Inactivation of Ca^{2+} Release Channels (Ryanodine Receptors RyR1 and RyR2) with Rapid Steps in $[Ca^{2+}]$ and Voltage

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ABSTRACT The transient responses of sheep cardiac and rabbit skeletal ryanodine receptors (RyRs) to step changes in membrane potential and cytosolic $[Ca^{2+}]$ were measured. Both cardiac and skeletal RyRs have two voltage-dependent inactivation processes ($\tau \approx$ 1–3 s at +40 mV) that operate at opposite voltage extremes. Approximately one-half to two-thirds of RyRs inactivated when the bilayer voltage was stepped either way between positive and negative values. Inactivation was not detected (within 30 s) in RyRs with P_o less than 0.2. Inactivation rates increased with intraburst open probability (P_o) and in proportion to the probability of a long-lived, RyR open state (P_{OL}). RyR inactivation depended on P_{OL} and not on the particular activator (Ca^{2+} (μM), ATP, caffeine, and ryanodine), inhibitor (mM Ca^{2+} and Mg^{2+}), or gating mode. The activity of one-half to two-thirds of RyRs declined (i.e., the RyRs inactivated) after $[Ca^{2+}]$ steps from subactivating (0.1 μM) to activating (1–100 μM) levels. This was due to the same inactivation mechanism responsible for inactivation after voltage steps. Both forms of inactivation had the same kinetics and similar dependencies on P_o and voltage. Moreover, RyRs that failed to inactivate after voltage steps also did not inactivate after $[Ca^{2+}]$ steps. The inactivating response to $[Ca^{2+}]$ steps (0.1–1 μM) was not RyRs "adapting" to steady $[Ca^{2+}]$ after the step, because a subsequent step from 1 to 100 μM failed to reactivate RyRs.

INTRODUCTION

In striated muscle, intracellular Ca^{2+} concentration, and hence force production, is regulated by the release and subsequent reuptake of Ca^{2+} by the sarcoplasmic reticulum (SR), with the ryanodine receptor (RyR) forming the major calcium release channel. In a cardiac muscle cell, depolarization opens L-type calcium channels (dihydropyridine receptors, DHPRs) in the surface membrane, and the inflowing Ca^{2+} activates nearby RyRs by the so-called Ca^{2+}-induced Ca^{2+} release (CICR) mechanism. In vertebrate skeletal muscle, Ca^{2+} inflow is not required for Ca^{2+} release, as the DHPRs in some way directly activate adjacent RyRs (Melzer et al., 1995), and the importance of CICR is uncertain (Endo, 1985; Lamb and Stephenson, 1990; Schneider, 1994; Pape et al., 1995).

In the absence of Mg^{2+}, both cardiac and skeletal RyRs (RyR1 and RyR2, respectively) can be activated by μM Ca^{2+}. Where CICR operates, it would seem that there must also be some negative control mechanism present that prevents Ca^{2+} release from being continuously self-perpetuating. In both cardiac and skeletal muscle, Ca^{2+} inactivation has been reported, in which cytoplasmic Ca^{2+} apparently decreases further Ca^{2+} release by binding to a high-affinity site (Fabiato, 1985; Simon et al., 1991) or, alternatively, to a low-affinity site close to the Ca^{2+} efflux pathway (Jong et al., 1995). It is commonly thought that such Ca^{2+} inactivation could be mediated by the Ca^{2+} inactivation mechanism observed in SR vesicles and isolated RyRs at high myoplasmic $[Ca^{2+}]$. However, this is evidently not the case, because the latter process has an almost equal affinity for Ca^{2+} and Mg^{2+} (Soler et al., 1992; Meissner et al., 1986, 1997; Laver et al., 1997a,b), so that even a very high local rise in $[Ca^{2+}]$, in the presence of physiological $[Mg^{2+}]$, will only cause a relatively small decrease in channel activity via this mechanism. In view of the low specificity of this type of inhibition, it is referred to as "Ca^{2+}/Mg^{2+} inhibition" (see Lamb, 1993).

Studies on isolated RyRs have also described a number of other processes that cause a decline in RyR activity. First, three apparently distinct, voltage-dependent inactivation phenomena have been reported when RyRs are subject to step changes in bilayer potential:

1. Inactivation has been observed at positive potentials in chicken skeletal RyRs (Percival et al., 1994), sheep cardiac RyRs (Laver et al., 1995), and recombinant skeletal RyRs expressed in HEK cells (Imredy et al., 1996; Chen et al., 1997). The RyRs inactivated within seconds by entering long-lived closed states, and could be reactivated in milliseconds by voltage steps to negative potentials. Increasing the bilayer potential increased the RyR inactivation rate.

2. Another, much faster (10–100×) inactivation at positive potentials has been identified in 20% of rabbit skeletal RyRs (Ma et al., 1995). This type of inactivation could be removed by the presence of perchlorate ions and restored by exogenous FK-506 binding protein (FKBP).

3. Inactivation at negative potentials has also been reported by Ma (1995) in rabbit skeletal RyRs, where half the

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RyRs inactivated to 55% of their initial activity at −100 mV with a mean time constant $\tau = 3.9$ s.

Furthermore, when isolated RyRs are subjected to rapid rises in cytosolic [Ca^{2+}] from subactivating levels, they activate and then, under some circumstances, show a marked decrease in activity (Györke and Fill, 1993; Valdivia et al., 1995; Sitsapesan et al., 1995; Schiefer et al., 1995; Laver and Curtis, 1996a,b). However, these studies have to be subdivided into two distinct groups because of the large differences in both the actual stimuli used and the conclusions drawn. Using flash photolysis of caged Ca^{2+}, Györke and Fill (1993) and Valdivia et al. (1995) reported that "step" rises in [Ca^{2+}] rapidly activated RyRs ($\tau = 1.3$ ms), and then activity slowly declined to a lower steady-state level ($\tau = 3.4$ s). Importantly, Györke and Fill (1993) found that when the activity had declined after a [Ca^{2+}] step from 0.1 to 0.2 μM, the RyR could be immediately reactivated by a second [Ca^{2+}] step from 0.2 to 0.5 μM. On the basis of this behavior, they described the declining phase of RyR activity as "adaptation," to distinguish it from "inactivation," in which the RyR remains unresponsive to further stimulation. However, it is now generally recognized that such flash photolysis of caged Ca^{2+} produces a large leading-edge spike at the start of the [Ca^{2+}] step. In view of this, it has been argued that RyRs in such experiments are not adapting to a steady [Ca^{2+}] stimulus, as recently reiterated by Velez et al. (1997), but rather they are deactivating (i.e., closing after withdrawal of the activating stimulus) in a manner peculiar to the spike stimulus (Lamb and Stephenson, 1995; Lamb, 1997).

When the RyRs are instead stimulated by [Ca^{2+}] steps produced by solution change methods, they rapidly activate ($\tau = 2.4$ ms at 1 μM [Ca^{2+}]; Schiefer et al., 1995) and, under some conditions, show a decline in activity with τ on the order of 1 s. Sitsapesan et al. (1995) and Schiefer et al. (1995) attributed this declining phase to an inactivation mechanism, and no evidence for adaptation was found. Using sheep cardiac RyRs, Sitsapesan et al. (1995) observed inactivation mainly in channels that were activated to a high open probability ($P_o > 0.75$ using Ca^{2+} plus another channel activator, i.e., ATP or EMD41000) rather than in RyRs activated to lower P_o (~0.2) by Ca^{2+} alone. The sheep cardiac RyRs studied by Laver and Curtis (1996b) could be activated to high P_o in the presence of Ca^{2+} alone, and inactivation was seen much more frequently than under the same conditions in the study of Sitsapesan and colleages. Sitsapesan et al. (1995) and Schiefer et al. (1995) obtained evidence for a refractory period after inactivation (adaptation effects should lack a refractory period). They showed this by demonstrating that repeatedly lowering and reapplying Ca^{2+} failed to reactivate channels that had inactivated. Sitsapesan et al. (1995) found that inactivation occurred at +40 mV but not at −40 mV, and suggested that inactivation may be related to a voltage-dependent inactivation mechanism. Schiefer et al. (1995), on the other hand, found that RyRs inactivated at −50 mV (their study states +50 mV, by the reverse convention). None of the above-mentioned studies, however, used flow methods to perform the double [Ca^{2+}] step protocol used in the flash photolysis studies (Györke and Fill, 1993) to support the adaptation hypothesis.

This study investigates the mechanisms of declining activity of rabbit skeletal and sheep cardiac RyRs after steps in both voltage and [Ca^{2+}]. The response of RyRs to flow-induced [Ca^{2+}] steps confirms the findings of the two other laboratories that have used similar methods to produce steps in [Ca^{2+}]: RyRs inactivate in response to [Ca^{2+}] steps. Furthermore, the double [Ca^{2+}] step protocol, similar to that used in the flash photolysis studies, is carried out here for the first time, using the flow technique, and shows no evidence for an adaptation process in RyRs. This study also shows that individual RyRs inactivate after voltage steps to positive and negative values, and that the same inactivation mechanism underlies RyR responses to steps in either voltage or [Ca^{2+}]. Several novel properties of this mechanism are elucidated, and the possible relevance of inactivation to SR Ca^{2+} release is considered.

