

# Oxidation and Reduction of Pig Skeletal Muscle Ryanodine Receptors

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**ABSTRACT** Time-dependent effects of cysteine modification were compared in skeletal ryanodine receptors (RyRs) from normal pigs and RyR<sub>MH</sub> (Arg<sup>615</sup> to Cys<sup>615</sup>) from pigs susceptible to malignant hyperthermia, using the oxidizing reagents 4,4'-dithiodipyridine (4,4'-DTDP) and 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) or the reducing agent dithiothreitol (DTT). Normal and RyR<sub>MH</sub> channels responded similarly to all reagents. DTNB (1 mM), either cytoplasmic (*cis*) or luminal (*trans*), or 1 mM 4,4'-DTDP (*cis*) activated RyRs, introducing an additional long open time constant. 4,4'-DTDP (*cis*), but not DTNB, inhibited channels after >5 min. Activation and inhibition were relieved by DTT (1–10 mM). DTT (10 mM, cytoplasmic or luminal), without oxidants, activated RyRs, and activation reversed with 1 mM DTNB. Control RyR activity was maintained with 1 mM DTNB and 10 mM DTT present on the same or opposite sides of the bilayer. We suggest that 1) 4,4'-DTDP and DTNB covalently modify RyRs by oxidizing activating or inhibiting thiol groups; 2) a modified thiol depresses mammalian skeletal RyR activity under control conditions; 3) both the activating thiols and the modified thiols, accessible from either cytoplasm or lumen, reside in the transmembrane region; 4) some cardiac sulfhydryls are unavailable in skeletal RyRs; and 5) Cys<sup>615</sup> in RyR<sub>MH</sub> is functionally unimportant in redox cycling.

## INTRODUCTION

Contraction of striated muscle depends on Ca<sup>2+</sup> release from the sarcoplasmic reticulum (SR), through ryanodine receptor (RyR) calcium release channels. Oxidants are important ligands in the physiological activity of RyRs and act by oxidation of free sulfhydryl (SH) groups, because their effects are prevented by sulfhydryl reducing agents (Boraso and Williams, 1994; Favero et al., 1995; Abramson et al., 1995). Oxidants activate and can inhibit RyRs (Holmberg et al., 1991; Holmberg and Williams, 1992; Boraso and Williams, 1994; Favero et al., 1995; Stoyanovsky et al., 1997). Oxidation-induced activation proceeds under *in vivo* conditions, in the presence of glutathione (GSH) (Koshita et al., 1993), and is enhanced during ischemia or reperfusion, in which oxygen free radicals increase and the ratio of GSH:GSSG falls (Sies et al., 1972; Curello et al., 1985).

Reagents that react specifically with free SH groups are used as model compounds to look at the effects of oxidation and the importance of cysteine residues in RyR channel function. Reactive disulfides 4,4'- or 2,2'-dithiodipyridine (4,4'-DTDP or 2,2'-DTDP) activate and then block cardiac RyRs when applied to the cytoplasmic (*cis*) side of the channels (Eager et al., 1997; Eager and Dulhunty, 1998). Skeletal RyRs have also been reported to be activated, but not inhibited, by *cis* 4,4'- or 2,2'-DTDP and by GSSH (Nagura et al., 1988; Marengo et al., 1998) and to be inhibited by the reducing agents GSH, dithiothreitol (DTT),

or  $\beta$ -mercaptoethanol (BME). The domain of the RyR on which oxidants act is not clear. Both lipid-soluble and water-soluble reagents can partition into the membrane, to a greater or lesser extent, depending on their pK<sub>a</sub> values and could react with -SH groups on either the side to which they are added or in the transmembrane parts of the RyR protein. It is unlikely that reagents would act rapidly on the opposite side of the bilayer, because reagent crossing the bilayer would be diluted in the large volume of the opposite solution and would take a long time to reach activating concentrations. Water-soluble oxidants appear to react with SH groups only in the cytoplasmic or transmembrane domains of the mammalian RyRs. Methanethiosulfonate derivatives block skeletal muscle RyRs only from the *cis* side (Quinn and Ehrlich, 1997). Some residues (-<sub>a</sub>SH and -<sub>i</sub>SH) responsible for activation or inhibition of cardiac RyRs are accessible to thimerosal from either side of the bilayer and are located in the transmembrane domain, while other activating (-<sub>a</sub>\*SH) residues are confined to the cytoplasmic domain (Eager and Dulhunty, 1999). There have been no similar reports of the long-term effects of thiol-specific oxidizing agents added to the luminal or cytoplasmic sides of mammalian skeletal RyR channels.

The present study examines the effects on single skeletal RyRs of long exposure to 2–10 mM concentrations of the thiol-specific 4,4'-DTDP or 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) or DTT. The experiments tested the hypothesis that, as in cardiac RyRs (Eager et al., 1999), three classes of -SH (-<sub>a</sub>SH and -<sub>i</sub>SH, within the transmembrane domain, or -<sub>a</sub>\*SH in the cytoplasmic domain) are available in skeletal RyRs for oxidation by specific sulfhydryl reagents. It was possible that the same thiols could be oxidized in cardiac and skeletal RyRs, because 71 of >80 cysteine residues are conserved between the two isoforms (Otsu et al., 1990). The experiments tested an additional

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hypothesis that the Arg<sup>615</sup>-to-Cys<sup>615</sup> substitution in RyR<sub>MH</sub> from pigs susceptible to malignant hyperthermia (MH) provides an additional -SH group for oxidation. MH is an inherited skeletal muscle disorder, characterized by increased Ca<sup>2+</sup> release from SR (Ohta et al., 1989; Carrier et al., 1991; Mickelson and Louis, 1996) and reduced inhibition of RyRs by Ca<sup>2+</sup> (Fill et al., 1991; Shomer et al., 1993) or Mg<sup>2+</sup> (Laver et al., 1997).

Novel findings are 1) that the water-soluble DTNB (in either *cis* or *trans* solutions) and the lipid-soluble 4,4'-DTDP (in the *cis* solution) activate skeletal RyRs in a similar way; 2) 4,4'-DTDP added to the *cis* solution, but not DTNB (*cis* or *trans*), inhibits skeletal RyRs after >5 min; 3) in the absence of an oxidizing reagent, 10 mM DTT activates skeletal RyRs from the *cis* or *trans* solution; 4) "control-like" channel activity is maintained when 1 mM DTNB and 10 mM DTT are present together on either the same or opposite sides of the bilayer; and 5) effects of oxidation and reduction are the same in normal RyRs and RyR<sub>MH</sub>. Our conclusions are that 1) -<sub>a</sub>SH is located in the skeletal RyR transmembrane domain, as in cardiac RyRs; 2) -<sub>a</sub>\*SH, in cardiac RyRs, is not available for oxidation in skeletal RyRs; 3) a modified thiol in the transmembrane domain (-<sub>ab</sub>S-R, where R is either a protein S if the modified thiol is a disulfide, or an N if the modified group is nitrosylated; Xu et al., 1998) normally suppresses skeletal RyR activity; and 4) the Arg<sup>615</sup>-to-Cys<sup>615</sup> substitution in MH does not provide an additional SH group for oxidation by thiol-specific reagents.

## MATERIALS AND METHODS

### Biological material and caffeine-halothane contracture test for MH susceptibility

The methods for genetic testing, muscle dissection, caffeine-halothane contracture testing, preparation of SR vesicles, and single-channel recording have been described previously (Otsu et al., 1992; Owen et al., 1997; Laver et al., 1997). Muscle and blood samples were obtained from three homozygous normal pigs (one Belgium Landrace and two Landrace) and three homozygous MH pigs (two Belgium Landrace and one Landrace) aged ~4 months. Each animal was genetically tested for a normal or MH RyR allele (containing either Arg<sup>615</sup> or Cys<sup>615</sup>). The SR preparations were from the same animals as those used in Laver et al. (1997). The description of anesthetic techniques, muscle dissection, and halothane/caffeine contracture tests are given by Laver et al. (1997). All fiber bundles from the three homozygous normal animals failed to respond to halothane or 2 mM caffeine, while all fiber bundles from the three homozygous MH animals developed tension in response to both drugs.

### Isolation of SR vesicles

The preparation of crude SR vesicles was based on the methods of Meissner (1984) and Ma et al. (1995). The freshly dissected back and leg muscle was washed in cold phosphate-buffered saline containing 2 mM EGTA (pH 7.0), trimmed of fat and connective tissue, cubed, and either frozen in liquid N<sub>2</sub> and stored at -70°C or freshly processed. The fresh or thawed muscle cubes were suspended in (mM) 5 Tris maleate, 100 NaCl, 2 EDTA, 0.1 EGTA (pH 6.8) (5 ml/g of tissue). The muscle was homogenized in a Waring blender in four 15-s high-speed bursts. The homogenate was centrifuged at 2600 × g for 30 min, and the supernatant was filtered

through cotton gauze and centrifuged at 10,000 × g for 30 min. The pellet (P2) was collected, and the supernatant was centrifuged again at 35,000 × g and the pellet (P3) collected. Pellets P2 and P3 were resuspended in (mM) 5 Tris-2-(N-morpholino)ethanesulfonic acid (Tris-MES), 300 sucrose, 100 KCl, 2 DTT (pH 6.8). Aliquots of the suspensions were frozen in liquid nitrogen and stored at -70°C. All buffers contained the protease inhibitors phenylmethylsulfonyl fluoride (0.7 mM), leupeptin (1 μg/ml), pepstatin A (1 μM), and benzamide (1 mM).

