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Response of Ryanodine Receptor Channels to Ca^{2+} Steps Produced by Rapid Solution Exchange

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ABSTRACT We used a flow method for Ca^{2+} activation of sheep cardiac and rabbit skeletal ryanodine receptor (RyR) channels in lipid bilayers, which activated RyRs in <20 ms and maintained a steady $[Ca^{2+}]$ for 5 s. $[Ca^{2+}]$ was rapidly altered by flowing Ca^{2+}-buffered solutions containing 100 or 200 μM Ca^{2+} from a perfusion tube inserted in the *cis*, myoplasmic chamber above the bilayer. During steps from 0.1 to 100 μM, $[Ca^{2+}]$ reached 0.3 μM (activation threshold) and 10 μM (maximum P_o) in times consistent with predictions of a solution exchange model. Immediately following rapid RyR activation, P_o was 0.67 (cardiac) and 0.45 (skeletal) at a holding voltage of +40 mV (*cis/trans*). P_o then declined (at constant $[Ca^{2+}]$) in 70% of channels ($n = 25$) with time constants ranging from .5 to 15 s. The mechanism for P_o decline, whether it be adaptation or inactivation, was not determined in this study. *cis*, 2 mM Mg^{2+} reduced the initial P_o for skeletal RyRs to 0.21 and marginally slowed the declining phase. During very rapid falls in $[Ca^{2+}]$ from mM (inhibited) to sub-μM (sub-activating) levels, skeletal RyR did not open. We conclude the RyR gates responsible for Ca^{2+}-dependent activation and inhibition of skeletal RyRs can gate independently.

INTRODUCTION

Ryanodine receptor channels play a central role in excitation-contraction (E-C) coupling by mediating Ca^{2+} release from the sarcoplasmic reticulum (SR). In both cardiac and skeletal muscle, a tissue-specific dihydropyridine (DHP) receptor (L-type calcium channel) is the voltage sensor for E-C coupling. In cardiac muscle, Ca^{2+} flux through DHP receptors is believed to trigger the ryanodine receptors (RyRs) via the mechanism of Calcium Induced Calcium Release (CICR). In skeletal muscle, however, there may also be a direct link between DHP receptors and RyRs; an influx of extracellular Ca^{2+} through the DHP receptors does not appear to be a prerequisite for muscle contraction (Ashley, Mulligan and Lea, 1991). Curtis (1994) described a source of Ca^{2+} within the transverse tubular system that was not lost in zero Ca^{2+} and entered the SR during subsequent contracture, possibly activating CICR on the way. CICR may also act as a secondary Ca^{2+} source from those RyRs not in apposition to DHP receptors (Rios and Pizarro, 1991).

During muscle contraction, the RyRs function in response to changes in $[Ca^{2+}]$ on a millisecond time scale. Until recently all measurements of RyR kinetics in lipid bilayers were carried out at steady or slowly altering $[Ca^{2+}]$. Measurements of Ca^{2+} release from the SR of skinned cardiac muscle fibers (Fabiato, 1985) showed that RyR kinetics under the nonstationary conditions found in vivo differ from their steady-state properties. $[Ca^{2+}]$ rise times of <1 ms can be achieved by the flash-induced release of caged Ca^{2+} (Gyorke and Fill, 1993). When $[Ca^{2+}]$ was elevated rapidly to activating levels, RyRs opened quickly and open probability (P_o) subsequently declined (Gyorke et al., 1994; Valdivia et al., 1995). The nature of declining activity following activation in RyRs is controversial because the presence of a large leading edge spike on the flash-induced $[Ca^{2+}]$ steps could also explain the declining activity (Escobar et al., 1995; Lamb and Stephenson, 1995). By rapidly moving a pipette tip containing a cardiac RyR between two flowing solutions, Sitsapesan et al. (1995a,b) were able to rapidly activate cardiac RyRs to low P_o and then maintain steady $[Ca^{2+}]$. Occasional decline in RyR activity was observed at +40 mV but not at −40 mV.

We introduced a beveled tube above a RyR-containing bilayer. By squirting Ca^{2+}-buffered solutions we were able to measure the response of cardiac and skeletal RyRs to rapid rises and falls in $[Ca^{2+}]$ to new steady levels. We could infer the time course of $[Ca^{2+}]$ at the bilayer/channel from bilayer surface potentials and RyR conductance changes induced by changing $[Cs^+]$ (Laver and Curtis, 1996). In response to 5-s puffs of 100 μM Ca^{2+}, at a +40 mV holding voltage (*trans* bath at ground), P_o for both cardiac and skeletal RyRs increased very rapidly and then declined with a time constant, τ_d, of several seconds. The presence of 2 mM Mg^{2+} and 500 mM CsCl during the Ca^{2+} step reduced the skeletal RyR P_o during the initial and declining phases by ~50%.

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METHODS

RyRs from rabbit skeletal muscle and sheep hearts were isolated and reconstituted as previously described (Laver et al., 1995). Bilayers (80 μm diameter) separating two, 1.5-ml, aqueous baths (*cis* and *trans*) were

formed from a mixture of palmitoyl-oleoyl-phosphatidylethanolamine (PE), palmitoyl-oleoyl-phosphatidylserine (PS) and palmitoyl-oleoyl-phosphatidylcholine (PC; 5:3:2, by weight) in *n*-decane using the film drainage technique of Mueller et al. (1962). The bilayer aperture in the wall of the delrin cup was located at the base of a broad recess that was machined to ~100 μm thickness in the 2–3 mm diameter region bordering the aperture. SR vesicles added to the *cis* bath fused with the bilayer so that their myoplasmic surface (ryanodine receptor) faced the *cis* (myoplasmic) bath (Laver et al., 1995).

In all bathing solutions 10 mM TES was used to buffer pH and BAPTA was used to buffer pCa. Solutions were adjusted to pH 7.4 with CsOH, and to a range of pCa with $CaCl_2$. Free [Ca^{2+}] was measured using an ion meter. The luminal (*trans*) bath contained (in mM) 50 CsCl, 0.1 $CaCl_2$. The myoplasmic (*cis*) bath usually contained either 250 or 500 CsCl and variable free [Ca^{2+}] and [Mg^{2+}]. Before vesicle fusion, *cis* [Ca^{2+}] was either 0.1 or 1 mM. After fusion, the cis chamber was perfused with pCa 7 solution. At least 95% exchange of *cis* or *trans* solutions could be achieved by gradually perfusing the bath with at least four volumes (6 ml) of [Ca^{2+}]-buffered solution. Perfusion was done using a back-to-back syringe system configured such that bath perfusion and waste withdrawal could be maintained at equal rates. Slow raising of *cis* [Ca^{2+}] or [Mg^{2+}] was done by adding stock solutions to the bath while stirring vigorously.

Details of the methods for achieving and evaluating rapid solution exchange are given by Laver and Curtis (1996). Rapid exchange of solutions at the *cis* face of the bilayer was achieved by puffing solutions from a beveled tube located above and within 50 μm of the *cis* face of the bilayer. The design of the bilayer aperture (see above) facilitated the placement of the 0.7-mm outside diameter tube near the membrane and the rapid solution exchange at the bilayer surface. Care was taken that during experiments, accumulation of the puffing solution in the *cis* bath did not significantly alter the bath composition. To avoid Ca contamination, the myoplasmic chamber was gradually reperfused with fresh solution, usually after 10, 5-s puffs.