MATERIALS AND 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 palmitoyloleoylphosphatidylethanolamine (PE), palmitoyloleoylphosphatidylserine (PS), and palmitoyloleoylphosphatidylcholine (PC) (5:3:2 by weight) in *n*-decane, using the film drainage technique of Mueller et al. (1962). Incorporation of ion channels with the bilayers was achieved by fusion with SR vesicles, as described by Miller and Racker (1976). SR vesicles were added to the *cis* bath, and the cytoplasmic side of the SR membrane faced the *cis* solution when fused with the bilayer.

In all bathing solutions, 10 mM *N*-tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) was used to buffer pH. 1,2-Bis(2-aminophenoxy)ethane-*N,N,N,N*-tetraacetic acid (BAPTA) (2 mM) was used to buffer [Ca^{2+}] to 0.1 μM, and dibromo-BAPTA (2mM) was used to buffer [Ca^{2+}] between 1 and 10 μM. Solutions were adjusted to pH 7.4 with CsOH, and to a range of [Ca^{2+}] with $CaCl_2$. Free [Ca^{2+}] was measured with an ion meter. The luminal (*trans*) bath usually contained (in mM) either 50 or 250 CsCl plus 0.1 $CaCl_2$. The myoplasmic (*cis*) bath usually contained either 250 or 500 CsCl and variable free [Ca^{2+}].

Details of the methods for achieving and evaluating rapid solution exchange are given by Laver and Curtis (1996a). Briefly, single rapid-step changes in [Ca^{2+}] at the *cis* face of the bilayer were achieved by puffing solutions from a beveled tube located above and within 50 μm of the *cis* face of the bilayer. Before each series of [Ca^{2+}] steps, the *cis* chamber was perfused with solution buffered to 0.1 μM Ca^{2+}. Each [Ca^{2+}] step was produced by squirting solutions containing [Ca^{2+}] in the range 1–200 μM for periods of 5–10 s. Care was taken that during these experiments accumulation of the puffing solution in the *cis* bath did not significantly alter the bath composition. To avoid Ca^{2+} contamination, the myoplasmic chamber was gradually perfused with fresh solution, usually after ten 5-s puffs. At least 95% exchange of *cis* or *trans* solutions was achieved by gradually perfusing the bath with at least four volumes (6 ml) of [Ca^{2+}]-buffered solution. This was done by using a back-to-back syringe system configured such that bath perfusion and waste withdrawal could be maintained at equal rates.

A two-step [Ca^{2+}] staircase was applied to RyRs by funneling solutions from each of three tubes, in turn, into a single tube near the bilayer. The "dead space" in this perfusion system meant that solution exchange times (100–500 ms) were considerably slower that those obtained using the single-step method.

Slow changes in the concentrations of *cis* Ca^{2+}, Mg^{2+}, AMP-PNP (a nonhydrolyzable ATP analog), ryanodine, and caffeine were made by adding stock solutions to the bath. In experiments in which rapid solution exchange was not used, a local perfusion technique could be used to change ligand concentrations near the bilayer. For the duration of each measurement (lasting 30 s to several minutes), the solution at the *cis* face of the bilayer was exchanged with any one of 16 available recording solutions by flowing solutions from a vinyl tube directly onto the bilayer surface. This method evolved from the fast perfusion technique that is used to produce [Ca^{2+}] steps; it is described more fully by Laver et al. (1997a).

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. During the experiments, the bilayer current was recorded at a bandwidth of 5 kHz on videotape. 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 Prof. P. W. Gage and Mr. M. M. Smith).

Open probability within bursts was calculated from the time-averaged current divided by the unitary current and the number of channels. Bursts were defined as periods of activity separated by gaps lasting longer than 1–3 s. Open and closed dwell-time distributions were obtained from single-channel recordings with steady baseline and apparently stationary gating kinetics (i.e., no gating mode changes were apparent). Details of the methods used to determine dwell times are given by Laver et al. (1997a). Unless otherwise stated, the scatter in the data is given by the standard deviation (SD).

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 (Laver and Curtis, 1996a). Individual episodes were separated by 15-s intervals of stirring to allow the *cis* bath to equilibrate near the bilayer surface. The bilayer survival time was usually limited in experiments to ~10 episodes, which was sufficient to reveal the broader features of the ensemble RyR activity. Episodes from the recordings were edited in synchrony with switching of the solution flow, and averaged to produce "ensemble" RyR responses to rapid solution changes. Ensembles of RyR responses to voltage steps were compiled from episodes lasting up to 30 s. These episodes were edited from recordings in synchrony with the voltage steps.

RESULTS

Steady-state properties of cardiac and skeletal RyRs

RyRs were activated by μM Ca^{2+} and inhibited by mM Ca^{2+} and Mg^{2+}. Both cardiac and skeletal RyRs were half-maximally activated by ~1 μM cytoplasmic Ca^{2+}, and their threshold for steady-state activation in Cl^- containing solutions was ~0.3 μM Ca^{2+}. Skeletal RyRs were maximally activated by 10–200 μM Ca^{2+} and cardiac RyRs by 10–1000 μM Ca^{2+}. At higher concentrations Ca^{2+} inhibited skeletal and cardiac RyRs by Ca^{2+}/Mg^{2+} inhibition (see Introduction). The half-inhibiting [Ca^{2+}] (K_I) depended on the CsCl concentration and varied considerably between individual channels, as described previously by Laver et al. (1995, 1997b). The K_I values for skeletal RyRs, in the presence of 250 mM CsCl, had a mean of 700 μM, and ~60% of individual channels had K_I values between 400 μM and 1000 μM. Cardiac RyRs were less sensitive to Ca^{2+} inhibition, having a mean K_I of 15 mM, with K_I values in ~60% of channels lying between 5 mM and 20 mM. Mg^{2+} and Ca^{2+} in the *cis* bath had effectively identical inhibiting effects on RyRs, provided that [Ca^{2+}] exceeded 10^{-3} times the K_I for Ca^{2+} inhibition (see Laver et al., 1997a,b, for details of Mg^{2+} inhibition).

The gating kinetics of cardiac and skeletal RyRs depend on the bilayer potential. The open probability of RyRs within bursts was larger at positive voltages (e.g., Fig. 1 B). However, the overall open probability of RyRs at positive potentials was lower than at negative potential, because of a lower burst probability.

Response of RyRs to steps in membrane potential

The bursting activity of both cardiac and skeletal RyRs commonly showed a marked decline and occasionally ceased completely after the bilayer potential was stepped to either positive or negative values larger than ~40 mV. Reversing the polarity of the membrane potential would

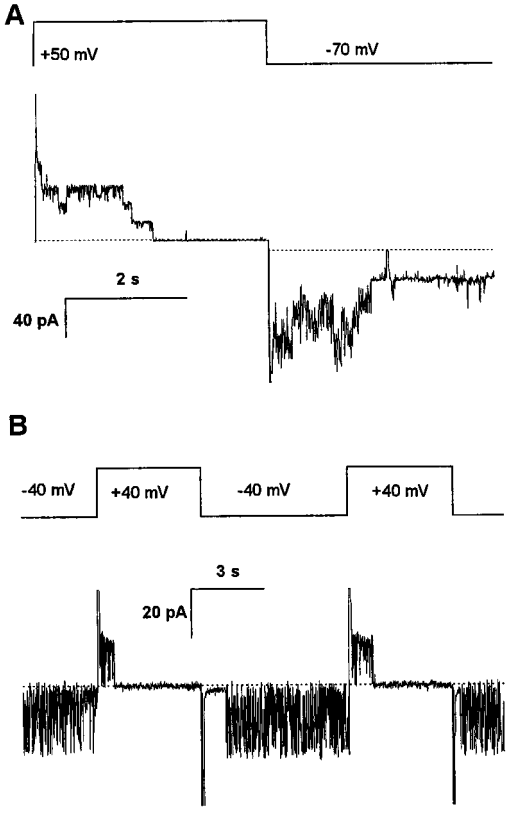


FIGURE 1 The response of RyRs to voltage steps in the presence of activating cytosolic Ca^{2+}. (*A*) A bilayer with four cardiac RyRs (*bottom trace*) undergoing voltage steps between +50 mV and −70 mV (*top trace*). Both *cis* and *trans* baths contained 250 mM CsCl. The *cis* bath also contained 1 mM Ca^{2+} and 500 mM mannitol. No Ca^{2+} was added to the *trans* bath. The dashed line in the current record shows the baseline level. The RyRs were transiently activated by voltage steps in either direction. The large capacitance-induced current spikes are associated with the voltage steps. (*B*) A single skeletal RyR activating and inactivating in response to a series of voltage steps between +40 mV and −40 mV. The *cis* bath contained 250 mM CsCl plus 1 mM Ca^{2+}, and the *trans* bath contained 450 mM CsCl and 0.1 mM Ca^{2+}.

rapidly activate RyRs and then inactivate them within several seconds (Fig. 1 *A*). The voltage step produced an initial burst of RyR activity, followed by long-lived closed periods separating occasional bursts of activity. RyR activation was usually fast, occurring within 50 ms of the voltage step. The capacitance current spike induced by voltage steps obscured RyR currents for ~50 ms, so that the channel activation rate usually could not be measured. However, in a few instances activation rates could be measured from slowly activating RyRs, such as that shown in Fig. 1 *B* at negative potentials.

