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Regulation of the Gating of the Sheep Cardiac Sarcoplasmic Reticulum Ca^{2+}-Release Channel by Luminal Ca^{2+}

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Abstract. We investigated the effects of changes in luminal [Ca^{2+}] on the gating of native and purified sheep cardiac sarcoplasmic reticulum (SR) Ca^{2+}-release channels reconstituted into planar phospholipid bilayers. The open probability (P_o) of channels activated solely by cytosolic Ca^{2+} was greater at positive than negative holding potentials. Channels activated solely by 10 μM cytosolic Ca^{2+} exhibited no change in steady-state P_o or in the relationship between P_o and voltage when the luminal [Ca^{2+}] was increased from nanomolar to millimolar concentrations. In the absence of activating concentrations of cytosolic Ca^{2+}, the channel can be activated by the phosphodiesterase inhibitor sulmazole (AR-L 115BS). However, cytosolic Ca^{2+}-independent activation of the channel by sulmazole requires luminal Ca^{2+}. In the presence of sulmazole, at picomolar luminal [Ca^{2+}] the channel remains completely closed. Increasing the luminal [Ca^{2+}] to millimolar levels markedly increases the P_o via an increase in the duration of open events. The P_o and duration of the sulmazole-activated, luminal Ca^{2+}-dependent channel openings are voltage dependent. In the presence of micromolar luminal Ca^{2+}, the P_o and duration of sulmazole-activated openings are greater at negative voltages. However, at millimolar luminal [Ca^{2+}], long openings are also observed at positive voltages and the P_o appears to be similar at positive and negative voltages. Our findings indicate that the regulation of channel gating by luminal Ca^{2+} depends on the mechanism of channel activation.

Key words: Ca^{2+}-release channels — Sarcoplasmic reticulum — Cardiac — Sulmazole

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Introduction

In the mammalian cardiac cell, Ca^{2+} entering the cytoplasmic space during the action potential triggers the release of Ca^{2+} from the sarcoplasmic reticulum. As expected for a channel predicted to release SR Ca^{2+}, the Ca^{2+}-release channel from cardiac SR exhibits marked sensitivity to cytosolic Ca^{2+} changes after incorporation into planar lipid bilayers. If the channel is opened by micromolar concentrations of cytosolic Ca^{2+}, the gating of the channel is characterized by very brief opening events, and Ca^{2+} alone cannot fully activate the channel [1]. The primary mechanism for a Ca^{2+}-induced increase in P_o is an increase in the frequency of channel opening.

The channel can also be opened in a cytosolic Ca^{2+}-independent manner by other agents—for example, the phosphodiesterase inhibitors caffeine and sulmazole [18, 27]. In the absence of activating cytosolic [Ca^{2+}], these agents elicit openings which are long in comparison to cytosolic Ca^{2+}-dependent open events.

There are few reports of modification of gating by agents acting from the luminal face of cardiac or skeletal Ca^{2+}-release channels. Only annexin VI has been shown to modulate the P_o of the rabbit skeletal channel from the luminal side [5]. However, in skinned cardiac cells, Fabiato and Fabiato [6] demonstrated that the amount of SR Ca^{2+} released was graded according to the level of Ca^{2+} preloading of the SR. As the Ca^{2+} level in the SR was reduced, the amount of Ca^{2+} released for a given amount of trigger Ca^{2+} was also decreased. In isolated skeletal SR, a threshold level of Ca^{2+} loading of the vesicles is required before Ca^{2+}-induced Ca^{2+} release will occur [8, 14]. This led to the suggestion that an intraluminal Ca^{2+} binding site must be regulating release of Ca^{2+}. At the single channel level, changes in luminal [Ca^{2+}] have been shown to mod-

ify single channel conductance [23, 24], but does the level of luminal [Ca^{2+}] also directly affect the open probability of the Ca^{2+} release channel? Several groups report that millimolar luminal Ca^{2+} closes the Ca^{2+}-release channels from skeletal muscle [7, 10, 12]; however, preliminary work indicates that this is not the case with the cardiac isoform of the channel [19–21].

Previously, the gating of native SR Ca^{2+}-release channels was investigated using Ca^{2+} as the permeant ion [1, 22] to avoid current flow through the SR K^+ and Cl^- channels. High concentrations of luminal Ca^{2+} (40–80 mM) were used, and current flow from the luminal to the cytosolic channel side was recorded. Under these conditions, it was impossible to investigate the effects of changing the luminal [Ca^{2+}] in the micromolar to low millimolar range. In addition, the dependence of P_o on voltage could only be investigated over a very narrow range under asymmetrical conditions. We have overcome these problems by using Cs^+ as the permeant ion. In symmetrical 250 mM Cs^+, the conductance of the SR K^+ channel is very low in comparison with that of the Ca^+-release channel and does not interfere with the analysis of gating [4, 7]. We have therefore carried out experiments to investigate how luminal Ca^{2+} modifies channels activated by different mechanisms; either by cytosolic Ca^{2+} alone or by sulmazole in the absence of activating cytosolic Ca^{2+} levels.

Half of the studies on the effects of changes in luminal [Ca^{2+}] on skeletal SR Ca^{2+}-release channels were performed on purified channels [9, 12]. However, no comprehensive study of the gating of purified SR Ca^{2+}-release channels (either skeletal or cardiac) has been reported. It is possible that purification may lead to altered gating behavior either because of damage caused during the isolation procedure or due to the absence of other regulatory proteins or agents associated with the channel. We have therefore investigated if the effects of changing the [Ca^{2+}] at the luminal face of cardiac SR Ca^{2+}-release channels are altered in purified channels.

Our experiments indicate that the P_o and voltage dependence of channels activated by cytosolic Ca^{2+} alone do not appear to be altered by changing the luminal [Ca^{2+}]. In contrast, cytosolic Ca^{2+}-independent channel openings are highly dependent on the luminal [Ca^{2+}] and exhibit marked voltage dependence which is modified by increasing luminal [Ca^{2+}]. Similar results were obtained with native and purified channels.

Materials and Methods

Preparation of SR Membrane Vesicles

SR membrane vesicles were prepared from sheep hearts obtained from a local abattoir as previously described [17]. Membrane vesicles were frozen rapidly in liquid nitrogen and stored at −80°C.

Purification of the Ca^{2+}-Release Channel

The ryanodine receptor was solubilized by 3-((3-cholamidopropyl)-dimethylammonio)-1-propane sulphonate (CHAPS) as previously described [11]. Heavy SR membrane vesicles were suspended in 1 M NaCl, 0.1 mM EGTA, 0.15 mM $CaCl_2$, and 25 mM PIPES-NaOH (pH 7.4) at a protein concentration of 1.5–2 mg protein/ml in the presence of 0.5% (wt/vol) CHAPS and 2.5 mg/ml L-α phosphatidylcholine. After incubation for 1 hr on ice, unsolubilized material was sedimented by centrifugation for 45 min at 36,000 rpm in a Sorvall T-875 rotor.

The ryanodine binding protein was separated from other solubilized membrane components by sedimentation on a 5–25% (wt/vol) continuous linear sucrose gradient overnight at 28,000 rpm in a Sorvall AH-629 rotor. Gradient fractions were drawn from the base of the tube and those containing the ryanodine receptor were identified by comparison with an identical gradient which contained material incubated in the presence of 5 nM [^{3}H] ryanodine during the solubilization period. The solubilized receptor was reconstituted into unilamellar liposomes as previously described [11] before incorporation into planar lipid bilayers.

