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Cardiac Ryanodine Receptor Activity is Altered by Oxidizing Reagents in Either the Luminal or Cytoplasmic Solution

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Abstract. The location of reactive cysteine residues on the ryanodine receptor (RyR) calcium release channel was assessed from the changes in channel activity when oxidizing or reducing reagents were added to the luminal or cytoplasmic solution. Single sheep cardiac RyRs were incorporated into lipid bilayers with 10^{-7} M cytoplasmic Ca^{2+}. The thiol specific-lipophilic-4,4′-dithiodipyridine (4,4′-DTDP, 1 mM), as well as the hydrophilic thimerosal (1 mM), activated and then inhibited RyRs from either the *cis* (cytoplasmic) or *trans* (luminal) solutions. Activation was associated with an increase in the (a) mean channel open time and (b) number of exponential components in the open time distribution from one (~2 msec) to three (~1 msec; ~7 msec; ~15 msec) in channels activated by *trans* 4,4′-DTDP or *cis* or *trans* thimerosal. A longer component (~75 msec) appeared with *cis* 4,4′-DTDP. Activation by either oxidant was reversed by the thiol reducing agent, dithiothreitol. The results suggest that three classes of cysteines are available to 4,4′-DTDP or thimerosal, SHa or SHa* activating the channel and SHi closing the channel. SHa is either distributed over luminal and cytoplasmic RyR domains, or is located within the channel pore. SHi is also located within the transmembrane domain. SHa* is located on the cytoplasmic domain of the protein.

Key words: Ryanodine receptor — Luminal oxidation — Reactive disulfides — Sarcoplasmic reticulum — Sulfhydryl oxidation

Introduction

The ryanodine receptor (RyR) is the calcium release channel in the sarcoplasmic reticulum (SR) of cardiac and skeletal muscle, as well as in the endoplasmic reticulum of smooth muscle and non-muscle cell types where it shares a functional role with IP_3 receptor calcium channels (Coronado et al., 1994; Dulhunty et al., 1996). Ca^{2+} efflux through the RyR is thought to contribute to the detrimental increase in cytoplasmic [Ca^{2+}] which occurs under pathological conditions, e.g., ischemia and reperfusion (Sies et al., 1972; Curello et al., 1985) and perhaps during aging (Stadtman, 1992). Reactive oxygen species, whose concentrations increase under these pathological conditions, can open RyR channels and release Ca^{2+} from the internal Ca^{2+} stores (Holmberg et al., 1991; Holmberg & Williams, 1992; Boraso & Williams, 1994; Favero, Zable & Abramson, 1995). Cysteine residues on the RyR are likely to be the targets for the reactive oxygen species because oxidation of sulfhydryl (SH) groups induces Ca^{2+} release from SR vesicles and increases single RyR channel activity (Abramson & Salama, 1989; Boraso & Williams, 1994; Eager et al., 1997).

Evidence that oxidants act by oxidizing sulfhydryl groups on cysteine residues is that their effects are reversed by reducing agents (Boraso & Williams, 1994; Abramson et al., 1995; Favero et al., 1995). However, SH oxidation has been confirmed in only three studies of the effects on single RyR channel activity of oxidizing reagents which react specifically with free -SH groups (Nagura et al., 1988; Eager et al., 1997; Eager & Dulhunty, 1998). Eager et al. (1997) showed that the thiol-specific reactive disulfides, 4,4′-dithiodipyridine (4,4′-DTDP) and 2,2′-dithiodipyridine (2,2′-DTDP), induce a transient activation of cardiac RyR channels followed by an irreversible loss of activity. The actions of the DTDPs are not seen when reducint agent, dithiothreitol (DTT), is added to the solution before 4,4′-DTDP, and activation by 4,4′-DTDP is reversed by addition of DTT (Eager et al., 1997).

Because of the potential for in vivo modulation of

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RyRs by oxidants (Eley et al., 1991), it is important to understand the mechanisms by which oxidation of sulfhydryl residues alters RyR activity. Information about the influence of SH oxidation on channel activity is also important in understanding the mechanisms that regulate RyR opening, the way in which cysteine residues are involved in gating the channel and which cysteine residues can modify channel activity. There is, at present, very little information about which of the 89 cysteine residues on the cardiac RyR (Otsu et al., 1990) can influence channel activity. A previous study suggested that an SH group near the ATP-binding domain on the cytoplasmic surface of the RyR may be involved in channel activation (Eager & Dulhunty, 1998). However, there are many other cysteine residues located on both the putative luminal and cytoplasmic sides of the RyR channel (Otsu et al., 1990), some of which may also influence channel function. Indeed, multiple classes of sulfhydryls modulate the skeletal muscle ryanodine receptor (Aghdasi et al., 1997). Since most reactive oxygen species and reactive disulfides are lipid soluble it is not clear where their functional reactions occur, although the reagents are generally added to solutions in contact with the cytoplasmic face of the channel. The presence of reactive sulfhydryls on the luminal domain of mammalian RyRs has not yet been demonstrated. Neither glutathione, nor glutathione disulfide, alter rabbit RyR activity when added to the luminal side of the channel (Zable, Favero & Abramson, 1997). Conversely, frog skeletal muscle RyRs are activated when H_2O_2 is added to the luminal solution, with catalase present in the cytoplasmic solution (Oba, Ishikawa & Yamaguchi, 1998). In the present study we compare the effects on cardiac RyR activity of adding the lipophilic 4,4′-DTDP to the luminal or cytoplasmic solutions. We then look at changes in RyR channel activity following cytoplasmic or luminal addition of the membrane impermeable organomercurial, thimerosal. Thimerosal reacts specifically with SH groups on cysteine residues (Sayers et al., 1993) and is used as a hydrophilic oxidizing agent (Chiamvimonvat et al., 1995). The results show that residues accessible to the luminal as well as the cytoplasmic solutions bathing the cardiac RyR are susceptible to modulation by oxidation reactions.

