Sulfhydryl Oxidation Modifies the Calcium Dependence of Ryanodine-Sensitive Calcium Channels of Excitable Cells

Juan José Marengo,* Cecilia Hidalgo,#§ and Ricardo Bull*
*Programa de Fisiología y Biofísica and #Programa de Biología Molecular y Celular, Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, Santiago, and §Centro de Estudios Científicos de Santiago, Santiago, Chile

ABSTRACT The calcium dependence of ryanodine-sensitive single calcium channels was studied after fusing with planar lipid bilayers sarcoendoplasmic reticulum vesicles isolated from excitable tissues. Native channels from mammalian or amphibian skeletal muscle displayed three different calcium dependencies, cardiac (C), mammalian skeletal (MS), and low fractional open times (low P_o), as reported for channels from brain cortex. Native channels from cardiac muscle presented only the MS and C dependencies. Channels with the MS or low P_o behaviors showed bell-shaped calcium dependencies, but the latter had fractional open times of <0.1 at all $[Ca^{2+}]$. Channels with C calcium dependence were activated by $[Ca^{2+}] < 10\ \mu M$ and were not inhibited by increasing *cis* $[Ca^{2+}]$ up to 0.5 mM. After oxidation with 2,2′-dithiodipyridine or thimerosal, channels with low P_o or MS dependencies increased their activity. These channels modified their calcium dependencies sequentially, from low P_o to MS and C, or from MS to C. Reduction with glutathione of channels with C dependence (native or oxidized) decreased their fractional open times in 0.5 mM *cis* $[Ca^{2+}]$, from near unity to 0.1–0.3. These results show that all native channels displayed at least two calcium dependencies regardless of their origin, and that these changed after treatment with redox reagents.

INTRODUCTION

Calcium release from internal stores contributes to the transient increments in intracellular free calcium concentration ($[Ca^{2+}]$) that underlie many responses of excitable cells, such as synaptic plasticity and gene expression in neurons (Gosh and Greenberg, 1995), and contraction in skeletal and cardiac muscle (Meissner, 1994). Calcium is released from these stores through two separate pathways, the inositol 1,4,5-trisphosphate receptor channels (Furuichi et al., 1994) and the ryanodine-sensitive calcium release channels (RyR channels) (Meissner, 1994; Zucchi and Ronca-Testoni, 1997).

Different species and different excitable cells express diverse ryanodine receptor (RyR) isoforms (Furuichi et al., 1994; Ogawa, 1994; Sutko and Airey, 1996). In mammals, the RyR-1 isoform is the main isoform found in adult skeletal muscle cells (Furuichi et al., 1994), whereas RyR-2 is the main isoform of cardiac muscle cells (Coronado et al., 1994; Furuichi et al., 1994). Neurons contain the RyR-3 isoform in addition to the RyR-1 and RyR-2 isoforms (Giannini and Sorrentino, 1995). Small amounts of RyR-3 are also found in adult diaphragm muscle (Murayama and Ogawa, 1997). Amphibian, avian, and fish skeletal muscle cells express two different RyR isoforms, α and β (Ogawa, 1994; Sutko and Airey, 1996). The amphibian and avian α and β isoforms have a significant extent of homology with the RyR-1 and the RyR-3 mammalian isoforms, respectively (Ogawa, 1994; Oyamada et al., 1994; Ottini et al., 1996).

The physiological mechanisms of activation of these diverse RyR channels are not well understood at the present time (Furuichi et al., 1994; Giannini and Sorrentino, 1995; Melzer et al., 1995; Zucchi and Ronca-Testoni, 1997). Only for the cardiac muscle RyR-2 isoform is there general consensus that calcium is the physiological agonist that triggers calcium release from sarcoplasmic reticulum (SR) after plasma membrane depolarization (Melzer et al., 1995). Yet all single RyR channels studied so far show activation by μM cytosolic $[Ca^{2+}]$ (Coronado et al., 1994; Meissner, 1994; Melzer et al., 1995). In the particular case of the RyR-3 isoform, functionally expressed single RyR-3 channels from mammalian smooth muscle are activated by μM $[Ca^{2+}]$ (Chen et al., 1997), and ryanodine binding to RyR-3 obtained either from mammalian brain (Murayama and Ogawa, 1996) or from diaphragm muscle is calcium dependent (Murayama and Ogawa, 1997). Thus it is likely that calcium activation of channel activity, the underlying mechanism of calcium-induced calcium release, is a general feature of all the RyR isoforms present in excitable cells.

Different single-channel responses to changes in *cis* (cytosolic) $[Ca^{2+}]$ have been reported. Channels present in SR vesicles isolated from mammalian cardiac muscle show sigmoidal activation by *cis* μM $[Ca^{2+}]$. Most reports show a lack of inhibition by increasing $[Ca^{2+}]$ up to 1–2 mM (Rousseau et al., 1986; Anderson et al., 1989; Holmberg and Williams, 1990; Chu et al., 1993). This type of calcium dependence will be named the C calcium dependence (for cardiac). Yet cardiac channel activity decreases after changing pCa from 8 to 4 or 3 in a time-resolved bilayer system (Schiefer et al., 1995). Furthermore, variable inhibition of

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Address reprint requests to Dr. Juan Jose Marengo, Programa de Fisiología y Biofísica, ICBM, Facultad de Medicina, Universidad de Chile, Casilla 70005, Correo 7, Santiago, Chile. Tel.: 56-2-678-6313; Fax: 56-2-777-6916; E-mail: jmarengo@canela.med.uchile.cl.

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cardiac channel activity recorded in steady-state conditions was reported when *cis* $[Ca^{2+}]$ was increased to 2 mM or beyond (Laver et al., 1995; Copello et al., 1997). These results suggest that cardiac RyR channels present more than one type of response to increases in cytoplasmic $[Ca^{2+}]$.

The ryanodine-sensitive calcium channels of mammalian skeletal muscle SR display a bell-shaped calcium dependence (which will be named the MS calcium dependence (for mammalian skeletal)) with activation by μM $[Ca^{2+}]$ (Smith et al., 1986) and inhibition by $[Ca^{2+}] \geq 0.1$ mM (Fill et al., 1990; Chu et al., 1993). However, recent studies have shown that these channels also display more than one type of calcium dependence (Copello et al., 1997). Single RyR channels obtained from amphibian, avian, and fish skeletal muscle display two types of calcium dependencies, C and MS (Bull and Marengo, 1993; Percival et al., 1994; O'Brien et al., 1995).

We have described how RyR channels derived from rat brain cortex neurons exhibit three types of responses to changes in *cis* $[Ca^{2+}]$ (Marengo et al., 1996). In addition to the C and MS calcium dependencies, a third response, the low P_o calcium dependence, was observed with the highest frequency. This low P_o behavior is characterized by a bell-shaped calcium dependence with fractional open times (P_o) less than 0.1 in the $[Ca^{2+}]$ range 0.1 μM to 0.5 mM (Marengo et al., 1996). A calcium dependence with the same characteristics as the low P_o dependence has recently been reported for mammalian skeletal RyR channels (Copello et al., 1997).

In addition to cytosolic $[Ca^{2+}]$, many other agents modify RyR channel activity (Coronado et al., 1994; Meissner, 1994), among them, agents that modify sulfhydryl (SH) groups. Thus SH reagents (Abramson et al., 1995), free radicals (Stoyanovsky et al., 1994), and hydrogen peroxide (Favero et al., 1995) activate RyR channels incorporated into planar lipid bilayers. Heavy metals (Abramson et al., 1983; Trimm et al., 1986; Salama et al., 1992), mercurials (Bindoli and Fleischer, 1983), dithiodipyridines (Nagura et al., 1988; Prabhu and Salama, 1990; Donoso et al., 1997), and derivatives of nitric oxide (Stoyanovsky et al., 1997) trigger calcium release from isolated SR vesicles as well. Furthermore, SH reagents shift the calcium activation curve of ryanodine binding to the left (Stuart et al., 1992; Favero et al., 1995), and heavy metals enhance the calcium sensitivity of tension development in skinned fibers from rabbit psoas muscle (Salama et al., 1992). These findings suggest that SH reagents modify the activation of mammalian RyR channels by cytosolic $[Ca^{2+}]$.

In this work we studied in steady-state conditions the responses to changes in *cis* $[Ca^{2+}]$ of single RyR-channels present in SR isolated from rabbit or frog skeletal muscle, or from rabbit cardiac muscle. All native RyR channels studied displayed more than one calcium dependence, despite the channel isoform(s) present in the SR vesicles. Furthermore, we found that 1) oxidation of SH residues modified the calcium dependencies of all single RyR channels, regardless of their origin, and 2) reduction of channels with the C calcium dependence (native or oxidized) caused a decrease in channel activity. We propose that the oxidation state of the channel protein is a decisive factor in determining the calcium dependence of the channel activity exhibited by any given isoform.