Planar Lipid Bilayer Methods

Planar phospholipid bilayers containing phosphatidylethanolamine (Avanti Polar Lipids, Birmingham, AL) in decane (35 mg/ml) were formed across a 200 μm diameter hole in a polystyrene partition separating two fluid-filled compartments; the *cis* (volume 0.5 ml) and *trans* (volume 0.7 ml) chambers. The *trans* chamber was held at ground and the *cis* chamber was clamped at various potentials relative to ground using Ag-AgCl electrodes via 2% agar bridges in 3 M LiCl. Current flow through the bilayer was measured using an operational amplifier as a current-voltage converter [13].

Bilayers for incorporation of SR vesicles containing native Ca^{2+}-release channels were formed in solutions containing 250 mM CsOH and 140 mM PIPES, pH 7.2. For incorporation of proteoliposomes containing the purified ryanodine receptor, the following solution was used: 200 mM KCl, 20 mM HEPES, and KOH and HCl to pH 7.2 to give a solution of 210 mM K^+. Experiments were performed at 23 ± 1°C. Following bilayer formation, an osmotic gradient was established by the addition of 100 μl of 3 M KCl to the *cis* chamber. Either SR vesicles or proteoliposomes were added to the *cis* chamber and stirred. To aid fusion, a second aliquot (100 μl) of 3 M KCl was added to the *cis* chamber and stirred. After incorporation, the *cis* chamber was perfused with either the solution containing 250 mM Cs^+ for recording from native Ca^{2+}-release channels or 210 mM K^+ for recording from purified ryanodine receptors. The contaminant free [Ca^{2+}] in these solutions was 9.7 ± 1.1 μM (n = 8, SEM). The free [Ca^{2+}] and pH of the solutions were determined at 23°C using a calcium electrode (Orion 93-20) and Ross-type pH electrode (Orion 81-55) as described previously [17]. The [Ca^{2+}] on either side of the bilayer was increased by adding $CaCl_2$. The [Ca^{2+}] was lowered with EGTA and the free [Ca^{2+}] calculated using the computer program EQCAL (Biosoft, Cambridge).

Data Acquisition and Analysis

Single channel current fluctuations were displayed on an oscilloscope and recorded on Digital Audio Tape (DAT). The number of channels incorporated into a bilayer could be assessed at the end of an experiment by maximally activating the channels by sulmazole in the presence of 10 μM cytosolic Ca^{2+}. For analysis, data were low-

pass filtered at 2.5 kHz and digitized at 5 kHz using an AT-based system (Intracel, Cambridge, UK). This represents the level of filtering required to resolve the single channel events reduced in amplitude by addition of 4 mM Ca^{2+} to the luminal channel face. Channel P_o and the lifetimes of open and closed events were monitored by 50% threshold analysis. Under the above conditions, lifetimes with durations of less than 0.3 msec were not fully resolved and were therefore excluded from the fitting procedure. Lifetimes accumulated from approximately 3 min of steady-state recordings were stored in sequential files and displayed in noncumulative histograms. Individual lifetimes were fitted to a probability density function (pdf) using the method of maximum likelihood [3]. A missed events correction was applied [3] and a likelihood ratio test was used to compare fits to up to six exponentials [2]. Single channel current amplitudes were measured from digitized data using manually controlled cursors. Channel P_o values were obtained from three minutes of steady-state recording with the exception of P_o values used for P_o *vs.* voltage relationships where recordings of 30 sec were used.

Materials

All solutions were prepared using MilliQ deionized water (Millipore UK). ^{3}[H] ryanodine was obtained from New England Nuclear (Boston, MA) and aqueous counting scintillant from Amersham International (Amersham, UK). Sulmazole (AR-L 115BS) was a gift from Boehringer Ingelheim.

Results

The Channel Activated by Cytosolic Ca^{2+}

Effects of Luminal Ca^{2+} on Conductance

Figure 1 illustrates how increasing the luminal $[Ca^{2+}]$ from micromolar to millimolar concentrations reduces the single channel conductance. In symmetrical 250 mM Cs^+ with a cytosolic free Ca^{2+} of 10 μM, the conductance of the native channel is 462 ± 4.3 pS (SEM; n = 10). Increasing the luminal free $[Ca^{2+}]$ to 100 μM did not produce any measurable change in conductance. With millimolar luminal $[Ca^{2+}]$, deviation from the ohmic relationship occurs and the decrease in conductance is slightly more marked at negative potentials. A similar modification by Ca^{2+} of the conductance of the purified channel in symmetrical K^+ has been reported [23].

Effects of Luminal Ca^{2+} on Open Probability (P_o)

Figure 2 illustrates the effects of changing luminal $[Ca^{2+}]$ on native SR Ca^{2+}-release channels activated by 10 μM cytosolic Ca^{2+}. At least three channels have incorporated into the bilayer. Increasing luminal $[Ca^{2+}]$ from 10 to 100 μM and 2 mM and then reducing $[Ca^{2+}]$ to approximately 30 nM by addition of EGTA did not change P_o. Channels were completely closed by lowering the cytosolic free $[Ca^{2+}]$ to approximately 100 pM.

Figure 3 illustrates the general relationship between P_o and holding potential from six experiments where native SR Ca^{2+}-release channels were activated solely by 10 μM Ca^{2+}. The figure illustrates that the P_o of the cytosolic Ca^{2+}-activated channel is highly dependent on voltage. An approximately 6–10 fold increase in P_o occurs on changing the holding potential from −50 to +50 mV. Due to the instability of the bilayers containing HSR vesicles in Cs^+ solutions when held at potentials > +70 mV or < −70 mV, open probabilities for this relationship were monitored over 30 sec of recording.

The P_o at any voltage was not significantly altered by increasing luminal Ca^{2+} from 10 μM to 4 mM. To obtain a more accurate result of the possible differences in P_o at low and high luminal $[Ca^{2+}]$, we have monitored P_o over 3 min at −40 mV, before and after changing the luminal $[Ca^{2+}]$. At 10 μM luminal Ca^{2+}, the P_o of the cytosolic Ca^{2+}-activated channel was 0.065 ± 0.04 (SEM; n = 6) compared with 0.061 ± 0.03 (SEM; n = 6) at 2 mM luminal Ca^{2+}. Therefore, increasing the luminal $[Ca^{2+}]$ does not significantly alter open probability or the relationship between P_o and holding potential.

The Channel Activated by Sulmazole in the Absence of Cytosolic Ca^{2+}

At a subactivating cytosolic $[Ca^{2+}]$ (approximately 100 pM), the sheep cardiac SR Ca^{2+}-release channel remains completely closed. Previous experiments carried out with Ca^{2+} as the permeant ion (50 mM luminal) have demonstrated that the channel can then be opened in a cytosolic Ca^{2+}-independent manner by agents such as caffeine and sulmazole [18, 27]. The mechanisms involved in cytosolic Ca^{2+}-independent activation and cytosolic Ca^{2+} activation of the channel appear to be different. The cytosolic Ca^{2+}-activated channel is characterized by very brief openings, whereas the channel activated by caffeine or sulmazole at subactivating $[Ca^{2+}]$ exhibits long opening events. In this study, we have used sulmazole rather than caffeine to activate the channel in the absence of activating cytosolic Ca^{2+}, as it produces more reproducible effects at lower concentrations.

Conductance of the Channel Activated by Sulmazole in the Absence of Activating Ca^{2+}

Figure 4 illustrates a typical current-voltage relationship of the native channel in symmetrical 250 mM Cs^+ and 4 mM luminal Ca^{2+} activated by 10 μM cytosolic Ca^{2+}. No modification of single channel conductance was observed after reducing cytosolic $[Ca^{2+}]$ to picomolar levels and adding sulmazole (5 mM).