Materials and Methods

Preparation of SR Microsomes

Methods, based on Sitsapesan et al. (1991), are described in detail in Laver et al. (1995). 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. The crude fraction and heavy SR were stored in liquid N_2 or at −70°C.

Lipid Bilayers and Solutions

Bilayers were formed from phosphatidylethanolamine, phosphatidylserine and phosphatidylcholine (5:3:2) (Avanti Polar Lipid, AL) across a 150–200 μm diameter aperture in a Delrin cup. 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[hyroxymethyl]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* $[Ca^{2+}]$ 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 minimize Cl^- currents. Experiments were performed at room temperature (21–24°C).

Data Acquisition and Analysis

Channel activity was displayed on an oscilloscope and stored on video tape 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 and closed times and mean open time (T_o) 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). Open time distributions were displayed as described by Sigworth & Sine (1987) for data obtained during a 2 min control period, and during the 5 min immediately after drug addition. For display of average trends in the distributions, open time constants were allocated to: τ_1, <3 msec; τ_2, 3–12 msec; τ_3, 12–50 msec; or τ_4, 50–500 msec (Eager et al., 1997). No time constants were measured in τ_5 (>500 msec) in the present study. Sampling at double the filter frequency was considered sufficient for the analysis performed in the present study, since we have previously 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 mean of the logarithms of paired variables was tested. A value of <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. Thimerosal and DTT (Sigma, St. Louis, MO) were dissolved in deionized water.

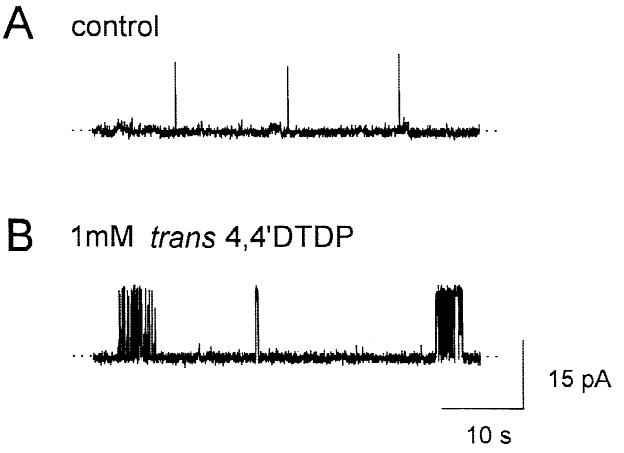

Fig. 1. Effect of 1 mM 4,4′-DTDP addition to the *trans* chamber on the single channel activity of a cardiac RyR incorporated into an artificial lipid bilayer from native SR vesicles. (*A*) Control activity immediately before 4,4′-DTDP addition. (*B*) During the period of maximum activation within 3 min of adding 1 mM 4,4′-DTDP. Channel activity was recorded at +40 mV with 10^{-7} M/10^{-3} M Ca^{2+} *cis/trans.* The dotted line shows the zero current level. Channel opening is upward.

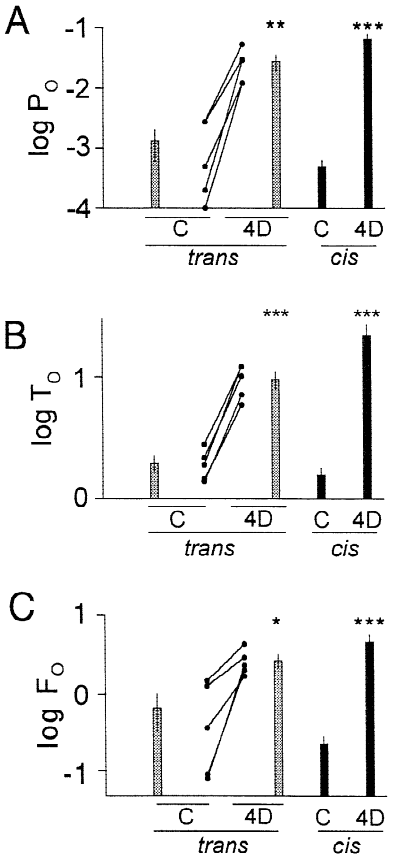

Fig. 2. Effects of 1 mM 4,4′-DTDP on P_o (*A*), T_o (*B*) and F_o (*C*) in 5 single channels under control conditions (2 min immediately before 4,4′-DTDP addition, *C*), and after adding 4,4′-DTDP to the *trans* chamber (during the 30 sec of highest channel activity, 4*D*). The single channel data are shown as the filled circles connected by lines. Stippled bars — mean ± SEM for control and with *trans* 4,4′-DTDP. Solid bars — included for comparison, control activity (*C*) and after 4,4′-DTDP addition (4*D*) to the *cis* chamber (Eager & Dulhunty, 1998). Asterisks indicate the significance of the difference between the control data and the data obtained with 4,4′-DTDP. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Results

4,4′-DTDP Activates Cardiac RyRs When Added to the *Trans* Chamber

Addition of the lipophilic 4,4′-DTDP to the *cis* chamber (cytoplasmic side of the channel (Eager et al., 1997; Eager & Dulhunty, 1998)) causes an initial increase in cardiac RyR channel activity followed by an irreversible loss of activity which occurs after ~5 min with 1 mM 4,4′-DTDP (Eager et al., 1997). Application of 4,4′-DTDP to the *trans* chamber (luminal channel surface) also caused an increase in activity at +40 mV, with 10^{-7} M *cis* Ca^{2+} and 10^{-3} M *trans* Ca^{2+} (Fig. 1). The occasional brief openings that characterized control channel activity at 10^{-7} M *cis* Ca^{2+} were replaced, for a period of up to 3 min after adding 1 mM 4,4′-DTDP, by bursts of activity lasting several seconds and containing long channel openings (Fig. 1). Long closed times separated both the occasional openings in control activity, and the bursts of openings in the 4,4′-DTDP-activated channels. The increase in channel activity occurred with an average delay of 37 ± 16 sec ($n = 5$) after 4,4′-DTDP addition to the *trans* chamber, a delay that was similar ($P = 0.85$) to the 42 sec reported when the reactive disulfide was applied to the *cis* chamber (Eager & Dulhunty, 1998).