The *cis* chamber was electrically grounded to prevent electrical interference from the flow tube. To retain convention, electrical potentials are reported here with respect to the *trans* chamber as ground and positive current as directed from the *cis* to *trans* bath. All records were taken with the bilayer potential clamped at +40 mV; our original rational was to suppress voltage gated Cl^- channels, though it became evident that RyRs are also voltage sensitive. During the experiments, the bilayer current was recorded at a bandwidth of 5 kHz on video tape. Before analysis, the current signals were replayed through a 1-kHz, low-pass, 8-pole Bessel filter, sampled at 2 kHz and displayed using an in-house program (Channel2, developed by Professor P.W. Gage and Mr. M. Smith).

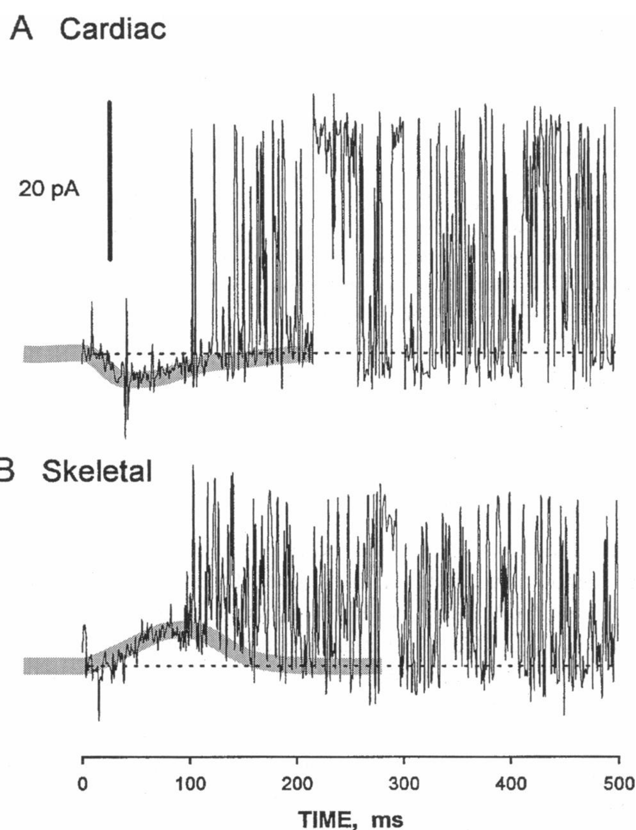

FIGURE 1 Examples of the gating response of cardiac (*A*) and skeletal (*B*) RyRs to a rapid rise in *cis* [Ca^{2+}] from subactivating levels by exchanging solutions under a pressure of 15 cm H_2O. The period of solution exchange is indicated by the transient in the current baseline, the wide stippled lines, which start at $t = 0$ and continue for 100–200 ms. The bilayer potential was held at +40 mV and the bathing solutions contained 250/50 mM CsCl (*cis/trans*). Before $t = 0$, the channels were inactive and the dashed lines indicate the steady current baseline in each record.(*A*) Activation of a single cardiac RyR (D17018) by a [Ca^{2+}] step from 0.1 to 1 μM.(*B*) Activation of a single skeletal RyR (D14244) by a [Ca^{2+}] step from 0.1 to 200 μM in the presence of 2 mM Mg^{2+}.

Tracking [Ca^{2+}] during rapid solution exchange

Here we outline two methods we use for simultaneously measuring the time course of solute concentrations and ion channel gating during solution exchange. Detailed evaluation of these methods is given by Laver and Curtis (1996).

1) Exchange of solution against the bilayer produced a capacitive current transient (Fig. 1, *stippled band*), which was driven by an ion-dependent change in the bilayer surface potential. The start of the capacitive current was used to mark the beginning of solution exchange at the bilayer. We used changes in the bilayer surface potential to probe the ionic composition of the solution at the bilayer surface. The surface potential depended on the ion species and bilayer composition and was accurately described by the Stern-Gouy-Chapman theory.

2) Changes in [Cs^+] and [Ca^{2+}] (applied separately or together) measured using method 1 could be described by an exponential exchange (Eq. 1) of bath and puffing solutions. This relationship was used to predict the time course of *cis* [Ca^{2+}] near RyRs. The concentration of any ion species, [*A*] at a time (*t*) after the onset of solution exchange is given by:

$$[A](t) = [A]_0 \cdot \{\exp(-t/\tau)\} + [A]_\infty \cdot \{1-\exp(-t/\tau)\} \quad (1)$$

where τ is the exponential time constant (mixing time) of the solution exchange. Thus we were able to calculate the time course of free [Ca^{2+}] from the total [Ca^{2+}] and [BAPTA] predicted by Eq. 1. The mixing times, τ, ranged from 20 to 110 ms (depending on the flow pressure), and these were inferred from measurements of RyR conductance during changes in [Cs^+]. A reservoir height of 5 cm allowed moderate rates of solution exchange (τ = 110 ms), while conserving bilayer integrity. At 15 cm ($\tau <$ 55 ms) bilayer rupture was more common, whereas at 30 cm ($\tau <$ 20 ms) flow could only be applied for <1 s.

Ensemble analysis

Ensembles of RyR responses to upward [Ca^{2+}] steps were compiled from episodes consisting of a 5 s, continuous flow of solution over the bilayer. Individual episodes were separated by 15-s intervals of stirring to allow the *cis* bath to equilibrate near the bilayer surface. Obtaining significant current ensembles from the stochastic signals in each episode required compilation of many sweeps so that the total recording time is long (~50 s from 10 episodes) compared with the longest channel open or closed periods (~1 s). The significance of mean channel parameters determined from limited segments of stochastic data has been evaluated elsewhere (e.g., Draber et

al., 1993; and Yeo et al., 1988). The bilayer survival time usually limited experiments to ~10 episodes which was sufficient to reveal the broader features of the ensemble RyR activity. Channel open probability, P_o, was calculated from the ensemble current divided by the open channel current.

Episodes from the recordings were edited in synchrony with solution flow (see above) and averaged to produce ensemble RyR responses to rapid solution changes. The limit to the temporal resolution of this method is determined by the size of the bilayer. Whereas the beginning of the capacitive transient indicates change of solutions at some point on the bilayer, this may not coincide with the location of the channel being studied. This uncertainty limits the synchrony of episodes in our experiments to ~25 ms. Consequently very brief, transient RyR activity can be significantly blurred or obliterated by the ensemble averaging process.