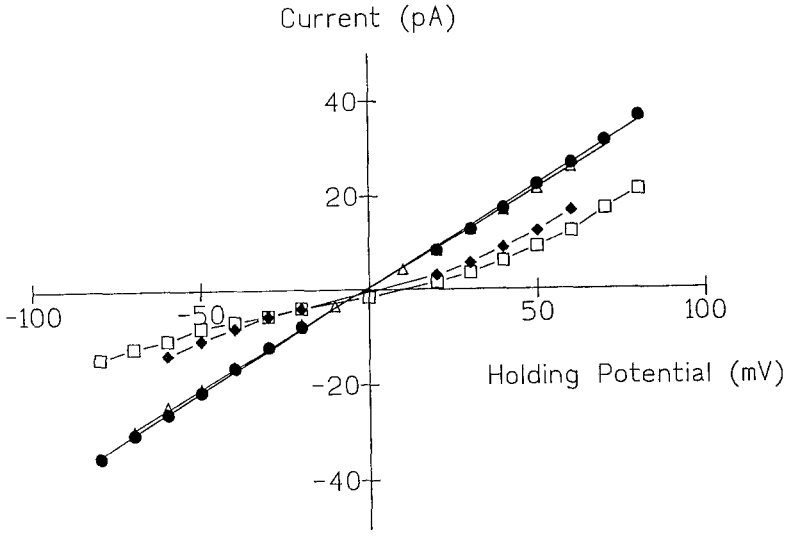

Fig. 1. Current-voltage relationship of the native Ca^{2+}-release channel in symmetrical 250 mM Cs^+ with the following luminal [Ca^{2+}]: 10 μM (circles), 100 μM (triangles), 4 mM (diamonds), 10 mM (squares).

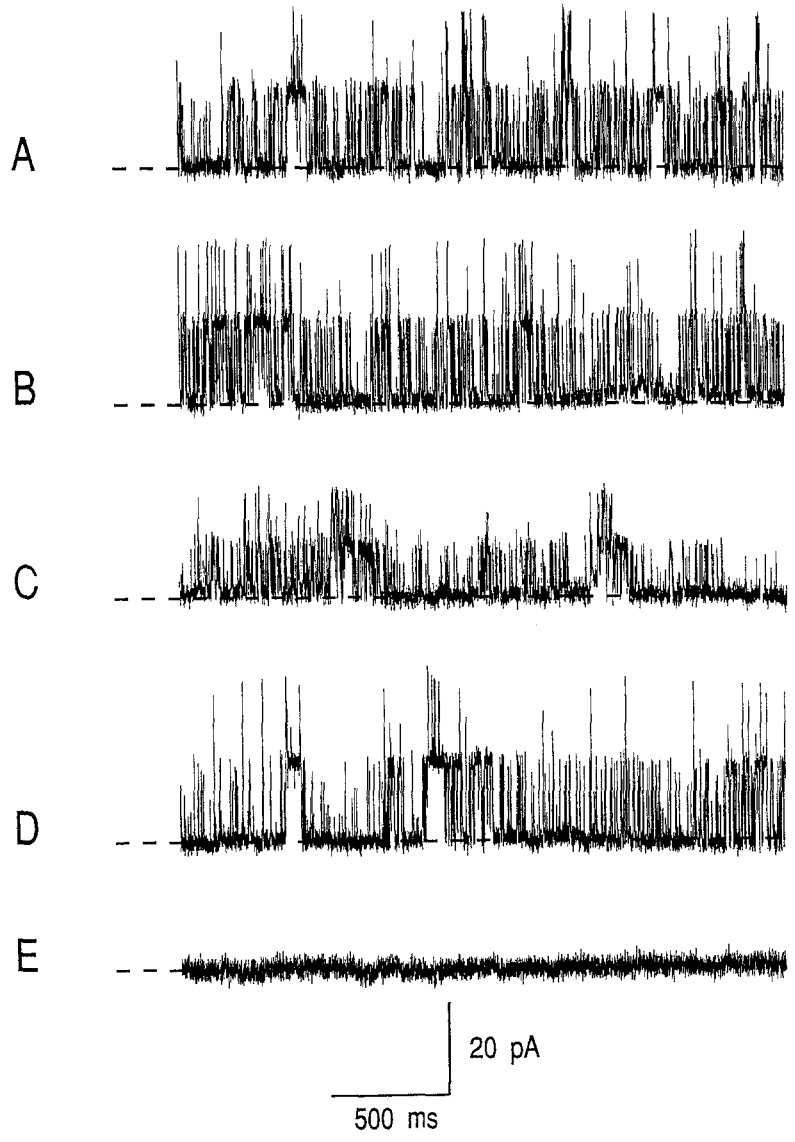

Fig. 2. Current fluctuations through native Ca^{2+}-release channels recorded from a single representative experiment in symmetrical 250 mM Cs^+ and voltage-clamped at −40 mV are shown. The dotted lines indicate the closed channel level. In the first trace (*A*), both the cytosolic and the luminal [Ca^{2+}] were 10 μM. Sequential changes to the luminal [Ca^{2+}] were made as follows: (*B*) 100 μM, (*C*) 2 mM, (*D*) approximately 30 nM (addition of 12 mM EGTA). Finally, the cytosolic [Ca^{2+}] was lowered to approximately 100 pM by addition of 12 mM EGTA (*E*).

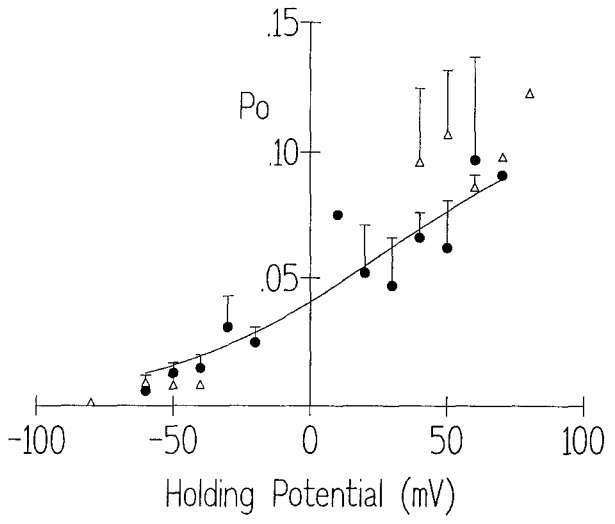

Fig. 3. Shows the P_o-voltage relationship of native Ca^{2+}-release channels in symmetrical 250 mM Cs^+ activated solely by cytosolic Ca^{2+} (10 μM) in the presence of 10 μM (circles) or 4 mM luminal Ca^{2+} (triangles). P_o was measured for 30 sec at each voltage. Each point is the mean of 3–6 experiments; standard error bars are shown on points where $n \geq 4$. The unbroken line represents the best fit to the control data (10 μM luminal Ca^{2+}) according to the equation $P_o = P_{o_{max}}/[1 + \exp(-BV/RT/F - C]$, where $P_{o_{max}}$ is the limiting P_o, B is the gating charge, C is the balance of equilibrium at zero mV and R, T and F have their usual meanings. The best fit gave a limiting P_o value of 0.124, a gating charge of −0.60 and the balance of equilibrium at 0 mV is −0.71. In view of the limited voltage range of these data, no significance should be given to the magnitude of the calculated value of the gating charge.