Analysis of single channel records showed that the values of P_o, T_o and F_o were increased above control in each of five channels examined, during the 30-sec period with the highest activity after the channel had been exposed to *trans* 4,4′-DTDP, with an increase in each average value (Fig. 2). The increase in P_o was due to a ~5-fold increase in mean channel open time and a ~4 fold increase in event frequency. The solid bars in Fig. 2, showing average P_o, T_o and F_o before and after adding 4,4′-DTDP to the *cis* chamber (Eager & Dulhunty, 1998), have been included for comparison. Although control values for the *cis* experiment were ~2.5 times lower than those for the *trans* experiment, the maximum P_o and T_o with 1 mM *cis* 4,4′-DTDP were ~2 to 3 times higher ($P < 0.05$) than those measured after *trans* addition of the drug. The differences between the control values reflect normal variations between control channel recordings. The microsomal preparation used in the present experiments was the same as that used in the previously reported experiments. DTT was present in the storage solution for all vesicles. Thus the redox state of

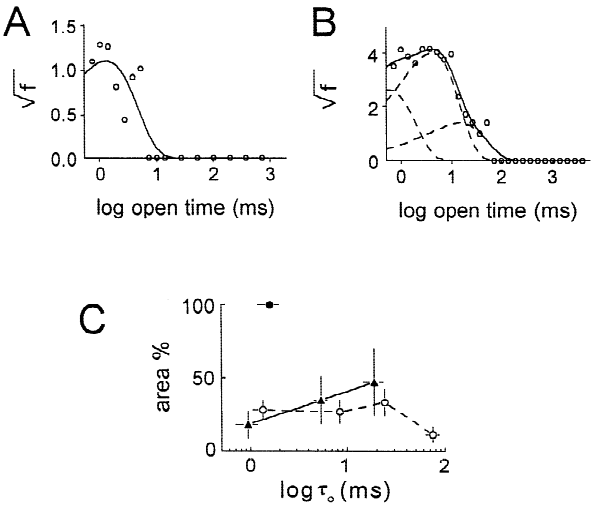

Fig. 3. Effect of 1 mM *trans* 4,4′-DTDP on the open time distribution. The square root of the frequency of open times is plotted in logged bins: *A* and *B* — open time distributions for a single channel; (*A*) control (2 min immediately before 4,4′-DTDP addition) and (*B*) during the 5 min after adding *trans* 4,4′-DTDP. In *A* and *B:* open symbols — data points; continuous line — best multiple exponential fit to the data; broken lines — individual exponential components (superimposed on the continuous line in *A*). (*C*) Average % of events (area %) in each component, having an average open time constant time constant (τ_o). Filled circle — control data ($n = 5$). Filled triangles — after *trans* 4,4′-DTDP addition ($n = 5$). Open circles — after *cis* 4,4′-DTDP addition ($n = 21$) (Eager & Dulhunty, 1998), included for comparison. Vertical bars indicate ±1 SEM of the mean area and horizontal bars indicate ±1 SEM of the mean open time constant.

the channels should have been the same at the start of all experiments.

The effect of *trans* 4,4′-DTDP on open times was examined further by examining the open time distributions for individual channels, plotted in logged bins (Sigworth & Sine, 1987) to illustrate exponential components over a wide range of times. The peaks in the distribution correspond to the time constants of the exponential components (Fig. 3). Although the scatter of the control data is large because there were very few channel openings, the openings clearly fell into a single exponential component with a time constant, τ_1, of 1.2 msec. In contrast, 3 exponential components were required to fit the data with *trans* 4,4′-DTDP, with time constants $\tau_1 = 0.5$ msec, $\tau_2 = 4.0$ msec and $\tau_3 = 14.2$ msec. On average ($n = 5$), the number of components increased from one (τ_1, filled circle) in control, to three (τ_1, τ_2 and τ_3, filled triangles) after adding 4,4′-DTDP (Fig. 3*C*). 4,4′-DTDP induced openings in τ_1, τ_2 and τ_3, but not in τ_4, in 8 other channels in which the reactive disulfide was added to the *trans* chamber with DTT present in the *cis* chamber (*see* Results below). The restriction of open times to the first three time constant components is in contrast to openings seen in τ_1–τ_4 ($n = 21$, open circles) after 4,4′-DTDP addition to the *cis* chamber with 10^{-7} M *cis* Ca^{2+} (Eager & Dulhunty, 1998). Seven of the 21 channels exposed to *cis* 4,4′-DTDP had openings in τ_4.

To summarize, activation by 4,4′-DTDP applied to the *cis* or *trans* chamber differs in (a) the magnitude of increase in P_o and T_o and (b) the number of exponential components in the open time distribution. These differences suggest that some SH groups, initially oxidized by *trans* 4,4′-DTDP, differ from the SH groups oxidized by 4,4′-DTDP in the *cis* solution. The different actions of *cis* and *trans* 4,4′-DTDP further suggests that activation within 3 min of 4,4′-DTDP addition to the *trans* chamber is not due to oxidation of cytoplasmic residues after drug equilibration across the bilayer.

