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Ryanodine Interferes with Charge Movement Repriming in Amphibian Skeletal Muscle Fibers

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ABSTRACT Cut twitch muscle fibers mounted in a triple Vaseline-gap chamber were used to study the effects of ryanodine on intramembranous charge movement, and in particular on the repriming of charge 1. Charge 1 repriming was measured either under steady-state conditions or by using a pulse protocol designed to study the time course of repriming. This protocol consisted of repolarizing the fibers to −100 mV from a holding potential of 0 mV, and then measuring the reprimed charge moving in the potential range between −40 and +20 mV. Ryanodine at a high concentration (100 μM) did not affect the maximum amount of movable charge 1 and charge 2, or their voltage dependence. This indicates that the alkaloid does not interact with the voltage sensor molecules. However, ryanodine did reduce the amount of reprimed charge 1 by ~60% suggesting the possibility of a retrograde interaction between ryanodine receptors and voltage sensors.

INTRODUCTION

Intramembrane charge movement is thought to trigger calcium release from the sarcoplasmic reticulum (SR), (Schneider and Chandler, 1973; Chandler et al., 1976; Rios and Brum, 1987; Rios and Pizarro, 1991). This triggering signal occurs at the level of the dihydropyridine receptors (DHPr), which are localized in the transverse-tubule (T-t) membranes (Fossett et al., 1983). On the other hand, calcium release occurs through calcium channels, which are localized in the SR membrane at the level of the feet structures that extend in the gap between the T-t system and the SR (Inui et al., 1987; Block et al., 1988). Calcium release channels have been identified as ryanodine receptors (Ryr) (Inui et al., 1987; Imagawa et al., 1987). The mechanism of transmission between the DHPr and Ryr is still unclear in spite of its importance for excitation-contraction coupling (ECC) (Fleischer and Inui, 1989; Rios and Pizarro, 1991; Rios et al., 1993).

The spatial (Block et al., 1988) and stoichiometric (Bers and Stiffel, 1993) organization of the DHPr and Ryr in the region of apposition of the T-t and SR systems (T-SR junctions), strongly suggests a direct interaction between the two molecular moieties. Biochemical studies also strongly support the idea of direct interaction between DHPr and Ryr (Brandt et al., 1990; Marty et al., 1994; Lu et al., 1994). Recently it has been proposed that perchlorate, a known contractile potentiator (Lüttgau et al., 1983) may act primarily at the level of the SR release channels and affect the voltage sensor by retrograde signaling via an allosteric mechanism based on a direct mechanical interaction between the two molecular species (Gonzalez and Rios, 1993; Ma et al., 1993; Rios et al., 1993). On the other hand, ryanodine, a specific probe of the calcium release channels (Inui et al., 1987; Meissner and El-Hashem, 1992), has been reported to cause only minor changes in the intramembrane charge movement in normally polarized fibers (Fryer et al., 1989; Garcia et al., 1991). In the present study we have investigated the effect of ryanodine on the repriming of charge movement. Prolonged (seconds) depolarization causes the disappearance of inactivation of the charge that is normally observed in polarized fibers, charge 1, and the appearance of a charge that shows a different voltage distribution, charge 2 (Adrian and Almers, 1976; Chandler et al., 1976; Brum and Rios, 1987). Analogous to the removal of inactivation of ionic conductances, membrane repolarizations cause the reappearance, or repriming, of charge 1 (Adrian et al., 1976; Chandler et al., 1976; Brum and Rios, 1987). The data presented in the present study demonstrate that ryanodine does not affect the voltage dependence or the maximum movable charge of polarized fibers, but does greatly diminish charge movement repriming. These results could be explained by retrograde signaling between the calcium release channels and the voltage sensors, which agrees with a recent proposal of allosteric interaction between DHPr and Ryr (Gonzalez and Rios, 1993; Ma et al., 1993; Rios et al., 1993). A preliminary account of these experiments has been presented elsewhere (Gonzalez and Caputo, 1994).

