RyR. When channels were activated solely by cis ATP, increases in luminal Ca\(^{2+}\) from (sub)micromolar to high micromolar or millimolar concentrations increased channel activity and increased the duration of open events (Sitsapesan and Williams, 1995a; Tripathy and Meissner, 1996) (see section 1.4.2a).

In this section, it has been shown that the RyR channels incorporated are modulated by the reagents that are commonly used to identify RyRs. The effects of these reagents have been in general agreement with the literature. The differences in the properties of channel activation that were raised are of interest, but do not bring into question the integrity of the channel.

### 3.12 Open time distribution analysis

The analysis of event lifetime can reveal kinetic components in the stochastic process that are inherent in channel gating. The duration of open times were analysed for channels before and after ATP addition. The duration of almost all open events at 10\(^{-7}\) M Ca\(^{2+}\) (in the absence of other ligands) was less than 3 ms, and their distribution was best described by a single exponential component. In this low [Ca\(^{2+}\)], the number of events is not high. On average, over a 2 min period, the number of events that occurred was less than the number that were required to ascertain the mean lifetime with a 10% degree of accuracy (Colquhoun and Sigworth, 1983). However, because the data were distributed according to the single exponential function \(\exp(-t/\tau)\), the average open time \((T_o)\) is equivalent to \(\tau\) (the exponential time constant) (Hille, 1992). In the twelve channels (in 10\(^{-7}\) M Ca\(^{2+}\)) to which ATP was added, the value of \(T_o\) and \(\tau\) were compared. Because two of these channels did not have a sufficient number of events to allow lifetime analysis, only the values of the remaining ten were calculated for comparison. The value of \(T_o\) was 1.49 ± 0.16 ms. The average time constant obtained for the same channels was 1.59 ± 0.21 ms. The similarities in values suggest the assumption that the data were distributed according to a single exponential function was accurate. However, durations of closed times in subactivating Ca\(^{2+}\) were widely
spread (data not shown), and would require more than a single exponential component to fit their distribution. Because of the relatively few events in the low [Ca\(^{2+}\)], and the consequent difficulty in obtaining consistent control values, closed times were not routinely analysed.

ATP increased the mean open time, in general, by increasing the value of each time constant rather than adding further components to the distribution. The result of this was to have the time constants from some of the channels falling into the shortest time constant component ($\tau_1$: <3 ms), and time constants from other channels in the second component ($\tau_2$: 3 - 12 ms). These experiments were conducted in subactivating Ca\(^{2+}\) concentrations. Ca\(^{2+}\) appears to influence the effects of adenine nucleotides on open times. In Ca\(^{2+}\)-activated channels ($10^{-5}$ M) McGarry and Williams (1994a) found that adding millimolar adenosine to cardiac RyRs added an exponential component to the open time distribution. Consequently, the distribution was described by three exponential components with time constants ~3, 14 and 57 ms, which corresponded to three open states.

### 3.13 Substate activity in the purified RyR

The purified RyR incorporates into the bilayer with the same orientation as the native channel (Sitsapesan and Williams, 1994b). The properties of the purified RyR including Ca\(^{2+}\) activation, the I/V relationship and the sensitivity of the channel to ryanodine were indistinguishable from those of the native RyR. This is consistent with other reports comparing the native and the purified RyR. However, the proportion of substate activity shown by the purified RyR was higher than that seen in the native channel, in which substate activity is not common (Williams, 1992). The reason for the different levels of substate activity has not been determined.

Substate activity occurs in many channels, including other SR ion channels (Hals et al., 1989). Apparent substate activity may be due to stable subconducting levels, or it could be due to recording artefacts in which improperly resolved events during rapid gating are indicative of subconductance levels (Hals et al., 1989). FKBP12 deficient skeletal RyRs have prominent conductance levels that are ~0.25, 0.5 and 0.75 of maximum channel conductance (Brillantes et al., 1994; Ahern et al., 1997). This suggests that the RyR has four conducting subunits that are unable to work cooperatively in the absence of FKBP12. The effects of CHAPS -solubilisation on subconductance activity may be due to the disruption of proteins that are associated with the native RyR, such as the FK506-binding proteins and triadin, thereby leading to altered RyR function. In addition to the effects on substate activity, CHAPS -solubilisation removes the Ca\(^{2+}\) -inhibition properties of the RyR (Laver et al., 1995). Interestingly, Ca\(^{2+}\) -inhibition is also disrupted by 500 mM cis Cs\(^{+}\), although this does not appear to increase the levels of substate
activity. Hence, RyR purification is likely to affect at least two different sites on the protein.
EFFECTS OF SULFHYDRYL REAGENTS ON THE RYANODINE RECEPTOR
4.1 INTRODUCTION

The reactive disulfide (RDS) 4,4'-dithiodipyridine (4,4'-DTDP), and its closely related compound 2,2'-dithiodipyridine (2,2'-DTDP), are particularly useful reagents for determining the influence of SH groups on biological function. They are sulfur containing pyridines that react rapidly and completely with thiols (Grassetti and Murray, Jr. 1967) for which they are specific (Brocklehurst, 1979). A consequence of their interaction with thiols is the stoichiometric production of thiopyridone, whereby 1 mol of 4-thiopyridone or 2-thiopyridone is produced per mol of oxidised SH (Zaidi et al., 1989). This is determined from the ultraviolet spectra of 4-thiopyridone and 2-thiopyridone which are different from that of their corresponding disulfides (4,4'- and 2,2'-DTDP). Therefore, thiopyridone formation, and the disappearance of the disulfide, can be spectrophotometrically followed (Grassetti and Murray, Jr. 1967).

The potential number of accessible SH groups/mg of skeletal HSR was estimated at up to 185 nmol (based on the binding of Ag⁺ to SR membrane) (Salama and Abramson, 1984). However, taking into consideration the binding of Ag⁺ to SH groups on other SR proteins, such as the Ca²⁺.Mg²⁺-ATPase, less than 42 nmol of Ag⁺ was thought to bind to the Ca²⁺ release protein of the SR (Salama and Abramson, 1984). Since this estimate is likely to include the binding of Ag⁺ to residues other than cysteines (van Iwaarden et al., 1992), the number of SH groups that may be bound by Ag⁺ is small. To determine the maximum number of SH groups in SR membrane that have been oxidised by the RDSs, thiopyridone production was measured. Based on total thiopyridone production, when RDSs were added to solutions containing HSR vesicles, 2 - 5 nmol of SH groups/mg HSR protein were oxidised by RDS (Zaidi et al., 1989). This thiopyridone production was associated with Ca²⁺ release from the vesicles. During a fast phase of thiopyridone production, which slightly preceded Ca²⁺ efflux, ~2 nmol of thiopyridone/mg HSR was produced. Consequently, only ~2 - 3% of the sites on the HSR membrane that were bound by Ag⁺ are likely to be oxidised by RDS, which indicates that the number of SH groups involved in the Ca²⁺ release mechanism is small (Zaidi et al., 1989). A similar conclusion was drawn by Liu et al. (1994) who showed in skeletal and cardiac HSR that a class of hyperreactive cysteine residues (≤1 nmol/mg SR protein) on both the RyR and triadin are involved in the gating of Ca²⁺ efflux from the SR.

The ability of the RDSs to induce Ca²⁺ efflux from skeletal and cardiac SR vesicles, and inhibit, with higher concentrations, the binding of [³H]ryanodine to its receptor, has previously been discussed (section 1.5.3b). No studies using the RDSs, or any other SH specific oxidising agent, have been carried out to see how such reagents alter the activity of an individual RyR. In this chapter the actions of...
4,4'-DTDP on the activity of the single cardiac RyR are examined. The aims of the chapter are to determine the general characteristics of the RyR activated by 4,4'-DTDP, and the potential ability of 4,4'-DTDP to inhibit RyR activity. This includes an investigation of aspects of 4,4'-DTDP-induced activation such as the general properties of channel activity, channel conductance and the concentration dependence of the RDS, as well as the conditions under which channel inhibition may occur. In addition, the mechanism by which 4,4'-DTDP induces Ca\textsuperscript{2+} efflux from SR vesicles (an oxidation reaction) will be examined using the powerful reducing agent dithiothreitol.

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RESULTS

4.2 4,4'-DTDP alters activity & kinetics of the RyR

4.2.1 Biphasic effects on RyR activity induced by 4,4'-DTDP

Unless stated otherwise, the experiments in this chapter were carried out at a cis [Ca^{2+}] equivalent to the resting intracellular levels - 10^{-7} M. Under these conditions the RyR is essentially inactive (P_o = 0.0005 ± 0.0001; n = 21). Because of the variability in the levels of open probability and gating patterns of the Ca^{2+}-activated RyR (Smith et al., 1986; Shomer et al., 1993; Zahradniková and Palade, 1993; Sitsapesan and Williams, 1994b; Laver et al., 1995), the conditions chosen for these experiments provide control values with a low variance as well as having a physiological relevance.

a) Transient activation

Addition of 1 mM 4,4'-DTDP to the cis chamber activated 100% of RyRs within 3 min (n = 21). The average delay before activation, or "latency", was 42.1 ± 12.3 s (n = 21). The channel was only transiently activated: the time from onset of the 4,4'-DTDP-induced activation until the return to control levels, or cessation of activity, ranged from 2 - 284 s with an average of 80.4 ± 17.2 s (n = 21). Because of this transient activation induced by 4,4'-DTDP, the most accurate way to quantitatively assess the activation was to compare the P_o in the 2 min period immediately before 4,4'-DTDP with the 30 s period of highest P_o within 2 min of adding 4,4'-DTDP. Use of these periods gave minimum bias to the results because control activity in 10^{-7} M Ca^{2+} was reasonably consistent over a 2 min period, while the maximum effect of 4,4'-DTDP was usually evident within a 30 s section even though increased levels of activity in some channels could be observed for up to 2 - 3 min (Eager et al., 1997). This method of analysis showed that 4,4'-DTDP significantly increased the P_o of the RyR from 0.0005 ± 0.0001 to 0.0654 ± 0.0129 (P < 0.0001, n = 21).

b) Irreversible loss of channel activity

In the first 2 - 3 min after 1 mM 4,4'-DTDP addition there were long channel closures in-between the bursts of activity (see Fig. 4.1Aii). After 5 min exposure to 1 mM 4,4'-DTDP a voltage pulse (a 3 - 5 s change in holding potential to -40 mV) briefly activated 6 of 18 RyRs. None of these 6 (nor any of the other 12 RyRs) were activated by a 2nd voltage pulse 1 min later, or by any subsequent voltage pulses. In addition, increasing the [Ca^{2+}]_ca to 1 mM, 7 - 8 min after adding 4,4'-DTDP, did not activate the channel (n = 8). In some experiments, in which
Fig. 4.1 Effects of 4,4'-DTDP on channel activity of an individual RyR in 10^{-7} M Ca^{2+}. A single native RyR recorded in 250/50 mM CsCl at a holding potential of +40 mV. Openings are upward deflections from baseline (dotted line).

A - Left - Records of 1 min continuous channel activity selected from three different sections of an experiment. Right - All points histogram of the 1 min of channel activity.  
i) Control activity. ii) Activity immediately after the addition 1 mM 4,4'-DTDP cis. iii) 9 min after 4,4'-DTDP addition, and 2 min after increasing the cis [Ca^{2+}] to 1 mM. B - Time course of P_o and T_o, for the duration of the experiment shown above (A), for consecutive 30 s sections of activity. The first four points are control values before 4,4'-DTDP was added (time 0). The channel was not activated by brief voltage pulses (VP) to -40 mV or by increasing the [Ca^{2+}]_cis to 1 mM after 4,4'-DTDP was added.
A

i) 

ii) 

iii) 

B

\[ P_0 \]

\[ 4.4'-DTDP \]

\[ 1 \text{ mM Ca}^{2+} \]

\[ \text{Time (s)} \]

\[ \text{Ai} \quad \text{Aii} \quad \text{Aiii} \]

\[ \text{To} \]

\[ \text{VP} \]

\[ \text{VP} \]

\[ \text{VP} \]

\[ \text{VP} \]
the bilayers lasted long enough, other methods were also used in an attempt to reactivate the channel. These included the washout of 4,4'-DTDP from the cis chamber and the addition of millimolar concentrations of ATP. Both methods failed to induce channel activity. Hence, within 8 - 10 min of adding 1 mM 4,4'-DTDP to the RyR, channel activity was irreversibly lost i.e. P_o = 0.

An example of the transient activation, and subsequent loss of channel activity, is shown in the activity of a single channel exposed to 4,4'-DTDP in Figure 4.1. The continuous control activity over a 60 s period for a single RyR in 10^-7 M Ca^{2+} is shown (Fig. 4.1Ai). Immediately after 1 mM 4,4'-DTDP was added to the cis chamber a burst of activity occurred, followed by 2 subsequent bursts within 40 s (Fig. 4.1Aii). The complete cessation of channel activity is shown 9 min after the addition of 4,4'-DTDP, when an increase in the cis [Ca^{2+}] to 1 mM failed to activate the channel (Fig. 4.1Aiii). The time course of the values of P_o and T_o during the experiment is shown in Figure 4.1B. The filled circles show P_o over a 30 s period while the open circles show the mean open time for events within the corresponding 30 s section. This channel provides a good example of the pattern of changes in RyR activity following addition of 1 mM 4,4'-DTDP, with transient activation followed by the cessation of activity and irretrievable loss of channel function.

4.2.2 Effects on channel activity of 4,4'-DTDP

As seen from the recordings of a single channel activated by 4,4'-DTDP (Fig. 4.1) it appears that there is an increase in both the frequency of events (F_o) as well as the mean open time (T_o). The properties of channel activity were analysed after exposure to the RDS.

a) Mean open time and event frequency

The average T_o in 10^-7 M Ca^{2+} of 1.52 ± 0.23 ms was significantly increased during the period of maximum activation by 4,4'-DTDP to 22.2 ± 5.1 ms (P < 0.0001, n = 21). Average F_o was also significantly increased from 0.23 ± 0.05 s^-1 to 4.6 ± 1.2 s^-1 (P < 0.0001, n = 21) following the addition of 4,4'-DTDP. The single channel values, from individual experiments, and the mean values for each of P_o, T_o and F_o before and after 4,4'-DTDP are shown in Figure 4.2. The figure shows that 1 mM 4,4'-DTDP caused increases in all three parameters for each of the 21 channels.

b) Open & closed time constants

Open time constants, before and after 4,4'-DTDP, were obtained for each of the 21 channels. The distribution of open times for a single channel before (left) and after (right) the addition of 4,4'-DTDP is shown in Figure 4.3A. The control open times (open circles) were fitted, using the least squares method, by a single
Fig. 4.2 Effects of 4,4'-DTDP on $P_o$, $T_o$, and $F_o$ for single RyRs in $10^{-7}$ M Ca$^{2+}$. 1 mM 4,4'-DTDP was added to individual RyRs in 21 experiments. $P_o$, $T_o$ and $F_o$ were measured during a 2 min control period and compared to the values measured during the 30 s period with the highest $P_o$ during the 2 min after addition of 4,4'-DTDP (see section 4.2.1a). Values of $P_o$, $T_o$ and $F_o$ for each channel, before and after 4,4'-DTDP, were plotted and linked by lines. Mean ± SEM for control and 4,4'-DTDP are shown alongside individual data. Asterisks indicate significant differences from control: *** $P < 0.001$. 
Fig. 4.3 Effects of 4,4'-DTDP on the distribution of open and closed times. RyRs were recorded at +40 mV over a 2 min period in either 10^{-7} M Ca^{2+} before 1 mM 4,4'-DTDP was added to the cis chamber, or in 10^{-5} M Ca^{2+} alone. A - Open times (○) of a single RyR in 10^{-7} M Ca^{2+} before (left) and after (right) the addition of 4,4'-DTDP. Control values were fitted by a single exponential function (solid line) with a time constant of 0.95 ms. Values after 4,4'-DTDP required the sum of three exponential functions (solid line) to fit the distribution of open times during the 5 min period immediately after 4,4'-DTDP addition. The three exponentials (broken lines) had time constants of 1.2, 8.5 and 23.8 ms. B - Top - Open time constants obtained (as shown in A) from 21 channels in 10^{-7} M Ca^{2+} before and after 4,4'-DTDP was added to the cis chamber, and from 5 channels in 10^{-5} M Ca^{2+}, were allocated to groups (see section 2.6). In 10^{-7} M Ca^{2+}, control values (○) fell into the shortest time constant component (τ_1). After 4,4'-DTDP addition (●) the values fell into four components (τ_1 - τ_4) - see text for values. The open time constants of channels in 10^{-5} M Ca^{2+} (●) also had average values in τ_1 - τ_4. Bottom - Closed time constants in 10^{-7} M Ca^{2+}, obtained from 5 channels during the 2 min period before and after 4,4'-DTDP, were not consistently affected by 4,4'-DTDP (see text). Average closed time constants in 10^{-5} M Ca^{2+} were obtained from the 5 channels for which the open times were displayed (above).
Effects of SH reagents

Exponential function (solid line). This gave a time constant of \( \tau_1 = 0.95 \text{ ms} \). After the addition of 4,4'-DTDP, the sum of three exponentials was required to fit the data (\( \tau_1 = 1.2 \text{ ms}, \tau_2 = 8.5 \text{ ms} \) and \( \tau_3 = 23.8 \text{ ms} \)). The scatter in the data from control experiments was, in some channels, relatively large because there were few open events in \( 10^{-7} \text{ M Ca}^{2+} \). Time constants, calculated from each of the 21 channels, were allocated to groups (see section 2.6) to obtain the average results. The control open times were each fitted by a single exponential component with an average time constant of \( 1.8 \pm 0.3 \text{ ms} \) (\( n = 21 \)). Open times measured during the 5 min period immediately after 1 mM 4,4'-DTDP were fitted by the sum of up to three exponentials, which fell into groups \( \tau_1 - \tau_4 \) with time constants \( \tau_1 = 1.3 \pm 0.3 \text{ ms} \) (\( n = 14 \)), \( \tau_2 = 8.4 \pm 0.9 \text{ ms} \) (\( n = 9 \)), \( \tau_3 = 24.0 \pm 2.9 \text{ ms} \) (\( n = 14 \)) and \( \tau_4 = 75 \pm 11 \text{ ms} \) (\( n = 7 \)) (Fig. 4.3B - top). The number of time constants required to fit the open time distribution was variable. Of the 21 channels to which 1 mM 4,4'-DTDP was added, the open times of 7 channels fell into 3 exponentials, 9 fell into 2 exponentials and 5 channels were adequately fit by only a single exponential. Of the latter five RyRs, the time constants were in either \( \tau_2 \) or \( \tau_3 \) (3 - 50 ms), in contrast to the time constants for the control recordings which were all in the shortest group - \( \tau_1 \) (<3 ms).

Analysis of closed times was not routinely performed on channels recorded in \( 10^{-7} \text{ M Ca}^{2+} \) because of the relatively low frequency, and wide distribution, of events during control recordings (see section 3.12). Although the closed times appeared shorter in some recordings (e.g. Fig. 4.1Aii), the closed time distributions were, on average, not affected by 4,4'-DTDP -activation. The time constants of five channels were obtained and plotted (Fig. 4.3 - bottom; compare filled and open circles). Apart from the addition of one closed time constant after 4,4'-DTDP (with the occurrence of events in \( \tau_1 \)), both the average values of the other time constants and the percentage of events allocated to each component were similar. These results indicate that changes in closed times after 4,4'-DTDP -activation in \( 10^{-7} \text{ M Ca}^{2+} \) are unlikely to contribute to significant changes in RyR activity following SH oxidation.

The method used to display the average time constants, in which the time constants from individual channels were allocated to groups (\( \tau_1 - \tau_5 \)), show changes in the average value of the time constant and the percentage of events within each group. It was an interesting observation that the average open time constant values, and the proportion of events in each group, were very similar for the channels activated by either 1 mM 4,4'-DTDP (in \( 10^{-7} \text{ M Ca}^{2+} \)) or \( 10^{-5} \text{ M Ca}^{2+} \) (\( n = 5 \); Fig. 4.3B, top). This indicates that a common mechanism may underlie \( \text{Ca}^{2+} \) and 4,4'-DTDP -induced activation. It is possible that the 4,4'-DTDP -induced RyR activation occurs through an increase in the sensitivity of the channel to \( \text{Ca}^{2+} \). This was suggested for the mechanism of \( \text{H}_2\text{O}_2 \) -induced channel activation of the skeletal RyR (Favero et al., 1995a). However, two lines of
evidence suggest this is not the case. Firstly, the relative percentage of events in the average closed time constants (τ₁ - τ₃) differs considerably between channels activated by 1 mM 4,4'-DTDP and 10⁻³ M Ca²⁺ (Fig. 4.3, bottom). Secondly, there was a significant difference in the value of Pₒ for the 4,4'-DTDP -activated RyR (~0.065, n = 21) compared to the channels activated by 10⁻⁵ M Ca²⁺ (~0.146, n = 23) (P < 0.05).

c) Conductance

It has been shown in this section that 4,4'-DTDP increases Pₒ of the RyR by increasing the values of Tₒ and Fₒ. However, an increase in Pₒ is not the only way that 4,4'-DTDP could cause the observed increase in Ca²⁺ efflux from SR vesicles - an increase in channel conductance could also be involved. This possibility was examined by measuring the current-voltage (I/V) relationship in the absence and presence of 4,4'-DTDP. In these experiments it was necessary to activate the channel so the full conductance could be readily be measured. This was done by increasing the [Ca²⁺]ₙᵢ to 1 mM. A potential problem encountered when designing these experiments was that channel activity could cease before the data for a full I/V curve were collected, since 1 mM 4,4'-DTDP causes the irreversible loss of channel function within 8 - 10 min. However, a lower concentration of 4,4'-DTDP may not cause the loss RyR activity - a study investigating the effects of 4,4'-DTDP on [³H]ryanodine binding showed that while high concentrations of 4,4'-DTDP inhibited binding, lower concentrations did not (Zaidi et al., 1989). Ca²⁺ efflux from SR vesicles was induced with concentrations of 4,4'-DTDP from 1 - 200 µM (Zaidi et al., 1989; Prabhu and Salama, 1990b), therefore similarly low concentrations of 4,4'-DTDP were used to examine the effects of the drug on the I/V relationship.

The I/V relationship under normal experimental conditions (250/50 mM CsCl cis/trans) was non-linear and had a Eᵱᵣᵥ of -17.8 ± 0.3 mV (n = 3) (Fig. 4.4A). The Eᵱᵣᵥ was calculated from a third order polynomial fit to the control data (dashed line). Addition of 100 µM 4,4'-DTDP to the cis chamber of the same three RyRs produced no change in conductance between -80 and +80 mV. The Eᵱᵣᵥ was essentially unchanged at -18.1 ± 0.3 mV. A fit to these data (third order polynomial - dotted line) was indistinguishable from the fit to the control data. In two of the channels, the 4,4'-DTDP concentration was increased to 1 mM after the I/V data were collected (3 min post 100 µM 4,4'-DTDP). Conductance in both channels remained unchanged at a holding potential of +40 mV (data not shown).

d) Vehicle for 4,4'-DTDP does not alter channel activity

The effects on channel activity of ethanol, the vehicle for 4,4'-DTDP, were examined following its addition to the cis chamber. As for the I/V experiments,
Fig. 4.4 **4,4'-DTDP has no effect on the single channel current-voltage relationship, and the vehicle for 4,4'-DTDP does not affect single channel activity.** Channels recorded in usual control solutions: 250/50 mM CsCl, 1 mM CaCl$_2$. A - I/V relationship under control conditions and during activation by 100 µM 4,4'-DTDP. Each point is the mean single channel current obtained in three experiments. The dashed line is a third order polynomial fit to the control data. The dotted line (obscured by the dashed line) is a third order polynomial fit to the data in the presence of 4,4'-DTDP. SEM for the ordinate fall within the symbol. B - Left - Averaged normalised parameters P'$_o$ (●), T'_o (○), and F'_o (□) ± SEM for consecutive 30 s periods of activity in four channels before and after the *cis* addition of 10 µl ethanol (time 0), and after addition of 1 mM 4,4'-DTDP at 300 s. Values of P$_o$, T$_o$ and F$_o$ for each 30 s period were normalised to the mean value obtained for the 2 min control period. Right - Average time constants, obtained from the distribution of open times, for the four RyRs before and after addition of ethanol (see legend for Fig. 3.5 for description of graph).
the channel was first activated with 1 mM [Ca\(^{2+}\)]\(_{cis}\) to clearly observe any depressing or activating effects of ethanol on channel activity. Addition of 10 µl ethanol alone (1% of bath volume: the maximum volume added to 1 ml of cis solution to yield 1 mM 4,4'-DTDP) had no effect on average P\(_o\), T\(_o\), F\(_o\) or open time distributions during 5 min continuous recording, in contrast to the rapid changes upon the addition of 1 mM 4,4'-DTDP (Fig. 4.4B). The highest concentration of ethanol added to the cis chamber during RDS addition (i.e. 1% of total bath volume) had no effect on single channel activity of the skeletal RyR (Oba et al., 1997).

### 4.2.3 Concentration dependence of 4,4'-DTDP actions on the RyR

#### a) RyR activation

Concentrations of 4,4'-DTDP from 1 - 10 µM had no effect on RyR activity within the lifetime of the bilayer at 10\(^{-7}\) M Ca\(^{2+}\) (n = 7). A 4,4'-DTDP concentration of 50 µM was required before activation was observed, but only 3 of 5 channels were activated. Increasing the concentration of 4,4'-DTDP to 100 µM, in separate RyRs, activated the same proportion of channels (6 of 10) that were activated by 50 µM 4,4'-DTDP. Only when the concentration of 4,4'-DTDP was increased to 1 mM were all channels activated (n = 21) (Fig. 4.5A). While the initial activation of the channel by 4,4'-DTDP was dependent on the concentration of the drug, the degree of activation was not affected by the concentration. The increase in P\(_o\) from 0.0024 ± 0.0009 to 0.0681 ± 0.0464 (P < 0.01) in the six channels that were activated by 100 µM 4,4'-DTDP was no different from the maximum activation induced by 1 mM 4,4'-DTDP (P\(_o\) = 0.0654 ± 0.0129, n = 21; P > 0.95). Examples of RyRs activated by different 4,4'-DTDP concentrations from 50 µM to 1 mM are shown in Figure 4.5B. The records show that maximum activation over a 30 s period is not dependent on the 4,4'-DTDP concentration. The similarities in the degree of channel activation when using either 100 µM or 1 mM 4,4'-DTDP are also seen in the distribution of open times: addition of 100 µM 4,4'-DTDP increased the number of exponential components required to fit the open time distribution from a single exponential for control data (t\(_1\) = 2.6 ± 0.4 ms (n = 6)) to three exponentials (t\(_1\) = 1.8 ± 0.4 ms (n = 6), t\(_2\) = 6.4 ± 0.9 ms (n = 4) and t\(_3\) = 22.3 ± 9.5 ms (n = 4)). The average values of t\(_1\), t\(_2\) and t\(_3\) after the addition of 1 mM 4,4'-DTDP (see section 4.2.2b) were very similar to those seen with 100 µM 4,4'-DTDP. However, with the higher concentration of the drug, a fourth time constant (t\(_4\) ~75 ms) was seen in 7 of 21 channels. These long open events in t\(_4\) were not seen in any channels after adding lower concentrations of 4,4'-DTDP. In addition, the delay before channel activation was longer (>1 min) with lower concentrations of 4,4'-DTDP (≤100 µM) than it was after adding 1 mM 4,4'-DTDP (~42 s).
Fig. 4.5 Activation of the RyR by 4,4'-DTDP depends on [4,4'-DTDP], but degree of activation is independent of [4,4'-DTDP]. Channels were recorded in 10⁻⁷ M Ca²⁺ at a holding potential of +40 mV. A - Different concentrations of 4,4'-DTDP (1 µM to 1 mM) were added to RyRs to find the minimum concentration of 4,4'-DTDP required to activate the channel. B - Records of channel activity after the addition of 50, 100 or 1000 µM 4,4'-DTDP. The degree of RyR activation by low and high concentrations of 4,4'-DTDP shows a lack of concentration -dependence. Two channels were active in bilayer (ii) - records of channels with > 1 active RyR in the bilayer were not analysed. Dotted line is the closed current level.
The minimum 4,4’-DTDP concentration of 50 µM that was required to activate single RyRs was higher than the 2.5 - 10 µM reported to induce Ca\(^{2+}\) release from skeletal and cardiac SR vesicles (Zaidi et al., 1989; Prabhu and Salama, 1990b). This was surprising since the reconstituted channel in lipid bilayers is considered to be more sensitive to pharmacological reagents than channels in SR vesicles (Lu et al., 1994, Favero et al., 1995a). The level of channel activity can influence the receptiveness or availability of SR SH groups to the SH probe, 7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin (Liu et al., 1994). The possibility that RyR activity may alter the threshold 4,4’-DTDP concentration for activation was tested by activating the channel with 10\(^{-5}\) M \(\text{cis Ca}^{2+}\) before adding the RDS. The results (Fig. 4.6) are expressed as the mean logarithm (ln) for the normalised parameters, \(P'_o\), \(T'_o\) and \(F'_o\), i.e. the logarithm of the variables for each parameter measured over a 30 s segment within the period of highest \(P_o\) with the drug, normalised to that in the 2 min before drug application (Eager et al., 1997). The horizontal line drawn at zero indicates the experimental parameters equal to control: positive values show an increase in \(P'_o\), \(T'_o\) or \(F'_o\); negative values a decrease. Expressing the results in this way compensates for the variation in levels of control activity (Laver et al., 1995) and clearly illustrates the drug effects. 1 µM 4,4’-DTDP did not affect channel activity. However, concentrations ≥10 µM did alter RyR activity: \(T_o\) was significantly increased and \(F_o\) significantly decreased in the 3 channels with 10 µM 4,4’-DTDP - the increase in \(P_o\) was not significant. These results show that RyRs in 10\(^{-5}\) M \(\text{Ca}^{2+}\) are more sensitive to 4,4’-DTDP than RyRs in 10\(^{-7}\) M \(\text{Ca}^{2+}\). This may be due to the increased activity of the RyR in activating concentrations of \(\text{Ca}^{2+}\), or due to a specific influence of \(\text{Ca}^{2+}\). As was seen in 10\(^{-7}\) M \(\text{Ca}^{2+}\), there was no consistent dependence on the concentration of 4,4’-DTDP for \(P_o\), \(T_o\) or \(F_o\) in the channel maximally activated by 4,4’-DTDP and \(\text{Ca}^{2+}\). Because of this, the data from all 14 channels in 10\(^{-5}\) M \(\text{Ca}^{2+}\) that were exposed to concentrations of 4,4’-DTDP between 10 µM and 1 mM were combined. \(P_o\) increased in 12 of the 14 channels, while \(T_o\) increased and \(F_o\) fell in all 14 channels. When the data from these 14 channels were pooled, \(P_o\) increased from 0.14 ± 0.04 to 0.25 ± 0.08 (\(P < 0.01\)); \(T_o\) increased from 14 ± 3 to 156 ± 33 ms (\(P < 0.0001\)); and \(F_o\) decreased from 26 ± 13 to 2.8 ± 1.0 s\(^{-1}\) (\(P < 0.0001\)).