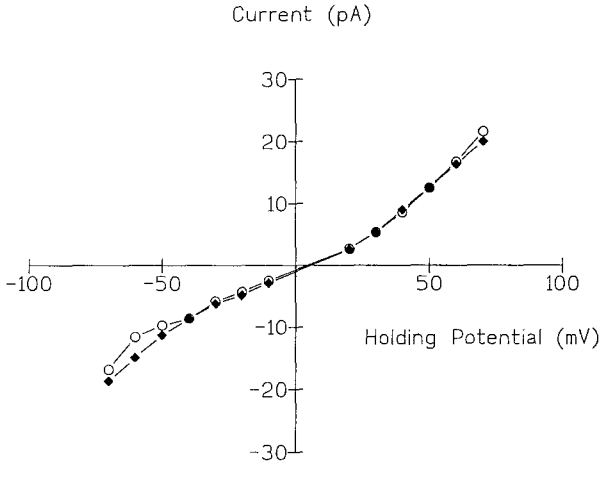

Fig. 4. The current-voltage relationship of a native Ca^{2+}-release channel in symmetrical 250 mM Cs^+ and 4 mM luminal Ca^{2+} activated by cytosolic Ca^{2+} (10 μM) (circles) or by sulmazole (5 mM) with approximately 100 pM cytosolic Ca^{2+} (diamonds).

Effects of Luminal Ca^{2+} on Open Probability

Figure 5 shows a typical example of a purified SR Ca^{2+}-release channel held at +40 mV and compares activation by cytosolic Ca^{2+} alone and activation by sulmazole in the absence of activating cytosolic [Ca^{2+}]. Initially, the luminal [Ca^{2+}] was 10 μM and the channel was activated by 10 μM cytosolic Ca^{2+}. The figure demonstrates the brief openings resulting from activation with Ca^{2+} as the sole ligand. The open probability and mean open time were 0.064 and 1.26 msec, respectively. Increasing the cytosolic [Ca^{2+}] to 20 μM elevated the P_o to 0.50 and resulted in only a small increase in the mean open lifetime to 2.65 msec. After reducing the cytosolic [Ca^{2+}] to approximately 100 pM by adding 12 mM EGTA, the channel completely closed. After adding sulmazole, very brief openings could again be observed similar to those occurring when the channel was activated by cytosolic Ca^{2+}. The P_o remained low even at 5 mM sulmazole and no increase in the duration of open times was observed (mean open lifetimes at 1, 2 and 5 mM sulmazole were 0.83, 0.8 and 0.64 msec). These experiments contrast with those of Williams and Holmberg [27] and Sitsapesan et al. [17] where Ca^{2+} was the permeant ion and sulmazole elicited long opening events in the absence of activating cytosolic Ca^{2+}. At picomolar cytosolic Ca^{2+}, 10 μM luminal Ca^{2+} and +40 mV, increasing the concentration of sulmazole up to 16 mM did not increase the duration of the open lifetimes any further. However, after addition of 2 mM Ca^{2+} to the luminal channel face, the P_o increased to 0.268 and long opening events similar to those reported by Williams and Holmberg [27] could be observed (mean open time 3.85 msec). This behavior occurred in 100% of the channels (6 purified channels; 13 native channels).

Lifetime analysis has demonstrated that the primary mechanism for a Ca^{2+}-induced increase in P_o is an increase in the frequency of channel opening [1]. Unless P_o approaches a near maximal value of 0.5, no increase in the duration of open events is observed in either native or purified channels [16, 19]. Even at high P_o values, the contribution by the increase in open lifetime duration to the increase in P_o is small. With a monovalent cation as the permeant ion, at least three open and between three to five closed states can be detected when native or purified sheep cardiac SR Ca^{2+}-release channels are activated by cytosolic Ca^{2+} alone. Under these conditions, P_o is low and open lifetimes are brief (for example, at 10 μM cytosolic Ca^{2+} $P_o = 0.038 \pm 0.011$ and the mean open lifetime duration = 0.96 ± 0.213 msec. SEM; $n = 6$). A typical example of the open and closed lifetime distributions of a Ca^{2+}-activated channel is shown in Fig. 6. At +40 mV and with 10 μM luminal Ca^{2+}, when the channel is activated by sulmazole at subactivating [Ca^{2+}], the distributions of open

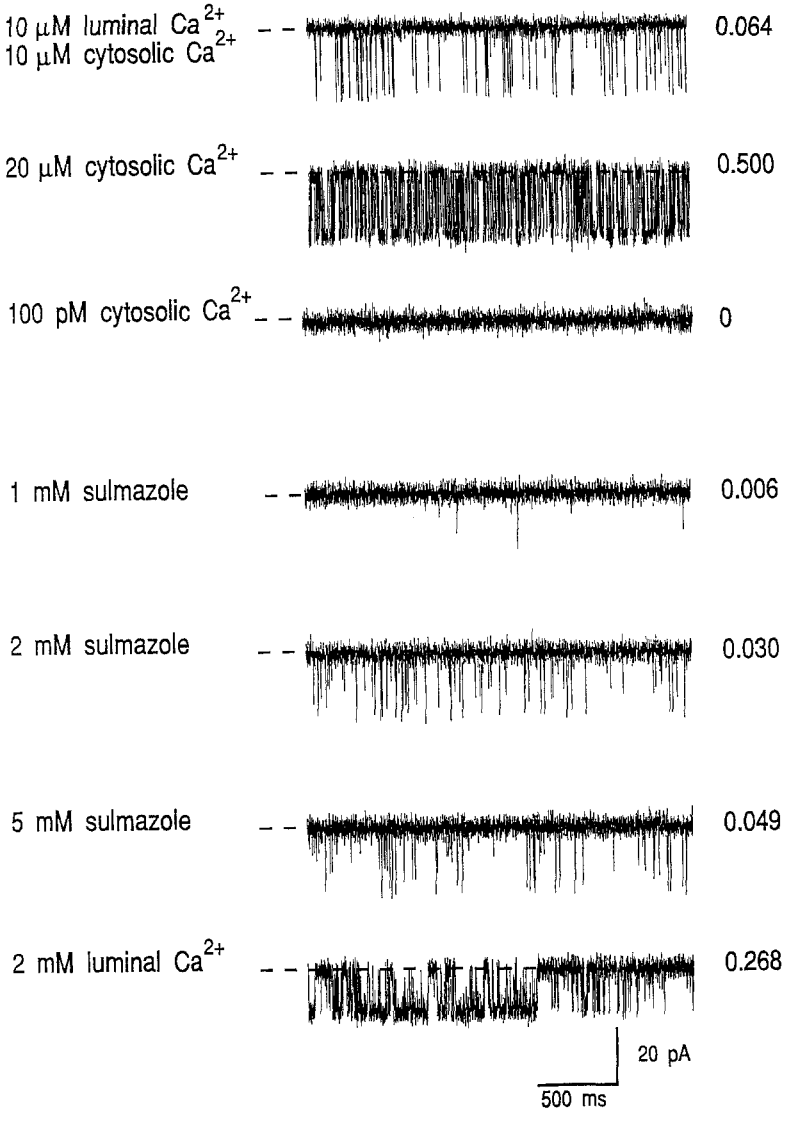

Fig. 5. Recordings from a typical single purified channel in symmetrical 210 mM K^+, voltage-clamped at +40 mV are shown. The dotted lines indicate the closed channel level. The channel was initially activated by 10 μM cytosolic Ca^{2+} in the presence of 10 μM luminal Ca^{2+}. Increasing the cytosolic [Ca^{2+}] increased P_o by increasing the frequency of channel opening with very little effect on the duration of open events. Addition of 12 mM EGTA to the cytosolic channel face lowered the free [Ca^{2+}] to approximately 100 pM and abolished channel openings. Increasing the concentration of sulmazole resulted in brief cytosolic Ca^{2+}-independent channel openings similar to those observed with cytosolic Ca^{2+} as the sole activator. After the addition of 2 mM luminal Ca^{2+}, a large increase in P_o was observed and many long open events occurred. Millimolar luminal Ca^{2+} resulted in a decrease in single channel conductance as shown in Fig. 2.