RyR activation by 4,4′-DTDP was due to thiol oxidation. Channel activity returned to normal levels when 2 mM DTT was added to the *trans* chamber within 30–60 sec after RyR activation by 1 mM *trans* 4,4′-DTDP ($n = 5$). A similar reversal of activation was seen when *cis* application of 1 mM 4,4′-DTDP was followed by either DTT or glutathione (Eager et al., 1997). Furthermore, the activation by 4,4′-DTDP depended on the oxidation of -SH groups on the side of the bilayer to which the drug was added. Activation by 1 mM *cis* 4,4′-DTDP was seen in 7 out of 7 channels when 2 mM DTT was present in the *trans* chamber during the experiment. Similarly, 1 mM 4,4′-DTDP added to the *trans* chamber activated RyRs in 7 out of 8 experiments in which DTT was present in the *cis* chamber.

Channel activation with *trans* 4,4′-DTDP was transient and all activity was irreversibly lost within 10 min of adding the drug in 3 out of 3 experiments where reversibility was tested. A similar biphasic effect of *cis* 4,4′-DTDP (1 mM) was seen on cardiac RyR activity (Eager et al., 1997). Channels could not be re-activated by voltage pulses or by increasing *cis* [Ca^{2+}] to 1 mM when activity was lost after long exposures to either *cis* or *trans* 4,4′-DTDP.

As with activation, the loss of activity was also due to an oxidation reaction since it was not seen when 2 mM DTT was added to the *trans* chamber within 4 min of adding *trans* 4,4′-DTDP ($n = 4$) or when *cis* DTT was added prior to *cis* 4,4′-DTDP (Eager et al., 1997). Interestingly, if the *cis* solution contained 2 mM DTT when 1 mM 4,4′-DTDP was added to the *trans* chamber, channel activation was not followed by the usual loss of activity after 10 min in 5 out of 6 experiments. In the converse situation, activity in 4 of 4 experiments was lost in the usual way after adding 1 mM *cis* 4,4′-DTDP with 2 mM DTT in the *trans* chamber. These results suggest that the loss of activity with 4,4′-DTDP depends on the oxidation of an SH group that is located on the cytoplasmic side of the channel protein.

Thimerosal Activates the RyR from the Luminal and Cytoplasmic Sides

The lipid impermeable organomercurial, thimerosal, was used to further explore the effects of SH oxidation by

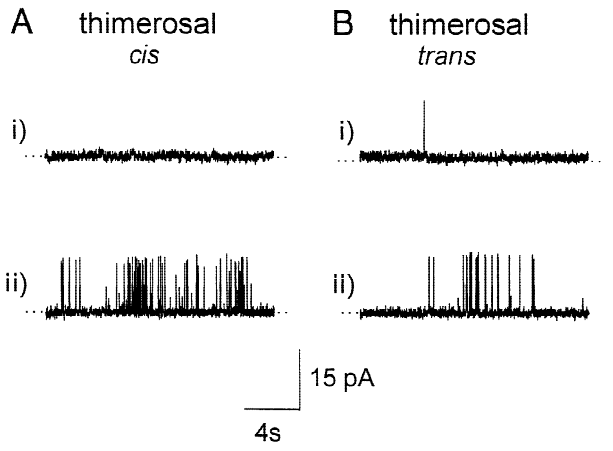

Fig. 4. Effect of adding 100 μM thimerosal to the *cis* (*A*) or *trans* (*B*) chamber on the single channel activity of separate cardiac RyRs. (i) Control activity immediately before thimerosal addition. (ii) During the period of maximum activation after adding thimerosal. Channel activity was recorded at +40 mV with 10^{-7} M/10^{-3} M Ca^{2+} *cis*/*trans*. The dotted line shows the zero current level. Channel opening is upward.

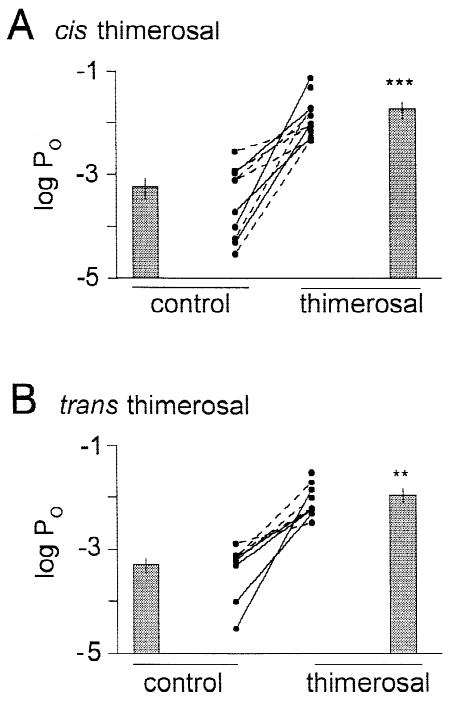

Fig. 5. The effects on P_o of adding thimerosal to the *cis* ($n = 12$, *A*) or *trans* ($n = 9$, *B*) chamber. Control data (control) was recorded during the 2 min immediately before thimerosal addition. Data in thimerosal (thimerosal) were recorded during the 30 sec of highest activity after adding 100 μM or 1 mM of the oxidizing reagent. The broken lines connect data obtained with 100 μM thimerosal and the continuous lines connect data obtained with 1 mM thimerosal. The two sets of data were lumped together to obtain average values. The stippled bars show the mean ± 1 SEM for control and in the presence of thimerosal. Asterisks show the significance of the difference between control and thimerosal data: $^{**}P < 0.01$; $^{***}P < 0.001$.

using a class of oxidizing agent that was distinctly different from the reactive disulfides. Different classes of oxidizing agents can have different accessibility to reactive thiol groups and have the potential to oxidize different thiol residues (van Iwaarden, Driessen & Konings, 1992). Thimerosal activated the RyR, when added to either the *cis* ($n = 12$) or the *trans* ($n = 9$) chamber (*cis* and *trans* additions were performed in separate experiments), with 10^{-7} M *cis* Ca^{2+} and 10^{-3} M *trans* Ca^{2+} (Fig. 4). The increase in activity with *cis* thimerosal had an average latency of 54 ± 16 sec (1 mM, $n = 6$) or 190 ± 36 sec (100 μM, $n = 6$). This 54 sec delay with 1 mM thimerosal was not significantly different ($P > 0.6$) from the 42 sec delay when 1 mM 4,4′-DTDP was added to the *cis* chamber (Eager & Dulhunty, 1998). In contrast, *trans* thimerosal activated the RyRs after a latency of 14 ± 12 sec (1 mM, $n = 4$) or 16 ± 2 sec (100 μM, $n = 5$). The delay of 16 sec before activation by 100 μM *trans* thimerosal was shorter ($P < 0.01$) than the 190 sec when the drug was added to the *cis* chamber. Activation by 1 mM *trans* thimerosal also tended to be more rapid than after *cis* application.

