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Kinetics of Cardiac RyR Channel Gating Studied at High Temporal Resolution

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Summary

Measurements of ryanodine receptor (RyR) activity during dynamic changes of calcium concentration have suggested that RyR has at least four calcium binding sites, and that activation transpires as an increase in the activity within the high open probability H-mode. Binding of several Ca^{2+} ions within the H-mode should manifest itself in the steady-state RyR activity by the presence of multiple closed times. However, previously only two closed times were detected in the H-mode of RyR activity. Here we recorded steady-state activity of single cardiac RyRs with high temporal resolution and compared it to data simulated under the same conditions using our previously published model of RyR gating. At a 10 kHz resolution, the closed time histograms of both experimental and simulated data had three exponential components. The closed times of simulated data were not significantly different from those obtained experimentally. After filtering at 2 kHz, only two exponential closed time components with time constants not significantly different from those previously published could be detected in both experimental and simulated records. The conformity of the steady-state experimental data to the model derived from the dynamic data provides further support for the idea that RyRs need binding of multiple Ca^{2+} ions to open.

Key words

Ryanodine receptor • Gating • Kinetics • Calcium release • Cardiac muscle

Introduction

Calcium ions play a central role in cardiac muscle contraction. Increasing intracellular concentration of Ca^{2+} causes a release of further Ca^{2+} ions from sarcoplasmic reticulum (SR) through the ryanodine receptor calcium release channels (RyRs). This process, called calcium-induced calcium release, is believed to be controlled mainly by the entry of Ca^{2+} through the voltage sensitive Ca^{2+} channels (DHPR receptors) on the sarcolemma (Bers 2002, Noble 2002).

The homotetrameric RyR protein is localized in discrete junctional domains of the SR near specialized domains of the surface membrane and T tubules that contain DHPR receptors (Franzini-Armstrong *et al.* 1999). Considering the intracellular RyR localization, the most direct and compelling data about SR Ca^{2+} release channel regulation come from measurements of single RyR channels reconstituted in planar lipid bilayers (BLMs). Ryanodine receptor channels exhibit several patterns of activity under steady-state conditions (Ashley and Williams 1990, Percival *et al.* 1994, Zahradníková

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and Zahradník 1995, Armisén *et al.* 1996, Copello *et al.* 1997). Analysis of these distinct kinds of gating revealed three modes of RyR channel activity: H (high open probability) mode, L (low open probability) mode and I (inactivated) mode (Zahradníková and Zahradník 1995, Armisén *et al.* 1996). It was suggested that the existence of different modes of activity could elucidate some phenomena such as adaptation (prevalence of H-mode after Ca^{2+} binding) (Zahradníková and Zahradník 1995, 1996, Zahradníková *et al.* 1999a,b) or modulation of RyR activity by distinct ligands (Zahradníková and Palade 1993, Percival *et al.* 1994, Copello 1997). Other studies dealing with the measurements of RyR activity under non-steady-state conditions suggest that the activity of the channel can also be described as modal under dynamic conditions (Zahradníková *et al.* 1999a,b). Zahradníková *et al.* (1999b) showed that the kinetics of channel activation in response to brief Ca^{2+} stimuli could be well described by a gating scheme incorporating four independent Ca^{2+} binding sites in the channel. According to this model, sequential binding of four Ca^{2+} ions to the fast-access H-mode states of the channel is necessary to enable the channel to open. This fact was manifested by a delay in channel opening with respect to the time of calcium elevation, and by the steepness of the calcium dependence of peak open probability. In the steady-state, the existence of several closed states with a different number of Ca^{2+} ions bound should result in the occurrence of multiple exponential components in the closed time histogram for channel gating in the H-mode. However, our previous experiments at a lower time resolution (Zahradníková and Zahradník 1995) detected only two closed time components in this mode of activity. In the present study we re-examined channel activity at a higher temporal resolution (100 kHz sampling frequency, 10 kHz filtering) to determine whether the steady-state behaviour of RyR is consistent with the gating model of Zahradníková *et al.* (1999b).

Methods

Bilayer Experiments

Heavy sarcoplasmic reticulum microsomes were prepared from canine left ventricles by standard procedures (Zahradníková and Palade 1993). In brief, hearts were removed under pentobarbital anesthesia. Left ventricles, cleaned of connective tissue and vessels, were minced and then homogenized with a Polytron homogenizer in 3 volumes of 0.9 % NaCl buffered with 10 mM tris(hydroxymethyl)-aminomethane (pH 7.3). The homogenate was centrifuged at 4000 x *g* for 20 min, then recentrifuged at 8000 x g (20 min) and 40 000 x *g* (30 min) to obtain two crude microsomal fractions that were resuspended in the above saline + 10 % sucrose, frozen and stored in liquid nitrogen.