EXPERIMENTAL PROCEDURES

Membrane isolation

Triad-enriched SR vesicles were isolated from fast skeletal muscles of rabbit (New Zealand) and frog (*Caudiverbera caudiverbera*), as reported elsewhere (Hidalgo et al., 1993). Endoplasmic reticulum vesicles from rat (Sprague-Dawley) brain cortex were obtained as described previously (Marengo et al., 1996). All membranes were isolated with or without 3–5 mM dithiothreitol (DTT) present throughout the isolation procedure, as indicated in the text. SR from rabbit cardiac muscle was obtained by using a modification of the procedure developed to isolate triads from skeletal muscle (Hidalgo et al., 1993). Briefly: the ventricles of one rabbit heart were cleaned of blood and connective tissue, and were placed in 5 volumes of ice-cold buffer (0.15 M KCl, 5 mM $MgS0_4$, 20 mM 3-[*N*-morpholino]propanesulfonic acid/Tris, pH 6.8, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 0.4 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, plus or minus 3 mM DTT). The tissue was finely minced and homogenized twice in a Polytron homogenizer (Heidolph Diax 600) for 45 s at 13,500 rpm. Cardiac SR vesicles sedimenting between 1,500 and 17,000 $\times$ *g* were collected by differential centrifugation and were resuspended in the same buffer used for homogenization. To remove contractile proteins, the suspension was made 0.6 M in KCl by the addition of solid salt, and the sediment obtained at 1500 $\times$ *g* was discarded. Cardiac SR vesicles were collected by sedimentation at 17,000 $\times$ *g* and were resuspended in a small volume of homogenization buffer plus 0.3 M sucrose. Small aliquots were quickly frozen in liquid N_2 and stored at -80°C.

Channel recording and analysis

Channel recording and analysis were performed as described previously (Bull and Marengo, 1994). Values of P_o were calculated from single-channel records lasting at least 180 s. All experiments were carried out at room temperature (22–24°C). The recording conditions were 40 mM Ca^{2+}-HEPES, 15 mM HEPES/Tris, pH 7.4, in the *trans* compartment; 225 mM HEPES/Tris, pH 7.4, and variable $[Ca^{2+}]$ in the *cis* compartment. To set the desired *cis* $[Ca^{2+}]$, 0.5 mM total Ca^{2+} and sufficient *N*-(2-hydroxyethyl)-ethylenediamine-triacetic acid (HEDTA) or EGTA were added to the *cis* compartment. Resulting *cis* $[Ca^{2+}]$ values were routinely checked with a calcium electrode.

After channel incorporation into the bilayer and establishment of recording conditions (Bull and Marengo, 1994), oxidation was carried out by the addition of 2,2′-dithiodipyridine (DTDP) or thimerosal to the *cis* compartment. SH oxidation was stopped by removal of the nonreacted reagent through extensive perfusion of the *cis* compartment (5–10 times the *cis* volume) with a solution containing 225 mM HEPES/Tris (pH 7.4). Native or oxidized channels were treated with SH-reducing agents by essentially the same procedure. The *cis* $[Ca^{2+}]$ used during incubation with SH reagents is specified in the text. The pseudo-steady states reached during oxidation or reduction were defined as periods of time during which the slope of the change in P_o versus time, calculated in successive frames of 1.024 s, did not differ from zero.

Theoretical analysis of calcium dependence

To fit the experimental data, the following general function (Bull and Marengo, 1993; Marengo et al., 1996) was used for the three different

calcium dependencies:

$$P_o = P_{o,max}/(1 + (K_a/[Ca^{2+}])^n + ([Ca^{2+}]/K_i)^n) \quad (1)$$

In this equation the parameter $P_{o,max}$ corresponds to the theoretical P_o value of maximum activation by calcium; K_a and K_i correspond to the calcium concentrations for half-maximum activation and inhibition of channel activity, respectively; and n is the Hill coefficient for activation and inhibition. Data fitting yielded the particular cases described in detail in the Results section.

Materials

Lipids were obtained from Avanti Polar Lipids (Birmingham, AL). Protease inhibitors and other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

RESULTS

High-conductance calcium channels (~100 pS with Ca^{2+} as permeant ion) were obtained after fusion with planar lipid bilayers of vesicles isolated from the different excitable tissues used in this work. The addition of μM ryanodine (not shown) always induced the characteristic subconductance open state produced by this alkaloid (Rousseau et al., 1987).

Effect of *cis* $[Ca^{2+}]$ on the activity of native RyR channels from skeletal or cardiac muscle

Native RyR channels derived from rabbit (Fig. 1 *A*) or frog (Fig. 1 *B*) skeletal muscle SR presented the same three calcium dependencies: low P_o (*solid diamonds*), MS (*open circles*), and C (*solid circles*), displayed by the RyR channels of brain cortex neurons (Marengo et al., 1996). The native RyR channels of SR vesicles isolated from rabbit ventricular muscle cells presented the MS calcium dependence (Fig. 1 *C*, *open circles*), in addition to the most commonly reported C response (Fig. 1 *C*, *solid circles*). Channels with the low P_o behavior were not observed in cardiac SR vesicles ($N = 19$ experiments).

The presence of DTT as a reducing agent during isolation of SR vesicles affected the probability of finding the different calcium dependencies. Whereas SR vesicles isolated from cardiac muscle with DTT yielded with comparable frequency native channels with the MS or the C calcium dependencies ($N = 14$ experiments), cardiac vesicles isolated without DTT revealed only channels with the C calcium dependence ($N = 5$). Skeletal SR vesicles isolated in the presence of DTT lacked channels with C calcium dependence ($N = 14$, rabbit; $N = 11$, frog); this calcium dependence was only observed in channels from skeletal SR vesicles isolated without DTT ($N = 3$, rabbit; $N = 37$, frog). A detailed description of each calcium dependence follows.

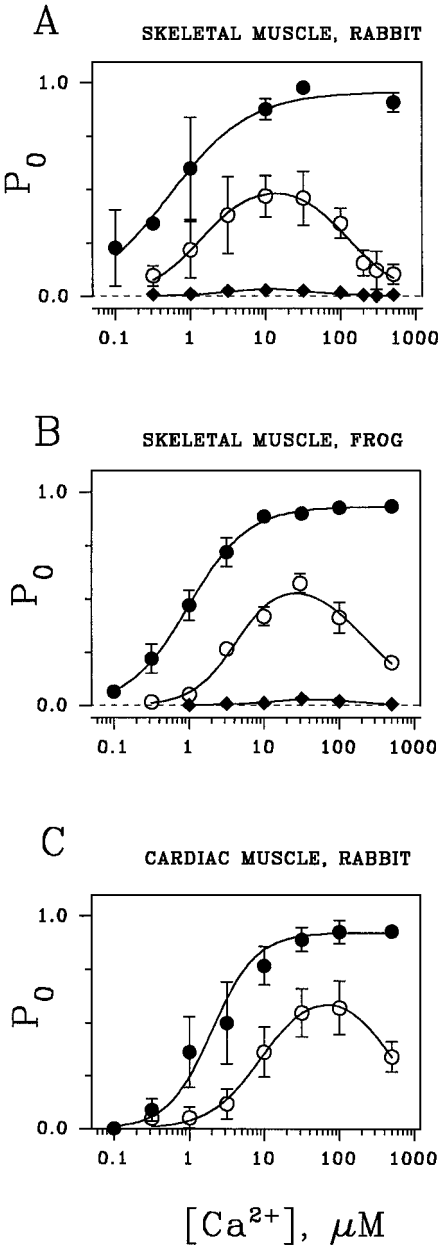

FIGURE 1 Effect of *cis* $[Ca^{2+}]$ on the activity of RyR-channels derived from muscle SR vesicles. Data obtained with RyR-channels from SR vesicles isolated with or without DTT are presented. The panels show the changes in P_o as a function of *cis* $[Ca^{2+}]$; values are given as mean ± SEM. For RyR channels that presented the low P_o calcium dependence (♦), the solid line through the data was obtained by the best fit of the experimental points to Eq. 2, as defined in the text. For RyR channels that displayed the MS (○) or the C (●) calcium dependence, the lines through the experimental points represent the best fits of the data to Eqs. 1 or 3, respectively, as defined in the text. The best fits of the experimental points to these equations yielded the parameters given in Table 1. Data for MS and C calcium dependencies of SR from frog skeletal muscle, as well as theoretical curves for the C calcium dependence, were taken from Bull and Marengo (1993), and are included for comparison purposes.

Low P_o calcium dependence

As mentioned above, this calcium dependence, first observed in RyR channels from brain cortex neurons, was found only in RyR channels from skeletal muscle (Fig. 1, *solid diamonds*). In the case of the low P_o calcium dependence, data fitting to Eq. 1 yielded a Hill coefficient near unity and essentially equal values for K_a and K_i. Therefore

the following particular equation was used:

$$P_o = P_{o,max}/(1 + K/[Ca^{2+}] + [Ca^{2+}]/K) \quad (2)$$

In this equation K represents the $[Ca^{2+}]$ for half-maximum activation and half-maximum inhibition of channel activity, so that $K = K_a = K_i$. As shown in Table 1, the values of K and $P_{o,max}$ obtained for native skeletal muscle channels are within the same range as the corresponding values of K and $P_{o,max}$ reported for channels from brain cortex neurons (Marengo et al., 1996).

MS calcium dependence

This particular kind of calcium dependence was displayed by all native RyR channels studied, regardless of their origin (Fig. 1, *open circles*). The best fit to Eq. 1 yielded the values of $P_{o,max}$, n, K_a, and K_i given in Table 1. The values for these parameters were again comparable to the values obtained for brain RyR channels.