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MATERIALS AND METHODS

Experiments were performed on single fibers dissected from semitendinosus muscles of the tropical toad, *Leptodactylus insularis*, using the triple Vaseline-gap voltage clamp technique (Hille and Campbell, 1976). The experimental procedure followed in this work was similar to that already described in detail in Caputo and Bolaños, 1989, except that the Axon Instrument (Foster City, California) Digidata 1200 interface system with

the Pclamp 6 software was used in some experiments instead of the UCLAWAD acquisition system. In the present experiments the external solution contained (in mM): TEA-methanesulphonate, 120; magnesium methanesulphonate, 10; 3,4-diaminopyridine, 1; TTX, 0.01; and Hepes buffer, 10; pH 7.0. The composition of the internal solution was (in mM): caesium-aspartate, 93; calcium aspartate, 0.8; magnesium-aspartate, 0.5; magnesium-ATP, 5.5; creatine phosphate-Tris, 5; glucose, 5; EGTA, 15.9; and Hepes buffer, 10; pH 7.0. After mounting the fibers in the chamber, their cut ends were exposed to 0.01% saponin for 1 min, after which the saponin was washed out. The A pool gap width was 500 μm to reduce the contribution of the membrane segment under the Vaseline seals. Experiments were usually started 30 min after the last solution change in the internal pools. In polarized fibers (−100 mV), the asymmetric currents were measured by subtracting currents elicited by control pulses to −130 mV, while in depolarized fibers (0 mV), the control pulses were to +40 mV. To study the time course of repriming of charge 1 the pulse protocol shown in Fig. 3 *a* was used. This protocol was designed to measure reprimed charge 1 with as little contamination as possible from charge 2 (Gonzalez and Caputo, 1993). In brief, it consisted of a conditioning pulse to −100 mV, followed it by a prepulse to −40 mV. During this prepulse, both charge 1 and charge 2 moved without the possibility of distinguishing them. In fact, charge 2 spanned the range between −40 and −150 mV, whereas charge 1 was visible between −80 to +50 mV (Brum and Rios, 1987). The duration of this pedestal at −40 mV was 100 ms, time enough for all the charge to be moved but not for inactivation to take place (Chandler et al., 1976; Brum and Rios, 1987). From the −40 mV potential a test pulse was applied to +20 mV. This value was chosen because in the range between −40 and +20, only charge 1 is mobilized, whereas at more positive potentials a different charge movement responsible for the gating of slow calcium currents could be evoked (Shirokova et al., 1995). For the subtraction procedure, the control and test runs were the same except that in the latter the repriming duration was variable. A short duration (usually 100 ms) was used for the control runs, whereas longer times were used for the test runs. Because both test and control pulses were applied with the same protocol, the subtraction of both transients would cancel the linear capacitive current plus some small fraction of the total asymmetric current, which may be charge movement in the activated or inactivated state (Huang, 1993). In these repriming experiments the fibers were allowed to rest for at least 2 min between measurements.

RESULTS

Effects of ryanodine on charges 1 and 2

In agreement with previous work (Fryer et al., 1989; Garcia et al., 1991) we have found that ryanodine at a high concentration, 100 μM, does not greatly affect charge 1. In addition, this paper shows that charge 2 is also basically not affected by this drug. It is important to stress that ryanodine even at this high concentration does not affect the functions of the dihydropyridine receptor, either as a calcium channel or as a voltage sensor for ECC. Fig. 1 shows some representative records of charge 1 and charge 2 obtained from two fibers in the absence and presence of 100-μM ryanodine. Ryanodine appears to cause only minor kinetic effects on the charge signal without affecting its other characteristics. This is better demonstrated in Fig. 2, which illustrates the voltage dependence of charge 1 and charge 2 in the presence and