### Lipid bilayer techniques

The lipid bilayer and single-channel recording technique are described by Ahern et al. (1994) and Laver et al. (1995). Bilayers were formed from phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine (5:3:2 w/w) (Avanti Polar Lipids, Alabaster, AL) across an aperture with a diameter of 200–250 μm in the wall of a 1.0-ml Delrin cup (Cadillac Plastics, Australia). TC vesicles (final concentration 10 μg/ml) were added to the *cis* chamber and stirred until vesicle incorporation was observed. The cytoplasmic side of channels incorporated into the bilayer faced the *cis* solution. The bilayer potential was controlled and single-channel activity was recorded with an Axopatch 200A amplifier (Axon Instruments, Foster City, CA). For experimental purposes, the *cis* chamber was held at ground and the voltage of the *trans* chamber was controlled. Bilayer potential is expressed in the conventional way as  $V_{cis} - V_{trans}$  (i.e.,  $V_{cytoplasm} - V_{lumen}$ ).

Bilayers were formed and vesicles incorporated into the bilayer, using *cis* and *trans* solutions containing (mM) 230 Cs methanesulfonate (CsMS), 20 CsCl, 0.1 CaCl<sub>2</sub>, and 10 N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES) (pH 7.4 adjusted with CsOH). The *cis* solution also contained 500 mM mannitol to aid SR vesicle fusion and RyR incorporation into the bilayer.

### Recording and analysis of single-channel data

Channel activity was recorded at -40 mV. In general activity was recorded for a 2-min control period before and 1 min after 0.5-s voltage pulses to +40 mV. Drugs were then added to either the *cis* or *trans* chamber with a ~10-s stirring period, and then activity was recorded for several minutes. Voltage pulses to +40 mV were occasionally applied to the bilayer after drug addition to determine whether channels could be activated by the change in bilayer potential. Because RyR channel activity can increase immediately after a voltage pulse (Zahradnikova and Meszaros, 1998; Laver and Lamb, 1998), the first 30 s after each voltage pulse was excluded from analysis.

Channel activity was filtered at 1 kHz (10-pole low-pass Bessel, -3 dB) and digitized at 2 kHz. Analysis of single-channel records (using Channel 2, written by P. W. Gage and M. Smith) yielded channel open probability ( $P_o$ ), frequency of events ( $F_o$ ), open times, closed times, and mean open or closed times ( $T_o$  or  $T_c$ ), as well as mean current ( $I'$ ). The open discriminator was set at ~25% of the maximum current, and the closed discriminator at 50% of the open discriminator, so that openings to both subconductance and maximum conductance levels were included in the analysis. Single-channel parameters were measured during the 30 s, showing maximum  $I'$  during the control period and then the period of maximum  $I'$  after the addition of drugs. Dwell-time distributions were plotted as the frequency of openings in logged bins (Sigworth and Sine, 1987) to display the large range of open times seen in control recordings and after the addition of oxidizing reagents. The fit of a multiple exponential function to the data was assessed using a least-squares fit.

### Statistics

Average data are given as mean ± SEM. The significance of the difference between control and test values was evaluated with a Student's *t*-test, either one or two sided and for independent or paired data, as appropriate. Differences were considered to be significant when  $p \leq 0.05$ .

## RESULTS

### Effects of DTNB or 4,4'-DTDP on normal RyRs

#### Activation by DTNB

RyRs from homozygous normal pig muscle were activated by addition of the hydrophilic DTNB (1 mM) to either the *cis* or *trans* chamber. Control RyR activity was characterized by brief openings to the maximum conductance and to lower conductance levels (Figs. 1, *A* and *B*, and 2, *A* and *B*). Longer openings appeared with DTNB (Figs. 1 *A* and 2 *B*). Channel activity increased with a delay of  $\sim 1$  min after DTNB was added to the *cis* chamber in 13 of 14 channels, with a delay of  $\sim 3$  min after *trans* DTNB was added to 10 of 11 RyRs. Average mean current ( $I'$ ) increased from a control value of  $-1.43 \pm 0.12$  pA to  $-3.91 \pm 0.19$  pA ( $n = 10$ ) or from  $-2.73 \pm 0.38$  pA to  $-5.81 \pm 0.64$  pA ( $n = 6$ ). When the average ratio of mean current before and after addition of the drugs for individual channels was determined, there was an approximately fivefold increase with *cis* DTNB and an approximately threefold increase with *trans* DTNB (Table 1). Note that the delay was assessed by

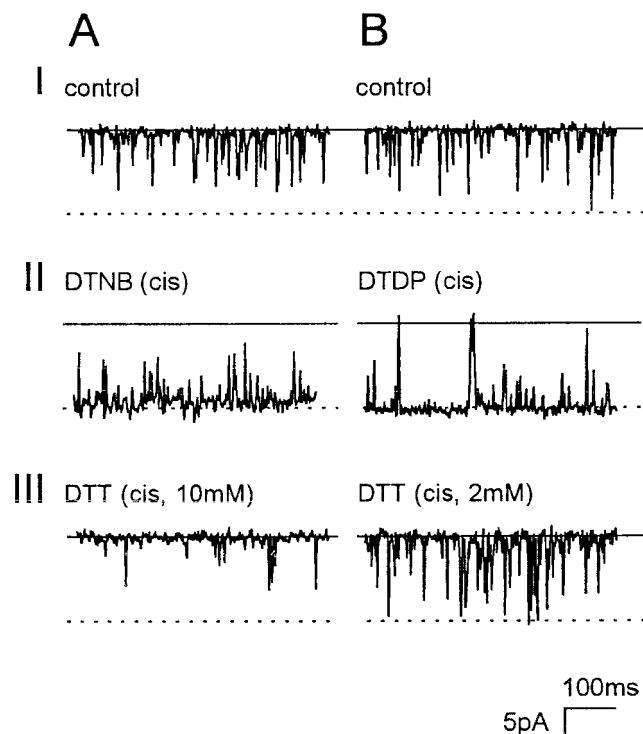


FIGURE 1 Activation of normal RyRs by 1 mM DTNB or 1 mM 4,4'-DTDP added to the *cis* chamber and reversal of activation by DTT. Channel activity in this and subsequent figures was recorded at  $-40$  mV with symmetrical 250 mM  $\text{Cs}^+$  and 100  $\mu\text{M}$   $\text{Ca}^{2+}$ . (A) A single RyR under control conditions (I), during activation by 1 mM *cis* DTNB (II) and then after addition of 10 mM DTT to the *cis* chamber (III). (B) A single RyR under control conditions (I), during activation by 1 mM *cis* 4,4'-DTDP, added from a 10 mM stock dissolved in *cis* solution (II), and then after the addition of 2 mM DTT to the *cis* chamber (III). The zero current level is indicated by solid lines, and the maximum open conductance is shown by broken lines.

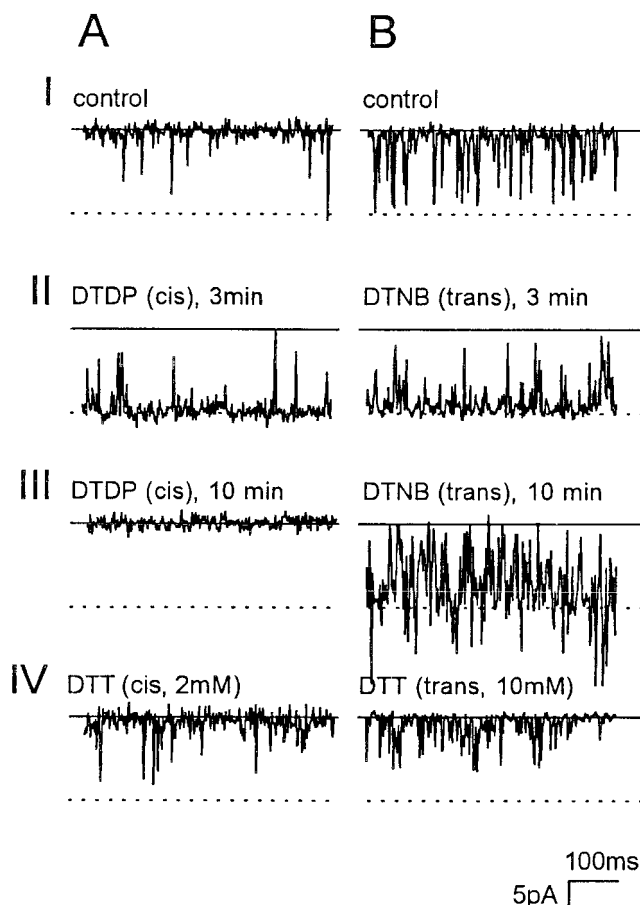


FIGURE 2 Inhibition of normal RyRs by 1 mM *cis* 4,4'-DTDP, but not by 1 mM *trans* DTNB. (A) A bilayer containing one RyR channel under control conditions (I), activation 3 min after the addition of 1 mM *cis* 4,4'-DTDP dissolved in ethanol (II), inhibition 10 min after the addition of 4,4'-DTDP (III), and recovery from inhibition after a final addition of 2 mM DTT (IV). (B) A bilayer containing two normal RyR channels with only one channel active under control conditions (I), increased activity in one channel 3 min after the addition of 1 mM *trans* DTNB (II), two channels active after a 10-min exposure to *trans* DTNB (III), and return to control activity in one channel after a final addition of 10 mM DTT (IV). The zero current level is indicated by solid lines, and the maximum open conductance is shown by dotted lines.

eye from channel records. The numbers of channels for  $I'$  in Table 1 are fewer than the number for the delay because  $I'$  was measured using Channel2 analysis of low-noise recordings only.