Because of the slow activation by 100 μM *cis* thimerosal, the 30 sec of maximum activation selected for analysis was during the 5 min period after drug addition, rather than within the usual 2 min. However, P_o, T_o or F_o during maximum activation, as well as the open time distributions, were not significantly different ($P > 0.15$) with either 100 μM or 1 mM thimerosal and data obtained with the two concentrations were combined for presentation in Figs. 5 and 6 and in Tables 1 and 2. P_o increased with thimerosal, in each of 12 experiments after *cis* addition and in each of 9 experiments after *trans* addition (Fig. 5), due to significant increases in both T_o and F_o (Table 1). Average parameter values were not significantly different ($P > 0.05$) when thimerosal was added to the *cis* or *trans* chamber.

The increases in the mean open time with *cis* and *trans* thimerosal were due to longer time constant components in the open time distribution. Open times fell into the usual single exponential (τ_1) in control, and into three components (τ_1, τ_2 and τ_3) after drug addition (Fig. 6). The average percentage of events in the shortest component (τ_1) of 73 ± 6.3% after *cis* addition ($n = 12$) was greater ($P < 0.01$) than the 34 ± 10% ($n = 9$) after *trans* application and was greater than that in τ_1 after either *cis* or *trans* additions of 4,4′-DTDP (*see* Fig. 3 above). Long openings in τ_4 (50 to 500 msec), characteristic of 4,4′-DTDP-activation from the *cis* side, were not seen in channels activated by *cis* or *trans* thimerosal.

The increase in P_o induced by drug addition to either the cytoplasmic or luminal side of the channel was significantly less with thimerosal than with 4,4′-DTDP and this difference in P_o was most apparent after drug addition to the *cis* chamber, due to long mean open times with *cis* 4,4′-DTDP (Table 2). The longer mean open time with *cis* 4,4′-DTDP was due to long channel open-

Table 1. Effects of *cis* and *trans* thimerosal on P_o, T_o and F_o in 10^{-7} M Ca^{2+}

	Cis (n = 12)			*Trans* (n = 9)		
	Control	Thimerosal	P	Control	Thimerosal	P
P_o	0.0006 ± 0.0003	0.0189 ± 0.0065	<0.0001	0.0005 ± 0.0002	0.0111 ± 0.0031	<0.01
T_o	1.6 ± 0.4	4.2 ± 1.0	<0.01	1.5 ± 0.2	6.1 ± 1.1	<0.001
F_o	0.3 ± 0.1	5.8 ± 1.8	<0.001	0.3 ± 0.1	2.0 ± 0.6	<0.01

Mean ± SEM for n channels under control conditions and after thimerosal addition. P_o, open probability. T_o, mean open time (msec). F_o, number of events (sec^{-1}). P values: significance of difference between means for control and thimerosal.

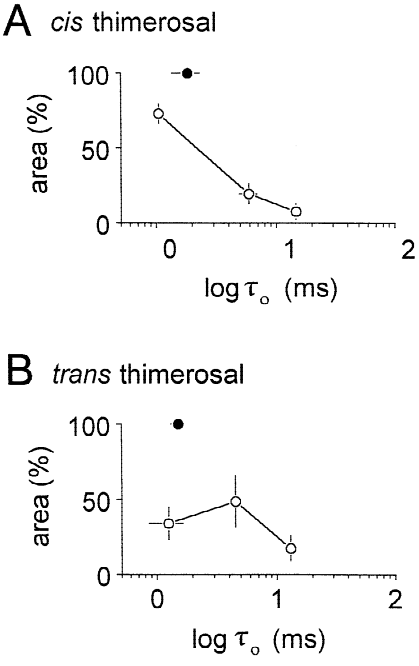

Fig. 6. Effects of adding 100 μM or 1 mM thimerosal to the *cis* (*A*) or *trans* (*B*) chamber on the open time distributions of RyRs at 10^{-7} M *cis* Ca^{2+}. Open times were plotted as the square root of frequency in logged bins and fitted with a multiple exponential function (*see* Fig. 3 above). The open time distributions were the same with 100 μM and 1 mM thimerosal and the results at the two concentrations have been lumped together. The graphs show the average % of events (area %) in each exponential component having an average open time constant time constant (τ_o). Filled circle — control data, 2 min before thimerosal addition. Open circles — the 5-min period following 4,4′-DTDP addition. Vertical bars indicate ±1 SEM of the mean area and horizontal bars indicate ±1 SEM of the mean open time constant.

ings in τ_4, which were not seen with either *trans* 4,4′-DTDP or with thimerosal in either chamber.

As with 4,4′-DTDP, activation by thimerosal was due to oxidation of SH groups. Addition of 2–5 mM DTT to either the *cis* (n = 3) or *trans* (n = 3) solution, within 40 sec to 4 min after adding 1 mM thimerosal to either the *cis* or *trans* solution respectively, reversed the thimerosal-induced activation and restored channel activity to control values. Furthermore, channel activity was not altered by 1 mM *cis* thimerosal if the *cis* chamber contained 5 mM DTT before the oxidant was added (n = 3).