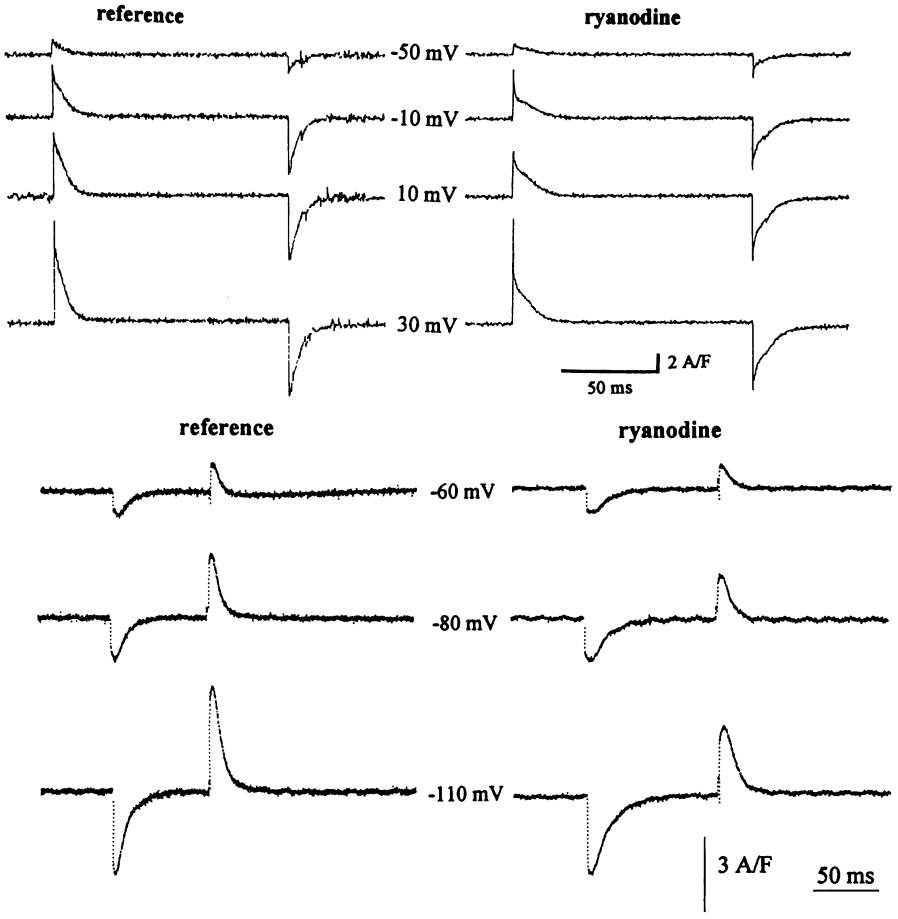

FIGURE 1 Nonlinear membrane currents corresponding to charge 1 (*upper records*) and charge 2 (*lower records*) obtained in two fibers in the absence and presence of ryanodine (100 μM). The records, in the presence of ryanodine, were obtained after more than 30 min exposure to the drug. In the experiment measuring charge 2 the pulse duration was 75 ms for the reference records and 100 ms for the records obtained after addition of ryanodine.

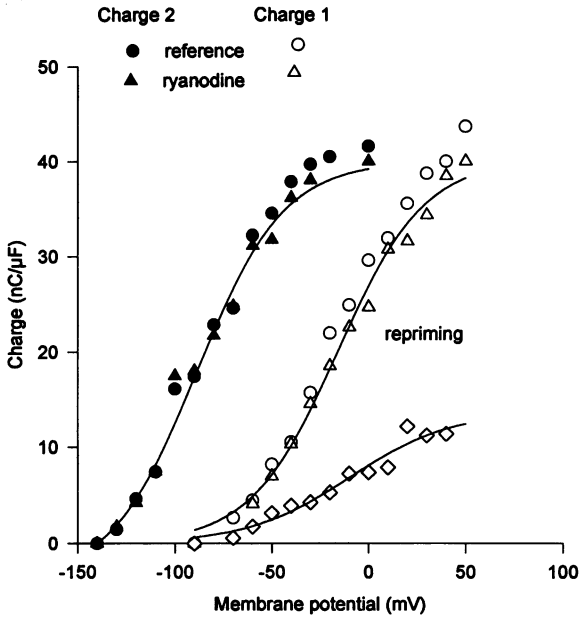

FIGURE 2 Voltage dependence of charge 2 (*filled symbols*) and of charge 1 (*empty symbols*) in the absence (*circles*) and presence (*triangles*) of ryanodine. Charge movement was first measured with the fiber held at 0 mV, where the asymmetric currents corresponding to charge 2 were obtained (*filled circles*). After repolarization of the fiber to −100 mV, charge 1 currents were recorded (*empty circles*). The addition of 100 μM ryanodine did not affect charge 1 (*empty triangles*). The fiber was then depolarized to 0 mV, and the points represented by the filled triangles were obtained. The empty diamonds represent the results obtained after repolarizing the fiber in the presence of ryanodine. The curves were calculated according to the equation: $Q/Q_{max} = 1/\{1 + \exp - (V - V_{1/2})/k\}$, (Chandler and Schneider, 1973), using the following parameter values: Charge 2 without ryanodine (*filled circles*): Q_{max} = 44.0 nC/μF; $V_{1/2}$ = −88.5 mV; k = 22.3 mV. Charge 2 with ryanodine (*filled triangles*): Q_{max} = 44.6 nC/μF; $V_{1/2}$ = −92.7 mV; k = 24.5 mV. Charge 1 without ryanodine (*empty circles*): Q_{max} = 40.3 nC/μF; $V_{1/2}$ = −15.8 mV; k = 22.4 mV. Charge 1 with ryanodine (*empty triangles*): Q_{max} = 39.0 nC/μF; $V_{1/2}$ = −14.2 mV; k = −14.3 mV. Reprimed charge in the presence of ryanodine (*empty triangles*): Q_{max} = 13.8 nC/μF; $V_{1/2}$ = −9.4 mV; k = 26.7 mV.

