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Effect of Ryanodine on Cardiac Calcium Current and Calcium Channel Gating Current

A. Lacampagne,* C. Caputo,‡ and J. Argibay*

*Laboratoire de Physiologie des Cellules Cardiaques et Vasculaires, Faculté des Sciences, 37200 Tours, France, and ‡Laboratorio de Biofísica del Músculo, Centro de Biofísica y Bioquímica, Instituto Venezolano de Investigaciones Científicas, Caracas 1020A, Venezuela

ABSTRACT The effects of 100 μM ryanodine on the L-type calcium channel were studied using the pacth-clamp technique in isolated guinea pig ventricular myocytes. The inactivation kinetics of the calcium current were slowed down in the presence of ryanodine in agreement with the blockade of the release of calcium from the sarcoplasmic reticulum by the drug. The I-V and steady-state inactivation curves of the calcium current were shifted to negative values by ryanodine. A similar shift was observed in the activation and inactivation curves of the intramembrane charge movement associated with the calcium channel. Due to this shift, ryanodine slightly reduced the maximal amount of displaced charge although it did not modify the transition from the inactivated to the activated state (i.e., charge movement repriming). This result is in notable contrast with that obtained in skeletal muscle, where it has been found that ryanodine interferes with charge movement repriming. These results provide additional evidence of the postulated differences between the architecture of the excitation-contraction coupling system in cardiac and skeletal muscle.

INTRODUCTION

Excitation-contraction coupling (ECC) in cardiac and skeletal muscle occurs by different mechanisms, although the molecular components of the coupling machinery are basically the same. In skeletal muscle fibers, dihydropyridine receptors (DHPRs) localized in the transverse tubule membrane operate as voltage sensors and generate the intramembrane charge movement, providing the gating signal for primary calcium release from the ryanodine receptors (RYRs) in the sarcoplasmic reticulum (SR) (Rios and Pizarro, 1991). The molecular architecture of the transverse tubules (T)-SR junction in skeletal muscle strongly suggests a direct interaction between the two receptors (Block et al., 1988). Such interaction has recently been demonstrated in vitro (Marty et al., 1994) and in reconstituted systems (Lu et al., 1994), but an in vivo demonstration at the cellular level is still lacking.

In cardiac muscle, the different spatial relationship and stoichiometry of DHPRs and RYRs determines a different type of coupling (Bers and Stiffel, 1993). Here, DHPRs localized in the sarcolemma function as voltage-dependent calcium channels and allow calcium entry into the cells (see references in McDonald et al., 1994). This calcium triggers additional calcium release from the SR via SR calcium channels, which have been identified as RYRs (Fabiato, 1985; Meissner, 1986). Thus, in heart muscle, intramembrane charge movement does not participate in ECC as a voltage sensor (Näbauer et al., 1989) but instead it is thought to provide the voltage-dependent gating of sarcolemmal sodium and calcium channels (Bean and Rios, 1989; Hadley and Lederer, 1989, 1991a; Shirokov et al. 1992).

Ryanodine is a natural alkaloid that binds with high specificity and affinity to the calcium release channels of SR of both skeletal and cardiac muscle cells (Meissner, 1986; Meissner and El Hashem, 1992). It has been shown in normally polarized skeletal muscle fibers that ryanodine does not affect the basic properties of intramembrane charge movement (Fryer et al., 1989; Garcia et al., 1991). However, it does interfere with the repriming of charge movement after prolonged membrane depolarization (Gonzalez and Caputo, 1994, 1995). It has been suggested that ryanodine binding to its receptor locks the voltage sensor in its inactivated state and does not allow the transition from the inactivated to the available state when the fibers are repolarized (Gonzalez and Caputo, 1995). This would imply a retrograde action of ryanodine effective from the intracellular calcium release channel of the SR to the DHPR in the sarcolemma, consistent with the mechanical hypothesis of ECC (Rios and Pizarro 1991; Rios et al., 1993). However, ryanodine could act directly on the DHPR in its depolarized state without involving the RYR.