C calcium dependence

All vesicles isolated without DTT had channels that displayed the C calcium dependence (Fig. 1, *solid circles*). To fit Eq. 1 to the data obtained from channels that displayed the C calcium dependence, values of $K_i > 5$ mM were required. Because these values are outside the $[Ca^{2+}]$ range tested, the following simplified equation was used:

$$P_o = P_{o,max}/(1 + (K_a/[Ca^{2+}])^n) \quad (3)$$

The parameter values obtained from this analysis are given in Table 1. All native channels with the C dependence displayed similar values of K_a and $P_{o,max}$, whereas n varied in the range 0.9–1.9.

Sulfhydryl oxidation modified the activity of single RyR-channels from brain cortex neurons, skeletal, or cardiac muscle

The observation that SR vesicles isolated with or without DTT presented different calcium dependencies suggests that SH reagents modify the channel response to changes in *cis* $[Ca^{2+}]$. To test this hypothesis, the effects of two different SH-oxidizing reagents, DTDP and thimerosal, on channels from skeletal and cardiac muscle and from brain cortex neurons were investigated. DTDP reacts with free SH groups, forming a covalent disulfide bond, and this reaction can be reversed by reducing agents; thimerosal, in contrast, reacts irreversibly with SH groups (Brocklehurst, 1979).

The addition of DTDP ($N = 35$ experiments) or thimerosal ($N = 10$ experiments) to the *cis* compartment caused an increase in P_o in all native channels that spontaneously displayed either the low P_o or the MS behavior, as detailed below. With a delay that depended on channel previous activity, new pseudo-steady-state P_o values, lasting several minutes, became apparent. These new pseudo-steady states had P_o values significantly higher ($p < 0.001$) than the preceding stationary P_o values, and the slope of the change in the new P_o values versus time was equal to zero (see Experimental Procedures). No changes in channel P_o were observed after the addition of water-soluble thimerosal to the *trans* (luminal) compartment ($N = 4$), indicating that the SH residues involved in the observed P_o changes were only accessible to the *cis* solution. Long incubations or the addition of high concentrations of SH reagents caused a reduction in the activity of all channels tested, rendering them insensitive to the *cis* addition of caffeine, ATP, and calcium (data not shown); these results suggest that extensive oxidation caused irreversible loss of channel activity. The RyR

TABLE 1 Fitting parameters for the three types of $[Ca^{2+}]$ dependencies exhibited by native ryanodine-sensitive calcium channels

	Low P_o calcium dependence				
	K (μM)			$P_{o,max}$	N
Skeletal muscle, rabbit	11.3 ± 2.30			0.11 ± 0.01	26
Skeletal muscle, frog	42.1 ± 8.20			0.09 ± 0.01	16
Brain cortex, rat*	26.3 ± 1.90			0.13 ± 0.01	22
	MS calcium dependence				
	K_a (μM)	K_i (μM)	n_{Hill}	$P_{o,max}$	N
Skeletal muscle, rabbit	1.40 ± 0.45	124 ± 39	1.21 ± 0.26	0.54 ± 0.0	16
Skeletal muscle, frog	4.50 ± 1.34	226 ± 84	1.46 ± 0.25	0.57 ± 0.09	35
Brain cortex, rat*	6.90 ± 1.60	152 ± 10	1.81 ± 0.08	0.49 ± 0.09	14
Cardiac muscle, rabbit	9.20 ± 2.80	505 ± 145	1.26 ± 0.28	0.68 ± 0.10	6
	C calcium dependence				
	K_a (μM)		n_{Hill}	$P_{o,max}$	N
Skeletal muscle, rabbit	0.55 ± 0.12		0.94 ± 0.15	0.96 ± 0.04	3
Skeletal muscle, frog*	1.10 ± 0.03		1.42 ± 0.06	0.94 ± 0.01	37
Brain cortex, rat*	1.20 ± 0.17		1.90 ± 0.54	0.73 ± 0.04	6
Cardiac muscle, rabbit	2.00 ± 0.70		1.46 ± 0.24	0.92 ± 0.10	8

The numbers shown represent the values obtained from the nonlinear fit ± SD. N stands for the number of independent single channels studied in each case.
*The values obtained for these channels were taken from Bull and Marengo (1993) and Marengo et al. (1996).

channels from cardiac muscle were more susceptible to irreversible inhibition by SH reagents than channels from brain or skeletal muscle, as described further in the text.

Oxidation of RyR channels from brain cortex neurons

Channels with the low P_o calcium dependence. A single RyR channel derived from rat brain cortex, which initially displayed low P_o calcium dependence, within the stirring period of 30 s after the addition of 50 μM DTDP in 30 μM *cis* $[Ca^{2+}]$, increased its P_o from 0.006 ± 0.001 to >0.1 (see Fig. 2 *A*, *interval I* and *record I* taken in this interval). Three minutes after DTDP addition, a new pseudo-steady state, with P_o = 0.14 ± 0.05, was attained (Fig. 2 *A*, *interval II* and *record II*). In the continuous presence of DTDP, the channel stayed in this pseudo-steady state for ~5 min. Then it exhibited a further increase in P_o, reaching a second pseudo-steady state that lasted the entire recorded