absence of the drug. In this figure the symbols in the graph correspond to the mean of the on and off values of the time integrals of the nonlinear capacitive currents similar to those shown in Fig. 1. In this particular experiment, the fiber was initially depolarized to 0 mV, and the time integral of the currents were measured (*filled circles*). Next, the fiber was polarized, and the voltage dependence of charge 1 was obtained (*empty circles*). The fiber was then exposed to 100-μM ryanodine for about 25 min, after which the experimental points represented by the empty triangles were obtained. This was followed by depolarization to 0 mV, which allowed measurement of charge 2 in the presence of ryanodine, as noted by the filled triangles. Ryanodine does not affect the voltage distribution or the maximum value of charge 1 and charge 2. The continuous lines were calculated using the equation for a two-state Boltzmann distribution model (Schneider and Chandler, 1973) with the parameters given in the legend to Fig. 2. The experiment shown in Fig. 2 was continued by again repolarizing the fiber to −100 mV. In this case however, charge movement was greatly reduced (*empty diamonds*), suggesting that the repriming of charge movement was blocked by ryanodine. Table 1 summarizes the results obtained from several experiments which were similar to that shown in Fig. 2. The upper part of the table shows the parameters for charge 1 and charge 2 obtained in reference. These results demonstrate the similarity of the maximum charge 1 and charge 2, in agreement with the interconversion hypothesis (Brum and Rios, 1987). While depolarized to 0 mV, the fibers were exposed to 100-μM ryanodine for about 30 min, and charge 2 was measured again. After this, the fibers were repolarized and 10 min later, measurements of charge 1 were carried out. The results are summarized in the bottom part of the table. These results demonstrate that after the repriming process in the presence of ryanodine, charge 1 is reduced by 62%. In agreement with published results (Brum and Rios, 1987; Caputo and Bolanos, 1989), control experiments carried out in the absence of ryanodine showed that reprimed charge 1 was the same as charge 2 or as charge 1 before depolarization.

TABLE 1

	Charge 1 reference			Charge 2 reference			Charge 2 ryanodine			Reprimed ryanodine		
Fiber	Q_{max} (nC/μF)	$V_{1/2}$ (mV)	K (mV)	Q_{max} (nC/μF)	$V_{1/2}$ (mV)	K (mV)	Q_{max} (nC/μF)	$V_{1/2}$ (mV)	K (mV)	Q_{max} (nC/μF)	$V_{1/2}$ (mV)	K (mV)
1	39.26	−28.68	11.47	35.15	−87.97	16.93	39.49	−79.96	14.55	9.86	−30.46	11.11
2	37.37	−30.26	8.23	41.67	−80.39	18.66	37.60	−77.08	15.47	7.31	−31.04	13.29
3	34.94	−28.50	9.29	34.51	−80.83	13.82	35.54	−89.66	14.61	15.71	−25.64	11.10
4	49.98	−22.75	12.28	49.24	−74.10	22.28	47.05	−72.31	20.31	14.48	−36.81	15.35
5	34.07	−9.03	11.79	41.50	−79.00	15.99	41.57	−76.99	17.11	10.47	−15.49	16.51
6	38.97	−14.24	21.00	44.04	−88.58	22.31	41.59	−92.74	24.54	13.79	−9.39	26.71
7	36.81	−19.67	11.46	43.70	−92.34	14.20	43.68	−89.79	13.39	12.01	−21.40	16.78
8	53.10	−20.25	14.81	28.81	−80.59	17.90	27.68	−82.77	16.78	33.08	−21.46	13.53
9	42.25	−15.09	12.15							11.72	−34.97	12.90
X	40.94	−19.97	12.63	39.83	−82.98	17.76	39.28	−82.66	17.10	14.27	−24.53	15.77
std. err	2.49	2.55	1.39	2.31	2.13	1.15	2.08	2.60	1.30	2.50	3.36	1.70