In cardiac muscle the organization of the T-SR junction is different and no mechanical interaction is expected to occur between DHPRs and RYRs. Thus, the effect of ryanodine on the repriming of charge movement in cardiac cells constitutes a crucial test for the retrograde hypothesis of ryanodine action on skeletal muscle. In this work we have measured the effect of ryanodine at a concentration that would be maximally effective in skeletal muscle (Gonzalez and Caputo, 1995) on the charge movement associated with the gating of calcium channels in cardiac myocytes. Although ryanodine slightly diminishes charge movement in normally polarized cells, it does not affect the transition of charge

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Address reprint requests to Dr. J. A. Argibay, Laboratoire de Physiologie des Cellules, Cardiaques et Vasculaires, CNRS EP 2, Parc Grandmont, 37200 Tours, France. Tel.:33-47-36-7009; Fax: 33-47-36-7112; E-mail: argibay@univ-tours.fr.

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movement from the inactivated to the available state, i.e., repriming.

MATERIALS AND METHODS

Cell preparation

Cardiac myocytes were isolated from guinea pig ventricles using the procedure described by Le Guennec et al. (1990). Isolated cells were placed in a 1-ml experimental chamber on the stage of an inverted microscope (TMS, Nikon). They were continuously perfused at the rate of 1 ml/min with a Tyrode solution (in mM): 140 NaCl, 5.4 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 11 glucose, 0.33 NaH_2PO_4, 10 Hepes; pH adjusted to 7.3 with NaOH.

Experimental solutions

Cells were internally dialyzed with a pipette solution (mM): 110 CsCl, 30 tetraethylammonium chloride (TEA-Cl), 5 Mg-ATP, 10 EGTA, 10 Hepes; pH adjusted to 7.3 (CsOH). Calcium current was measured with an extracellular solution containing (mM): 140 TEA-Cl, 6 CsCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 11 glucose, 10 Hepes; pH adjusted to 7.3 with TEA-OH. Intramembrane charge movements were measured with an extracellular solution containing (mM): 140 TEA-Cl, 1 $MgCl_2$, 1.0 $CaCl_2$, 2 $CdCl_2$, 0.1 $GdCl_3$, 10^{-3} tetrodotoxin, 11 glucose, 10 Hepes; pH adjusted to 7.3 with TEA-OH. Gadolinium was employed as calcium channel blocker (Lacampagne et al., 1994). External solutions were applied to the cells by superfusion with microcapillaries. Ryanodine (Sigma Chemical Co., LaVerpilliere, France) was added to both external solutions at the final concentration of 100 μM from a stock solution. Experiments were performed at room temperature (22–25°C).

Experimental procedures

Voltage clamp experiments were performed in the whole-cell configuration of the patch-clamp technique (Hamill et al., 1981), with an Axopatch 200A (Axon Instruments, Foster City, CA). The pipettes, with a resistance from 2 to 3 MΩ, were pulled with a PB7 puller (Narishige, Tokyo, Japan). To eliminate the pipette fast capacitance transient, the pipettes were coated with dental wax. In all experiments the pipette and cell capacitance were compensated. Small cells were selected for the experiments and the mean cell capacitance was 108 ± 6.4 pF. Except for experiments in which calcium current (I_{Ca}), current-voltage, and steady-state inactivation were measured, the series resistance was compensated by 70–80%. The data were filtered by an 8-pole low-pass Bessel filter at 5 kHz, or 1 kHz when series resistance was compensated. In addition, a P/5 subtraction protocol was used to remove any residual leak and capacitance currents. The cell capacitance was measured by integration of the capacitance transient elicited by a 10-mV hyperpolarization pulse from a holding potential of −80 mV (in every case the value measured was similar to that given by the amplifier after compensation). I_{Ca} was measured as the difference between the peak current and the current recorded at the end of the depolarization. Gating currents were analyzed by integrating the first 25 ms of the depolarization (Q_{ON}) after defining the steady-state current level as that recorded during the last 5 ms of the pulse. Q_{OFF} was measured by integrating the current recorded during the first 25 ms of repolarization and subtracting the baseline of the steady-state current recorded at the end of this period and is expressed as the absolute value. Voltage-dependent relationships of gating currents were fitted by the following Boltzmann equation:

$$Q_{Ca} = Q_{Camax}/\{1 + \exp[(V - V_{Ca})/k]\} \quad (1)$$

where Q_{Camax} is the maximal charge, k the slope factor and V_{Ca} the voltage for the half-maximal activation.