**b) Loss of RyR activity**

The results in the previous section showed that increasing the concentration of 4,4’-DTDP above the threshold concentration for activation did not enhance the activation. However, this was not the case for the loss of channel activity that occurred at longer times in the presence of 4,4’-DTDP. In 10\(^{-7}\) M \(\text{Ca}^{2+}\), the addition of 1 mM 4,4’-DTDP to the \(\text{cis}\) chamber caused the cessation of all channel activity within 8 - 10 min (see section 4.2.1). Lower concentrations (≤100 µM) either did not cause the loss of channel function over the lifetime of the
Fig. 4.6  **The degree of channel activation in 10^{-5} M Ca^{2+} is independent of 4,4'-DTDP concentration.** 4,4'-DTDP was added to the cis chamber at concentrations ranging from 1 - 500 µM (n = 3 at each concentration) and 1 mM (n = 5). The ordinate shows the logarithm (ln) of the normalised parameters P', T', and F'. (see text). The mean of the logarithms of the variables is shown. The vertical lines are ± SEM. Experimental parameters are equal to control when their value is zero (horizontal line): positive values indicate an increase in the parameters; negative values a decrease. Asterisks indicate values significantly different from control: * P < 0.05; ** P < 0.01.
Effects of SH reagents or took well in excess of 10 min (in the majority of channels) to produce the effect. Of the five RyRs to which 100 μM 4,4'-DTDP was added, activity in three of the channels ceased after 12 to 30 min. The remaining two channels were still spontaneously active, and were activated by voltage pulses, for 26 and 30 min after drug addition: activity may have eventually ceased had the bilayers survived longer. Similar results were also seen in the experiments with 10⁻⁵ M Ca²⁺: no loss of activity was seen with ≤100 μM 4,4'-DTDP in RyRs that were activated by 4,4'-DTDP (n = 6; four of these bilayers lasted >10 min), whereas 1 mM 4,4'-DTDP caused the loss of function in all RyRs within 6 min (n = 5).

4.2.4 Addition of oxidising agents to the luminal face of the RyR

A small number of agents have been shown to affect RyR activity when added to the luminal face of the channel (trans chamber) (see section 4.2.2). Only one of these agents was a SH oxidising agent. Xiong et al. (1992) showed that the photooxidising xanthine dye, rose bengal, activated the skeletal RyR irrespective of the side of the bilayer to which the dye was added. However, the reactive species liberated upon exposing rose bengal to light readily cross the bilayer before decaying (Xiong et al., 1992). Therefore, the activation of the RyR could be due to the oxidation of thiols on either side of the bilayer. Another study showed no effects on channel activity when the membrane impermeable SH reagents GSH and GSSG were added to the luminal side of the skeletal RyR (Zable et al., 1997). No studies have investigated the luminal effects of redox reagents on the cardiac RyR. Consequently, 4,4'-DTDP and thimerosal were added to the trans chamber to examine whether the oxidation of a SH group(s) on the luminal face of the RyR can affect channel activity. Thimerosal was used because the lipid membrane is impermeable to the agent (Chiamvimonvat et al., 1995) whereas the RDSs are lipid soluble.

a) 4,4'-DTDP

Addition of 1 mM 4,4'-DTDP to the trans chamber in 10⁻⁷ M cis Ca²⁺ (Fig. 4.7A) produced changes in RyR activity that were generally consistent with those observed after cis addition of 4,4'-DTDP. The values of Pₐ, T₀ and F₀ for five channels increased after exposure to trans 4,4'-DTDP: Pₐ from 0.0013 ± 0.0007 to 0.0270 ± 0.0084 (P < 0.01), T₀ from 1.9 ± 0.3 to 9.5 ± 1.4 ms (P < 0.0001) and F₀ from 0.7 ± 0.3 to 2.7 ± 0.5 s⁻¹ (P < 0.05) (Fig. 4.7B). The increase in channel activity after the trans addition of 4,4'-DTDP occurred following a delay of 37.0 ± 16.2 s (n = 5). This was similar to the 42.1 ± 12.3 s (n = 21) delay following the cis addition of the reagent (P = 0.85). There was also an increase in the number of exponential components required to fit the distribution of open times. Control open times were fitted by a single exponential component with the average time constant τ₁ = 1.6 ± 0.4 ms (n = 5). The addition of trans
Fig. 4.7 4,4'-DTDP addition to the trans chamber activates the RyR.
A - Channel activity in 10^{-7} M Ca^{2+} before (i) and after (ii) the addition of 1 mM 4,4'-DTDP to the trans chamber. B - Values of $P_o$, $T_o$ and $F_o$ obtained during a 2 min control period were compared to the values in the 30 s period with the highest $P_o$ in the 2 min after addition of 4,4'-DTDP ($n = 5$). Asterisks indicate values significantly different from control: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. C - Average time constants, obtained from the distribution of open times of the five RyRs shown in (B), during the 2 min control period (○) and the 5 min period immediately after 4,4'-DTDP was added to the trans chamber (●).
4,4'-DTDP added a further two time constants to the distribution: 
\[ \tau_1 = 0.9 \pm 0.2 \text{ ms} \ (n = 3), \ \tau_2 = 5.4 \pm 0.8 \text{ ms} \ (n = 3) \] 
and 
\[ \tau_3 = 18.9 \pm 4.2 \text{ ms} \ (n = 4) \] 
(Fig. 4.7C). Like the effects of 4,4'-DTDP when added to the cis chamber, the increased channel activity after trans addition of the reagent was transient: activity was irretrievably lost within 10 min of the addition of trans 4,4'-DTDP (n = 3). However, while the overall trends in each parameter were similar whether 4,4'-DTDP was added to the cis or the trans chamber, quantitative differences existed. Firstly, the maximum values obtained for \( P_o \) and \( T_o \) after 4,4'-DTDP was added to the trans chamber were significantly less than the corresponding values after 4,4'-DTDP was added to the cis chamber in 21 experiments (\( P_o = 0.065 \pm 0.01; \ T_o = 22.2 \pm 5.1 \) (P < 0.05). Secondly, the trans addition of 4,4'-DTDP did not produce the very long open events, which added a fourth component (\( \tau_4 \)) to the open time distribution after the cis addition of 4,4'-DTDP.

The overall characteristics of 4,4'-DTDP -activation from the trans chamber were similar to those after cis -activation, suggesting the possibility that the same SH group(s) are oxidised by 4,4'-DTDP. This could occur because 4,4'-DTDP is lipid soluble. Thus it could cross the bilayer and interact with free SH groups in the trans-membrane region or on either side of the membrane. However, significant differences in the values of \( P_o \) and \( T_o \) between the cis and trans additions of 4,4'-DTDP, along with the lack of events in the fourth time constant group (\( \tau_4 \)) after trans 4,4'-DTDP raise doubt about a common oxidation site. Thus, there may be more than one SH group on the Ca\(^{2+}\) release channel complex with which 4,4'-DTDP interacts to produce channel activation. It is known that water soluble SH reagents added to the cytoplasmic face of the channel activate the RyR (Abramson et al., 1995). Free SH groups susceptible to oxidation may also exist on the luminal side of the protein, since conserved cysteine residues are present on the putative M3 - M4 luminal loop (Hakamata et al., 1992). To test this possibility, the lipophobic SH oxidising agent, thimerosal, was used.

b) Thimerosal

Thimerosal (100 \( \mu \text{M} \) or 1 mM) was added to both the cis \((n = 12)\) and the trans \((n = 9)\) chambers in separate experiments. Unlike the RDSs, thimerosal is not specific for free SH groups (Brocklehurst, 1979). Consequently, its effects cannot necessarily be attributed to an action on SH groups alone. Nonetheless, the addition of thimerosal to RyRs, in 10\(^{-7}\) M cis Ca\(^{2+}\), caused a transient increase in channel activity in each experiment. The delay before channel activation after the addition of thimerosal to the cis and trans chamber was examined. The delay after the cis addition of 1 mM thimerosal was 53.5 \pm 16.5 s \((n = 6)\) which was no different to the \(-42 s\) delay with the cis addition of 1 mM 4,4'-DTDP \((n = 21; \ P > 0.6)\). When a lower concentration of thimerosal was added to the cis chamber (100 \( \mu \text{M} \)), the delay of 190 \pm 36 s \((n = 6)\) was significantly longer than with 1 mM thimerosal (P < 0.01). Because of this longer delay before channel activation after
Adding 100 µM thimerosal, the period during which the 30 s of maximum channel activity was measured was extended from the usual 2 min after drug addition to 5 min. Interestingly, thimerosal activated the RyR more rapidly when it was added to the trans chamber, compared to the rate of activation after thimerosal was added to the cis chamber. In addition, following trans thimerosal, there was no difference (P > 0.08) in the rate of activation between the two different concentrations of the reagent (1 mM or 100 µM). The delay after adding 1 mM thimerosal (trans) was 13.5 ± 1.1 s (n = 4), while the delay after 100 µM (trans) was 16.2 ± 3.2 s (n = 5). Channel activation after the trans addition of thimerosal appeared to occur more rapidly than the activation that occurred after adding 1 mM 4,4'-DTDP to the trans chamber (~37 s), although the differences were not statistically significant.

Examples of channel activity following the addition of 100 µM thimerosal are shown in Figure 4.8Aii&Bii. Increasing the thimerosal concentration from 100 µM to 1 mM produced no significant difference in the values of P0, T0 or F0 (P > 0.15). Therefore, the data obtained from the analysis of experiments in which thimerosal was added to the cis chamber at concentrations of 100 µM (n = 6) and 1 mM (n = 6) were combined. The data were also combined from the experiments in which 100 µM (n = 5) and 1 mM (n = 4) thimerosal were added to the trans chamber. The characteristics of channel activity were compared before and after thimerosal by measuring P0, T0 and F0, along with the distribution of open times. The distributions of closed times were not investigated (see section 3.12). P0 was increased in each channel after adding thimerosal to either the cis or the trans chamber (Fig. 4.8Aiii&Biii). Details of the average changes in P0, T0 and F0 induced by thimerosal are given in Table 4.1. Thimerosal significantly increased each of the three parameters after cis or trans addition. Interestingly, the average values for each of P0, T0 and F0 were the same whether thimerosal was added to either the cis or the trans chamber (P > 0.05).

Thimerosal was not as strong an activator of the RyR as 4,4'-DTDP. Table 4.2 compares the average maximum values recorded after addition of either thimerosal or 4,4'-DTDP to either the cis or the trans chamber in 10−7 M Ca2+ cis. Addition of 4,4'-DTDP produced a significantly greater increase in P0 than thimerosal after both cis or trans addition, although there appears to be less difference between the two reagents after trans addition. Interestingly, maximum values for F0 were very similar, irrespective of which agent was added, but T0 was more strongly influenced by 4,4'-DTDP. This could be due to 4,4'-DTDP having preferential access to SH groups unobtainable by thimerosal because of its water solubility, or the ability of 4,4'-DTDP to act on free SH groups from both sides of the bilayer thus enhancing its effect. To ascertain whether similar mechanisms were involved in RyR activation by both reagents, the effects of thimerosal (added
Fig. 4.8 Effects of thimerosal on RyR activity after its addition to either the cis or the trans chamber. 100 µM and 1 mM thimerosal were added to the cis (A, n = 12) or the trans (B, n = 9) chamber in 10^{-7} M [Ca^{2+}]_{cis}. i) Channel activity in the absence (top) and presence (bottom) of 100 µM thimerosal. ii) Values of P_o for individual channels and mean values in control conditions (2 min) and after thimerosal addition (maximum activation over 30 s). Thimerosal concentration given to each channel is indicated by the straight line connecting the values of P_o before and after drug addition: broken line - 100 µM; solid line - 1 mM. Values of mean P_o combine the data from RyRs exposed to 100 µM and 1 mM thimerosal. Asterisks indicate values significantly different from control: ** P < 0.01; *** P < 0.001. iii) Average time constants, obtained from the distribution of open times of each RyR (Aii & Bii), during the 2 min period before (○) and the 5 min immediately after (●) thimerosal addition.
Tab. 4.1 Effects of *cis* and *trans* thimerosal on $P_o$, $T_o$ and $F_o$ in $10^{-7}$ M Ca$^{2+}$.

<table>
<thead>
<tr>
<th></th>
<th><em>Cis</em> (n=12)</th>
<th></th>
<th></th>
<th><em>Trans</em> (n=9)</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
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<td>$P$</td>
<td>Control</td>
<td>Thimerosal</td>
<td>$P$</td>
</tr>
<tr>
<td>$P_o$</td>
<td>.0006±.0003</td>
<td>.0189±.0065</td>
<td>&lt;.0001</td>
<td>.0005±.0002</td>
<td>.0111±.0031</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>$T_o$</td>
<td>1.61 ± 0.42</td>
<td>4.23 ± 1.01</td>
<td>&lt;.01</td>
<td>1.48 ± 0.18</td>
<td>6.14 ± 1.05</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>$F_o$</td>
<td>0.29 ± 0.10</td>
<td>5.76 ± 1.79</td>
<td>&lt;.001</td>
<td>0.32 ± 0.08</td>
<td>1.97 ± 0.58</td>
<td>&lt;.01</td>
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</table>

Mean ± SEM for $n$ channels under control conditions and after thimerosal addition. $P_o$, open probability. $T_o$, mean open time (ms). $F_o$, number of events ($s^{-1}$). $P$ values: significance of difference between means.
Tab. 4.2 Comparison of the effects of thimerosal and 4,4'-DTDP on RyR activity in subactivating Ca\(^{2+}\).  

<table>
<thead>
<tr>
<th></th>
<th>Cis</th>
<th></th>
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<tr>
<td></td>
<td>Thimerosal (n=12)</td>
<td>4,4'-DTDP (n=21)</td>
<td>P</td>
<td>Thimerosal (n=9)</td>
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<tr>
<td>(P_o)</td>
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<td>.0025</td>
<td>.0111±.0031</td>
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<tr>
<td>(T_o)</td>
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<td>22.2 ± 5.1</td>
<td>.0018</td>
<td>6.1 ± 1.1</td>
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<tr>
<td>(F_o)</td>
<td>5.8 ± 1.8</td>
<td>4.6 ± 1.2</td>
<td>.5727</td>
<td>2.0 ± 0.6</td>
</tr>
</tbody>
</table>

Mean ± SEM for \(n\) channels. P values: significance of difference between means.

4.3 Fortified RyR modified by 4,4'-DTDP

Pooled RyR was used to determine whether 4,4'-DTDP was acting on the RyR in the subactive portion of the subactive state or in the active state. The selection of 4,4'-DTDP above the purified RyR caused the transient activation and the slow-exit block of channel activity that was observed in the active state. The delay before activation was in the same range as that seen in the recycled channels - between 2.8 ± 1.7 s (\(n\=31\)). However, the period before channel activity was lost was considerably longer in 2 of the 3 purified RyRs than that measured in
to the *cis* and *trans* chambers) on the distribution of open times were examined, and compared to the effects of 4,4'-DTDP.

There was no difference between the *cis* or the *trans* addition of thimerosal in the average values of the open time constants (Fig. 4.8Aii&Bi). However, the percentage of events in the shortest time constant ($\tau_1$) was significantly greater after *cis* addition of thimerosal (73.0 ± 6.3%, n = 12) compared to the percentage of events in $\tau_1$ after: a) the *trans* addition of thimerosal (34.1 ± 10.9%, n = 9; P < 0.01) or b) the *cis* or *trans* addition of 4,4'-DTDP (see Fig. 4.3 and 4.7). In addition, channel activation by thimerosal, following its addition to the *cis* or the *trans* chamber, did not produce any of the long openings in $\tau_4$ that occurred during activation by high concentrations of *cis* 4,4'-DTDP. However, the average values for the shorter time constants ($\tau_1 - \tau_3$) were comparable with those seen for *cis* or *trans* 4,4'-DTDP. Overall, the properties of channel activation by thimerosal are similar to the general properties of RyR activation by 4,4'-DTDP, and the characteristics of thimerosal activation following *cis* and *trans* addition are very similar. However, slight, but measurable differences in the kinetics of activation were observed between the actions of thimerosal on the *cis* and *trans* sides of the RyR (Eager et al., 1995).

Thimerosal, like 4,4'-DTDP, also caused irreversible loss of activity when added to the RyR from either the *cis* or the *trans* chamber. Of the nine RyRs in which thimerosal was added to the *trans* chamber, four were assessed for loss of activity by increasing the $[\text{Ca}^{2+}]_{\text{cis}}$ to 1 mM and applying voltage pulses to the bilayer. All four lost channel function within 10 min of adding 100 µM (n = 2) or 1 mM (n = 2) thimerosal. Similar results were seen after addition of thimerosal to the *cis* chamber where 7 of 12 RyRs were assessed for the loss of activity. After adding 1 mM thimerosal (n = 4), three channels had lost channel function within 10 min while the fourth was still active after 17 min when the bilayer broke. With 100 µM thimerosal (n = 3), two RyRs showed loss of channel activity within 20 min of drug addition. The third was still active 15 min post thimerosal when the bilayer broke. Thimerosal appeared to be more effective at inducing the loss of channel activity when added to the *trans* chamber.

### 4.3 Purified RyR modified by 4,4'-DTDP

Purified RyRs were used to determine whether 4,4'-DTDP was acting on the RyR or on another protein in the SR which could modify RyR activity. The addition of 1 mM 4,4'-DTDP to the purified RyR caused the transient activation and the subsequent loss of channel activity that was observed in the native channel. The delay before activation was in the same range as that seen in the native channels - between 21 - 170 s (n = 3). However, the period before channel activity was lost was considerably longer in 2 of the 3 purified RyRs than that measured in
native channels. An example of channel activity before and after 4,4'-DTDP in 10^{-7} M Ca^{2+} is shown in Figure 4.9A. Each trace shows a continuous 30 s period of activity: control activity (i) is typical of the native RyR in 10^{-7} M Ca^{2+} (P_o = 0.0001). The addition of 1 mM 4,4'-DTDP increased P_o to 0.17 (over the 30 s period) within 1 min (ii), and the channel was still active in the presence of 1 mM Ca^{2+} (and 4,4'-DTDP) 25 min after addition of the RDS (iii). The activity of the channel eventually ceased but, in comparison to the native RyR, a much greater period elapsed (35 min) before this occurred (iv).

The activity of the purified RyR following the addition of 4,4'-DTDP (Fig. 4.9A) appears different to that of the native RyR in the presence of 4,4'-DTDP (Figs 4.1 & 4.5). Comparing the values of P_o, T_o and F_o for the purified RyR with those of the native RyR after activation by 4,4'-DTDP showed significant differences in some parameters. The 4,4'-DTDP-induced activation of the purified RyR was due to a significant increase in F_o with no effect on T_o (Tab. 4.3 & Fig. 4.9B). In contrast, activation of the native RyR by 4,4'-DTDP was due to significant increases in both T_o and F_o. The high F_o after 4,4'-DTDP-activation of the purified RyR was evident from the channel trace (Fig. 4.9Aii) and in the distribution of open times: there was an increase in the number of exponential components required to fit the distribution of open times from one to three after 4,4'-DTDP, which is similar to that seen in native channels. However, for the purified RyR, a very high percentage of events (>70%) fell into the shortest time constant (\tau_i), compared to <30% of events in \tau_i after 4,4'-DTDP-activation of the native RyR (after either cis or trans addition). While there are differences in channel kinetics after 4,4'-DTDP -activation of the purified RyR compared to the native channel, solubilisation of the cardiac RyR does not alter the ability of 4,4'-DTDP to activate the channel and cause its eventual and irreversible loss of activity. This suggests that the SH residue(s) oxidised by 4,4'-DTDP is on the RyR or on a tightly associated protein that is not removed during CHAPS-solubilisation.

4.4 4,4'-DTDP activation occurs by an oxidation reaction

Glutathione (GSH) is present in the myoplasm at relatively high concentrations (see section 1.5.4). Brunder et al. (1988) concluded that its presence prevents \textit{in vivo} SH oxidation of the SR Ca^{2+} release protein, thus refuting the hypothesis of oxidation -induced Ca^{2+} release proposed by Trimm et al. (1986). However, Koshita et al. (1993) demonstrated oxidation -induced Ca^{2+} efflux from skeletal SR vesicles in the presence of either GSH or dithiothreitol (DTT), and concluded that SH oxidation in the presence of reducing agents could occur if the reaction between the oxidant and the SH group occurred more quickly than the reaction between the oxidant and the reducing agents.
Fig. 4.9 *Activation of the purified RyR by 4,4'-DTDP.* Channels recorded in usual solutions with $10^{-7}$ M [Ca$^{2+}$]$_{cis}$. A - (i) Control. (ii) Within 1 min of adding 1 mM 4,4'-DTDP to the cis chamber. (iii) 25 min after adding 4,4'-DTDP and 4 min after increasing [Ca$^{2+}$]$_{cis}$ to 1 mM. (iv) 35 min after 4,4'-DTDP addition. B - Values of $P_o$, $T_o$ and $F_o$ from individual channels, mean ± SEM plotted alongside individual data. Asterisks indicate values significantly different from control: ** $P < 0.01$; *** $P < 0.001$. 
Tab. 4.3 Effects of 4,4'-DTDP on the purified RyR and a comparison with the effects of 4,4'-DTDP on the native RyR.

<table>
<thead>
<tr>
<th></th>
<th>Purified (n=3)</th>
<th>Native (n=21)</th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cont</td>
<td>4,4'-DTDP</td>
<td>P</td>
</tr>
<tr>
<td>( P_0 )</td>
<td>0.0006±0.0003</td>
<td>1.955±1.357</td>
<td>0.001</td>
</tr>
<tr>
<td>( T_0 )</td>
<td>0.66±0.07</td>
<td>2.6±1.1</td>
<td>0.10</td>
</tr>
<tr>
<td>( F_0 )</td>
<td>0.11±0.05</td>
<td>59.9±28.5</td>
<td>0.0009</td>
</tr>
</tbody>
</table>

Mean ± SEM for \( n \) channels. \( \text{cis} \ [\text{Ca}^{2+}] \), \( 10^{-7} \) M. \( P \) values: significance of difference between means of the purified RyR (\( n = 3 \)). \( P' \) values (“Pur vs Nat”): significance of difference between means of the 4,4'-DTDP -activated purified RyRs (\( n = 3 \)) and the 4,4'-DTDP -activated native RyRs (\( n = 21 \)).
DTT is a strong reducing agent with a redox potential of -330 mV at pH 7.0 (Cleland, 1964). It is a more powerful reducing agent than the physiological reducing agents, GSH or cysteine, which have higher redox potentials: -260 mV and -210 mV respectively (Cleland, 1964; Lundstrom Ljung and Holmgren, 1995). DTT inhibits the 2,2'-DTDP-induced Ca\(^{2+}\) efflux from skeletal and cardiac SR vesicles (Zaidi et al., 1989; Prabhu and Salama 1990b) suggesting that RDS-induced Ca\(^{2+}\) release occurs via an oxidation reaction. However, reducing agents have not been added to single cardiac RyRs activated by SH oxidising agents. DTT was used in the following experiments to determine whether: a) 4,4'-DTDP could activate the channel in the presence of a reducing agent; b) 4,4'-DTDP -induced RyR activation could be reversed with DTT and c) the loss of channel activity induced by 1 mM 4,4'-DTDP could be reversed by the addition of DTT.

4.4.1 DTT protects the RyR from effects of 4,4'-DTDP

RyRs in 10\(^{-7}\) M Ca\(^{2+}\) were exposed to 2 mM DTT (cis) for 5 min before 1 mM 4,4'-DTDP was added to the cis chamber (Fig. 4.10). DTT, itself, produced no significant change in RyR activity from control levels for P\(_o\), T\(_o\) or F\(_o\) (P > 0.05; n = 8). The addition of 4,4'-DTDP had no effect on channel activity in 8 of 9 RyRs in the presence of DTT. Although one channel appeared to be activated in the usual way by the addition of 4,4'-DTDP, the values of P\(_o\), T\(_o\) and F\(_o\) in the other eight channels were unaffected (P > 0.25). The open time distributions for control, DTT and DTT + 4,4'-DTDP treatments were each described by an averaged single exponential function (\(\tau_1 = 1.82 \pm 0.24\) ms; \(\tau_1 = 1.89 \pm 0.37\) ms; and \(\tau_1 = 1.51 \pm 0.51\) ms respectively).

The addition of 4,4'-DTDP (1 mM) causes the loss of RyR activity within 10 min (see section 4.2.1b). Its ability to cause this effect in the presence of DTT (2 mM) was examined from the records of the nine experiments described above. All channels to which 1 mM 4,4'-DTDP was added remained active for the duration of the experiment: seven of the nine experiments lasted >10 min, and five of these beyond 20 min. Interestingly, even the one channel that was activated by 4,4'-DTDP (in the presence of DTT) did not lose its channel activity over the >30 min duration of the experiment following the addition of 4,4'-DTDP. This indicates that the activation, usually induced by 4,4'-DTDP, and the subsequent loss of channel activity may be due to separate processes i.e. the oxidation of different SH groups.

In these experiments, the concentration of reducing agent was double that of the oxidising agent so that excess DTT remained after stoichiometric reactions with free 4,4'-DTDP. It was likely that DTT reduced the RDS, rendering it incapable of oxidising protein thiols. The resulting inability of the reduced RDS to activate the RyR suggests that: a) 4,4'-DTDP activates the RyR through an
Fig. 4.10  Dithiothreitol prevents the activation of the native RyR by 4,4'-DTDP.  2 mM DTT (cis) was added to RyRs in 10^{-7} M Ca^{2+}.  After 5 min 1 mM 4,4'-DTDP was added to the cis chamber in the presence of DTT.  Values of P_o, T_o and F_o for the eight individual channels during control (2 min), DTT (5 min) and DTT + 4,4'-DTDP (30 s) are shown on the left of each graph; mean values ± SEM on the right.  Statistical significance was evaluated using the paired t-test: there were no differences in the mean values of P_o, T_o or F_o when comparing a) DTT with control or b) 4,4'-DTDP with DTT.
Effects of SH reagents

oxidation reaction; b) 4,4'-DTDP cannot oxidise thiols in the presence of excess concentrations of a strong reducing agent and c) the RDS must be in its disulfide form to cause channel activation and/or subsequent loss of function.