Time course of charge 1 repriming: effect of ryanodine

The results described above indicate that ryanodine interferes with the repriming process of charge 1. To investigate this further, we studied the effect of ryanodine on the time course of repriming. A special protocol was designed for these experiments to minimize complications that might arise from 1) the contamination of charge 1 by residual charge 2 moving during the test pulses, and 2) the subtraction of the charge moving during the test pulses from that moving during the control pulses. These complications could be particularly significant in experiments in which the time course of repriming is measured. To overcome these complications, repriming of charge 1 was studied using the pulse protocol shown in Fig. 3 *a*. The superimposed records in Fig. 3 *b* show asymmetric transient currents obtained after a 2 min repriming period in the absence of ryanodine and an 8 min repriming period in the presence of ryanodine (100 μM). Fig. 4 shows reprimed charge records obtained after various repriming periods in the absence (*left*) and presence (*right*) of 100-μM ryanodine. The effect of ryanodine on the time course of charge movement repriming in 5 fibers is summarized in Fig. 5. The graph displays the normalized reprimed charge as a function of the repriming time in the absence (*circles*) and presence (*squares*) of ryanodine. In agreement with recent results obtained in our laboratory (Gonzalez and Caputo, 1996), we find that under the conditions used here and in the absence of ryanodine, repriming of charge 1 may be described by a double exponential function with time constants of 1.8 and 12.3 s. In the presence of ryanodine, repriming is reduced to ~44%, and the double exponential fit has time constants of 1.7 and 18 s. The results shown in Fig. 5 confirm those presented in Fig. 2 and Table 1, indicating that ryanodine effectively interferes with the transition process in which charge 2 (charge in the inactivated state) is reprimed to charge 1. It is important to note that the total reprimed charge is less than the total charge 1, because the protocol allows only the measurement of charge that is moved in the potential range between −40 and +20 mV. These results indicate that a component of the reprimed charge appears to be affected by ryanodine.

To test the viability of the dihydropyridine receptor after the repriming process in the presence of ryanodine we carried out a few additional experiments to test whether calcium currents were still present. Fig. 6 shows the results of one such run. The current traces were obtained after measuring the reprimed charge in the presence of ryanodine, and then changing the external solution to one containing 10-mM Ca^{2+}. Although a systematic study was not carried out, calcium currents measured after the repriming procedure were smaller (~30%) than currents measured in the absence of ryanodine. This decrease could probably be explained by rundown or inappropriate washout of the charge movement recording external solution. However, a direct effect on the calcium current gating sensor cannot be discarded. On the other hand, in cardiac muscle such an effect was not observed (Lacampagne et al., 1995).

