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Activation of the Cardiac Ryanodine Receptor by Sulfhydryl Oxidation is Modified by Mg^{2+} and ATP

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Abstract. The reactive disulfide 4,4′-dithiodipyridine (4,4′DTDP) was added to single cardiac ryanodine receptors (RyRs) in lipid bilayers. The activity of native RyRs, with cytoplasmic (*cis*) [Ca^{2+}] of 10^{-7} M (in the absence of Mg^{2+} and ATP), increased within ~1 min of addition of 1 mM 4,4′-DTDP, and then irreversibly ceased 5 to 6 min after the addition. Channels, inhibited by either 1 mM *cis* Mg^{2+} (10^{-7} M *cis* Ca^{2+}) or by 10 mM *cis* Mg^{2+} (10^{-3} M *cis* Ca^{2+}), or activated by 4 mM ATP (10^{-7} M *cis* Ca^{2+}), also responded to 1 mM *cis* 4,4′-DTDP with activation and then loss of activity. P_o and mean open time (T_o) of the maximally activated channels were lower in the presence of Mg^{2+} than in its absence, and the number of openings within the long time constant components of the open time distribution was reduced. In contrast to the reduced activation by 1 mM 4,4′-DTDP in channels inhibited by Mg^{2+}, and the previously reported enhanced activation by 4,4′-DTDP in channels activated by Ca^{2+} or caffeine (Eager et al., 1997), the activation produced by 1 mM *cis* 4,4′-DTDP was the same in the presence and absence of ATP. These results suggest that there is a physical interaction between the ATP binding domain of the cardiac RyR and the SH groups whose oxidation leads to channel activation.

Key words: Reactive disulfides — Sarcoplasmic reticulum — Sulfhydryl oxidation — Calcium-induced activation — Calcium-induced inhibition

Introduction

Ryanodine receptors (RyRs) form the calcium release channels in the sarcoplasmic reticulum (SR) of cardiac and skeletal muscle, and are found in the endoplasmic reticulum of nonmuscle cell types (Coronado et al., 1994; Dulhunty et al., 1996). Opening of the RyR ion channel in vivo is modulated by numerous ligands including Ca^{2+}, Mg^{2+} and ATP (Smith, Coronado & Meissner, 1985) which bind to the RyR, by coproteins such as FKBP12 (Dulhunty et al., 1996) and triadin (Knudson et al., 1993*b;* Knudson et al., 1993*a*), and by sulfhydryl (SH) oxidation reactions involving protein cysteine residues (Eager, Roden & Dulhunty, 1997). The RyR contains several cysteine residues in its ''regulatory'' domain and in the putative Ca^{2+} channel region (Otsu et al., 1990). These cysteine residues are thought to interact with the gating mechanisms of the RyR Ca^{2+} channel because oxidation of SH groups stimulates Ca^{2+} release from the SR vesicles and increases single RyR channel activity (Abramson & Salama, 1989; Boraso & Williams, 1994; Eager et al., 1997). Many reactive oxygen species activate RyR channels (Holmberg et al., 1991; Holmberg & Williams, 1992; Boraso & Williams, 1994; Favero, Zable & Abramson, 1995), and can cause the long term loss of activity in skeletal RyRs (Holmberg & Williams, 1992; Favero et al., 1995). Nitric oxide (NO), which is produced in skeletal (Nakane et al., 1993) and cardiac muscle (Schulz et al., 1991; Schulz, Nava & Moncada, 1992), alters the activity of single skeletal and cardiac muscle RyRs in lipid bilayers (Mészáros, Minarovic & Zahradníková, 1996; Stoyanovsky et al., 1996; Zahradníková et al., 1997), possibly by the formation of S-nitrosothiol groups (Lei et al., 1992).

The actions of the reactive oxygen species and other oxidizing agents are prevented or reversed by reducing agents (Boraso & Williams, 1994; Favero et al., 1995; Abramson et al., 1995), suggesting that they act via oxidation of SH groups on cysteine residues. However, this has been confirmed in only two studies which have examined the effects of reagents which react specifically with free SH groups on single RyR channel activity. Nagura et al. (1988) found that the reactive disulfide

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4,4′-dithiodipyridine (4,4′-DTDP) increased current through a bilayer containing multiple skeletal RyRs. Eager et al. (1997) showed that 4,4′-DTDP and 2,2′-dithiodipyridine (2,2′-DTDP) induced a transient activation of cardiac RyR channels, followed by an irreversible loss of activity, both of which could be prevented if the channels were exposed to the reducing agent dithiothreitol (DTT) before exposure to the reactive disulfide. The biphasic effect of 4,4′-DTDP was seen at *cis* [Ca^{2+}] from 10^{-9} to 2×10^{-2} M, but occurred most rapidly when channels were activated by either 10^{-5} or 10^{-3} M *cis* Ca^{2+}.

The ability of oxidants to influence RyR channels in vivo has been questioned because the reducing agent glutathione (GSH) is present at ~5 mM in most cells (Meister & Anderson, 1983). However, oxidation reactions do occur under in vivo conditions. Koshita et al. (1993) observed an oxidation-induced Ca^{2+} efflux from skeletal SR vesicles in the presence of GSH or DTT and concluded that thiol oxidation can proceed if the affinity of the oxidant for the -SH group is higher than its affinity for the reducing agent. Oxidation reactions are enhanced if the ratio of GSH to GSSG falls when the concentration of oxygen free radicals increases under pathological conditions such as ischaemia and reperfusion (Sies et al., 1972; Curello et al., 1985), and possibly in aged cells (Stadtman, 1992). Therefore, defining the mechanisms by which specific sulfhydryl reagents alter RyR gating is essential for understanding (i) the involvement of cysteine residues in RyR gating processes and (ii) the in vivo modulation of RyRs by oxidants under normal and pathological conditions.