Statistical analysis was done by comparison of the fitting parameters obtained for each cell (see Table 1) using an unpaired t-test ($p<0.05$ was considered significant). The results are expressed as the mean ± SE.

RESULTS

It is known that ryanodine at 10 μM does not affect calcium currents in skeletal muscle (Garcia et al. 1991). In cardiac muscle it has been reported that ryanodine causes small changes in the inactivation properties of I_{Ca}. Thus, this effect is possibly an indirect one, caused by calcium released from the SR due to the action of ryanodine (Boyett et al., 1988; Balke and Wier, 1991). A first series of experiments was carried out to test the effect of ryanodine at high concentration, 100 μM, on I_{Ca}. This concentration keeps the RYR at the closed configuration (Lindsay et al., 1994) and was shown by Gonzalez and Caputo (1995) to significantly decrease the repriming of intramembrane charge movement in skeletal muscle. At this concentration, ryanodine caused a 10-mV shift in the I-V relationship as it is shown in Fig. 1 *A*. The steady-state inactivation curve (Fig. 1 *B*) was also shifted and the degree of inactivation increased over the whole range of explored voltages. Similar results were obtained in three cells. Cohen and Lederer (1988) found an opposite shift but only in the inactivation curve. Ryanodine also affected the inactivation kinetics of I_{Ca}. As shown in Fig. 2 *A*, the time course of inactivation was slowed down. This result was obtained in three other cells. A similar effect has been obtained at lower concentrations of ryanodine (Mitchell et al., 1984; Boyett et al., 1988; Cohen and Lederer, 1988; Balke and Wier, 1991) and was attributed to ryanodine inhibiting SR calcium release.

TABLE 1 Effect of 100 μM ryanodine on calcium channel charge movement

	Control (n = 8)			Ryanodine (100 μM; n = 6)			Repriming (100 μM ryanodine; n = 5)		
	Q_{Camax} (nC/μF)	V_{Ca} (mV)	k (mV)	Q_{Camax} (nC/μF)	V_{Ca} (mV)	k (mV)	Q_{Camax} (nC/μF)	V_{Ca} (mV)	k (mV)
Q_{ON}	8.68 ± 0.49	−13.74 ± 1.74	10.32 ± 0.77	7.99 ± 0.52	−18.16 ± 2.01	9.88 ± 1.02	6.15 ± 0.71	−17.61 ± 4.53	9.99 ± 1.21
Q_{OFF}	7.46 ± 0.5	−10.81 ± 1.3	12.14 ± 0.74	6.17 ± 0.76	−16.95 ± 2.36	10.12 ± 1.02	5.13 ± 1.05	−22.15 ± 2.16	7.32 ± 0.73

Cell data were individually fitted to Eq. 1 and values of the fitting parameters of Q_{ON} and Q_{OFF} are given as mean ± SE. Statistical analysis compared data from control and ryanodine and from ryanodine and repriming ryanodine. There was no significant difference in the second comparison, indicating that ryanodine is without effect on the repriming of calcium channel charge movement. From the first comparison there was a significant decrease of Q_{Camax} and a significant shift of the activation curves induced by ryanodine.