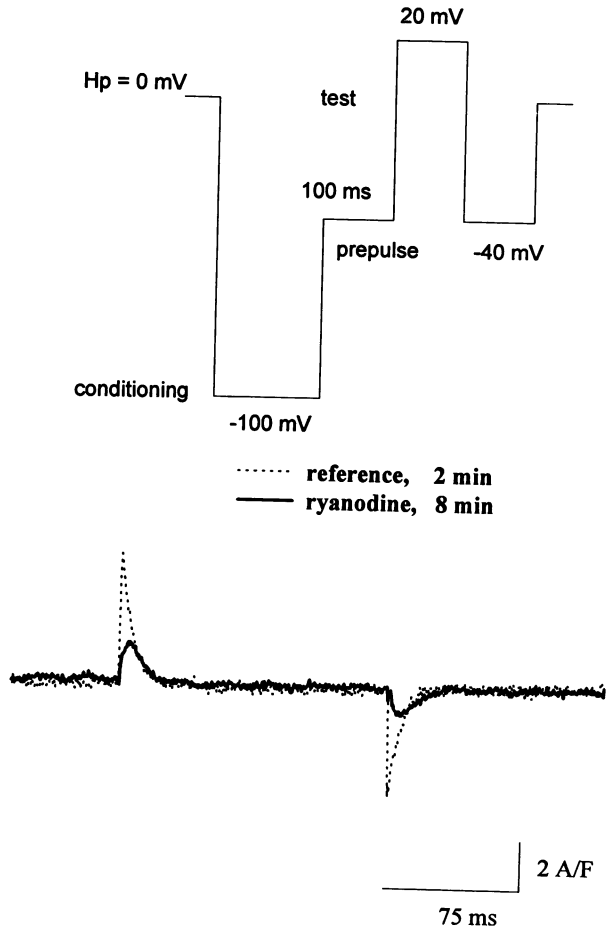

FIGURE 3 (*a*) Pulse protocol used for the measurement of charge 1 repriming. This protocol was designed to minimize the contribution of charge 2 to the measured reprimed charge 1. Starting from the holding potential of 0 mV, the pulse protocol consisted of a hyperpolarizing step usually to −100 mV where repriming would occur, followed by a pedestal to −40 mV to reach the level where a net separation exists between the voltage distribution of charge 1 and charge 2 (see Fig. 2), concluding with the test pulse from −40 mV to +20 mV, which is a membrane potential range where only charge 1 is observed. The asymmetric current was obtained from the subtraction of the reference and test runs. In the reference pulse the duration of the membrane polarization to −100 mV was 100 ms, which does not allow sufficient time for repriming. In the test run this duration was varied. (*b*) Effects of ryanodine on charge 1 repriming. The figure shows superimposed reprimed asymmetric current records obtained at 15°C in the absence (*dotted trace*) and presence (*continuous heavy trace*) of 100 μM ryanodine. The repriming periods in the absence and presence of ryanodine were 2 and 8 min, respectively. The results clearly indicate that in the presence of ryanodine the amount of reprimed charge 1 is appreciably reduced regardless of the longer repriming duration.

Ryanodine dose-effect relationship

The experiments described demonstrate that a substantial fraction of the reprimable charge (~40%) is not affected by ryanodine at a relatively high concentration. This suggests the presence of a ryanodine-insensitive charge movement. To test this possibility the dose-response relationship for this effect was studied. The results indicate that the relationship between the normalized reprimed charge and the ryanodine concentration can be described by a single expo-

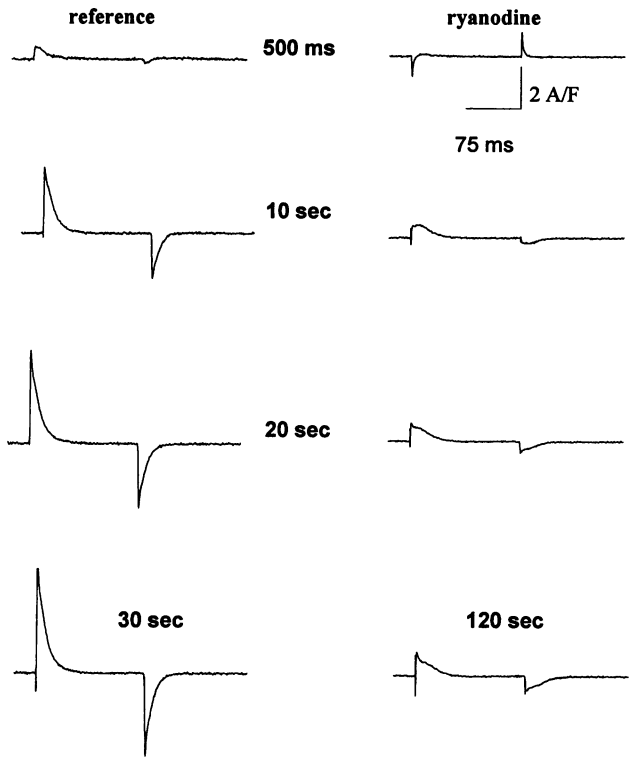

FIGURE 4 The effect of ryanodine (100 μM) on reprimed charge movement after different repriming periods. The records on the right were obtained without the drug, and those on the left were obtained in the presence of ryanodine.