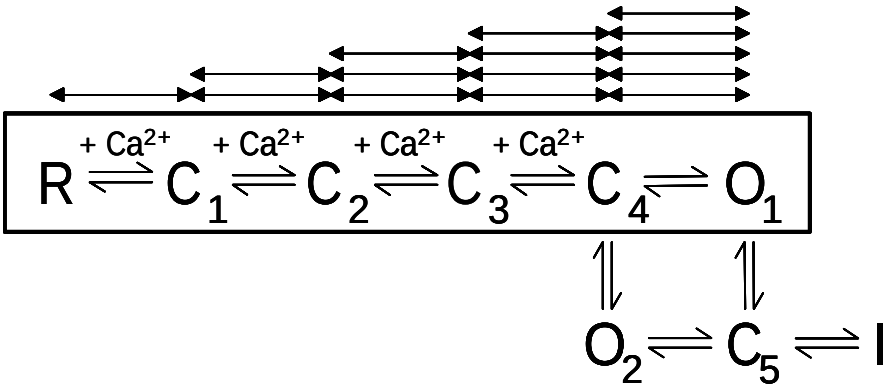

Fig. 1. *Extended minimal gating model of the RyR. R, resting state; C1-C5, closed states, O1, O2, open states; I, inactivated (long-lived closed) state. The H-mode states are outlined. The arrows at the top represent all possible transitions between the open state and the closed/resting states – these different transitions should be manifested as five exponential components in closed time histograms of the H-mode. The values of the rate constants used for simulations are given in Table 1.*

Single SR Ca^{2+} release channels were reconstituted by fusing heavy sarcoplasmic reticulum microsomes into planar lipid bilayers. Planar lipid bilayers were formed across a 100 μm aperture in a Delrin chamber, which separated the *cis* and *trans* bathing solutions, corresponding to the cytoplasmic and

luminal milieu, respectively. The bilayer chamber was designed to minimize the background current noise during recordings with high temporal resolution. The small bilayer aperture resulted in a bilayer capacitance of 50-70 pF. Bilayers were prepared from a mixture of phosphatidyl ethanolamine and phosphatidyl choline (Avanti Polar Lipids, USA) in a ratio of 7 : 3. Fusion was promoted by an osmotic gradient (400/20 mM $CsCH_3SO_3$ *cis*/*trans*) and detected by the appearance of single-channel activity with the appropriate single-channel amplitude at membrane potentials of +50 and -50 mV (cytoplasmic vs. luminal side). After incorporation of a single Ca^{2+} release channel, the osmotic gradient was eliminated. The experimental solution contained 400 mM $CsCH_3SO_3$, 10 mM CsHEPES, pH 7.4. Cardiac RyRs were activated by 20 μM Ca^{2+} from the cytoplasmic (*cis*) side. The chemicals, except when noted otherwise, were from Sigma (USA).

Data acquisition, analysis and single-channel simulation

The incorporated RyRs were held at 0 mV and single channel currents were recorded on depolarization to +40 mV (cytoplasmic vs. luminal side) using the Axopatch 200A patch-clamp amplifier (Axon Instruments, USA) in 400 ms data segments, filtered at 10 kHz, and digitized at 100 kHz. A Pentium computer with Digidata 1200A and pClamp software (Ver. 8.0, Axon Instruments, USA) was used for data acquisition. Only records containing activity of a single ryanodine receptor were analyzed.

Simulation of cardiac ryanodine receptor activity was performed on a Pentium computer using the program SCESim (Ver. 2.3, iii21, Slovakia) and the previously published extended minimal model of RyR gating (Zahradníková *et al.* 1999b, Fig. 1). Current amplitude was set to 20 pA, noise level to 3 pA (r.m.s.), filter to 10 kHz, sampling to 100 kHz. The on and off rates for calcium binding and the rate constants of individual transitions of the model are given in Table 1.