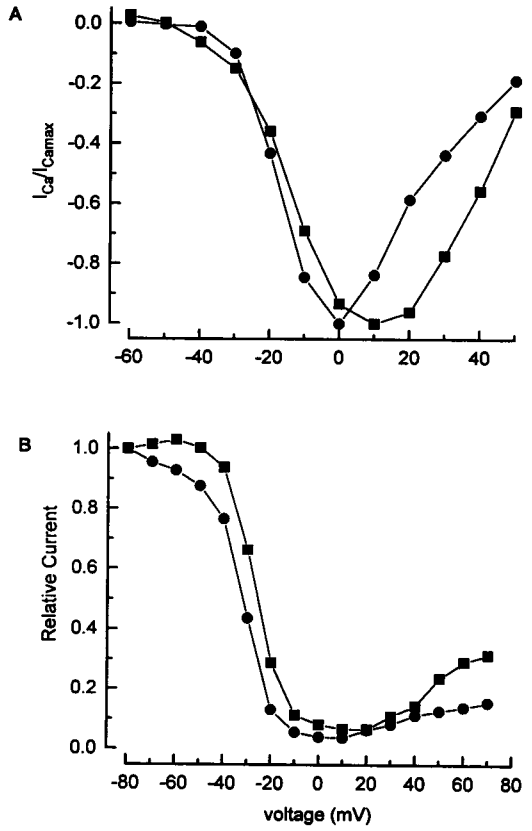

FIGURE 1 I-V and steady-state inactivation curves of I_{Ca} were obtained with a double-pulse voltage-clamp protocol. A 450-ms depolarizing test pulse from a holding potential of −80 to 0 mV was preceded by 450-ms depolarizing prepulse from −80 to 80 mV in 10-mV increments. In both curves the currents are normalized to the maximal value of I_{Ca} (■, control; ●, 100 μM ryanodine). (*A*) 5 min after application of ryanodine the I-V curve is shifted by ~10 mV to the left. (*B*) Under the same conditions we can see the loss of the potentiation observed in control between −80 and −40 mV, a 10-mV shift to the left and an increase in the inactivation between 20 and 80 mV.

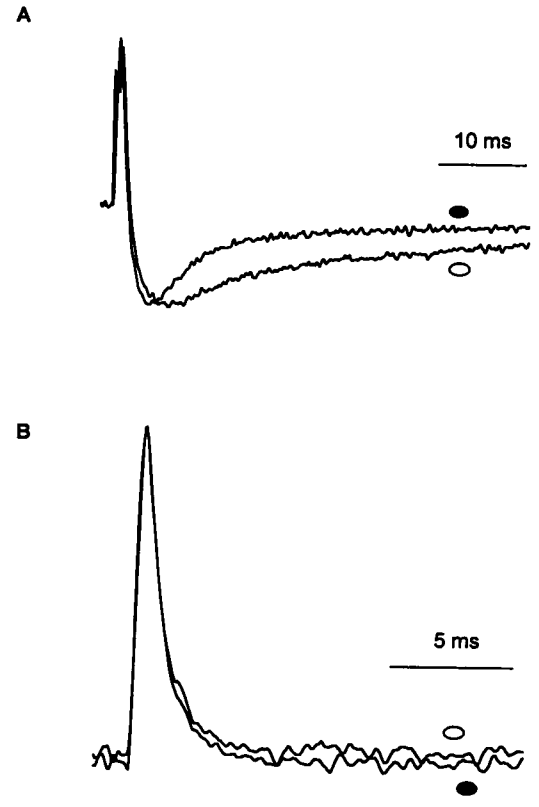

FIGURE 2 (*A*) Normalized I_{Ca} elicited by a 100-ms depolarization pulse from a holding of −80 to 0 mV every 10 s, sampled at 10 kHz. Ryanodine slows down the time course of inactivation of the current. (*B*) Normalized Q_{ON} elicited by a 20-mV depolarization pulse according to the protocol described in Fig. 3. Ryanodine was without effect upon the kinetics of the gating currents. (●, control; ○, 5 min after application of 100 μM ryanodine).