RESULTS

Steady-state, [Ca^{2+}]-dependent gating of cardiac and skeletal RyRs

Skeletal RyRs were activated by slow addition of Ca^{2+} to the myoplasmic face (*cis* bath); their threshold for activation was ~0.3 μM Ca^{2+}, with ~1 μM Ca^{2+} for half activation and ~10 μM Ca^{2+} for maximal activation. Higher [Ca^{2+}] half-inhibited skeletal RyRs at 700 μM in 250 mM CsCl, at ~2 mM in 500 mM CsCl and cardiac RyRs were half-inhibited at 15 mM in 250 mM CsCl as shown previously (Laver et al., 1995). Skeletal RyRs slowly activated by 10–200 μM Ca^{2+} and sampled in the next 60 s had P_o of 0.32 ± 0.05 (seven channels) and cardiac RyRs slowly activated by 10 μM–1 mM Ca^{2+} had P_o of 0.47 ± 0.07 (five channels). (Note: The open probability values quoted here are slightly lower than those reported previously by Laver et al. (1995) who excluded long interburst closures whereas here these closures are included.) These values are similar to the [Ca^{2+}]-dependent open probabilities observed in other bilayer studies (Chu et al., 1993; Rousseau and Meissner, 1989). The gating activity of skeletal RyRs consisted of bursts of brief openings (~1 ms), whereas the cardiac RyRs had longer openings.

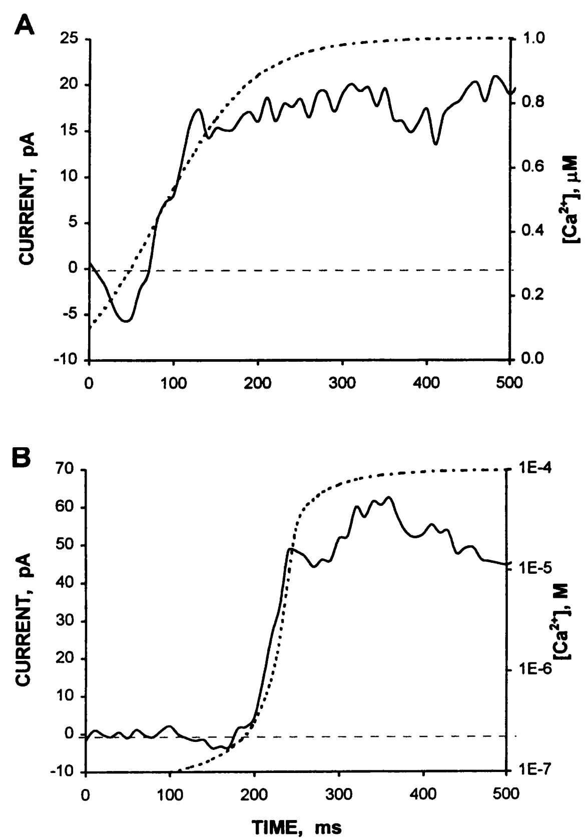

FIGURE 2 The mean activation of cardiac (*A*) and skeletal (*B*) RyRs in response to a rapid rise in *cis* [Ca^{2+}] from subactivating levels by exchanging solutions under a pressure of 15cm H_2O. Solid line: The mean current from several episodes; dashed curve: the model prediction (see Eq. 1) of the [Ca^{2+}] time course based on a mixing time of 55 ms. The dashed horizontal lines indicate the steady current baseline before $t = 0$.(*A*) (D17043) Average of 11 episodes of a single cardiac RyR activated by a [Ca^{2+}] step from 0.1 to 1 μM starting at $t = 0$. (*B*) (D15032) Average of five episodes of a single skeletal RyR activated by a [Ca^{2+}] step from 0.1 to 100 μM starting at $t = 100$ ms.

Response of RyR gating to rapid rises in cis [Ca^{2+}]

Fig. 1 shows single episodes of individual cardiac and skeletal RyRs activated by flowing Ca^{2+}-buffered solutions past the bilayer. The time interval over which the solution is changing to a new steady state is manifest by the small baseline transient at the beginning of each trace (*wide stippled line*). Rapid changes in myoplasmic [Ca^{2+}] from 0.1 to 100 or 200 μM activated the skeletal channels in the time predicted for [Ca^{2+}] to rise to threshold levels. A [Ca^{2+}] of 100 or 200 μM was chosen to activate skeletal RyR rapidly, yet not inhibit the channel when complete solution exchange had been achieved. Averages of several of these episodes are shown in Fig. 2. The time course of [Ca^{2+}] (*dashed curves*) was calculated using the solution exchange model with a mixing time constant of 55 ms (15 cm H_2O puffing pressure, see Methods). The beginning of the capacitive transient marked the onset of solution exchange. Both skeletal and cardiac RyRs started to open when the predicted [Ca^{2+}] was 0.3 μM and the skeletal RyR attained maximal activation (~10 μM Ca^{2+}) within 40 ms for a 100-μM-[Ca^{2+}] step and 20 ms when using 200-μM-[Ca^{2+}] steps (data not shown). In 91 trials in six skeletal RyRs (15 cm H_2O puffing pressure), channels opened in 83% of trials before the time 10 μM Ca^{2+} was expected. Immediately after channel activation, cardiac RyRs attained a P_o of 0.67 ± 0.08 (SE of five channels) and skeletal RyRs attained a P_o of 0.45 ± 0.12 (six channels).

Following activation, the behavior of individual skeletal and cardiac RyRs at steady [Ca^{2+}] was quite variable (Figs. 3 and 4). The P_o of some RyRs remained steady or increased, whereas for 70% of RyRs, P_o declined over the 5 s sampled. The frequency distribution of the relative P_o changes following rapid activation (Fig. 4) was determined from ensemble currents of 4–18 episodes on individual channels. Declines in P_o could be described by an exponen-

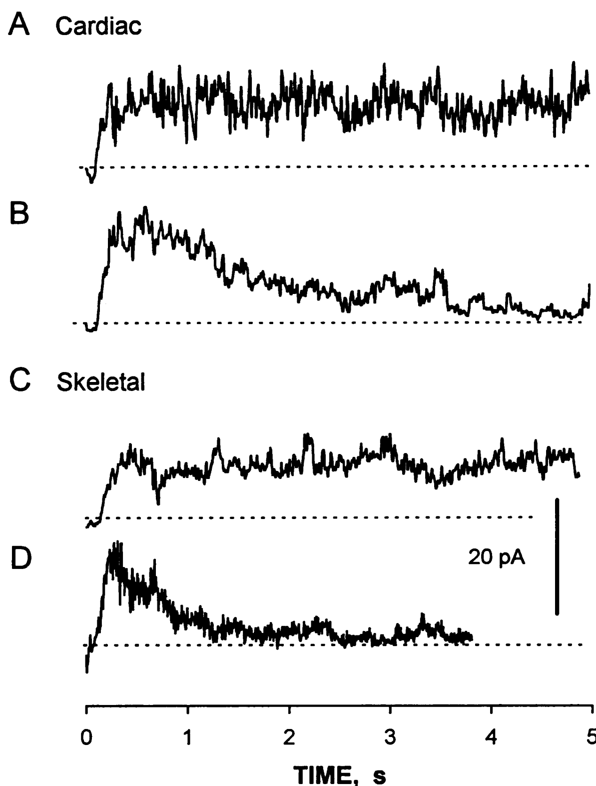

FIGURE 3 Examples of the long-term response of cardiac (*A* and *B*) and skeletal (*C* and *D*) RyRs to a rapid rise in *cis* [Ca^{2+}] starting at t = 0. Two channels that exhibited decline are shown in *B* and *D*, whereas two others that did not decline are shown in *A* and *C*. The dashed horizontal lines indicate the steady current baseline before t = 0.(*A*) (D17018) Average of eight episodes of a single cardiac RyR activated by a [Ca^{2+}] step from 0.1 to 1 μM. (*B*) (D17113) Mean of nine episodes of a single cardiac RyR activated by a [Ca^{2+}] step from 0.1 to 1 μM. (*C*) (D15103)) Mean of 11 episodes of a single skeletal RyR activated by a [Ca^{2+}] step from 0.1 to 100 μM. (*D*) (D16143) Mean of 14 episodes of two to three skeletal RyRs activated by a [Ca^{2+}] step from 0.1 to 100 μM.