Experimental and simulated data were analyzed, including digital filtering, using the TRANSIT software (VanDongen 1996) as described previously (Zahradníková and Zahradník 1995, 1996). In brief, the data segments were idealized, the open probability in each of the segments was calculated, and the segments were categorized into H-mode ($p_o > 0.08$), L-mode ($0 < p_o < 0.08$), and I-mode ($p = 0$). Only H-mode segments were used for subsequent analysis. The closed times from all segments were pooled, and their frequency histograms were fitted using Origin (Ver. 7.0, OriginLab, USA), in which also all secondary analyses, calculations, and statistics were performed. The results are presented as mean ± S.E.M.

Table 1. Rate constants of the extended minimal model of RyR channel gating.

Rate constant	Value	Unit
k_{on}	712	$\mu M^{-1}.s^{-1}$
k_{off}	3000	s^{-1}
k_{C4O1}	10000	s^{-1}
k_{C4O2}	1	s^{-1}
k_{O1C4}	500	s^{-1}
k_{O2C4}	0.5	s^{-1}
k_{O1C5}	2	s^{-1}
k_{O2C5}	3000	s^{-1}
k_{C5O1}	0.6666	s^{-1}
k_{C5O2}	100	s^{-1}
k_{C5I}	0.5	s^{-1}
k_{IC5}	1.5	s^{-1}

$k_{RC1} = 4 \times k_{on}$; $k_{C1R} = k_{off}$; $k_{C1C2} = 3 \times k_{on}$; $k_{C2C1} = 2 \times k_{off}$; $k_{C2C3} = 2 \times k_{on}$; $k_{C3C2} = 3 \times k_{off}$; $k_{C3C4} = k_{on}$; $k_{C4C3} = 4 \times k_{off}$;

Results

Steady-state channel activity at high and low temporal resolution

To investigate gating kinetics of the RyR under steady-state conditions, we recorded channel activity at 20 μM Ca^{2+} at a temporal resolution of 10 μs and 10 kHz filtering. Examples of channel activity are shown in Figure 2A. The same data after digital refiltering at 2 kHz are shown in Figure 2B. Three distinct types of behavior can be distinguished in the records of steady-state activity of the ryanodine receptor: the H mode with high P_O and long openings separated by short closures, the L mode with low P_O and short isolated openings, and the I mode, which is without openings (Zahradníková and Zahradník 1995).

According to the extended minimal model of RyR gating (Zahradníková *et al.* 1999b, Fig. 1), the presence of several Ca^{2+} binding sites should affect specifically the closed times in the H mode of RyR activity. Therefore, we analyzed only segments displaying H mode activity, which could be distinguished by having open probability higher than 0.08 (Zahradníková and Zahradník 1995).