Although it has been established that sulfhydryl reagents strongly modify the activity of cardiac RyR channels with Ca^{2+} as the sole ligand (*see* Introduction), the effects of oxidation by sulfhydryl reagents in the presence of other ligands has not yet been examined. Therefore, we have compared the activation and depression of cardiac RyR activity by 1 mM *cis* 4,4′-DTDP at 10^{-7} M *cis* Ca^{2+} with the activation and depression of RyRs with physiological concentrations of Mg^{2+} (1 or 10 mM) or Na_2ATP (4 mM). Addition of 4,4′-DTDP caused the activation and subsequent loss of activity in native RyR channels under control conditions and when channels were inhibited by mM Mg^{2+} or activated by mM Na_2ATP. The degree of channel activation by 4,4′-DTDP was reduced in the presence of 1 or 10 mM Mg^{2+}. Surprisingly, the level of channel activation by 4,4′-DTDP was not enhanced by ATP. The results show that (i) the activity of the cardiac RyR channel is susceptible to activation by oxidation reactions in the presence of Mg^{2+} and ATP at in vivo concentrations, (ii) that inhibition by Mg^{2+} and activation by 4,4′-DTDP are additive, while (iii) activation by ATP and by 4,4′-DTDP are not additive.

Materials and Methods

All Methods are described in detail in Laver et al. (1995) and Eager et al. (1997).

Preparation of SR Microsomes

Methods were based on Sitsapesan et al. (1991). Ventricular muscle from fresh sheep heart was differentially centrifuged to yield a crude microsomal fraction which was run on a discontinuous sucrose gradient. Heavy SR vesicles were collected from the 35–40% (wt/vol) interface.

Lipid Bilayers and Solutions

Bilayers were formed from phosphatidylethanolamine, phosphatidylserine and phosphatidylcholine (5:3:2) (Avanti Polar Lipids, Alabama) across a 150–200 μm diameter aperture in a Delrin cup (Cadillac Plastics, Australia). Bilayer potential was controlled and currents recorded using an Axopatch 200A amplifier (Axon Instruments). Bilayer potential is expressed as $V_{cis} - V_{trans}$, i.e., $V_{cytoplasm} - V_{lumen}$.

The normal *cis* solution contained (in mM): 250 CsCl, 1 $CaCl_2$ and 10 N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES, pH 7.4 with CsOH) and the *trans* solution contained (in mM): 50 CsCl, 1 $CaCl_2$ and 10 TES (pH = 7.4). *Cis* Ca^{2+} concentration was changed by perfusion with solutions containing (in mM): 250 CsCl, 10 TES and 2 BAPTA (titrated with $CaCl_2$ to the required free [Ca^{2+}], measured with a Ca^{2+}-selective electrode—Radiometer ION83). SR vesicles were added to the 1 ml *cis* chamber to a final concentration of ~10 μg/ml. Vesicle incorporation was sometimes facilitated by adding 500 mM mannitol to the *cis* solution, increasing the *cis* [CsCl] to 500 mM and/or increasing *cis* [$CaCl_2$] to 5 mM. To prevent multiple fusions, the *cis* chamber was perfused with normal *cis* solution when channel activity was observed. Cs^+ was used as the conducting ion because RyRs have a high Cs^+ conductance and because Cs^+ blocks SR K^+ channels (Cukierman, Yellen & Miller, 1985; Coronado et al., 1992). RyR activity was recorded at the Cl^- equilibrium potential (+40 mV) to minimise Cl^- currents. Experiments were performed at room temperature (21–24°C).

Data Acquisition and Analysis

Channel activity was displayed on an oscilloscope, stored on videotape and later digitized for analysis. Current was recorded at 1 kHz (10-pole low pass Bessel, −3 dB) and digitized at 2 kHz (Labmaster 125 MHz Interface, Axon Instruments). Channel open probability (P_o), frequency of events (F_o), open times, closed times and mean open or closed times (T_o or T_c) were determined using an analysis program, *Channel2* (developed by P.W. Gage and M. Smith). A 50% threshold discriminator was used to detect channel opening and closing, since subconductance levels were seldom observed in cardiac RyRs (Laver et al., 1995). P_o, T_o, T_c and F_o were measured during the 2 min of channel activity recorded immediately before 4,4′-DTDP addition, and during the 30-sec period having the highest P_o within 2 min of 4,4′-DTDP addition (Eager et al., 1997). Open and closed time distributions were displayed as described by Sigworth and Sine (1987) for data obtained during a 2-min control period, and during the 5 min immediately after drug addition. Each distribution was fitted by a multiple exponential function. For display of average trends in the distributions, open time constants were allocated to one of the following time constant groups:

τ_1, <3 msec; τ_2, 3–12 msec; τ_3, 12–50 msec; τ_4, 50–500 msec; and τ_5, >500 msec; similar time constant groups were used for closed time distributions (Eager et al., 1997). Sampling at the Nyquist frequency (double the filter frequency) was considered sufficient for the analysis performed in the present study, since we have shown that the effects of Ca^{2+}, 4,4′-DTDP or caffeine on the open time distribution of the cardiac RyR are not influenced by possible ''undersampling'' (Eager et al., 1997).

Statistics

The 2-tailed students *t*-test was used to test the significance of paired and independent data. The significance of the difference between the logarithms of paired variables was tested. Logarithms were used because control values vary widely and values in 4,4′-DTDP- or ATP-activated channels were proportional to original values, rather than absolute values. In addition, increments in values were normally several orders of magnitude. A value of $P < 0.05$ was considered significant. Data are presented as mean ± 1 SEM.