4.4.2 **DTT reverses 4,4'-DTDP induced activation but not the loss of activity**

If RyR activation and the loss of activity induced by 4,4'-DTDP occur via oxidation reactions, then addition of DTT to the 4,4'-DTDP -activated channel, and to the channel in which activity has ceased, might be expected to restore the control activity. To test this assumption for activation, DTT (2 mM) was added to four channels in 10 M cis Ca^{2+} ~ 30 s after 4,4'-DTDP addition. DTT was only added to channels that were activated by 4,4'-DTDP within 30 s of adding the RDS. Adding DTT 30 s after 4,4'-DTDP addition allowed for the usual delay in activation by the RDS in many channels, and it was also well outside the period in which the RDS -induced loss of channel activity usually occurs. 4,4'-DTDP produced the expected increases in \( P_\infty \), \( T_\infty \), and \( F_\infty \) prior to the addition of DTT: \( P_\infty \) increased from 0.0006 ± 0.0005 to 0.0363 ± 0.0275; \( T_\infty \) from 2.2 ± 0.6 to 10.6 ± 2.8 ms and \( F_\infty \) from 0.18 ± 0.13 to 5.6 ± 5.3 s\(^{-1}\) \((P < 0.05; n = 4)\). Immediately after the addition of DTT, channel activity was restored to control levels. The value of each parameter fell significantly in the 2 min after the addition of DTT: \( P_\infty \) fell to 0.0002 ± 0.0001; \( T_\infty \) fell to 1.1 ± 0.3 ms and \( F_\infty \) fell to 0.15 ± 0.06 s\(^{-1}\) \((P < 0.05; n = 4)\) (Fig. 4.11). The average values for each of the three parameters after the addition of DTT (in the presence of 4,4'-DTDP) were no different to the control values \((P > 0.2)\). In addition, the usual loss of activity seen after 4,4'-DTDP -induced activation was not observed in these experiments. All four RyRs displayed typical control activity after addition of both 4,4'-DTDP and DTT for the duration of the experiments. However, three of the bilayers lasted only 5 - 6 min after DTT with only one still intact after 30 min. Because three of four experiments were terminated within 10 min of drug addition, it is not certain that DTT addition would have prevented the loss of activity in all cases.

The rapid and complete restoration of control activity, after addition of DTT to the 4,4'-DTDP -activated RyR, is considerably different to the normal RyR activity in the 5 min after 4,4'-DTDP addition. To highlight this point, the time courses for \( P_\infty \), \( T_\infty \) and \( F_\infty \) in 10 M Ca^{2+} (cis) were plotted for four RyRs exposed to 1 mM 4,4'-DTDP (Fig. 4.12A). These were compared to the same parameters in the four RyRs to which DTT was added 30 s after 1 mM 4,4'-DTDP (Fig. 4.12B). This comparison graphically demonstrates the usual time course for individual RyRs during the 5 min after 1 mM 4,4'-DTDP. \( P_\infty \) falls to zero for long periods after the transient activation, occasionally interspersed with a brief burst of activity. In contrast, after the addition of DTT to the 4,4'-DTDP -activated RyRs, the average values of \( P_\infty \), \( T_\infty \) and \( F_\infty \) were immediately restored to control levels.
Fig. 4.11  Dithiothreitol reverses the 4,4'-DTDP-induced activation of RyRs.  
1 mM 4,4'-DTDP cis was added to four RyRs in 10^{-7} M Ca^{2+}. After 30 s, 2 mM DTT was added to the cis chamber in the presence of 4,4'-DTDP. Values of P_o, T_o and F_o for single channels during control (2 min), 4,4'-DTDP (20 - 28 s) and 4,4'-DTDP + DTT (2 min) are shown on the left of each graph; mean values ± SEM on the right. Asterisks - significant differences between the mean and the mean in the preceding bin: * P < 0.05; ** P < 0.01.
Fig. 4.12 Comparison of the time course of channel activity from four RyRs activated by 4,4'-DTDP with the time course of another four RyRs exposed to DTT after 4,4'-DTDP-activation. Channel activity in $10^{-7}$ M Ca$^{2+}$ is displayed over a 2 min control period and a 5 min period after addition of redox reagents. A - Channels A - D were exposed to 1 mM 4,4'-DTDP alone. B - Channels E - H (the same four channels described in Fig. 4.11) were exposed to 1 mM 4,4'-DTDP for ~30 s before 2 mM DTT was added. The values of $P_0$, $T_0$, and $F_0$ for consecutive 30 s periods were plotted. Each number on the horizontal axis represents a 30 s period: the negative numbers (-4 to -1) represent the 2 min of control activity; the positive numbers (1 to 10) represent the 5 min period following addition of either 4,4'-DTDP (A) or DTT (B). The addition of 4,4'-DTDP (44) or DTT is indicated by an arrow.
The irreversible loss of RyR activity by 4,4'-DTDP has been clearly shown (section 4.2.1b). If the loss of channel activity is also the result of an oxidation reaction between 4,4'-DTDP and the RyR, then the addition of a reducing agent would be expected to restore RyR activity. To test this, nine channels were exposed to 1 mM 4,4'-DTDP; after 8 - 10 min the level of channel activity was tested by increasing the [Ca$^{2+}$]$_{cis}$ to 1 mM (n = 5) and applying voltage pulses (n = 9). No activity was observed. 10 min after 4,4'-DTDP was added, 2 mM DTT was added to the cis chamber. DTT addition failed to restore activity to the expected control levels in all nine channels. Six of the channels remained completely inactive after DTT, while three displayed low levels of activity over a 30 - 150 s period before activity, once again, returned to zero. Therefore, either the loss of activity does not depend directly on thiol oxidation, or the mixed disulfide between the RyR and the pyridyl ring is inaccessible to DTT.

**4.4.3 Effects of DTT and GSH on RyR activity**

Boraso and Williams (1994) reported that DTT decreases $P_o$ of the cardiac RyR in a concentration dependent manner, with 10 mM DTT completely closing the channel. Activity of the skeletal RyR is depressed with millimolar concentrations of GSH (Zable et al., 1997). 2 mM DTT was earlier shown to have no effect on $P_o$, $T_o$ or $F_o$ in 10$^{-7}$ M Ca$^{2+}$ over a 5 min period (section 4.4.1). The results of these experiments are considered in more detail in the present section, and the effects of DTT and GSH on the Ca$^{2+}$-activated channel are described. In addition, it is shown that 10 mM DTT does not alter channel conductance, although this higher concentration of DTT reduced $P_o$ in two channels by 60 - 64%.

The channel activity of eight RyRs in 10$^{-7}$ M Ca$^{2+}$ was measured during a 2 min period before and after the addition of 2 mM DTT. The values of $P_o$, $T_o$ and $F_o$ were compared. Of the eight channels, the value of $P_o$ increased in four, decreased in three and remained constant in one (Fig. 4.13Ai). There were no significant changes in any of the three measured parameters after the addition of DTT (Fig. 4.13Aii; $P > 0.3$; $n = 8$). With activating concentrations of Ca$^{2+}$ (10$^{-3}$ M), the 2 min before and after addition of either DTT (2 mM, $n = 4$) or GSH (2 mM, $n = 8$) to the RyR were compared. Neither of the reducing agents significantly affected the values of $P_o$, $T_o$ or $F_o$ (data not shown). However, after increasing the GSH concentration to 5 mM in six of the eight channels, the activity in three of the channels completely ceased while the activity of the remaining three were not significantly altered ($P > 0.05$, $n = 3$). These results show that low concentrations of DTT or GSH ($\leq$2 mM) do not affect activity of the cardiac RyR, but higher concentrations ($\geq$5 mM) appear to depress channel activity.

The effect of DTT on RyR conductance was also examined. To obtain clear channel openings to the maximum conductance (section 4.2.2c), the RyR was
Fig. 4.13  **2 mM dithiothreitol does not affect RyR activity or the current-voltage relationship.**  A - 2 mM DTT was added to eight RyRs in $10^{-7}$ M Ca$^{2+}$. RyR activity in the 2 min immediately after DTT addition was compared to channel activity during the 2 min control by showing: (i) values of $P_o$ for individual channels before and immediately after the addition of DTT and (ii) mean ± 2 SEM of the logarithm of the normalised values (ln(D/C)) of $P_o$, $T_o$ and $F_o$ i.e. the logarithm of the value of $P_o$, $T_o$ and $F_o$ for each channel after DTT (D) was normalised to the control value (C) in the same channel. The horizontal line drawn at zero indicates where the experimental parameters are equal to control: a positive value shows an increase in the parameter, a negative value a decrease. 2 SEM shows the lack of statistical significance at the 95% level if the error bars pass through zero.  B - channels were recorded in control solutions with 1 mM CaCl$_2$. Data for control ($n = 3$) were taken from the experiments shown in Fig. 4.4A. Data in the presence of 10 mM DTT ($n = 2$) were obtained from separate channels. Each point is the mean single channel current obtained in $n$ experiments. A third order polynomial was fitted to control (dashed line) and DTT (dotted line) data. ± SEM for the ordinate fall within the symbol.
Evidence has been presented which strongly suggests that
4,4'-DTDP reduces the number of cells via an oxidation reaction. The effects of an oxidant on the cell membrane can not be reversed when the reducing agent is removed from the bathing solution. The presence of the reducing agent in the control solution (10mM DTT) was therefore determined. After 30s, the cell membrane was protected from the oxidant by the reducing agent. The oxidant was then added to the bathing solution and the oxidized membrane was returned to the control solution. An increase in the amount of oxidation was observed when the reducing agent was subsequently removed from the bathing solution.

The holding potentials were measured in a 500mM KCl bathing solution, either the solution and subsequent addition of 4,4'-DTDP or dimethyl sulfoxide (DMSO) were compared. No significant differences were seen when comparing the holding potentials when no agents were present. Interestingly, the presence of 4,4'-DTDP or DMSO did not appear to have a significant effect on the holding potentials when a low KCl solution was used. However, during an oxidation reaction with a high KCl and low CaCl2, the holding potential was increased when 4,4'-DTDP was added to the bathing solution. This increase was more pronounced after the oxidant was removed from the bathing solution.

The average value of the three constants were similar to those seen when the distribution of impulse timing was measured from several experiments, in the presence of 4,4'-DTDP and dimethyl sulfoxide (see Figs 4 A and B).
activated with 1 mM Ca\textsuperscript{2+}_cis in the otherwise normal experimental solutions (250/50 mM CsCl cis/trans). The I/V data for controls were taken from the same three RyRs used in Figure 4.4A which had a E\textsubscript{rev} of -17.8 ± 0.3 mV. I/V data, in the presence of 10 mM DTT cis, but otherwise identical conditions to control experiments, were obtained from two other RyRs. The E\textsubscript{rev} of -18.05 ± 0.03 mV (n = 2) was close to the control value, and the curve fitted to the DTT data (a third order polynomial - dotted line) closely matched the fit to the control values (dashed line). Therefore, DTT does not affect RyR conductance.

4.4.4 Effects of 4,4'-DTDP and thimerosal are not washed out

Evidence has been presented (above) which strongly suggests that 4,4'-DTDP-induced RyR activation occurs via an oxidation reaction. The effects of an oxidation reaction should not be reversed when the oxidising agents are removed from solution. To test whether activation of the RyR by 4,4'-DTDP (1 mM, n = 3) or thimerosal (100 μM, n = 4) was reversed by removal of the reagent from the cis chamber, each reagent was added to the cis chamber. After 30 s, the cis solution was perfused with at least 5 volumes of oxidising agent -free control solution (10\textsuperscript{-7} M Ca\textsuperscript{2+}_cis). The 30 s period between the addition of either 4,4'-DTDP or thimerosal and perfusion, was allowed so that the reagent/protein-thiol reaction could occur. The important observation in these experiments was that activation of the RyR that occurred when the reagent was added, remained when the reagent was subsequently removed from the cis chamber. Examples of maximum activation induced by both agents, after perfusion, are shown in Figure 4.14Ai&Bi. P\textsubscript{o} was significantly increased from a control of 0.0006 ± 0.0007 to a maximum value of 0.1597 ± 0.0594 following the perfusion of 4,4'-DTDP from the cis chamber (P < 0.05; n = 3), and from 0.0022 ± 0.0015 to a maximum value of 0.0397 ± 0.0357 following the perfusion of thimerosal from the cis chamber (P < 0.05; n = 4) (Fig. 4.14Aii&Bii).

The levels of channel activity that were measured during maximum activation, after the addition and subsequent removal of 4,4'-DTDP or thimerosal (cis), were compared to a separate group of channels in which the reagents were present when maximum channel activity was measured (Tab. 4.4). Interestingly, the average maximum values obtained for P\textsubscript{o} and F\textsubscript{o} were higher, and T\textsubscript{o} lower after the reagents were removed from the chamber (only the differences for P\textsubscript{o} and T\textsubscript{o} after 4,4'-DTDP were statistically significant - see Tab. 4.4). Curiously, a similar pattern of activity, with a high F\textsubscript{o} and low T\textsubscript{o}, was seen when 4,4'-DTDP was added to the purified RyR (see section 4.3). The number of time constants required to fit the open time distribution, after the reagents were washed from the cis chamber, increased from one during control to three after drug washout (Fig. 4.14Aiii&Biii). The average values of the time constants were similar to those seen when the distribution of open times was measured from separate experiments, in the presence of 4,4'-DTDP and thimerosal (see Figs. 4.3 and 4.8).
Fig 4.14 Effects of 4,4'-DTDP and thimerosal on channel activity are not reversed by removal of the agents from the cis chamber. Channels were recorded in $10^{-7}$ M Ca$^{2+}$. 1 mM 4,4'-DTDP (A, $n = 3$) or 100 µM thimerosal (B, $n = 4$) were added to the cis chamber. After 30 s the reagents were removed from the cis chamber by perfusion with control solution. i) Sections of channel activity under control conditions (a), after addition of oxidising agents and subsequent perfusion (b) and 20 min after perfusion of the cis chamber (c). ii) Values of $P_o$ for individual channels during control, and during maximum activation following perfusion. iii) Average time constants, obtained from the distribution of open times from each RyR (Aii & Bii), during control (o) and after removal of the oxidising agents by perfusion (●).
Tab. 4.4 Comparison of the values of $P_0$, $T_0$ and $F_0$ in the presence of oxidising agents with the values after removal of oxidising agents from the cis solution by perfusion.

<table>
<thead>
<tr>
<th></th>
<th>4,4'-DTDP</th>
<th></th>
<th>Thimerosal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No wash</td>
<td>After wash</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>(n=21)</td>
<td>(n=3)</td>
<td></td>
</tr>
<tr>
<td>$P_0$</td>
<td>0.0654±0.0129</td>
<td>0.1597±0.0594</td>
<td>0.0191</td>
</tr>
<tr>
<td>$T_0$</td>
<td>22.2 ± 5.1</td>
<td>3.5 ± 1.1</td>
<td>0.0013</td>
</tr>
<tr>
<td>$F_0$</td>
<td>4.6 ± 1.2</td>
<td>57.4 ± 34.2</td>
<td>0.1994</td>
</tr>
</tbody>
</table>

Mean ± SEM for $n$ channels. *cis* $[\text{Ca}^{2+}]$, $10^{-7}$ M. P values: significance of difference between means in the presence of the oxidising agent (no wash) and after removal of the oxidising agent from the *cis* chamber by perfusion (after wash).
Effects of SH reagents

However, there was an unusually high proportion of events in the shortest component ($\tau_1 \geq 75\%$) for the open times that were obtained following the washout of the reagents. Such a high percentage of events in $\tau_1$ was only previously observed after 4,4'-DTDP -activation of the purified RyR or after thimerosal was added to the cis chamber.

Not only did channel activity remain high after removal of the oxidising agents from the cis chamber but, subsequently, channel activity was irreversibly lost in the usual way (Fig. 4.1Aic&Bic), albeit after a longer delay. Interestingly, longer than usual delays, before the loss of channel activity occurred, were also seen in the purified RyRs exposed to 4,4'-DTDP and native RyRs activated by the cis addition of thimerosal. Following 4,4'-DTDP addition to the cis chamber and its subsequent washout, all activity had ceased after 7 - 14 min and could not be restored by increasing the $[\text{Ca}^{2+}]_{cis}$ to 1 mM or by applying voltage pulses. This also occurred with thimerosal: no activity was seen 10 - 18 min after washout. These results show that the effects of the interactions between either 4,4'-DTDP or thimerosal and the RyR are not simply reversed upon exchange of solution and, hence, support the conclusion that their effects occur by SH oxidation.
DISCUSSION

The primary findings described in this section are that 4,4'-DTDP and thimerosal both caused activation and subsequent irreversible loss of activity of the native RyR when added to the cis or the trans chambers. The purified RyR was affected by 4,4'-DTDP in the same qualitative way as the native channel. When the RyR was activated by either 4,4'-DTDP or thimerosal, the degree of channel activation was independent of drug concentration, but the reagent concentration was an important factor in the loss of channel activity. Channel activation by 4,4'-DTDP was due to thiol oxidation since it was prevented and reversed by DTT. However, the loss of channel activity that occurred within 10 min of the addition of millimolar concentrations of 4,4'-DTDP could not be reversed when the reducing agent was added after the loss of channel activity had occurred.

4.5 Oxidation -induced RyR -activation

4.5.1 4,4'-DTDP -induced activation

The ability of 4,4'-DTDP to induce Ca$^{2+}$ efflux from Ca$^{2+}$ loaded SR vesicles is thought to be due to the oxidation of thiol groups on the RyR Ca$^{2+}$ release channel (see section 1.5.3b). The present study has shown that 4,4'-DTDP activates the cardiac RyR by the oxidation of cysteine residues on the RyR itself, or on a protein closely associated with the RyR. Channel activation was due to an increase in the mean open time and event frequency. 4,4'-DTDP does not affect channel conductance. An increase in $P_o$ with no effect on conductance was consistent with other non-specific SH reagents such as H$_2$O$_2$, thimerosal and doxorubicin (Ondrias et al., 1990; Boraso and Williams, 1994; Abramson et al., 1995). This suggests that thiol oxidation increases Ca$^{2+}$ flux across SR membrane by an effect on the gating of the RyR and not the permeation mechanism. Such an influence on the gating mechanism could be due to a direct or an indirect effect of thiol oxidation. Two different mechanisms have been proposed for the H$_2$O$_2$ -induced activation of skeletal and cardiac RyRs: a H$_2$O$_2$ -induced increase in Ca$^{2+}$ sensitivity of the skeletal RyR (Favero et al., 1995a), and a direct influence of H$_2$O$_2$ on channel gating in the cardiac RyR (Boraso and Williams, 1994). The evidence presented in this chapter suggests that the 4,4'-DTDP -induced RyR activation does not occur by an increase in the sensitivity of the channel to Ca$^{2+}$. Firstly, while the open time constants and the proportion of events in each component are very similar for the channel activated by either 10$^{-5}$ M Ca$^{2+}$ or 1 mM 4,4'-DTDP, the distribution of events in the closed time components are considerably different. Secondly, the level of channel open probability was significantly different between the RyRs activated by Ca$^{2+}$ and 4,4'-DTDP. This evidence is not sufficiently
conclusive to dismiss the possibility that 4,4′-DTDP -induced RyR activation occurs as a result of an increase in Ca$^{2+}$ sensitivity. An investigation of the effects of 4,4′-DTDP on the activity of the RyR at different cis Ca$^{2+}$ concentrations was conducted to clarify this point, and is described in the following chapter.

Concentrations of RDS between ~1 and 100 μM have a dose -dependent effect on the rate and amount of Ca$^{2+}$ efflux from skeletal and cardiac SR vesicles (Zaidi et al., 1989; Prabhu and Salama, 1990b). At subactivating concentrations of cis Ca$^{2+}$, the minimum 4,4′-DTDP concentration that activated the single cardiac RyR was 50 μM, but this decreased to 10 μM at an activating [Ca$^{2+}$]_{in}. The fact that a lower concentration of 4,4′-DTDP activated RyRs in the presence of Ca$^{2+}$, suggests that the Ca$^{2+}$ may influence the reactivity of the SH group(s) oxidised by the RDS, just as the cytoplasmic [Ca$^{2+}$] influences the reactivity of cysteine residues on the SR Ca$^{2+}$,Mg$^{2+}$-ATPase (Murphy, 1976; Thorley-Lawson and Green, 1977). The rate at which a thiol reacts with a reagent is influenced by its immediate environment i.e. the properties of its neighbouring residues and its position in relation to the membrane (Brocklehurst, 1979). A change in the conformation of the RyR may alter the “immediate” environment of the free SH group, thereby affecting its reactivity. It is possible that in the presence of Ca$^{2+}$, the reactivity of the relevant SH group on the RyR was increased because of the conformation of the open channel. Alternatively, the reactivity of the relevant SH group may be increased due to the specific binding of Ca$^{2+}$, irrespective of whether the channel is open or not. Either of these possibilities may allow 4,4′-DTDP to react more readily with the relevant SH group on the RyR. Evidence suggests that some SH groups involved in Ca$^{2+}$ efflux from the SR are more reactive than others. The addition of RDSs to Ca$^{2+}$ loaded skeletal SR vesicles induced thiopyridone production at two different rates (Zaidi et al., 1989). The fast rate marginally preceded Ca$^{2+}$ efflux. In summary, it appears that Ca$^{2+}$ may increase the sensitivity of the RyR to 4,4′-DTDP by increasing the reactivity of certain critical SH groups. This concept is considered in the following chapter using ATP and caffeine to activate the RyR in subactivating Ca$^{2+}$ before adding 4,4′-DTDP.

The degree of channel activation by 4,4′-DTDP was independent of the reagent concentration. This was shown at subactivating and activating cis Ca$^{2+}$ concentrations. However, it is interesting to note that, in subactivating Ca$^{2+}$, none of the channels activated by <1 mM 4,4′-DTDP had events greater than 50 ms in duration (i.e. in $\tau_4$ or $\tau_6$). In comparison, ~33% of channels activated by 1 mM 4,4′-DTDP had events in $\tau_4$. The reason for this difference is not clear, but an additional SH group with a low level of reactivity may be oxidised only in the presence of high concentrations of 4,4′-DTDP. This may introduce the additional kinetic component into the channel activity without significantly affecting $P_0$. The overall concept of concentration -independent activation by 4,4′-DTDP is consistent with the nature of an interaction between a protein thiol and a RDS; the
formation of a mixed disulfide would form a stable bond and not be expected to be influenced by the surrounding RDS concentration. This was supported by the results of 4,4'‐DTDP removal from the cis chamber by perfusion, ~30 s after its addition. Removal of the reagent did not prevent or reduce the usual 4,4'-DTDP-induced channel activation. In addition, removal of the oxidising agent, H₂O₂, by the addition of catalase does not reduce the H₂O₂-stimulated binding of [³H]ryanodine to its receptor (Fawer et al., 1995a). The stimulated binding of [³H]ryanodine was only returned to normal levels after the addition of DTT. In apparent contrast to the concentration-independent effect of 4,4'-DTDP on channel activation, studies investigating the rate and amount of Ca²⁺ efflux from skeletal and cardiac SR vesicles induced by RDSs show a dose-dependent effect of the reagents (Zaidi et al., 1989; Prabhu and Salama, 1990b). This discrepancy can be explained by the percentage of RyRs that are activated by a given RDS concentration. It was shown in the present study that millimolar concentrations of 4,4'-DTDP activated all RyRs, but concentrations ≤100 μM activated only up to 60% of the channels. Therefore, at low RDS concentrations, the percentage of Ca²⁺ efflux from vesicles may be due to only a small proportion of channels being oxidised. The low rate of Ca²⁺ release may be due to the gradual oxidation of RyRs over time. Another study appears to conflict with the concept that oxidation induced RyR activation is a concentration-independent reaction. Ondrias et al. (1990) found that the activation of the single cardiac RyR by doxorubicin was concentration-dependent. However, the presence of DTT did not prevent the activation of the RyR by doxorubicin suggesting that doxorubicin may not be activating the channel by an oxidation reaction. Although doxorubicin is reported to activate the RyR by the cross linking of vicinal thiols due to an oxidation reaction (Abramson and Salama, 1989; Pessah et al., 1990), it is also possible that the RyR was activated by an alkylation reaction. This is because: 1) doxorubicin is a quinone, and this class of reagent is able to alkylate SH groups as well as oxidise them (van Iwaarden et al., 1992) and 2) alkylation of SH groups does occur in the presence of DTT (Maness and Orengo, 1976; Yurewicz and Moghissi, 1981; Blanchard et al., 1982).

### 4.5.2 Luminal effects of oxidising agents

SH reagents have previously been applied only to the cytoplasmic face of the cardiac RyR, with little attention given to the location of the thiol group(s) being modified. The addition of a membrane impermeable thiol oxidising agent (glutathione disulfide) to the luminal face of the skeletal RyR had no effect on channel activity (Zable et al., 1997). A model of the skeletal RyR suggests that four putative transmembrane regions in each homoterramer (M1 - M4) comprise the channel region (Takeshima et al., 1989; Hakamata et al., 1992). There are two conserved cysteine residues on M2 and another two on the luminal loop between M3 and M4. Each pair of cysteines are separated by five amino acid residues. The
Effects of SH reagents

Sequence for the skeletal and cardiac RyR for each of these pairs are CIIGYXC (M2) and CDDMXTC (luminal M3 - M4 loop). These conserved transmembrane and luminal cysteine residues mean that the influence of SH reagents on RyR activity may not necessarily be mediated solely through sites located on the cytoplasmic face of the channel.

a) 4,4'-DTDP

In the present study, 4,4'-DTDP was added to the trans chamber for two reasons. Firstly, to see whether 4,4'-DTDP could activate the channel when added to the trans chamber. If channels were activated by the trans addition of 4,4'-DTDP, the second aim was to see whether there were any differences in the levels of activation or channel kinetics between RyRs activated by trans and cis 4,4'-DTDP. The RyR was activated following the addition of 4,4'-DTDP to the trans chamber. There were considerable qualitative similarities in the overall characteristics of channel activity after the addition of 4,4'-DTDP to either the cis or the trans chamber. These included an increase in P0 due to increases in both T1 and F0, an increase in the number of open time constant components and a similar delay prior to channel activation. However, quantitatively, the values of both P0 and T0 were lower after the addition of 4,4'-DTDP to the trans chamber than those recorded when the drug was added to the cis chamber. In addition, none of the very long open events that were seen after addition of 1 mM 4,4'-DTDP to the cis chamber (i.e. τo), were seen after the trans addition of 1 mM 4,4'-DTDP. It was suggested, above, that this longer kinetic component may be introduced into channel activity only when the RDS concentration is sufficiently high to oxidise an additional thiol group with a low reactivity. If this is the case, then it is likely that the particular thiol is located on the cytoplasmic side of the RyR, because the equivalent RDS concentration in the trans chamber did not activate it. Overall, the results of this experiment did not provide conclusive evidence to support a hypothesis that oxidation of different SH groups on the cytoplasmic and luminal sides of the RyR could be responsible for channel activation. The actions of cis or trans 4,4'-DTDP were sufficiently similar to suggest that diffusion across the bilayer may have allowed the oxidation of the same thiols. Other SH reagents are known to activate the RyR by oxidising residues specifically on the cytoplasmic side of the bilayer (Abramson et al., 1995; Zable et al., 1997). This is because the reagents used were added to the cis chamber, and were unable to cross the bilayer.

b) Thimerosal

Thimerosal activated the RyR after its addition to either the cis or the trans chamber. Because of its inability to cross the bilayer, this result strongly suggests that there are at least two separate sites on the cardiac RyR that, when modified by a thiol oxidising agent, activate the channel. It also confirms that there are sites on the luminal and cytoplasmic sides of the bilayer that influence channel activity when
Effects of SH reagents

Thimerosal increased the $P_o$ of the RyR, from either side of the bilayer, by increasing both $T_o$ and $F_o$. The overall properties of channel activity after cis or trans thimerosal-induced activation were surprisingly similar. Comparison of the effects of thimerosal and 4,4'-DTDP on RyR activity revealed that 4,4'-DTDP increased $P_o$ to a higher value than did thimerosal. The mean open time was increased more by 4,4'-DTDP than it was by thimerosal (contributing to the larger 4,4'-DTDP-induced increase in $P_o$). Event frequency was increased to similar levels by both reagents. Interestingly, when the reagents were added to the cis chamber, the differences in $P_o$ and $T_o$ between the two reagents were larger than when they were added to the trans chamber. The large influence of cis 4,4'-DTDP on $T_o$, compared to the effect of thimerosal on $T_o$, was reflected in a difference in the distribution of open times. The 1 mM 4,4'-DTDP-activated RyR had open times that fell into four time constant groups $\tau_1 - \tau_4$. Thimerosal also increased the duration of open events, but the open times only fell into three groups, $\tau_1 - \tau_3$. The effect of thimerosal on open times was similar to that seen when: 1) low concentrations of 4,4'-DTDP (100 $\mu$M) were added to the cis chamber and 2) 4,4'-DTDP (1 mM) was added to the trans chamber. The long openings, with events in $\tau_4$, were only observed after the addition of 1 mM 4,4'-DTDP to the cis chamber. It was suggested earlier in this section that a second thiol may be oxidised only by high concentrations of 4,4'-DTDP when added to the cytoplasmic face of the channel. The inability of millimolar concentrations of thimerosal to induce this additional kinetic component ($\tau_4$) in the channel activity suggests that, if this "other" thiol does exist, it may be located in a site that is more readily accessible to 4,4'-DTDP than thimerosal.