Because of the different voltage sensitivity of the sodium and calcium channel gating currents, it is possible to isolate the latter by inactivating the former with conditioning depolarizations (Hadley and Lederer, 1989, 1991a; Bean and Rios, 1989; Shirokov et al. 1992). To measure intramembrane charge movement associated with the gating of calcium channels we used the pulse protocol shown in the upper part of Fig. 3 *A*. In this figure the records on the left were obtained under normal conditions, whereas those on the right were obtained after 5 min of exposure to 100 μM ryanodine. It appears that under these conditions ryanodine has little effect on the intramembrane charge movement, causing only a small decrease in Q_{Camax}. Fig. 3 *B* and Table 1 summarize the results obtained from several cells. The symbols in the graphs of Fig. 3 *B* represent, respectively, the Q_{ON} and Q_{OFF} mean values obtained from the time integrals of the nonlinear currents plotted against the membrane potential. The continuous lines were calculated using a two-state Boltzmann equation as described in Materials and Methods. The mean on and off values obtained in these cells were 7.46 and 8.78 nC/μF, respectively, in agreement with previously published values (Bean and Rios, 1989; Shirokov et al., 1992). Ryanodine reduced the maximal charge Q_{Camax} by ~10% and shifted the activation curve toward negative values by 5 mV, whereas the slope factor (k) was unchanged. These effects were not associated with changes in the kinetics of the gating currents (n = 4), as illustrated in Fig. 2 *B*.

Fig. 3 *C* shows the availability curves of the on charge movement, obtained in seven cells using a double-pulse protocol. The holding potential was −100 mV and under these conditions a double Boltzmann fitting showed the existence of two components of charge. In control conditions, the more negative component represents ~27% of the total charge and is probably associated with the inactivation of the sodium channel, which is completely inactivated at ~−60 mV (Bean and Rios, 1989). The more positive component corresponds to the inactivation of the calcium channels, which begins at around ~−50 mV and is maximal at 20 mV. After application of ryanodine no significant changes were observed either in the amount of sodium or calcium charge or in the slope factors. However, a shift in the availability curve of the calcium channel toward more