tial with τ_d ranging from 15 to 171 s. In general, we found that greater initial P_o was associated with faster decline. An example of this is shown by the ensemble traces in Fig. 5 where a cardiac RyR activated by 1 μM Ca^{2+} (Fig. 5 *A*, P_o = 0.5, 11 episodes) shows a much smaller decline over the subsequent 5-s period than the same channel activated by 10 μM Ca^{2+} (Fig. 5 *B*, P_o = 0.8, 13 episodes). To gain an estimate of how a whole population of RyRs in skeletal muscle respond to [Ca^{2+}] steps, we produced a grand ensemble current compiled from all experiments where [Ca^{2+}] was rapidly changed from 0.1 μM to 100 μM or 200 μM (Fig. 6, *solid line* "Ca^{2+}"). P_o increased rapidly and then declined for the 5-s duration of the puff, with a τ_d of 12 s; skeletal RyR activity clearly declined even though [Ca^{2+}] remained constant. In individual experiments where P_o declines exceed 20% in 5 s, the τ_d = 1.9 ± 0.9 s (five channels). The relatively slow τ_d of the grand ensemble was a product of the averaging process, which included 30% of RyRs that did not show P_o decline.

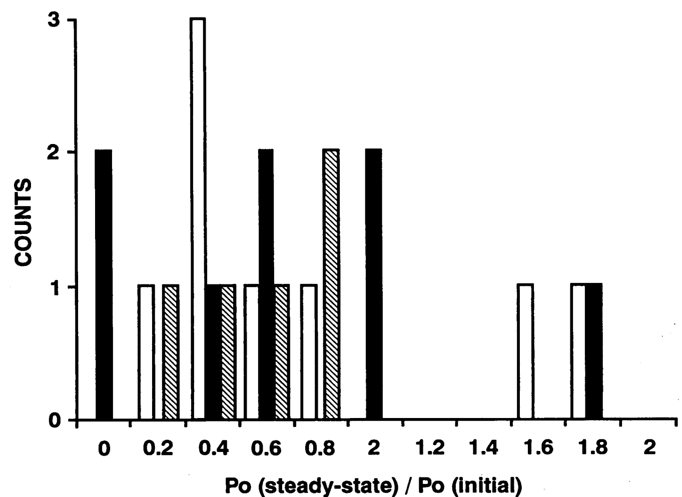

FIGURE 4 The frequency distribution in the relative declines in RyR activity following activation by rapid [Ca^{2+}] steps from 0.1 to 1 μM for cardiac RyR and from 0.1 to 100 μM for skeletal RyR. Solid: Skeletal RyRs in the absence of Mg^{2+}; Filled: Skeletal RyRs in the presence of 2 mM Mg^{2+}, Cross-hatched: Cardiac RyRs in the absence of Mg^{2+}. The ratio of RyR open probabilities at steady state and within 100 ms of activation were calculated from exponential fits to the current ensembles from each experiment. Even though there was considerable variation among RyR, most channels showed a significant decline in P_o after activation.

Effects of Mg^{2+}

Addition of physiological Mg^{2+} to the myoplasmic bath inhibited skeletal RyRs that had been slowly activated by 100 or 200 μM Ca^{2+}. The sensitivity of skeletal RyRs to

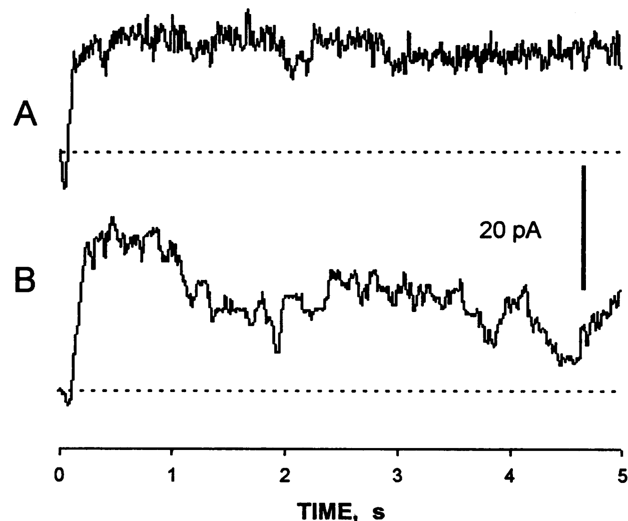

FIGURE 5 Ensemble currents from a single cardiac RyR in response to a rapid rise in *cis* [Ca^{2+}] from subactivating levels by exchanging solutions under a pressure of 15 cm H_2O, commencing at t = 0 (D17043). The dashed horizontal lines indicate the current baseline before t = 0. (*A*) ensemble of 11 episodes of the RyR activated by a [Ca^{2+}] step from 0.1 to 1 μM. The RyR activates with a mean P_o = 0.5 and shows no decline. (*B*) ensemble of 13 episodes of the same channel activated by a [Ca^{2+}] step from 0.1 to 10 μM. Maximum P_o = 0.8 and RyR activity declined over 5 s.

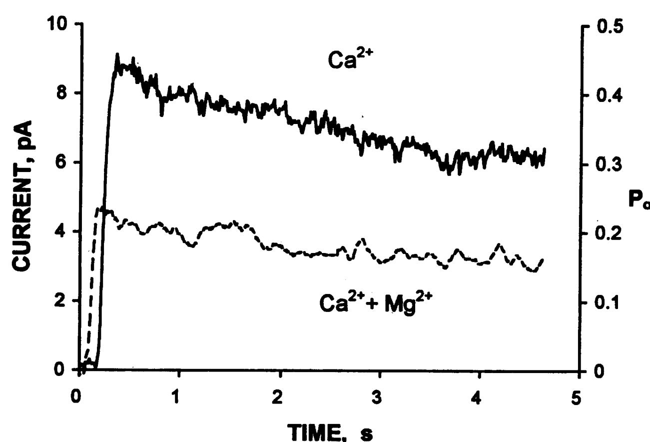

FIGURE 6 The ensemble skeletal RyR currents from several experiments representing the mean current per RyR and P_o during $[Ca^{2+}]$ steps by exchanging solutions under a pressure of 15 cm H_2O. P_o is approximately equal to the average current divided by 25 pA, which was the open channel current at 40 mV. The $[Ca^{2+}]$ step from 0.1 to >100 μM starts at $t = 0$. (*Solid line*, Ca) $[Ca^{2+}]$ rises to 100 μM (64 episodes from 24 channels in 7 bilayers). (*Dashed line*, Ca^{2+} + Mg^{2+}) $[Ca^{2+}]$ rises to 200 μM in the presence of 2 mM Mg^{2+} before and during the solution exchange (26 episodes from 6 different channels in 4 bilayers). Our method did not allow changing the puffing solution on the same bilayer, or for practical purposes on the same day. The faster rise from channels in the presence of Mg^{2+} is due to the higher $[Ca^{2+}]$ in the puffing solution, which halved the duration of the $[Ca^{2+}]$ rise. Exponential fits to the declining phase of the current responses (not shown) had time constants of 12.3 s ($[Mg^{2+}]$ = 2 mM) and 11.2 s ($[Mg^{2+}]$ = 0).