In contrast to the notable differences in activity between channels activated by the cis additions of 4,4'-DTDP or thimerosal, there were relatively minor differences between channels activated by trans additions of either reagent. Although the addition of 4,4'-DTDP to the trans chamber increased $P_o$ to a higher level than did thimerosal, the difference in $P_o$ between the two reagents was not as great as it was when they were added to the cis chamber. In addition, there were no significant differences in the average values of $T_o$ or $F_o$ between the two reagents when added to the trans chamber, and the distribution of open times where each had time constants with average values in $\tau_1 - \tau_3$. These similarities suggest that the same thiol group(s) may be oxidised by both reagents. If this is the case, then the more rapid rate of channel activation by thimerosal than 4,4'-DTDP (following their trans addition) indicates that the SH group oxidised by the reagents may be located in a relatively hydrophilic environment.

The addition of glutathione disulfide (GSSG) to the luminal face of the skeletal RyR had no effect on channel activity (Zable et al., 1997). GSSG can oxidise SH groups to form disulfides (Gilbert, 1984). Its addition to the cytoplasmic side of single skeletal RyRs activated the channels in micromolar concentrations of GSSG.
concentrations of Ca\(^{2+}\) by increasing event frequency (Zable et al., 1997). This GSSG-induced activation was reversed upon addition of GSH. Unlike the effects of 4,4′-DTDP or thimerosal on cardiac RyRs GSSG did not: a) activate channels in subactivating concentrations of Ca\(^{2+}\); b) increase channel open times or c) activate RyRs following \textit{trans} addition. There was also no report of a loss of channel activity that is often associated with oxidation of RyRs (see section 1.5.5). Evidence suggests that GSSG is a less powerful oxidising agent than thimerosal: micromolar concentrations of thimerosal (\textit{cis}) increased \(P_0\) of skeletal RyRs (Abramson et al., 1995) to values which were considerably higher than those recorded after the addition of millimolar concentrations of GSSG (\textit{cis}) under similar experimental conditions (Zable et al., 1997). A reduced ability to oxidise thiols may account for the inability of GSSG to activate the skeletal RyR when added to its luminal face. Alternatively, the inability of a thiol oxidising agent to activate the skeletal RyR following the \textit{trans} addition of GSSG may reveal differences in the skeletal and cardiac RyR isoforms.

Thimerosal is an organomercurial and, as such, is not a thiol specific oxidising agent. However, organomercurials react preferentially with thiols (Brocklehurst, 1979). \(Ca^{2+}\) currents from many cell types are affected by thimerosal in reactions that are prevented or reversed by DTT. These include the stimulation of cytosolic \(Ca^{2+}\) oscillations in myometrium via an interaction with the IP\(_3\) receptor (Phillippe, 1995), inhibition of the smooth muscle L-type \(Ca^{2+}\) channel (Chiamvimonvat et al., 1995) and the activation of the skeletal RyR (Abramson et al., 1995). Thus, the effects of thimerosal on the cardiac RyR are likely to be due to thiol oxidation. There are sites on both the cytoplasmic and luminal face of the RyR, and even in the putative pore of the channel, that could potentially be modified after oxidation by thimerosal. The RyR has eight conserved cysteine residues located between the start of the putative channel region (M1) and the cytoplasmic carboxyl terminal. They are arranged in four pairs with between 2 - 8 amino acid residues separating each pair. One pair is located on M2 (the second putative transmembrane segment), the second pair is on the luminal M3 - M4 loop and the remaining two pairs are on the cytoplasmic carboxyl-terminal tail. It was suggested that cysteine residues on the carboxyl-terminal tail of the IP\(_3\) receptor are likely to be the targets for the thimerosal -induced modification of channel activity (Kaplin et al., 1994). Antibodies directed against the carboxyl-terminal on the IP\(_3\) receptor stimulated IP\(_3\) -activated \(Ca^{2+}\) currents in cultured lobster olfactory receptor neurons (Fadool and Ache, 1992). Interestingly, the carboxyl-terminal tails of both the IP\(_3\) and the ryanodine receptors contain two cysteine residues in a highly conserved sequence (TXCFICG) (Berridge, 1993), and both receptors are activated by thimerosal (Kaplin et al., 1994; Abramson et al., 1995; Eager et al., 1995). Therefore, the cysteine residues in the conserved sequence on the carboxyl-terminal tail of the cardiac RyR (4887 - 4895), as well as the corresponding
sequence on the IP₃ receptor, may be the target for channel modification by thimerosal and other SH oxidising agents (Thrower et al., 1996).

4.5.3 The CHAPS-solubilised RyR

The CHAPS-solubilised RyR was also activated by 4,4'-DTDP. Activation of the purified cardiac or skeletal RyR has not previously been shown with any SH reagent. Interestingly, there were differences in aspects of channel activity between purified and native RyRs following oxidation by 4,4'-DTDP. 4,4'-DTDP-induced activation of the purified RyR was mainly due to increases in event frequency with no significant change in mean open time. The differences in channel activity between the purified and the native channel could be due to disruption of the protein during solubilisation, although the purified RyR shares most of the characteristics of the native RyR (see section 3.4.3). The differences may also be due to proteins associated with the native RyR that are removed during the solubilisation procedure. However, this is speculative. It is not known what proteins remain associated with the RyR after CHAPS-solubilisation. The coprecipitation of the FKBP with the CHAPS-solubilised cardiac RyR (Kaftan et al., 1996) shows that not all associated proteins are removed. Consequently, other SR proteins associated with the RyR, such as triadin or calmodulin, may still be attached to the CHAPS-solubilised RyR. Testing the effects of 4,4'-DTDP on the cloned RyR would show whether the effects were due solely to interactions with the RyR. However, even then, post-translational changes in the RyR protein may occur in muscle cells, that do not occur in the cells in which the receptor is expressed. Such changes could affect channel function, including protein thiol/reagent interactions.

4.5.4 Reducing agents

Reducing agents including DTT and GSH prevent the RDS-induced Ca²⁺ efflux from skeletal and cardiac SR vesicles in a dose-dependent manner (Zaidi et al., 1989; Prabhu and Salama, 1990b), prevent the activation of cardiac RyRs by H₂O₂ (Boraso and Williams, 1994) and reverse the activation of single skeletal RyRs induced by thimerosal, H₂O₂ and GSSG (Abramson et al., 1995; Favero et al., 1995a; Zable et al., 1997). While these studies have shown that reducing agents protect thiol groups on the RyR from the effects of oxidising agents under various experimental conditions, the effects of reducing agents on the single cardiac RyR, added in the presence of oxidising agents, had not been examined. The results from the present study showed, firstly, that 4,4'-DTDP could not activate the cardiac RyR in the presence of excess concentrations of DTT. Secondly, the addition of DTT to the channel, soon after activation by 4,4'-DTDP, could restore control activity. This confirms that RyR activation, induced by 4,4'-DTDP, is due to thiol oxidation. Because of the specificity of the RDSs in oxidising SH groups, there was little doubt that their effects were due to
interactions with SH groups on the RyR. Analysis of the properties of single channel activity after the additions of both DTT and 4,4'-DTDP showed that there were no lasting effects on the activity of the RyR if: 1) sufficient reducing agent was present when the channel was exposed to a thiol oxidising agent and 2) a reducing agent was added soon enough after the oxidation of the channel.

Neither DTT nor GSH (≤2 mM) had any effect on channel activity at the concentrations used in the experiments with 4,4'-DTDP. However, higher concentrations of the reducing agents (≥5 mM) were found to lower, or abolish, channel activity. Similar effects on the ability of reducing agents to inhibit Ca\(^{2+}\) currents in the RyR (Boraso and Williams, 1994; Zable et al., 1997) and the IP\(_3\) receptor (Kaplin et al., 1994) have been reported. Since the range of concentrations over which these effects have been observed (low millimolar) is comparable to the intracellular concentration of GSH (see section 1.5.4), it is likely that reducing agents in vivo may exert a tonic inhibition of the RyR. Interestingly, inhibition of the cardiac RyR induced by DTT did not prevent the activation of the channel by ATP or caffeine (Boraso and Williams, 1994).

The implications of the current findings, combined with previous studies, suggest that if the myocyte is exposed to oxidising agents, as can occur during ischaemia, reperfusion, fatigue, etc, the activity of the RyR should not be affected if the high ratio of GSH:GSSG is maintained. However, if the GSH concentration is sufficiently depleted by a large oxidative onslaught then the channel may become activated thereby increasing the [Ca\(^{2+}\)]. Under usual conditions, increases in the [Ca\(^{2+}\)], would activate the SR Ca\(^{2+}\),Mg\(^{2+}\)-ATPase along with sarcolemmal Ca\(^{2+}\) transporters to maintain Ca\(^{2+}\) homeostasis. However, the Ca\(^{2+}\),Mg\(^{2+}\)-ATPase is inhibited by oxidising agents (Murphy, 1976; Castilho et al., 1996; Viner et al., 1996). The combined effects of the oxidising agents on these SR Ca\(^{2+}\) transporters could contribute to the large increase in [Ca\(^{2+}\)], that occurs when the myocyte is exposed to oxidative stress (Eley et al., 1991b; Fukui et al., 1994). Damage to the mitochondria by oxidative stress has also been shown to result in mitochondrial Ca\(^{2+}\) efflux (Richer and Frei, 1988; Masaki et al., 1989). This may contribute to the increase in [Ca\(^{2+}\)], during an oxidative assault, but the relative contribution from SR and mitochondria to the increased [Ca\(^{2+}\)], is not known. Indeed, a high cytoplasmic [Ca\(^{2+}\)] can damage the mitochondria by causing an increase in the matrix [Ca\(^{2+}\)] (Vercesi, 1993). This can result in the inhibition of oxidative phosphorylation (Vercesi, 1993) and cause the production of ROS, such as \(\cdot\)OH, which can damage the mitochondria (Vercesi et al., 1994) and cause Ca\(^{2+}\) efflux. Hence, an increase in [Ca\(^{2+}\)], due to SR Ca\(^{2+}\) efflux may actually precede the mitochondrial damage. In summary, exposure of the cell to oxidising agents, combined with a decrease in the intracellular concentration of reducing agents, may contribute to a disruption of Ca\(^{2+}\) flux across the SR and cause mitochondrial dysfunction, thereby resulting in an elevation of [Ca\(^{2+}\)]. The results of the present
Effects of SH reagents

study indicate that, under such conditions, the rapid administration of reducing agents may restore usual RyR function. The activity of the Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase is restored with the addition of reducing agents (Viner et al., 1996). Hence, the administration of reducing agents at a time when the cell is initially exposed to oxidative stress may prevent the usual increase in Ca\(^{2+}\), that is associated with such an onslaught. If levels of Ca\(^{2+}\), have already risen due to oxidative stress, then the rapid administration of reducing agents may be able to reestablish Ca\(^{2+}\) homeostasis by increasing the GSH:GSSG ratio, scavenging the radical elements and restoring SR function.

4.6 Loss of channel activity

The loss of channel activity that followed the transient activation of the RyR by either 4,4'-DTDP or thimerosal is a time and concentration-dependent process. The native RyR lost all channel activity within 10 min of the addition of 4,4'-DTDP (1 mM) to the cis or trans chamber, and within 10 - 20 min of the addition of thimerosal to either chamber. After this time, none of the methods that would usually activate the RyR were able to restore channel activity. These methods included changing the bilayer holding potential (voltage pulses) and the addition of ATP and/or activating concentrations of Ca\(^{2+}\). Removal of the RDS from the cis chamber by perfusion was also ineffectual in restoring channel activity. Additions of lower concentrations of 4,4'-DTDP (≤100 μM) to the cis chamber rarely caused the loss of channel activity within 10 min of RDS addition, and the loss of activity was not always observed within the lifetime of the bilayer (often >15 min). The loss of single channel activity after the addition to the cis chamber of other membrane permeable (Holmberg et al., 1991) and membrane impermeable (Abramson et al., 1995) SH reagents has been shown in both the skeletal and cardiac RyR. However, this is the first report that a thiol oxidising agent, which is incapable of penetrating the lipid bilayer, caused the loss of channel activity when added to the trans chamber. This was a particularly intriguing finding because the membrane impermeable reagent caused an almost identical biphasic effect from either side of the bilayer. Whether the causes of these effects, following the addition of cis or trans thimerosal, are due to common or separate mechanisms has yet to be investigated.

The possibility that the loss of channel activity, induced by oxidising agents, is due to the oxidation of a cysteine residue on the RyR, or on a protein closely associated with the RyR, is supported by the evidence from the present study. The addition of 4,4'-DTDP, in the presence of DTT, did not induce the loss of activity. However, the issue is confused by the fact that the loss of channel activity that occurred following the addition of 4,4'-DTDP could not be reversed by the addition of reducing agents. Therefore, the direct cause for the loss of RyR activity cannot necessarily be attributed per se to the oxidation of a thiol group.
The activation of the channel by SH oxidation may be the trigger of an event, or a series of events, that results in the eventual loss of channel activity. In this case, the loss of channel activity is dependent on oxidation-induced activation. On the other hand, if the loss of channel activity is primarily due to the oxidation of a second thiol group, then it could be argued that channel activity was not restored after the addition of DTT because the oxidised group was inaccessible to reducing agents. A conformational change in the protein after oxidation could sterically shield the oxidised thiol from reduction. This concept is in agreement with the suggestion that oxidation-induced activation and "inactivation" are due to separate biochemical mechanisms (Ondrias et al., 1990). This suggestion was based on the finding that DTT prevented the channel "inhibition" by doxorubicin, while doxorubicin-induced activation occurred in the presence of the reducing agent. Other evidence supports the suggestion that the activation and the loss of channel activity are due to independent reactions. 1) The degree of channel activation by 4,4'-DTDP is concentration-independent. If the degree of channel activation influenced the subsequent loss of channel activity then, as long as the channel was activated, the loss of channel activity should proceed irrespective of the concentration of 4,4'-DTDP. This was not the case. 2) Even after 4,4'-DTDP-induced activation had occurred, the addition of DTT (after 30 s) to the 4,4'-DTDP-activated channel prevented subsequent loss of channel activity. 3) The loss of channel activity does not always occur after channel activation by an oxidation reaction (see section 4.4.1 and Refs. Boraso and Williams, 1994; Zable et al., 1997). Because many different types of SH reagent cause the loss of channel activity, and DTT prevents this from happening, strongly suggests that the loss of channel activity that occurs in the presence of oxidising agents is due to thiol oxidation.

The biphasic effects of oxidising agents on RyR activity suggests that there are at least two distinct classes of cysteine residues on the protein. The oxidation of a cysteine residue(s) with a relatively high reactivity causes the activation of the RyR within seconds of reagent addition. The oxidation of a second class of cysteine residue with lower levels of reactivity results in the loss of channel activity which takes several minutes. There is evidence for a third class of cysteine residue in the skeletal RyR. Alkylation of the skeletal RyR with N-ethylmaleimide caused channel inhibition prior to activation and a subsequent loss of channel activity (Aghdasi et al., 1997b). This inhibitory phase (3-10 min) prior to channel activation did not occur upon the oxidation of the cardiac RyR by thimerosal or the thiol specific 4,4'-DTDP.
OXIDATION OF THE RYANODINE RECEPTOR BY SULFHYDRYL REAGENTS IN THE PRESENCE OF CHANNEL AGONISTS AND ANTAGONISTS
5.1 INTRODUCTION

The results from Chapters 3 and 4 showed that the channel incorporated into the lipid bilayer is the RyR, and that the oxidation of cysteine residues on the RyR, or on a protein closely associated with the RyR, activates the channel. In addition, the properties of channel activity of the 4,4'-DTDP -activated RyR were analysed, revealing additional exponential components in the open time distribution of the oxidised channel. The next step was to consider the changing environment within the cell. Modulators of RyR activity such as Ca\(^{2+}\), ATP, Mg\(^{2+}\) and pH are all subject to fluctuation either during usual muscle action or under extreme physiological or pathological states such as fatigue (Favero et al., 1995b), ischaemia (Xu et al., 1996) or cardiac reperfusion injury (Mubagwa, 1995). Thus, it was considered important to examine the effects of altering the environment at the myoplasmic face of the RyR by changing the [Ca\(^{2+}\)], adding ATP or Mg\(^{2+}\) or lowering pH before oxidising the channel with RDSs. This would show to what degree oxidation -induced activation is dependent on ligand binding to the channel.

Various techniques have been used to investigate the relationship between physiological modulators of RyR activity and SH reagents in the function of the Ca\(^{2+}\) release channel. However, apparent inconsistencies in the results suggest the need for further examination. Firstly, different techniques used to study skeletal and cardiac RyRs have implicated two separate mechanisms for the activation of the channels by H\(_2\)O\(_2\). H\(_2\)O\(_2\) appeared to activate the skeletal RyR by increasing its sensitivity to Ca\(^{2+}\) (Favero et al., 1995a) while activation of the cardiac RyR by H\(_2\)O\(_2\) is apparently due to a direct effect on the gating mechanism (Boraso and Williams, 1994). This suggests important differences between RyR isoforms. However, this is yet to be confirmed. Secondly, low concentrations of Mg\(^{2+}\) stimulate RDS-induced Ca\(^{2+}\) release in skeletal and cardiac SR vesicles while higher concentrations inhibit the release (Zaidi et al., 1989; Prabhu and Salama, 1990b). Thirdly, the presence of adenine nucleotides appears to stimulate Ag\(^{+}\)-induced Ca\(^{2+}\) efflux from cardiac SR vesicles (Prabhu and Salama, 1990a) but inhibit RDS-induced Ca\(^{2+}\) efflux from skeletal and cardiac SR vesicles (Zaidi et al., 1989; Prabhu and Salama, 1990b).

The inconsistencies (mentioned above) suggest a need to gain a greater understanding of the interrelationships between the physiological modulators of the RyR and thiol oxidation. The only single channel study to have addressed this issue is that of Boraso and Williams (1994). They showed that the non-specific oxidising agent H\(_2\)O\(_2\) could activate the cardiac RyR in nanomolar as well as micromolar [Ca\(^{2+}\)]\(_{cis}\), and that the activity of the H\(_2\)O\(_2\) -activated RyR was potentiated by ATP and caffeine, and inhibited by Mg\(^{2+}\). In this chapter, the effects on channel activity of SH-specific oxidising agents, added to the RyR already
modulated by physiological and pharmacological reagents, are described. This includes descriptions of: 1) the Ca\(^{2+}\) -dependence of thiol oxidation and 2) the oxidation of channels modulated by either Mg\(^{2+}\), ATP, caffeine or low pH.

5.2 The Ca\(^{2+}\) dependence of 4,4\(^{-}\)-DTDP activation

In the previous chapter the effects of 4,4\(^{-}\)-DTDP were described primarily at a [Ca\(^{2+}\)]\(_{0}\) of 10\(^{-5}\) M, where the channel was in a resting or a non-active state. However, since the cytoplasmic [Ca\(^{2+}\)] in cardiac muscle cells is often carried over a wide range during muscle contraction and relaxation, and Ca\(^{2+}\) influences RyR activity (see section 5.1), it was of interest to add 4,4\(^{-}\)-DTDP to RyRs exposed to different concentrations of [Ca\(^{2+}\)]. The [Ca\(^{2+}\)] in the cell channels was changed from 10\(^{-5}\) M, required for channel incorporation, to concentrations that varied from the highest visible at Ca\(^{2+}\) (10\(^{-7}\) M) to very low (estimatory) levels of Ca\(^{2+}\) C x 10\(^{-5}\) M, prior to the addition of 1 mM 4,4\(^{-}\)-DTDP. Figure 5.1 shows that 4,4\(^{-}\)-DTDP affected channel activity at each [Ca\(^{2+}\)] tested. The degree of channel activation and the pattern of channel activity changed as the [Ca\(^{2+}\)]\(_{0}\) was increased from 10\(^{-5}\) M. The effects of 4,4\(^{-}\)-DTDP -induced RyR activation on various parameters of channel activity were measured at different Ca\(^{2+}\) concentrations.

5.2.1 4,4\(^{-}\)-DTDP activates the RyR over a wide range of [Ca\(^{2+}\)]\(_{0}\)

Under certain conditions activated from 4,4\(^{-}\)-channels, at one of 3 different Ca\(^{2+}\) concentrations (between 10\(^{-6}\) to 10\(^{-5}\) M), was recorded over a 2 min period and the average values of P, T, and F were measured. The response of the channels to the increasing [Ca\(^{2+}\)]\(_{0}\) (Fig. 5.2 - upper middle) is consistent with that shown, from a greater number of channels in Figure 5.3. As the [Ca\(^{2+}\)]\(_{0}\) was increased from subsaturating (10\(^{-6}\) and 10\(^{-5}\) M) to saturating levels (10\(^{-5}\) and 10\(^{-4}\) M) event frequency and open times increased, resulting in an increase in P. A further increase in [Ca\(^{2+}\)]\(_{0}\) to inhibiting levels (2 x 10\(^{-5}\) M) produced decreases in each of the three parameters.

After the current recording, 1 mM 4,4\(^{-}\)-DTDP was added to the cell chamber. P, T, and F were measured during the 30 s period of highest P, within 2 min of adding the BDI. Average values of each parameter were plotted for comparison with control values (Fig 5.2). Average P, and T, increased at each [Ca\(^{2+}\)]\(_{0}\). F also increased at 10\(^{-6}\), 10\(^{-5}\) and 2 x 10\(^{-5}\) M Ca\(^{2+}\) but decreased at 10\(^{-5}\) and 10\(^{-4}\) M Ca\(^{2+}\). Values for each of these parameters after addition of 1 mM 4,4\(^{-}\)-DTDP were significantly different from control, except for F at 10\(^{-5}\) M Ca\(^{2+}\) and F, at 2 x 10\(^{-5}\) M Ca\(^{2+}\) off the irreversible loss of channel activity that occurred within 10 mins of addition of 1 mM 4,4\(^{-}\)-DTDP at 10\(^{-7}\) M Ca\(^{2+}\), also occurred at each [Ca\(^{2+}\)] level. The delay before channel activity was lost was not influenced by the [Ca\(^{2+}\)]\(_{0}\). For the purposes of calculating in this series of experiments, the
RESULTS

5.2 The Ca^{2+} dependence of 4,4’-DTDP activation

In the previous chapter the effects of 4,4’-DTDP were described primarily at a [Ca^{2+}]_{cis} of 10^{-7} M, where the channel is in a resting or a non-active state. However, since the cytoplasmic [Ca^{2+}] in cardiac muscle fibres in vivo varies over a wide range during muscle contraction and relaxation, and Ca^{2+} influences RyR activity (see section 1.4.2a), it was of interest to add 4,4’-DTDP to RyRs exposed to different concentrations of cis Ca^{2+}. The [Ca^{2+}] in the cis chamber was changed from 10^{-3} M, required for channel incorporation, to concentrations that ranged from the virtual absence of Ca^{2+} (10^{-9} M) to very high (inhibitory) levels of Ca^{2+} (2 \times 10^{-2} M), prior to the addition of 1 mM 4,4’-DTDP. Figure 5.1 shows that 4,4’-DTDP affected channel activity at each [Ca^{2+}] tested. The degree of channel activation and the patterns of channel activity changed as the [Ca^{2+}]_{cis} was increased from 10^{-9} M. The effects of 4,4’-DTDP-induced RyR activation on various parameters of channel activity were measured at different Ca^{2+} concentrations.

5.2.1 4,4’-DTDP activates the RyR over a wide range of [Ca^{2+}]_{cis}

Under control conditions activity from 4 - 5 channels, at one of 5 different Ca^{2+} concentrations (between 10^{-9} to 2 \times 10^{-2} M), was recorded over a 2 min period and the average values of P_o, T_o, and F_o were measured. The response of the channels to the increasing [Ca^{2+}]_{cis} (Fig. 5.2 - open circles) is consistent with that shown, from a greater number of channels, in Figure 3.3. As the [Ca^{2+}]_{cis} was increased from subactivating (10^{-9} and 10^{-7} M) to activating levels (10^{-5} and 10^{-3} M) event frequency and open times increased, resulting in an increase in P_o. A further increase in [Ca^{2+}]_{cis} to inhibiting levels (2 \times 10^{-2} M) produced decreases in each of the three parameters.

After the control recording, 1 mM 4,4’-DTDP was added to the cis chamber. P_o, T_o and F_o were measured during the 30 s period of highest P_o within 2 min of adding the RDS. Average values of each parameter were plotted for comparison with control values (Fig. 5.2). Average P_o and T_o increased at each [Ca^{2+}]_{cis}. F_o also increased at 10^{-9}, 10^{-7} and 2 \times 10^{-2} M Ca^{2+} but decreased at 10^{-5} and 10^{-3} M Ca^{2+}. Values for each of the three parameters after addition of 1 mM 4,4’-DTDP were significantly different from control, except for P_o in 10^{-5} M Ca^{2+} and F_o in 2 \times 10^{-2} M Ca^{2+}. The irreversible loss of channel activity that occurred within 10 min of addition of 1 mM 4,4’-DTDP in 10^{-7} M Ca^{2+} also occurred at each [Ca^{2+}] tested. The delay before channel activity was lost was not influenced by the [Ca^{2+}]_{cis}. For the purposes of consistency in this series of experiments, the
<table>
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<th>4,4'-DTDP 1 mM</th>
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<tr>
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<tr>
<td>E 2x10^-2</td>
<td><img src="image" alt="Control E" /></td>
<td><img src="image" alt="4,4'-DTDP 1 mM E" /></td>
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Fig. 5.1 **Records of RyRs activated by 1 mM 4,4'-DTDP at different cis Ca\(^{2+}\) concentrations.** Channels were incorporated into the bilayer in 250/50 mM CsCl 1 mM CaCl_2 and 10 mM TES; the [Ca\(^{2+}\)]\(_{cis}\) was then adjusted to give the desired free [Ca\(^{2+}\)]. Separate channels are shown at each [Ca\(^{2+}\)]. Holding potential was +40 mV and channel openings are upward deflections from baseline (dotted line). Records shown for control (left) and within 2 min of 4,4'-DTDP addition (right) were selected from periods of high channel activity.
Fig. 5.2 Ca\(^{2+}\) -dependence of 4,4'-DTDP -activation. Average values of \(P_o\), \(T_o\) and \(F_o\) for control (2 min; ○) and after maximum 1 mM 4,4'-DTDP -induced activation within 2 min of drug addition (30 s; ●) at the indicated [Ca\(^{2+}\)]\(_{cyt}\). The number of channels at each [Ca\(^{2+}\)]\(_{cyt}\) are given in brackets. Channels were recorded under the conditions described in the legend to Figure 5.1. The vertical bars represent the SEM where this is greater than the size of the symbol. Lines link data points. Asterisks indicate significant differences from control: * \(P < 0.05\); ** \(P < 0.01\).
five channels recorded in $10^{-7}$ M Ca$^{2+}$ were obtained during experiments which were additional to the 21 channels recorded under identical conditions and described in Chapter 4.