Thimerosal, like 4,4′-DTDP, induced irreversible loss of channel activity within several minutes of its application to either the *trans* or the *cis* chamber. All activity was lost within 10 min of *trans* additions of either 100 μM (n = 2) or 1 mM (n = 2) thimerosal, in 4 RyRs tested with voltage pulses and by increasing *cis* [Ca^{2+}] to 1 mM. In separate experiments, adding thimerosal to the *cis* chamber caused the loss of activity in each of 7 experiments tested: 3 of 4 lost activity within 10 min after 1 mM thimerosal, while 2 of 3 lost activity within 20 min of adding 100 μM thimerosal.

Activation of the RyR by 4,4′-DTDP or Thimerosal Was Not Reversed When the Drug Was Washed Out of the Cis Chamber

The effect of washing the oxidizing agents out of the *cis* chamber was tested in 3 channels activated by 1 mM 4,4′-DTDP and in 4 channels activated by 100 μM thimerosal. The channels selected for this experiment showed an increase in activity within 30 sec of adding the oxidizing reagents to the *cis* chamber. The drugs were then perfused out of the *cis* chamber 30 sec after their addition. Channel activity remained elevated after removal of either drug, in much the same way as it had in the presence of the drugs. The average P_o increased significantly from 0.0006 ± 0.0007 in control to 0.16 ± 0.06 during the 30 sec of maximal activity after 4,4′-DTDP addition and removal (P < 0.05), and from 0.002 ± 0.002 to 0.04 ± 0.04 during the 30 sec of maximum activity after addition and removal of thimerosal (P < 0.05). Activity was irreversibly lost after the drugs were added and then removed, in the same way as it was when the reagents remained in the solution.

The fact that the effects of the two oxidizing reagents on the RyR could not be reversed by removing the reagents from the *cis* chamber is consistent with the hypothesis that the reagents alter channel activity as a result of oxidation of protein SH groups, rather than by binding to the RyR. Since inactivation continued after removal

Table 2. Comparison of the effects of thimerosal and 4,4′-DTDP on RyR activity in subactivating Ca^{2+}

	Cis			*Trans*		
	Thimerosal (n = 12)	4,4′-DTDP (n = 21)	*P*	Thimerosal (n = 9)	4,4′-DTDP (n = 5)	*P*
P_o	0.0189 ± 0.0065	0.0654 ± 0.0129	0.0025	0.0111 ± 0.0031	0.0270 ± 0.0084	0.0353
T_o	4.2 ± 1.0	22.2 ± 5.1	0.0018	6.1 ± 1.1	9.5 ± 1.4	0.0631
F_o	5.8 ± 1.8	4.6 ± 1.2	0.5727	2.0 ± 0.6	2.7 ± 0.5	0.4169

Mean ± SEM for n channels. *P* values: significance of difference between means for thimerosal and 4,4′-DTDP.

of the drug, it might well be that the modification that leads to inactivation occurred within 30 sec in these channels, but a lag period was required before the effects of modification altered channel activity.

Discussion

Both 4,4′-DTDP and thimerosal transiently activated native cardiac RyR channels when added to either the *cis* or the *trans* chamber. Since channel activation was prevented or rapidly reversed by DTT, both the reactive disulfide and the organomercurial activated cardiac RyRs by oxidizing protein thiol groups (*see also* Eager et al., 1997). In agreement with the present results, the hydrophilic 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) activates skeletal RyRs by oxidation of thiol groups, when added to either the luminal or cytoplasmic solutions (C.S. Haarmann, R.A. Fink & A.F. Dulhunty, *unpublished*). Interestingly, H_2O_2 activates frog skeletal RyRs by oxidizing luminal, but not cytoplasmic, thiols (Oba et al., 1998). In contrast, glutathione disulfide (GSSG) does not activate skeletal RyRs from the *trans* chamber (Zable et al., 1997). GSSG may be a weaker oxidizing agent that 4,4′-DTDP, thimerosal, DTNB or H_2O_2 since, unlike each of these four oxidizing agents, GSSG in the *cis* chamber does not activate RyRs at subactivating *cis* [Ca^{2+}] (Zable et al., 1997).

How Many Populations of SH Groups Are Oxidized to Alter Channel Activity?

The observed differences in channel activity with addition of sulfhydryl reagents to the *cis* and *trans* chambers allows an estimate of the minimum number of SH residues that must be oxidized in order to explain the results. Multiple populations of SH residues have been shown to activate and inhibit skeletal RyRs (Aghdasi et al., 1997). We postulate that *separate* classes of SH groups are responsible for activation of the RyR and for the loss of activity: SHa (oxidation/activation sites) and SHi (oxidation/closure sites). Evidence for separate sites is that low concentrations of 4,4′-DTDP (100 μM) activate the RyR, but do not always abolish activity (Eager et al., 1997) and that channel activity in the presence of 4,4′-DTDP can occasionally be lost without the channel being initially activated (Eager & Dulhunty, 1998). Further, at least two classes of SH group must be involved in channel activation. Long open events in τ_4 (>50 msec), seen when 1 mM 4,4′-DTDP is added to the *cis* chamber, are not seen when channels are activated by ⩽100 μM *cis* 4,4′-DTDP, with *cis* Ca^{2+} ⩽10^{-7} M (Eager et al., 1997), and are not seen with 4,4′-DTDP added to the *trans* chamber, or with *cis* or *trans* thimerosal. The long open events are likely to depend on oxidation of an additional class of SH group (SHa*) that is accessible only to 4,4′-DTDP from the cytoplasmic side of the channel. Thus at least three classes of cysteine residue (two activating — SHa and SHa* — and one abolishing channel activity — SHi), must be accessible to 4,4′-DTDP in the cytoplasmic solution to explain the channel's response *cis* application of the drug.