Ca^{2+} and Mg^{2+} inhibition depended on *cis* $[Cs^+]$ (e.g., for Ca^{2+} see Laver et al., 1995). 2 mM Mg^{2+} in the presence of 500 mM CsCl halved the P_o of skeletal RyRs from 0.32 (above) to 0.14 ± 0.03 (seven channels), whereas 1 mM Mg^{2+} could half-inhibit them in the presence of 250 mM CsCl (six channels). A rapid increase in $[Ca^{2+}]$, in the presence of Mg^{2+} and 500 mM CsCl was achieved by flowing Ca^{2+}-buffered solutions containing Mg^{2+} onto the bilayer with Mg^{2+} also present in the *cis* bath. The initial P_o of rapidly activated skeletal RyRs was also 50% inhibited (to 0.24) by 2 mM Mg^{2+} (Fig. 6, *dashed line* "Ca^{2+} + Mg^{2+}"). The rate of decline of P_o after activation did not change significantly (11.2 s in the absence of Mg^{2+} to 12.3 s in the presence of Mg^{2+}). The τ_d among RyRs, which showed a more than 20% decline in 5 s, was 1.6 ± 0.7 s (three channels). In two experiments, where Mg^{2+} (2 mM) was present only in the puffing solution, initial P_o was also reduced.

Response of RyR gating to rapid falls in *cis* $[Ca^{2+}]$

To further investigate the non-steady-state properties of skeletal RyRs, we measured their gating activity during sudden falls in *cis* $[Ca^{2+}]$ from inhibiting levels (3–10 mM) to subactivating levels (<0.1 μM). The solution exchange model predicts that rapid $[Ca^{2+}]$ plunges of such large magnitudes require both high solution flow rates and high [BAPTA] (5 to 50 mM) in the puffing solution. Bilayers could withstand repeated puffs with pressures of 30 cm H_2O provided that they were brief (~1 s). We found that the response of RyRs to $[Ca^{2+}]$ plunges varied according to the rate of $[Ca^{2+}]$ decrease. Fig. 7 shows representative sweeps from two experiments on skeletal RyRs where $[Ca^{2+}]$ was plunged from mM to sub-μM levels, and the current ensembles are shown in Fig. 8. During a relatively slow $[Ca^{2+}]$ decrease when it took longer than 30 ms to reach subactivating levels, the RyR activity increased during the $[Ca^{2+}]$ plunge (Figs. 7 *A* and 8 *C*). This behavior is in keeping with the steady-state, $[Ca^{2+}]$-dependence of RyR gating where the transient increase in P_o indicates when $[Ca^{2+}]$ is in the range of 10–100 μM. However, skeletal RyRs did not show a transient activation during fast $[Ca^{2+}]$ plunges when subactivating levels were reached in ~2 ms (10 channels, e.g., see Figs. 7 *A*, and 8, *A* and *B*). The $[Ca^{2+}]$-dependence of the same channels near steady-state appeared to be normal because they showed a transient increase in P_o when $[Ca^{2+}]$ rose slowly from subactivating back to inhibiting levels after each plunge (Fig. 8 *A*, cf. *left* and *right traces*). The omission of transient activation seen with skeletal RyRs could not be observed in cardiac RyRs because we were unable to remove the higher $[Ca^{2+}]$ quickly enough (10–40 mM is required to inhibit cardiac RyRs).

DISCUSSION

The time course of the $[Ca^{2+}]$ step during solution exchange

We report a flow or puffing method capable of either activating or deactivating RyR channels in bilayers in <20 ms. The usual activating step for skeletal RyR was 0.1 to 100 or 200 μM $[Ca^{2+}]$. Smaller $[Ca^{2+}]$ steps resulted in slower change of $[Ca^{2+}]$ at the bilayer/channel, whereas $[Ca^{2+}]$ steps >500 μM caused a decline in open probability because of Ca^{2+}-inhibition. After a flow delay, $[Ca^{2+}]$ rose with a 20- to 50-ms time constant to a level that remained steady for the remainder of the 5-s flow period (pCa traces, Fig. 2). This method has the advantage of simplicity and is applicable to changing the concentration of one or more dissolved ions or compounds over a wide range. It is most useful when the critical concentration change (for skeletal RyR from 0.3 to 10 μM Ca^{2+}) is a small fraction of the total concentration jump (here 0.1 to 100 or 200 μM Ca^{2+}). A solution-mixing model predicted $[Ca^{2+}]$ during a puff; the baseline transient marked the onset of solution change.

Sitsapesan et al. (1995a) formed a bilayer across the tip of a pipette and changed solutions by rapidly moving that pipette from one solution stream to another. Solution exchanges within 10 ms were estimated from current induced by changes in the liquid junction potential at the pipette tip in the absence of a bilayer. Schiefer et al. (1995), using 1 μm pipettes, reported a time constant of 0.07 ms using the same method. However, liquid junction potentials may se-