### 5.2.2 Cytoplasmic Ca$^{2+}$ influences [4,4'-DTDP] required to activate the RyR

In the previous chapter, it was shown that the concentration of 4,4'-DTDP required to activate the RyR depended on the cis [Ca$^{2+}$]. In $10^{-7}$ M Ca$^{2+}$, the minimum concentration of 4,4'-DTDP that activated the channel was 50 µM, but this was reduced to 10 µM after the channel activity was increased by $10^{-5}$ M Ca$^{2+}$ (see section 4.2.3a). This influence of Ca$^{2+}$ was investigated to find out whether the higher $P_o$ of the channel (at an activating [Ca$^{2+}$]$_{cis}$) or the presence of Ca$^{2+}$ itself, were important factors in lowering the minimum concentration of 4,4'-DTDP required to activate the channel. To do this, RyRs were exposed to inhibitory levels ($2 \times 10^{-2}$ M) of cis Ca$^{2+}$. This would reduce the $P_o$ of the channel while maintaining the presence of Ca$^{2+}$. Low concentrations of 4,4'-DTDP were then added to the cis chamber to determine the lowest concentration of the RDS that could activate the channel. Interestingly, when the channel was inhibited by Ca$^{2+}$ ($2 \times 10^{-2}$ M), 4,4'-DTDP activated the channel with lower concentrations than those required when the channel was active in $10^{-5}$ M Ca$^{2+}$. 2 of 3 channels were activated by 10 µM 4,4'-DTDP, 2 of 3 with 1 µM 4,4'-DTDP and 1 of 3 channels was activated with 100 nM 4,4'-DTDP. This suggests that it is not the conformation of the open channel that allows lower concentrations of 4,4'-DTDP to activate the channel, since $P_o$ is low in Ca$^{2+}$-inhibited channels. The binding of Ca$^{2+}$ to the Ca$^{2+}$-activation site, or another site, may alter the conformation of the channel such that the reactivity of the SH group that is oxidised by 4,4'-DTDP to activate the channel is increased.

### 5.2.3 Cytoplasmic Ca$^{2+}$ affects the rate of 4,4'-DTDP-induced activation

The [Ca$^{2+}$] not only influenced the levels of 4,4'-DTDP -induced activation and the sensitivity of the channel to the reagent, but the rate of activation was also affected by the [Ca$^{2+}$]$_{cis}$ (Fig. 5.3). When 1 mM 4,4'-DTDP was added to RyRs in activating concentrations of Ca$^{2+}$ ($10^{-3}$ and $10^{-3}$ M), the delay before an increase in channel activity was $2.2 \pm 0.9$ s ($n = 9$). In contrast, the delay was significantly longer ($37.7 \pm 9.5$ s, $n = 30$) when 4,4'-DTDP was added to RyRs at subactivating concentrations of Ca$^{2+}$ ($10^{-9}$ and $10^{-7}$ M) ($P < 0.001$). The reduced latency in activating concentrations of Ca$^{2+}$ could be due to the relatively high open probability of the RyR prior to drug addition i.e. a SH group may become exposed when the channel is open thereby potentially increasing its reactivity. Alternatively, the reduced latency may be due to the presence of Ca$^{2+}$ itself. It was shown above (section 5.2.2) that high concentrations of Ca$^{2+}$ increase the sensitivity of the RyR to oxidation by 4,4'-DTDP. Based on this finding, activation of the RyR in high concentrations of Ca$^{2+}$ could be expected to occur rapidly. However, this did not
Fig. 5.3 Cytoplasmic Ca\(^{2+}\) affects the rate of channel activation by 4,4'-DTDP. Changes in mean (± SEM) P\(_o\), T\(_o\), and F\(_o\) during sequential 30 s periods before and after addition of 1 mM 4,4'-DTDP (time 0), with 10\(^{-3}\) M Ca\(^{2+}\) (left axis, ■, n = 4) and 10\(^{-9}\) M Ca\(^{2+}\) (right axis, □, n = 3). Channels were recorded under the conditions described in the legend to Figure 5.1.
occurs. The average delay of 34.5 ± 21.5 s (n = 5), when 1 mM 4,4'-DTDP was added to RyRs inhibited by 2 x 10^{-2} M Ca^{2+}, was no different to the ~37 s delay seen at subactivating concentrations of Ca^{2+} (P = 0.90). This suggests that the higher open probability in the presence of activating concentrations of Ca^{2+} may be a more important factor in influencing the rate of channel activation by 4,4'-DTDP than the presence of Ca^{2+}.

It was observed that Ca^{2+} efflux from SR vesicles, induced by RDSs, was delayed when low concentrations of RDSs were added (Zaidi et al., 1989). This increased delay also occurred when micromolar concentrations of RDSs were added to RyRs in lipid bilayers. Channel activation took ~30 s following the addition of 50 µM 4,4'-DTDP (n = 3) in activating Ca^{2+} (10^{-5} M). The delay was longer when channels were not activated by Ca^{2+}. Channel activation took >2 min when ≤50 µM 4,4'-DTDP was added to RyRs in subactivating (n = 3) or inhibitory (n = 5) concentrations of Ca^{2+}.

5.2.4 Effects of Ca^{2+} and 4,4'-DTDP on distribution of event durations

a) Open time distributions

The effects of 4,4'-DTDP on RyR activation were examined more closely by looking at the distribution of open and closed times; multiple exponential equations were fitted to the distributions. Histograms were compiled for the distribution of open times, recorded from a single channel in 10^{-5} M Ca^{2+} over a 2 min period, both before and immediately after 1 mM 4,4'-DTDP (Fig. 5.4). Histograms on the left show the square root of the frequency of open events in bins with logged durations (Sigworth and Sine, 1987). Histograms on the right, displaying the same data, show the probability of events in 20 ms bins. Exponential functions were obtained from the best least squares fit to data plotted in logged bins (Fig. 5.4Ai&Bi - dashed lines). The control open time histograms contained 7,669 events and a good fit required two exponential components which had time constants of 3.1 and 10.8 ms (Fig. 5.4A). After 4,4'-DTDP, four exponential components (0.3, 5.9, 49 and 274 ms) were required to adequately fit the 583 events (Fig. 5.4B). The sum of the exponential components (solid line) was re-drawn on a graph plotting probability verses duration on linear axes (Fig. 5.4Ai&Bi). The fit to the data using the logged bin technique also adequately fit the data when displayed on the linear histogram. Examples of channel activity from the same experiment (Fig. 5.4Ai&Bi) were obtained during periods of high activity in the absence and presence of 4,4'-DTDP.

To look at the average effects of 4,4'-DTDP on both the open and the closed times at different Ca^{2+} concentrations, exponential components for the open and closed time distributions from individual channels were calculated using the method of Sigworth and Sine (1987). The time constants were allocated to groups
**A Control**

![Control graph](image)

**B 4,4'-DTDP**

![4,4'-DTDP graph](image)

**Fig. 5.4 4,4'-DTDP affects the distribution of open times.** Open times for an individual RyR in 10^-5 M Ca^{2+} during the 2 min before (A) and the 5 min after (B) the addition of 1 mM 4,4'-DTDP. Graphs on the left show the v'frequency of open times (o) plotted in bins of logged duration. Control data were fitted by two exponential components (broken lines); four components were obtained after 4,4'-DTDP (see text for time constants). The sum of the exponential components (solid line) is shown on a graph of the same data plotted in bins of 20 ms (Aii & Bii). Records of channel activity were taken during a period of high activity under control conditions (Aiii) and after 4,4'-DTDP addition (Biii).
(see section 2.6), and plotted to display the average values and the average percentage of events in each group (Fig. 5.5). There was some variability between channels in the number of time constants: for example, the open times for control channels with 10−5 M Ca2+ could be fitted by two to three exponentials in most cases, but one channel required four. However, on average, Figure 5.5A illustrates the following important characteristics of open time distributions for control data at different Ca2+ concentrations, as well as the effects of 1 mM 4,4′-DTDP. 1) Ca2+ induced increase in the number of exponential components from one at 10−9 M Ca2+ (τ1), to as many as four at 10−5 M Ca2+ (τ1 - τ4). 2) 4,4′-DTDP induced increase in the number of components to either 4 or 5 at each [Ca2+]. 3) >70% of all events in the shortest component (τ1) at each [Ca2+] (except 10−5 M) under control conditions. 4) After 1 mM 4,4′-DTDP, <36% of all events in τ1 at each [Ca2+] (except 2 x 10−2 M).

b) Closed time distributions

The time constants for the distribution of closed times, both before and after 1 mM 4,4′-DTDP (Fig. 5.5B), were obtained and presented in the same way as the open time constants. The exponential components were allocated to one of five groups, τ1 - τ5 (see section 2.6). The graphs (Fig. 5.5B) show the average time constant of each group and the percentage of events that fell into each time constant group. The main feature of the closed time distributions as the [Ca2+]cn is increased from 10−9 M to 2 x 10−2 M is a change in the percentage of events in the shortest component (τ1). As the [Ca2+] rises from 10−9 to 10−3 M there is a progressive increase in the percentage of closures in τ1 from 5% to 92%. This falls to 22% of events with inhibition by 2 x 10−2 M Ca2+. Unlike the open time distribution, there was no consistent effect of 4,4′-DTDP on the average closed time constants or the relative weighting of each component.

The effect of 4,4′-DTDP on the distribution of open times, together with no consistent effect on the closed time distributions, suggests that there is a preferential interaction between 4,4′-DTDP and free SH groups that are exposed when the channel is open, or thiol groups that interact with mechanisms that regulate channel opening (Eager et al., 1997).

5.3 Comparable effects of 2,2′-DTDP and 4,4′-DTDP

2,2′-DTDP is a closely related compound to 4,4′-DTDP (see section 1.5.3b). Zaidi et al. (1989) showed that both of these compounds induce Ca2+ efflux from skeletal SR vesicles from concentrations as low as 2.5 µM. However, a compound, which is similar in structure to the RDSs but has methyl groups between the pyridyl ring and the disulfide (pyrithioxin), did not induce Ca2+ efflux at concentrations up to 100 µM. The presence of DTT resulted in a concentration dependent inhibition of 2,2′- or 4,4′-DTDP -induced Ca2+ efflux from cardiac SR
Fig. 5.5 Average effects of Ca\(^{2+}\) and 1 mM 4,4'-DTDP on open and closed time distributions. Time constants were calculated for 4 to 5 individual channels at each [Ca\(^{2+}\)] during the 2 min before (○) and after (●) 4,4'-DTDP addition. Each time constant was grouped into one of five time constant groups (τ\(_1\) - τ\(_5\); see section 2.6). The values in each group were averaged and plotted. Open time constants (A). Closed time constants (B).
vesicles (Prabhu and Salama, 1990b), suggesting strongly that both reagents induce their effects by oxidising SH groups on proteins in SR membrane. Therefore, it was likely that 2,2'-DTDP would have a similar effect to 4,4'-DTDP on the single cardiac RyR. The effects of 2,2'-DTDP on the Ca\(^2+\) -activated channel were examined to test: 1) whether 2,2'-DTDP did, in fact, activate the cardiac RyR; 2) whether the properties of channel activity after adding 2,2'-DTDP differed from those of the 4,4'-DTDP -activated RyR and 3) if channel activity was irreversibly lost following the addition of 2,2'-DTDP, as it was after adding 1 mM 4,4'-DTDP.

1 mM 4,4'-DTDP (Fig. 5.6A) and 1 mM 2,2'-DTDP (Fig. 5.6B) appear to have similar effects on RyR activity. In the channel shown, gating was altered within seconds of the addition of 2,2'-DTDP to the cis chamber, and activity of the channel was irreversibly lost after 5 min. 100 µM (n = 3) and 1 mM (n = 3) 2,2'-DTDP were added to RyRs (cis) in the presence of 10^{-3} M Ca\(^2+\) and the parameters of channel activity were measured (Fig. 5.6C). As with 4,4'-DTDP, no difference in channel activation between the two concentrations was observed so the data were combined for statistical analysis. While there was a 2,2'-DTDP -induced increase in P\(_o\) from 0.20 ± 0.09 to 0.29 ± 0.08 the difference was not significant (P = 0.07, n = 6). However, the changes in T\(_o\) and F\(_o\) after 2,2'-DTDP were significantly different to control values: T\(_o\) was increased from 16.4 ± 4.5 to 259 ± 134 ms (n = 6, P < 0.01) while F\(_o\) decreased from 16.2 ± 6.4 to 4.1 ± 2.0 s\(^{-1}\) (n = 6, P < 0.01) (Fig. 5.6C).

The irreversible loss of RyR activity, seen within 8 min of 1 mM 4,4'-DTDP addition (see section 4.2.1b), was also seen with 1 mM 2,2'-DTDP. Of the eight channels to which 1 mM 2,2'-DTDP was added (in 10^{-5} and 10^{-3} M Ca\(^2+\)), the bilayers of seven lasted sufficiently long to test for the loss of channel activity. Channel activity ceased between 3 - 7 min of adding 1 mM 2,2'-DTDP (n = 7). Voltage pulses, which usually activate RyRs (Eager et al., 1997), failed to induce channel activity when applied to the bilayer ~5 min after drug addition. Lower concentrations of 2,2'-DTDP did not produce the loss of activity within the lifetime of the bilayers. Each of three channels to which 100 µM 2,2'-DTDP was added were still spontaneously active 9 - 20 min after drug addition, and channel activity could be invoked by voltage pulses. These results are consistent with the concentration dependent effects of 4,4'-DTDP.

The distributions of open and closed times, following RyR activation by 2,2'-DTDP (100 µM and 1 mM, n = 6; 10^{-5} M *cis* Ca\(^2+\)), were similar to the distributions of event durations following 4,4'-DTDP -activation (see section 5.2.4a and Fig. 5.5). The changes in open times after 2,2'-DTDP included: 1) an increase in the number of exponential components from three to five; 2) the appearance of events in the longest time constant group (τ\(_s\) - >500 ms) and 3) a reduction in the percentage of events in the shortest two groups (τ\(_1\) and τ\(_2\) - <12 ms) from 57.9% under control conditions to 15.3% after 2,2'-DTDP. The
Fig. 5.6 **Effects of 2,2'-DTDP on channel activity of the Ca\(^{2+}\) -activated RyR are comparable to the effects of 4,4'-DTDP.** Channels were recorded in 250/50 mM CsCl, 1 mM trans Ca\(^{2+}\) at +40 mV. A & B - Addition of 1 mM 4,4'-DTDP (A) or 2,2'-DTDP (B) to the cis chamber in 10\(^{-3}\) M Ca\(^{2+}\). *Left* - Maximum activity during control. *Centre* - Maximum activity recorded within 30 s of adding reactive disulfides. *Right* - Lack of channel activity 5 min after adding RDSs; a voltage step to -40 mV did not activate channels. Bilayer potential is indicated by the line below the current traces. C - Values of \(P_o\), \(T_o\) and \(F_o\) for individual channels before and after 100 \(\mu\)M (broken lines) or 1 mM (solid lines) 2,2'-DTDP.
values of the average open time constants under control conditions were: \( \tau_1 = 2.0 \pm 1.4 \) (n = 2), \( \tau_2 = 8.3 \pm 1.5 \) (n = 5) and \( \tau_3 = 36 \pm 2.3 \) ms (n = 5). After 2,2'-DTDP the values of \( \tau_1 = 2.6 \) (n = 1), \( \tau_2 = 3.7 \pm 0.2 \) (n = 2), \( \tau_3 = 26 \pm 18 \) (n = 2), \( \tau_4 = 116 \pm 31 \) (n = 3) and \( \tau_5 = 582 \pm 108 \) ms (n = 2) were obtained. The distributions of closed times for each channel, before and after 2,2'-DTDP, were fitted by the sum of 2 - 3 exponentials. These fell into four time constant groups with \( \sim 90\% \) of events in \( \tau_1 \). The actions of 2,2'-DTDP on the distributions of open and closed times, taken together with its effects on \( P_o, T_o, F_o \) and its ability to cause the loss of channel function, strongly suggest that 2,2'-DTDP acts at the same site as 4,4'-DTDP to induce changes in RyR activity.

While the general effects on RyR activity with 2,2'-DTDP were comparable with the effects of 4,4'-DTDP, the increase in \( P_o \) following the addition of 2,2'-DTDP was not as large as the increase in \( P_o \) with 4,4'-DTDP. Interestingly, while 4,4'-DTDP caused a faster rate of \( \text{Ca}^{2+} \) efflux in cardiac SR vesicles, the overall \( \text{Ca}^{2+} \) release from the vesicles was similar with 4,4'-DTDP and 2,2'-DTDP (Prabhu and Salama, 1990b).

### 5.4 Functional RyR retained after 4,4'-DTDP activation

Reactive oxygen species (ROS) and 4,4'-DTDP affect the activity of the single cardiac RyR in a similar way i.e. transient activation followed by an irreversible loss of channel activity (Holmberg et al., 1991; Eager et al., 1997). It was proposed that the changes in RyR gating, after addition of ROS, may be due to degradation of a section of the channel that specifically affects control of gating (Holmberg et al., 1991). If this occurs, the 4,4'-DTDP -induced changes in RyR gating may also be due to degradation of the protein. This does not appear to be the mechanism by which 4,4'-DTDP activates the channel since the addition of DTT reversed the 4,4'-DTDP -induced activation and restored control activity in \( 10^{-7} \) M \( \text{Ca}^{2+} \) (see section 4.4.2). The ability of DTT and GSH to reverse the oxidation -induced activation of the RyR was investigated at different \( \text{Ca}^{2+} \) concentrations. DTT (1 - 10 mM, n = 3) and GSH (1 mM, n = 2) were added to both the \( \text{Ca}^{2+} \) -activated (10\(^{-3}\) M, n = 2) and the \( \text{Ca}^{2+} \) -inhibited (2 \( \times 10^{-2}\) M, n = 3) channel during activation by 4,4'-DTDP (0.1 - 0.5 mM). The reversal of 4,4'-DTDP -induced activation by the reducing agents was apparent, from the changes in channel activity, within 20 s of adding either DTT or GSH (Fig. 5.7A). In all experiments the concentration of reducing agent was 2 to 100 -fold greater than the concentration of 4,4'-DTDP, so that excess reducing agent remained after stoichiometric reactions with free RDS. These results, together with those presented earlier (section 4.4), clearly show that reduction of the oxidised thiols restores control activity over a wide range of \([\text{Ca}^{2+}]_{\text{cyt}}\). Therefore, RDS -induced activation does not fundamentally alter the conduction or gating mechanism(s) of
Fig. 5.7  Reversal of 4,4'-DTDP-induced activation by glutathione, and usual actions of ryanodine and ruthenium red on the 4,4'-DTDP-activated RyR. Channels were recorded in 250/50 mM CsCl, 1 mM trans Ca²⁺ at +40 mV. A - Effect of GSH on the 4,4'-DTDP-activated RyR: (i) activity of an individual RyR partially inhibited by 2 x 10⁻² M Ca²⁺; (ii) subsequent activation with 100 µM 4,4'-DTDP and (iii) control activity restored after addition of 1 mM GSH to the cis chamber in the presence of 4,4'-DTDP. B - Effects of ryanodine on the 4,4'-DTDP-activated RyR: (i) channel activity of a RyR activated by 100 µM 4,4'-DTDP in the presence of 2 x 10⁻² M Ca²⁺ (left) and an all points histogram of the 10 s trace (right). (ii) Within 5 s of adding 10 µM cis ryanodine to the same channel in the presence of 4,4'-DTDP (left) and an all points histogram of the 10 s trace (right). Dotted line indicates current baseline; arrows indicate maximum conductance. C - Channel activity showing the 1 mM 4,4'-DTDP-activated RyR in 10⁻³ M Ca²⁺ before (i), and 2 s after (ii), addition of 10 µM ruthenium red.
the RyR. This is in contrast to the eventual loss of channel activity which is irreversible.

Reduction of the 4,4'-DTDP-oxidised RyR reverses channel activation and restores control activity. The question remains whether the channel responds in the usual way to channel ligands when it is activated by RDSs i.e. when the RyR is disulfide linked to one or more pyridine rings. This was tested by adding ryanodine and ruthenium red to the 4,4'-DTDP-activated channel. Ryanodine (10 µM) locked 5 of 5 4,4'-DTDP-activated channels into the usual ryanodine subconductance level at ~50% of the maximum conductance (Fig. 5.7B) in 10^{-7} M (n = 2) and 2 x 10^{-2} M Ca^{2+} (n = 3). Ruthenium red (10 µM) blocked channel activity of all 1 mM 4,4'-DTDP-activated RyRs, within 5 s, in 10^{-7} M (n = 5) and 2 x 10^{-2} M Ca^{2+} (n = 1) (Fig. 5.7C). Hence, the formation of a mixed disulfide bond(s) between the RyR and a pyridyl ring(s) did not appear to alter the interaction between the RyR and ryanodine or ruthenium red.

5.5 Oxidation of the pharmacologically modified RyR

The final section of this chapter shows how the oxidation of the RyR affects channel activity in the presence of physiological (Mg^{2+}, ATP or pH) or pharmacological (caffeine) reagents. The cis [Ca^{2+}] was either 10^{-7} or 10^{-3} M. Mg^{2+}, ATP or caffeine were added individually to the cis chamber ~2 min before adding 1 mM 4,4'-DTDP. To examine the effects of pH, the cis chamber was perfused with a low pH solution prior to adding 4,4'-DTDP.

5.5.1 4,4'-DTDP activation of the Mg^{2+}-inhibited RyR

The presence of Mg^{2+} in the myoplasm, and a proposed role for Mg^{2+} in regulating Ca^{2+} release from the SR, have been discussed (see section 1.4.2c). The inhibitory effects of low (1 mM) and high (10 mM) concentrations of Mg^{2+} on the single cardiac RyR have been presented (see section 3.4.2b) and were shown to be consistent with the findings in the literature. If oxidation-induced channel activation is of physiological consequence 4,4'-DTDP would have to activate the channel in the presence of physiological concentrations of Mg^{2+}. This was tested by adding 1 mM 4,4'-DTDP to the cis chamber under two different conditions: a) when channel activity was inhibited by 1 mM Mg^{2+} (in 10^{-7} M Ca^{2+}) and b) in the Ca^{2+}-activated channel (10^{-3} M) with activity subsequently inhibited by 10 mM Mg^{2+}.

a) Mg^{2+}-inhibition at the Ca^{2+}-activation site: 4,4'-DTDP-activation

P_o, T_o and F_o of the RyR in 10^{-7} M Ca^{2+} were significantly reduced by the addition of 1 mM Mg^{2+} (see section 3.4.2b & Tab 5.1). Under these conditions, the concentrations of both ions are close to the levels recorded in the quiescent
myocyte i.e. during diastole (Fabiato, 1983; Murphy et al., 1989); Mg\(^{2+}\) inhibits RyR activity by competitively binding to the Ca\(^{2+}\)-activation site (Nagasaki and Kasai, 1983; Laver et al., 1997). Addition of 4,4'-DTDP to the Mg\(^{2+}\)-inhibited RyR (n = 5) significantly increased P\(_0\), T\(_0\) and F\(_0\) (Fig. 5.8A & Tab. 5.1). Channel records show the effects of Mg\(^{2+}\)-inhibition and subsequent 4,4'-DTDP-activation (Fig. 5.8B). However, while 4,4'-DTDP activated the Mg\(^{2+}\)-inhibited RyR, the increase in P\(_0\) to 0.0066 was 10-fold lower than the increase in P\(_0\) obtained by 4,4'-DTDP in the absence of Mg\(^{2+}\) (P\(_0\) = 0.065). Figure 5.9 shows a comparison of the mean change in P\(_0\), T\(_0\) and F\(_0\) after 1 mM 4,4'-DTDP in the absence (n = 21) and presence (n = 5) of 1 mM Mg\(^{2+}\). While 4,4'-DTDP significantly increased each parameter in the absence or presence of Mg\(^{2+}\), the mean values of the 4,4'-DTDP-activated RyRs in 1 mM Mg\(^{2+}\) (n = 5) were significantly less than the 4,4'-DTDP-activated RyRs without Mg\(^{2+}\) (P\(_0\) (P < 0.001), T\(_0\) (P < 0.01) and F\(_0\) (P < 0.01)). Thus, while 4,4'-DTDP activated the Mg\(^{2+}\)-inhibited channel, its actions were still inhibited by Mg\(^{2+}\).

The influence of Mg\(^{2+}\) on 4,4'-DTDP-induced activation of the RyR is illustrated by the distribution of open times. In the presence of 1 mM Mg\(^{2+}\) and 10\(^{-7}\) M Ca\(^{2+}\), the numbers of events were too few to fit exponential components to the open time distribution. After the addition of 4,4'-DTDP, openings in four of the five channels fell into the two shortest exponential components, while the fifth channel had events only in \(\tau_2\). On average, \(\tau_1 = 1.4 \pm 0.1\) ms (n = 4) and \(\tau_2 = 9.5 \pm 1.6\) ms (n = 5). Each group contained approximately equal percentages of events: 46.7 \pm 13.9\% and 53.3 \pm 13.9\% respectively. In contrast, the addition of 4,4'-DTDP to RyRs in 10\(^{-7}\) M Ca\(^{2+}\) (n = 21), in the absence of Mg\(^{2+}\), resulted in open events falling into four time constant groups (\(\tau_1 - \tau_4\)) as described previously (see section 4.2.2b). Interestingly, the two shortest time constants obtained with 4,4'-DTDP in the absence of Mg\(^{2+}\), with average values of \(\tau_1 = 1.3 \pm 0.3\) ms and \(\tau_2 = 8.4 \pm 0.9\) ms, were close to the values obtained for 4,4'-DTDP-activation in the presence of Mg\(^{2+}\). The ability of Mg\(^{2+}\) to inhibit the oxidised RyR may be due, in part, to preventing the introduction of the longer kinetic components.

b) Mg\(^{2+}\)-inhibition of the Ca\(^{2+}\)-activated channel: 4,4'-DTDP-activation

In contrast to the opposing actions of Ca\(^{2+}\) and Mg\(^{2+}\) at the high affinity Mg\(^{2+}\)-binding site, the binding of either Ca\(^{2+}\) or Mg\(^{2+}\) to the low affinity sites results in channel inhibition with very similar effects on channel activity. This is shown in the comparison of channel records where RyRs from two separate experiments are inhibited by Mg\(^{2+}\) (Fig. 5.10Ai) and Ca\(^{2+}\) (Fig. 5.10Bi). The distribution of channel open times were obtained from a 1 - 2 min period after increasing the Ca\(^{2+}\) or Mg\(^{2+}\) concentration to inhibitory levels: under each condition the data fell into two exponential components, with >94\% of events falling into the shortest time constant component (Fig. 5.10Ai&Bii). The Mg\(^{2+}\)-induced reduction in the number of exponential components from four (in 1 mM Ca\(^{2+}\)) to
Fig. 5.8 4,4'-DTDP increases activity of the RyR inhibited by 1 mM Mg$^{2+}$ in subactivating Ca$^{2+}$. A - Channel activity of five RyRs in $10^{-7}$ M Ca$^{2+}$ was recorded for 2 min before cis additions of 1 mM Mg$^{2+}$ followed after 2 min by 1 mM 4,4'-DTDP. Values of $P_0$, $T_0$, and $F_0$ for control and Mg$^{2+}$ were measured over a 2 min period; values for 4,4'-DTDP were measured during the 30 s of highest $P_0$ within 2 min of drug addition. Values obtained for individual channels (left) and mean ($\pm$ SEM) data (right) are shown. Only three data points are shown for single channels in the presence of Mg$^{2+}$ because there were no openings in the other two channels in the presence of Mg$^{2+}$. Asterisks indicate significant differences between the mean and the mean in the preceding bin: ** $P < 0.01$; *** $P < 0.001$. B - Records from a single experiment showing activity in $10^{-7}$ M Ca$^{2+}$ (i), no activity after additions of 1 mM Mg$^{2+}$ (ii) and activity after 1 mM 4,4'-DTDP (iii).
Fig. 5.9 **Comparison of 4,4'-DTDP -activation in subactivating Ca\textsuperscript{2+} in the absence and presence of 1 mM Mg\textsuperscript{2+}.** Mean values of 21 channels (from Fig. 4.2) before and after 4,4'-DTDP -activation in the absence of Mg\textsuperscript{2+} (hatched bars) are compared to the mean values of 5 channels (from Fig. 5.8A) before and after 4,4'-DTDP -activation in the presence of Mg\textsuperscript{2+} (filled bars). Asterisks indicate significant differences between mean values in the absence and presence of Mg\textsuperscript{2+} for: a) the two sets of control data and b) the maximum activation induced by 4,4'-DTDP addition: ** P < 0.01; *** P < 0.001.
Tab. 5.1 Effects of 4,4'-DTDP on $P_o$, $T_o$ and $F_o$ of the $Mg^{2+}$-inhibited RyR in subactivating and activating concentrations of $Ca^{2+}$.