Channel activity with 1 mM *cis* DTNB was not altered by subsequent addition of 2 mM *cis* DTT (Results below) but returned toward control when 10 mM DTT was added to the *cis* side of nine of nine RyRs activated by *cis* DTNB or to the *trans* side of three of three RyRs activated by *trans* DTNB (Figs. 1 and 2). This fall in activity was presumably due to reduction of -SH groups oxidized by DTNB, because DTT (10 mM *cis* or *trans*) added without DTNB activated RyRs (Results below). The similar magnitude and time course of effects of *cis* or *trans* DTNB support the hypothesis that DTNB oxidizes  $-\text{aSH}$ , located in the transmembrane domain.

**TABLE 1** Average effect of drugs on mean current,  $I'$ , in normal RyRs (normal) and RyR<sub>MH</sub> (MH)

	Normal				MH			
	$I'_{\text{drug}}/I'_{\text{control}}$	(n)	Delay [s]	(n)	$I'_{\text{drug}}/I'_{\text{control}}$	(n)	Delay [s]	(n)
DTNB ( <i>cis</i> )	$5.28 \pm 1.47^{**}$	(10)	$68 \pm 13$	(13)	$3.91 \pm 0.92^{**}$	(9)	$198 \pm 38^{##}$	(14)
DTNB ( <i>trans</i> )	$2.87 \pm 0.73^{**}$	(6)	$207 \pm 50$	(10)	$4.70 \pm 1.42^{**}$	(9)	$141 \pm 25$	(16)
DTDP (ethanol)	$6.56 \pm 3.34^{**}$	(12)	$138 \pm 48$	(15)	$4.22 \pm 1.53^*$	(7)	$214 \pm 35$	(8)
Ethanol (1%)	$2.65 \pm 1.03^*$	(5)	$168 \pm 21$	(8)	$1.79 \pm 0.51$	(6)	$150 \pm 13$	(5)
DTDP (H <sub>2</sub> O)	$3.41 \pm 1.64^*$	(8)	$100 \pm 15$	(18)	$3.02 \pm 0.87$	(13)	$156 \pm 27$	(13)

Relative changes in  $I'$  ( $I'_{\text{drug}}/I'_{\text{control}}$ ) are shown. DTNB and 4,4'-DTDP (DTDP) were added to a final concentration of 1 mM. Data are given as mean  $\pm$  1 SEM.

$I'_{\text{drug}}$  is significantly different from  $I'_{\text{control}}$  with:  $*p < 0.05$ ,  $**p < 0.01$ .

$I'_{\text{drug}}/I'_{\text{control}}$  for RyR<sub>MH</sub> is significantly different from  $I'_{\text{drug}}/I'_{\text{control}}$  for normal RyRs with:  $^{##}p < 0.01$ .

DTDP (ethanol), DTDP dissolved in ethanol.

DTDP (H<sub>2</sub>O), DTDP dissolved in *cis* solution.

### Activation by 4,4'-DTDP

4,4'-DTDP (1 mM *cis* added in ethanol, 1% v/v) caused an approximately sevenfold increase in  $I'$ , which began  $\sim$ 2 min after its addition to 15 of 15 channels (Table 1). In contrast to sheep cardiac (Eager et al., 1997) or rabbit skeletal (Ahern et al., 1997a) RyRs, 1% ethanol (alone) increased  $I'$  after  $\sim$ 3 min in eight of eight RyRs (Table 1). Therefore 4,4'-DTDP was dissolved in *cis* solution at 10 mM and added to the *cis* chamber to a final concentration of  $\sim$ 1 mM. In this situation,  $I'$  increased in 18 of 21 channels, after a delay of  $\sim$ 1.5 min (Table 1). Activity returned toward control after 2 mM *cis* DTT addition to each of four single 4,4'-DTDP-activated RyRs (Figs. 1 B and 2 A). Thus 4,4'-DTDP also activated RyRs via oxidation of -SH groups, which could also be -<sub>a</sub>SH in the transmembrane domain.

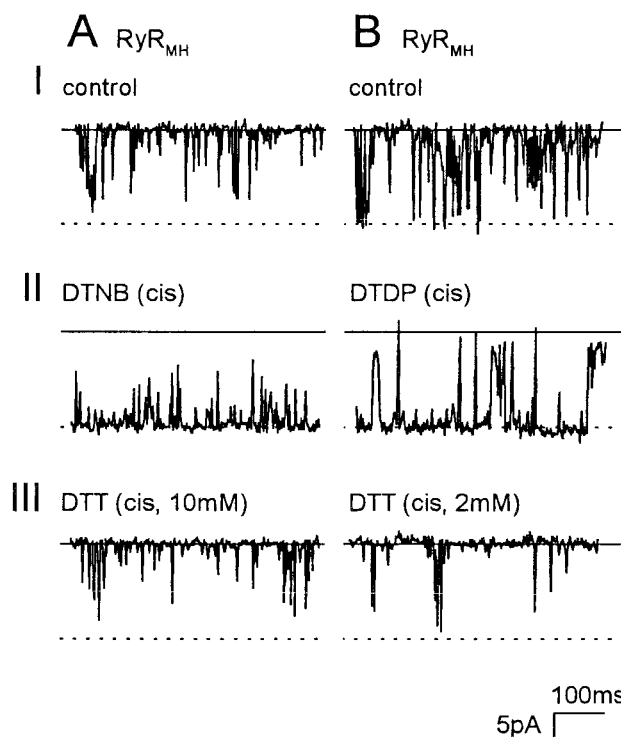
### Inhibition by 4,4'-DTDP

*Cis* 4,4'-DTDP (1 mM) abolished activity in seven of 10 RyRs, after  $334 \pm 52$  s (4,4'-DTDP plus ethanol, Fig. 2 AIII), or in five of nine channels after  $456 \pm 82$  s (4,4'-DTDP added in *cis* solution). Inactivated channels were not reactivated by four pulses to +40 mV (0.03 Hz). Infrequent activity remained in seven of 19 channels, for 26 min in one case. RyRs were not inactivated after 5–30 min with 1% ethanol alone ( $n = 9$ ). *Cis* DTT (2 mM) restored activity after  $129 \pm 62$  s in three of five RyRs inactivated by 4,4'-DTDP plus ethanol (Fig. 2 AIV) or after  $240 \pm 165$  s in three of five RyRs inactivated by 4,4'-DTDP alone. Recovery with DTT shows that inactivation is due to -SH oxidation and that -<sub>i</sub>SH is present in skeletal RyRs and accessible to DTT. In contrast to 4,4'-DTDP, 1 mM DTNB (either *cis* or *trans*) did not inactivate RyRs after 5–20 min (Fig. 2 B, II and III). If activity fell, it increased again after pulses to +40 mV.

### Comparison of RyR and RyR<sub>MH</sub>

The characteristics of normal RyRs and RyR<sub>MH</sub> (from homozygous MH pigs) were similar under control conditions

at  $-40$  mV, with  $100 \mu\text{M Ca}^{2+}$  and symmetrical 250 mM CsMS (Fig. 3). Single-channel conductance was  $452 \pm 9$  pS ( $n = 4$ ) for normal RyRs and  $470 \pm 11$  pS ( $n = 5$ ) for RyR<sub>MH</sub>, and control  $I'$  for RyR<sub>MH</sub> was  $1.76 \pm 0.13$  pA ( $n = 39$ ), compared with  $\sim$ 2.1 pA for 16 normal RyRs (Results above). In addition, both types of channel were 1) locked into a submaximum conductance state with  $10$ – $15 \mu\text{M cis}$  ryanodine (RyR,  $n = 11$ ; RyR<sub>MH</sub>,  $n = 12$ ), 2) blocked by 5



**FIGURE 3** RyR<sub>MH</sub> is activated by 1 mM *cis* DTNB and 1 mM *cis* 4,4'-DTDP. (A) A single RyR<sub>MH</sub> under control conditions (I) and during activation by 1 mM *cis* DTNB (II), and recovery after the addition of 10 mM DTT to the *cis* chamber (III). (B) A single RyR<sub>MH</sub> under control conditions (I), during activation by 1 mM *cis* 4,4'-DTDP, and added from a 10 mM stock dissolved in *cis* solution (II), and recovery after the addition of 2 mM DTT to the *cis* chamber (III). The zero current level is indicated by solid lines, and the maximum open conductance is shown by the broken lines.



$\mu\text{M}$  *cis* ruthenium red (RyR,  $n = 3$ ; RyR<sub>MH</sub>,  $n = 2$ ), and 3) activated by 5 mM ATP (RyR,  $n = 4$ ; RyR<sub>MH</sub>,  $n = 5$ ).

### Effects of reactive disulfides on RyRs from MH pigs

#### Activation by DTNB or 4,4'-DTDP

Activity increased fourfold in 14 of 14 RyR<sub>MH</sub> channels  $\sim 3$  min after the addition of 1 mM *cis* DTNB or approximately fourfold in 16 of 17 channels  $\sim 2$  min after *trans* DTNB (Fig. 3, Table 1). Activation of RyR<sub>MH</sub> by *cis* DTNB was significantly slower than activation of normal RyRs ( $p < 0.01$ ). There were no other differences in activation by DTNB between normal RyRs and RyR<sub>MH</sub>. 4,4'-DTDP added to RyR<sub>MH</sub>, with ethanol or alone (in *cis* solution), induced three- to fourfold increases in average  $I'$  (Fig. 3), after 2.5–3.5 min, while ethanol alone caused an approximately twofold increase in  $I'$  after  $\sim 2.5$  min (Table 1). RyR<sub>MH</sub> activity returned to control levels within 1 min after 10 mM DTT was added to the *cis* side of channels activated by *cis* DTNB (four of four) or *trans* DTNB (three of three) or after 2 mM *cis* DTT was added to *cis* 4,4'-DTDP-activated channels (five of five). The similar results with normal RyRs and RyR<sub>MH</sub> show that the Arg<sup>615</sup>-to-Cys<sup>615</sup> substitution in MH does not alter the ability of sulfhydryl-specific reagents to activate RyRs by oxidizing  $-\text{aSH}$ . The differences between RyR<sub>MH</sub> and normal RyRs in the rate of activation by *cis* DTNB could be explained by structural changes that reduce the accessibility of  $-\text{aSH}$  in RyR<sub>MH</sub>.