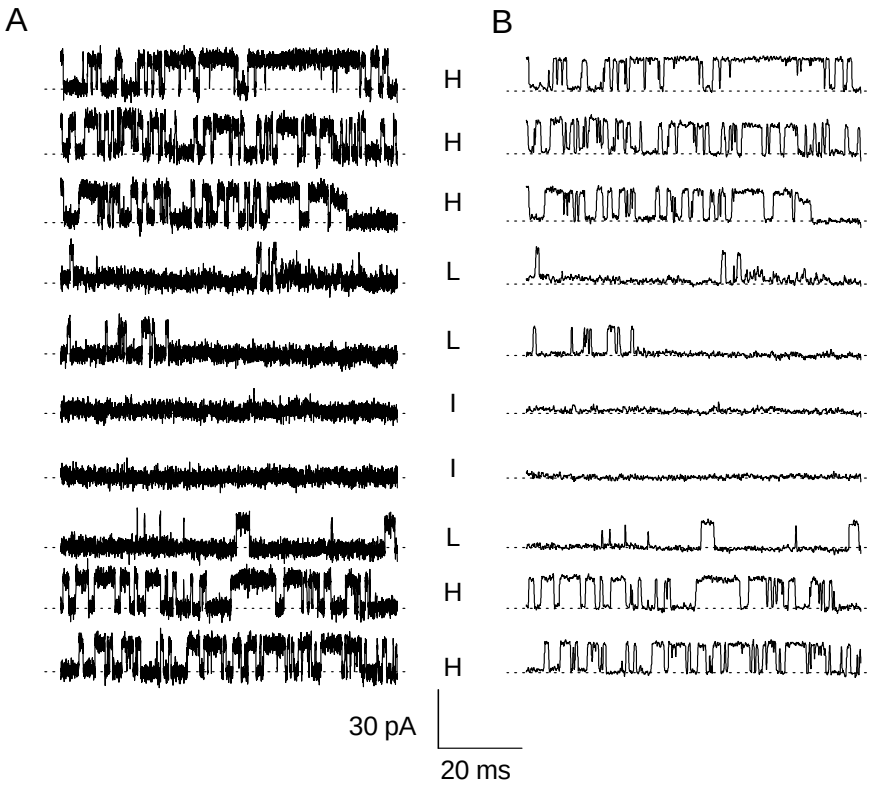

Fig. 2. *Typical traces of the steady-state activity of the cardiac calcium release channel. Dotted lines denote closed levels. Single channel openings are plotted as upward deflections. The amplitude of single-channel current is about 20 pA. The type of the RyR gating mode (H, L or I) is indicated by the respective letter next to each trace. A – Original records filtered at 10 kHz. B – The same data as in A digitaly re-filtered at 2 kHz.*

For each individual segment of activity, we determined the number and duration of single-channel closures and fitted the distributions with a sum of 2 or 3 exponential components (Fig 3). Using 10 kHz filtering, three exponential components could be detected in the closed time histogram with time constants of $\tau_{C1} = 0.05 \pm 0.01$ ms, $\tau_{C2} = 0.56 \pm 0.10$ ms and $\tau_{C3} = 2.24 \pm 0.16$ ms (three independent experiments; 53 s of total time analyzed). Attempts to approximate the closed time histograms with two exponentials provided substantially higher χ^2 values than did the three-exponential fit. To compare these results with our previous analysis of channel kinetics measured at low time resolution (Zahradníková and Zahradník 1995), we reanalyzed the data after applying a 2 kHz digital filter. In this case, only two exponential components could be distinguished in the closed time histogram with time constants of $\tau_{C1} = 0.36 \pm 0.07$ ms and $\tau_{C2} = 2.27 \pm 0.50$ ms. Approximation of the closed time histograms with three exponentials was not possible. The closed times determined after filtering at 2 kHz were not significantly different from those reported by Zahradníková and Zahradník (1995), suggesting that the differences between our present results and those of Zahradníková and Zahradník (1995) were not due to differences in the properties of RyRs or in the analysis of data. Rather, these results suggest that the use of 2 kHz filtering is not sufficient for detecting the shortest closed times (< 100 μs) and therefore it underestimates the number of closed states in the H-mode.

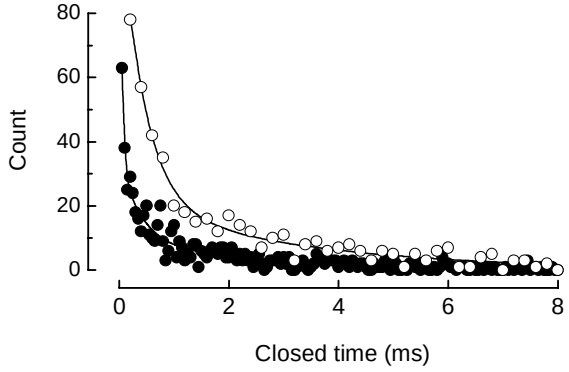

Fig. 3. *Closed time distributions of experimental data. Distribution of closed times fitted with a sum of exponential functions. At 10 kHz filtering (full circles), three exponential components were detected in the closed time histogram. After digital filtering at 2 kHz (open circles), only two exponential components were detected. The respective values of the time constants are given in the Results.*