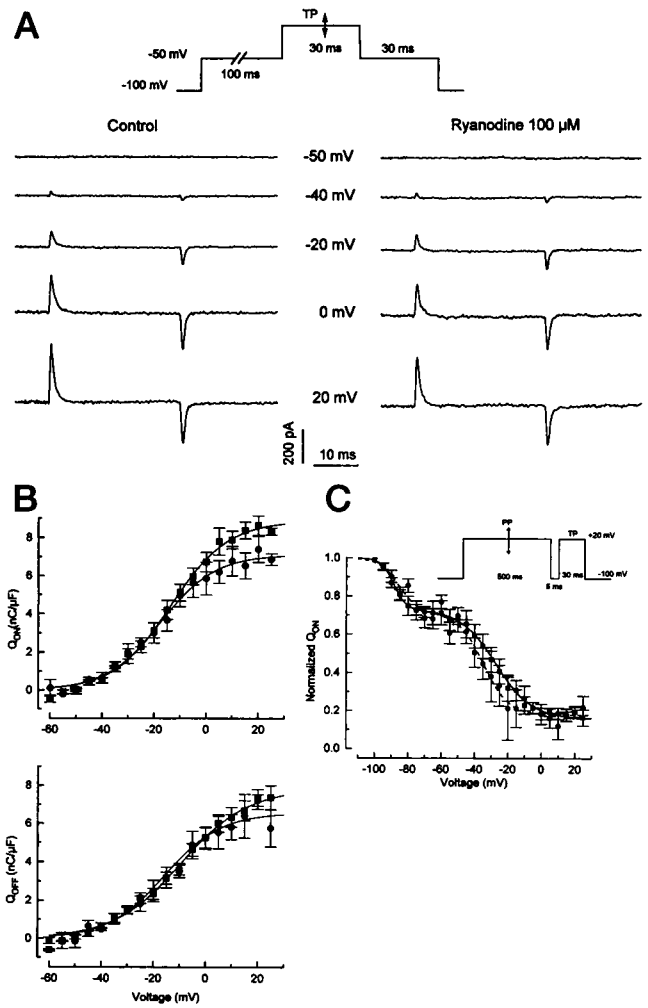

FIGURE 3 Effect of ryanodine on the activation and inactivation of intramembrane charge movement. (*A*) Protocol used to measure activation of L-type calcium channel charge movement. The cells were maintained at a holding potential of −100 mV, depolarized to −50 mV for 100 ms to inactivate sodium channel charge movement, depolarized for 30 ms to test pulse voltages (TP) from −60 to +25 mV, and repolarized to −50 mV for 30 ms. The signals were sampled at 16.5 kHz; the protocol was repeated every 6 s. To diminish high freqency noise the records shown are the averages of three runs. The left column illustrates gating currents recorded under control conditions and the right column shows the currents after a 5-min exposure to 100 μM ryanodine. (*B*) Effect of ryanodine on the voltage dependence of charge movement (*top*, Q_{ON}; *bottom*, Q_{OFF}). ■, mean ± SE of the charge density in control (n = 8); ●, after a 5-min exposure to ryanodine (n = 6). The lines were obtained by fitting all individual data to Eq. 1. Ryanodine induced a small decrease of gating current, as shown by the following fitting parameters. In the upper graph (Q_{ON}), Q_{Camax} = 8.86 ± 0.34, V_{Ca} = −13.62 ± 1.4, k = 10.94 ± 1.02 in control and Q_{Camax} = 7.09 ± 0.38, V_{Ca} = −17.55 ± 2.04, k = 10.53 ± 1.59 in ryanodine. In the lower graph (Q_{OFF}), Q_{Camax} = 7.85 ± 0.45, V_{Ca} = −9.43 ± 2.17, k = 13.0 ± 1.35 in control and Q_{Camax} = 6.58 ± 0.46, V_{Ca} = −15.75 ± 2.66, k = 11.0 ± 2.01 in ryanodine. (*C*) Inactivation of charge movement obtained with the protocol shown in the inset. Holding potential, −100 mV; prepulse (PP) duration, 500 ms; interpulse, 5 ms; test pulse, 30 ms at 20 mV; n = 7. ■ (——), control; ● (- - - -), 100 μM ryanodine. Values fitted to the equation $Q = Q_0 + Q_{Na}/\{1 + \exp[(V_{Na} - V)/K_{Na}]\} + Q_{Ca}/\{1 + \exp[(V_{Ca} - V)/K_{Ca}]\}$. Fitting parameters (mean ± SE): Q_0 = 0.16, Q_{Na} = 0.27 ± 0.0, Q_{Ca} = 0.57 ± 0.0, V_{Na} = −88.8 ± 2.0, V_{Ca} = −27.0 ± 2.1, K_{Na} = 3.78 ± 1.8, and K_{Ca} = 10.8 ± 1.9 in control; Q_0 = 0.19, Q_{Na} = 0.28 ± 0.0, Q_{Ca} = 0.53 ± 0.0, V_{Na} = −85.5 ± 3.5, V_{Ca} = −34.5 ± 2.4, K_{Na} = 5.97 ± 2.9, K_{Ca} = 7.56 ± 1.9 in ryanodine.

negative potentials was obtained, which was similar to the shift observed in Fig. 1, *A* and *B* and Fig. 3 *B*. This suggests that the 10% inhibition of Q_{Camax} (Fig. 3 *B* and Table 1) is the consequence of partial inactivation of the calcium channels at −50 mV in the presence of ryanodine.

In view of the finding that ryanodine significantly reduced charge movement repriming in skeletal muscle (Gonzalez and Caputo, 1995) it was important to test whether this was also the case for cardiac cells. To study charge movement repriming, the pulse protocol illustrated in Fig. 4 *A* was used. Repriming at the steady state was studied using a repolarization interval (Δt) of more than 30 s after the inactivation depolarization of 2 min at 0 mV. The degree of repriming was tested with a second depolarization (TP) to different voltages. Fig. 4, *A* and *B*, and Table 1 show that ryanodine has no significant effect on the steady-state repriming of charge movement. We also explored the time course of repriming in the absence and presence of ryanodine (Fig. 4 *C*). This time, we used a constant test pulse (TP = 0 mV) and Δt was varied from 250 ms to 60 s. Fig. 4 *Ca* shows the results expressed in charge density (nC/μF) and confirms the lack of effect of ryanodine on the repriming process. Fig. 4 *Cb* gives the normalized values and shows the absence of effect of ryanodine on the kinetics of the time course of repriming.