<table>
<thead>
<tr>
<th></th>
<th>$10^{-7}$ M $Ca^{2+}$ + 1 mM $Mg^{2+}$ (n=5)</th>
<th>$10^{-3}$ M $Ca^{2+}$ + 10 mM $Mg^{2+}$ (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$10^{-7}$ M $Ca^{2+}$</td>
<td>+ 1 mM $Mg^{2+}$</td>
</tr>
<tr>
<td>$P_o$</td>
<td>.0008 ± .0004</td>
<td>.00001 ± .000007***</td>
</tr>
<tr>
<td>$T_o$</td>
<td>1.19 ± 0.12</td>
<td>0.51 ± 0.02***</td>
</tr>
<tr>
<td>$F_o$</td>
<td>0.59 ± 0.30</td>
<td>0.02 ± 0.01***</td>
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Mean ± SEM for $n$ channels. Asterisks indicate values significantly different from the value in the preceding column: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. 
Oxidation of ligand modified RyRs

two after adding 10 mM Mg$^{2+}$ (Fig. 5.10Aii) reflected a significant decrease in $T_0$. The mean open time for each of seven Mg$^{2+}$-inhibited channels was reduced to an average value of $0.77 \pm 0.04$ ms (Fig. 5.11A, Tab. 5.1). The corresponding decrease in $P_o$ was not significant, primarily due to a non-significant increase in $F_0$ (see section 3.4.2b).

The subsequent addition of 1 mM 4,4'-DTDP to the Mg$^{2+}$-inhibited channels produced significant increases in both $P_o$ and $T_o$ with no significant effect on $F_0$ (Fig. 5.11A & Tab. 5.1). The changes in channel activity described here can be seen in the channel recordings of a single experiment (Fig. 5.11B). The 4,4'-DTDP -induced increases in $P_o$ and $T_o$ in the presence of Mg$^{2+}$ were significantly less than the average values when 4,4'-DTDP was added to the 1 mM Ca$^{2+}$-activated channels in the absence of Mg$^{2+}$ (Tab. 5.2). Interestingly, the values of $P_o$, $T_o$ and $F_0$ were similar for the channels oxidised by 4,4'-DTDP whether they were inhibited by 10 mM Mg$^{2+}$ ($n = 6$) or 20 mM Ca$^{2+}$ ($n = 5$) (Tab. 5.2). One channel included in Figure 5.11A and Table 5.1 was not included in the average data (Tab. 5.2) because its response to 4,4'-DTDP was well outside the values of the other Mg$^{2+}$-inhibited channels: $P_o$ increased to ~51 and $T_o$ increased to ~600 ms.

The open times for the Mg$^{2+}$-inhibited channels activated by 4,4'-DTDP had exponential components in each of the five groups with average time constants: $\tau_1 = 1.5 \pm 0.1$ ms ($n = 7$), $\tau_2 = 6.8 \pm 0.9$ ms ($n = 7$), $\tau_3 = 36.0 \pm 5.6$ ms ($n = 6$), $\tau_4 = 240$ ms ($n = 1$) and $\tau_5 = 745 \pm 85$ ms ($n = 2$). The majority of events were in the shortest two time constant groups with only ~15% of events in $\tau_3 - \tau_5$. The increased number of time constants was comparable with the four, after 4,4'-DTDP, in 1 mM Ca$^{2+}$ and zero Mg$^{2+}$ ($\tau_1$ and $\tau_3 - \tau_5$; see section 5.2.4a). However, in these experiments i.e. zero Mg$^{2+}$, there was a much greater percentage of events (~64%) in the longer time constants ($\tau_3 - \tau_5$) compared to the ~15% in the presence of Mg$^{2+}$. Thus, while the longest kinetic components were activated by 4,4'-DTDP in the presence of Mg$^{2+}$, the frequency of events in the long components was limited by Mg$^{2+}$.

5.5.2 4,4'-DTDP potentiation of the ATP-activated RyR

ATP activates the cardiac RyR in $10^{-7}$ M Ca$^{2+}$ by increasing both $T_o$ and $F_o$ (see section 3.4.2c). However, ATP apparently inhibits Ca$^{2+}$ release induced by RDSs from skeletal and cardiac SR vesicles (Zaidi et al., 1989; Prabhu and Salama, 1990b). Because of the possible antagonistic actions of ATP and the RDSs on SR Ca$^{2+}$ efflux, 4,4'-DTDP was added to the ATP-activated RyR to examine the combined effects of the two reagents at the single channel level. The experiments were carried out in the usual control solutions with $10^{-7}$ M [Ca$^{2+}$]$_{cis}$. After a 2 min control period 4 mM ATP was added to the $cis$ chamber and 1 mM 4,4'-DTDP was added 1 - 2 min later. 4,4'-DTDP significantly increased $P_o$ of the ATP
**Fig. 5.10** 10 mM Mg\(^{2+}\) or Ca\(^{2+}\) causes channel inhibition with similar distributions of open times. Channels were recorded in 250/50 mM CsCl, 1 mM CaCl\(_2\). i) 20 s records when the channel was active after additions to the *cis* chamber of 10 mM Mg\(^{2+}\) (A) or 9 mM Ca\(^{2+}\) (B). Aii) Open time constants were obtained for seven channels in 1 mM Ca\(^{2+}\) before and after adding 10 mM Mg\(^{2+}\). The average time constants plotted (Aii) had the following values: control - \(\tau_1 = 1.8 \pm 0.4\) ms (\(n = 6\)), \(\tau_2 = 5.5 \pm 1.6\) ms (\(n = 6\)), \(\tau_3 = 18.9 \pm 1.0\) ms (\(n = 2\)) and \(\tau_4 = 159 \pm 97\) ms (\(n = 2\)) and Mg\(^{2+}\) - \(\tau_1 = 0.66 \pm 0.03\) ms (\(n = 7\)) and \(\tau_2 = 2.7 \pm 0.3\) ms (\(n = 6\)). Bii - Open time constants were obtained for three channels in 10 mM Ca\(^{2+}\). The average values plotted (Bii) had the following time constants: \(\tau_1 = 0.81 \pm 0.17\) ms (\(n = 3\)) and \(\tau_2 = 2.9 \pm 0.7\) ms (\(n = 3\)). Most channel openings of the Ca\(^{2+}\) or Mg\(^{2+}\)-inhibited channels were very brief. To accurately represent the two components, that were evident after obtaining time constants from the distribution of open times for individual channels, all time constants of \(<2\) ms were included in \(\tau_1\) (usually \(<3\) ms) and all time constants between 2 - 12 ms were in \(\tau_2\) (usually 3 - 12 ms). The control data shown in Bii (o) are the same as the control values in Aii.
Fig. 5.11 4,4'-DTDP-induced activation of the Ca\textsuperscript{2+}-activated RyR inhibited by 10 mM Mg\textsuperscript{2+}. A - Channel activity of seven RyRs in 1 mM Ca\textsuperscript{2+} was recorded for 2 min before 10 mM Mg\textsuperscript{2+} was added to the cis chamber followed 2 min later by 1 mM 4,4'-DTDP. Data for control and Mg\textsuperscript{2+} were obtained over a 2 min period; data for 4,4'-DTDP were obtained during the 30 s of highest P\textsubscript{o} within 2 min of drug addition. Values of P\textsubscript{o}, T\textsubscript{o} and F\textsubscript{o} for individual channels (left) and mean (± SEM) data (right) are shown. Changes in mean values after Mg\textsuperscript{2+} and 4,4'-DTDP additions were tested for significance: * P < 0.05; ** P < 0.01.

B - Channel traces from one experiment show periods of high activity during activation by 1 mM Ca\textsuperscript{2+} (i), during inhibition by 10 mM Mg\textsuperscript{2+} (ii) and after activation by 1 mM 4,4'-DTDP (iii).
Tab. 5.2 Comparison of maximum RyR activity after 4,4’-DTDP was added to Ca\(^{2+}\) -activated, Ca\(^{2+}\) -inhibited or Mg\(^{2+}\) -inhibited channels.

<table>
<thead>
<tr>
<th></th>
<th>1 mM 4,4’-DTDP +</th>
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<tr>
<td></td>
<td>1 mM Ca(^{2+}) (n=4)</td>
</tr>
<tr>
<td>Po</td>
<td>.9939 ± .0017</td>
</tr>
<tr>
<td>To</td>
<td>218 ± 39</td>
</tr>
<tr>
<td>Fo</td>
<td>4.9 ± 1.0</td>
</tr>
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</table>

Mean ± SEM for \(n\) channels. Asterisks indicate significant differences between the means of channels in the presence of 1 mM Ca\(^{2+}\) and those in 20 mM Ca\(^{2+}\) or 10 mM Mg\(^{2+}\): ** \(P < 0.01\); *** \(P < 0.001\). There were no differences in mean values of Po, To or Fo for 4,4’-DTDP -activated channels inhibited by either 20 mM Ca\(^{2+}\) or 10 mM Mg\(^{2+}\).
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-activated RyR in 9 of 12 channels from $0.0153 \pm 0.0039$ to $0.0742 \pm 0.0270$ (P < 0.05; n = 9) (Fig. 5.12). The increase in $P_o$, in 7 of 9 channels, was due to an increase in $T_o$ (9 of 9 channels) from $3.1 \pm 0.5$ to $21.7 \pm 6.7$ ms (P < 0.0001). The decrease in $F_o$ from $4.8 \pm 0.6$ to $3.5 \pm 1.1 s^{-1}$ (n = 9) was not significant. Interestingly, the values of $P_o$, $T_o$ and $F_o$ obtained after 4,4'-DTDP addition were the same in the presence (n = 9) or absence (n = 21) of ATP (P > 0.49) (Fig. 5.12). In the three channels not activated by the addition of 4,4'-DTDP, the ATP-induced activation was depressed (i.e. channel activity returned to low levels) and the usual 4,4'-DTDP-induced bursts of long openings did not occur.

The 4,4'-DTDP-induced increase in $P_o$ of the ATP-activated RyR was due to an increase in $T_o$, $F_o$ was unchanged. The records from a single experiment, displaying the effects on channel activity after ATP and 4,4'-DTDP addition, clearly show the 4,4'-DTDP-induced change in $T_o$ (Fig. 5.13A). The exponential components in the distribution of open times were obtained (Fig. 5.13B). The top graph shows that the control open times ($10^{-7} M Ca^{2+}$) fell into the shortest time constant group with an average time constant of $\tau_1 = 1.6 \pm 0.2$ ms. After adding ATP, the average time constants fell into two groups ($\tau_1$ and $\tau_2$). However, the open times of each of the nine channels were still fitted by a single exponential component: $\tau_1 = 2.2 \pm 0.3$ ms (n = 3) or $\tau_2 = 4.5 \pm 0.8$ ms (n = 6). The addition of 4,4'-DTDP added a further two components to the distribution: $\tau_1 = 1.9 \pm 1.0$ ms (n = 2), $\tau_2 = 8.6 \pm 1.0$ ms (n = 5), $\tau_3 = 26.6 \pm 6.5$ ms (n = 3) and $\tau_4 = 76.8 \pm 16.2$ ms (n = 3). As for $P_o$, $T_o$ and $F_o$, the average time constants after 4,4'-DTDP-activation, either in the presence or absence of ATP, were almost identical. The four time constants fitted to the open time distributions after 1 mM 4,4'-DTDP alone (n = 21) (Fig. 5.13B - bottom) were: $\tau_1 = 1.3 \pm 0.3$ ms (n = 14), $\tau_2 = 8.4 \pm 0.9$ ms (n = 9), $\tau_3 = 24.0 \pm 2.9$ ms (n = 14) and $\tau_4 = 74.7 \pm 11.4$ ms (n = 7). Similarly, there were no differences in the average closed time constants after 4,4'-DTDP-activation in the absence or the presence of ATP (data not shown).

While most aspects of RyR activation by 4,4'-DTDP were unaffected by ATP, two elements of the activation phase were changed by the presence of ATP. Firstly, the percentage of open events in $\tau_1$ after ATP/4,4'-DTDP-activation was $5.5 \pm 4.4\%$ (n = 9). This was significantly less (P < 0.01) than the $28.3 \pm 6.3\%$ of events in $\tau_1$ for the 21 channels activated by 4,4'-DTDP alone (Fig 5.13B). Secondly, the rate of activation by 4,4'-DTDP in the presence of ATP was $2.9 \pm 1.4$ s (n = 9). The rate of activation was significantly faster than the delay of $42.1 \pm 12.3$ s (n = 21) (P < 0.01) after 4,4'-DTDP was added to channels in the absence of ATP. These two changes in the activation phase of the oxidised RyR that were brought about by the presence of ATP, were also seen in the 4,4'-DTDP-induced activation of the $Ca^{2+}$-activated RyR. After the $[Ca^{2+}]_{e\in}$ was raised from subactivating to activating concentrations, there were reductions in both the delay
Fig. 5.12 Effects of 4,4'-DTDP on channel activity of the ATP -activated RyR. Channels were recorded in 10^{-7} M Ca^{2+}. ATP (4 mM) was added to the cis chamber (n = 9) to activate the channel before 1 mM 4,4'-DTDP was added. Left - Values of P_o, T_o and F_o for individual channels for control (2 min), ATP (1-2 min) and 4,4'-DTDP (30 s). Right - Filled bars show the mean ± SEM from the data of the single channels (left). Asterisks - significance of difference between each mean and the mean in the preceding bin: * P < 0.05; *** P < 0.001. Hatched bar shows the mean values (± SEM) of P_o, T_o and F_o for 21 channels (from Fig. 4.2) activated by 1 mM 4,4'-DTDP in the absence of ATP. ATP did not significantly change the mean values of P_o, T_o or F_o after RyR-activation by 4,4'-DTDP (P > 0.49; see text).
Addition of 4,4'-DTDP to ATP-activated RyRs: effects on single channel activity and average open time constants. RyRs were recorded in 10^{-7} M Ca^{2+}. A - Single channel records from one experiment. (i) Control. (ii) After addition of 4 mM ATP (cis). (iii) After addition of 1 mM 4,4'-DTDP. B - Top - Average open time constants were obtained from seven RyRs (data from channels shown in Fig. 5.12) activated by ATP and 4,4'-DTDP. Bottom - Average open time constants from analysis of 21 RyRs (data from Fig. 4.3B) activated by 4,4'-DTDP in the absence of ATP.
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before 4,4'-DTDP -induced activation and the percentage of events in the shortest time constant (see sections 5.2.3 and 5.2.4a).

In summary, the effects of ATP and Ca\(^{2+}\) on the 4,4'-DTDP -activated channel were similar in that they both increased the rate of activation of the channel by 4,4'-DTDP, and they both reduced the percentage of events in the shortest open time constant component. However, there were differences in the effects of the two ligands on the 4,4'-DTDP -induced activation of the RyR. First, 4,4'-DTDP added to Ca\(^{2+}\) -activated channels (10\(^{-2}\) and 10\(^{-3}\) M, n = 9) caused a significantly greater increase in P\(_o\) and T\(_o\) than the increase that occurred when 4,4'-DTDP was added to the channels in 10\(^{-7}\) M Ca\(^{2+}\) (n = 21) (P < 0.001). In contrast, ATP addition did not alter the degree of activation by 4,4'-DTDP. Second, after 4,4'-DTDP -activation, events in the longest time constant component (\(\tau\)) were seen in activating concentrations of Ca\(^{2+}\), but not at subactivating concentrations of Ca\(^{2+}\) and not in the presence of ATP. Because both ATP and Ca\(^{2+}\) increase P\(_o\), it is likely to be P\(_o\), rather than either of the ligands, that influences the rate of activation by 4,4'-DTDP. On the other hand, Ca\(^{2+}\) per se, or the high P\(_o\) induced by Ca\(^{2+}\), appears to be an important factor(s) in determining the degree of 4,4'-DTDP -induced activation and the long open times. The generality of these conclusions was tested by activating the channel with caffeine, which increased P\(_o\) to a similar level to that seen with ATP -activation (see section 3.4.2c), before adding 4,4'-DTDP.

5.5.3 4,4'-DTDP actions of the caffeine -activated RyR

Caffeine (2 mM) was added to the channel in 10\(^{-7}\) M Ca\(^{2+}\). 2 min before the addition of 1 mM 4,4'-DTDP. An example of RyR activation by caffeine, and the increase in the caffeine -activated RyR by 4,4'-DTDP is shown in Figure 5.14A. Caffeine significantly increased P\(_o\) of the RyR in 10\(^{-7}\) M Ca\(^{2+}\) to 0.032 ± 0.01 by 3 -fold increases in both T\(_o\) and F\(_o\) (see section 3.4.2c). There was a further increase in P\(_o\) to 0.15 ± 0.06 (n = 6, P < 0.01) after 1 mM 4,4'-DTDP was added. This increase in P\(_o\) occurred because of a 20 -fold increase in T\(_o\) from 6.3 ± 0.9 ms in the caffeine -activated RyR to 134 ± 49 ms (n = 6, P < 0.001) after 4,4'-DTDP addition, despite a decrease in F\(_o\) from 4.6 ± 1.6 to 1.7 ± 1.1 s\(^{-1}\) (n = 6, P < 0.05) (Fig. 5.14B). Analysis of previous results, showing RyRs in the presence of ATP and activating concentrations of Ca\(^{2+}\), suggested that when the channel is in an activating environment, the rate of activation by 4,4'-DTDP is faster than that seen when the channel is in an inactive state (i.e. 10\(^{-7}\) M Ca\(^{2+}\)). Surprisingly, the 46.5 ± 21.2 s (n = 6) delay before the channel was activated by 4,4'-DTDP in RyRs activated by caffeine, was no different to the 42.0 ± 12.3 s (n = 21) delay seen when 4,4'-DTDP was added to channels in the absence of caffeine (P = 0.86). This suggests that an elevated P\(_o\) alone is not sufficient to increase the rate of 4,4'-DTDP -induced activation.
Fig. 5.14  **Effects of 4,4'-DTDP on channel activity of the caffeine-activated RyR.**  
A - Activity from an individual channel in 10^-7 M Ca^{2+} before (i), and after consecutive additions to the *cis* chamber of 2 mM caffeine (ii) and 1 mM 4,4'-DTDP (iii).  
B - Values of P_o, T_o and F_o from single channels (n = 6) recorded in 10^-7 M Ca^{2+} with additions to the *cis* chamber of caffeine and 4,4'-DTDP (*top*).  
*Bottom* - Filled bars show the mean ± SEM from the data of the single channels. Asterisks - significance of difference between each mean and the mean in the preceding bin: *P < 0.05; **P < 0.01; ***P < 0.001. The hatched bar shows mean ± SEM of P_o, T_o and F_o for 21 channels (from Fig. 4.2) in 10^-7 M Ca^{2+} with 1 mM 4,4'-DTDP in the absence of caffeine. There were no significant differences in the mean values of P_o, T_o or F_o after 4,4'-DTDP-induced activation of channels in the presence or absence of caffeine (P > 0.05; see text).  
C - Average open time constants obtained from RyRs activated by caffeine and 4,4'-DTDP.
While the properties of channel activity of the RyR activated by 4,4'-DTDP alone or by ATP/4,4'-DTDP share many similarities, channel activation by caffeine appeared to alter the subsequent activation by 4,4'-DTDP. Table 5.3 lists the values of $P_o$, $T_o$ and $F_o$ for the 21 channels activated by 4,4'-DTDP (in $10^{-7}$ M Ca$^{2+}$) and compares them with activation by ATP/4,4'-DTDP ($n = 9$) and caffeine/4,4'-DTDP ($n = 6$). The main points to note are the ~2-fold difference in $P_o$ and the ~5-fold difference in $T_o$ after the activation by 4,4'-DTDP in the presence of caffeine compared to the effects of 4,4'-DTDP in the absence of caffeine. Although the differences were not statistically significant, similar trends were seen in all channels (see Fig. 5.14). The long $T_o$ induced by 4,4'-DTDP in the caffeine -activated RyR was approaching the values seen after 4,4'-DTDP addition to the Ca$^{2+}$ -activated RyRs ($T_o = 218 \pm 39$ ms in $10^{-3}$ M Ca$^{2+}$ and $249 \pm 54$ ms in $10^{-5}$ M Ca$^{2+}$). This shows that the very long open times are not necessarily uniquely associated with the Ca$^{2+}$ -activation mechanism or with Ca$^{2+}$ per se.

The distribution of channel open times when the RyR was activated by caffeine alone and caffeine/4,4'-DTDP were measured. Figure 5.14C shows that the control distribution was fitted by a single exponential: $\tau_1 = 1.9 \pm 0.3$ ms ($n = 6$). Caffeine introduced an additional two components into the distribution, with the average time constants: $\tau_1 = 1.4 \pm 0.5$ ms ($n = 4$), $\tau_2 = 7.4 \pm 1.5$ ms ($n = 5$) and $\tau_3 = 18.7 \pm 6.5$ ms ($n = 3$). Following the addition of 4,4'-DTDP, all openings fell into two groups: $\tau_3 = 29.0 \pm 9.1$ ms ($n = 4$) and $\tau_4 = 227 \pm 74.5$ ms ($n = 5$). No events fell into the two shortest groups i.e. < 12 ms. This was not seen under any other conditions examined, suggesting a unique influence of caffeine on channel activation by SH oxidation. The effects of oxidation on the kinetics of the RyR appear to be largely influenced by the nature of the activating ligand, not simply the $P_o$ of the channel.

### 5.5.4 Influence of pH on RyR-activation by 4,4'-DTDP

Intracellular pH is generally stable due to the exceptional buffering capabilities within striated muscle, which maintains the pH between 7.2 - 7.4 (see section 1.4.2d). However, under extreme conditions, such as fatigue, ischaemia or during reperfusion of the ischaemic heart, the intracellular pH can fall to <6.5 (Dennis et al., 1991; Favero et al., 1995b). These are also conditions whereby the cell may be exposed to oxidants (Kaneko et al., 1994). A reduction in pH from 7.4 to 6.6 caused significant decreases in $P_o$ in skeletal and cardiac RyRs (Rousseau and Pinkos, 1990). Consequently, it was considered important to determine whether oxidising agents could activate the RyR when it was inhibited by low pH. To test this, the pH in the cis chamber was reduced to 6.5 (by perfusion with a low pH solution), in the presence of an activating [Ca$^{2+}$]$_{cis}$ ($10^{-3}$ M), before adding 1 mM 4,4'-DTDP. The buffer used in all previous experiments (TES) could not be used at pH 6.5 because, with a pK$_a$ of 7.4, its effective range (~6.8 - 8.2) did not
Tab. 5.3 Values of $P_o$, $T_o$ and $F_o$ for channels activated by 4,4'-DTDP alone compared to the values of channels activated by ATP + 4,4'-DTDP and caffeine + 4,4'-DTDP.

<table>
<thead>
<tr>
<th></th>
<th>4,4'-DTDP alone (n=21)</th>
<th>ATP + 4,4'-DTDP (n=9)</th>
<th>Caff + 4,4'-DTDP (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean value</td>
<td>Mean value</td>
<td>Mean value</td>
</tr>
<tr>
<td>$P_o$</td>
<td>.0654 ± .0129</td>
<td>.0742 ± .0270</td>
<td>.1454 ± .0597</td>
</tr>
<tr>
<td>$T_o$</td>
<td>22.18 ± 5.08</td>
<td>21.65 ± 6.74</td>
<td>133.7 ± 49.3</td>
</tr>
<tr>
<td>$F_o$</td>
<td>4.63 ± 1.17</td>
<td>3.54 ± 1.14</td>
<td>1.69 ± 1.09</td>
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</table>

Mean ± SEM for $n$ channels. cis [Ca$^{2+}$], 10$^{-7}$ M. P values: significance of the difference between the means of channels activated by 4,4'-DTDP alone with channels activated by a) ATP + 4,4'-DTDP and b) caffeine + 4,4'-DTDP.
include the required pH. Hence, 10 mM MES (with a pK\textsubscript{a} of 6.1) was used to buffer the solutions to pH 6.5. MES (≤60 mM) had no effect on the binding of \([^3H]ryanodine\) to the skeletal RyR in activating concentrations of Ca\textsuperscript{2+} (pH 7.2) (Meissner et al., 1997). To ensure that MES had no effect on the activity of the cardiac RyR, 10 mM MES was added to channels in 10\textsuperscript{-3} M Ca\textsuperscript{2+} (pH 7.4). The values of P\textsubscript{o}, T\textsubscript{o} and F\textsubscript{o} were measured during a 2 min control period and compared to those during the 2 min period immediately after MES addition: there was no significant difference in P\textsubscript{o}, T\textsubscript{o} or F\textsubscript{o} after the addition of MES (P > 0.05, n = 5).

In the initial experiments, lowering the pH to 6.5 totally inhibited all RyR activity within ~10 s to 2 min (n = 4). The addition 1 mM 4,4'-DTDP to the cis chamber, between 40 s to 3 min after the last activity had been observed, failed to reactivate any of the four channels (Fig. 5.15Ai). This was unlikely to be due to the loss of effectiveness of 4,4'-DTDP in the more acidic environment since 2,2'-DTDP was found to be effective over the pH range of 3.4 - 8.1 (Grassetti and Murray, Jr. 1967). Because of this failure by 4,4'-DTDP to activate the RyR in a low pH environment, the ability of the channel to withstand a low pH was investigated by testing whether channel activity could be restored after 6 min in pH 6.5. The complete loss of channel activity that was induced by reducing the pH to 6.5 could not be reversed upon perfusion of control solutions (pH 7.4; n = 4). This is contrary to previous reports in which RyR activity was restored by returning pH to physiological levels (Rousseau and Pinkos, 1990; Ma and Zhao, 1994). The reason for this discrepancy was not explored further. Because these channels appeared to be very sensitive to changes in pH, 4,4'-DTDP was added soon after the pH was reduced. 4,4'-DTDP was added to the cis chamber, after the pH had been lowered to 6.5, either while channel activity was still observed or within 20 s of observing the last events. Because 4,4'-DTDP was added so soon after the perfusion of low pH solutions (15.4 ± 4.8 s in 5 of 6 channels), the control values in low pH were measured during the 10 s period containing channel activity with the highest P\textsubscript{o}. Low pH rapidly inhibited channel activity: the average values of six channels with pH 6.5 for P\textsubscript{o} (0.04 ± 0.02), T\textsubscript{o} (2.2 ± 0.9 ms) and F\textsubscript{o} (16.3 ± 7.0 s\textsuperscript{-1}) were significantly lower (P < 0.01) than the values measured from 36 RyRs during control conditions in pH 7.4 and 1 mM Ca\textsuperscript{2+} (see Fig. 3.3).

The addition of 4,4'-DTDP to RyRs at pH 6.5 affected channel activity (Fig. 5.15Ai) in 6 of 8 experiments after a delay of 13.4 ± 7.2 s (n = 6). While the average P\textsubscript{o} after 4,4'-DTDP (recorded over a 30 s period during maximum 4,4'-DTDP -induced activation) was not significantly greater than control (pH 6.5), P\textsubscript{o} increased from 0.04 ± 0.02 to 0.18 ± 0.06 (P = 0.09, n = 6) (Fig. 5.15B). This 4,4'-DTDP -induced change in P\textsubscript{o} was due a significant increase in T\textsubscript{o} from 2.2 ± 0.9 to 39.7 ± 12.1 ms (P < 0.01, n = 6) even though there was a decrease in F\textsubscript{o} from 16.3 ± 7.0 to 6.3 ± 2.8 s\textsuperscript{-1} (which was not significant). The values of P\textsubscript{o} and T\textsubscript{o} after 4,4'-DTDP -activation in pH 6.5 were significantly less
Fig. 5.15 4,4′-DTDP -activation of the RyR inhibited by low pH. Channels were recorded in 250/50 mM CsCl, 1 mM Ca^{2+}, and trans pH 7.4. Cis pH was lowered to 6.5 after channel incorporation (see text). A - Records of channel activity in the absence (left) and presence (right) of 1 mM 4,4′-DTDP. B - Values of P_o and T_o for individual channels (n = 6) and mean ± SEM are shown for control activity in pH 6.5 (10 s) and after 4,4′-DTDP addition (30 s). Asterisks - significance of difference between means: ** P < 0.01. C - Average open time constants obtained from six RyRs in pH 6.5 during the 10 s period of maximum activity before (○) and the 5 min period after (●) 4,4′-DTDP addition.
than the corresponding values after 4,4'-DTDP -activation in pH 7.4 (P_o = 0.99 ± 0.002 and T_o = 218 ± 39 ms; n = 4) (P < 0.01). While the interpretation of these differences may be complicated by the fact that P_o was initially higher for the channels in pH 7.4 (see Fig. 5.2), the 4,4'-DTDP -induced increase in T_o of >50 -fold in pH 7.4 compared to the -18 -fold increase in pH 6.5 suggests that the low pH suppresses the effects of 4,4'-DTDP on channel activity.