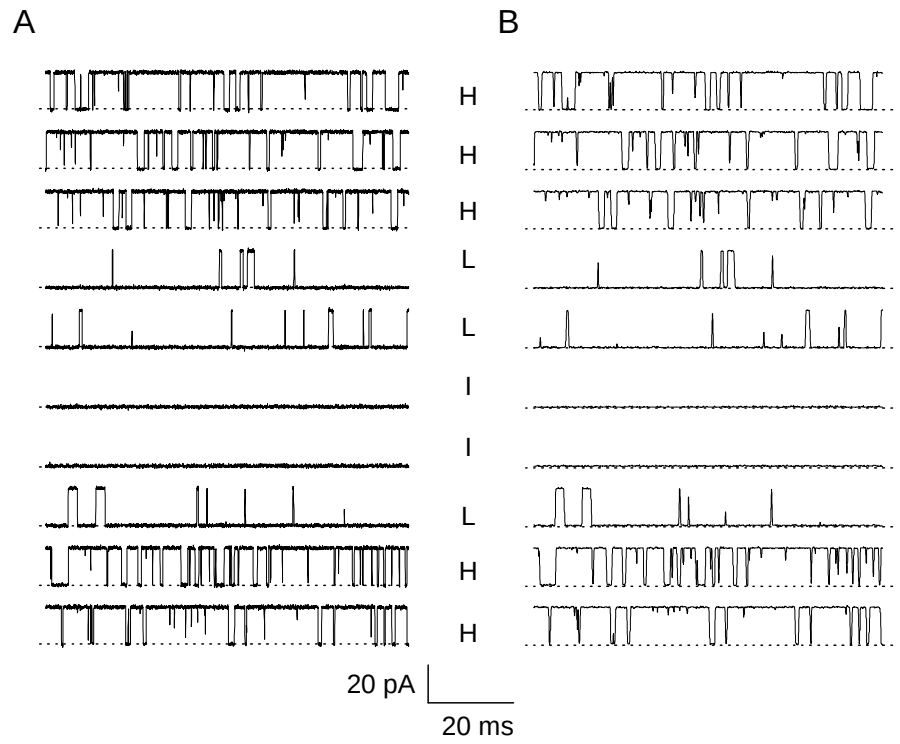

Fig. 4. *Typical traces of simulated activity of the cardiac calcium release channel. Dotted lines denote closed levels. Single channel openings are plotted as upward deflections. The amplitude of single-channel current is 20 pA. The type of the RyR gating mode (H, L or I) is indicated by the respective letter next to each trace. A – Original data filtered at 10 kHz. B – The same data as in A digitaly re-filtered at 2 kHz.*

Simulation of channel activity at high and low temporal resolution

To test whether the H-mode activity of RyRs measured at high temporal resolution is consistent with the extended minimal gating model, we have compared the experimental data to simulated steady-state RyR activity at 20 μM Ca^{2+}, using the extended minimal gating model of RyR (Fig. 1, Table 1). Examples of simulated activity are shown in Fig. 4 after filtering at 10 kHz (Fig 4A) and after refiltering at 2 kHz (Fig 4B). Again, the three distinct types of modal gating (H mode, L mode and I mode) were apparent in the simulated steady-state records.

The simulated data were analyzed in the same manner as the experimental data. As in the experiment, records with $p_o > 0.08$, i.e., those of H-mode activity, were selected for analysis. Three closed time components were apparent in the closed time distribution after filtering at 10 kHz (Fig. 5). The closed time constants of simulated data, $\tau_{C1} = 0.026 \pm 0.004$ ms, $\tau_{C2} = 0.50 \pm 0.10$ ms, $\tau_{C3} = 3.8 \pm 1.7$ ms (three independent simulations; 63 s of total time analyzed), were neither substantially nor significantly different ($p>0.05$) from those obtained experimentally. On the other hand, only 2 components with $\tau_{C1} = 0.54 \pm 0.02$ ms and $\tau_{C2} = 2.17 \pm 0.59$ ms could be detected in simulated data after 2 kHz filtering. Again, the time constants were neither substantially nor significantly different ($p>0.05$) from those obtained experimentally. These results show that even in the simulated data, free of experimental variation, one of the three short-lived closed states is detectable with 10 kHz filtering, but cannot be detected after filtering at 2 kHz. Moreover, the results suggest that the number as well as the time constants of exponential components in the steady-state H-mode activity of the RyR is in good agreement with the extended minimal gating model.

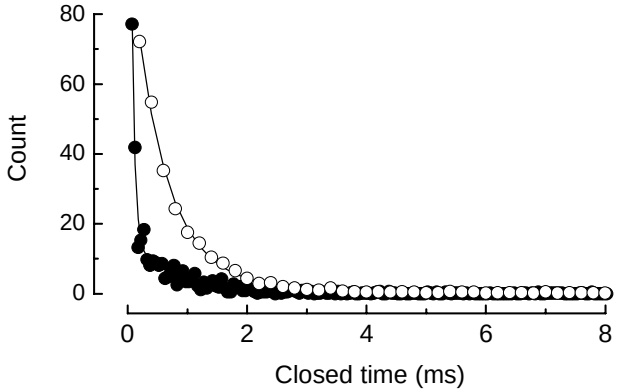

Fig. 5. *Closed time distributions of simulated data. Distributions of closed times fitted with a sum of exponential functions. At 10 kHz filtering (full circles), three exponential components were detected in the closed time histogram. After digital filtering at 2 kHz (open circles), only two exponential components were detected. The respective values of the time constants are given in the Results.*

Discussion

The aim of this study was to test whether the steady-state behavior of RyRs can be reconciled with its dynamic behavior after brief Ca^{2+} elevations. This was achieved by comparing the experimental data obtained at a steady Ca^{2+} concentration with the data generated by the model based on high-resolution dynamic Ca^{2+} data, but run at a steady Ca^{2+} concentration.