Drugs

All solutions were prepared using MilliQ deionized water. 4,4′-DTDP (Sigma, St Louis, MO) was dissolved in ethanol. Na_2ATP (Sigma, St Louis, MO) was dissolved in deionized water plus 10 mM TES and adjusted to pH 7.4 using a 500 mM stock CsOH solution.

Results

Biphasic Action of 4,4′-DTDP on RyRs in the Absence of Added Ligands

All experiments were performed at a bilayer potential of +40 mV and most with 10^{-7} M *cis* Ca^{2+}. Under these control conditions, addition of 1 mM 4,4′-DTDP to the *cis* chamber activated each of 21 RyRs within 3 min (e.g., Fig. 1), with an average delay of 42 ± 12 sec before an increase in activity was seen.

Activity in 18 out of 18 of the channels returned to control levels at 80.4 ± 17.2 sec after 4,4′-DTDP addition and was irreversibly lost between 6 and 10 min (i.e., activity could not be restored by voltage pulses to −40 mV or by increasing *cis* $[Ca^{2+}]$ to 1 mM). It has previously been shown (Eager et al., 1997) that (i) channel conductance is unaltered during the ''activation'' phase of the 4,4′-DTDP effect, (ii) addition of the vehicle for 1 mM 4,4′-DTDP alone, i.e., 10 μl of ethanol, does not alter RyR activity.

Activation of the 21 control channels by 4,4′-DTDP was measured quantitatively by comparing P_o, T_o and F_o during the 2 min of channel activity recorded immediately before adding 1 mM 4,4′-DTDP, with parameter values during the 30-sec period having the highest P_o within 2 min of 4,4′-DTDP addition (Materials and Methods). On average P_o in the 21 channels increased ($P < 0.0001$) from 0.0005 ± 0.0001 to 0.065 ± 0.013.

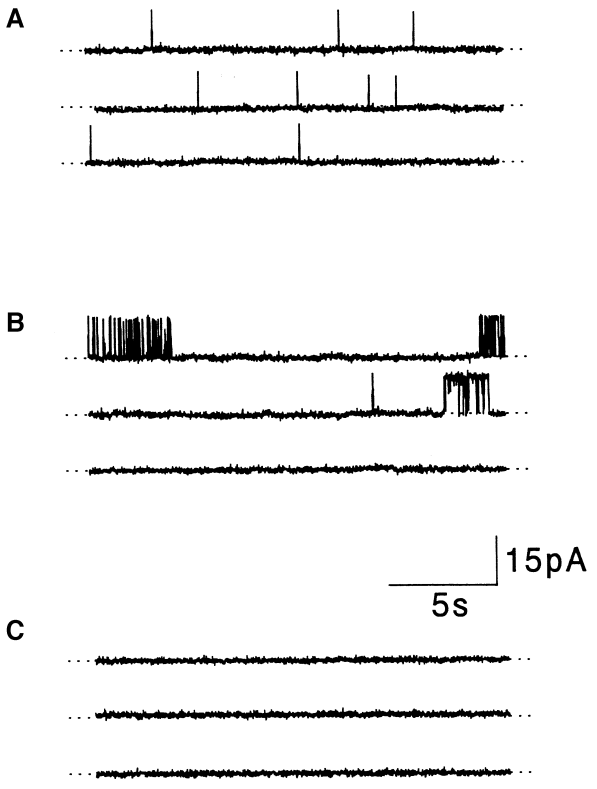

Fig. 1. An example of the effects of 4,4′-DTDP on a cardiac RyR at 10^{-7} M *cis* Ca^{2+}. Continuous 1-min recordings from a single native RyR at a holding potential of +40 mV. Channel opening is upward from the baseline (dotted lines). (*A*) Control; (*B*) activated channel immediately after adding 1 mM 4,4′-DTDP to the *cis* chamber; (*C*) after irreversible loss of channel. The record in *C* was obtained 9 min after adding 4,4′-DTDP and 30 sec after increasing *cis* Ca^{2+} concentration to 1 mM.

The higher P_o was due to increases in duration and frequency of channel opening. On average T_o increased from 1.52 ± 0.23 msec to 22.2 ± 5.1 msec ($P < 0.0001$), while F_o increased from 0.23 ± 0.05 sec^{-1} to 4.6 ± 1.2 sec^{-1} ($P < 0.0001$). The increase in T_o reflected the addition of long time constant components to the open time distribution, with no consistent change in the closed time distributions (Eager et al., 1997). Control open times were in a single exponential (Materials and Methods) with an average $\tau_1 = 1.8 \pm 0.3$ msec. One to three exponentials were seen in open times recorded during the 5 min after 4,4′-DTDP addition, with time constants that fell into 1 to 3 of τ_1, τ_2, τ_3 or τ_4: average $\tau_1 = 1.3 \pm 0.3$ msec ($n = 14$), $\tau_2 = 8.4 \pm 0.9$ msec ($n = 9$), $\tau_3 = 24.0 \pm 2.9$ msec ($n = 14$) and $\tau_4 = 75 \pm 11$ msec ($n = 7$). Although open times in 5 of the 21 RyRs were still described by a single exponential in the presence of 1 mM 4,4′-DTDP, the time constants were in τ_2 or τ_3 (3–50 msec), in contrast to control openings which were within τ_1 (<3 msec).