DISCUSSION

The main point of this study is that ryanodine does not interfere with repriming of intramembrane charge movement in cardiac myocytes. This contrasts with recent work showing that ryanodine reduces repriming of intramembrane charge movement in skeletal muscle (Gonzalez and Caputo, 1994, 1995). Thus, in skeletal muscle, ryanodine binding to its receptor severely disrupts repriming of charge movement whereas in cardiac muscle it does not. The results presented here weaken the possibility that ryanodine might act directly on the DHPR molecule during the transition from the inactivated to the reprimed state. Taken together the results reported here and those of Gonzalez and Caputo (1995) support the mechanical coupling model proposed for skeletal muscle (Chandler et al, 1976; Rios and Pizarro, 1991; Gonzalez and Rios 1993; Rios et al., 1993). This model is based on a protein-protein interaction between DHPRs and RYRs that is favored by the molecular disposition and stoichiometry of the two macromolecules at the level of the T-SR junction (Block et al., 1988; Bers and Stiffel, 1993). This arrangement is not found in cardiac muscle.

There are varying reports of the effect of ryanodine on the function of sarcolemmal calcium channels in cardiac muscle. It is generally thought that the effect is not direct but mediated by calcium released from the SR. However, we show that high concentrations of ryanodine induce some changes in the voltage dependence of the calcium channel. We have also found a small decrease in the maximal acti-