The channel inactivation rate (τ_i^{-1}) at positive potentials depended on the intraburst open probability, P_o (Figs. 2 and 3), and the bilayer potential (Fig. 4) and showed considerable variability between channels. Neither skeletal nor cardiac RyRs with P_o values less than 0.2 ever showed inactivation. Of the channels with higher P_o, 56% of cardiac RyRs (33 of 59) and 80% of skeletal RyRs (12 of 15) showed inactivation within 30 s of voltage steps. RyR inactivation rates were slowed by any treatment that reduced P_o, whether it was via Ca^{2+} deactivation (i.e., dropping cytosolic [Ca^{2+}] to subactivating levels) or Ca^{2+}/Mg^{2+} inhibition (mM cytosolic Ca^{2+} and/or Mg^{2+}), or it happened spontaneously as a result of gating mode changes. Examples of these effects in an individual cardiac RyR are shown in Fig. 2, and the results from nine experiments are summarized in Fig. 3. It can be seen in Fig. 3 that there was also considerable variation in the inactivation rate between individual RyRs with P_o values in excess of 0.4.

In several experiments it was clear that the inactivation rate showed a supralinear dependence on P_o (Fig. 3 *A*, Experiments 4, 7, and 9), rather than the direct proportional relationship that would be expected if inactivation were dependent simply on the RyRs being open. Given that RyRs have several open states, such a supralinear dependence may arise because RyRs do not inactivate equally from each open state. To investigate this possibility, the inactivation rates were plotted against the probabilities of different types of channel opening. Dwell-time histograms of channel openings had up to three exponential components (not shown), indicating the presence of at least three different open states. The probability of a channel being in any of these open states was estimated from the parameters derived from triple-exponential fits to the open and closed dwell-time histograms. Fig. 3 *B* shows the inactivation rates versus the probability of RyRs being in their longest open state (OL; $\tau \approx 3$–8 ms for skeletal RyRs, and $\tau = 1$–10 ms for cardiac RyRs). The inactivation rates within each experiment showed a nearly linear dependence on P_{OL}. The data from all of the experiments in which dwell-time histograms could be obtained (i.e., 1, 4–7, and 9) were more tightly correlated to P_{OL} ($r^2 = 0.93$) than to P_o ($r^2 = 0.70$).

RyR inactivation was strongly voltage dependent, proceeding faster as the bilayer voltage was stepped to more extreme values. For a given magnitude of bilayer voltage, the average inactivation rate was slightly faster at positive values than at negative values (e.g., see Fig. 1 *B*). During experiments, inactivation usually became noticeable when voltages were stepped beyond +40 mV or −60 mV. Fig. 4 shows the voltage dependencies of cardiac RyR inactivation rates (log scale) at opposite polarities. The magnitudes of the slopes of these plots were not significantly different from each other, and when interpreted in terms of charged

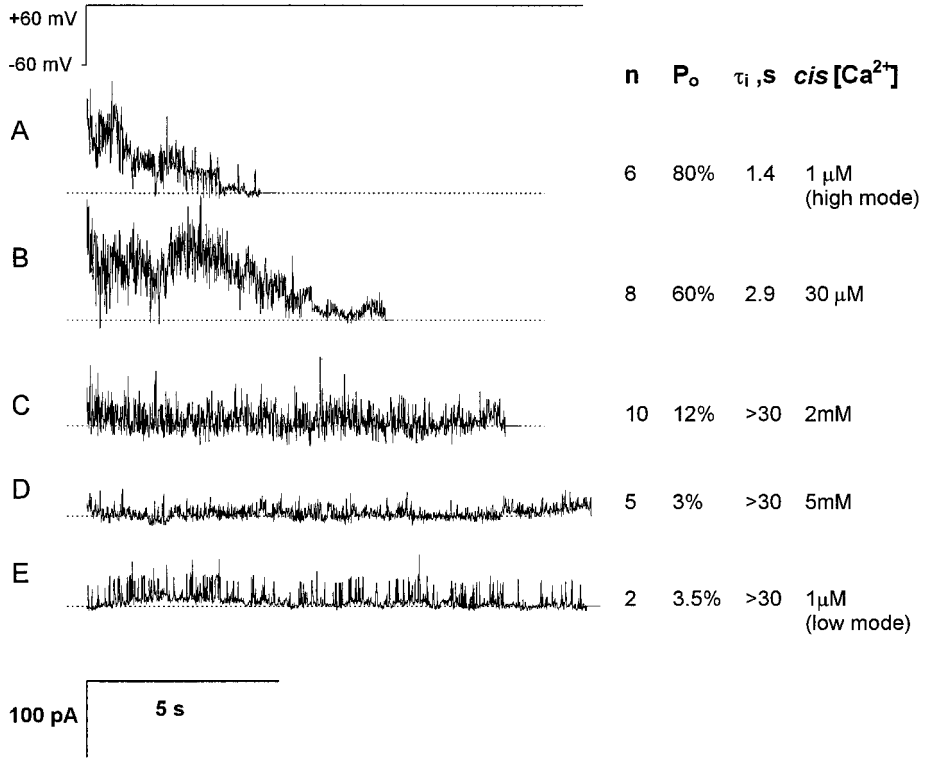


FIGURE 2 The rate of RyR inactivation depends on channel open probability. Ensemble summations of the current through a representative cardiac RyR from several episodes (*n*) are shown in which the bilayer potential was stepped from −60 mV to +60 mV at time = 0 (*top trace*). The records were fitted with an exponential decay with a time constant of τ_i. Solutions contained 250 mM/50 mM CsCl (*cis*/*trans*). Records *A*–*E*, ranked here in descending order of intra-burst open probability (P_o), were obtained from a single cardiac RyR at various *cis* [Ca^{2+}]. In 19 min of recording, the superposition of unitary currents, which would indicate the presence of multiple RyRs, was not observed. In the presence of 1 μM Ca^{2+}, this channel showed two distinct and sustained modes of gating, one with a P_o of 0.8 (*A*, high mode), and the other with a P_o of 0.035 (*E*, low mode). Increasing *cis* [Ca^{2+}] above 1 mM reduced P_o via the Ca^{2+}/Mg^{2+} inhibition mechanism. When P_o was relatively high, the channel inactivated within several seconds of a voltage step to positive potentials, whereas no RyR inactivation was observed when the intraburst open probability was low.