#### Inhibition of RyR<sub>MH</sub>

RyR<sub>MH</sub> channels were inactivated after  $458 \pm 30$  s exposure to 1 mM *cis* 4,4'-DTDP (added with 1% ethanol,  $n = 10$ ), and nine of the 10 channels were reactivated  $160 \pm 30$  s after adding 2 mM DTT. Similarly, 1 mM *cis* 4,4'-DTDP added alone (in *cis* solution) abolished RyR<sub>MH</sub> activity in three of six channels after  $383 \pm 156$  s, and the three

channels recovered  $50 \pm 26$  s after 2 mM DTT was added. No RyR<sub>MH</sub> channels were inhibited during 3.5–32-min exposure to DTNB (*cis* or *trans*,  $n = 13$ ) or 4–30-min exposure to 1% *cis* ethanol ( $n = 5$ ). Thus inhibition of RyR channels via oxidation of  $-\text{aSH}$  was not altered by the MH mutation.

There were some curious exceptions to the general observations reported above. One normal and two RyR<sub>MH</sub> channels were inactivated 2–7 min after 4,4'-DTDP was added, without initial activation, and inhibition was relieved 40–90 s after *cis* DTT was added. This confirmed independent channel activation and inhibition and supported separate  $-\text{aSH}$  and  $-\text{iSH}$  residues (see also Eager et al., 1998). Activity in three RyR<sub>MH</sub> and two normal RyRs fell after initial activation by *cis* 4,4'-DTDP, and activity in two normal RyRs fell 5 min after *cis* 4,4'-DTDP was added, without any initial activation. There was no further change in activity in these seven channels when 2 mM *cis* DTT was added, suggesting that the disulfide formed upon exposure to 4,4'-DTDP ( $-\text{aS-Sr}$ , where  $-\text{Sr}$  is contributed either by the reactive disulfide or by the channel protein) was reduced during exposure to 4,4'-DTDP and hence that the affinity of  $-\text{aSH}$  for 4,4'-DTDP in these channels was less than normal.

### Single-channel properties of RyR and RyR<sub>MH</sub>

There was no significant difference between normal RyR ( $n = 48$ ) and RyR<sub>MH</sub> ( $n = 45$ ) in the steady-state parameter values for  $P_o$ ,  $F_o$ ,  $T_o$ , and  $T_c$  measured over 30-s periods (Table 2). The two types of channel showed similar modes of activity, transitions between modes and responses to voltage pulses to +40 mV (Fig. 4). Predominant modes were either low activity or high activity, unaltered after the voltage pulse (Fig. 4, *I* and *II*) or voltage-activated increase in activity immediately after the voltage pulse, which then decayed after 10–20 s to a lower level that was also seen before the voltage pulse (Fig. 4 *III*). Strong submaximum conductance activity was also seen in both normal RyRs and

**TABLE 2** Effects of oxidizing reagents and ethanol on single-channel parameters open probability ( $P_o$ ), mean open time ( $T_o$ ), mean closed time ( $T_c$ ), and frequency of openings ( $F_o$ )

Condition ( $n$ (normal, MH))	$P_o$		$T_o$ (ms)		$T_c$ (ms)		$F_o$ ( $\text{s}^{-1}$ )	
	Normal	MH	Normal	MH	Normal	MH	Normal	MH
Control (8, 9)	$0.15 \pm 0.04$	$0.20 \pm 0.06$	$1.17 \pm 0.07$	$2.75 \pm 0.12$	$8.82 \pm 1.08$	$12.41 \pm 1.03$	$97 \pm 19$	$71 \pm 17$
DTNB ( <i>cis</i> )	$0.27 \pm 0.05^*$	$0.41 \pm 0.06^{**}$	$5.26 \pm 0.81^{**}$	$6.68 \pm 0.22^{**}$	$11.22 \pm 1.22$	$11.19 \pm 1.24$	$62 \pm 21$	$64 \pm 10$
Control (6, 6)	$0.23 \pm 0.06$	$0.25 \pm 0.08$	$1.65 \pm 0.10$	$5.35 \pm 0.71$	$6.55 \pm 0.77$	$13.27 \pm 0.98$	$139 \pm 31$	$60 \pm 14$
DTNB ( <i>trans</i> )	$0.48 \pm 0.12^{**}$	$0.56 \pm 0.09^{**}$	$4.72 \pm 0.72^*$	$11.46 \pm 1.17^{**}$	$5.74 \pm 0.97$	$8.39 \pm 0.82^*$	$125 \pm 34$	$64 \pm 16$
Control (6, 6)	$0.32 \pm 0.13$	$0.19 \pm 0.06$	$2.57 \pm 0.40$	$2.02 \pm 0.15$	$5.62 \pm 0.70$	$11.75 \pm 2.24$	$88 \pm 18$	$95 \pm 24$
DTDP (eth)	$0.67 \pm 0.13^*$	$0.38 \pm 0.11$	$13.23 \pm 3.35^{**}$	$5.85 \pm 0.78^*$	$5.83 \pm 1.58$	$10.17 \pm 1.55$	$74 \pm 24$	$73 \pm 18$
Control (4, 5)	$0.24 \pm 0.08$	$0.38 \pm 0.12$	$1.86 \pm 0.26$	$3.43 \pm 0.50$	$6.43 \pm 0.75$	$6.60 \pm 1.18$	$125 \pm 18$	$113 \pm 28$
Ethanol (1%)	$0.44 \pm 0.10^*$	$0.49 \pm 0.12$	$4.10 \pm 0.69^*$	$4.13 \pm 0.43^*$	$4.65 \pm 0.57$	$4.52 \pm 0.57$	$118 \pm 22$	$119 \pm 14$
Control (7, 5)	$0.17 \pm 0.06$	$0.20 \pm 0.07$	$1.20 \pm 0.19$	$1.05 \pm 0.57$	$7.65 \pm 1.26$	$4.15 \pm 1.81$	$72 \pm 17$	$78 \pm 31$
DTDP ( $\text{H}_2\text{O}$ )	$0.42 \pm 0.11^*$	$0.40 \pm 0.09^*$	$8.32 \pm 2.37^{**}$	$2.59 \pm 1.06$	$9.84 \pm 2.78$	$6.87 \pm 8.50$	$50 \pm 17$	$86 \pm 26$

DTNB and 4,4'-DTDP (DTDP) were added to a final concentration of 1 mM. Results are given as mean  $\pm$  1 SEM.

Significant differences between parameters under control conditions and after addition of the reagent are indicated by:  $^*p < 0.05$ .  $^{**}p < 0.01$ .

DTDP (ethanol), DTDP dissolved in ethanol.

DTDP ( $\text{H}_2\text{O}$ ), DTDP dissolved in *cis* solution.

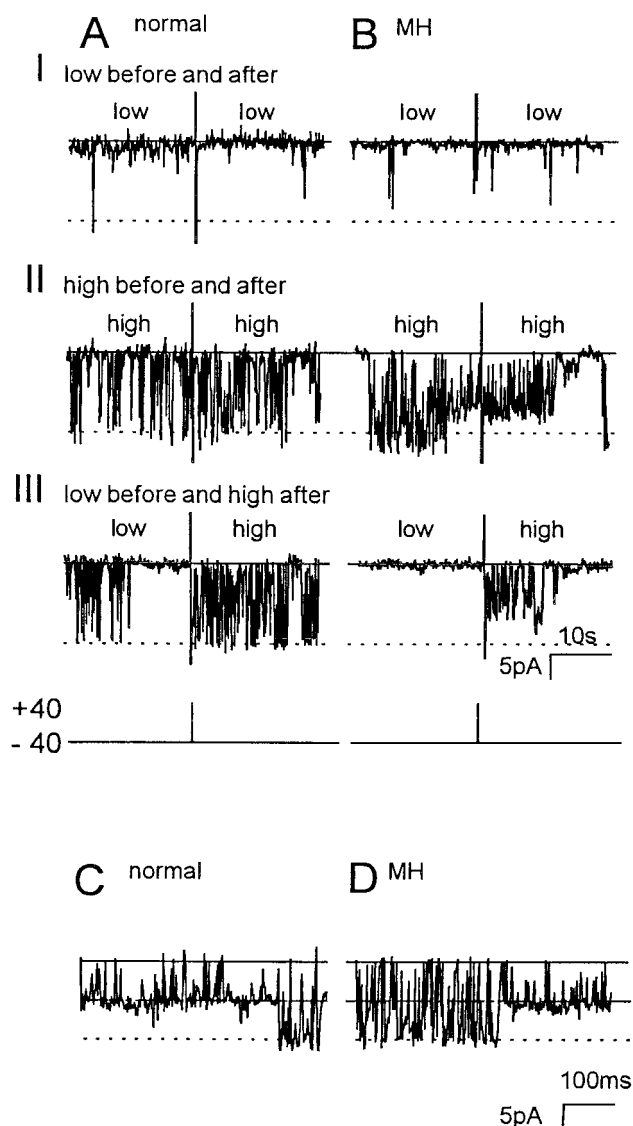


FIGURE 4 Modes of activity and openings to submaximum conductance levels in normal RyRs and RyR<sub>MH</sub>. (A and B) Examples of channels showing low activity before and after a voltage pulse to +40 mV (I), high activity before and after a pulse to +40 mV (II), or low activity before and high activity after the voltage pulse (III). (A) Normal RyRs. (B) RyR<sub>MH</sub>. (C and D) A single normal RyR and single RyR<sub>MH</sub>, respectively, both showing long openings to a submaximum conductance level. The zero current level is indicated by solid lines, and the maximum open conductance is shown by dotted lines.