and closed lifetimes are similar to those of the cytosolic Ca^{2+}-activated channel (Fig. 7*A* and *B*). Under these experimental conditions, sulmazole is not very effective at opening the channel (P_o = 0.043 ± 0.019; mean open time 0.77 ± 0.07 msec. SEM; n = 7) and any small increase in P_o results solely from an increase in the frequency of channel opening; no increase in the duration of open lifetimes can be detected. This is similar to the mechanism of cytosolic Ca^{2+} activation of the channel and quite different to that previously reported for cytosolic Ca^{2+}-independent activation [18, 27]. However, this mechanism is uncovered following the addition of 2 mM Ca^{2+} to the luminal side of the channel. The resultant large increase in P_o is caused by an increase in the duration of open events in addition to a decrease in the duration of closed lifetimes. This mechanism is demonstrated in Fig. 7*C* (P_o = 0.244 ± 0.06; mean open lifetime 6.41 ± 2.48 msec. SEM; n = 7). Not only is the longest open time constant increased but a higher proportion of the overall number of open lifetimes now occurs to this time constant.

Voltage Dependence of Cytosolic Ca^{2+}-independent Channel Openings

As described above, the voltage dependence of the cytosolic Ca^{2+}-activated channel is such that the channel is more open at positive potentials regardless of the luminal [Ca^{2+}]. In contrast, at micromolar luminal [Ca^{2+}], when the channel is activated by sulmazole at subactivating [Ca^{2+}], the P_o of the channel is higher at negative holding potentials. At 10 μM luminal Ca^{2+}, the P_o of the sulmazole-activated channel was 0.061 ± 0.03

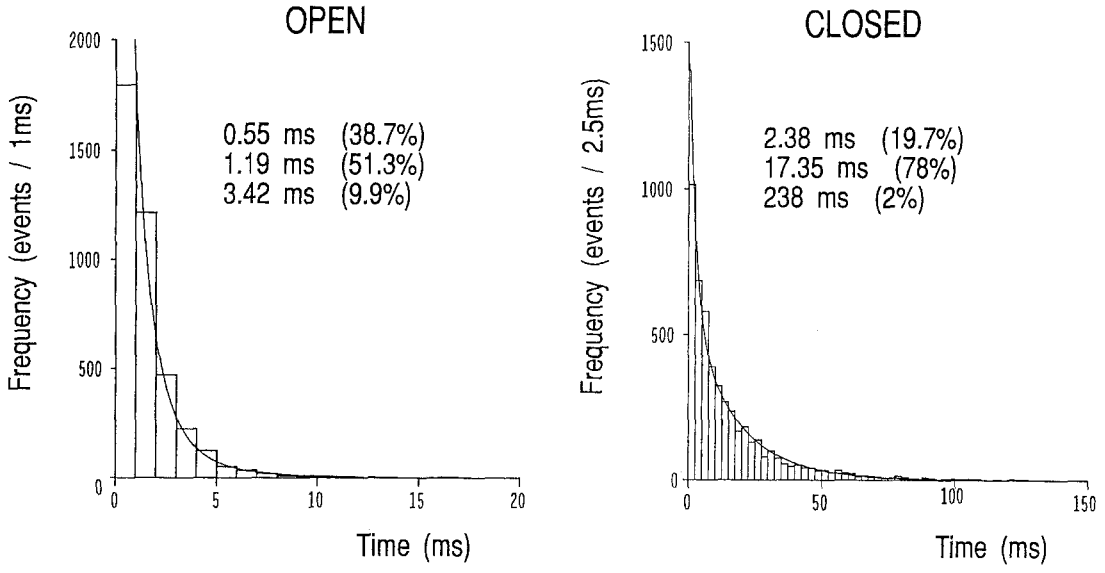

Fig. 6. Lifetime distributions and probability density functions (pdf) from the channel illustrated in Fig. 5 when activated by 10 μM cytosolic Ca^{2+} (P_o = 0.064). The best fit to the data was obtained with three exponentials to both the open and closed lifetime data. Open and closed time constants and percentage areas are shown. Lifetimes were determined as described in Materials and Methods. Probability density functions were fitted by the method of maximum likelihood according to the equation: $f(t) = a_1(1/\tau_1)\exp(-t/\tau_1) + \ldots + a_n(1/\tau_n)\exp(-t/\tau_n)$ with areas a and time constants τ.

at +40 mV compared to 0.182 ± 0.08 at −40 mV (SEM; n = 4). However, at millimolar luminal [Ca^{2+}], the P_o at all the voltages measured was substantially increased and was similar at positive and negative voltages (P_o = 0.387 ± 0.12 at +40 mV and 0.247 ± 0.081 at −40 mV, SEM; n = 6). The voltage dependence and concentration-dependent nature of the luminal Ca^{2+} effect is illustrated in Figs. 8 and 9. In Fig. 8, the current fluctuations through a bilayer containing three native channels are compared at + and −40 mV. At subactivating cytosolic [Ca^{2+}] (approximately 100 pM), the P_o is zero at all holding potentials (Fig. 8*B*). With the luminal [Ca^{2+}] still maintained at 10 μM, addition of sulmazole (3 mM, Fig. 8*C*) produced only occasional brief openings at +40 mV and P_o remained effectively zero. At −40 mV, again only occasional openings were observed but these were of longer duration and unlike the openings caused by cytosolic Ca^{2+} activation of the channel at + or −40 mV (*cf.* Fig. 8*A*). Increasing the luminal [Ca^{2+}] to 100 μM (Fig. 8*D*) resulted in an increase in the frequency and duration of the open events at both positive and negative voltages. At millimolar luminal [Ca^{2+}] (Fig. 8*E*), there was no further increase in the P_o at −40 mV, whereas at +40 mV a large increase in the open probability was observed to a level similar to that at −40 mV. Chelation of the luminal [Ca^{2+}] to approximately picomolar levels completely abolished the long openings and the P_o at all voltages became zero (Fig. 8*F*). Figure 9 summarizes how the P_o-voltage relationship of the channel activated by sulmazole in a cytosolic Ca^{2+}-independent manner can be modified by the luminal [Ca^{2+}]. Sulmazole activation of the channel at subactivating cytosolic [Ca^{2+}] exhibits an absolute requirement for luminal Ca^{2+}. At 100 pM luminal Ca^{2+}, the P_o at all voltages between + and −60 mV is zero. The concentration of luminal Ca^{2+} required to maximally activate the channels is lower at negative voltages.

Can Other Cations Mimic the Effects of Luminal Ca^{2+}?

Addition of luminal barium (Ba^{2+}) in concentrations up to 10 mM did not increase the P_o of the channel activated by sulmazole at subactivating cytosolic [Ca^{2+}]. Figure 10 illustrates a typical example. The top traces show the channel recordings at ±40 mV with 10 μM luminal [Ca^{2+}] in the presence of 5 mM sulmazole and subactivating cytosolic [Ca^{2+}]. Luminal Ba^{2+} (10 mM) does not increase P_o at positive or negative voltages. However, after subsequently adding 2 mM Ca^{2+} to the luminal side of the channel, a large increase in P_o was observed at both ±40 mV. This result was seen in three experiments. High luminal concentrations of the monovalent cations Cs^+ (1.1 M; n = 3) or K^+ (1.2 M; n = 3) (*results not shown*) also did not increase the P_o of channels activated solely by sulmazole in the presence of 10 μM luminal Ca^{2+}.