The distribution of open times, for the six channels inhibited by pH 6.5, was fitted by two exponential components with average time constants: τ_1 = 1.3 ± 0.4 ms (n = 5) and τ_2 = 6.1 ± 1.6 ms (n = 2). Addition of 4,4'-DTDP added a further two components to this distribution with the shortest two remaining essentially unchanged: τ_1 = 1.2 ± 0.3 ms (n = 4), τ_2 = 6.8 ± 2.0 ms (n = 5), τ_3 = 25.6 ± 11.5 ms (n = 3) and τ_4 = 187 ± 42 ms (n = 6). The 4,4'-DTDP -induced increase in the number of time constants at pH 6.5 is similar to the 4 - 5 time constants required to fit the data in the Ca^{2+} -activated channels after 4,4'-DTDP addition in pH 7.4 (see Fig. 5.3). However, unlike the channels activated by 10^{-5} or 10^{-3} M Ca^{2+} in pH 7.4, there were no events in τ_5 for the channels inhibited by low pH. The low pH appears to have inhibited the ability of 4,4'-DTDP to activate the longest kinetic component (τ_5).

5.5.5 4,4'-DTDP -induced loss of RyR activity

The presence of the physiological and pharmacological reagents did not alter the ability of 4,4'-DTDP to cause the usual irreversible loss of channel activity. This supports the previous evidence (see section 4.6) that the activation induced by 4,4'-DTDP and the subsequent loss of channel activity are induced by independent mechanisms. In the presence of Mg^{2+}, ATP, caffeine and a low pH environment, the activation of the RyR was transient. This was followed by long closures with the subsequent irreversible loss of channel activity in most channels within 10 min. Of the channels that were assessed for loss of activity after the addition of 1 mM 4,4'-DTDP, 7 of 8 could not be activated in the presence of Mg^{2+}, 7 of 8 in the presence of ATP and all of those in caffeine (n = 6) and the low pH (n = 5). The loss of channel activity in low pH cannot necessarily be attributed to the RDS since it may have been due to the acidic environment. The two channels in which activity was not lost in the presence of millimolar 4,4'-DTDP were both activated by the RDS, and retained channel activity for >45 min (in 10 mM Mg^{2+}) and >20 min (in ATP) after RDS addition. The fact that these two channels did not lose activity after RDS addition suggests that the loss of channel activity, usually seen within 10 min of the addition of 1 mM 4,4'-DTDP, is not inseparably associated with oxidation -induced RyR activation. Further evidence supporting the concept that oxidation -induced activation and the subsequent loss of channel activity are due to separate processes (Ondrias et al., 1990), came from the experiments in which the RyR was ATP activated. Three of twelve RyRs in the presence of ATP were not activated by the addition of 1 mM 4,4'-DTDP.
However, all activity was lost in each of the three channels within the usual 5 - 10 min after the addition of the RDS.

The results have shown that the oxidation of the RYR by 4,4'-DTDP defines over a wide range of free Ca\(^{2+}\) concentrations. However, the [Ca\(^{2+}\)]\(_{c}\) effects various aspects of channel activity that follows exposure of the RYR to the SH oxidizing agent. 4,4'-DTDP can activate channels inhibited by Mg\(^{2+}\) by a fine part as well as increase the activity of channels already activated by ATP- or caffeine. Physiological concentrations of Ca\(^{2+}\) and Mg\(^{2+}\) modulate the effects of 4,4'-DTDP on RyR activity. In contrast, ATP exerts little influence on channel activity of the RYR activated by 4,4'-DTDP. None of the physiological or pharmacological responses had any substantial effect on the ability of millimolar concentrations of 4,4'-DTDP to induce a loss of channel activity within 5 - 10 min of addition.

5.6 Cytoplasmatic Ca\(^{2+}\) influences activity of the oxidised RyR

In the previous chapter the effects of 4,4'-DTDP on RyR activity were described primarily in submicromolar Ca\(^{2+}\). The [Ca\(^{2+}\)]\(_{c}\) was increased to 10^(-7) M in a series of experiments to assess the concentration dependence of the RYR, but other variables between Ca\(^{2+}\) and the reagent were not investigated. In the present chapter, the relationship between Ca\(^{2+}\) and 4,4'-DTDP was described in experiments using millimolar or millimolar concentrations of 4,4'-DTDP in concentrations of Ca\(^{2+}\) (apo) from 10^{-7} M to 2 x 10^{-5} M. The data were analysed to assess the influence of Ca\(^{2+}\) on the activation of the RYR by 4,4'-DTDP, and to find out if the oxidation of the RYR activated the channel by increasing the sensitivity to Ca\(^{2+}\). An increase in receptor sensitivity to its primary activating ligand was shown to be the mechanism by which transient activation of the Ca\(^{2+}\)-dependent m-calpain (Prater et al., 1993; Kaptin et al., 1994) has also been suggested as the mechanism by which H\(_2\)O\(_2\) activates the skeletal RyR (Faveri et al., 1994).

The oxidation of the RYR by 4,4'-DTDP increased the open probability of almost the proportion of channel activity at each (Ca\(^{2+}\)) level. Channel activation by 4,4'-DTDP occurred in the absence of Ca\(^{2+}\) (10^{-7} M), and in the presence of inhibitory concentrations of Ca\(^{2+}\) (2 x 10^{-5} M). These findings support the preliminary results (section 4.2.2b) which suggested that RYRs do not increase the sensitivity of the channel to Ca\(^{2+}\). Another study which investigated oxidation of the cardiac RyR by H\(_2\)O\(_2\) also showed that the channel could be activated in the absence of Ca\(^{2+}\) (Kreutz and Williams, 1994), but no studies have previously shown oxidation-induced activation of the Ca\(^{2+}\) activated RyR. Additional evidence that the effects of cardiac RyR activation by RYRs are not due to an increase in RyR Ca\(^{2+}\) sensitivity is provided by the studies of 4,4'-DTDP as it may also activate the RyR (P. -- G 10^{-7} M Ca\(^{2+}\), Ca\(^{2+}\) alone is unable to fully
DISCUSSION

The results have shown that the activation of the RyR by 4,4'-DTDP occurs over a wide range of cis Ca\(^{2+}\) concentrations. However, the [Ca\(^{2+}\)]\(_{cis}\) affects various aspects of channel activity that follows exposure of the RyR to the SH-specific oxidising agent. 4,4'-DTDP can activate channels inhibited by Mg\(^{2+}\) or a low pH as well as increase the activity of channels already activated by ATP or caffeine. Physiological concentrations of Ca\(^{2+}\) and Mg\(^{2+}\) modulate the effects of 4,4'-DTDP on RyR activity. In contrast, ATP exerts little influence on channel activity of the RyR activated by 4,4'-DTDP. None of the physiological or pharmacological reagents had any substantial effect on the ability of millimolar concentrations of 4,4'-DTDP to induce a loss of channel activity within 5 - 10 min of addition.

5.6 Cytoplasmic Ca\(^{2+}\) influences activity of the oxidised RyR

In the previous chapter the effects of 4,4'-DTDP on RyR activity were described primarily in subactivating Ca\(^{2+}\). The [Ca\(^{2+}\)]\(_{cis}\) was increased to 10\(^{-5}\) M in a series of experiments to assess the concentration-dependence of the RDS, but other interactions between Ca\(^{2+}\) and the reagent were not investigated. In the present chapter, the relationship between Ca\(^{2+}\) and 4,4'-DTDP was described for experiments using nanomolar to millimolar concentrations of 4,4'-DTDP in concentrations of Ca\(^{2+}\) (cis) from 10\(^{-9}\) M to 2 x 10\(^{-2}\) M. The data were analysed to assess the influence of Ca\(^{2+}\) on the activation of the RyR by 4,4'-DTDP, and to find out if the oxidation of the RyR activated the channel by increasing its sensitivity to Ca\(^{2+}\). An increase in receptor sensitivity to its primary activating ligand was shown to be the mechanism by which thimerosal activated the IP\(_3\) receptor (Sayers et al., 1993; Kaplin et al., 1994), and has also been suggested as the mechanism by which H\(_2\)O\(_2\) activates the skeletal RyR (Favero et al., 1995a).

The oxidation of the RyR by 4,4'-DTDP increased the open probability and altered the properties of channel activity at each [Ca\(^{2+}\)] tested. Channel activation by 4,4'-DTDP occurred in the absence of Ca\(^{2+}\) (10\(^{-9}\) M), and in the presence of inhibitory concentrations of Ca\(^{2+}\) (2 x 10\(^{-2}\) M). These findings support the preliminary results (section 4.2.2b) which suggested that RDSs do not increase the sensitivity of the channel to Ca\(^{2+}\). Another study which investigated oxidation of the cardiac RyR by H\(_2\)O\(_2\) also showed that the channel could be activated in the absence of Ca\(^{2+}\) (Boraso and Williams, 1994), but no studies have previously shown oxidation-induced activation of the Ca\(^{2+}\)-inhibited RyR. Additional evidence that the effects of cardiac RyR oxidation by RDSs are not due to an increase in RyR Ca\(^{2+}\) sensitivity includes, firstly, the ability of 4,4'-DTDP to maximally activate the RyR (P \(_{o} - 1\)) in 10\(^{-3}\) M Ca\(^{2+}\). Ca\(^{2+}\) alone is unable to fully
activate the channel (Williams, 1992). Therefore, sensitising the channel to Ca\(^{2+}\) is unlikely to increase P\(_o\) to the level seen following 4,4'-DTDP -activation. Secondly, the activation of the channel by 4,4'-DTDP in activating concentrations of Ca\(^{2+}\) was achieved by increases in T\(_o\) while F\(_o\) fell. In contrast, Ca\(^{2+}\) -activation has been characterised by increases in both T\(_o\) and F\(_o\) (see Fig. 3.3). Thirdly, 4,4'-DTDP activated the RyR when the cis [Mg\(^{2+}\)] (10\(^{-3}\) M) was 10\(^4\) times greater than the [Ca\(^{2+}\)] (10\(^{-7}\) M). Under these conditions, Mg\(^{2+}\) is competitively binding to the Ca\(^{2+}\) -activation site so changing the Ca\(^{2+}\) -sensitivity of the channel would be unlikely to affect channel activity. Because 4,4'-DTDP does not activate the RyR by increasing its sensitivity to Ca\(^{2+}\), or by an effect on channel conductance (Eager et al., 1997) it is possible that the oxidation of the channel affects the gating mechanism, either directly or indirectly. This is consistent with other reports suggesting that the modification of thiol groups on the RyR directly influences channel gating (Abramson and Salama, 1988; Boraso and Williams, 1994; Liu et al., 1994).

The activation of the RyR by 4,4'-DTDP is affected to a large extent by the cis [Ca\(^{2+}\)]. In subactivating concentrations of Ca\(^{2+}\) the following observations characterised 4,4'-DTDP -induced RyR activation.

1) Maximum P\(_o\) obtained after 4,4'-DTDP was, on average, <0.1.
2) Average T\(_o\) during maximum activation was <25 ms.
3) Open times fell into four groups (\(\tau_1 - \tau_4\)) with less than 10\% of events in \(\tau_4\).
4) An average latency before channel activation of ~37 s with 1 mM 4,4'-DTDP, or >2 min with <100 \(\mu\)M 4,4'-DTDP.

When 4,4'-DTDP was added to the Ca\(^{2+}\) -activated channel (10\(^{-5}\) or 10\(^{-3}\) M Ca\(^{2+}\)), the characteristics of channel activity were as follows.

1) A P\(_o\) of >0.48.
2) Average T\(_o\) >200 ms.
3) An open time distribution with events in \(\tau_1 - \tau_5\), and >30\% of events in \(\tau_4\) & \(\tau_5\).
4) A delay before activation of ~2 s (1 mM 4,4'-DTDP), or ~30 s (<100 \(\mu\)M 4,4'-DTDP).

When the channel was inhibited by high Ca\(^{2+}\) concentrations, the characteristics of 4,4'-DTDP -induced activation were similar to those in subactivating Ca\(^{2+}\). Interestingly, the presence of Ca\(^{2+}\) (\(\geq 10^{-5}\) M) also increased the sensitivity of the RyR to the RDSs. Thus, altering the [Ca\(^{2+}\)]\(_{cis}\) produced considerable differences in the activation parameters of the RyR in response to 4,4'-DTDP. The influence of Ca\(^{2+}\) on the activity of the oxidised channel could be due to specific interactions between Ca\(^{2+}\) and the RyR. Alternatively, the changes induced by Ca\(^{2+}\) may be due to the level of channel open probability prior to the addition of the oxidising agent.
The influence of channel $P_o$ is indicated by: a) the shift in the $Ca^{2+}$-activation curve for $P_o$, following the oxidation of RyRs (see Fig. 5.2 - top) and b) the more rapid activation by 4,4'-DTDP of the $Ca^{2+}$-activated RyRs compared to those in subactivating and inhibitory $Ca^{2+}$ concentrations.

Irrespective of the cis $[Ca^{2+}]$, activation of the RyR by 4,4'-DTDP resulted in an increase in the mean open time. This was due to an increase in the proportion of openings in longer time constants. Similar results were found with the activation of the cardiac RyR in $10^{-5}$ M $Ca^{2+}$ with 2,2'-DTDP (see section 5.3) and $H_2O_2$ (Boraso and Williams, 1994). The consistent effects of RDSs on channel open times were not matched by other parameters of channel activity. Event frequency and closed times of the oxidised channels at the different $Ca^{2+}$ concentrations were not consistency altered, thereby suggesting the oxidation of a thiol(s) group on the RyR has a preferential effect on the channel activation mechanism. Although the activation mechanism of the RyR is considerably influenced by thiol oxidation, the ability of the protein to function normally does not appear to be diminished. This was shown by the reversal of the oxidation -induced activation with DTT or GSH. In addition, ligand binding by ryanodine and ruthenium red (Eager et al., 1997), as well as ATP and caffeine (Boraso and Williams, 1994), produced their usual effects on the oxidised channel. However, after time, exposure of the RyR to oxidising agents totally abolishes channel function (Ondrias et al., 1990; Holmberg et al., 1991; Eager et al., 1997).

$Ca^{2+}$ had a strong influence on the activation phase of RyRs oxidised by RDSs, but had no apparent effect on the RDS -induced loss of channel activity. The loss of channel activity, when exposed to 2,2'- or 4,4'-DTDP and other SH reagents, has a latency of several minutes. Increasing the $[Ca^{2+}]_{o}$ neither reduced this latency nor decreased the minimum concentration of 4,4'-DTDP that routinely caused the loss of channel activity within 10 min. Results from the previous chapter suggested that RDS -induced loss of channel activity is due to the oxidation of a SH group, but that this thiol oxidation is in addition to that which causes the activation of the channel. Findings from the present chapter show a strong $Ca^{2+}$ -dependence for 4,4'-DTDP -induced activation and a lack of $Ca^{2+}$ -dependence in the RDS -induced loss of channel activity. This supports the finding that the two mechanisms are separate, and is in accord with the model of Laver et al. (1997) showing an independent inhibition gate.

There is evidence which indicates that at least two SH groups are involved in modulating the activity of the RyR following exposure to thiol oxidising agents. Firstly, the phthalocyanine dyes are thought to activate the channel by oxidising a pair of endogenous vicinal thiols to form a disulfide bridge (Abramson et al., 1988b). Secondly, the oxidation of a second SH group by 4,4'-DTDP was suggested to be involved in the introduction of an additional kinetic component following the addition of millimolar concentrations of the reagent to the cis
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chamber (see section 4.5.1). Abramson and Salama (1988) proposed a model to explain the observations that heavy metals, RDSs and other oxidising agents induced Ca\(^{2+}\) efflux from SR vesicles (Abramson and Salama, 1989) and caused the contraction of skinned skeletal muscle fibres (Pike et al., 1987). The model postulated the involvement of three SH groups to control physiological gating of the channel. The cross linking of two of these groups (i.e. formation of a disulfide) opened the channel. A subsequent thiol disulfide exchange reaction involving the third SH group caused the rapid closure of the channel. A mechanism widely accepted as being integral to the physiological activation of the cardiac RyR has been termed Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) (Fabiato and Fabiato, 1975; Stern, 1992). CICR was speculated to be compatible with the model because it was suggested that Ca\(^{2+}\) could be involved in the cross linking of two free SH groups by the formation of a Ca\(^{2+}\) dithiolate complex, or that Ca\(^{2+}\) may control the rate of the oxidation reaction (Abramson and Salama, 1989). Thus, it was suggested that Ca\(^{2+}\) facilitates thiol oxidation to activate the channel. However, this is not consistent with the findings of the present study. Ca\(^{2+}\) and 4,4'-DTDP activate the RyR by apparently separate mechanisms; each reagent alone is able to activate the channel. The activity of the channel, activated by either Ca\(^{2+}\) or 4,4'-DTDP, can be distinguished by differences in the properties of channel activity. However, there is cooperativity of the two reagents in channel activation. 4,4'-DTDP increases the activity of the Ca\(^{2+}\) -activated RyR, and it also activates the channel faster, and at lower reagent concentrations when the channel is Ca\(^{2+}\) -activated. The results suggest a synergism between Ca\(^{2+}\) and 4,4'-DTDP.

The model (Fig. 5.16) summarises some of the results of the interactions between Ca\(^{2+}\) and 4,4'-DTDP and presents a scheme to explain the effects of 4,4'-DTDP on the RyR. The RyR has two independent gates as described by Laver et al. (1997): an activation gate (a) and an inactivation gate (i). Each gate is located in the channel pore and can swing between an open and a shut position. The gates swing with a frequency that is modulated by ligand binding and each has an open probability, P\(_o\), and P\(_i\). The position of the gate indicates its open probability. When parallel to the pore (0°) there is a high probability of the gate being open (P\(_o\) ~1). When at 90° to the pore, there is a low probability of the gate being open (P\(_o\) ~0). The channel P\(_o\) is the product of the open probability of each gate (P\(_o\), P\(_i\)). When both gates are fully open the P\(_o\) of the channel is at unity. However, because of the independence of the gates, only one needs to be closed for channel activity to be lost. The hypothesis is that, in the absence of ligands, the activation gate is shut and the inactivation gate is open. Consequently, P\(_o\) is ~0. The binding of Ca\(^{2+}\) to C\(_1\) and the oxidation by 4,4'-DTDP of SH\(_i\), and possibly also SH\(_o\), individually or jointly modulate the activation gate to increase P\(_o\). The binding of Ca\(^{2+}\) to its low affinity site (C\(_2\)) decreases P\(_i\), thereby reducing the probability that the inactivation gate is open. Channel activity is irreversibly lost when RDSs oxidise the less reactive SH group(s) (SH\(_{3,4}\)) and decrease P\(_i\) to 0.
Fig. 5.16 Model depicting the actions of RDSs and Ca\(^{2+}\) on the RyR and a summary of results. The RyR is shown in the membrane of the SR, with binding sites on the RyR for RDSs (SH\(_{1-4}\)) and Ca\(^{2+}\) (C\(_{1-2}\)) on the cytoplasmic (cis) side of the bilayer. The central channel has two independently controlled gates: an activation gate (a) and an inactivation gate (i). The probability that the gates are open determines the \(P_o\) of the channel (shown beneath each diagram). The probability that the gate will be open is 1.0 when the gate is vertical. The probability of the gates being open is influenced by ligand binding. The RyRs in the top row show the influence of Ca\(^{2+}\) on gate open probability. The addition of 4,4'-DTDP (cis) is indicated on the left column: concentration of the RDS influences the rate of activation and subsequent loss of channel activity. The remainder of the matrix illustrates the interactions between Ca\(^{2+}\) and 4,4'-DTDP on the gates to control channel \(P_o\). The model is described fully in the text (section 5.6).
\[ \leq 10^{-7} \text{M Ca}^{2+} \]

- **cis**: C\(_1\), C\(_2\), SH\(_1\), SH\(_2\)
- **trans**: SH\(_{30}\)

\( P_0 \approx 0.005 \)

\[ 10^{-5} \text{M/}10^{-3} \text{M Ca}^{2+} \]

- **Ca-activated**: C\(_1\), C\(_2\), SH\(_1\), SH\(_2\), Ca

\( P_0 \approx 0.5 \)

\[ 2 \times 10^{-2} \text{M Ca}^{2+} \]

- **Ca-inhibited**: C\(_1\), C\(_2\), SH\(_1\), SH\(_2\), Ca

\( P_0 \approx 0.01 \)

**4,4'-DTDP** ↔ DTT

- **DTDP-activated**: P\(_0 \approx 0.1\)
  - \( \mu\text{M} > 2 \text{min} \)
  - \( \text{mM} < 50 \text{s} \)

- **No activity**: P\(_0 = 0\)
  - \( \mu\text{M} > 20 \text{min} \)
  - \( \text{mM} 5-10 \text{min} \)
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This decrease P; to 0 is shown to occur via an interaction with the inactivation gate (i). However, it is possible that the cause of the loss of channel activity following the oxidation of SH_sub_3.4 is not due to the closure of the inactivation gate, but due to a separate, independent, inhibition mechanism. Two SH groups (SH_sub_1-2) are shown in the model to be associated with the activation gate, and another two (SH_sub_3-4) with the inactivation gate. This is because the formation of a cystine from vicinal cysteine residues is a mechanism by which some thiol oxidising agents may affect channel gating (Abramson and Salama, 1988; Ondrias et al., 1990; Boraso and Williams, 1994) (see section 1.5.1).

The model makes four assumptions. Firstly, that separate cysteine residues are responsible for the two different effects of the RDSs i.e. activation and the loss of channel activity. Secondly, that the SH groups associated with channel gating (SH_sub_1-4) are located on the RyR. Thirdly, that Ca^2+ and the RDSs bind at different sites. Fourthly, that SH_sub_3-4 are associated with the inactivation gate (i).

Communication between the ligand binding sites for SH reagents and the gates could be due to direct interactions if the two sites are close. Alternatively, if the binding site are some distance from the gating mechanism, then the communication may be due to a long range allosteric interaction, as was indicated for the binding of calmodulin and FKBP12 to the skeletal RyR (Wagenknecht et al., 1994; Samso et al., 1997) or the binding of acetylcholine to its receptor (Wang et al., 1987). The model accounts for the synergistic effects on the activation of the channel following the binding of Ca^2+ and the oxidation by 4,4'-DTDP. The binding of Ca^2+ to its activation site, combined with thiol oxidation, influences RyR activity via separate mechanisms or pathways that converge on the activation gate to produce an additive effect on channel open probability. Thiol oxidation of SH_1 and/or SH_2 by agents that bind to the individual groups e.g. RDSs or thimerosal, or agents that oxidise both SH groups to form a disulfide bridge (according to Eq. 1.4), could equally influence the activation gate according to the model. The model also explains the biphasic effects of the RDSs on RyR activity. This is depicted by the oxidation of separate SH groups which have independent effects on the activation and inactivation gates. However, the proposed mechanism does not explain how the presence of Ca^2+ influences other aspects of 4,4'-DTDP-induced channel activation. Firstly, concentrations of Ca^2+ which activate the RyR decrease the latency of 4,4'-DTDP-induced activation. Secondly, Ca^2+ increases the sensitivity of the channel to lower concentrations of 4,4'-DTDP. These effects may be due to the channel being open in the presence of Ca^2+, thereby potentially increasing the accessibility of the specific SH group to the RDS. Alternatively, it may be due specifically to the binding of Ca^2+ to its activation site, or another site, which increases the reactivity of the relevant SH group thereby increasing its sensitivity to the RDS and allowing a faster rate of activation.
5.7 Oxidation of the inhibited RyR

5.7.1 Mg$^{2+}$

In skeletal and cardiac SR vesicles, physiological concentrations of Mg$^{2+}$ inhibit the rate and percentage of Ca$^{2+}$ efflux induced by RDSs (Zaidi et al., 1989; Prabhu and Salama, 1990b) and Ag$^+$ (Salama and Abramson, 1984). The inhibition by Mg$^{2+}$ occurred in a dose dependent manner at concentrations $>$ 1 mM. Ca$^{2+}$ release from SR vesicles did not appear to be stimulated by RDSs or Ag$^+$ in the presence of $>$ 5 mM Mg$^{2+}$. In the present study, the binding of Mg$^{2+}$ to either the high or low affinity Mg$^{2+}$-inhibition sites did not prevent the subsequent activation of the channel by 4,4'-DTDP. However, the presence of Mg$^{2+}$ (millimolar) considerably reduced the degree of 4,4'-DTDP-induced activation and altered some of the usual characteristics of channel activity following subsequent activation by 4,4'-DTDP. This was an important finding for two reasons. Firstly, oxidation of thiol groups on the RyR can activate the channel in the presence of concentrations of Mg$^{2+}$ that are present under physiological and, potentially, under pathological conditions (in which [Mg$^{2+}$], may rise well above normal (see section 1.4.2c)). Secondly, because physiological concentrations of Mg$^{2+}$ limited the degree of activation by 4,4'-DTDP, Mg$^{2+}$ may be an important factor in limiting Ca$^{2+}$ efflux from the SR when the cell is exposed to oxidative stress.

In the presence of concentrations of Ca$^{2+}$ and Mg$^{2+}$ ($10^{-7}$ and $10^{-3}$ M, respectively) equivalent to those in the resting myocyte, Mg$^{2+}$ exerts an inhibitory influence on the ability of 4,4'-DTDP to activate the RyR. This occurs because Mg$^{2+}$ binds to the Ca$^{2+}$-activation site. However, the inhibitory influence of Mg$^{2+}$ appears to be more complex than simply preventing the binding of Ca$^{2+}$ to the activation site. This was apparent because the $P_o$ of the oxidised RyR in the presence of Mg$^{2+}$ and $10^{-7}$ M Ca$^{2+}$ was several-fold lower than the $P_o$ of the oxidised RyR in the absence of Mg$^{2+}$ and $10^{-9}$ M Ca$^{2+}$. In $10^{-9}$ M Ca$^{2+}$ there is essentially no free Ca$^{2+}$ to bind to the site, yet there is still a considerably higher degree of oxidation-induced activation than in the presence of Mg$^{2+}$. Therefore, Mg$^{2+}$ must inhibit gating via an additional mechanism. This may be due to the Mg$^{2+}$ binding to the low affinity Ca$^{2+}$/Mg$^{2+}$-inhibition site. Even at the relatively low [Mg$^{2+}$] of 1 mM, Mg$^{2+}$ can exert an influence on channel gating by binding to this low affinity site (Laver et al., 1997). An alternative explanation for this inhibitory effect of Mg$^{2+}$, is that the Mg$^{2+}$ bound to the Ca$^{2+}$-activation site prevents conformational changes in the protein that usually occur during channel activation i.e. after Ca$^{2+}$ binding or SH oxidation. Consequently, the usual effects on the channel of SH oxidation may be limited by the conformational restrictions imparted by the bound Mg$^{2+}$. The overall effect of Mg$^{2+}$ was to reduce the open probability of the 4,4'-DTDP-activated RyR, and to prevent the long channel openings usually seen with 4,4'-DTDP activation. In contrast, when the channel
was activated by 1 mM Ca\(^{2+}\), the subsequent inhibition by high concentrations of Mg\(^{2+}\) did not have such a strong inhibitory effect on activation by 4,4'-DTDP. \(P_0\) was increased to relatively high levels following the addition of 4,4'-DTDP (\(P_0 \approx 0.38\), and long open events were seen. However, the relative frequency of events in the longer time constants was reduced by the presence of Mg\(^{2+}\). Consequently, Mg\(^{2+}\) appears to inhibit 4,4'-DTDP -induced activation by two different mechanisms depending on the cis [Ca\(^{2+}\)]. This is because there are two separate Mg\(^{2+}\) -inhibition sites on the RyR (Laver et al., 1997). In a low [Ca\(^{2+}\)], Mg\(^{2+}\) binds to the Ca\(^{2+}\) -activation site (C\(_1\) - Fig. 5.16) to inhibit the channel. In activating Ca\(^{2+}\) concentrations, Ca\(^{2+}\) competitively binds to the Ca\(^{2+}\) -activation site in favour of Mg\(^{2+}\). However, higher concentrations of Mg\(^{2+}\) can inhibit the channel via the second Mg\(^{2+}\) -inhibition mechanism whereby Mg\(^{2+}\) binds to the low affinity Ca\(^{2+}/\text{Mg}^{2+}\) binding site (C\(_2\) - Fig. 5.16). Because of this, channel activation by 4,4'-DTDP in the presence of 10 mM Mg\(^{2+}\) is similar to that of the channel inhibited by 2 x 10\(^{-2}\) M Ca\(^{2+}\). The different effects of 4,4'-DTDP on channel activity in 10\(^{-7}\) M Ca\(^{2+}\) and 1 mM Mg\(^{2+}\) or 1 M Ca\(^{2+}\) and 10 mM Mg\(^{2+}\) support the existence of separate high and low affinity Mg\(^{2+}\) binding sites on the cardiac RyR (Nagasaki and Kasai, 1983; Meissner et al., 1986; Laver et al., 1997).