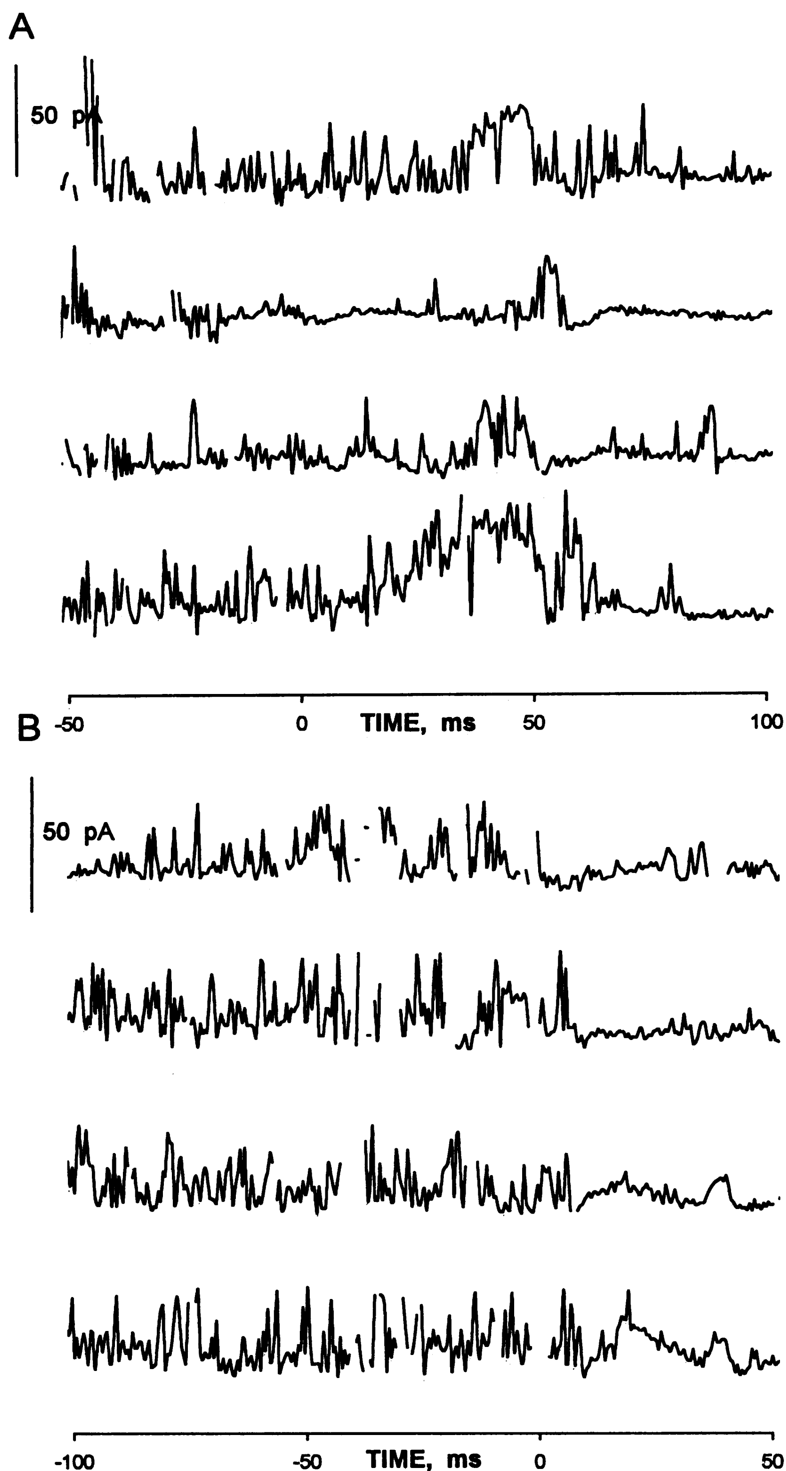

FIGURE 7 Skeletal RyR channel activity during rapid *cis* [Ca^{2+}] plunges from mM to nM levels. Baseline transients were subtracted from the data and large electrical or acoustic clicks were deleted from each record. (*A*) Four out of 11 episodes of a single RyR (D15316) during a [Ca^{2+}] plunge, starting at $t = 0$, from 5 mM to <100 nM in ~40 ms. The *cis* bath contained 500 mM CsCl + 5 mM $CaCl_2$ and the puff solution, under 15 cm H_2O pressure, contained 500 mM CsCl + 5 mM BAPTA. The time course of *cis* [Ca^{2+}] for the experiment is shown as a dashed curve in Fig. 8 *C*. Under these conditions RyR activity transiently increased during the [Ca^{2+}] plunge. (*B*) Four out of seven episodes of a single RyR (D16158), which initially are partially inhibited Ca^{2+}. The *cis* bath contained 3 mM $CaCl_2$ plus 500 mM CsCl. At $t = 0$ the Ca^{2+}-free solution containing 50 mM BAPTA and 500 mM CsCl reached the bilayer (flow pressure = 30 cm H_2O). The time course of the [Ca^{2+}] plunge is shown in Fig. 8 *B* and [Ca^{2+}] was predicted to reach less than 100 nM in ~2 ms. Under these conditions no transient opening of RyR could be detected during the [Ca^{2+}] plunge.

riously underestimate the rate of solution exchange at the bilayer surface. Unstirred regions adjacent to the bilayer limit the half-time for solution exchange to ~400 ms under conditions of extreme conventional stirring and to ~40 ms when solutions are squirted directly at the membrane surface (Barry and Diamond, 1981). Sitsapesan et al. (1995b) in their study do not report time constants for RyR activation but Scheifer et al. (1995) quoted an activation time constant, τ_a, of 2.4 ms. The fine pipettes used by Scheifer et al. (1995) are likely to have had relatively thin unstirred layers, which would have allowed faster [Ca^{2+}] steps at the channel than could be obtained in our study or that of Sitsapesan et al. (1995a,b).

Fast [Ca^{2+}] rise times, of the order of 1 ms or less, have been accomplished with flash induced release of caged Ca^{2+} where the rise times are not limited by effects of

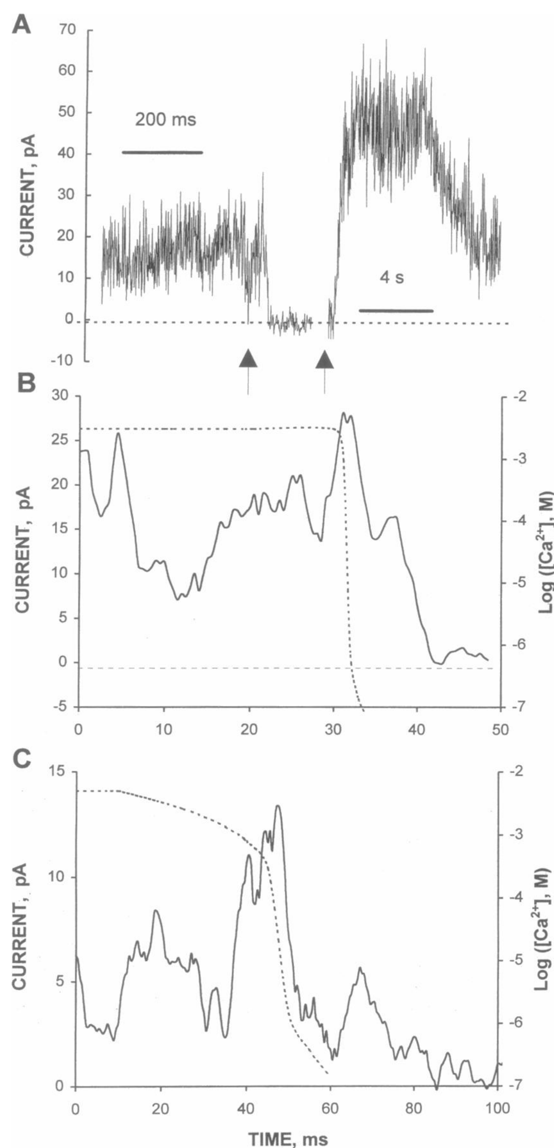

FIGURE 8 Ensemble averages of skeletal RyR channel activity during rapid *cis* [Ca^{2+}] plunges from mM to nM levels. (*A*) D16207; an ensemble average of six episodes of several RyR that initially are partially inhibited Ca^{2+} (*left trace*). The *cis* bath contained 3 mM $CaCl_2$ + 500 mM CsCl. The left arrow indicates when the flow valve released Ca^{2+}-free solution containing 50 mM BAPTA and 500 mM CsCl onto the bilayer under a pressure of 30 cm H_2O. Right trace: 10–20 s after the flow was shut off RyR activity began to appear (*right arrow*). As [Ca^{2+}] near the bilayer slowly returned to 3 mM (note the longer time scale), RyR currents reached a maximum and then declined as the higher [Ca^{2+}] inhibited the channels. (*B*) Experiment D16207, shown in *A*, shown on an expanded time scale (*solid line*). Solution exchange began at t = 30 ms and channel activity ceased within 10 ms. During this time RyR activity showed no significant increase like that seen during the slow recovery (*A, right trace*). Dashed curve: The time course of *cis* [Ca^{2+}] during the experiments calculated using the solution exchange model (Eq. 1). (*C*) An ensemble average of 11 episodes of a single RyR (D15316) during a [Ca^{2+}] plunge that was slower than that for experiment D16207 (see *B*). The *cis* bath contained 500 mM CsCl + 5 mM $CaCl_2$ and the puff solution, under 15 cm H_2O pressure, contained 500 mM CsCl + 5 mM BAPTA. Dashed curve: The time course of cis[Ca^{2+}] for the experiment. The slower rate of [Ca^{2+}] decrease under these conditions allowed the RyR activity to transiently increase before channel deactivation set in at low [Ca^{2+}] increased during the [Ca^{2+}] plunge.

unstirred layers (Gyorke et al., 1994; Valdivia et al., 1995). However, with flash photolysis [Ca^{2+}] changes are restricted to steps (or transients) in the μM range and [Ca^{2+}] steps begin with a ~1-ms spike followed by a slowly declining plateau (Escobar, Cifuentes and Vergara, 1995; Lamb and Stephenson, 1995).