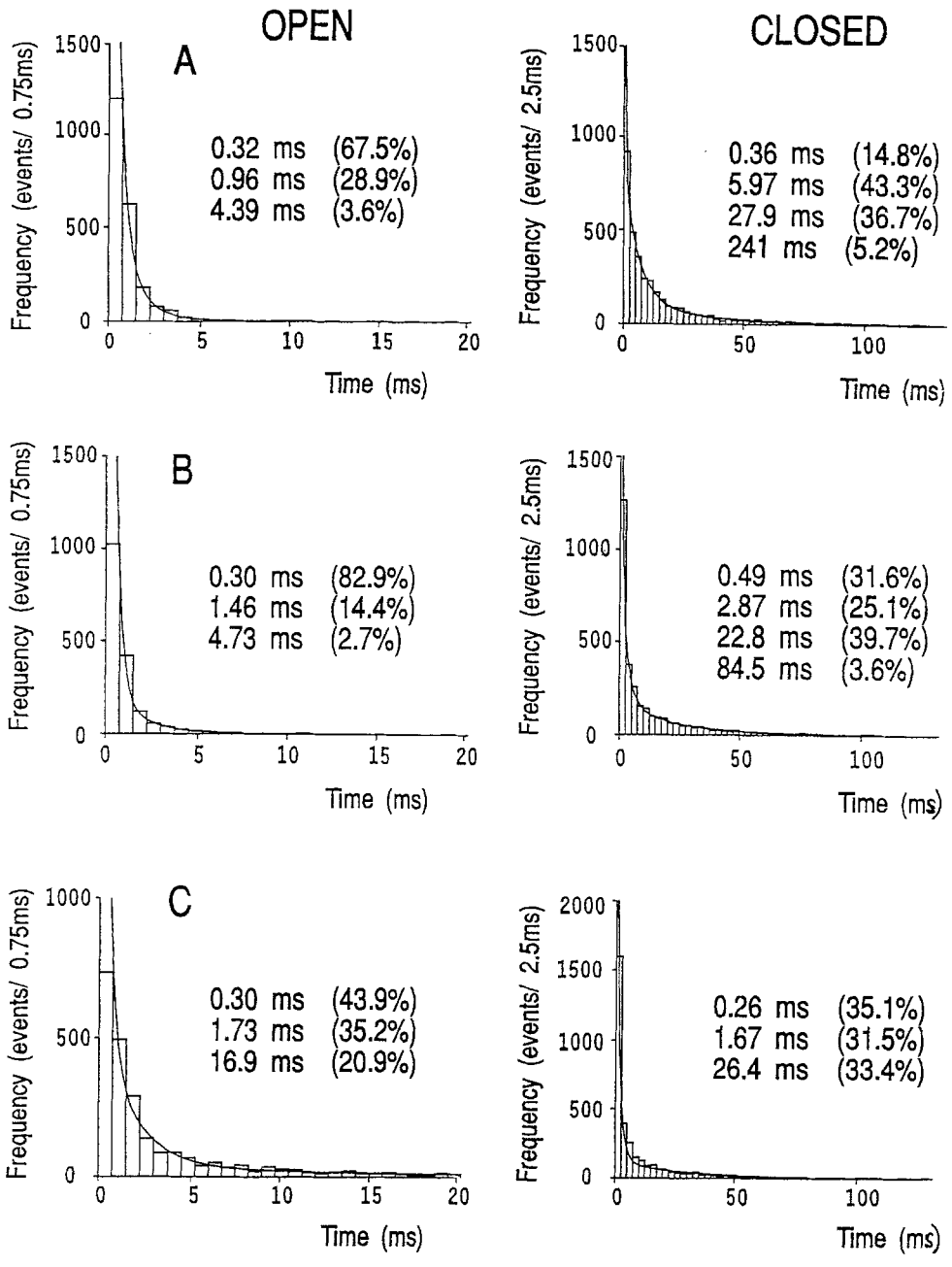

Fig. 7. Open and closed lifetime histograms and pdf's obtained from the channel illustrated in Fig. 5 when activated by sulmazole in the absence of activating [Ca^{2+}]. Time constants and percentage areas are shown at 2 mM sulmazole and 10 μM luminal Ca^{2+} (*A*), 5 mM sulmazole and 10 μM luminal Ca^{2+} (*B*) and 5 mM sulmazole and 2 mM luminal Ca^{2+} (*C*).

Discussion

We have examined the effects of changing luminal [Ca^{2+}] in both native and purified cardiac SR Ca^{2+}-release channels. Similar results were obtained in each case. These experiments confirm that the purified channel is not functionally damaged during our purification procedure and that the effects of luminal Ca^{2+} on the native channel are not dependent on other SR proteins or associated agents. The unexpected finding of this study is that the gating of channels activated solely by micromolar cytosolic Ca^{2+} is apparently unmodified by the luminal [Ca^{2+}], whereas the P_o of channels activated by sulmazole in the absence of cytosolic Ca^{2+} is completely dependent on the luminal [Ca^{2+}]. We have also demonstrated that the P_o of the Ca^{2+}-activated chan-