Experimental steady-state data characterizing the activity of the RyR within the H-mode published so far (Zahradníková and Zahradník 1995, Armisén *et al.* 1996, Saftenku *et al.* 2001) were obtained at a lower time resolution and could be well approximated by a model containing only a single Ca^{2+} binding site (Zahradníková and Zahradník 1996). Therefore, we had to elucidate the effect of filtering on the kinetic characteristics of RyR activity, for which rapid transitions and short closed times are typical.

We compared the closed time constants of the experimental data with those provided by the extended minimal gating model (Zahradníková *et al.* 1999b) under the same conditions. Regardless of whether we analyzed experimental or simulated data for RyR channel gating, the analysis yielded three closed time components at high-time resolution. The closed time constants determined in the experimental and simulated data sets were not significantly different, suggesting that the closed times within the H-mode are well approximated by the model. The equivalence of the closed times imply that the steady-state and dynamic behavior of RyRs can be described using the same model, as in the present simulations and those used to characterize the dynamic behavior of RyRs during brief Ca^{2+} elevations the same sets of rate constants were used.

The extended minimal gating model has a total of seven closed states, out of which the five shorter-lived states belong to the H-mode, and the two long-lived states belong to the L- and I-mode, respectively. The presence of three out of the five theoretical exponential components in the experimental as well as the simulated H-mode data suggests that the 10 kHz time resolution used in the present study sets the detection limit above the two shortest closed time components, given that the model is correct. This finding is consistent with the lifetime analysis of RyR channel activity done by Sitsapesan and Williams (1994) using 4 kHz filtering, who observed two to three short-lived closed states and two long-lived closed states in the overall steady-state activity of RyRs (i.e., where L- and I-mode activity was included). In this work we showed that all three short closed states belong to the H-mode of RyR activity.

The data measured or simulated with a 10 kHz resolution but digitally refiltered at 2 kHz provided only two closed time components, in agreement with the data measured previously at a lower time resolution (Zahradníková and Zahradník 1995). Individual lifetime constants obtained in these three cases – experimental, simulated and the published low-resolution data – were not significantly different ($p>0.05$). They were also in good agreement with the short closed times of high-activity data at 50 μM Ca^{2+} and at 2 kHz filtering reported by Saftenku *et al.* (2001).

The presence of three fast exponential components of the closed time in H-mode activity of the RyR at high temporal resolution provides evidence that the H-mode contains at least three closed states. Neither in the experimental data, nor in the simulations additional closed time components, expected from the model, could be detected at 10 kHz filtering. This apparent paradox results from the different experimental approach used in the dynamic experiments (Zahradníková *et al.* 1999b), in which the number of Ca binding steps was estimated from the calcium dependence of peak open probability, which is independent of the time resolution.

It is noteworthy that the data of Zahradníková and Zahradník (1995) were measured by selecting H-mode segments from continuous single channel activity records at 0 mV, while in the present study we used discontinuous data segments of RyR activity measured following a step voltage change to +40 mV. In spite of the different membrane potentials, the closed time constants were not significantly different ($p>0.05$). These observations suggest that the membrane potential does not significantly affect the observed gating properties of RyRs within the H-mode of activity. However, it has been observed previously that the activity of RyRs is much higher and more stable when the membrane potential is periodically changed (Fill *et al.* 1990). In skeletal RyRs, periodic changes in the membrane potential were shown to increase the prevalence of the H-mode at the expense of the I-mode without changing the open probability within individual modes (Zahradníková and Mészáros 1998). It is therefore possible that a similar effect may also be operative in the cardiac ryanodine receptors.

In conclusion, the presence of three closed time components in steady-state activity of the RyR gating in the H-mode demonstrate the presence of at least three closed states in this mode of activity. The good agreement between the experimental results and the simulations at both high and low time resolution is in strong support of our model, in which the closed states represent the RyR conformations with different numbers of bound calcium ions. Taken together, these results give further support to the idea that the ryanodine receptor has multiple Ca^{2+} binding sites. Occupation of these sites by calcium ions precedes channel opening and release of calcium from the sarcoplasmic reticulum.

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