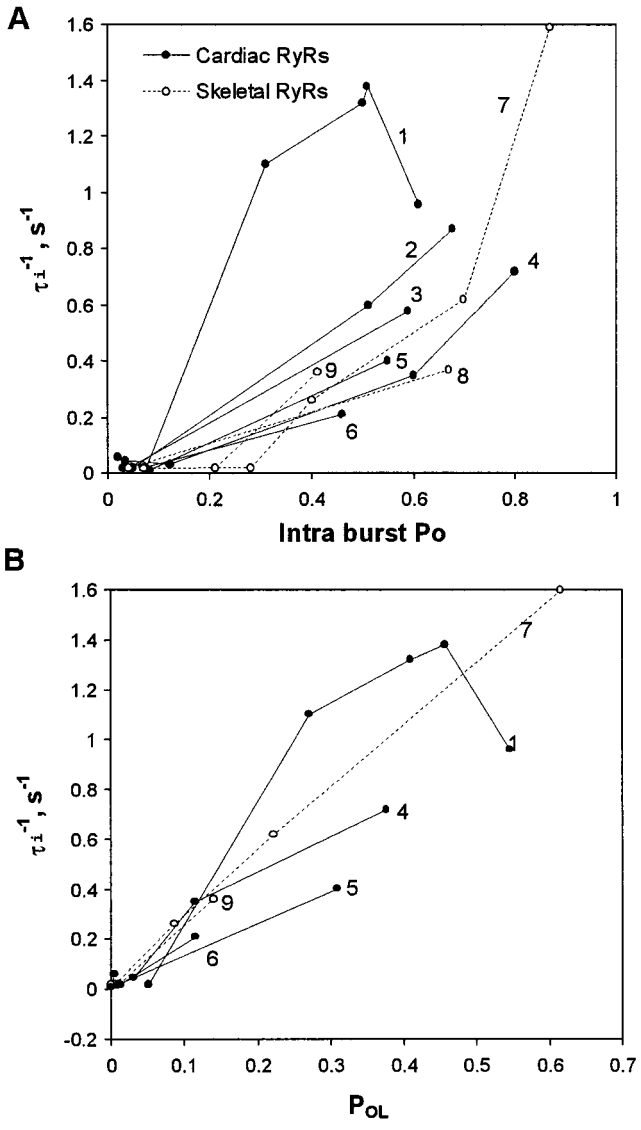


FIGURE 3 The inactivation rate (τ_i^{-1}) of cardiac (●) and skeletal (○) RyRs and its dependence on the RyR open probabilities. In experiments 1–9, inactivation occurred after voltage steps from −40 to +40 mV, except in experiment 4, where voltages were stepped from −60 to +60 mV. The inactivation rates were determined from exponential fits to current ensembles like those shown in Fig. 2. The data shown here are for RyRs that showed significant inactivation at high, intraburst P_o after voltage steps. (*A*) In all experiments, a decrease in P_o reduced the RyR inactivation rate. This happened when P_o was reduced by reducing *cis* [Ca^{2+}] to subactivating levels (Exp. 5), increasing [Ca^{2+}] to inhibiting levels (Exps. 3–7) or by the addition of mM Mg^{2+} (Exps. 1–3, 8, 9). Stochastic gating mode changes in which P_o was reduced also slowed inactivation (Exp. 4; the ensemble currents for this experiment are shown in Fig. 2). (*B*) The inactivation rates from several experiments, shown in *A*, from which dwell-time histograms could be obtained. The inactivation rates are plotted against the probability of RyRs being in a long open state (OL). P_{OL} was estimated from the triple exponential fits to open-time distributions.

particle movement had a $z\delta$ of 1.14 ± 0.25 at positive potentials and −0.8 ± 0.5 at negative potentials. (Inactivation has been interpreted in terms of a charged particle moving within the transmembrane electric field to plug the channel. Within this framework, z is the amount of charge

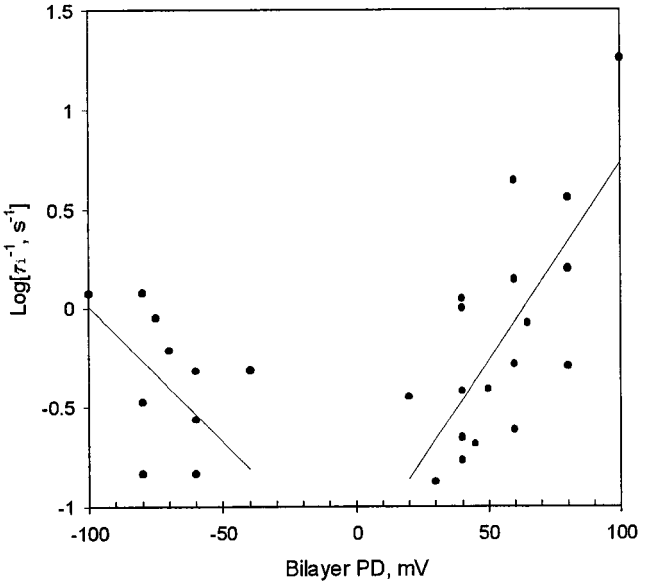


FIGURE 4 The inactivation rate (τ_i^{-1}) of cardiac RyRs and its dependence on bilayer potential (with respect to the *trans* chamber as ground). These results have been compiled from six experiments on a total of 12 RyRs that showed inactivation and were subject to a range of voltage steps. Experiments were carried out in symmetrical 250 mM Cs^+, and RyRs were activated to $P_o > 0.8$, either by Ca^{2+} alone or by Ca^{2+} plus the additional stimulatory action of mM ATP and caffeine. The inactivation rates were determined from exponential fits to current ensembles like those shown in Fig. 2. The lines show linear fits to the data at positive and negative potentials. The fit parameters are (slope and intercept at $V = 0$) 20 V^{-1} and 0.056 s^{-1} for the line at positive potentials and −14 V^{-1} and 0.045 s^{-1} at negative potentials.

and δ is the fraction of the transmembrane potential difference through which the charge moves to block the pore.) Reactivation rates, where they could be measured at negative potentials, increased by approximately fourfold between −20 and −80 mV.

The inactivation properties seen in Figs. 1–4 were obtained from RyRs in the presence of activating cytosolic [Ca^{2+}]. The Ca^{2+} requirement for inactivation at both positive and negative potentials was investigated by measuring the response of RyRs to voltage steps in the virtual absence of Ca^{2+}. Fig. 5 shows the response of cardiac RyRs activated by a combination of caffeine and ATP (~10 nM Ca^{2+}) or by ryanodine modification (~1 nM Ca^{2+}). These RyRs showed similar inactivation rates at low and high [Ca^{2+}] at both positive voltages (Fig. 5) and negative voltages (not shown). Thus Ca^{2+} is not a requirement for RyR inactivation.

Response of RyRs to single steps in cytosolic [Ca^{2+}]

RyRs were rapidly activated by [Ca^{2+}] steps produced by allowing Ca^{2+}-buffered solutions to flow over the bilayers. The time course of [Ca^{2+}] was estimated from the flow-induced current transient in conjunction with a solution exchange model. The details of this method are given by

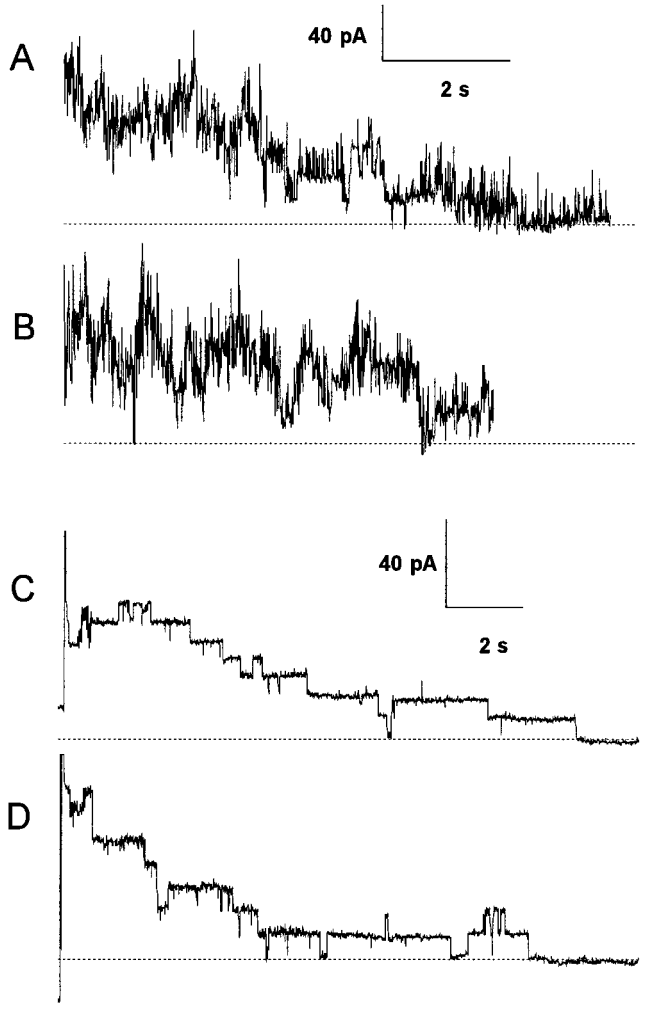


FIGURE 5 The response to voltage steps of cardiac RyRs that have been activated by ligands (ATP plus caffeine and ryanodine) in the presence and virtual absence of cytosolic Ca^{2+}. (*A*) An ensemble of four sweeps from two RyRs activated by 2 mM ATP and 5 mM caffeine in the presence of 10 nM Ca^{2+}. The declining activity shows the onset of inactivation after voltage steps from −60 mV to +60 mV. (*B*) An ensemble of five sweeps of the same RyRs in as in *A*, inactivating in response to the same voltage steps, but in the presence of 1 μM Ca^{2+} and 6 mM caffeine. (*C*) A single sweep of seven RyRs responding to voltage steps from −40 mV to +40 mV in the presence of 30 μM ryanodine and 1 mM Ca^{2+}. (*D*) The same experiment and the same situation as in *C*, except that free $[Ca^{2+}]$ was lowered to ~1 nM by perfusing the *cis* chamber with Ca^{2+}-buffered solutions. The RyR inactivation rate did not appear to be sensitive to cytoplasmic $[Ca^{2+}]$.