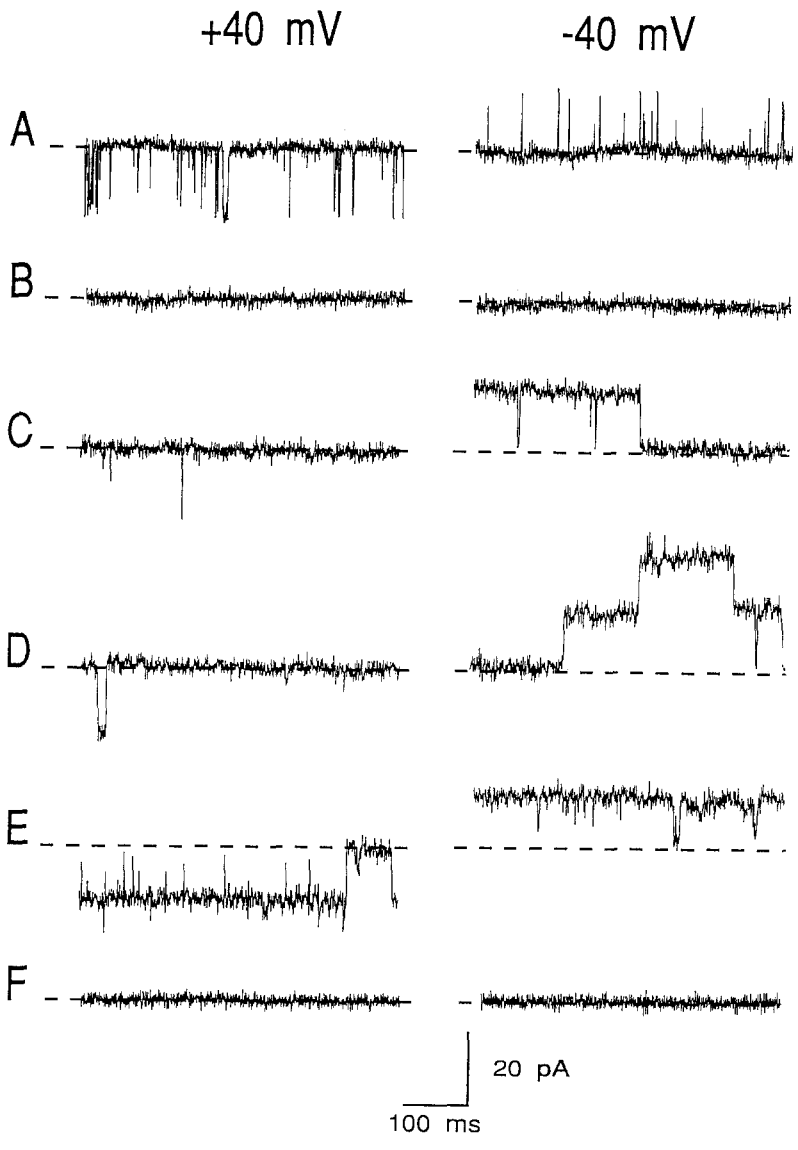

Fig. 8. A single representative experiment is shown at ±40 mV where three channels have incorporated into the bilayer. The dotted lines indicate the closed channel level. Initially, the channels were activated by 10 μM cytosolic Ca^{2+}; luminal $[Ca^{2+}]$ 10 μM (*A*). After addition of 12 mM EGTA (free $[Ca^{2+}]$ approximately 100 pM) to the cytosolic channel face, the P_o becomes zero (*B*). Under the conditions, addition of 3 mM sulmazole produced very occasional brief openings at +40 mV and long openings at −40 mV (*C*). Increasing the luminal $[Ca^{2+}]$ to 100 μM resulted in longer openings at both positive and negative potentials (*D*). At 2 mM luminal Ca^{2+}, a further increase in P_o occurred at +40 mV but not at −40 mV (*E*). After addition of 12 mM EGTA to the luminal side of the channel, all channel openings were abolished at positive and negative potentials (*F*).

nel is dependent on the holding potential and that this relationship is altered when the channel is activated by sulmazole in the absence of activating cytosolic Ca^{2+}.

Increasing the luminal $[Ca^{2+}]$ does not exert any significant effect on the P_o of sheep cardiac SR Ca^{2+}-release channels activated solely by cytosolic Ca^{2+}. In contrast, other groups record either a reduction in skeletal muscle Ca^{2+}-release channel P_o when the luminal Ca^{2+} levels are increased [7, 10, 12] or an increase followed by a decrease in P_o in a concentration-dependent manner [9]. Thus, the gating of skeletal and cardiac channels may be modified differently by changes in luminal $[Ca^{2+}]$. However, the experiments reported by Ma et al. [12] were performed on a channel displaying a single channel conductance of 400 pS. Under the ionic conditions of their experiments (250 mM KCl), the full conductance of the skeletal muscle ryanodine-receptor channel is closer to 800 pS (for example: [25, 28]). It is quite possible that the full conductance, purified ryanodine-receptor channel from skeletal muscle may not be modified by luminal Ca^{2+} to the same extent as the subconductance state. The report by Fill et al. [7] does not show conclusively that high levels of luminal Ca^{2+} close the skeletal muscle channel. Only three of five channels were closed by millimolar luminal $[Ca^{2+}]$. In addition, the effect of luminal Ca^{2+} was only investigated on a selected population of channels, those with a high control P_o. Therefore, these results may not be indicative of the normal regulatory effects of luminal Ca^{2+}.

The high dependence of the P_o of the native Ca^{2+}-activated cardiac Ca^{2+}-release channel on holding potential has not previously been demonstrated. Rousseau and Meissner [15] reported only a slight increase in P_o with increasing voltage. However, their experiments were performed with Ca^{2+} as the permeant ion and the

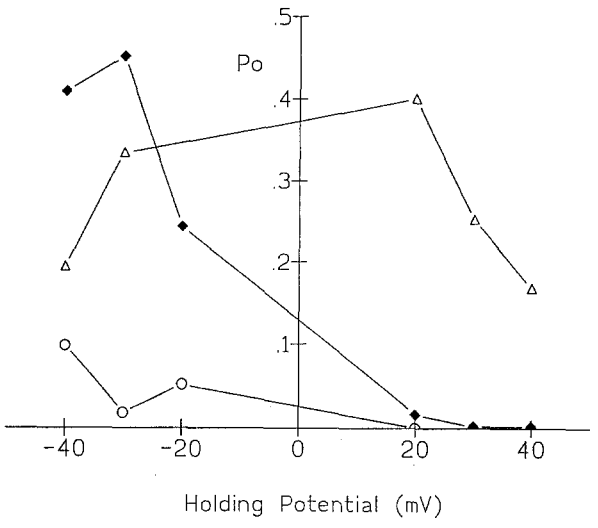

Fig. 9. Effect of luminal Ca^{2+} on the P_o-voltage relationship of the channel activated by sulmazole (3 mM) in the absence of activating cytosolic [Ca^{2+}] (100 pM free Ca^{2+}). At 10 μM luminal Ca^{2+}, the P_o is greater at negative potentials than at positive potentials (circles). Note that this relationship is different to that of the cytosolic Ca^{2+}-activated channel (Fig. 3). At 100 μM luminal Ca^{2+}, a very marked increase in P_o is observed at negative potentials with only a slight increase at positive potentials (diamonds). At 2 mM luminal Ca^{2+}, the P_o at all potentials are similar (triangles).

voltage range over which they monitored P_o (−40 to +20 mV) was very narrow. Since the single channel current reversed at approximately 30 mV, their data should be compared to our data over the range −70 to −10 mV. Over this limited voltage range, no marked change in P_o occurs. Ma et al. [12] report an increase in P_o with increasing voltage between +60 and +120 mV of a channel purified from skeletal SR. However, as discussed previously, their channel does not exhibit the full conductance of the Ca^{2+}-release channel and therefore the relevance of their data is questionable.

Our data also indicates that the P_o-voltage relationship of the cardiac SR Ca^{2+}-release channel may depend on the mechanism of channel activation. When the channel is activated by sulmazole in the presence of 10 μM luminal Ca^{2+} and picomolar cytosolic Ca^{2+}, P_o is higher at negative voltages. This relationship can then be modified by changing the luminal [Ca^{2+}] such that at 100 μM luminal [Ca^{2+}] the relationship is very steep.

Unlike the channels activated solely by cytosolic Ca^{2+}, channels activated by sulmazole alone are dependent on the presence of luminal Ca^{2+}. At picomolar [Ca^{2+}] at both the cytosolic and the luminal side of the channel, sulmazole, in concentrations up to 10 mM was unable to open the channel. However, as the luminal [Ca^{2+}] was increased, the channels could be opened in a concentration-dependent manner. At 10 μM Ca^{2+}, the open events at +40 mV were very brief and increasing the sulmazole concentration increased only the frequency of opening not the duration. No long open events were observed. Under these conditions, the mechanism of channel activation by sulmazole appears to be similar to that of Ca^{2+} activation; Ca^{2+} increases P_o by increasing the frequency of channel opening with very little effect on the duration of the open events.

If the luminal Ca^{2+} is subsequently increased to millimolar levels, then long open events can be observed which are quite different from those occurring when the channel is activated solely by cytosolic Ca^{2+}. The P_o-voltage relationship of the channel activated in a cytosolic Ca^{2+}-independent manner by sulmazole appears to be markedly altered by increasing the luminal [Ca^{2+}]. Lower concentrations of Ca^{2+} are required at negative voltages to lengthen the open events and increase P_o than are required at positive potentials. Therefore, the interaction of luminal Ca^{2+} with the sulmazole-bound conformation of the channel may be modifying the voltage dependence of the channel. However, as the voltage range over which P_o can be monitored is limited, it is not possible to comment on whether the Boltzmann relationship is merely shifted to the right as the [Ca^{2+}] is increased or if it is altered in some other respect. One possible explanation for the change in the P_o-voltage relationship that occurs when luminal Ca^{2+} is increased is that luminal Ca^{2+} binds to a gating regulatory site within the channel pore. The [Ca^{2+}] at this site would be increased at negative holding potentials as Ca^{2+} is driven through the channel.