RyR<sub>MH</sub> (Fig. 4, C and D), giving an average open channel conductance that was  $0.50 \pm 0.05$  of the maximum conductance in normal RyRs or  $0.37 \pm 0.09$  in RyR<sub>MH</sub>.

#### Effects of oxidizing reagents and ethanol on single-channel parameters of normal RyRs and RyR<sub>MH</sub>

The oxidizing reagents increased mean open time ( $T_o$ ) in all normal RyRs and all RyR<sub>MH</sub> channels, with significant increases in average  $T_o$  under most conditions (Table 2). The effect on other aspects of channel activity was complex,

with an increase in mean closed time ( $T_c$ ) in some channels and a decrease in  $T_c$  in others, so that the changes in average  $F_o$  and  $P_o$  were not significant (Table 2).

DTT at 10 mM reversed the effects of DTNB, and at 2 mM reversed the effects of 4,4'-DTDP in normal RyRs and RyR<sub>MH</sub>. Data were pooled from the few channels that were suitable for analysis and were exposed first to an oxidizing reagent and then to DTT.  $I'$ ,  $P_o$ , and  $T_o$  fell significantly after DTT was added to the oxidation-activated channels (Fig. 5). The similar actions of *cis* DTNB, *trans* DTNB, and *cis* 4,4'-DTDP on the single-channel parameters during activation and the similar reversal of these actions by DTT provide further evidence that one class of cysteine residues ( $-_aSH$ ) is oxidized in each of the three situations.

The average open channel conductance was higher after oxidation, being  $0.50 \pm 0.05$  of the maximum conductance before and  $0.57 \pm 0.09$  after oxidation in normal RyRs ( $n = 25$ ), or  $0.37 \pm 0.05$  before and  $0.49 \pm 0.06$  after in RyR<sub>MH</sub> ( $n = 24$ , pooled data for *cis* DTNB, *trans* DTNB, and *cis* 4,4'-DTDP), although the increase was significant only in RyR<sub>MH</sub>. This suggests that there was an increased fraction of openings to the maximum conductance and to higher submaximum conductance levels after oxidation-induced activation.

#### Effects of DTNB and 4,4'-DTDP on open and closed time distributions

Open times for normal RyR and RyR<sub>MH</sub> fell into two exponential components under control conditions and three exponential components after oxidation-induced activation (Figs. 6 and 7). Closed times for both normal RyRs and RyR<sub>MH</sub> channels fell into three exponential components under control conditions and after oxidation-induced activation. Data for normal RyRs and RyR<sub>MH</sub> were combined (Fig. 7) because there were no consistent differences in the average time constants or probability of events between the two channel types. Under control conditions and during oxidation-induced activation, the shortest open time constant,  $\tau_{o1}$ , was  $\sim 1$ –2 ms, and the second time constant,  $\tau_{o2}$ , was 7–13 ms. The longest time constant,  $\tau_{o3}$ , was seen only in "activated" channels; it was 50–100 ms and contained  $<10\%$  of openings. The increase in  $T_o$  was largely due to the appearance of  $\tau_{o3}$ .

Closed time constants under control and oxidation-induced activation conditions were  $\tau_{c1}$  (2–4 ms),  $\tau_{c2}$  (9–23 ms), and  $\tau_{c3}$  (90–480 ms) (Figs. 6 and 7). The longest component ( $\tau_{c3}$ ) contained very few events and did not change in any consistent way during oxidation-induced activation. The similar open and closed time distributions with *cis* DTNB, *trans* DTNB, and *cis* 4,4'-DTDP are consistent with the hypothesis that the same cysteine residues are oxidized with each reagent. An additional fourth long open time constant in cardiac RyRs exposed to *cis* 4,4'-DTDP (Eager et al., 1999) was not seen in skeletal RyRs.

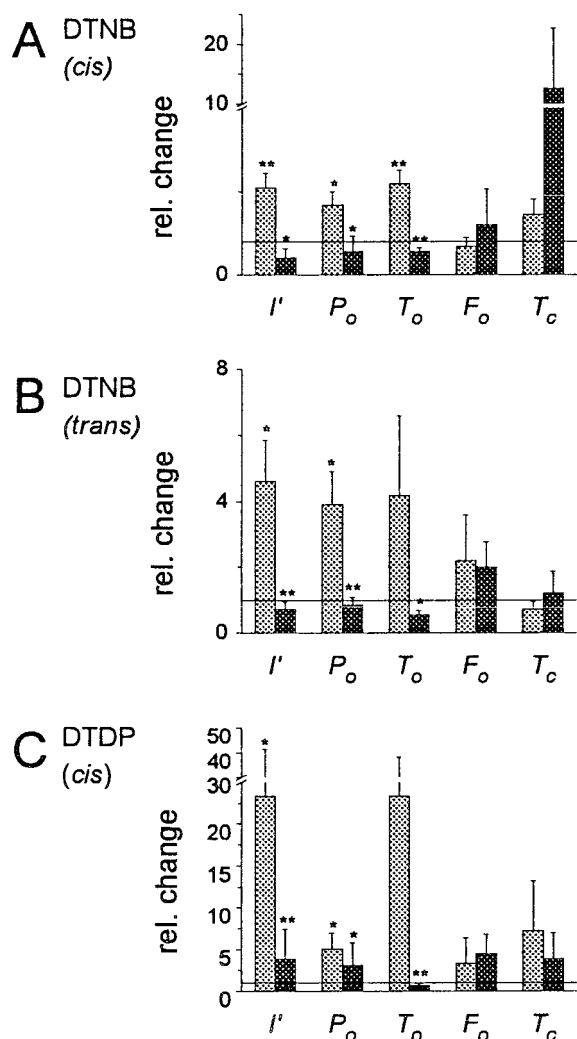


FIGURE 5 Effects of DTNB (*cis* or *trans*) or *cis* 4,4'-DTDP on average channel parameters and reversal of the effects by DTT. Average relative changes are shown for a subset of channels that were exposed first to an oxidizing reagent (light gray bins) and then to DTT (dark gray bins). Pooled data from normal RyRs and RyR<sub>MH</sub> are shown. Channels were exposed to (A) 1 mM *cis* DTNB and then 10 mM *cis* DTT ( $n = 6$ ), (B) 1 mM *trans* DTNB and then 10 mM *trans* DTT ( $n = 4$ ); (C) 1 mM *cis* 4,4'-DTDP (added in *cis* solution) and then 2 mM DTT ( $n = 4$ ). Channels were exposed to DTT 2–3 min after exposure to DTNB or 4,4'-DTDP, at times when channel activity was high. Single-channel parameters shown are mean current ( $I'$ ), open probability ( $P_o$ ), mean open time ( $T_o$ ), frequency of opening ( $F_o$ ), and mean closed time ( $T_c$ ). The bins show mean values and the vertical lines indicate  $\pm 1$  SEM. Asterisks indicate the significance of differences of test data from control (during activation) or from the activated level (after the addition of DTT). \*  $p < 0.05$ ; \*\*  $p < 0.01$ .

### An effect of oxidizing reagents on burst behavior

Channel openings in 20–50% of normal or RyR<sub>MH</sub> channels under control conditions was continuous, while activity in the other channels was clustered into bursts, separated by closures of 0.5–60 s (not included in Figs. 6 and 7). Continuous or burst activity was observed in each of the three modes (high, low, or voltage-activated activity) defined in the text description of Fig. 4 (I–III) above. When either 1

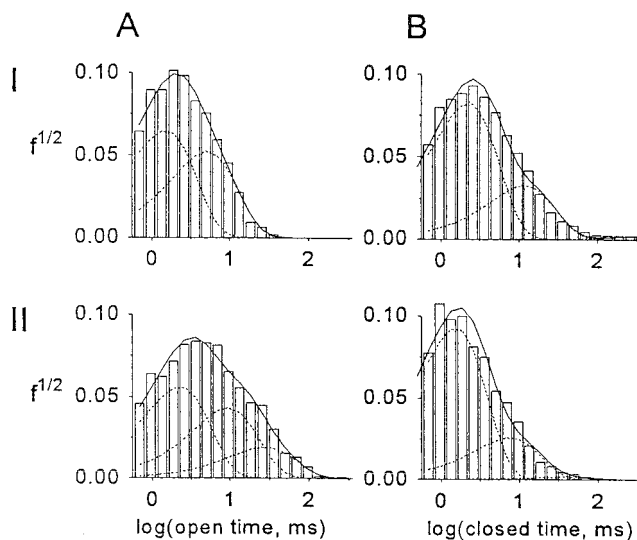


FIGURE 6 Effect of 1 mM *cis* DTNB on open and closed time distributions for a single RyR<sub>MH</sub>. Open and closed times are collected into logged bins, and the square root of the relative frequency of events ( $f^{1/2}$ ) is plotted against the logarithm of the open or closed times in ms. (A) Open time distribution. (B) Closed time distribution. In A and B, distributions are shown for control data (I) and during activation by 1 mM *cis* DTNB (II). The continuous lines in each graph show the multiple exponential function fitted to the data, and the broken lines show the individual exponential components (2 in AI and 3 in each of AII and BII).

mM DTNB or 1 mM 4,4'-DTDP was added to the *cis* chamber, 97% of channels adopted burst behavior, in addition to the increase in channel open time (Table 3). Only 59% of normal RyRs or RyR<sub>MH</sub> had burst behavior with *trans* DTNB-induced activation, or 45% during activation with *cis* or *trans* DTT (10 mM, Results below) or with ethanol (Table 3). The stabilization of burst behavior by oxidizing reagents in the *cis* chamber was not reversed by DTT. Bursting behavior remained in 94% of channels when 10 mM DTT was added to the *cis* or *trans* bath after 1 mM *cis* DTNB, and remained in 50% of channels when 10 mM DTT was added to the *trans* bath after 1 mM *trans* DTNB.