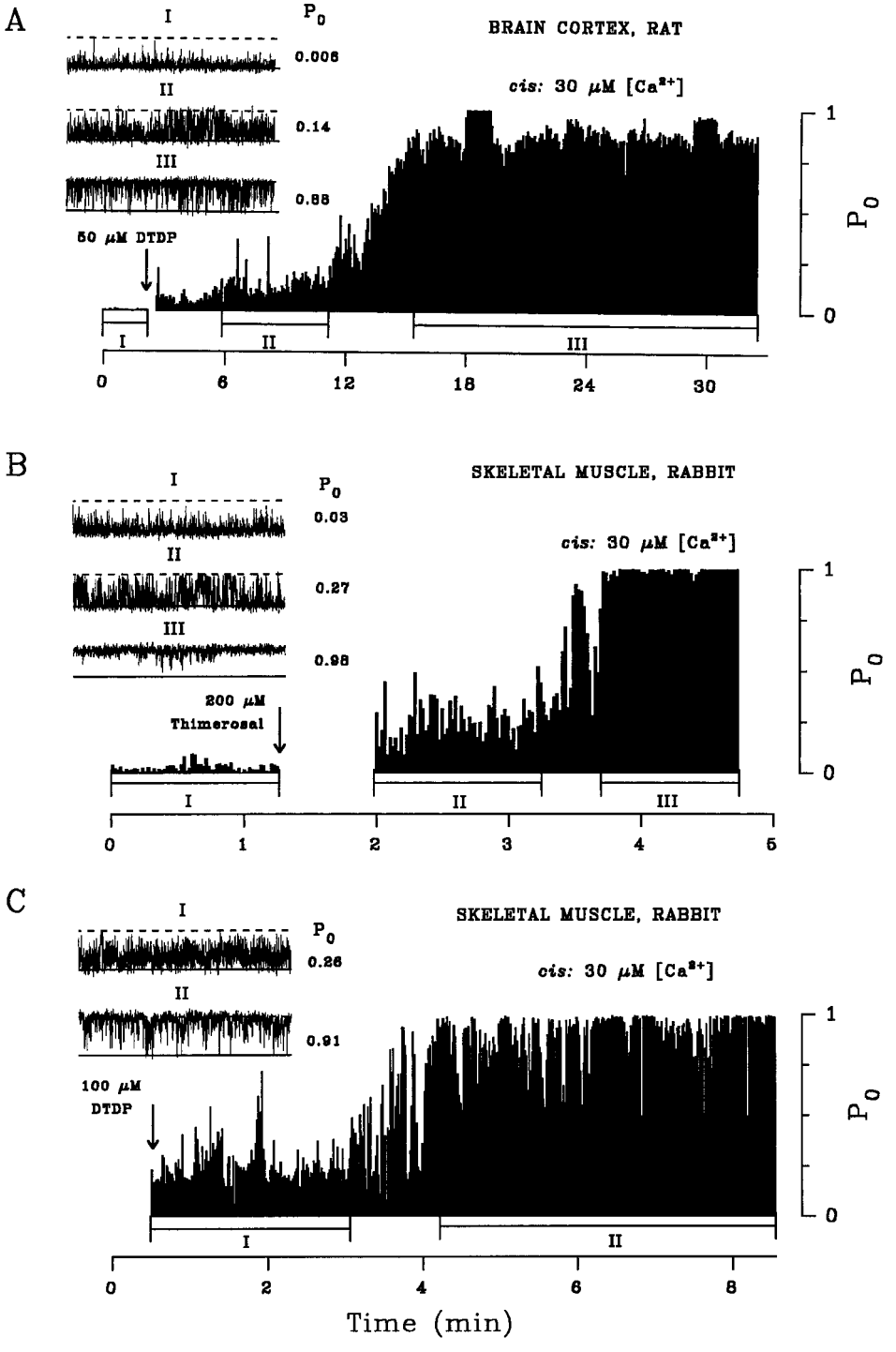

FIGURE 2 Time course of activation of single RyR-channels from brain cortex neurons (*A*) or mammalian skeletal muscle (*B* and *C*) during sustained incubation with DTDP or thimerosal. The values of channel P_o, calculated in successive periods of 1.024 s, are displayed as a function of time. (*A*) Effects of DTDP on the activity of a single RyR channel from rat brain cortex that displayed the low P_o calcium dependence. At the arrow, 50 μM DTDP was added to the *cis* compartment that contained 30 μM $[Ca^{2+}]$. Representative current traces of 10-s duration, obtained at 0 mV, are displayed in the inset for the intervals marked I, II, and III above the *x* axis; the average P_o value for each interval is also indicated. The channel opened upward with an amplitude of 3.1 pA. (*B*) Effects of thimerosal on the activity of a single RyR channel from rabbit skeletal muscle that displayed the low P_o calcium dependence. At the arrow, 200 μM thimerosal was added to the *cis* compartment that contained 30 μM $[Ca^{2+}]$. Representative current traces of 10-s duration obtained at 0 mV during intervals I, II, and III are displayed in the inset; the average P_o values for all periods are given. The channel opened upward with an amplitude of 3.1 pA. (*C*) Effects of DTDP on the activity of a single RyR channel from rabbit skeletal muscle that displayed the MS calcium dependence. At the arrow, 100 μM DTDP was added to the *cis* compartment that contained 30 μM $[Ca^{2+}]$. Representative current traces of 10-s duration obtained at 0 mV during intervals I and II are displayed in the inset; the average P_o values for both periods are given. The channel opened upward with an amplitude of 3.2 pA.

period (18 min), with a value of $P_o = 0.88 \pm 0.05$ (Fig. 2 *A*, *interval III* and *record III*). This same general behavior was observed in five independent single-channel experiments.

The increase in channel activity caused by oxidation was also observed after sequential additions of DTDP. Several single RyR channels that presented the low P_o behavior ($N = 13$) were incubated with 50–100 μM DTDP in 30 μM *cis* [Ca^{2+}] until P_o increased to values within the range 0.1–0.4 during incubation. In four of these experiments, after the oxidation reaction was stopped by washing (see Experimental Procedures), 50–200 μM DTDP was added in 0.5 mM *cis* [Ca^{2+}], causing a second increase in P_o to steady-state values near unity (not shown).

Channels with the MS dependence. In addition to increasing the activity of channels with the low P_o dependence, oxidation also increased the activity of a native single RyR channel from brain cortex neurons that spontaneously displayed the MS behavior. After the addition of 100 μM DTDP in 10 μM *cis* [Ca^{2+}], the channel reached P_o values of >0.8 (data not shown).

Oxidation of RyR channels from mammalian or amphibian skeletal muscle

Channels with the low P_o calcium dependence. As observed with channels from brain, native channels from rabbit ($N =$ 12 experiments) or frog ($N = 7$ experiments) skeletal muscle that exhibited the low P_o behavior also increased their activity after the addition of DTDP or thimerosal. Fig. 2 *B* shows the effects of thimerosal on a single RyR channel derived from rabbit skeletal muscle that initially displayed the low P_o calcium dependence. After the addition of 200 μM thimerosal in 30 μM *cis* [Ca^{2+}], channel P_o increased within the stirring period of 45 s. From a value of $P_o = 0.03 \pm 0.01$ (see Fig. 2 *B*, *interval I* and *record I* taken in this interval), the channel increased its activity to a new pseudo-steady state, with $P_o = 0.27 \pm 0.09$ (Fig. 2 *B*, *interval II* and *record II*). In the continuous presence of thimerosal, the channel exhibited a further increase in P_o, reaching a second pseudo-steady state, with $P_o = 0.98 \pm 0.01$ (Fig. 2 *B*, *interval III* and *record III*). Similar sequential increases in P_o were observed in two experiments on channels from rabbit and in four experiments on channels from frog.

Channel activity also increased after sequential incubations with DTDP, as observed in channels from brain cortex neurons. Single channels with the low P_o dependence from rabbit skeletal muscle ($N = 3$ experiments) were incubated with DTDP for 2–3 min, causing a P_o increase to values greater than 0.1, after which the *cis* compartment was extensively washed. A second addition of 100–200 μM DTDP induced in all cases a new pseudo-steady state, with P_o values near unity.

Channels with the MS calcium dependence. A native single channel from rabbit skeletal muscle SR that displayed the MS calcium dependence exhibited a stepwise increase in activity 4 min after the addition of 100 μM DTDP in 30 μM *cis* [Ca^{2+}]. Channel P_o increased from a value of 0.26 ± 0.01 (Fig. 2 *C*, *interval I*) to a new pseudo-steady-state value of 0.91 ± 0.01 (Fig. 2 *C*, *interval II*). Representative current traces obtained before (*record I*) and after (*record II*) the P_o increase are depicted in the inset to Fig. 2 *C*. Similar activation was observed with two other native single channels that initially presented the MS behavior, one from rabbit and the other from frog skeletal muscle SR.

Oxidation of RyR channels from cardiac muscle

The effects of oxidation on the activity of cardiac SR channels were tested in channels that displayed the MS or the C calcium dependencies, because the low P_o behavior was not observed in these channels.

Cardiac RyR channels ($N = 3$) that spontaneously displayed the MS behavior responded to the addition of 50–200 μM DTDP to the *cis* solution containing 3 μM [Ca^{2+}] with an immediate increase in P_o, from 0.29 ± 0.01 to values near unity. However, within 2–4 min after the addition of 50–200 μM DTDP, an irreversible P_o decay to values near zero was observed. Similar concentrations of thimerosal had the same effects. Likewise, a single RyR channel derived from rabbit cardiac muscle SR, previously characterized as displaying the C calcium dependence, after the addition of 50 μM DTDP in 30 μM *cis* [Ca^{2+}], presented a drastic decrease in P_o, from 0.877 ± 0.009 to 0.023 ± 0.001 (Fig. 3 *A*, *intervals I* and *II* and *records I* and *II*). This new pseudo-steady-state condition was attained 4 min after DTDP addition and lasted for the rest of the recorded period (6 min). After removal of the nonreacted DTDP, the channel did not increase its P_o after sequential *cis* addition of 0.5 mM [Ca^{2+}] and 20 mM glutathione (GSH). This irreversible inhibition of cardiac RyR channels by oxidation differs from the responses of channels with the low P_o or the MS dependencies derived from brain cortex and skeletal muscle. The latter underwent irreversible inhibition only after incubation with SH reagent at concentrations greater than 200 μM and for periods longer than 30 min. Therefore, to avoid irreversible loss of cardiac channel activity, lower concentrations of DTDP and thimerosal were used.

Channels with the MS calcium dependence. A cardiac channel previously characterized as displaying the MS response underwent an increase in P_o to near unity during the 30-s stirring period after *cis* addition of 10 μM thimerosal in 0.5 mM *cis* [Ca^{2+}] (Fig. 3 *B*). Representative current traces obtained before and after thimerosal addition are depicted in the inset to Fig. 3 *B*. After the addition of either DTDP or thimerosal, a comparable increase in P_o was observed in four other independent single cardiac RyR channels that initially displayed the MS response.