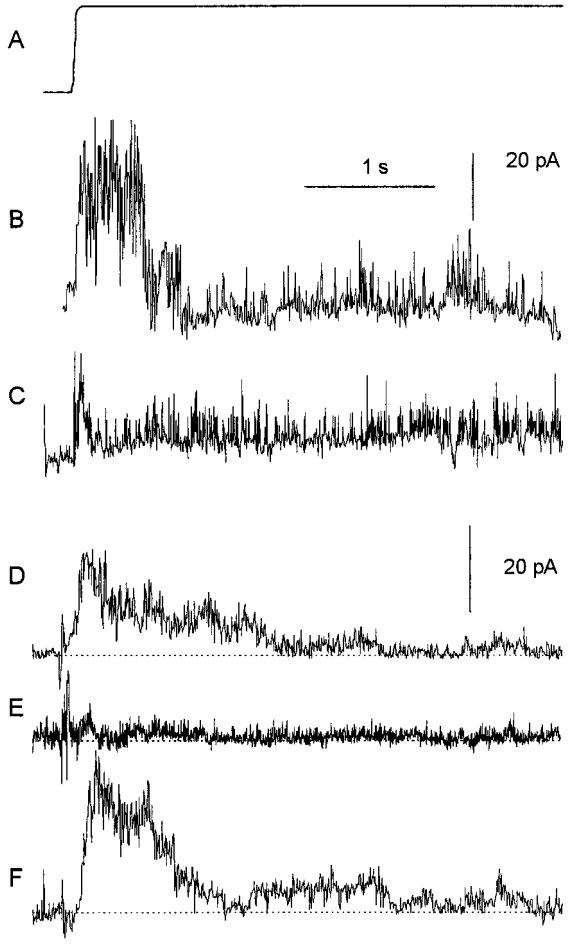


FIGURE 6 The response of three skeletal RyRs at +40 mV to steps in *cis* $[Ca^{2+}]$ from 0.1 to 200 μM lasting for 4 s (*trace A*). The $[Ca^{2+}]$ time course (shown on a log scale) was calculated with the solution exchange model of Laver and Curtis (1996a), using a solution exchange time of 20 ms. (*Traces B* and *C*) Representative, individual sweeps of RyR current. (*Traces D–F*) Ensemble averages in which the baseline transients associated with solution flow had been subtracted from each trace. (*Trace B*) Of the three RyRs present in the bilayer, the two with $P_o \approx 0.5$ generally shut down within 2 s of the $[Ca^{2+}]$ step, whereas the one with a P_o of 0.05 did not shut down (note the continued brief openings). (*Trace D*) The corresponding ensemble average current ($n = 8$), showing the marked decline in RyR activity after $[Ca^{2+}]$ steps. Later in the same experiment, the puff solution had been replaced with another containing 200 μM Ca^{2+} plus 2 mM Mg^{2+} (*Trace C*). The solution exchange at the bilayer produced a current transient near the start of each trace, with a peak amplitude of ~15 pA and lasting for ~100 ms (this is most easily seen in *Trace C*). The channel activity in Trace C stems from the three RyRs, which where strongly inhibited by Mg^{2+} (average $P_o = 0.05$) and failed to shut down after $[Ca^{2+}]$ steps. This is also shown by the continued flickery openings in the ensemble average ($n = 10$) in Trace E under these conditions. When the $[Mg^{2+}]$ in the puff solution is returned to zero, the RyR inhibition was lifted ($P_o \approx 0.5$), and the channels once again shut down soon after the Ca^{2+} steps (*Trace F*, $n = 6$).

Laver and Curtis (1996a). RyRs activated in the time it took for $[Ca^{2+}]$ to rise to ~0.3 μM. As reported by Laver and Curtis (1996b), the bursting activity of cardiac and skeletal RyRs at +40 mV usually declined after $[Ca^{2+}]$ steps from 0.1 μM to potently activating levels (e.g., Fig. 6). Fig. 6 shows single sweeps and ensemble averages of RyR activity during $[Ca^{2+}]$ steps. The declining activity of RyRs was associated with long-lived channel closures that occurred within several seconds of activation.

The rate of declining burst activity (i.e., inactivation; see Discussion) after $[Ca^{2+}]$ steps (τ_d^{-1}) at +40 mV depended on intraburst open probability, P_o (Figs. 6 and 7), and showed considerable variability between channels (Fig. 7). All RyRs with P_o values less than 0.2 had values of τ_d^{-1} that were not significantly different from zero. In Fig. 7 it can be seen that of the channels with P_o of 0.2 or more, 73%

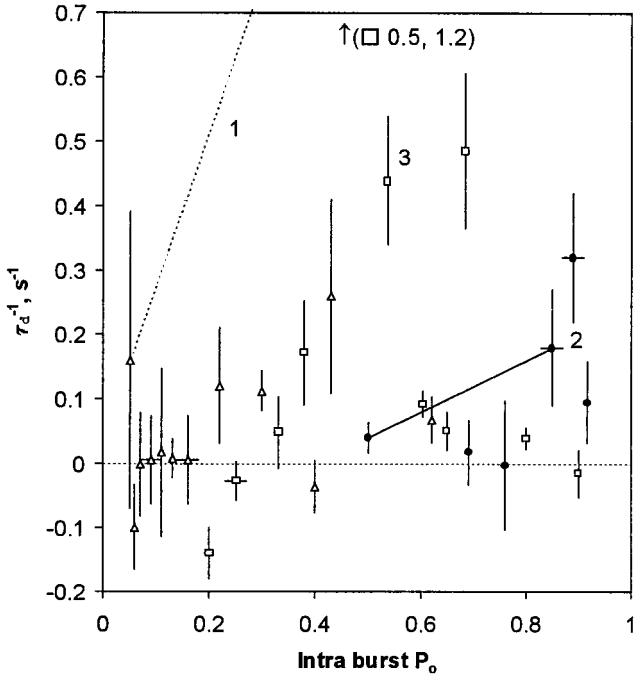


FIGURE 7 The rate of P_o decline at +40 mV, τ_d^{-1}, after activating [Ca^{2+}] steps ($t = 0$) from 0.1 μM in cardiac (●) and skeletal RyRs in the absence (□) and presence (△) of Mg^{2+}. The data marked with a horizontal bar indicate RyRs subject to [Ca^{2+}] steps to 10 μM. Other data show RyR responses steps to 1 μM (cardiac RyRs) and to 100–200 μM (skeletal RyRs). The datum point labeled 3 comes from a skeletal RyR stepped to 1 μM Ca^{2+}. Determination of $\tau_d \pm$ SD (*vertical error bars*) was made from exponential fits to current ensembles like those shown in Fig. 5. Many of the RyRs with intraburst P_o exceeding 0.5 showed significant declining activity, with τ_d ranging from 1 to 10s. RyRs with lower intraburst P_o tended to show a slower P_o decline, whereas those with $P_o < 0.2$ never showed a significant decline in activity within 5 s of a [Ca^{2+}] step. The lines connect data from the same RyRs: 1 indicates data obtained from the records shown in Fig. 6, which show that inhibition by Mg^{2+} slows P_o decline after [Ca^{2+}] steps, and 2 indicates data obtained from the records of Laver and Curtis (1996b; Fig. 5).

of skeletal RyRs (11 of 15) and 60% of cardiac RyRs (3 of 5) showed significant inactivation within 5 s of [Ca^{2+}] steps. In two experiments the response of RyRs to two different types of solution exchange were measured. Fig. 6 shows one experiment in which [Ca^{2+}] steps were produced in the absence and presence of 1 mM Mg^{2+}. Three skeletal RyRs were present in the bilayer. In the absence of Mg^{2+}, two appeared to have high P_o, and the other had much lower P_o. Only the RyRs with high P_o entered a long closed state shortly after the [Ca^{2+}] step. The presence of 1 mM Mg^{2+} had a strong inhibitory effect on at least the two RyRs with high P_o, and probably on the RyR with low P_o as well. Reducing the P_o of RyRs to less than 0.1 with Mg^{2+} prevented these RyRs from undergoing long closures during the 5 s of the solution flow. In the other experiment (shown by Laver and Curtis, 1996b, their figure 5, and further analyzed here in Fig. 7) a cardiac RyR was to subjected [Ca^{2+}] steps from 0.1 μM to either 1 μM or 10 μM. Steps to the higher [Ca^{2+}] activated the RyR to a higher intraburst P_o and sped up its inactivation rate. Thus RyR inactivation appears to be slowed by treatments that reduce P_o, and these were the same treatments that slowed voltage-dependent inactivation of RyRs (see above).