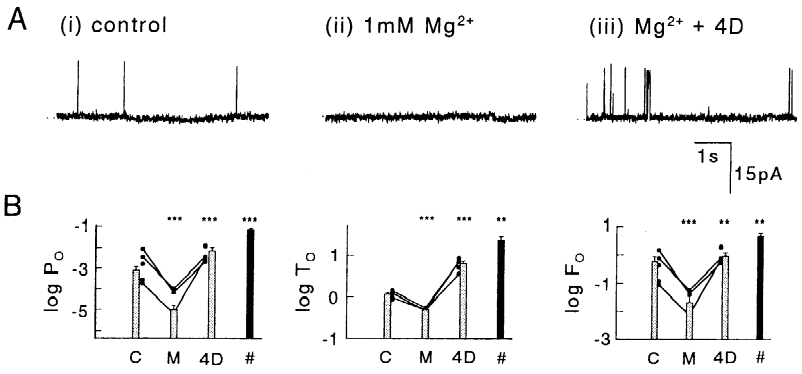

Fig. 2. Inhibition of RyRs by 1 mM *cis* Mg^{2+} (10^{-7} M *cis* Ca^{2+}) and subsequent activation by 4,4′-DTDP. Channels were exposed to Mg^{2+} for 2 min before 4,4′-DTDP was added. (*A*) Records from one channel: (i), with 10^{-7} M *cis* Ca^{2+}; (ii), within 10 sec of adding 1 mM *cis* Mg^{2+}; (iii) within 2 min of adding 1 mM *cis* 4,4′-DTDP to the Mg^{2+}-inhibited channel. (*B*) P_o, T_o and F_o for individual channels (filled circles, linked by lines), during the 2-min control period with 10^{-7} M *cis* Ca^{2+} (C), during 2-min exposure to 1 mM Mg^{2+} (M) and during the 30 sec with the highest P_o within 2 min of 4,4′-DTDP addition (4D). Histograms showing mean ± 1 SEM (vertical bars) for control, 1 mM Mg^{2+} and 1 mM Mg^{2+} plus 4,4′-DTDP are superimposed on the individual data. The bin labeled # is the mean data obtained from 21 channels exposed to 4,4′-DTDP in the absence of Mg^{2+}. Asterisks indicate the significance of differences between each mean and the mean in the preceding bin: $^{**}P < 0.01$; $^{***}P < 0.001$.

Effect of Ligands on 4,4′-DTDP-induced Activation of RyRs

Mg^{2+}-inhibited RyRs at 10^{-7} M cis Ca^{2+}

Mg^{2+} can inhibit cardiac RyRs by binding to either the high affinity Ca^{2+}-activation site, when *cis* Ca^{2+} concentration is low ($<10^{-6}$ M Ca^{2+}), or to the lower affinity Ca^{2+}/Mg^{2+}-inhibition site at higher *cis* Ca^{2+} concentrations (Laver et al., 1997). The effect of Mg^{2+} binding to the high affinity Ca^{2+}-activation site on 4,4′-DTDP-activation was examined by adding 1 mM *cis* Mg^{2+} with *cis* Ca^{2+} of 10^{-7} M and then adding 1 mM *cis* 4,4′-DTDP. Activity in 5 single RyRs was intermittent at 10^{-7} M *cis* Ca^{2+} and was reduced further after adding 1 mM Mg^{2+} (e.g., Fig. 2*A*).

There were significant reductions in the average P_o, T_o and F_o after addition of 1 mM *cis* Mg^{2+} (Fig. 2*B*). Subsequent addition of 1 mM 4,4′-DTDP caused an increase in all three parameters to values that were greater than those with Mg^{2+} alone. P_o and T_o in the 4,4′-DTDP-activated channel were also greater than the control values prior to adding Mg^{2+}. P_o, T_o and F_o were lower with 4,4′-DTDP in the presence of Mg^{2+}, than those recorded with 4,4′-DTDP alone (Fig. 2*B*), showing a summation of the inhibiting effects of Mg^{2+} with the activating effects of 1 mM 4,4′-DTDP.

Analysis of the open time distributions showed that Mg^{2+} prevented the long openings seen during 4,4′-DTDP-activation under control conditions (i.e., with 10^{-7} M *cis* Ca^{2+}, in the absence of Mg^{2+}, *see* Results above). Openings in 4 of 5 channels with 1 mM 4,4′-DTDP plus Mg^{2+}, fell into two components, τ_1 and τ_2, while the fifth channel had events only in τ_2. On average, $\tau_1 = 1.4 \pm 0.1$ msec (47 ± 14% of events, $n = 4$) and $\tau_2 = 9.5 \pm 1.6$ msec (53 ± 14% of events, $n = 5$). This was in contrast to 4,4′-DTDP alone, where events were in τ_1 to τ_4.

Mg^{2+}-inhibited RyRs at 10^{-3} M cis Ca^{2+}

The effect of Mg^{2+} binding to the low affinity Ca^{2+}/Mg^{2+} inhibition site was examined by increasing the *cis* Ca^{2+} concentration to 1 mM. When the high affinity Ca^{2+}-activation site is saturated by increasing the *cis* Ca^{2+} concentration, further addition of either Ca^{2+} or Mg^{2+} then inhibits the cardiac RyRs by binding to the low affinity Ca^{2+}/Mg^{2+} inhibition site (Laver, Baynes & Dulhunty, 1997). Activity in each of 7 single channels was high in the presence of 1 mM Ca^{2+} (e.g., Fig. 3*A*).

The open events became considerably shorter when 10 mM Mg^{2+} was added to the *cis* side of the Ca^{2+}-activated channels and there was a simultaneous increase in F_o in 4 of the 7 channels (Fig. 3). In 3 of the 4 channels showing an increase in F_o, P_o during the control period was low (0.05 to 0.09), despite the presence of 1 mM Ca^{2+}. Although P_o increased by 25 ± 4% in these 3 channels, because of the increase in F_o, mean open times showed the typical 10 mM Mg^{2+}-induced reduction. The increase in F_o in some channels was small and was not investigated further. The reduction in average T_o for all 7 channels was significant and analysis of the open time distributions showed that the number of exponential components fell from four (τ_1–τ_4) in Ca^{2+}-activated channels, to two (τ_1 and τ_2) in channels inhibited by 10 mM *cis* Mg^{2+} or by 10 to 20 mM *cis* Ca^{2+}, with >94% of events in τ_1 in both cases.