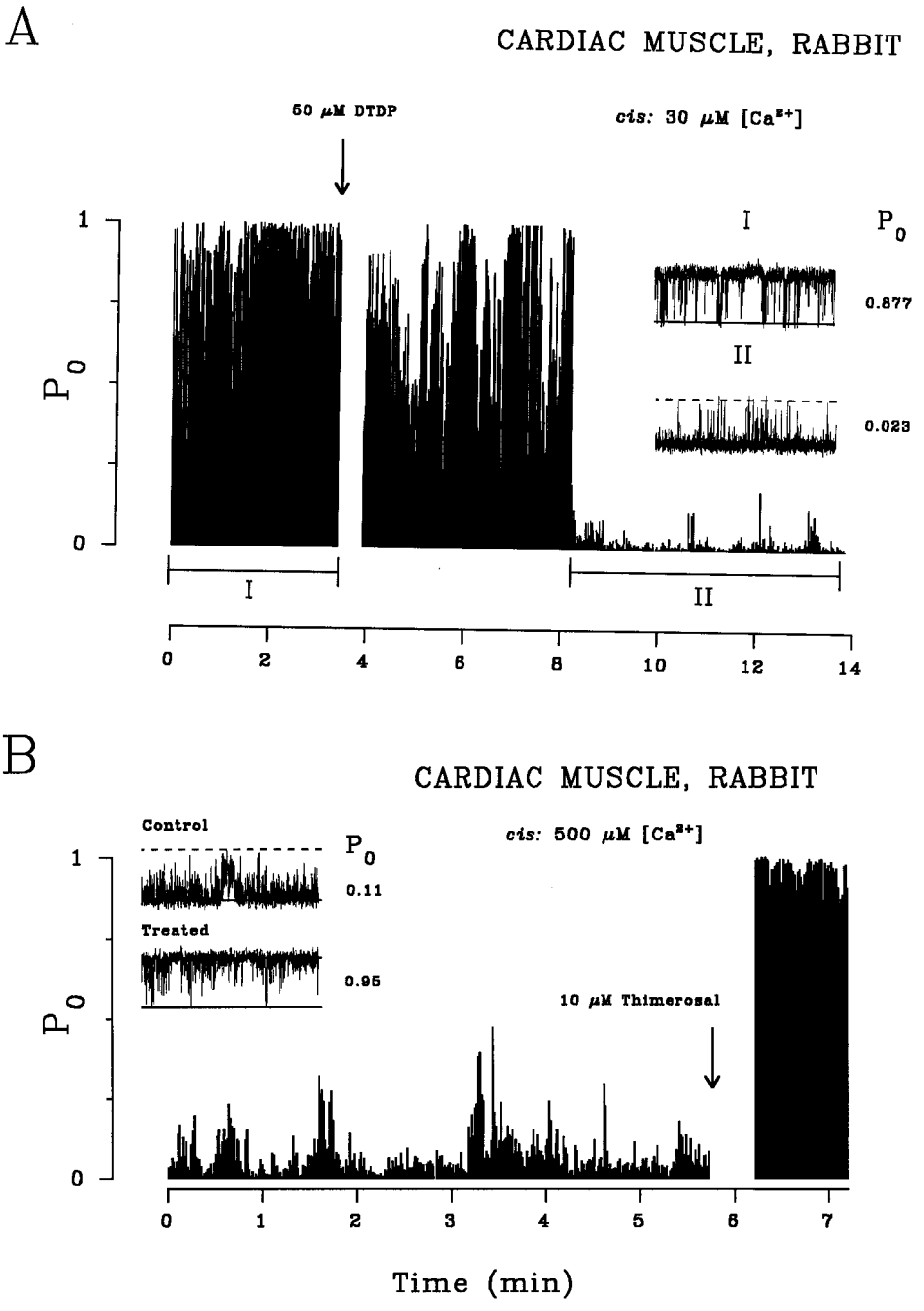

FIGURE 3 Time course of the effects of DTDP or thimerosal on single cardiac RyR channels. The values of channel P_o, calculated in successive periods of 1.024 s, are displayed as a function of time. (*A*) Effect of DTDP on a single native RyR channel with the C calcium dependence. At the arrow, 50 μM DTDP was added to the *cis* compartment that contained 30 μM [Ca^{2+}]. Representative current traces of 10-s duration, obtained at 0 mV, are displayed in the inset for the intervals marked I and II above the *x* axis; the average P_o values for each interval are also indicated. The channel opened upward with an amplitude of 3.0 pA. (*B*) Effect of thimerosal on a single native RyR channel with the MS calcium dependence. At the arrow, 10 μM thimerosal was added to the *cis* compartment that contained 500 μM [Ca^{2+}]. Representative current traces of 10-s duration obtained at 0 mV before and after the addition of thimerosal are displayed in the inset; the average P_o values for both periods are given. The channel opened upward with an amplitude of 2.9 pA.

Effects of SH oxidation on the Ca^{2+} dependence of RyR channels from brain cortex neurons or skeletal or cardiac muscle

As a next step in the characterization of the effects of oxidation on channel activity, SH oxidation was stopped during the incubation period with DTDP or thimerosal, and the effect of changing *cis* [Ca^{2+}] on the activity of the oxidized channels was studied.

RyR channels from brain cortex neurons

The calcium dependencies of single neuronal RyR channels, which before DTDP addition displayed low P_o calcium dependence and after oxidation underwent a change from low P_o to intermediate P_o values (e.g., see *record II* in the *inset* to Fig. 2 *A*), were investigated. After oxidation, all of these channels ($N = 6$) displayed the bell-shaped calcium dependence characteristic of the MS response. The changes in P_o values as a function of *cis* [Ca^{2+}] were adequately described by the curve generated for the native channels with the MS dependence (Fig. 4 *A*, *segmented line through open circles*). On the other hand, RyR channels ($N = 4$) that increased their P_o to values near unity after the addition of DTDP displayed an average calcium dependence that corresponded to the native C calcium dependence (Fig. 4 *A*, *segmented line through filled circles*).

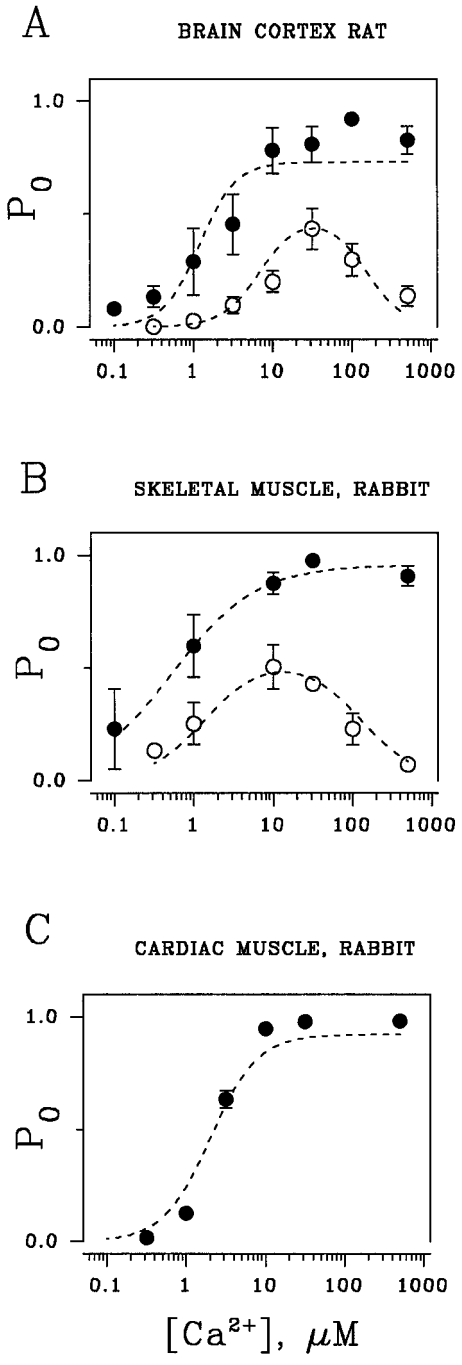

FIGURE 4 Effect of *cis* $[Ca^{2+}]$ on the activity of oxidized RyR channels from brain or muscle. The panels show the changes in P_o as a function of *cis* $[Ca^{2+}]$ for RyR channels from brain cortex neurons (*A*), rabbit skeletal muscle (*B*), and rabbit cardiac muscle (*C*). Values are given as mean ± SEM. The number of channels studied for each calcium dependence (○, MS; ●, C) is given in the text. The segmented lines represent the best fits obtained for the native channels, with the parameters given in Table 1.

Channel oxidation produced sequential modifications of calcium dependence. Thus the calcium dependence of single RyR channels derived from rat brain cortex was modified by oxidation from low P_o to MS in six experiments. In one of these experiments, a second incubation with DTDP was carried out; after this treatment the channel modified its calcium dependence from MS to C. Moreover, a native single neuronal channel that spontaneously exhibited the MS behavior also switched its calcium response to C after oxidation. Furthermore, after prolonged oxidation, two single RyR channels changed from low P_o to C calcium dependence.

Fig. 5 illustrates an example of a single neuronal RyR channel that sequentially switched its calcium dependence from low P_o (*upper traces*) to MS (*middle traces*), and from MS to C (*lower traces*), after one or two successive incubations with 100 μM DTDP.

RyR channels from mammalian or amphibian skeletal muscle

Oxidation of RyR channels obtained from rabbit and frog skeletal muscle SR produced the same changes described above for RyR channels from brain. After incubation with 100–200 μM DTDP, all channels with the low P_o dependence studied modified their calcium dependence. The channels that increased their P_o to intermediate values displayed the MS behavior after extensive washing of the *cis* compartment ($N = 5$, rabbit; $N = 2$, frog). The channels attaining P_o values near unity presented C calcium dependence ($N = 3$, rabbit; $N = 3$, frog). The corresponding calcium dependencies are illustrated in Fig. 4 *B* for channels from rabbit skeletal muscle. Again, their average P_o values were adequately described by the curves generated for the native channels (Fig. 4 *B*, *segmented lines*).

Representative current traces taken from a mammalian skeletal muscle RyR channel before (Fig. 6, *upper traces*) and after prolonged incubations with 100 μM DTDP (Fig. 6, *lower traces*) indicated that the channel changed its calcium dependence from low P_o to C.

RyR channels from cardiac muscle

Sulfhydryl oxidation with DTDP or thimerosal of RyR channels from cardiac muscle SR that displayed MS calcium dependence produced a change to C calcium dependence (Fig. 4 *C*, *solid circles*, $N = 2$). Representative traces taken from a single-channel experiment are shown in Fig. 7.

This overall behavior of the RyR channels present in rat cortex neurons, mammalian and amphibian skeletal muscle, and mammalian cardiac muscle indicates that channel oxidation modified their calcium dependence. Thus channels with low P_o behavior were changed after oxidation to MS dependence, and channels with MS dependence were changed by oxidation to C calcium dependence. Moreover, these changes in calcium dependence were obtained sequentially, from low P_o to MS and from MS to C. Oxidation of channels with C dependence caused their irreversible inactivation, because they failed to respond to $[Ca^{2+}]$ and other channel agonists.