Response of individual RyRs to steps in both voltage and cytosolic [Ca^{2+}]

The time course of the declining burst activity of RyRs with step changes in either voltage or [Ca^{2+}] shared several similar characteristics. In both situations, about half the RyRs entered long closed states within seconds of the step. The respective inactivation rates (τ_i^{-1} and τ_d^{-1}) had a similar dependence on P_o and showed considerable variation between individual channels. The possibility that a common mechanism underlies the inactivating response of RyRs to [Ca^{2+}] and voltage steps was investigated by comparing the responses of individual RyRs to combinations of these steps. Fig. 8 shows the degree of inactivation seen in three cardiac RyRs and six skeletal RyRs that were subjected, at different times, to steps in voltage (from −40 to +40 mV in the presence of activating [Ca^{2+}]) and steps in [Ca^{2+}] (at +40 mV). The data shown in Fig. 8 were obtained from RyRs with P_o values above 0.25, where inactivation was apparent in some channels. The wide range of inactivation rates was typical of interchannel variation seen in this study. A marked correlation was found between the ability of RyRs to show declining activity after voltage steps and after [Ca^{2+}] steps. In addition to this correlation,

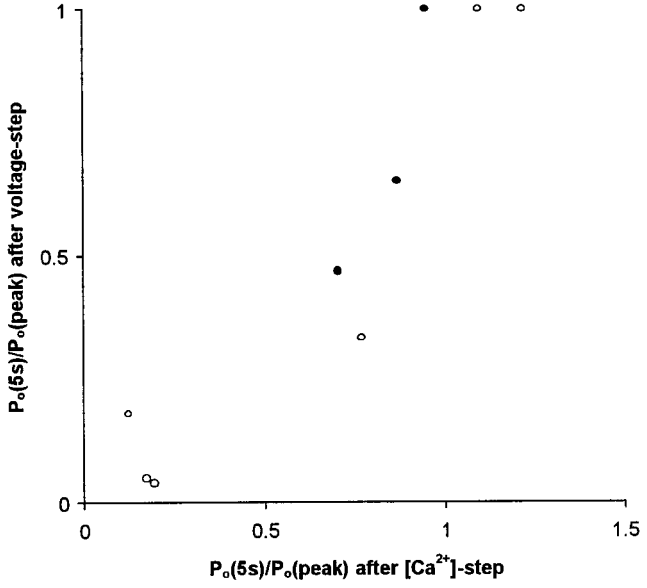


FIGURE 8 RyR responses to voltage and [Ca^{2+}] steps are correlated. The decline in activity of cardiac (●) and skeletal (○) RyRs after voltage steps from −40 mV to +40 mV is plotted against the decline in their activity after [Ca^{2+}] steps (at +40 mV) in the same experiment. The decline in activity was measured as the ratio of the ensemble average current at $t = 5$ s and at the time when the RyRs had fully activated in response to steps in either [Ca^{2+}] or voltage. There is a significant correlation ($r = 0.9$) between RyR responses to voltage and [Ca^{2+}] steps.

the RyR activity produced by $[Ca^{2+}]$ steps and voltage steps was also found to be interdependent. In five experiments (holding potential = +40 mV), it was consistently found that 1) brief (~1 s) voltage pulses to −40 mV (in subactivating $[Ca^{2+}]$) could prime RyRs to open during subsequent $[Ca^{2+}]$ steps (not shown), 2) voltage pulses to −40 mV (at activating $[Ca^{2+}]$) could reactivate RyRs that had inactivated after $[Ca^{2+}]$ steps (e.g., Fig. 9 *Ac*), 3) $[Ca^{2+}]$ steps could not activate RyRs after inactivation (Fig. 9, *Ab* and *Af*), 4) $[Ca^{2+}]$ steps could activate RyRs after deactivation brought about by lowering $[Ca^{2+}]$ to subactivating levels (Fig. 9 *Bj*). With regard to points 3 and 4, inactivation and deactivation processes could be readily distinguished on the basis of their different kinetic signatures. During inactivation, the RyR would abruptly cease high-P_o activity and enter a long closed state (e.g., Fig. 9, *Aa* and *Ad*). RyR deactivation by a gradual decline in $[Ca^{2+}]$ showed a gradual decrease in P_o and flickery channel activity (Fig. 9 *Bg–h*). It was unlikely that inactivation could have also occurred at the final stages of the decrease and gone unnoticed, because inactivation was never observed in channels with low P_o. The concentration of Ca^{2+} shown in Fig. 9 during its decline from 1 μM to 0.1 μM is due to the gradual equilibration of the solution near the bilayer with the bath solution after the puff. The $[Ca^{2+}]$ time course during this period is not known precisely, but the estimates shown are based on many observations of the $[Ca^{2+}]$-dependent activity of RyRs and SCl channels (Cl^- conductance of 70–100 pS) during equilibration of puff and bath solutions.

Response of RyRs to double steps in cytosolic $[Ca^{2+}]$

To test whether RyRs truly inactivate rather than adapt, cardiac RyRs were subjected to a rising staircase of two $[Ca^{2+}]$ steps. The first step from 0.1 to 1 μM was followed after 5–10 s by another step from 1 to 100 μM. In these experiments the main anion in the baths was methane sulfonate, and under these conditions the RyRs were often slightly active in the presence of 0.1 μM Ca^{2+}. After the flow was switched to the 1 μM Ca^{2+} solution, there was a lag of ~0.5 s before the new solution reached the bilayer. RyRs then activated over a period of 0.5 s, tracking the rising $[Ca^{2+}]$ time course at the bilayer surface. Fig. 10 shows one experiment, representative of all three, in which a cardiac RyR transiently activated in response to the first $[Ca^{2+}]$ step. Importantly, after the RyR had inactivated, it failed to open again in response to the second $[Ca^{2+}]$ step. It could nevertheless be reactivated by a brief voltage pulse to −40 mV, as in the single $[Ca^{2+}]$ step experiments.

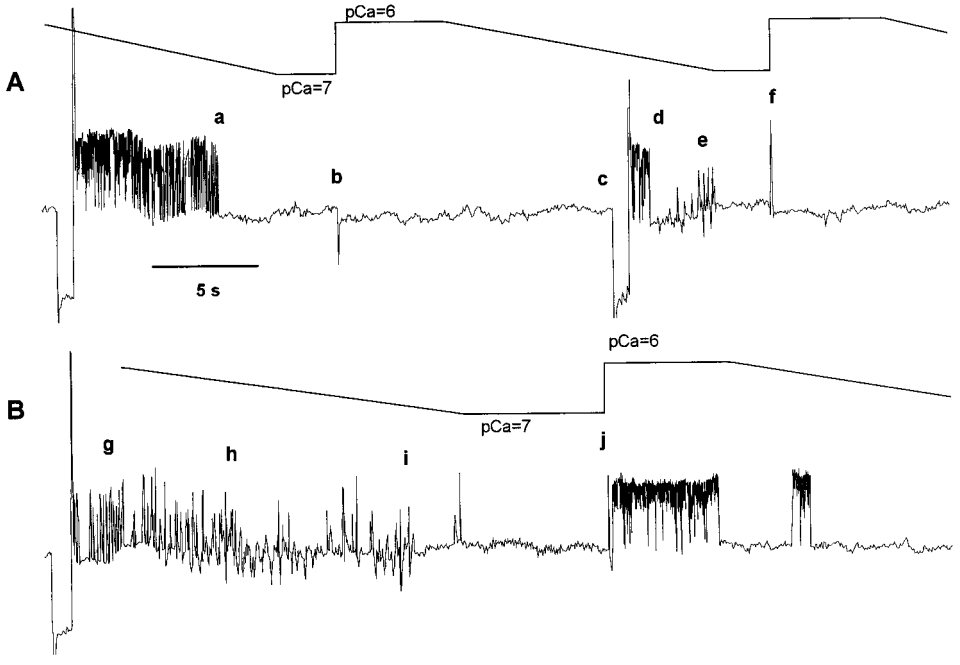


FIGURE 9 RyR responses to voltage and $[Ca^{2+}]$ steps are interdependent. Recording of a single cardiac RyR obtained at +40 mV that is representative of five experiments. The *cis* bath contained 0.1 μM Ca^{2+}, and the puff solution contained 1 μM Ca^{2+}. Upward $[Ca^{2+}]$ steps occurred when the puff solution was released onto the bilayer, and the slow decline in Ca^{2+} occurred when the flow of puff solution ceased and during the time when the solution near the bilayer equilibrated with the bath. In this experiment the RyR had a high intraburst open probability in 1 μM Ca^{2+} and was not active at 0.1 μM Ca^{2+}. (*Trace A*) The channel inactivating (*a*) ~6 s after it was activated with a brief voltage pulse to −40 mV at activating $[Ca^{2+}]$ levels. The inactivation could not be reversed by a subsequent $[Ca^{2+}]$ step from 0.1 to 1 μM at +40 mV (*b*), but could be relieved by a brief voltage pulse to −40 mV at activating $[Ca^{2+}]$ (*c*). Once again, the channel inactivated (*d*), and $[Ca^{2+}]$ was then lowered by stirring the bath, which produced the baseline noise (*e*). As before, a subsequent $[Ca^{2+}]$ step did not activate the RyR (*f*). (*Trace B*) The RyR open probability declining gradually to low levels, in response to a decrease in *cis* $[Ca^{2+}]$. The channel closure was not likely to have resulted from the inactivation mechanism seen in Trace A, because this never occurred when the intraburst P_o was this low (<0.1). The bath was stirred for several seconds (*h–i*), and a subsequent $[Ca^{2+}]$ step was able to open the channel (*j*).