[Ca^{2+}]-activation of RyRs

Gyorke et al. (1994) activated skeletal RyRs to an initial P_o of 0.95 by increasing [Ca^{2+}] to a nominal 1 μM with flash photolysis. Ensemble averages of their channel activity revealed a transient activation of RyR channel current (τ_a = 1.3 ms) in response to spike/step (see above) changes in [Ca^{2+}]. We report the first results of fast, flow-induced solution changes applied to the skeletal isoform. Skeletal RyRs were activated (τ_a ~ 30 ms) to a peak P_o of 0.45 in response to a [Ca^{2+}] step rising to 100 μM with a 50-ms time constant.

Gyorke et al. (1994) reported initial P_o of 0.9–1.0 in cardiac RyRs. Valdivia et al. (1995) reported a similar rapid activation (τ_a = 1.4 ms) of cardiac RyRs to an initial P_o of 0.6. Both groups report initial P_o increases with increasing rate of [Ca^{2+}] change. Sitsapesan et al. (1995b) reported Ca^{2+}-activation by fast, flow-induced changes in Ca^{2+}, but to a lower P_o (0.2), which was also the steady-state value. When they added ATP and EMD-41000, P_o was as high as 0.84. In spite of considerable differences in the [Ca^{2+}] transients generated by our flow method and flash photolysis, RyRs could reach an initial P_o (0.67) considerably greater than the steady-state P_o (0.34, Figs. 1 and 2).

Declining RyR activity following activation

Gyorke et al. (1994) report decline (we use the word to describe the data, rather than to imply mechanism) of skeletal RyR activity with τ_d = 3.4 s. Following rapid activation from 0.1 μM to 100 μM [Ca^{2+}] ensemble activity of skeletal RyRs declined (P_o decline) with τ_d between .5 and 15 s. A grand ensemble of all our experiments (Fig. 6) clearly showed a decline in P_o with τ_d = 12 s, even though faster rates of decline were observed for the individual ensembles. Because our method produces constant [Ca^{2+}] after the rapid rising phase, decline in skeletal RyR activity cannot be related to changing [Ca^{2+}], but is a basic channel property.

Table 1 summarizes the wide variety of RyR preparations, solutions, and holding voltages used by our lab and others. Studies using the flash photolysis method report P_o decline of cardiac RyR activity at holding potentials *(cis* relative to *trans)* of +40 mV (Valdivia et al., 1995; personal communication) and +30 mV (Gyorke et al., 1994; and personal communication). Gyorke and Fill (1993, Fig. 3) observed that after cardiac RyR channels activated by 0.2 μM Ca^{2+} had closed, they could be reactivated by a [Ca^{2+}] step from 0.2 to 0.5 μM Ca^{2+}. On the basis of this behavior,

Gyorke and Fill (1993) describe the declining phase of P_o as adaptation, as opposed to Ca^{2+}-dependent inactivation. Lamb and Stephenson (1995) suggested that the P_o decline might reflect the rate at which RyRs respond after being activated by the $[Ca^{2+}]$ spike. The decisive test for this mechanism would be to see if flow-induced $[Ca^{2+}]$ spikes produce adaptation-like responses in RyRs (this would be technically very difficult). However, our finding that RyRs close very rapidly (<10 ms, Fig. 8 *A*) when Ca^{2+} is removed places restraints on Lamb and Stephenson's (1995) suggestion.

Although our method has the disadvantage of a slow rise, we can be confident that $[Ca^{2+}]$ remains constant for the remainder of the puff. The variability in the rate and magnitude of the P_o declines among RyRs appears to be a property of RyRs rather than attributable to unavoidable variations in the $[Ca^{2+}]$ time course (see Methods). Variability in the stationary kinetics of RyRs is well documented (e.g., see citations in Laver et al., 1995). Moreover, we find that P_o decline following rapid activation by $[Ca^{2+}]$ steps is closely correlated with P_o decline following much more reproducible voltage steps (data not shown).

Schiefer et al. (1995) report declines at +50 mV holding potentials; $\tau_d \sim 1$ s at 1 mM Ca^{2+} and $\tau_d = 0.2$ s at 3 μM Ca^{2+}. Sitsapesan et al. (1995b) find P_o decline at +40 mV using ATP and EMD 41000 to achieve a P_o of 0.8. Cardiac RyRs, activated by Ca^{2+} alone to an initial P_o of 0.2, did not show P_o decline at −40 mV but occasionally did at +40 mV. In addition, their P_o decline at +40 mV was reversed by voltage steps to −40 mV but could not be reversed by repeated $[Ca^{2+}]$ steps from 0.1 to 100 μM. Sitsapesan et al. (1995b) attributed this P_o decline to an inactivation mechanism. The application of their findings to understanding the response of RyRs to flash-induced $[Ca^{2+}]$ changes was complicated by substantial differences in the steady-state Ca^{2+}-dependent P_o of cardiac RyRs from their sheep preparations and the dog preparations used in the flash photolysis studies. However, three lines of evidence indicate that non-steady-state RyR properties reported here and by Sitsapesan et al. (1995b) and Schiefer et al. (1995) are general properties of cardiac RyRs and are not restricted to RyRs from a particular species or tissue. 1) In our hands, the steady-state Ca^{2+}-activation properties of sheep cardiac RyRs were quite similar to those of canine RyRs (cf. Laver et al., 1995; and Chu et al., 1993) and yet we report similar non-steady-state findings to Sitsapesan et al. (1995b). 2) These results are similar to those obtained from canine cardiac RyRs (Schiefer et al., 1995). 3) The similar P_o declines observed in rabbit skeletal RyRs and sheep cardiac RyRs indicate that decline in RyR activity is not tissue specific.

As yet there appears to be no consensus on the nature of the mechanism (or mechanisms) underlying P_o decline seen after $[Ca^{2+}]$ steps using the flash and flow methods. Given the appearance of a P_o decline under some conditions in all RyR preparations (Table 1), it would be tempting to propose a common mechanism for the effect seen using the flash and flow methods. However, such a proposal would be premature as there is very little overlap in the measurements made using these techniques. Studies using flow-induced $[Ca^{2+}]$ steps measure the effects of membrane potential, but they do not repeat the double $[Ca^{2+}]$ step near threshold protocol used in the flash photolysis studies and vice versa. Because it has been established that flash-induced $[Ca^{2+}]$ steps have a large leading-edge spike (Escobar et al., 1995), whereas flow-induced $[Ca^{2+}]$ steps have a gradual, asymptotic, leading edge (e.g., Fig. 2), we cannot exclude the possibility that the different RyR responses obtained from flow- and flash-methods are due to differences in the $[Ca^{2+}]$ time course (Lamb and Stephenson, 1995).