Reversibility of the effects of SH oxidation

To further test the effects of changing the redox state of the channels on channel activity, we investigated whether SH-

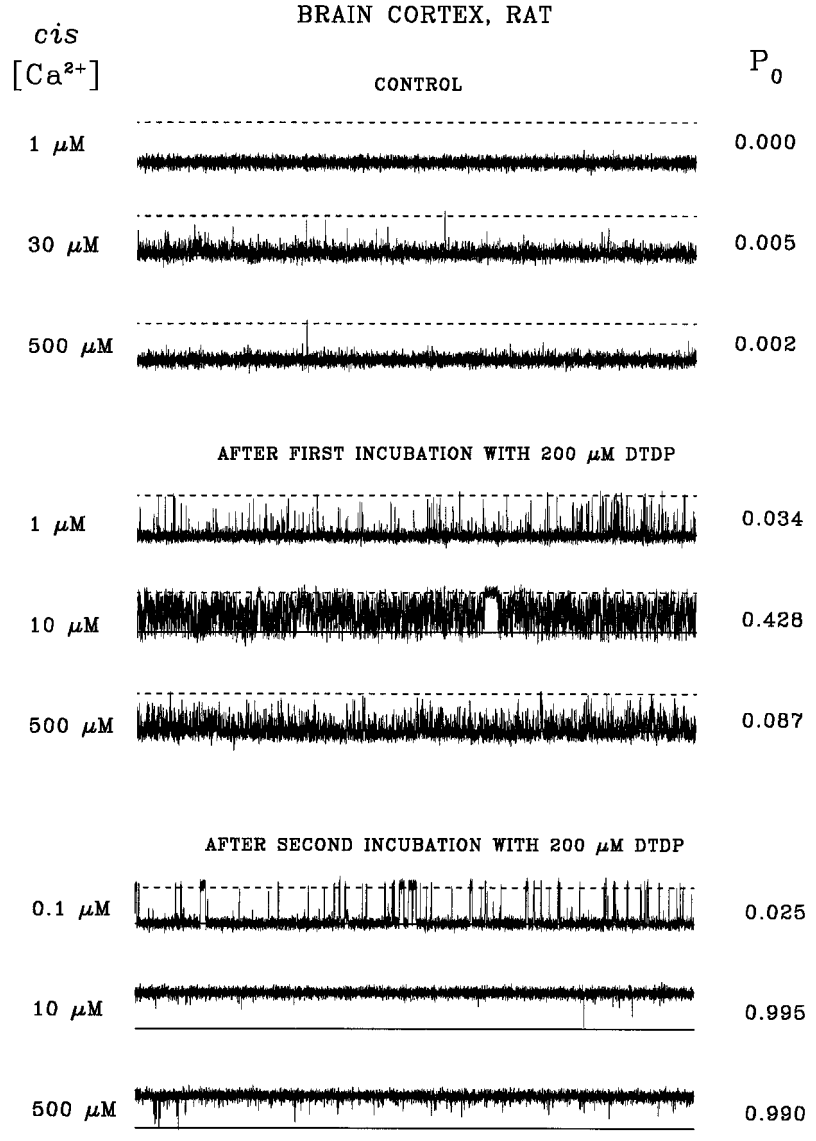

FIGURE 5 Effect of sequential incubation with DTDP on the calcium dependence of a native neuronal RyR-channel. The three top current records indicate that this native channel displayed the low P_o calcium dependence. After 7 min of oxidation with 200 μM DTDP, the reaction was stopped (see text), and the calcium dependence of the channel was characterized as MS, as demonstrated by the middle set of current records. After a second incubation with DTDP, the channel displayed the C behavior, as shown in the lower set of records.

reducing agents 1) modified the calcium dependencies of native channels and 2) reversed the changes in channel response to *cis* $[Ca^{2+}]$ produced by oxidation.

Native RyR channels from skeletal muscle

We investigated whether native skeletal muscle RyR channels that spontaneously displayed C calcium dependence changed their response to *cis* $[Ca^{2+}]$ after the addition of SH-reducing agents. As shown in Fig. 8 *A*, a single native channel derived from frog skeletal muscle, which initially displayed the C behavior, decreased its P_o from 0.98 $\pm$ 0.01 (*interval I*, Fig. 8 *A*) to an average P_o value of 0.71 $\pm$ 0.06 (Fig. 8 *A*) within the first minute after the addition of 20 mM GSH in 0.5 mM *cis* $[Ca^{2+}]$. A new pseudo-steady-state condition (*interval II*, Fig. 8 *A*), with $P_o = 0.27 \pm 0.09$, was attained 10 min after GSH addition. Current traces taken during intervals I and II, respectively, are shown in the inset to Fig. 8 *A*. The new value of $P_o \approx 0.30$, attained after channel reduction, is within the range of the activity that channels with the MS response exhibited in the presence of 0.5 mM *cis* $[Ca^{2+}]$ (see Fig. 1 *A*), suggesting that the reduced channel now displayed the MS behavior.

Reduction of native cardiac RyR channels

The addition of 20 mM GSH in 0.5 mM *cis* $[Ca^{2+}]$ to a native cardiac RyR channel with C behavior produced a reduction in its P_o, from 0.99 $\pm$ 0.01 to 0.26 $\pm$ 0.10 (Fig. 8 *B*), after 10 min of incubation. After this treatment with GSH, this cardiac single channel displayed MS calcium dependence (not shown).

Reduction of oxidized neuronal RyR channels

A neuronal RyR channel, which had switched from MS to C calcium dependence after treatment with 100 μM DTDP, was studied. After the addition of 10 mM β-mercaptoetha-

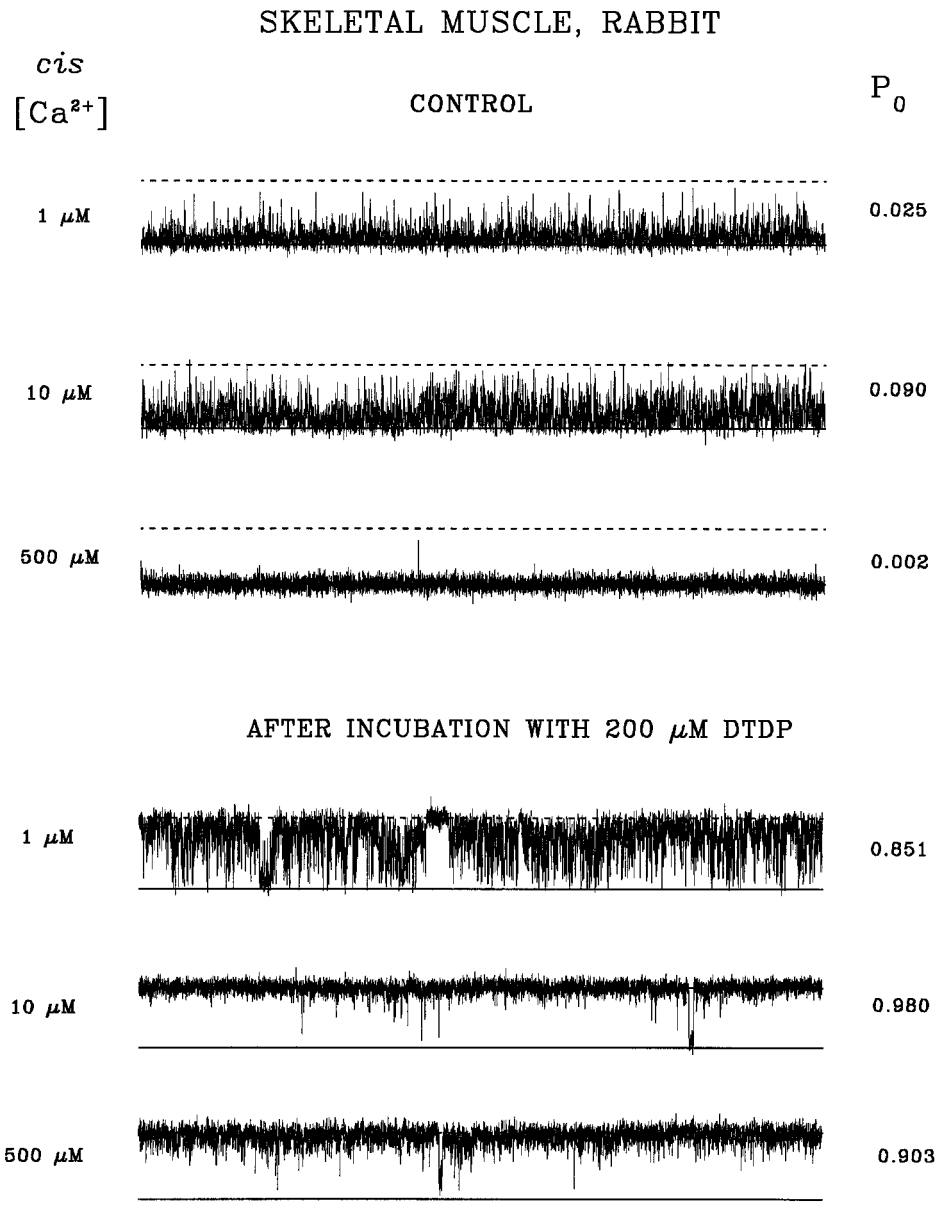

FIGURE 6 Effect of incubation with DTDP on the calcium dependence of a native rabbit skeletal RyR channel. The three top current records indicate that this native channel displayed the low P_o calcium dependence. After incubation with 200 μM DTDP for 12 min, the channel displayed the C behavior, as shown in the lower set of records.

nol, the channel decreased its activity, from a high P_o value of 0.78 ± 0.10 (*interval I* in Fig. 8 *C*) to an intermediate P_o value of 0.17 ± 0.09 (*interval II*, Fig. 8 *C*). This new pseudo-steady state was attained a few minutes after the addition of the reducing agent to the *cis* compartment containing 10 μM [Ca^{2+}] (Fig. 8 *C*). Representative current traces for the intervals marked as I and II, respectively, are shown in the inset to Fig. 8 *C*. Similar effects of β-mercaptoethanol were observed in two other independent single-channel experiments. Comparable changes from high P_o values (obtained by prior oxidation with DTDP) to intermediate P_o values were observed in single neuronal channels after the addition of either 5 mM DTT ($N = 2$) or 5–20 mM glutathione (GSH) ($N = 2$). In contrast, GSH failed to reverse the effects of oxidation caused by treatment with thimerosal, as expected from the irreversible SH oxidation caused by thimerosal ($N = 5$).