A substantial increase in activity was seen when 1 mM 4,4′-DTDP was added to channels that were inhibited by 10 mM Mg^{2+} (e.g., Fig. 3*A*). The increases in average P_o and T_o were significant (Fig. 3), but the values of the parameters were severalfold lower than those obtained when 4,4′-DTDP was added to channels activated by 1 mM *cis* Ca^{2+} (Table).

Thus, as seen with Mg^{2+} binding to the high affinity site (*see Results* above), there is summation of the inhibitory effects of Mg^{2+} binding to the low affinity

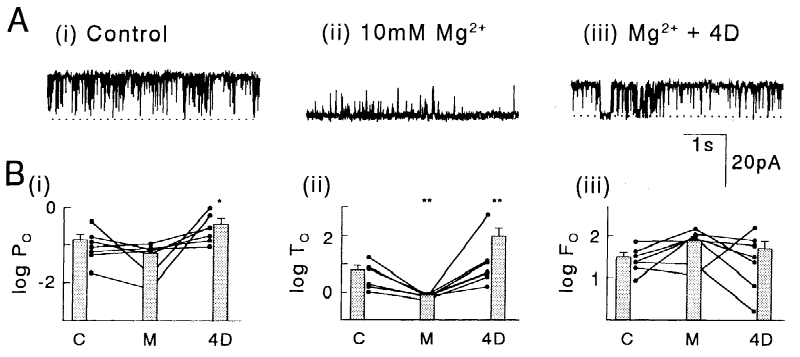

Fig. 3. Inhibition of RyRs by 10 mM *cis* Mg^{2+} (1 mM *cis* Ca^{2+}) and subsequent activation by 4,4′-DTDP. Channels were exposed to Mg^{2+} for 2 min before 4,4′-DTDP was added. (*A*) Records from one channel: (i), with 1 mM *cis* Ca^{2+}; (ii), within 10 sec of adding 10 mM *cis* Mg^{2+}; (iii) within 30 sec of adding 1 mM *cis* 4,4′-DTDP to the Mg^{2+}-inhibited channel. (*B*) P_o, T_o and F_o for individual channels (filled circles, linked by lines), during the 2-min control period with 1 mM *cis* Ca^{2+} (C), during 2-min exposure to 10 mM Mg^{2+} (M) and during the 30 sec with the highest P_o within 2 min of 4,4′-DTDP addition (4D). Histograms showing mean ± 1 SEM (vertical bars) for control, 10 mM Mg^{2+} and 10 mM Mg^{2+} plus 4,4′-DTDP are superimposed on the individual data. Asterisks indicate the significance of differences between each mean and the mean in the preceding bin: $*P < 0.05$; $**P < 0.01$.

Table 1. Comparison of RyR activity with 4,4′-DTDP added to Ca^{2+}-activated channels (1 mM Ca^{2+}), Ca^{2+}-inhibited channels (20 mM Ca^{2+}), or to Mg^{2+}-inhibited channels (1 mM Ca^{2+} + 10 mM Mg^{2+})

	1 mM 4,4′-DTDP +		
	1 mM Ca^{2+} (n = 4)	20 mM Ca^{2+} (n = 5)	1 mM Ca^{2+} + 10 mM Mg^{2+} (n = 6)
P_o	0.9939 (0.0017)	0.2501 (0.0870)***	0.2757 (0.0871)***
T_o	218 (39)	6.3 (2.9)**	7.2 (2.3)**
F_o	4.9 (1.0)	76 (44)	61 (26)

Mean ± SEM (in parentheses) for n channels. There were no differences in mean values of P_o, T_o (in msec) or F_o (in events sec^{-1}) for 4,4′-DTDP-activated channels inhibited by either 20 mM Ca^{2+} or 10 mM Mg^{2+}. Asterisks indicate the significance of differences between channels in the presence of 1 mM Ca^{2+} and those in 20 mM Ca^{2+} or 10 mM Mg^{2+} plus 1 mM Ca^{2+}: $**P < 0.01$; $***P < 0.001$.

Ca^{2+}/Mg^{2+} with the activating action of 4,4′-DTDP. Interestingly, increasing *cis* Ca^{2+} above 1 mM had the same effect on 4,4′-DTDP induced activation as the increase in Mg^{2+} concentration. P_o, T_o, and F_o in five 4,4′-DTDP-activated channels, that were inhibited by 20 mM Ca^{2+}, were similar to the parameters in channels inhibited by 10 mM Mg^{2+} (Table). One Mg^{2+}-inhibited channel included in the individual data in Fig. 3*B*, was not included in the average data in the Table because the increase in T_o to 600 msec upon addition of 4,4′-DTDP was well outside the T_o values recorded with 4,4′-DTDP in the other Mg^{2+}-inhibited channels.

Analysis of the open time distributions showed that, by binding to its low affinity site, Mg^{2+} reduced the number of long open events recorded during the 5 min after 1 mM 4,4′-DTDP: ~85% of events were in τ_1 and τ_2, although there were open events in each of the 5 exponential components and the average $\tau_1 = 1.5 \pm 0.1$ msec (n = 7); $\tau_2 = 6.8 \pm 0.9$ msec (n = 7); $\tau_3 = 36.0 \pm 5.6$ msec (n = 6); $\tau_4 = 240$ msec (n = 1) and $\tau_5 = 745 \pm 85$ msec (n = 2). The average time constants were also in τ_1–τ_5 when 4,4′-DTDP was added to channels activated by 1 mM *cis* Ca^{2+} in the absence of Mg^{2+}, but in contrast to the Mg^{2+} situation, ~64% of events were in τ_3 to τ_5. Note that, although 4,4′-DTDP-induced activation declined in the usual way (between 3 and 5 min), open events were recorded before activity was irreversibly lost between 6 and 10 min (*see* Results below).