Openings in τ_1, τ_2 and τ_3, but not in τ_4, were seen in channels activated by 1 mM *cis* or *trans* thimerosal and in channels activated by 100 μM *cis* or 1 mM *trans* 4,4′-DTDP. Thus SHa is accessible to both 4,4′-DTDP and thimerosal from the cytoplasmic and luminal side of the channel. However, SHa appears to be most easily accessed by thimerosal from the *trans* chamber, because the delay of 14 sec before activation by 100 μM *trans* thimerosal was shorter than the 190 sec delay when the drug was added to the *cis* chamber. Because both *cis* and *trans* thimerosal eventually abolished channel activity with the same delay, it is likely that SHi is also equally accessible to the drug from either the *cis* or *trans* solution.

The results thus clearly show at least three classes of cysteine residue are responsible for the biphasic actions of 4,4′-DTDP and thimerosal on RyR channel activity, but do not yield information about the number of residues included in each class. Since there is a minimum of one residue per class, a minimum of 3 residues per RyR is required to explain the results. However, the fourfold symmetry of the RyR, and the presence of binding sites for calmodulin and FKBP12 on each of the four subunits (Wagenknecht et al., 1997), suggests that each subunit contains each of the three classes of SH residues.

The Location of Thiol Groups That Influence Channel Activity

Since 4,4′-DTDP is lipophilic (Chiamvimonvat et al., 1995), it can partition into the bilayer and enter the opposite solution. Thus 4,4′-DTDP added to either the *cis* or the *trans* chamber could act on cytoplasmic, transmembrane or luminal channel domains. Experiments with DTT in one chamber, when 4,4′-DTDP was added to the opposite chamber, show that SHa is either located on the side of the bilayer to which 4,4′-DTDP was added, or located in the transmembrane domain. An intramembrane location is also suggested by the similar time course of activation of RyRs when 4,4′-DTDP was added to the *cis* or the *trans* chamber. Since thimerosal similarly activated RyRs from the *cis* and *trans* solutions, SHa must be located in a hydrophilic environment, perhaps within the channel pore. In contrast SHa* is located on the cytoplasmic domain of the channel. SHa* is oxidized by 4,4′-DTDP from the *cis* solution, and is not accessible to 4,4′-DTDP from the *trans* solution (*see* Discussion above). Thus reactive disulfide added to the *trans* solution cannot equilibrate in the *cis* solution in sufficient quantities to oxidize SHa* within 2 to 3 min of its addition.

Both 4,4′-DTDP and thimerosal, added to either the *cis* or the *trans* chamber, abolished channel activity after the initial activation. Since channel activity was not lost when 4,4′-DTDP was added to the *trans* chamber if the *cis* solution contained DTT, it is likely that 4,4′-DTDP added to either side of the bilayer gained access to SHi only from the cytoplasmic solution, i.e., 4,4′-DTDP added to the *trans* solution must have crossed the bilayer to abolish activity. In contrast, the lipid impermeable thimerosal abolished activity when added to either solution and thus had access to SHi from the *cis* or *trans* side. Since organomercurials penetrate proteins more effectively than other thiol agents (van Iwaarden et al., 1992), one explanation for these apparently conflicting results is the SHi is located in the transmembrane part of the protein and is separated from the pore by a hydrophilic region which allows thimerosal to pass, but excludes 4,4′-DTDP. Therefore thimerosal accesses SHi from the pore and thus from either solution. 4,4′-DTDP may reach SHi via a hydrophobic pocket is accessible only from the cytoplasmic solution.

If SHa is located in the channel pore, and accessible to 4,4′-DTDP and thimerosal from either side of the bilayer, then it should also be accessible to DTT from either solution. DTT in the *cis* chamber should prevent oxidation by 4,4′-DTDP or thimerosal from the *trans* chamber and *vice versa.* Since this does not happen, either the SHa is not located in the pore or the effect of adding DTT to the solution containing the oxidizing agent is not equivalent to adding DTT to the opposite solution. When DTT is added in excess concentration to a solution containing 4,4′-DTDP or thimerosal, it reduces the oxidant in the solution, as well as reducing protein thiol-oxidant bonds. Conversely, DTT from the opposite solution cannot rapidly reduce the bulk concentration of oxidant. In addition, the number of free oxidants in the pore is likely to be greater than the number of protein thiol-oxidant complexes. Thus DTT entering the pore will have a greater chance of interacting with free oxidant than with the protein thiol-oxidant complex. Reduced oxidants in the pore will rapidly be replaced by fresh oxidants from the bulk solution, which will again be available to interact with DTT entering from the opposite side and to, once again, oxidize the protein thiol if it was reduced by DTT. Oxidation reactions proceed in the presence of reducing agents if the interaction between the protein thiol and the oxidant is faster than that of the oxidant with the reducing agent (Koshita, Miwa & Oba, 1993).

The interactions of organic cations with the channel show that the cardiac RyR has a minimum pore radius of 3.4–3.5 Å and is permeable to ions as large as triethylamine and diethylmethylamine (Tinker & Williams, 1993). Other studies suggest that the skeletal RyR has a similar pore radius and show that organic cations such as $choline^+$ (Smith et al., 1988) or $Tris^+$ (Tinker, Lindsay & Williams, 1992), and sugars like glucose and xylose pass slowly through the channel (Meissner, 1986; Kasai, Kawasaki & Yamamoto, 1992). Molecules with formula weights (FW) similar to those of glucose and xylose (FW = 180 and 150, respectively) may also pass through the RyR pore. 4,4′-DTDP (FW = 220) and DTT (FW = 154) would fall into this category. Although thimerosal is larger (FW = 405), the organomercurials can penetrate proteins more deeply than other oxidizing reagents (van Iwaarden et al., 1992). Thus thimerosal or 4,4′-DTDP might enter the pore from either the luminal or cytoplasmic solution, oxidize SHa and activate the channel. Thimerosal could gain access to SHi from either side of bilayer, via the channel pore, and thus abolish channel activity.