This effect of oxidizing reagents on burst activity suggested that DTNB and 4,4'-DTDP modified a site on the cytoplasmic side of the channel that alters channel gating to stabilize bursting behavior. It is unclear whether the effect is due to -SH oxidation or an interaction between the oxidizing reagents and other sites on the channel, because the effect was not reversed by DTT.

### Further evidence that -aSH is located in the transmembrane domain

If the hypothesis that -aSH is located in the transmembrane domain and is accessible from either side of the bilayer is correct, then activation by DTNB on one side of the bilayer should be reversed by adding DTT to the opposite chamber. In this experiment, five of six RyR channels were activated when DTNB was added to the *cis* chamber, and activity fell in five of the channels when DTT was added to the *trans*

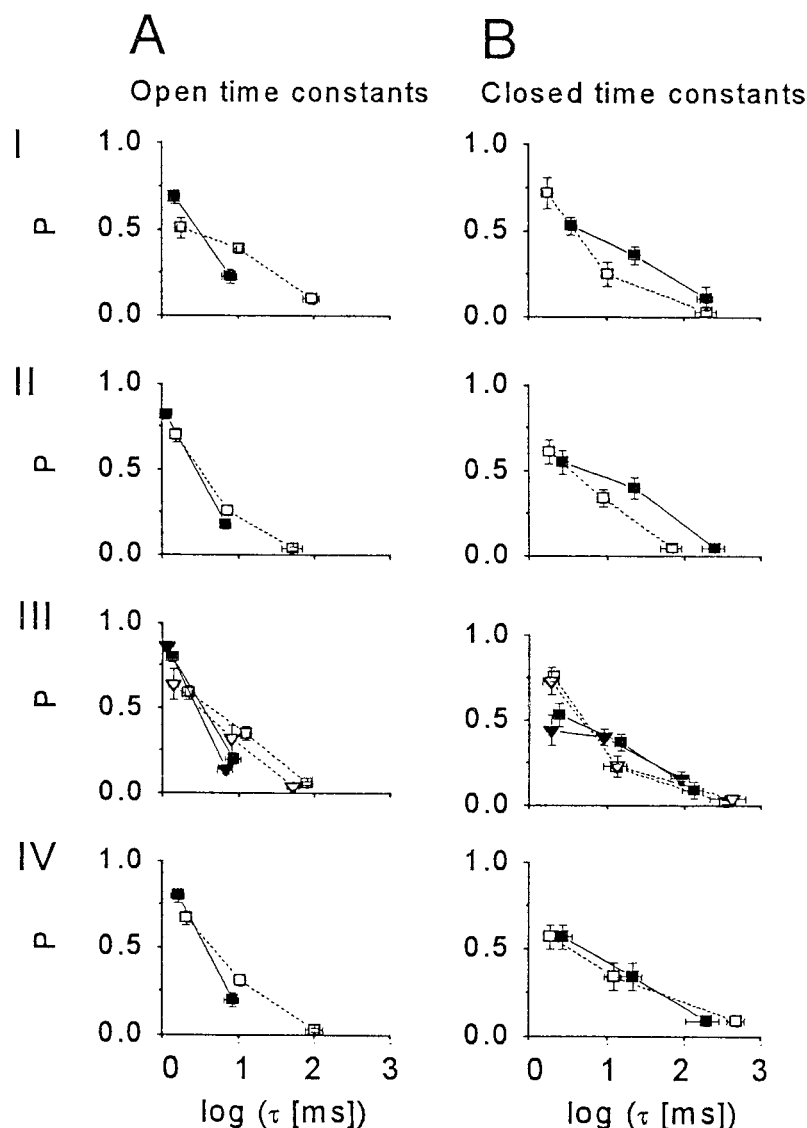


FIGURE 7 Effects of oxidizing reagents on the average time constants and fraction of events obtained from multiple exponential fits to the open and closed time distributions (e.g., Fig. 6). The probability of events falling into each time constant ( $p$ ) is plotted against the logarithm of the time constant in ms ( $\log$  [open time, ms] or  $\log$  [closed time, ms]). The average values are for combined data from normal RyRs and RyR<sub>MH</sub>. (A) Open time constants. (B) Closed time constants. In A and B data are shown before and after the addition of 1 mM *cis* DTNB (I); 1 mM *trans* DTNB (II); 1 mM *cis* 4,4'-DTDP with 1% ethanol or *cis* 1% ethanol alone (III); 1 mM *cis* 4,4'-DTDP (IV). Average values are shown for control conditions (■, ▼) and in the presence of the oxidizing reagent (□) or ethanol (▽, ▲III and BIII only).

chamber. Conversely, one of three other channels was activated when DTNB was added to the *trans* chamber, and activity fell in that channel when DTT was added to the *cis* chamber.

### Evidence for a modified thiol in the transmembrane domain

DTT at 2 mM, added in the absence of an oxidizing reagent, did not significantly alter RyR activity. The mean current, normalized to control, was  $3.6 \pm 2.8$  in five normal RyRs after 2 mM DTT addition and  $2.7 \pm 0.4$  in five RyR<sub>MH</sub> channels. On the other hand, 10 mM DTT significantly activated normal RyRs and RyR<sub>MH</sub> when added to either the *cis* or *trans* chamber in the absence of added oxidizing reagent; activity then fell toward control levels when 1 mM DTNB was added to the opposite side of the bilayer (Fig. 8). *Cis* DTT (10 mM) activated five of five RyRs (two normal; three RyR<sub>MH</sub>), while *trans* DTT activated two of four nor-

mal RyRs and three of three RyR<sub>MH</sub>. Data from normal RyRs and RyR<sub>MH</sub> is combined in Fig. 8. The two RyRs not activated by *trans* DTT were not included, because their control activity was high ( $I'$  of 4.0 and 6.8 pA) and outside the range of 0.2–1.2 pA in the other five channels. Average  $I'$  increased significantly with 10 mM DTT (*cis* or *trans*) and then fell significantly after DTNB addition to the opposite chamber (Fig. 9). Interestingly, activity in the two channels that were not activated by *cis* DTT fell to lower levels when DTNB was added to the *trans* chamber, with similar approximately sixfold reductions in  $I'$ . These channels may have already been in a reduced state before the addition of DTT.

These results suggest that a class of modified thiol ( $_{-ab}S-R$ , where R is either a protein S if the modified thiol is a disulfide, or a N if the modified group is nitrosylated; Xu et al., 1998) is available to 10 mM DTT from either the *cis* or *trans* chamber and that the reduced  $_{-ab}SH$  can be oxidized by DTNB from the opposite side of the bilayer. The acces-



**TABLE 3** Effects of oxidizing reagents and ethanol on burst behavior of normal RyRs and RyR<sub>MH</sub>

	Before → After Continuous → Continuous	Before → After Continuous → Bursting	Before → After Bursting → Bursting	Before → After Bursting → Continuous
DTNB ( <i>cis</i> )				
Normal RyR	0	8	10	0
RyR <sub>MH</sub>	0	2	16	0
DTNB ( <i>trans</i> )				
Normal RyR	8	0	4	1
RyR <sub>MH</sub>	3	0	15	1
4,4'-DTDP (eth) ( <i>cis</i> )				
Normal RyR	0	6	9	1
RyR <sub>MH</sub>	0	1	13	0
4,4'-DTDP (H <sub>2</sub> O) ( <i>cis</i> )				
Normal RyR	2	3	18	0
RyR <sub>MH</sub>	0	0	17	0
Ethanol (1%) ( <i>cis</i> )				
Normal RyR	1	1	7	1
RyR <sub>MH</sub>	0	1	13	1

Oxidizing reagents were added to a final concentration of 1 mM. The numbers of channels are shown with continuous or bursting activity before and after addition of the agents listed on the left-hand side, for normal RyRs and RyR<sub>MH</sub>.

4,4'-DTDP (eth), 4,4'-DTDP dissolved in ethanol.

4,4'-DTDP (H<sub>2</sub>O), 4,4'-DTDP dissolved in *cis* solution.

sibility from either side of the bilayer indicates that -<sub>ab</sub>S-R- and -<sub>ab</sub>SH are located in the transmembrane domain.

#### **“Control-like” channel activity is retained in the presence of 1 mM DTNB plus 10 mM DTT**

Channel activity returned to “control-like” levels when 10 mM DTT and 1 mM DTNB were present on either the same or opposite sides of the bilayer. This was confirmed in further experiments, in which the “control-like” activity was maintained if 10 mM DTT was present on both sides of the channel, with 1 mM DTNB on one side only (five of six experiments with *cis* DTNB, or three of three with *trans* DTNB). Average  $I'$  measured in four of the channels with *cis* DTNB or two of the channels with *trans* DTNB was  $-2.19 \pm 0.32$  pA. “Control-like” activity was retained when 10 mM DTT was removed from one chamber, leaving 10 mM DTT and 1 mM DTNB in the *cis* chamber in two cases, or in the *trans* chamber in a third case ( $I' = -1.33 \pm 0.51$  pA).

The assertion that channel activity in the presence of 10 mM DTT and 1 mM DTNB was “control-like” was supported by a final experiment ( $n = 2$ ) in which the *trans* chamber initially contained 1 mM DTNB plus 10 mM DTT and the *cis* chamber contained 1 mM DTNB ( $I' = -0.97 \pm 0.39$  pA). The *trans* chamber was perfused with normal *trans* solution, leaving 1 mM DTNB in the *cis* solution. In both cases, RyR activity increased after perfusion, as it usually did when DTNB was present alone in the *cis* chamber ( $I' = -5.50 \pm 0.33$  pA).