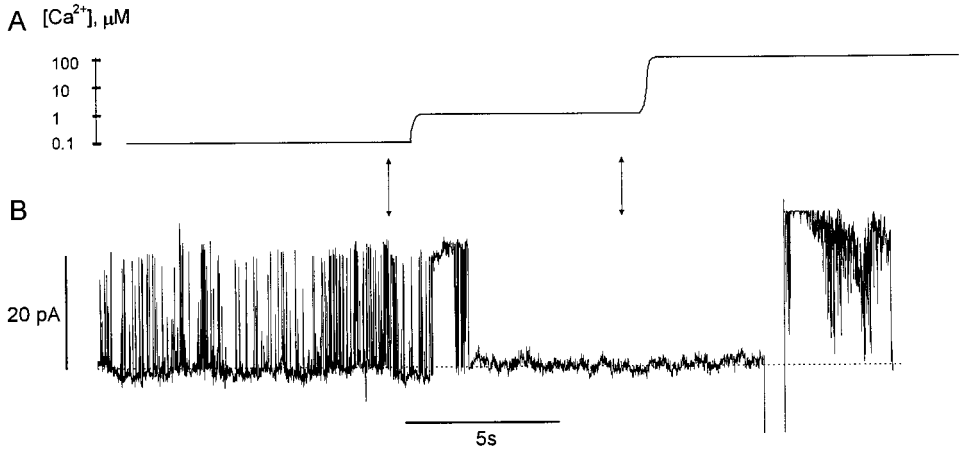


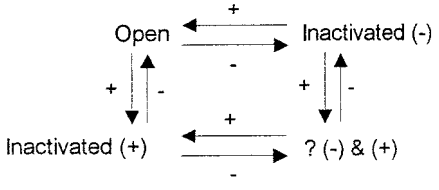
FIGURE 10 The response of a single cardiac RyR at +40 mV (*Trace B*) to sequential steps in *cis* [Ca^{2+}] from 0.1 to 1 μM and from 1 to 100 μM (*Trace A*). The [Ca^{2+}] time course was calculated using the solution exchange model of Laver and Curtis (1996a), with a time constant of 100 ms. The time constant was inferred from the duration of the baseline transient driven by the changing ionic concentrations near the bilayer. The arrows point to the times when the solution flow was switched from the different tubes. At 0.1 μM Ca^{2+}, the RyR had an open probability of 0.08, which rose to >0.9 when [Ca^{2+}] reached 1 μM. Approximately 1 s after this, the channel inactivated and remained closed until it was reactivated by a brief voltage pulse to −40 mV. Most importantly, while the RyR was inactivated, it could not be opened by raising [Ca^{2+}] to 100 μM.

DISCUSSION

Inactivation after voltage steps

This paper describes the transient responses of sheep cardiac and rabbit skeletal RyRs to step changes in membrane potential and cytosolic [Ca^{2+}]. In this study it has been shown that individual RyRs inactivate at both positive and negative voltages. Inactivation, seen here at positive potentials, is similar to that shown previously in sheep cardiac RyRs (Laver et al., 1995), chicken skeletal RyRs (Percival et al., 1994), and recombinant skeletal RyRs expressed in HEK cells (Imredy et al., 1996; Chen et al., 1997). Inactivation at negative potentials is similar to that seen by Ma (1995) in rabbit skeletal RyRs, where RyRs inactivated with a τ of 3.9 s (at −100 mV; cf. ~1 s, Fig. 4, in this study), and inactivation was reported in RyRs at potentials more negative than −80 mV (cf. −60 mV here). The fast inactivation seen at positive potentials by Ma et al. (1995) was not seen here.

The data shown in this study provide new information about the mechanism(s) of RyR inactivation. Individual RyRs inactivated at both positive and negative potentials, possibly indicating the existence of two different inactivation mechanisms. The inactivation processes have opposite voltage dependencies, which are summarized in the following gating scheme:

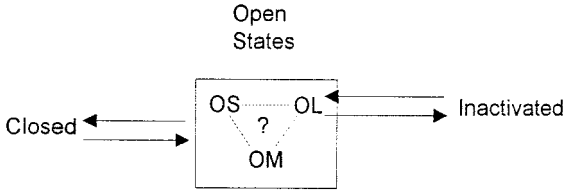


Scheme 1

The inactivation steps have rate constants on the order of 1 s^{-1}, and the activation steps typically have rate constants of 10^2 s^{-1}. The transition rates that are increased by more positive voltages are marked with a +, and those increased by more negative voltages are marked with a −. According to this scheme, stepping the membrane potential to positive values tends to put the RyRs into the inactivated (+) state. Reversing the potential would cause RyRs to rapidly assume the open state, followed by a relatively slow transfer to the inactivated (−) state. It is not yet clear if the two inactivation mechanisms can operate independently to allow the channel to be in a doubly inactivated state (hence the question mark in Scheme 1). It is worth noting that the presence of inactivation at opposite voltage extremes can also be explained by a single mechanism that responds to the magnitude of the membrane potential. An example of this would be a plug that gets pushed into the channel by dielectric forces (electrostriction force depends on the square of the membrane potential).

Opening of cardiac and skeletal RyRs at positive potentials, in itself, does not appear to be sufficient for inactivation to occur; it seems that the channel, in addition, needs to be in a long-lived open state. However, the precise open-state conditions for inactivation cannot be determined from simply fitting arbitrary exponential functions to dwell-time histograms. Inactivation of RyRs with voltage did not depend on the particular activating ligand. It was shown here for cardiac RyRs that the channel openers Ca^{2+} (μM), ATP plus caffeine, and ryanodine produced similar inactivation rates. Conversely, it was shown for both cardiac and skeletal RyRs that ligands that inhibited RyR opening (i.e., mM Ca^{2+} and Mg^{2+}) also slowed inactivation. Moreover, inactivation was sensitive to spontaneous changes in P_o resulting from gating mode changes that were not associated with any changes in ligand concentrations. Thus it appears that RyR inactivation, at least at positive potentials, depends strictly on the channel being in a long-lived open state.

Open and closed dwell-time distributions indicate the existence of at least three open and closed states, which is a number similar to that reported in other studies (see citations in Laver et al., 1995). Because it is not known how these states are interconnected, a simplified gating model was used to describe each inactivation mechanism:



Scheme 2

The model shows two nonconducting states of the channel, namely closed and inactivated. For simplicity, the Ca^{2+}- and Mg^{2+}-inhibited states are lumped together with the resting closed state of the channel. The inactivated state can represent inactivation at either positive or negative voltages. Three open states are shown (S, short; M, medium; L, long), but without their interconnections. According to the model, RyRs switch from their closed state to their open states when activated by ligands such as Ca^{2+}, ATP, and caffeine, or when they are modified by ryanodine. Inhibiting substances such as mM Ca^{2+} and Mg^{2+} tend to put RyRs into the closed state. Once the channel is in the long open state (OL), it can then inactivate. The transition rates between the inactivated and OL are voltage dependent, as described in Scheme 1.

A common mechanism for inactivation after voltage and [Ca^{2+}] steps

Several lines of evidence indicate that, at least at positive potentials, the mechanism for voltage-dependent inactivation in skeletal and cardiac RyRs also underlies the declining activity after [Ca^{2+}] steps:

1. The activity of RyRs after both voltage and [Ca^{2+}] steps declines with the same kinetics. RyRs show declining burst activity in which the channels undergo long-lived closures within seconds of the step.

2. Both inactivation processes have a similar dependence on P_o (cf. Figs. 3 and 7). Inactivation after voltage steps appears to depend on RyRs being in a specific open state, but it was technically difficult to discern whether inactivation after [Ca^{2+}] steps depended on P_o or a particular open state. Nevertheless, it has been shown by Sitsapesan et al. (1995) that inactivation after [Ca^{2+}] steps is promoted by RyR activators that increase channel open dwell times.

3. The occurrences of inactivation with steps in voltage and [Ca^{2+}] were closely correlated in RyRs (Fig. 8). Channel-to-channel variations were such that one-half to two-thirds of RyRs (at $P_o > 0.2$) inactivated after voltage or [Ca^{2+}] steps. Only those that showed inactivation after voltage steps inactivated after [Ca^{2+}] steps, and vice versa.

4. Both inactivation processes had the same voltage dependence, in that a transient reversal of bilayer voltage from +40 mV to −40 mV reactivated RyRs in both cases.

Therefore the model describing inactivation after voltage steps (Scheme 2) should also account for inactivation after [Ca^{2+}] steps. Scheme 2 is simplified and recast to specifically consider inactivation at positive potentials:

Scheme 3

Scheme 3 can explain the properties of RyR inactivation after [Ca^{2+}] steps. According to Scheme 3: 1) the rapid application of any activating ligand would put RyRs in the open state, which would then allow them to inactivate at positive voltages; 2) reversing the polarity of the membrane potential would reactivate RyRs that have inactivated (Fig. 9 *Ac*); 3) RyRs that are activated with high probabilities of being open will inactivate more quickly (Figs. 3 and 7); 4) RyRs that have inactivated will not be reactivated by further addition of activating ligands (Fig. 9, *Ab* and *Af*, and Fig. 10); 5) RyRs that have deactivated (i.e., closed upon the withdrawal of the activating stimulus rather than inactivated) can be reactivated by the addition of activating ligands (Fig. 9 *Bj*).