As Ba^{2+} is unable to substitute for Ca^{2+} and high concentrations of the monovalent cations K^+ or Cs^+ have no apparent effect on the sulmazole-bound channel, the effects of luminal Ca^{2+} are unlikely to be caused by changes in surface potential or due to an increase in the concentration of permeant ions.

We do not believe that the elevation of P_o of the sulmazole-bound channel brought about by luminal Ca^{2+} results from Ca^{2+} binding to the cytosolic activation site. If luminal Ca^{2+} had access to this site, it would not be possible to close channels in the absence of sulmazole simply by lowering the cytosolic free [Ca^{2+}] if the luminal [Ca^{2+}] was high. However, at 50–80 mM luminal Ca^{2+} in the absence of activating cytosolic ligands, the P_o of cardiac SR Ca^{2+}-release channels is zero [17, 18, 27]. If luminal Ca^{2+} had access to the cytosolic activation site, increasing the luminal [Ca^{2+}] to millimolar levels would be expected to increase the P_o of channels activated solely by 10 μM cytosolic Ca^{2+} as this is not a maximally activating concentration of cytosolic Ca^{2+}. Again, changing the luminal [Ca^{2+}] appears to exert no significant effect on P_o under these conditions.

There are other lines of evidence that indicate that a luminal to cytosolic flow of Ca^{2+} will not significantly modify the P_o of either the cardiac or skeletal SR Ca^{2+}-

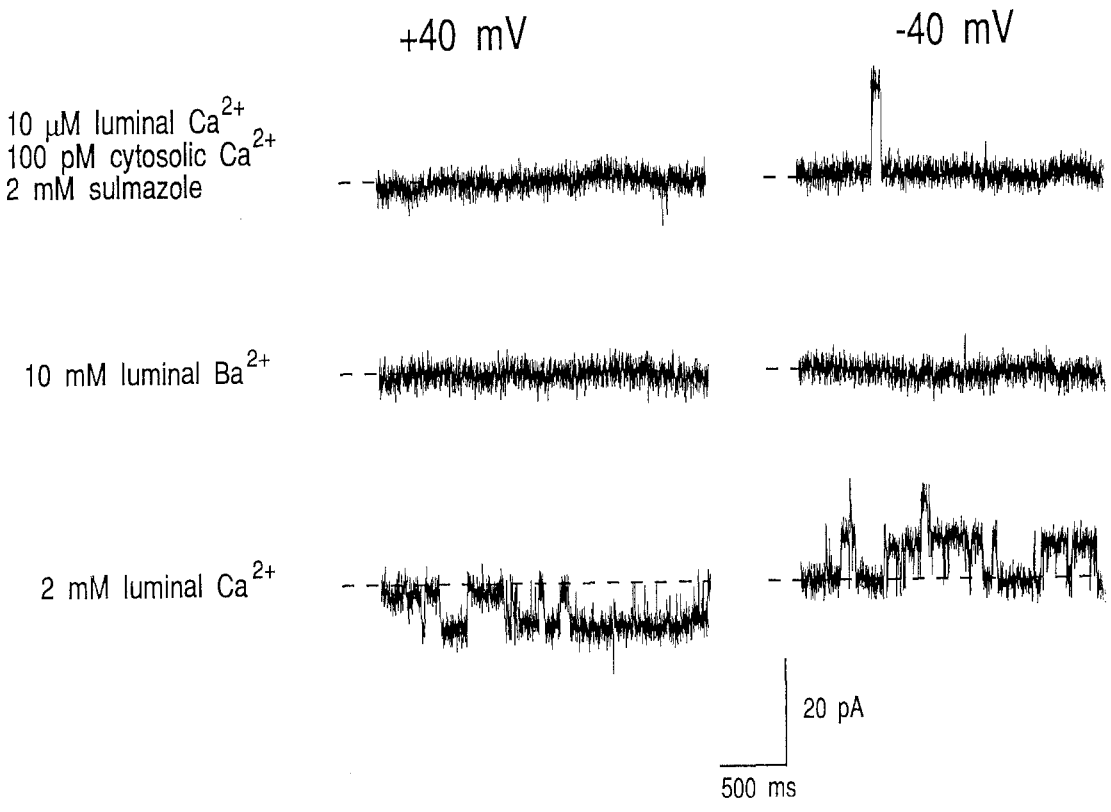

Fig. 10. Current fluctuations through native Ca^{2+}-release channels activated at subactivating cytosolic [Ca^{2+}] (approximately 100 pM) by 2 mM sulmazole in symmetrical 250 mM Cs^+ at ±40 mV. The dotted lines indicate the closed channel level. Luminal Ba^{2+} (10 mM) does not increase P_o at any voltage. After adding 2 mM luminal Ca^{2+}, long open events were observed at both positive and negative potentials.

release channel. When Mg^{2+} is added to the cytosolic side of cardiac or skeletal channels, P_o is reduced by competitive inhibition of Ca^{2+} at the Ca^{2+}-activation site [1, 22]. However, Mg^{2+} has a high relative permeability and carries significant luminal to cytosolic current in the Ca^{2+}-release channel [22, 26]. If luminal Mg^{2+} could reach the cytosolic site simply by flowing through the open channel, then one would expect channels activated by micromolar cytosolic Ca^{2+} to be closed when 50 mM luminal Mg^{2+} is used as the permeant ion; this is not the case [22, 26].

Why does luminal Ca^{2+} change the voltage dependence, the duration of open lifetimes and the open probability of channels activated by sulmazole in the absence of activating cytosolic Ca^{2+} without any apparent modification to the gating of channels activated solely by cytosolic Ca^{2+}? We can speculate that the binding of cytosolic Ca^{2+} and the binding of sulmazole may cause the channel to enter different conformational states, the sulmazole-bound conformation requiring the binding of Ca^{2+} for activation to occur. Another possibility is that the Ca^{2+}-binding site responsible for changes in open lifetime duration may not be accessible when the channel is in the cytosolic Ca^{2+}-bound conformational state.

What is the mechanism of sulmazole activation of the channel? It was previously suggested that sulmazole activation of the channel was complex and involved more than one mechanism [27]. What is clear from the present study is that Ca^{2+} is required for both the brief and the long channel openings. In the presence of picomolar levels of Ca^{2+} on both sides of the channel, sulmazole cannot elicit any openings. Therefore, what we previously termed Ca^{2+}-independent openings [18, 27] actually require the presence of luminal Ca^{2+} at micromolar or higher concentrations. The brief openings observed with sulmazole activation of the channel at picomolar cytosolic Ca^{2+} and 10 μM luminal Ca^{2+} may be explained by sensitization of the cytosolic Ca^{2+}-activation site, and the longer openings observed under different conditions may be caused by a different mechanism. However, we did not favor this explanation because it appears unlikely (as discussed previously) that Ca^{2+} from the luminal channel face can gain access to the cytosolic activation site. Alternatively, a single mechanism of activation may be occurring which de-

pends on at least three variables: the sulmazole and Ca^{2+} concentrations and the membrane potential.

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