ATP-activated RyRs

ATP is a potent activator of RyRs (Meissner, Darling & Eveleth, 1986). The activity in all of 12 channels increased substantially when 4 mM Na_2ATP was added to the *cis* chamber with 10^{-7} M *cis* Ca^{2+} (e.g., Fig. 4*A*), and the increases in the average P_o, T_o and F_o were significant (Fig. 4*B*).

Addition of 1 mM 4,4′-DTDP to the *cis* chamber, 1 to 2 min after ATP, induced a further increase in activity in 9 of the 12 channels (e.g., Fig. 4*A*), with significant increases in average P_o and T_o (Fig. 4*B*). Curiously, and in contrast to results obtained with Mg^{2+}, the effects of ATP and 4,4′-DTDP in these 9 channels were not additive because P_o, T_o and F_o values were the same in 4,4′-DTDP-activated channels, with or without ATP (Fig. 4*B*). Although P_o and T_o were significantly greater than they had been with ATP alone, the relative increases in the two parameters upon addition of 4,4′-DTDP were severalfold smaller than in the absence of ATP. Another unexpected observation was that, in the other 3 ATP-activated channels (not activated by 4,4′-DTDP, and not included in Fig. 4), 4,4′-DTDP depressed the ATP-induced activation, i.e., channel activity returned to low levels and the usual bursts of long openings were not observed.

Analysis of the open time distributions of the 9 channels activated by 1 mM 4,4′-DTDP in the presence

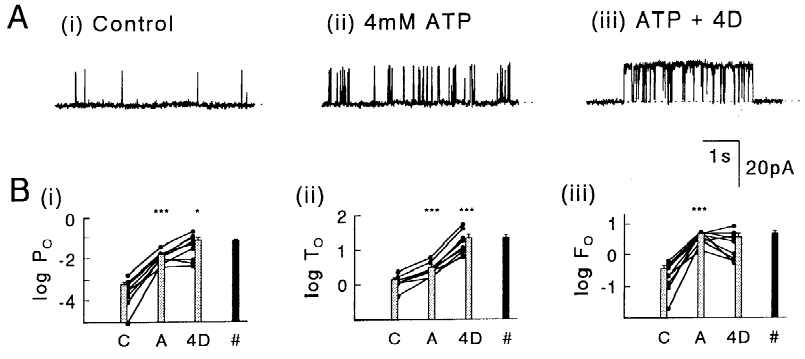

Fig. 4. Activation of RyRs by 4 mM *cis* ATP (10^{-7} M *cis* Ca^{2+}) and subsequent activation by 1 mM 4,4′-DTDP. Channels were exposed to ATP for 1–2 min before 4,4′-DTDP was added. (*A*) Records from one channel: (i), with 10^{-7} M *cis* Ca^{2+}; (ii), within 10-sec of adding 4 mM *cis* ATP; (iii) within 30 sec of adding 1 mM *cis* 4,4′-DTDP to the ATP-activated channel. (*B*) P_o, T_o and F_o for 9 individual channels (filled circles, linked by lines), during the 2-min control period with 10^{-7} M *cis* Ca^{2+} (*C*), during 2-min exposure to 4 mM ATP (M) and during the 30 sec with the highest P_o within 2 min of 4,4′-DTDP addition (4D). Histograms showing mean ± 1 SEM (vertical bars) for control, 4 mM ATP and 4 mM ATP plus 4,4′-DTDP are superimposed on the individual data. The bin labeled # is the mean data obtained from 21 channels exposed to 4,4′-DTDP in the absence of ATP. Asterisks indicate the significance of differences between each mean and the mean in the preceding bin: $*P < 0.05$; $***P < 0.001$. There was no difference between the mean values for 4,4′-DTDP-activated channels in the presence or absence of ATP ($P > 0.49$).

of ATP showed that openings fell into the usual single exponential with 10^{-7} M *cis* Ca^{2+}, before addition of ATP (Fig. 5*A*, filled circle).

Openings in individual channels were also in a single exponential after ATP had been added, but the time constants were either in τ_1 or τ_2 (open squares). Subsequent addition of 1 mM 4,4′-DTDP induced openings in τ_3 or τ_4, in addition to the openings in τ_1 and τ_2. The values of the average time constants (filled triangles, Fig. 5*A*) were similar to those with 4,4′-DTDP in the absence of ATP (Fig. 5*B*).

Two differences were noted between activation by 1 mM 4,4′-DTDP in the presence of ATP and activation by 4,4′-DTDP alone. Firstly, activity increased far more rapidly ($P < 0.01$) after addition of 1 mM 4,4′-DTDP in the presence of ATP (with a delay of only 2.9 ± 1.4 sec), than in its absence (delay = 42.1 ± 12.3 sec). Secondly, there were significantly fewer events ($P < 0.01$) in τ_1 with ATP (5.5 ± 4.4%) than in its absence (28.3 ± 6.3%). There were similar reductions in the latency of 4,4′-DTDP activation and the fraction of events in τ_1 when 1 mM 4,4′-DTDP was added to RyRs activated by *cis* Ca^{2+} (10^{-5} or 10^{-3} M), but the decrease in latency was not seen when 4,4′-DTDP was added to RyRs activated by caffeine (Eager et al., 1997).