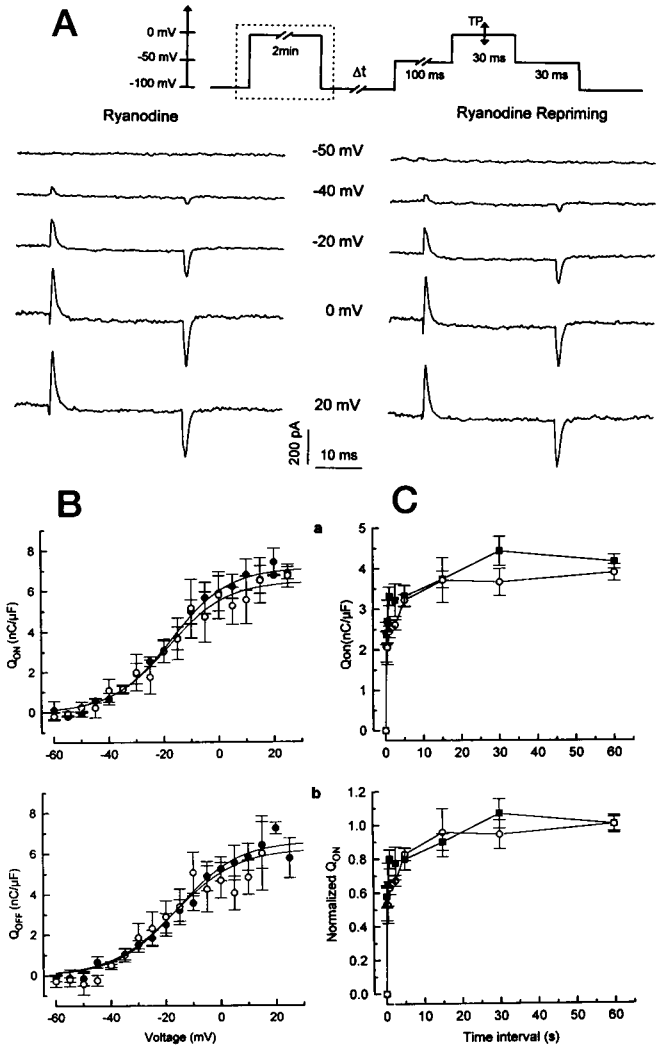

FIGURE 4 Effect of ryanodine on charge movement repriming. (*A*) Protocol used to measure the steady-state repriming of intramembrane charge movement. It is similar to that described in Fig. 3, but to test repriming, before stimulation the cells were maintained for 2 min at 0 mV to inactivate charge movement (surrounded by dotted lines) then repolarized for at least 30 s (Δt) to −100 mV to reprime the intramembrane charge movement. Gating currents were recorded after a 5-min exposure to 100 μM ryanodine before (*left panel*) and after (*right panel*) repriming. (*B*) Effect of ryanodine on the voltage dependence of charge movement (*top*, Q_{ON}, *bottom*, Q_{OFF}) before and after repriming. ●, mean ± SE of the charge density after a 5-min exposure to ryanodine (n = 6); ○, after repriming (n = 5). The lines were obtained by fitting all individual data to Eq. 1. Ryanodine is without significant effect on gating current repriming as shown by the following fitting parameters: upper graph (Q_{ON}) before repriming, Q_{Camax} = 7.09 ± 0.38, V_{Ca} = −17.55 ± 2.04, k = 10.53 ± 1.59; after repriming, Q_{Camax} = 6.42 ± 0.66, V_{Ca} = −18.58 ± 3.85, k = 10.63 ± 2.92; and lower graph ($-Q_{OFF}$) before repriming, Q_{Camax} = 6.58 ± 0.46, V_{Ca} = −15.75 ± 2.66, k = 11.0 ± 2.01; after repriming, Q_{Camax} = 6.23 ± 0.86, V_{Ca} = −16.9 ± 5.25, k = 11.48 ± 3.79. (*C*) Time course of repriming studied with the same protocol as in *A*. Test pulse was fixed at 0 mV and Δt was varied from 250 ms to 60 s (n = 5, for all Δt). ■, control; ○, ryanodine. (*a*) Represents values as charge density. (*b*) Values normalized to the 60-s value.

vation of charge movement (Q_{Camax}) that could be due to a decreased release of SR calcium (but see Hadley and Lederer, 1991b; Shirokov et al., 1993) but is more probably related to the recorded shift in the voltage dependence of the calcium channel. We have no explanation for this shift, but it can be interpreted as a consequence of a local increase in calcium concentration in the vicinity of the calcium channels. Our results are in contrast to those of Cohen and Lederer (1988) as they obtained a shift in the opposite direction. This shift cannot be interpreted by a local change in calcium concentration as it was obtained only for the inactivation curve.

It is known that the cytoplasmic loop region between transmembrane repeats II and III of the DHPR is a determining factor for skeletal-muscle-type ECC (Tanabe et al., 1988, 1990; Garcia et al., 1994). It has been shown that this loop region may activate SR calcium release channels (Lu et al., 1994). Interestingly, loop regions of cardiac and skeletal muscle were both effective in the activation of skeletal muscle release channels but not of cardiac release channels (Lu et al., 1994). These results are compatible with the finding that modification of the RYR by ryanodine differentially affects its interaction with the DHPR during repriming in cardiac and skeletal muscle.

Changes of the membrane potential cause transitions between different physical states in the sensor molecules (Bezanilla et al., 1982). In skeletal muscle it is possible that during these transitions the interactions between specific regions of the DHPR and RYR are favored, causing conformational changes in the RYR that lead to the opening of the SR calcium release channels. It has been proposed that, after steady membrane potential changes, the gating molecules occupy a different set of physical states that still allow transitions across the membrane but in a different potential range (Shirokov et al., 1992). In skeletal muscle, ryanodine binding to the RYR might cause the DHPR to remain locked in the conformation corresponding to the inactivated state, thus impeding repriming. This effect would be favored by the molecular arrangement of DHPRs and RYRs in the T-SR junction, which provides the structural basis for ECC in skeletal muscle. In contrast to the model proposed by Cohen and Lederer (1988), our results indicate that for cardiac muscle functional interactions between RYRs and DHPRs do not occur and intramembrane charge movement and its repriming are not affected by ryanodine. The calcium-mediated inactivation of DHPRs in cardiac muscle would be the equivalent of the negative feedback postulated above for skeletal muscle. In this sense one can suggest that the retrograde effect of RYRs on the repriming of DHPRs could be a mechanism that participates in the tonus modulation of skeletal muscle in case of strong or maintained depolarizations.

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