## **DISCUSSION**

We found that DTNB activated skeletal muscle RyRs from either the cytoplasmic or luminal side of the channel, while

4,4'-DTDP activated and then inhibited RyRs from the cytoplasmic solution. Activation by both reagents and inhibition by 4,4'-DTDP were reversed by DTT and thus are due to sulfhydryl oxidation. The results can be explained by oxidation of two classes of sulfhydryl, -<sub>a</sub>SH in the transmembrane domain for activation or -<sub>i</sub>SH in a hydrophobic environment for inhibition. Separate -<sub>a</sub>SH and -<sub>i</sub>SH residues have also been postulated for cardiac RyRs (Eager and Dulhunty, 1998, 1999). Additional novel findings were that 1) addition of the oxidizing reagents to the *cis* side of RyRs stabilized bursting channel activity, suggesting that cytoplasmic residues regulate burst activity; 2) addition of 10 mM DTT to either side of the channel caused activation, which was reversed when DTNB was added to the opposite side, suggesting that a modified thiol -<sub>ab</sub>S-R- in the transmembrane domain normally inhibits activity; and 3) “control-like” channel activity was maintained in the presence of 1 mM DTNB and 10 mM DTT. Finally, the effects of the oxidizing reagents on RyRs from normal and MH pigs were similar.

#### **Activation of skeletal RyRs by reactive disulfides**

Activation of skeletal RyRs by DTNB and 4,4'-DTDP confirms previous reports on skeletal RyRs (Nagura et al., 1988; Marengo et al., 1998; Zable et al., 1997). Similar activation, with a long time constant component introduced into the open time distribution, is seen in cardiac RyRs exposed to 4,4'-DTDP or thimerosal. An increase in open frequency is also seen in cardiac RyRs. A fourth long time constant component in the open time distribution of cardiac RyRs oxidized by 4,4'-DTDP was not seen in skeletal RyRs, suggesting that the -<sub>a</sub>\*SH class of sulfhydryl, postulated for the cardiac RyR (Eager and Dulhunty, 1998), either is not present or is not available for oxidation in

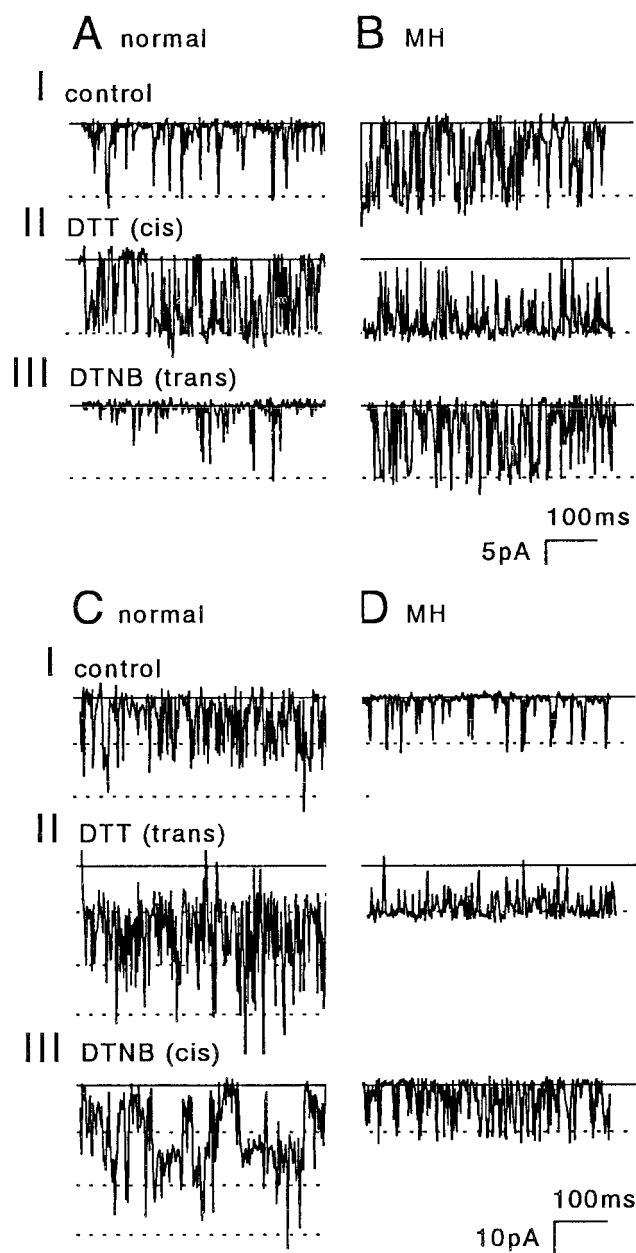


FIGURE 8 Activation of normal RyRs and RyR<sub>MH</sub> by 10 mM DTT added to the *cis* or *trans* chamber without oxidizing reagent, and the reversal of activation by the addition of 1 mM DTNB to the opposite chamber. (A and C) Normal RyRs. (B and D) RyR<sub>MH</sub>. Records in A–D show control activity (I); activity with 10 mM DTT (II); activity after addition of 1 mM DTNB (III). Drug additions were (A and B) *cis* DTT then *trans* DTNB and (C and D) *trans* DTT then *cis* DTNB. The zero current level is indicated by solid lines, and the maximum open conductance is shown by broken lines. Bilayers in A, B, and D contained one active channel, while the bilayer in C contained three active channel. The three broken lines in C indicate the maximum open conductance with one, two, or three channels open in the bilayer.

skeletal RyRs. The fact that reversal of activation by 1 mM DTNB required 10 mM DTT, while reversal of activation by 4,4'-DTDP or thimerosal requires 2 mM DTT (results above and Eager et al., 1999), suggests that DTNB has stronger oxidative properties than 4,4'-DTDP. The failure

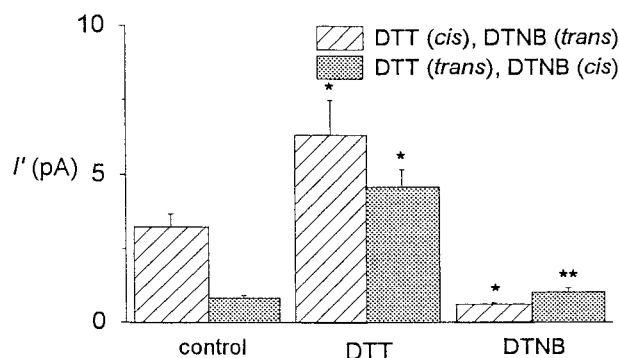


FIGURE 9 Average effects on  $I'$  of activation by 10 mM DTT added to the *cis* or *trans* chamber and reversal of the activation when 1 mM DTNB is added to the opposite chamber. The cross-hatched bins show results for *cis* DTT followed by *trans* DTNB. The stippled bins show data results for *trans* DTT followed by *cis* DTNB. The average  $I'$  (in pA) is given for control conditions (control), after the addition of DTT (DTT), and then after the addition of DTNB (DTNB). Asterisks indicate the significance of the difference between DTT and control, or between DTNB and DTT. \*  $p < 0.05$ ; \*\*  $p < 0.01$ .

of GSSG to activate skeletal RyRs from the luminal solution (Zable et al., 1997) might have been due to the weak oxidizing ability of GSSG.

#### What do similar changes to reagents added to either side of the RyR mean in terms of the location of target residues?

The ability of DTNB to activate RyRs from the luminal or cytoplasmic side and the reversal of activation by 10 mM DTT added to the opposite side of the channel suggest that  $-_a\text{SH}$  is accessible to DTNB and DTT from the cytoplasmic and luminal solutions. This accessibility to water-soluble reagents could suggest that target residues are located in the channel pore. However, DTT with a  $pK_a$  of 9.0–10 (Shaked et al., 1980) would be largely uncharged at pH 7.4 and would rapidly partition into the membrane. Similarly, 1–10% of DTNB with a  $pK_a$  of 5–6 (Houk et al., 1987) would enter the membrane. Thus the water-soluble agents could access residues located in the transmembrane domain, not in the pore, although activating residues in a transmembrane, rather than pore location, would see only 10–100  $\mu\text{M}$  DTNB. This is not an unreasonable [DTNB] for activation, because DTNB is a strong oxidizing reagent (above), and NO at  $\sim 40$  nM nitrosylates thiol groups and activates skeletal RyRs (Hart and Dulhunty, unpublished observations), while cardiac RyRs are activated by 100  $\mu\text{M}$  4,4'-DTDP (Eager et al., 1997).

It is unlikely that the reagents, either crossing the membrane or passing through the pore, could have targeted residues located on the opposite side of the channel and remote from the membrane, because dilution in the large volume of solution would mean that it would take a long time for their concentrations to increase to active levels. Thus activation with DTNB or recovery with DTT would

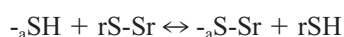
have been faster when the reagents were applied to the side of the channel containing the residues. Because similar rates of activation and deactivation were seen with *cis* and *trans* applications of both reagents, we conclude that DTNB and DTT act at a transmembrane (possibly pore) location.