The addition of GSH as a reducing agent caused a partial reversal of the effects of DTDP oxidation on channel calcium dependence. Thus a single neuronal channel previously modified with 200 μM DTDP from low P_o to C behavior changed from C calcium dependence to MS behavior after treatment for 20 min with 5 mM GSH (not shown). No changes from the MS behavior to the low P_o response were observed after the addition of SH-reducing agents to oxidized channels ($N = 7$ experiments).

DISCUSSION

Calcium dependencies of native RyR channels

The results presented in this work show that native RyR channels from mammalian and amphibian skeletal muscle exhibited the same three different calcium dependencies, C,

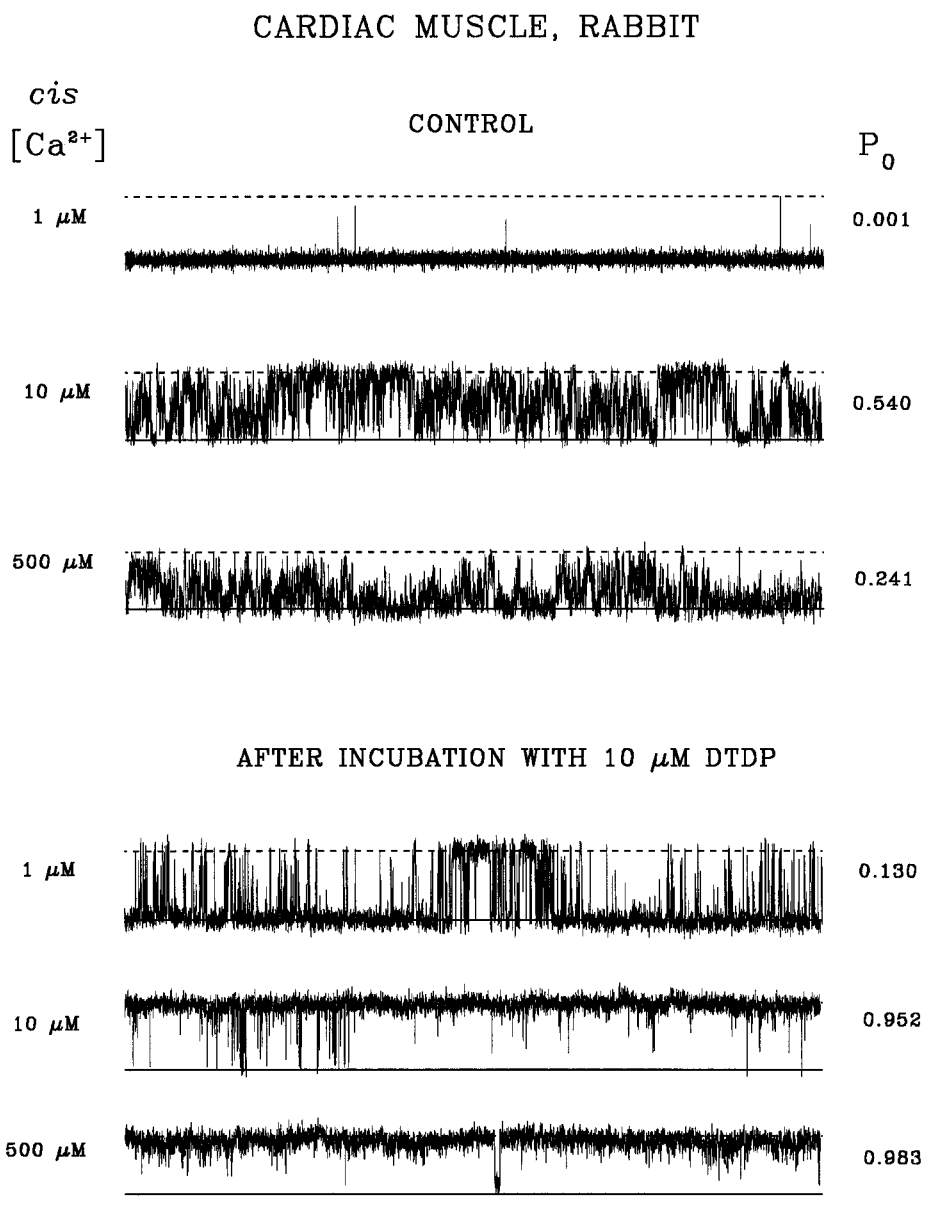

FIGURE 7 Effect of incubation with DTDP on the calcium dependence of a native cardiac RyR channel. The three top current records indicate that this native channel displayed the MS calcium dependence. After incubation with 10 μM DTDP for 2 min, the channel adopted the C behavior, as shown in the lower set of records.

MS, and low P_o, previously described for RyR channels from brain cortex neurons (Marengo et al., 1996). In addition, we found that native RyR channels from cardiac SR vesicles ($N = 19$) presented only two calcium dependencies, MS and C. These results suggest that cardiac RyR channels either do not present the low P_o behavior, or do so with low frequency ($<5.3\%$).

In our previous work we proposed tentatively that each calcium dependence exhibited by native channels from brain (Marengo et al., 1996) or frog skeletal muscle (Bull and Marengo, 1993) reflected the calcium dependence of each RyR isoform present in these tissues. However, the findings described in this work do not support that proposition. Channels from amphibian skeletal SR, which has two RyR isoforms, presented three different calcium dependencies. Likewise, channels with two or three different calcium dependencies, respectively, were found in mammalian cardiac and skeletal SR vesicles, which have only one RyR isoform. In agreement with these results, additional calcium dependencies that differ from the most commonly reported behaviors have been observed for RyR channels from cardiac muscle (Schiefer et al., 1995) or mammalian skeletal muscle (Coppello et al., 1997). Thus the presence of only one RyR isoform does not define a single calcium dependence for the corresponding RyR channel activity.

The above findings indicate that other factors determine the calcium dependence of the different RyR channels. Previous results (Stuart et al., 1992; Favero et al., 1995; Salama et al., 1992) suggested that SH reagents modify the activation of RyR channels by cytosolic $[Ca^{2+}]$. Accordingly, we studied the effects of SH reagents on channel activity, and we investigated whether the different channel