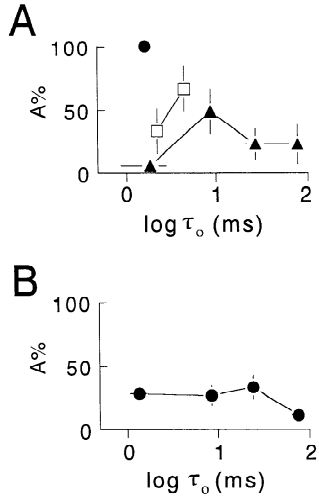

Fig. 5. Effects of *cis* ATP and 4,4′-DTDP on the open time constants of channels with 10^{-7} M *cis* Ca^{2+}. Data were obtained from the same 8 channels used for the average data in Fig. 4 above. Time constants for individual channels were allocated to time constant groups (τ_1 to τ_5, as defined in Materials and Methods), and the average percentage of events in each group (%A) is plotted against the average time constant of each group. (*A*) Time constants, from the same 9 channels used to obtained the average parameters in Fig. 4 above, were recorded with 10^{-7} M *cis* Ca^{2+} alone (filled circle), then after adding 4 mM ATP (open squares) and finally after adding 1 mM 4,4′-DTDP (filled triangles). (*B*) Data obtained from 21 single channels with 10^{-7} M *cis* Ca^{2+} after adding 1 mM 4,4′-DTDP (filled circles). The vertical bars show ±1 SEM for average %A, and the horizontal bars show ±1 SEM for the average time constants, where these exceed the dimensions of the symbols.

Effects of Ligands on the Long term 4,4′-DTDP-induced Loss of RyR Activity

The overall time course of channel activation and the irreversible loss of activity was essentially the same in the absence and in the presence of Mg^{2+} and ATP, with abolition of RyR activity seen within 10 min of adding 1 mM 4,4′-DTDP to the *cis* chamber in most channels in the presence of *cis* Mg^{2+} or ATP. Channels were tested for irreversible loss of activity with voltage pulses to negative potentials and by increasing *cis* Ca^{2+} concentration to 1 mM. Eight channels were tested for loss of activity in the presence of 4,4′-DTDP with 1 or 10 mM Mg^{2+} and 8 channels tested with 4,4′-DTDP and 4 mM ATP. Activity in 7 of the channels with Mg^{2+} was irreversibly lost within 10 min of adding 4,4′-DTDP, but one channel with 10 mM Mg^{2+} remained active for >45

min. Seven channels with ATP lost their activity irreversibly within 10 min of 4,4′-DTDP addition, while one channel remained active for >20 min. The significance of the failure of these two channels to irreversibly close was not explored further. Interestingly, the three channels that were not activated by 4,4′-DTDP in the presence of 4 mM ATP (*above*), lost their activity irreversibly by 8 min after adding 4,4′-DTDP. This observation is significant because it shows clearly that the loss of activity can occur without the 4,4′-DTDP-induced activation, and supports the hypothesis that channel activation and irreversible loss of activity are caused by oxidation of different SH residues.

Discussion

The sulfhydryl-specific reagent, 4,4′-DTDP at 1 mM, increases cardiac RyR activity at resting cytoplasmic [Ca^{2+}] (10^{-7} M *cis* Ca^{2+}), before the channel irreversibly closes after 5–10 min exposure to the reagent. These effects of 4,4′-DTDP depend on oxidation of thiol groups because they are prevented or reversed by DTT (Eager et al., 1997). Other non thiol-specific oxidizing agents have similar actions (Abramson et al., 1995; Favero et al., 1995). We show here that these effects of 4,4′-DTDP occur with physiological concentrations of Mg^{2+} or ATP, and that the degree of activation is altered by ligand binding to the RyR.

Effects of Mg^{2+}

Inhibition of RyRs by Mg^{2+} did not prevent their activation by 4,4′-DTDP. Binding of 1 mM Mg^{2+} to the high affinity Ca^{2+}-activation site, prevented the very long channel openings induced by 4,4′-DTDP in the absence of Mg^{2+}. On the other hand, binding of 10 mM Mg^{2+} to the low affinity Ca^{2+}/Mg^{2+} inhibition site reduced the number of long open events but did abolish them. Although the 4,4′-DTDP-induced activity is less when RyRs are inhibited by (millimolar) free Mg^{2+}, there is never-the-less a large increase in activity upon thiol oxidation.

The fact that the gating of the 4,4′-DTDP-activated channel differed, depending on whether the channel was inhibited by 1 mM Mg^{2+} at 10^{-7} Ca^{2+}, or by 10 mM Mg^{2+} at 1 mM Ca^{2+}, strongly supports separate high and low affinity Mg^{2+} binding sites on the cardiac RyR (Nagasaki & Kasai, 1983; Meissner et al., 1986; Laver et al., 1997). Further, similar changes in 4,4′-DTDP-activation in channels inhibited by either 10 mM Mg^{2+} or by 20 mM Ca^{2+} supports the contention that the Mg^{2+} binds with similar affinity to Ca^{2+}, to the low affinity Ca^{2+} binding site (Laver et al., 1997).

A Convergence Model for Mg^{2+} and 4,4′-DTDP modulation of the RyR

The biphasic action of 4,4′-DTDP on cardiac RyRs has been described by a ''convergence'' model (Eager et al., 1997) in which signals initiated by thiol oxidation or ligand binding, at sites remote from the ion channel, converge on ''gates'' in the channel pore. It is proposed that the oxidation of a reactive cysteine increases the probability of one gate being open, while oxidation of a less reactive thiol irreversibly shuts a second gate. The results of the present study are consistent with signals arising from Mg^{2+} binding and from SH oxidation converging on two separate gates. An alternative possibility is that different cysteine residues were oxidized in the presence of 1 mM and 10 mM Mg^{2+}. However, this would require gross conformational changes which are unlikely with the rapidly reversible effects of Mg^{2+}. The qualitatively similar effects with 1 and 10 mM Mg^{2+} suggest that the subtle quantitative changes are most likely to be due to modulation of the oxidation response, rather than a grossly different oxidation reaction.