Salama et al. (1992) proposed a model for skeletal ECC that involved the direct oxidation of the RyR by the DHP receptor. This was based on studies which showed that SH reagents induced Ca\(^{2+}\) efflux from SR vesicles (Abramson et al., 1983) and caused contraction of skinned skeletal muscle fibres (Salama et al., 1992). In the latter study, fibres were optimally primed for Ca\(^{2+}\) release. The fibres were heavily loaded with Ca\(^{2+}\) and the free [Mg\(^{2+}\)] was only 0.1 - 0.2 mM. This concentration of Mg\(^{2+}\) is below the physiological levels that are shown to inhibit RDS -induced Ca\(^{2+}\) efflux from skeletal SR vesicles (Zaidi et al., 1989). In the present study, activation of the cardiac RyR by 4,4'-DTDP in physiological concentrations of Ca\(^{2+}\) and Mg\(^{2+}\) (for the resting myocyte; 10\(^{-7}\) and 10\(^{-3}\) M respectively) did occur, but open probability was low (\(P_0 \approx 0.007\)). This level of channel open probability is comparable to that calculated from resting skinned mammalian skeletal muscle fibres exposed to a RDS under physiological conditions. Posterino and Lamb (1996) calculated an open probability for the RyR of \(\approx 0.003\) after exposure to 100 \(\mu\)M 2,2'-DTDP in nanomolar concentrations Ca\(^{2+}\) and millimolar Mg\(^{2+}\). They reasoned that such a level of Ca\(^{2+}\) release from RyRs was insufficient to induce a self propagating response, and concluded that ECC in skeletal muscle was unlikely to occur \emph{in vivo} by the "oxidative linkage" of DHP receptors to RyRs. Interestingly, the cardiac RyR is considerably less sensitive to Mg\(^{2+}\) -inhibition than the skeletal isoform (Meissner and Henderson, 1987; Laver et al., 1997). Under conditions when the cardiac RyR was Ca\(^{2+}\) -activated, Mg\(^{2+}\) (even at high levels) was less effective in limiting channel activation by SH oxidation than it was in subactivating concentrations of Ca\(^{2+}\). Open probability of the RyR increased to \(\approx 0.38\) during maximum 4,4'-DTDP -induced activation in
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1 mM Ca\(^{2+}\) and 10 mM Mg\(^{2+}\). This level of channel activity was considerably higher than that induced by caffeine in subactivating concentrations of Ca\(^{2+}\) (see section 3.4.2c), and caffeine alone triggers intracellular Ca\(^{2+}\) transients sufficient to cause the contraction of myocytes (Lipp and Niggl, 1994). Therefore, the level of Ca\(^{2+}\) efflux from oxidised RyRs in the presence of Ca\(^{2+}\) and Mg\(^{2+}\) may be sufficient to induce a regenerating response and/or contribute to an elevated [Ca\(^{2+}\)], especially if the SR Ca\(^{2+}\) pumps are inhibited due to oxidative stress.

5.7.2 pH

4,4'-DTDP activated 75% of RyRs when the pH in the cis solution was reduced to 6.5. This effect occurred when the channel was exposed to the RDS within ~1 min of lowering the pH. The qualitative characteristics of activation were similar to the usual effects of channel oxidation in the presence of activating concentrations of Ca\(^{2+}\) i.e. increases in P\(_o\), T\(_o\) and the number of components required to fit the open time distribution. However, the levels of P\(_o\) and T\(_o\) were lower than the comparable values when the pH of the Ca\(^{2+}\)-activated RyR was 7.4, and there were no open events in the longest component (\(\tau_s\)). The lower degree of activation by 4,4'-DTDP may be due to an influence of the high [H\(^+\)]. Alternatively, it could have been due to the low P\(_o\) of the channels when RDSs were added. The maximum P\(_o\) of the oxidised RyR appears to be constrained by the level of the P\(_o\) when the oxidising agent is added (see Fig. 5.2 - top). The P\(_o\) of the pH -inhibited and the caffeine -activated RyR was similar prior to the addition of 4,4'-DTDP (~0.03 - 0.04). The maximum activation induced by 4,4'-DTDP was similar in both sets of experiments (P\(_o\) ~0.15 - 0.18). However, while the maximum value of P\(_o\) under both conditions was comparable, the properties of channel activity were very different. These results suggest that the activation of the RyR by thiol oxidation is a complex function of P\(_o\) and the activating ligand.

5.8 Oxidation of the non-Ca\(^{2+}\)-activated RyR

5.8.1 ATP and 4,4'-DTDP - a shared link to the activation gate?

ATP is an intracellular ligand that has a major effect on the activity of the RyR (see section 1.4.2b). It is present in the cell in millimolar concentrations. Interestingly, the ability of micromolar concentrations of RDSs to oxidise SH groups is reduced in the presence of millimolar ATP. This is shown by a reduction in: 1) the percentage of Ca\(^{2+}\) efflux induced by RDSs from skeletal and cardiac SR vesicles and 2) the production of thiopyridone (the product obtained following the reduction of 2,2'- or 4,4'-DTDP) after RDSs were added to SR vesicles (Zaidi et al., 1989; Prabhu and Salama, 1990b). It was suggested that the access of RDSs to the SH group was sterically hindered by the binding of adenine nucleotides (Zaidi et al., 1989). This is conceivable since 2,2'- and 4,4'-DTDP are relatively
large (van Iwaarden et al., 1992). Alternatively, the reduction in RDS -induced Ca$^{2+}$ efflux from SR vesicles may be due to depleted Ca$^{2+}$ in the SR because of prior stimulation of Ca$^{2+}$ efflux by the adenine nucleotides. The results from the present study indicate that both of these mechanisms might contribute to the effects of adenine nucleotides on RDS -induced Ca$^{2+}$ efflux.

25% of ATP -activated RyRs were not activated by the addition of 4,4'-DTDP. The reason for the failure of activation could not be determined, but it is possible that the SH groups usually oxidised by the RDSs were not oxidised in these channels. This may be because ATP restricted the access of 4,4'-DTDP to the relevant SH group. This is in agreement with the findings, from studies using SR vesicles, that thiopyridone production was reduced in the presence of adenine nucleotides (Zaidi et al., 1989; Prabhu and Salama, 1990b). However, the addition of the RDS did influence the gating of these ATP -activated channels. Activity immediately declined upon the addition of 4,4'-DTDP. This was not due to a rapid irreversible loss of channel activity (i.e. an interaction with the inactivation gate) because there were low levels of activity until ~5 min after adding the RDSs in each of the three channels. The decline in activity may have been due to an interference with the ATP -activation mechanism. The remainder of the ATP -activated RyRs (n = 9) were transiently activated by the addition of 4,4'-DTDP. The effects of 4,4'-DTDP occurred rapidly. While activity of each of the nine channels were clearly affected by 4,4'-DTDP, the $P_o$ of only seven were increased above the levels of the ATP -activated channel. The overall effect of 4,4'-DTDP was to increase $P_o$. However, the level to which the channel was maximally activated by 4,4'-DTDP was the same in the presence or absence of ATP. In other words, the relative increase in channel activity, induced by 4,4'-DTDP, was reduced in the presence of ATP. This could explain the effects observed in SR vesicle studies (Zaidi et al., 1989; Prabhu and Salama, 1990b) in which RDS -induced Ca$^{2+}$ efflux was lower than usual in the presence of adenine nucleotides. Interestingly, other oxidising agents, alcian blue (Abramson et al., 1988b) and Ag$^+$ (Prabhu and Salama, 1990a), increased Ca$^{2+}$ efflux by ~50% in the presence of adenine nucleotides. This may be due to the non-specific effects of such reagents on the adenine nucleotide -modified protein (van Iwaarden et al., 1992; Stadtman, 1993), in addition to the effects of oxidation of the SH group oxidised by the thiol-specific RDSs.

Most of the measured parameters of channel activity, of the RyR oxidised by 4,4'-DTDP, were not affected by the presence of ATP. This is suggestive of a common site on the protein for the binding of ATP and the oxidation by 4,4'-DTDP. However, RDSs and ATP do not appear to act at the same site on the RyR, since effects of 4,4'-DTDP on single RyRs activated by millimolar concentrations of ATP were cooperative. In addition, adenine nucleotides stimulated Ca$^{2+}$ efflux from SR vesicles in the presence of RDSs (Zaidi et al., 1989;
Oxidation of ligand modified RyRs

Prabhu and Salama, 1990b). However, it is not known whether the adenine nucleotide stimulation was due to an additive effect of adenine nucleotides on oxidised RyRs or due to the activation of a population of RyRs that were not oxidised by the RDSs. It was shown (section 4.2.3a) that low concentrations of 4,4’-DTDP (50 - 100 μM) activated only ~60% of RyRs. Therefore, it is possible that a similarly low percentage of RyRs in SR vesicles are oxidised by micromolar concentrations of RDSs. Consequently, the subsequent addition of ATP could increase Ca\(^{2+}\) efflux from vesicles simply by activating those channels not oxidised by the RDSs. In other words, the stimulation of RDS -induced Ca\(^{2+}\) efflux by adenine nucleotides would not necessarily involve the activation of the oxidised RyRs.

The apparently cooperative actions of RDSs and adenine nucleotides on the RyR could be explained if the SH group oxidised by the RDS and the binding site for ATP were physically close together. A potential ATP binding site for the cardiac RyR has been identified on the cytoplasmic side of the channel. It is located at position 2619 - 2652, and contains the nucleotide binding motif GXGXXG (2627 - 2632; Otsu et al., 1990). A pair of conserved cysteine residues which are separated from each other by only four amino acid residues (2618 - 2623), are located very close to the putative ATP binding site. In addition to these two cysteine residues, another three (two of which are conserved) are located in the region of the cardiac RyR that has been designated the modulatory region (2619 - 3016; Otsu et al., 1990). The proposed modulatory region contains sites for the binding of ATP as well as calmodulin (Otsu et al., 1990) and the phosphorylation of serine (Otsu et al., 1990; Witcher et al., 1991). Because this region is also protease sensitive, it is likely that much of the modulatory region is surface orientated (Marks et al., 1990) and therefore accessible to the cytoplasmic milieu. This meets the physical requirement for the SH groups involved in Ca\(^{2+}\) efflux from SR vesicles since they appear to be located in a hydrophilic area of the protein (Abramson et al., 1983; Abramson et al., 1988b). Empirical evidence also supports the possibility that the binding site for ATP and the thiol group(s) involved in channel gating are closely positioned. Zaidi et al. (1989) suggested this to explain the effects of adenine nucleotides and their apparent ability to restrict access of RDSs to SH groups. The concept is also supported from the findings of the present study which showed that 25% of ATP -activated RyRs were not activated by 4,4’-DTDP. In addition, Tatsumi et al. (1988) found similar effects of Ag\(^+\) and ATP on Ca\(^{2+}\) efflux from skeletal SR vesicles, and concluded that the binding sites for both reagents were similar, if not the same. The similarities in the degree of maximum activation and the properties of single channel activity, when the channel is activated either individually by 4,4’-DTDP or jointly by ATP and 4,4’-DTDP, could be explained if the two agents modified a common structural element or “link” that was connected to the activation gate of the channel. The effects of either the RDSs or ATP could potentiate the activation induced by the
other if the link was not "saturated" (i.e. not maximally stimulated). The results of the present study suggest that the link is saturated when the channel is oxidised by 4,4'-DTDP, but not by ATP alone.

5.8.2 RyR agonists can independently influence activity of the oxidised RyR

The degree and the rate of RyR activation by 4,4'-DTDP were thought to be influenced by a combination of the level of channel activity prior to RDS addition and the presence of other channel ligands (see sections 5.6 and 5.7.2). The experiments with ATP revealed that this was not necessarily the case: while the rate of activation following the addition of 4,4'-DTDP was increased in the presence of ATP, the degree of activation and most other measured parameters of channel activity of the 4,4'-DTDP -activated RyR were unaffected by the presence of ATP. These results suggested that the levels of channel activity when the oxidising agent is added to the channel may not be important in determining the subsequent activity of the oxidised channel. In summary, the presence of activating concentrations of Ca\(^{2+}\), not merely an active channel, appears necessary in order to increase the subsequent levels of channel activity and/or influence the channel kinetics of the oxidised RyR. In addition, the rate of channel activation by 4,4'-DTDP appears to be faster when the \(P_o\) of the channel is elevated, irrespective of the activating ligand. To test the generality of these statements, the channel was activated by caffeine in subactivating concentrations of Ca\(^{2+}\) prior to the addition of 4,4'-DTDP. Caffeine was used because it is known to activate the channel by binding at a different site on the protein to ATP (McGarry and Williams, 1994a) and Ca\(^{2+}\) (Pessah et al., 1987).

The addition of either ATP or caffeine to RyRs, in subactivating concentrations of Ca\(^{2+}\), increased the values of \(P_o\) to a similar level. The oxidation of either the ATP or the caffeine -activated channels with 4,4'-DTDP increased \(P_o\) and \(T_o\), and introduced long components into the open time distribution. In contrast to ATP, which produced no additional effects on the activity of the oxidised RyR, the presence of caffeine increased the degree of activation and altered other channel properties of the oxidised RyR. Following the addition of 4,4'-DTDP, the \(P_o\) of the caffeine activated RyR was ~2 -fold greater than the \(P_o\) of oxidised RyRs in the presence or absence of ATP. This effect of caffeine on \(P_o\) is similar to the synergistic actions of Ca\(^{2+}\) and 4,4'-DTDP. The experiments with caffeine indicate that Ca\(^{2+}\) is not a prerequisite for increasing the degree of activation following oxidation by 4,4'-DTDP. However, caffeine is reported to increase the sensitivity of the RyR to Ca\(^{2+}\) (Pessah et al., 1987; Sitsapesan and Williams, 1990). Therefore, given the experimental conditions (\(10^{-7}\) M \(cis\) Ca\(^{2+}\)), it was possible that Ca\(^{2+}\) influenced the degree of 4,4'-DTDP -induced activation. This does not appear to have occurred because of considerable differences in the activity of the oxidised RyR activated by either Ca\(^{2+}\) or caffeine. These differences
are discussed below and include: a) the distribution of open times and b) the rate of channel activation.

When 4,4'-DTDP oxidised the caffeine -activated RyRs, mean open time increased to values that were an order of magnitude greater than channels activated jointly by ATP and 4,4'-DTDP. Values of $T_o >100$ ms were only previously seen after oxidation of the Ca$^{2+}$ -activated channels. This suggests that the long open times do not depend on Ca$^{2+}$ per se. However, when the distribution of open times for the oxidised RyR were analysed, considerable differences were found between those channels activated by either Ca$^{2+}$ or caffeine. Firstly, although the addition of 4,4'-DTDP to caffeine -activated RyRs added an additional component ($\tau_4$) to the distribution, only the Ca$^{2+}$ -activated channels oxidised by RDSs had events in the longest component ($\tau_5$). Secondly, the two shortest components ($\tau_1$ and $\tau_2$) of the caffeine -activated RyRs were abolished following thiol oxidation. While it is usual to see a decrease in the proportion of events in $\tau_1$ and $\tau_2$ following the addition of RDSs (see Fig. 5.5), the complete absence of events from all channels in the two shortest components had not previously been seen, other than when the channel was ryanodine -modified (data not shown). An additional difference between the Ca$^{2+}$ and the caffeine -activated RyRs was that the pre-activation of channels with Ca$^{2+}$, but not with caffeine, increased the rate of activation by 4,4'-DTDP. The delay before activation of $>40$ s in the absence or presence of caffeine was considerably longer than the $<3$ s delay when channels were activated by either Ca$^{2+}$or ATP. This strongly suggests that an increase in $P_o$ alone does not influence the rate of activation by 4,4'-DTDP, especially considering that the $P_o$ of the caffeine -activated channels was marginally greater than the $P_o$ of the ATP -activated channels prior to RDS addition.

The results have shown that the activation of the RyR by SH oxidation is influenced by the binding of RyR agonists. Caffeine, ATP or Ca$^{2+}$ each affected at least one of the following aspects of the activity of the oxidised RyR: a) the maximum degree of activation; b) the rate of activation and c) the distribution of open times. However, no single ligand was found, individually, to be responsible for influencing any specific aspect of the activation phase of the oxidised RyR. While the presence of activating concentrations of Ca$^{2+}$ affected all three parameters, the general effects of Ca$^{2+}$ could be replaced by either ATP or caffeine. This was shown by the ability of ATP alone to influence the rate of oxidation -induced activation, while caffeine alone influenced the other two parameters. The results suggest a synergism between the oxidation of the RyR by 4,4'-DTDP and the individual binding of Ca$^{2+}$ or caffeine. The effects of each agent individually or collectively converge on the activation gate via apparently separate pathways to produce a concerted action on channel gating. The model in Figure 5.17 represents a single RyR in the SR membrane. It is based upon the earlier model (Fig. 5.16) which was used to explain the synergistic actions of Ca$^{2+}$ and RDSs on
Fig. 5.17  Model of the RyR showing interactions between activation/inhibition gates and ligand binding sites on the cytoplasmic and luminal face of the RyR. The figure of a RyR in the SR membrane is based on the model described in Figure 5.16. The following ligand binding sites have been added:

a) putative SH groups on the luminal face of the RyR that are linked to both gates to account for the biphasic effects of the trans addition of 4,4'-DTDP and thimerosal; b) an independent caffeine (caff) link to the activation gate and c) an ATP binding site sharing a link to the activation gate with SH1.2. Mg\(^{2+}\) is depicted as sharing the Ca\(^{2+}\) binding sites Ca\(_a\) and Ca\(_i\). The inhibitory effect of Mg\(^{2+}\) binding to Ca\(_i\) is represented by the dashed link and the dashed activation gate.
channel activation, and the biphasic effects of the RDSs. Figure 5.17 expands on this scheme to show the relationship between the activation and inactivation gates on the channel (refer to section 5.6 and Fig. 5.16), the links to the binding sites for the ligands investigated in the present study and the interrelationships between the factors. The model explains the following points. 1) Channel inhibition by Mg\(^{2+}\) following its binding at the Ca\(^{2+}\)-activation site (Ca\(_a\)) or the low affinity Ca\(^{2+}\)-inhibition site (Ca\(_i\)). The connection of Ca\(_a\) to the activation gate is represented by separate links for the binding of Ca\(^{2+}\) or Mg\(^{2+}\). This is to indicate the opposing actions of the two ions. 2) The synergistic effects of Ca\(^{2+}\) and caffeine on the maximum activation induced by 4,4'-DTDP. All three links to the activation gate are separate. 3) The inability of ATP to influence channel activity (other than latency) of the oxidised RyR. This is indicated by the shared link to the activation gate. 4) The inability of any of the activating ligands to influence the loss of channel activity associated with channel oxidation because the activation and inactivation gates are independent.
6.1 CONCLUDING REMARKS

This study has described the effects of a thiol-specific oxidizing agent, 4,4'-DTDP, on single channel activity of the Kv1.5 RbK. The investigation has furthered the aims of the study by: 1) characterizing the effects of the reagent specific to RbK channel activity in the absence and presence of physiological and pharmacological reagents, 2) describing the mechanism by which sensitization of channel activity by the RDS occurs, and 3) presenting evidence that the biphasic effects of RbK were due to the oxidation of two separate classes of reactive cysteine residues in the RbK. The aims of this study were as follows:

1. Thiol oxidants, by 4,4'-DTDP, sensitized the RbK inhibited by Mg2+ low pH and high (lithium) concentrations of Ca2+

2. The loss of channel activity that occurred following thiol oxidation by RDSs could not be restored by reducing agents.

3. Oxidation of residues on the luminal side of the bilayer caused transient activation of the channel followed by the loss of channel activity.

4. 4,4'-DTDP added to the purified RbK caused channel activation before acidifying activity.

The first two points have important implications for the understanding of RbK function during pathological conditions, such as cardiac repolarization injury, during which time the concentrations of oxidizing agents, Mg2+, H+ and Ca2+ in the cytoplasm are elevated (Kamado et al., 1994; Xi et al., 1994). These two points indicate the location of reactive cysteine residues in the RbK. The study also implicates the conserved cysteine residues in the vicinity of the ATP binding site, including those at positions 2618 and 2619, as possible targets for the regulatory-induced activation by the RDSs.

4,4'-DTDP was found to produce similar qualitative effects on channel activity as many other non-specific oxidizing agents, such as reactive disulfide species i.e., channel activation followed by the loss of channel activity (Glitsch et al., 1994). This showed that the primary effects of oxidizing agents on RbK channel activity are likely to be mediated through the oxidation of reactive cysteine residues. Two separate classes of reactive cysteine residues mediate the biphasic effects on channel activity following oxidation by the RDSs. Oxidation of the more reactive residues caused channel activation within minutes of addition of 4,4'-DTDP, the oxidation of a less reactive residue caused the cessation of channel function within hours after adding the reagent. The effects of the RDS were not seen when they were added to the RbK in a reducing environment, i.e., in the presence of DTT. This implies that the reactive cysteine residues are, associated with
6.1 CONCLUDING REMARKS

This study has described the effects of a thiol specific oxidising agent, 4,4'-DTDP, on single channel activity of the cardiac RyR. The investigation has fulfilled the aims of the study by: 1) characterising the effects of the reactive disulfide on RyR channel activity in the absence and presence of physiological and pharmacological reagents; 2) describing the mechanism by which modification of channel activity by the RDS occurs and 3) presenting evidence that the biphasic effects of thiol oxidising agents on RyR activity are due to the oxidation of two separate classes of cysteine residue. The novel findings of the report were as follows.

1) Thiol oxidation, by 4,4'-DTDP, activated the RyR inhibited by Mg^{2+}, low pH and high (inhibitory) concentrations of Ca^{2+}.

2) The loss of channel activity that occurred following thiol oxidation by RDSs could not be restored by reducing agents.

3) Oxidation of residues on the luminal side of the bilayer caused transient activation of the channel followed by the loss of channel activity.

4) 4,4'-DTDP added to the purified RyR caused channel activation before abolishing activity.

The first two points have important implications for the understanding of RyR function during pathological conditions, such as cardiac reperfusion injury, during which time the concentrations of oxidising agents, Mg^{2+}, H^+ and Ca^{2+} in the myoplasm are elevated (Kaneko et al., 1994; Xu et al., 1996). The second two points indicate the location of reactive cysteine residues on the RyR. The study also implicates the conserved cysteine residues in the vicinity of the ATP binding site, including those at positions 2618 and 2623, as possible sites for the oxidation-induced activation by the RDSs.

4,4'-DTDP was found to produce similar qualitative effects on channel activity as many other non-specific oxidising agents, such as reactive oxygen species i.e. channel activation followed by the loss of channel activity (Holmberg et al., 1991). This showed that the primary effects of oxidising agents on RyR channel activity are likely to be mediated through the oxidation of reactive cysteine residues. Two separate classes of reactive cysteine residue mediate the biphasic effects on channel activity following oxidation by the RDSs. Oxidation of the most reactive residue caused channel activation within seconds of addition of 4,4'-DTDP; the oxidation of a less reactive cysteine caused the cessation of channel function ~5 min after adding the reagent. The effects of the RDSs were not seen when they were added to the RyR in a reducing environment i.e. in the presence of DTT. This implied that the reactive cysteine residues, associated with
RyR activity, may be protected from \textit{in vivo} oxidation due to the high intracellular concentrations of endogenous reducing agents. However, under pathological conditions, exposure of the myocyte to high levels of oxidants may reduce the GSH:GSSG ratio (Curello et al., 1985) thereby render the RyR susceptible to thiol oxidation. Interestingly, the oxidation of residues on the luminal face of the bilayer affected channel activity in a similar way to the effects observed following the oxidation of cysteine residues on the cytoplasmic face of the RyR. The GSH:GSSG ratio in the lumen of the endoplasmic reticulum is 10 to 100-fold lower than the levels in the cytoplasm (Hwang et al., 1992). If this is also the case for the GSH:GSSG ratio in the lumen of the SR, then oxidising agents that enter the SR lumen would be able to influence RyR activity.

Examination of the effects of 4,4'-DTDP on channel activity, when the RyR was modified by agonists and antagonists, provided support for the hypothesis that 4,4'-DTDP activates the RyR via an interaction with the channel activation mechanism. Oxidation of the class of highly reactive cysteine(s), that are associated with channel activation, produces an effect on channel activity that is independent of the Ca$^{2+}$-activation site and the binding site for caffeine. However, there is a close association between the ATP binding site and this class of cysteine residue(s). The effects on channel activity of cysteine oxidation, combined with the binding of one of Ca$^{2+}$, caffeine or ATP, showed that neither open probability alone, nor the specific RyR ligand, were individually responsible for a) the degree of 4,4'-DTDP -induced activation or b) the rate of 4,4'-DTDP -induced activation. This was indicated, firstly, by the synergistic effects on the degree of activation by a combination of 4,4'-DTDP and either Ca$^{2+}$ or caffeine, but not ATP. Secondly, the rate of 4,4'-DTDP -induced activation was rapid for the channel activated by Ca$^{2+}$ and ATP, but not caffeine. Both the degree and the rate of 4,4'-DTDP -induced activation are influenced by a complex function of ligand binding and channel open probability. The model (Figs. 5.16 and 5.17) was used to explain the independent but synergistic effects on channel activity of the combined actions of 4,4'-DTDP and one of Ca$^{2+}$ or caffeine, as well as the cooperative effects on channel activity of ATP and 4,4'-DTDP. The RDS -induced loss of channel activity was not influenced by any agents used to modify RyR activity. Oxidation of the cysteine residue(s) associated with the loss of channel activity appears to shut the “inactivation” gate associated with the Ca$^{2+}$/Mg$^{2+}$-inhibition mechanism, or an alternative, independent inhibition mechanism.

The location of the cysteine residues associated with both RyR activation and the loss of channel activity are not known. Two series of experiments provided information on the position of the cysteine residues associated with the modulation of channel activity. Firstly, exposure of the CHAPS -solubilised RyR to 4,4'-DTDP produced the similar biphasic effects on channel activity to those seen following the addition of RDSs to the native RyR. This suggested that the
reactive cysteine residues, associated with the alteration of channel activity, are located on the RyR itself. Interestingly, reactive cysteine residues located on the RyR-triadin complex are associated with channel gating (Liu et al., 1994; Liu and Pessah, 1994). It is likely that triadin, and possibly other proteins located in the SR membrane, remain associated with the native RyR after the incorporation of the SR membrane into the artificial bilayer (Liu et al., 1994). Consequently, it is possible that the oxidation of residues on triadin, not the RyR, causes the modification of RyR activity. Whether these RyR-associated proteins co-purify with the RyR is not known, but future experiments could use antibodies directed against the possible RyR-associated proteins to test for their presence following CHAPS-solubilisation of the protein. In the second series of experiments, ATP-activated RyRs were exposed to 4,4’-DTDP, and channel activity was examined. The results of these experiments indicated that the binding site for ATP, and the cysteine residue that is oxidised by 4,4’-DTDP to activate the RyR, may be in close proximity. If this is the case, then the cysteine residue could not be located on triadin because the ATP binding site, and hence the cysteine residue, is located on the cytoplasmic domain of the RyR, and there are no cysteine residues on the cytoplasmic domain of triadin (Guo et al., 1996). Therefore, this indicates that the cysteine residue associated with 4,4’-DTDP-induced activation is likely to be located on the RyR. Future studies using the cloned RyR could examine channel function, following the mutation of specific cysteine residues, to determine the location of the particular residues involved in channel activation and the loss of activity. Armed with this knowledge, experiments could be specifically designed to answer numerous questions on the role of redox reagents on RyR function, such as the involvement of the RyR in the Ca\(^{2+}\)-overload that is associated with cardiac reperfusion injury. Several conserved cysteine residues on the RyR have been implicated in the present study as sites which warrant closer investigation in relation to oxidation-induced channel activation. They would be ideal candidates for mutation studies, and include the residues: a) in the vicinity of the ATP binding site e.g. residues 2618 and 2623; b) in the putative pore of the channel i.e. on M2; c) on the putative M3-M4 luminal loop and d) on the cytoplasmic carboxyl-terminal tail.
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