The rates of P_o decline observed here and by others (see Table 1) are too slow for the declining phase of RyR activity to act as a regulatory mechanism during E-C coupling. The observation of a slow postactivation decline in SR calcium release in cardiac muscle ($\tau_d = 1.1$ s, Fabiato, 1985) suggests that P_o declines on the second time scale also occur in vivo. It is more likely that the initial, peak response of RyRs to rapid activation is important in E-C coupling.

Effect of Mg^{2+} on Ca^{2+}-activated skeletal RyRs

In cardiac RyRs, Valdivia et al. (1995) reported that 1 mM Mg^{2+} reduced the response to 1 μM Ca^{2+}, both in the steady state and in response to rapid activation (<1 ms). Decline (τ_d) became faster; Mg^{2+} inhibition increased with time. Magnesium's effect on decline was considerably greater than its effect on initial P_o. Using skeletal RyRs in bilayers, we report 1–2 mM Mg^{2+} reduced both steady-state P_o (0.32 to 0.14) and initial P_o (0.45 to 0.24, Fig. 6) in response to rapid Ca activation by the same 50% as in cardiac RyR. Rapid changes in Ca^{2+} did not alter the rate of decline; Mg^{2+} inhibition did not increase with time in skeletal RyR as reported in cardiac RyR. The different Mg^{2+} effects observed here and by Valdivia et al. (1995) indicate that different mechanisms underlie the P_o declines in the two studies. The different mechanisms may reflect either the different E-C coupling mechanisms operating in skeletal and cardiac muscle or the different Ca^{2+} time courses applied to the RyR using the flash and flow methods (see above).

Our observations of individual episodes comprising the current ensembles indicate that the Mg^{2+}-inhibition is mainly attributable to a decrease in P_o, rather than to changes in either the number of active channels or their conductance. Our observation that Mg^{2+} reduced P_o throughout the $[Ca^{2+}]$ step indicates that the RyRs' Mg^{2+}-sensitive gates were predominantly closed even before the RyRs were activated by Ca^{2+}, and/or the RyRs were very rapidly inhibited by Mg^{2+} after Ca^{2+}-activation. We can't rule out the latter because applying Mg^{2+} along with elevated Ca^{2+} had the same effect as when Mg^{2+} was present before and during the puff.

Mg^{2+} inhibits steady state (ΔCa^{2+}>1 s) Ca^{2+} release from skeletal SR (Meissner et l., 1986) and a variety of

TABLE 1 Sensitivity of RyRs to rapid [Ca^{2+}] steps

Laboratory	Gyorke	Valdivia	Sitsapesan		Schiefer	Laver
Anion	$MeSO_3^-$	$MeSO_3^-$	$PIPES^{2-}$		Cl^-	Cl^-
Cation (*trans/cis*)	Cs^+	Cs^+	Cs^+		K^+	Cs^+
Voltage (*cis* relative to *trans*)	+30	+40	−40	+40	+50	+40
CARDIAC	Canine	Canine	Sheep		Canine	Sheep
Initial P_o	0.9	0.6	0.2	0.8*	0.5–0.8	0.7
Declines	Yes	Yes	No	Yes	Yes	Yes
SKELETAL	Rabbit		*(+ ATP & EMD)			Rabbit
Initial P_o	0.9					0.5
Declines	Yes, τ_d = 3.4 s					Yes, $\tau_d = 2 \pm 1$ s#

#The mean is from experiments where P_0 decline exceeded 20% during a 5-s puff.

similar preparations to such an extent that Lamb and Stephenson (1994) concluded CICR could not be the major cause of Ca^{2+} release in skeletal E-C coupling. 1 mM Mg^{2+} reduced depolarization induced Ca^{2+} release in cut frog fibers by 30% (Jacquemond and Schneider, 1992) and in isolated triads by 50% (Ritucci and Corbett, 1995). Even though Mg^{2+}-inhibition would certainly reduce the sensitivity of any CICR coupling system, Valdivia et al.'s (1995) results in cardiac muscle suggest that when [Ca^{2+}] changes very rapidly, CICR can still be a major E-C coupling system. Our rapid changes in Ca^{2+} increased P_o of skeletal RyRs in 1 or 2 mM Mg^{2+} to a similar extent and hence do not preclude a role for CICR in skeletal muscle E-C coupling. Both the decline in channel activity at constant Ca^{2+} and the Mg^{2+} inhibition may well play a role in reducing positive feedback in this system (Rios and Pizzaro, 1991).

Ca^{2+} activation and Ca^{2+} inhibition mechanisms gate independently

Studies of Ca^{2+} regulation of skeletal RyRs under steady-state conditions (e.g., Chu et al., 1993; Laver et al., 1995) show that in mM Ca^{2+} they are inhibited because a [Ca^{2+}]-sensitive gate closes, which binds Ca^{2+} with an affinity of 700 μM and a Hill coefficient of ~2. At sub-μM [Ca^{2+}], RyRs are also inactive because a different gate requires Ca^{2+} to open and binds Ca^{2+} with an affinity of 1 μM. The Ca^{2+} sensitivities of these gates are sufficiently different to allow both activation and inhibition gates to be open at sustained [Ca^{2+}] levels in the range of 10–100 μM. The gating mechanisms appear to be associated with different parts of the RyR as their sensitivities to Ca^{2+} in many studies were seen to be modified independently (e.g., Mickelson et al., 1990; Boraso and Williams, 1994; Mack et al., 1994; Laver et al., 1995).

When [Ca^{2+}] is gradually reduced from levels that close the inhibition gate to levels that close the activation gate, RyRs are observed to transiently open when intermediate [Ca^{2+}] is sufficient to open both gates. However, when [Ca^{2+}] falls quickly so that it traverses the intermediate [Ca^{2+}] range within 2 ms, RyRs fail to open (Figs. 7 and 8). This can be understood in terms of a gating model in which the Ca^{2+} activation and inhibition gates operate in series and independently. By "independent gating" we mean that one gate can be either open or closed irrespective of the conformation of the other gate. This does not imply that Ca^{2+} binds independently at sites associated with these gates. If the inhibition gate opens in response to a [Ca^{2+}] decrease more slowly than the activation gate closes, then the channel will remain occluded. An ideal test for this model is to measure the response of each gate to [Ca^{2+}] steps. It is clear from Fig. 8 that the activation gate shuts within a few ms in response to a [Ca^{2+}] plunge, but the opening rate of the inhibition gate is unknown because it is occluded by the activation gate before it opens. Ideally we could measure the opening rate of the inhibition gate in response to [Ca^{2+}] steps from inhibiting levels (2–5 mM) to optimal levels (10–100 μM) so that the activation gate remains open. Unfortunately, using the flow method, we were unable to achieve [Ca^{2+}] transitions between these levels more quickly than ~100 ms, which is likely to be an order of magnitude slower than the opening rate of the inhibition gate.

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