Inactivation after [Ca^{2+}] steps at positive potentials

Several characteristics of sheep cardiac RyRs responding to steps in [Ca^{2+}] that are seen here confirm previous findings of Sitsapesan et al. (1995): 1) RyRs more frequently inactivated when they were activated to a high P_o. 2) Of those RyRs that had high P_o, a similar fraction in both studies inactivated, and they did so with similar burst kinetics and time course. 3) Repeated application of [Ca^{2+}] steps from subactivating to activating levels failed to reactivate channels that had inactivated. 4) Voltage steps to −40 mV could remove inactivation. Sitsapesan et al. (1995) noted that channel activators that increased open dwell times promoted inactivation. Here it is also shown that RyR inhibitors that lower P_o inhibit inactivation. Furthermore, inactivation in response to voltage steps depends on the RyRs being in a specific open state. The slowing effect of cytosolic Mg^{2+} (1 mM) on the inactivation response of the population average was not seen by Laver and Curtis (1996b). The reason for this is that Mg^{2+} affects the inactivation rate via its effect on P_{OL} and under the high ionic strength conditions used in some of the experiments here (Fig. 7) and reported by Laver and Curtis (1996b). Mg^{2+} had only mild a inhibitory effect on some RyRs, so that P_{OL} remained high (see Laver et al., 1997b).

Inactivation after [Ca^{2+}] steps at negative potentials

If indeed there is a common mechanism for inactivation after [Ca^{2+}] and voltage steps at positive potentials, a com-

mon mechanism may also cause inactivation after [Ca^{2+}] and voltage steps to negative potentials. On the other hand, if inactivations at positive and negative potentials are instead due to different mechanisms, then inactivations induced by [Ca^{2+}] steps at opposite polarities must also arise from different mechanisms.

Inactivation at negative potentials was studied here using voltage steps but not [Ca^{2+}] steps. Sitsapesan et al. (1995) did not detect inactivation with [Ca^{2+}] steps at −40 mV in sheep cardiac RyRs. The latter finding is consistent with the slow and infrequent inactivation rates detected here at −40 mV using voltage steps. Schiefer et al. (1995) did observe inactivation of dog cardiac RyRs after [Ca^{2+}] steps at −50 mV (they state +50 mV pipette potential, which is opposite the convention used here and by others). [Ca^{2+}] steps from 10 nM to 0.1–1 mM produced inactivation with τ of 200–500 ms, which is faster than that seen in sheep cardiac RyRs (this study and Sitsapesan et al., 1995). An interesting observation made by Schiefer et al. (1995), not seen for inactivation at positive potentials, was that after inactivation, lowering [Ca^{2+}] to subactivating levels was always followed by channel openings, indicating that RyRs recovered via their open state. A major difference between [Ca^{2+}] step inactivation reported by Schiefer et al. (1995) and inactivation at negative potentials seen here with voltage steps is in the proposed role for Ca^{2+}. In this study inactivation did not depend on [Ca^{2+}] per se, but rather on the level of RyR activation. Schiefer et al. (1995) detected inactivation at [Ca^{2+}] above 3 μM, which became more pronounced and faster as the [Ca^{2+}] was increased to 1 mM. The level of peak RyR activity over this range was relatively independent of [Ca^{2+}]. Consequently, the inactivation was considered to be Ca^{2+} dependent. The disparate findings might be reconciled if inactivation at negative potentials occurs only from a particular open state, as has been found at positive potentials. This possibility is suggested by single-channel recordings by Schiefer et al. (1995, their figure 5, *A* and *B*), which show that RyRs that are activated by 3 μM and 1 mM Ca^{2+} have similar P_o values, but apparently very different open dwell times. In that case, the inactivation seen in their study may not be Ca^{2+} dependent, but rather dependent on a long-lived open state that makes only a small contribution to the total P_o.

Inactivation versus adaptation

The adaptation phenomenon and the inactivation seen after flow-induced [Ca^{2+}] steps do have similar time courses. However, it appears that different mechanisms underlie these two processes. Three key pieces of evidence indicating that RyRs do indeed inactivate here, rather than adapt, are: 1) RyR inactivation is followed by a refractory period, on the order of seconds, in which repeated [Ca^{2+}] steps fail to activate RyRs (Sitsapesan et al., 1995; Schiefer et al., 1995; Fig. 9 in this study). 2) Inactivation of RyRs does not depend on [Ca^{2+}] per se (Fig. 5), whereas adaptation, by definition, is dependent entirely on the stimulus (in this case Ca^{2+} is the stimulus). 3) RyRs that inactivate in response to a [Ca^{2+}] step cannot be opened by a further rise in [Ca^{2+}] (Fig. 10), whereas RyRs that have adapted can be reopened this way (Györke and Fill, 1993). There are also a number of other differences between inactivation and the adaptation phenomenon. First, the conditions under which the two phenomena are observed are quite different. The adaptation phenomenon occurs with rising [Ca^{2+}] steps to as little as 0.2 μM (Györke and Fill, 1993), where such steady levels of Ca^{2+} do not activate RyRs. In contrast, inactivation is seen only at activating ligand concentrations (Sitsapesan et al., 1995; Schiefer et al., 1995; this study). Second, the kinetics of the two phenomena appear quite different. Inactivation always occurs by interruption of high P_o activity with long-lasting closures, whereas adaptation frequently appears more like a gradual decrease in RyR activity (Györke et al., 1994, their figure 5). Finally, Mg^{2+} inhibition of RyRs either slows or removes inactivation, whereas Mg^{2+} speeds up the adaptation phenomenon (Valdivia et al., 1995). Thus the declining RyR activity observed here, and in other studies using flow methods, is termed "inactivation" and is a phenomenon that is distinctly different from the "adaptation" observed in flash photolysis studies (see also Lamb, 1997, and Lamb and Laver, 1998).

Heterogeneity in RyR inactivation

Many single-channel studies have reported significant channel-to-channel differences in RyR gating and RyR sensitivity to regulating substances. Several studies of RyR inactivation have found that only approximately half of the RyRs exhibit inactivation. In this study, one-half to two-thirds of RyRs inactivated after steps in either voltage or myoplasmic [Ca^{2+}]. Ma (1995) found that 50–70% of skeletal RyRs inactivated after steps to negative potentials. Sitsapesan et al. (1995) reported that 56% of cardiac RyRs inactivated after steps in myoplasmic [Ca^{2+}] (in the presence of another agonist). The molecular basis for this heterogeneity is not clear. Interestingly, Hain et al., (1994, 1995) found that only about half the RyRs isolated from either skeletal or cardiac muscle are phosphorylated, which might suggest that the phosphorylation state of RyRs is important for inactivation. Another possibility considered by Ma (1995) and Laver et al. (1995) is that heterogeneity stems from the presence or absence of regulatory proteins (e.g., calmodulin, FK-506 binding protein) on different RyRs. Binding of exogenous FK-506 binding protein (FKBP12) to RyRs was found to cause "asymmetrical channel blockade" (Ma et al., 1995; Chen et al., 1997). However, a similar role for endogenous FKBP was not established. Furthermore, it seems unlikely that any of the RyRs examined here have simply lost their FKBP, given that when RyRs are stripped of the binding protein, their properties are distinctly altered (Ahern et al., 1994). Thus it remains to be seen how inactivation is regulated and how this regulation leads to behavioral heterogeneity of RyRs.

Physiological significance

This and previous bilayer studies show that RyRs possess an inactivation mechanism that can close them within seconds of being activated, be it by steps in voltage or [Ca^{2+}], or by any other activating stimulus. However, it is not clear what role, if any, this mechanism might play in regulating Ca^{2+} release in muscle. The results here indicate that RyRs would show relatively little inactivation at the presumed resting membrane potential of the SR (~0 mV; Somlyo et al., 1981). However, during rapid Ca^{2+} release, the SR potential might transiently become quite positive (i.e., negative in the lumen), which could cause activated RyRs to inactivate. Nevertheless, the rate of inactivation seen here seems to be much too slow for this phenomenon to be an important negative control mechanism during a muscle twitch. Perhaps instead it could contribute to inactivation of Ca^{2+} release during a tetanus or to the slow decline in sustained Ca^{2+} release that has been observed in some preparations (e.g., Dettbarn et al., 1994). Alternatively, noting that Ca^{2+} release in skeletal muscle is reported to inactivate substantially within ~10 ms after onset (Simon et al., 1991; Jong et al., 1995), perhaps the same inactivation mechanism operates in muscle in vivo and in bilayers, but in the latter it has been altered by the process of isolating the RyRs and reconstituting them with artificial lipid bilayers. Although it has been generally thought that Ca^{2+} inactivation in muscle is truly Ca^{2+} dependent, it has been suggested recently that such inactivation might depend on channel activation rather than Ca^{2+} per se (Pizarro et al., 1997), in which case a common mechanism for inactivation in muscle and in bilayers cannot be ruled out. Finally, RyRs are found in many cell types, yet their role in nonmuscle tissues is poorly understood. It is possible that the slow inactivation of RyRs seen here is not relevant to muscle function at all, but nevertheless has an important role in another cell type.

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