The thiols which alter RyR activity when oxidized are accessible to reagents in the cytoplasm and may reside on the protein surface. Calmodulin (Wagenknecht et al., 1994) and FKBP12 (Samso et al., 1997) bind to the outer margin of the skeletal RyRs, at least 10 nm from the central pore (Radermacher et al., 1994). Similar ligand binding sites probably exist on the cardiac RyR since it is 3 dimensionally similar to the skeletal RyR (Sharma et al., 1997). ''Links'' between surface sites and the pore could be the struts and bridges connecting the periphery of the RyR with its central domain (Wagenknecht et al., 1994).

Interaction between ATP and 4,4′-DTDP in activating RyRs—A Common Link to the Activation Gate

In contrast to the additive effects of Mg^{2+} (this report), or Ca^{2+} or caffeine (Eager et al., 1997), with 1 mM 4,4′-DTDP-induced activation, binding of ATP to the RyR did not alter the degree of activation by 4,4′-DTDP. Similarly, Ca^{2+} efflux from SR vesicles induced by reactive disulfides is reduced in the presence of adenine nucleotides (Zaidi et al., 1989; Prabhu & Salama, 1990*a*). Conversely, SR Ca^{2+} efflux induced by alcian blue (Abramson et al., 1988) or Ag^{+} (Prabhu & Salama, 1990*b*) is enhanced by adenine nucleotides, possibly because residues other than cysteines are oxidized (van Iwaarden, Driessen & Konings, 1992; Stadtman, 1993) or because a different population of thiols is oxidized.

A ''convergence'' model was suggested to explain additive activation of RyRs by 1 mM 4,4′-DTDP, and either Ca^{2+} or caffeine (Eager et al., 1997). The lack of

summation of activation by ATP and 4,4′-DTDP suggests a common mechanism in the action of these agents. 4,4′-DTDP does not oxidize the ATP binding site, because the binding site lacks cysteine residues (Otsu et al., 1990). However a cooperative action could be explained if (i) signals from SH oxidation and ATP binding acted on one ''link'' to the activation gate and (ii) the response of this link was nearly saturated by ATP binding and saturated after the oxidation reaction.

An interaction between ATP binding and SH oxidation site is also suggested by the fact that 4,4′-DTDP removed ATP-induced activation in some channels (although irreversible loss of activity proceeded within the usual time frame). The lack of 4,4′-DTDP-induced activation in these channels confirms an observed fall in thiopyridone production when reactive disulfide was added to SR vesicles in the presence of adenine nucleotides (Zaidi et al., 1989; Prabhu & Salama, 1990*a*). Both results could be explained if (i) the ATP binding site was physically close to the oxidation site and (ii) stearic interactions between 4,4′-DTDP and ATP prevented either molecule from properly accessing its target site (*see also* Zaidi et al., 1989).

At first sight, the hypothesis suggesting saturation of the ''link'' to the activation gate by SH oxidation is not supported by the observation that ATP induces Ca^{2+} release from SR vesicles after their oxidation by micromolar concentrations of reactive disulfides (Zaidi et al., 1989; Prabhu & Salama, 1990*a*). However, Ca^{2+} release is a population response, and a large fraction of channels are not oxidized by micromolar reactive disulfide (Eager et al., 1997). Thus the ATP evoked Ca^{2+} release can be attributed to activation of channels that are not oxidized.

Cysteine Residues that Might be Oxidized to Activate RyRs

The potential ATP binding region in the cardiac RyR at positions 2619–2652 contains the nucleotide binding motif GXGXXG (2627–2632; Otsu et al., 1990), as well as two conserved cysteine residues at 2618 and 2623 in skeletal and cardiac muscle. Four of six cysteines between 2462 and 2578 are also conserved. All residues are near a region that (i) is protease sensitive and possibly located on the surface of the molecule (Marks et al., 1990) and (ii) contains potential phosphorylation and calmodulin binding sites, and is proposed to be the modulator region for the RyR (Otsu et al., 1990).

4,4′-DTDP Oxidizes at Least Two Sites

A central assumption in the ''convergence'' model is that separate sites are oxidized to induce channel activation and the cessation of activity (Eager et al., 1997). However oxidation of one thiol group could trigger sequential events which activate the channel and then prevent its opening, so that activation *must* precede the loss of activity. This was not the case since activity ceased in ATP-activated RyRs that were not first activated by 4,4′-DTDP. Further evidence against serial events is that low concentrations of 4,4′-DTDP (100 nM–100 μM, depending on *cis* $[Ca^{2+}]$) activated the channel to the same extent as 1 mM 4,4′-DTDP, but did not abolish channel activity (Eager et al., 1997). The higher 4,4′-DTDP concentration required to abolish activity suggests that a less reactive SH group is involved in this second phase of the biphasic action of 4,4′-DTDP.

In conclusion, the reactive disulfide 4,4′-DTDP induced an increase in the activity of single cardiac RyRs at resting cytoplasmic Ca^{2+} concentrations (10^{-7} M), by oxidation of cysteine residues in the RyR protein complex. The oxidation-induced activation occurred when channels were inhibited by $\geq$1 mM *cis* Mg^{2+}, or activated by 4 mM *cis* ATP. In contrast to other activating ligands (Ca^{2+} or caffeine), which enhance the degree of activation by 4,4′-DTDP, the strongest channel activity induced by 4,4′-DTDP was the same in the presence or absence of ATP. This result suggested an interaction between the cysteine residues whose oxidation enhances channel activity, and the ATP binding domain in the cardiac RyR. Two conserved cysteine residues near the putative ATP binding domain of cardiac RyRs are suggested as potential oxidation/activation sites. The results are consistent with a convergence model for ligand regulation of RyR channel opening.

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