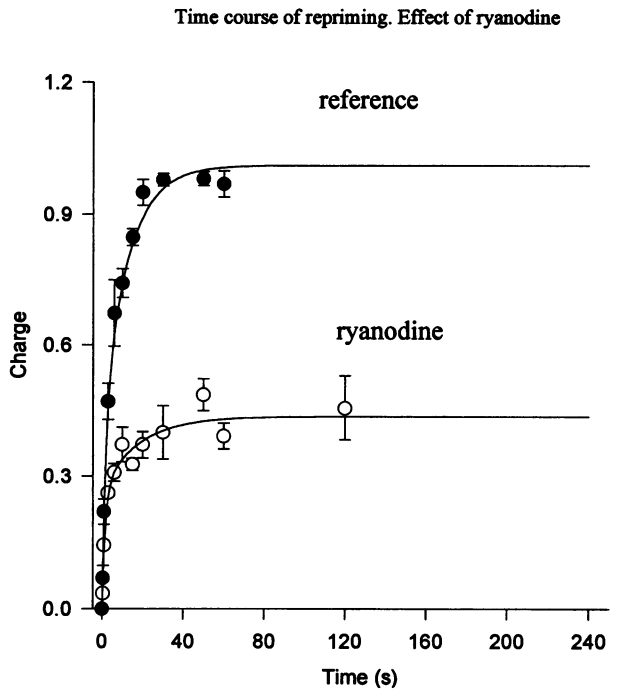

FIGURE 5 Effect of ryanodine on the repriming time course of charge 1 in different fibers. The graph shows the normalized reprimed charge as function of the repriming period. The data points represent the mean values (mean ± SE) obtained from five experiments similar to that shown in part a of the figure. The experimental data were fitted by a double exponential function using the following parameters, upper curve (*filled circles*): a = 0.4 and b = 0.6; τ_1 = 1.81 s, τ_2 = 12.3 s; lower curve (*empty circles*): a = 0.27 and b = 0.17; τ_1 = 1.68 s, τ_2 = 18.30 s.

nentially decaying function (Fig. 7). This suggests a single binding site, which is supported by Hill plot analysis (not shown). However, even at the high concentration used in these experiments the effect of ryanodine seemed to be maximum at ~40%, again suggesting the presence of a ryanodine-insensitive component.

DISCUSSION

This study focused on the repriming of charge movement following prolonged membrane depolarization using two experimental approaches. First, the steady state voltage distribution of charge movement was measured at different membrane potentials, in the absence and presence of ryanodine. Second, a pulse protocol was designed to measure the time course of repriming of charge 1 with little contamination from charge 2. Using this protocol, we found that charge movement repriming follows a double exponential time course with time constants of 4.16 and 25.0 s at 10°C (Gonzalez and Caputo, 1996).

Ryanodine, at 100 μM, reduces the repriming of charge 1 measured with either protocol by ~60%. The fact that ryanodine does not suppress all the reprimed charge may be because of the presence of more than one component of charge 1. This is also supported by the double exponential time course of repriming, and the ability of calcium currents to be reprimed following a depolarization episode in the presence of ryanodine (Fig. 6). The dose-effect relationship of ryanodine on the reprimed charge is also consistent with this hypothesis. Although the experimental data were fit by a single decaying exponential, which indicates a single binding site, the exponential seemed to level off at ~30–40%, suggesting a ryanodine-insensitive component. Another possible explanation for the partial effect of ryanodine on charge 1 is that the binding of extracellularly applied ryanodine to its cytoplasmic receptor was restricted despite the high concentration of the drug used.

Presently no direct information exists regarding the effects of ryanodine on contractile repriming. However, previous studies have shown that depolarization-induced calcium release is blocked after ryanodine treatment, whereas caffeine-induced calcium release is not affected (Lamb and Stephenson, 1990). These results could be interpreted in terms of the effect of ryanodine on charge movement repriming, which would lead to a blockade of contractile repriming.

Ryanodine does not modify the voltage distribution of charge movement (see also Fryer et al., 1989, and Garcia et al., 1991) or the activation of the slow calcium current (Garcia et al., 1991). These results indicate that ryanodine has no direct effect on the DHPr working as a voltage sensor for ECC or as a calcium channel (Rios and Brum, 1987; Beam et al., 1992). Furthermore, ryanodine at high concen-

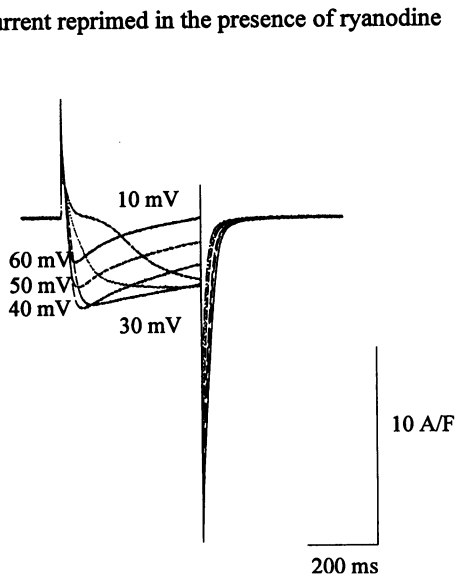

FIGURE 6 Presence of calcium current in a fiber after measurement of charge movement repriming in the presence of ryanodine. The current traces were obtained at different membrane potential values after testing the effect of ryanodine on the repriming of charge 1. After measurement of the reprimed charge signals (similar to those shown in Fig. 4), the external solution was changed to one containing 10 mM Ca^{2+}.

trations does not modify cardiac calcium currents and cardiac calcium channel gating currents (Lacampagne et al., 1995). This suggests that the effect of ryanodine on charge movement repriming in skeletal muscle fibers depends on some conformational change of the sensor for ECC that occurs during the depolarization-repolarization transition.