Models for the Distribution of SHa and SHi?

The results could be explained if SHa was either located on both the cytoplasmic and luminal domains of the RyR, or located in the transmembrane region and accessible to the *cis* and *trans* solutions. The model in Fig. 7*A* suggests that SHa is located on the luminal or cytoplasmic domains of the channel protein. The ''convergence'' model (Eager et al., 1997) suggests that ''activating'' or ''inhibiting'' cysteine residues (e.g., SHa, SHa*, or SHi) and ''activating'' or ''inhibiting'' ligand binding sites (La or Li) are located at a distance from the channel pore. The effects of reactions at these sites are transmitted to ''gates'' in the pore, via long range allo-

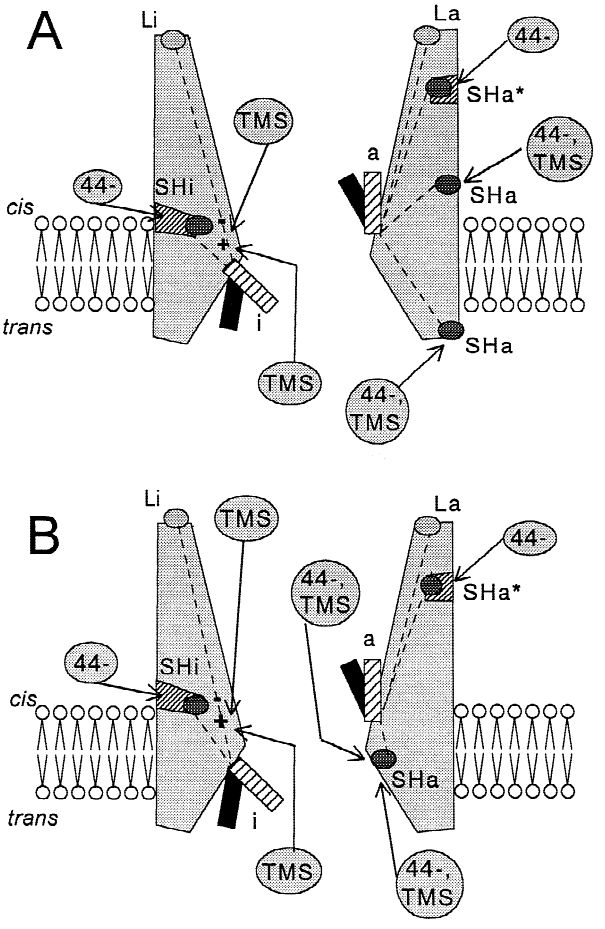

Fig. 7. Two models predicting the location of SH groups that are oxidized by 4,4′-DTDP or thimerosal. The models are based on the convergence model proposed by Eager, Roden and Dulhunty (1997) (*see* text). It is assumed that channel activity depends on the probability of gates ''a'' and ''i'' being open (in a position parallel to the pore). Binding of ligands to La or oxidation of SHa and SHa* increase the probability of gate ''a'' being open (the cross hatched position of ''a''). Binding of ligands to Li or oxidation of SHi decreases the probability that gate ''i'' is open (the cross hatched position of ''i''). The only difference between the two models is the location of SHa. (*A*) SHa is located on both the cytoplasmic and on the luminal domains of the channel. It is assumed that oxidation of SHa on either the cytoplasmic or the luminal domain have a similar action on gate ''a''. (*B*) SHa is located within the channel pore and is accessible to oxidizing reagents 4,4′-DTDP (44−) or thimerosal (TMS) from either the *cis* or *trans* solution. SHi and SHa* are in the same positions in *A* and *B* and both classes of residue are separated from the cytoplasmic solution by hydrophobic pocket (narrow cross-hatching) which allows 4,4′-DTDP to enter, but excludes thimerosal. Thimerosal gains access to SHi through a hydrophilic or polar region of the protein (+ −) which separates SHi from the channel pore.

steric effects. The mean position of the gate indicates its open probability, which is high when the gate is parallel to the pore. Reactions at activation sites increase the probability of gate ''a'' being open. In contrast, reactions at inhibition sites decrease the probability that gate ''i'' will be open. Although this model can explain the results, it could be argued that oxidation of residues on the luminal or the cytoplasmic domains of the protein would be unlikely to have identical actions on channel activity.

Figure 7*B* suggests that SHa is within the channel pore, but is consistent with the convergence model, since reactions at SHa must be transmitted to the channel gates. Thimerosal and 4,4′-DTDP enter the pore from the *cis* or *trans* solutions. SHa is shown on the luminal side of the pore because it is more accessible to thimerosal from the *trans* chamber than the *cis* chamber. In both models SHa* is within a hydrophobic environment on the cytoplasmic domain of the protein, because it is accessible only to 4,4′-DTDP and only from the *cis* solution. SHi is within the transmembrane part of the protein and separated from the pore by charged residues which can be penetrated by thimerosal but not by 4,4′-DTDP (*see* Discussion above). 4,4′-DTDP reaches SHi through a hydrophobic pocket accessible only from the *cis* solution.

The results of the present study do not allow us to distinguish between the two models, but argue in favor of that shown in Fig. 7*B.* The similar latency of channel activation, with openings in τ_1, τ_2 and τ_3, by agents added to both the cytoplasmic and the luminal solutions are most easily explained if the same residues are oxidized by reagents added to either solution.

In conclusion the results show that the cardiac RyR responds in a quantitatively similar manner, with activation followed by loss of channel activity, after lipid soluble (4,4′-DTDP) and lipid insoluble (thimerosal) oxidizing reagents are added to either cytoplasmic (*cis*) or luminal (*trans*) sides of the bilayer. An additional activating response is evoked by 4,4′-DTDP added to the cytoplasmic solution. The results suggest that a minimum of three classes of cysteine residues are evoked in the response of the RyR to 4,4′-DTDP and thimerosal.

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