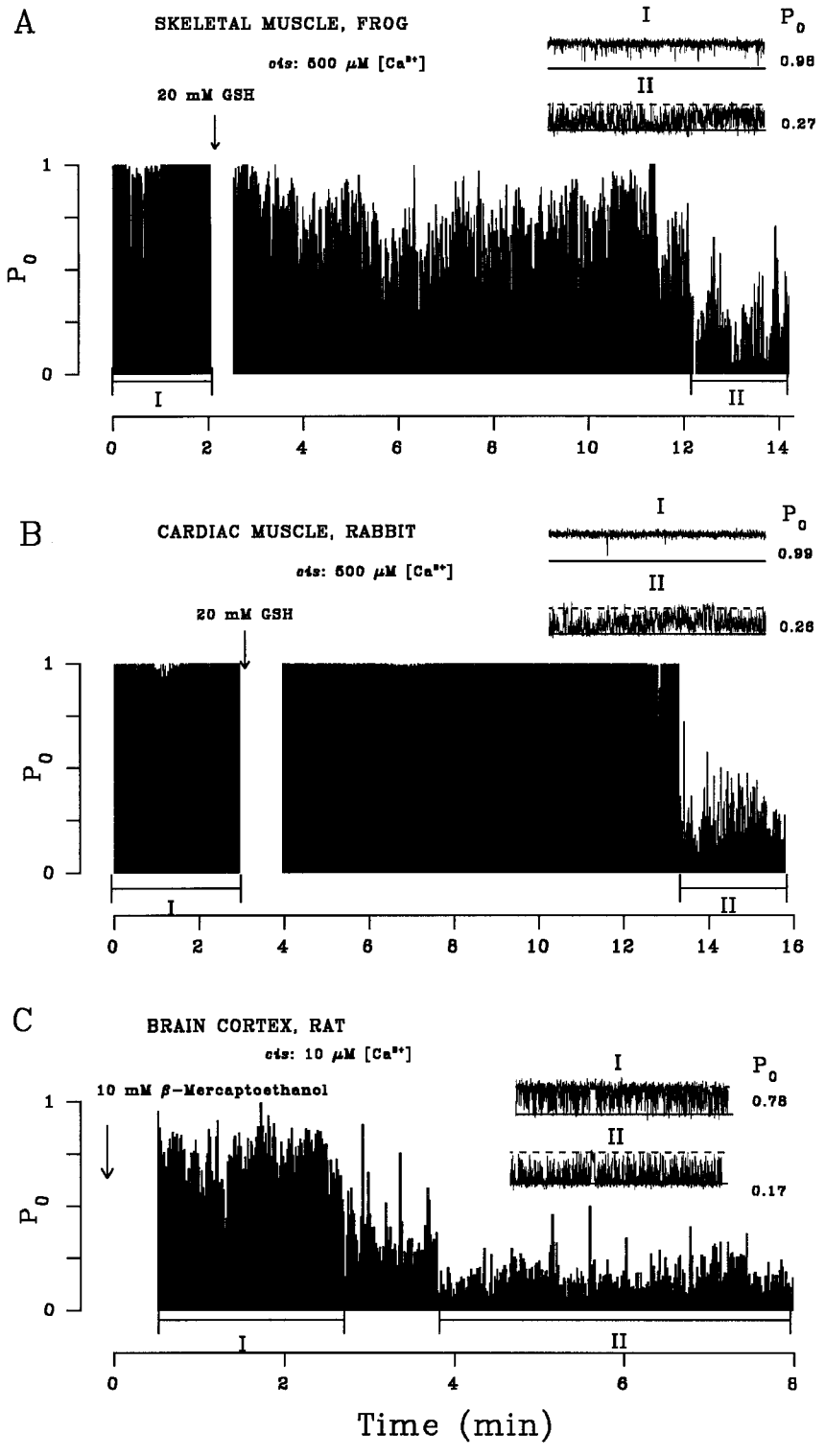

FIGURE 8 Effect of SH-reducing agents on RyR-channel activity. (*A*) Time course of changes in P_o after the addition of 20 mM GSH (*arrow*) to a native RyR channel from frog skeletal muscle in 0.5 mM *cis* $[Ca^{2+}]$, previously characterized as displaying the C calcium dependence. The values of P_o, calculated in successive periods of 1.024 s, are displayed as a function of time. Representative current traces (10 s) obtained from the intervals marked I and II, as well as the average P_o values for these entire periods, are displayed in the inset. The channel opened upward with an amplitude of 3.1 pA. (*B*) Time course of the effect of GSH on the activity of a native rabbit cardiac RyR channel, in 0.5 mM *cis* $[Ca^{2+}]$, with spontaneous C behavior. The values of P_o, calculated in successive periods of 1.024 s, are displayed as a function of time. Representative current records (10 s) obtained from the intervals marked I and II, as well as the average P_o values for these entire periods, are displayed in the inset. The channel opened upward with an amplitude of 3.2 pA. (*C*) Time course of changes in P_o after the addition of 10 mM β-mercaptoethanol (*arrow*) in 10 μM $[Ca^{2+}]$ to a neuronal RyR channel, which, after previous treatment with DTDP, had acquired the C calcium dependence. The values of P_o, calculated in successive periods of 1.024 s, are displayed as a function of time. Representative current traces (10 s) obtained at 0 mV from the periods marked as I and II, as well as average P_o values for these entire periods, are displayed in the inset. The channel opened upward with an amplitude of 3.0 pA.

responses to changes in *cis* $[Ca^{2+}]$ were affected by SH reagents.

Channel activity increased in response to treatment with SH-oxidizing reagents

We found that SH-oxidizing reagents significantly increased the activity of the different native RyR channels incorporated in lipid bilayers, as evidenced by a significant increase in P_o. These results confirm and extend to other tissues (brain) and species (frog) the previous findings reported for RyR channels from mammalian skeletal and cardiac muscle (Stoyanovsky et al., 1994; Favero et al., 1995). Moreover, the increase in RyR channel activity was produced stepwise, so that depending on the basal level of activity of the channel, one or two steps of increase in P_o

were observed after oxidation. We have also confirmed previous results, indicating that long incubations with SH reagents produced an irreversible inhibition of channel activity (Abramson et al., 1995).

Channel responses to changes in *cis* [Ca^{2+}] depended on the redox state of the RyR channels

The observation that oxidation or reduction of SH residues modified the type of response of the RyR channels to changes in *cis* [Ca^{2+}] is the most significant and novel finding of the present study. In general, regardless of their origin (skeletal or cardiac muscle, brain cortex), extensive oxidation led to either C behavior or to channel inactivation, whereas reduction induced lower channel activity and inhibition by 0.5 mM [Ca^{2+}]. These modifications in channel behavior could be effected either by the addition of oxidizing or reducing agents directly to the *cis* chamber during channel recording, or by the addition of DTT during all steps of membrane isolation. The calcium dependencies obtained after SH oxidations were comparable to those of the native channels. This concordance suggests that SH oxidation produced the same channel states found in native conditions.

The SH groups that are important in determining the calcium dependence of RyR channels are exposed to the cytosolic side of the bilayer, because thimerosal, a water-soluble SH reagent, increased channel P_o only when added to the *cis* compartment. The most likely target of SH modification are the many cysteine residues present in the large cytoplasmic domain of all the RyR isoforms characterized to date (Takeshima et al., 1989; Nakai et al., 1990; Otsu et al., 1990; Zorzato et al., 1990; Hakamata et al., 1992). Previous studies indicate that RyR channels from mammalian skeletal muscle have several highly reactive SH groups, and that conditions that favor channel closing increase by ~8-fold RyR channel labeling by a fluorescent thiol oxidizing reagent (Liu et al., 1994). These results indicate that these RyR channels have more free SH groups amenable to oxidation in the closed state. The changes in calcium dependence described in this work may involve molecular rearrangement of channel-protein segments produced by oxidation of specific SH residues, which somehow increase the calcium affinity of cytoplasmic activating sites and hinder calcium binding to the inhibiting sites. Thus when all specific SH residues are reduced, the channel would exhibit the low P_o calcium dependence. Partial oxidation of these residues would give rise to the MS calcium dependence. Further oxidation would induce C calcium dependence, and extensive oxidation would induce the irreversible loss of channel activity. According to this view, the fact that cardiac RyR channels did not exhibit low P_o behavior may be attributed to partial oxidation of the putative specific SH residues, even in vesicles isolated with DTT, because the cardiac channels were highly susceptible to oxidation. Alternatively, the RyR-2 isoform may lack the SH residues that, when reduced, give rise to low P_o behavior, because not all SH residues are conserved among isoforms (Takeshima et al., 1989; Nakai et al., 1990; Otsu et al., 1990; Hakamata et al., 1992).

However, as described by Liu et al. (1994), several other SR proteins, including triadin, were labeled with the fluorescent thiol-oxidizing reagent used by these authors. The extent of labeling of these proteins also increased when channels were closed by the addition of RyR channel blockers. We cannot rule out the possibility that RyR channels are incorporated into the planar lipid bilayers with other SR proteins, such as triadin, and that the redox state of these other proteins determines RyR channel calcium dependence. Yet, in the absence of direct experimental demonstration for the incorporation of such a complex into the bilayer, we favor the simpler interpretation that the redox state of the RyR channel protein itself conditions channel response to changes in *cis* [Ca^{2+}]. Experiments with the purified channel protein should help settle this issue.

Physiological and pathological implications of the present results

An important observation of the present work is that SH oxidation increased channel activity as well as the sensitivity to activation by calcium of the RyR channels from the three excitable tissues studied. As a consequence, oxidative stress should enhance calcium-induced calcium release in neurons and muscle. A concomitant increase in free radicals and cytoplasmic [Ca^{2+}] has been observed in conditions such as apoptosis (Wood and Youle, 1994), hypoxia (Bonfoco et al., 1995), reperfusion after ischemia (Kaneko et al., 1994), malignant hyperthermia (Duthie and Arthur, 1993), and neurodegenerative diseases (Bonfoco et al., 1995). If free radicals promote the oxidation of SH residues in the RyR channels of excitable cells, calcium-induced calcium release would be enhanced. As a consequence, an increase in cytoplasmic [Ca^{2+}] would take place, as observed in these conditions.

CONCLUSIONS

From the results reported in this work, it is proposed that the redox state of specific SH groups present in the cytoplasmic domain of RyR channels controls their responses to changes in *cis* [Ca^{2+}]. Channels with the low P_o behavior would have these SH groups in the reduced state. The addition of SH-oxidizing reagents would promote the rapid oxidation of these SH residues, producing a change from the low P_o to the MS behavior. In the continuous presence of SH-oxidizing reagents, additional SH groups would react, causing the observed change to C behavior. Because only this second oxidation reaction with DTDP was reversed by SH-reducing reagents, presumably the most highly reactive SH groups involved in the low P_o-to-MS transition undergo irreversible oxidation in vitro.

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