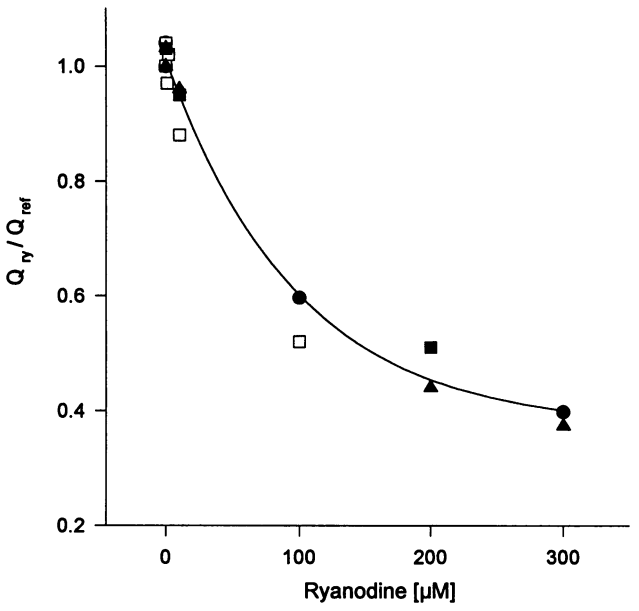

FIGURE 7 Dose dependence of the ryanodine effect. In the graph the reprimed charge measured in the presence of ryanodine at different concentrations has been normalized with respect to the reprimed charge measured without the drug. The different symbols represent results from four fibers. The parameters for the curve fitted to the experimental points are: τ = 82.98; a = 0.63.

It is known that alteration in state of the voltage sensor caused by membrane potential changes may affect its sensitivity to different compounds. For example, nifedipine (Rios and Brum, 1987) and D-600 (Caputo and Bolaños, 1989) bind preferentially to voltage sensors in the inactivated state. More recently it has been shown that heparin, acting intracellularly disrupts ECC only when the voltage sensors are activated for 1 s or more, and thus probably while undergoing the transition between "resting-activated-inactivated" (Lamb et al., 1994). These authors have proposed that heparin binds at the level of the intracellular loop between the second and third repeats of the DHP receptor molecule and thus hinders the normal coupling of this loop with the calcium release channels. This idea is based on the importance of the cytoplasmic loop between repeats II and III of the DHPr molecule for skeletal muscle ECC (Tanabe et al., 1990) and is supported by the recent demonstration by Lu et al. (1994) that this same loop may activate the calcium release channel of skeletal muscle SR.

Because ryanodine is a specific ligand for the calcium releasing channels of the SR (Meissner and El-Hashem, 1992), the most likely explanation for its effect on charge 1 repriming is based on its binding to specific sites on the SR calcium release channels. This interpretation would be consistent with the concept of a physical interaction between voltage sensors and release channels (i.e., ryanodine receptors), which is also supported by a substantial body of structural and biochemical evidence (Block et al., 1988; Brandt et al., 1990; Marty et al., 1994; Lu et al., 1995). Ryanodine may thus affect the voltage sensor in a secondary, retrograde way, comparable with the mechanism of an allosteric interaction between the voltage sensor and the ryanodine receptor that has been recently proposed to explain the action of perchlorate (Gonzalez and Rios, 1993; Ma et al., 1993; Rios et al., 1993). It is possible that in depolarized fibers, inactivation causes physical disengagement of the DHPr and Ryr, and the repriming process would then allow their reengagement. The binding of ryanodine to its receptor changes the spatial relationship between the voltage sensor and the calcium release channel. In turn, an altered relationship between the DHPr and Ryr would not allow state transitions of the DHPr associated with repriming of charge 1. Based on the model proposed by Rios et al. (1993), this would imply that ryanodine does not allow their reengagement, possibly by locking them in an immobilized configuration. This interpretation is supported by the demonstration that ryanodine has no effect on the repriming of cardiac calcium gating currents in cardiac muscle, where no mechanical coupling is expected between the DHPr and the Ryr (Lacampagne et al., 1995).

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