Another possibility is that separate actions of DTNB on cytoplasmic and luminal sulfhydryls result in similar functional effects on RyR activity (Eager et al., 1998). However, reversal of activation by DTT on the opposite side of the bilayer would not be expected if  $-_a\text{SH}$  were distributed over the luminal and cytoplasmic domains of the RyR. If  $-_a\text{SH}$  is located in the channel pore, then DTT, DTNB, and 4,4'-DTDP must penetrate the channel. Molecules of a mass similar to that of DTT (formula weight, FW 154) and 4,4'-DTDP (FW 220), such as glucose and xylose (FW 180 and 150, respectively), pass slowly through skeletal RyRs (Meissner, 1986; Kasai et al., 1992). DTNB (FW 396) and thimerosal (FW 405) have a greater mass but may nevertheless assume conformations that allow them to also pass through the channel. Curiously, neither DTNB nor 4,4'-DTDP nor DTT blocks the channel into the low-conductance states seen in the presence of the smaller (FW 110–172) methanethiosulfonate (MTS) derivatives (Quinn and Ehrlich, 1997). Although DTNB and 4,4'-DTDP are cleaved during oxidation of protein thiols, their half-masses of 110 and 198 remain equivalent to that of the MTS compounds. If DTT, DTNB, and 4,4'-DTDP enter the pore and interact with sulfhydryl residues to alter channel gating, they must do so without physically blocking the pore.

#### *Inhibition of skeletal RyRs by reactive disulfides*

We propose that oxidation of separate  $-_a\text{SH}$  and  $-_i\text{SH}$  leads, respectively, to activation and inhibition of RyRs, rather than oxidation of one class of sulfhydryl that first activates and then inhibits the channel. Evidence for separate residues is that 1) activation by DTNB was not followed by inhibition and 2) inhibition was observed without preceding activation in some channels (see also Eager et al., 1997). RyR inhibition by 4,4'-DTDP, but not DTNB, suggested that  $-_i\text{SH}$  may be located in a domain of the skeletal RyR that is inaccessible to DTNB. In contrast, the water-soluble thimerosal inhibited cardiac RyRs (Eager et al., 1999). This apparent difference may have been due to the use of thimerosal in cardiac and DTNB in skeletal RyRs, because thimerosal penetrates proteins more effectively than other thiol reagents (van Iwaarden et al., 1992).

An alternative possibility is that the different abilities of DTNB, 4,4'-DTDP, and thimerosal to inhibit RyRs are related to their different redox potentials. Sulfhydryl-specific oxidizing reagents can react with proteins in two steps (Glazer, 1970). The first step is the formation of a mixed disulfide between the protein thiol ( $-_a\text{SH}$ , for example) and the reagent (rS-Sr):



The reaction can then proceed under appropriate conditions, to cross-link  $-_a\text{S-}$  with another thiol group within the protein ( $-_i\text{SH}$ , for example):



In this case the cross-linking step would be responsible for inhibition. The second step may proceed with weak oxidizing reagents like 4,4'-DTDP and thimerosal, but not with stronger oxidants like DTNB. Stronger oxidation by DTNB is supported by the fact that 10 mM DTT is required to reduce  $-_a\text{S-Sr}$  when the oxidizing agent is 1 mM DTNB, while the thiols oxidized by 1 mM 4,4'-DTDP or thimerosal are reduced by 2 mM DTT. The hypothesis that inhibition depends on the formation of a disulfide bridge requires separate  $-_a\text{SH}$  and  $-_i\text{SH}$  residues but does not explain the observation that inhibition can proceed in the absence of activation (discussed above).

#### **Comparison between cardiac and skeletal RyRs**

It is possible that the same cysteine residues form  $-_a\text{SH}$  in cardiac and skeletal RyRs. Many cysteine residues in the channel domain are conserved between cardiac and skeletal RyRs (Otsu et al., 1990) and are in an appropriate location for  $-_a\text{SH}$ . The subtly different effects of oxidation on the gating of the cardiac and skeletal RyRs could be attributed to a difference in the connections between the  $-_a\text{SH}$  and the channel gating mechanisms. Such a difference may be imposed by sequence differences between the cardiac and skeletal RyRs (having only 66% sequence identity; Otsu et al., 1990), which could impose structural differences between the proteins.

Skeletal RyR channels recovered from inhibition when DTT was added to the *cis* solution, but the loss of activity in cardiac RyRs could not be reversed by DTT (Eager et al., 1997). Inhibition of skeletal RyRs by NO is also relieved by DTT (Hart and Dulhunty, unpublished observations). These observations suggest that the oxidized  $\text{xS-S}_i$ - (where  $-\text{xS}$  is contributed either by DTNB or 4,4'-DTDP or by the protein) is more accessible to DTT in skeletal RyRs than in cardiac RyRs.

#### *Effect of DTT alone on pig skeletal RyRs*

Addition of 10 mM DTT to either side of the bilayer (with oxidizing reagents absent from *cis* and *trans* solutions) increased RyR activity. Increased mammalian skeletal RyR activity with DTT is in contrast to a fall in  $P_o$  when 20 mM GSH was added to frog skeletal or rabbit cardiac RyRs (Marengo et al., 1998) or when 10 mM DTT was added to sheep cardiac RyRs (Eager et al., 1997). Zable et al. (1997) also showed a reduction in rabbit skeletal RyR activity with 6–10 mM GSH and reduced ryanodine binding with GSH, DTT, and BME. The reason for the difference between our results and those of Zable et al. may be partly due to sequence differences between pig and rabbit RyRs (see

below). However, we also find that rabbit skeletal RyR activity increases when 2 mM DTT alone is added to the *trans* chamber (Green, Hart, and Dulhunty, unpublished observations). In agreement with our findings, DTT induces contraction in intact frog skeletal muscle fibres by triggering  $\text{Ca}^{2+}$  release from SR (Oba et al., 1996).

Activation of skeletal RyRs by *cis* or *trans* DTT and its reversal with DTNB in the opposite chamber suggest that a class of modified thiols,  $-\text{abS-R}-$ , which normally suppresses channel activity, is present in the mammalian skeletal RyR transmembrane region. The R group could be contributed either by another protein cysteine if a disulfide is formed or could be a N if  $-\text{abS}$  is nitrosylated. Cardiac RyRs contain stable nitrosothiols if isolated in the absence of DTT, and nitrosylated thiols can be denitrosylated by 10 mM DTT (Xu et al., 1998). The pig SR used in the present experiments was isolated in the presence of 2 mM DTT, which may not have denitrosylated all nitrosothiols on the RyRs. Denitrosylation, however, is difficult to reconcile with the reversibility of the effects of DTT by DTNB, unless it is also suggested that modification per se depresses channel activity, independent of whether the thiol is nitrosylated or oxidized by DTNB. It seems more likely that the modified thiol is part of an intraprotein disulfide bridge.

Two cysteine residues in the skeletal RyR, one just outside the M1 region and one between M5 and M6, are not present in cardiac RyRs (Otsu et al., 1990). These cysteines are likely to be in the transmembrane region and could form disulfide bridges or be available for S-nitrosylation.

#### *"Control-like" channel activity with 1 mM DTNB and 10 mM DTT*

The observation that channel activity returned to "control-like" levels when 1 mM DTNB and 10 mM DTT were present, either on the same or opposite sides of the bilayer, provided further evidence that  $-\text{aSH}$  and  $-\text{abS-R}-$  are located in the transmembrane domain. The fact that removal of DTT, leaving only DTNB in one chamber, resulted in channel activation, which was similar to that seen when DTNB was added alone, supported the suggestion that channel activity was "control-like" when the two reagents were present together. The result suggests that redox cycling with 1 mM DTNB plus 10 mM DTT kept  $-\text{abS-R}-$  mostly in its modified form, and  $-\text{aSH}$  mostly in its reduced form.

#### *Effect of ethanol on the RyRs from pig muscle*

Activation of normal RyRs and RyR<sub>MH</sub> by 1% ethanol after 2–3 min was unexpected because exposure to ethanol for the same period does not alter single-channel activity of cardiac (Eager et al., 1997) or rabbit skeletal (Ahern et al., 1997b) RyRs. However, open times increase if rabbit skeletal RyRs are exposed to ethanol for 5 min (Dulhunty and Curtis, unpublished observations). The results may reflect intrinsic differences between pig and rabbit skeletal RyRs reported previously. The maximum rate of polylysine-in-

duced  $\text{Ca}^{2+}$  release is four times greater in pig skeletal SR than that in rabbit SR (El-Hayek et al., 1995; Cifuentes et al., 1989). In addition, pig RyRs are less sensitive than rabbit RyRs to activation by peptides corresponding to the loop between membrane-spanning segments II and III of the skeletal DHPR (Gallant, Pace, and Dulhunty, 1999). These functional differences are likely to be imposed by sequence differences between the pig and rabbit RyRs. There are 45 residues (two cysteines) that differ between the pig and rabbit RyRs in the first 1500 residues of the protein, with an overall 3% dissimilarity (Fujii et al., 1991).

#### *Effect of the RyR mutation in malignant hyperthermia*

RyR<sub>MH</sub> responded to oxidation and reduction in a way similar to that of normal RyRs. Therefore the additional cysteine residue in MH does not alter the response of the RyR to either oxidizing or reducing reagents. Either Cis<sup>615</sup> is buried in the protein and is not accessible to the redox reagents, or oxidation/reduction of the additional  $-\text{SH}$  or  $-\text{S-R}-$  does not effect channel activity under the conditions of our experiments. The response of RyRs to oxidation can depend on ligands bound to the protein (Eager et al., 1998; Xu et al., 1998); thus it remains possible that Cis<sup>615</sup> in RyR<sub>MH</sub> becomes available for oxidation or reduction, or that the modified residue is able to regulate activity, during increased  $\text{Ca}^{2+}$  release and increased metabolic activity, and can thus further enhance  $\text{Ca}^{2+}$  release from the SR during the MH response.

In conclusion, the similar effects of oxidizing reagents in cardiac and skeletal RyRs suggest that at least two of the cysteine residues whose modification alter channel gating are conserved between the two proteins. The results further suggest that free  $-\text{SH}$  residues and modified thiols are present in the transmembrane region of the skeletal RyR under control conditions and that their covalent modification under oxidizing or reducing conditions can significantly modify RyR channel gating.

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