# Gating of the Native and Purified Cardiac SR $Ca^{2+}$-Release Channel with Monovalent Cations as Permeant Species

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ABSTRACT The primary aim of this study was to characterize the steady-state gating of the native and the purified cardiac sarcoplasmic reticulum $Ca^{2+}$-release channel using monovalent cations ($K^+$ in the purified, $Cs^+$ in the native) rather than $Ca^{2+}$ as the permeant ions. The improved resolution of the single-channel events under these conditions has provided a more detailed and accurate description of channel gating than was previously possible. Micromolar cytosolic $Ca^{2+}$ activates the channel but in the absence of other activating ligands cannot fully open the channel. The relationship between the open probability (Po) and cytosolic free [$Ca^{2+}$] in both native and purified channels indicates the binding of at least three $Ca^{2+}$ ions for maximal activation. Lifetime analysis indicates a minimum of three open and five closed states for channels activated solely by $Ca^{2+}$ and demonstrates that the primary mechanism for the increase in Po is an increase in the frequency of channel opening. Burst analysis also indicates that $Ca^{2+}$ activates the channel by binding to closed states of the channel to increase the frequency of channel opening. Correlations between successive lifetimes suggest the existence of at least two pathways between the open and closed states. At a given activating [$Ca^{2+}$], the Po is lower at negative than at positive holding potentials; however, we find no change in the mechanisms of $Ca^{2+}$ activation at different voltages. Po measurements and lifetime analysis indicate that the gating of the purified channel when activated by $Ca^{2+}$ is indistinguishable from that of the native channel and indicate that the channels are not modified by the purification procedure.

## INTRODUCTION

The steady-state gating of cardiac and skeletal sarcoplasmic reticulum (SR) $Ca^{2+}$-release channels and activation by cytosolic $Ca^{2+}$ has been investigated in detail only under conditions where $Ca^{2+}$ is the permeant ion (Smith et al., 1986; Ashley and Williams, 1990). In such experiments, the mechanism for $Ca^{2+}$ activation in cardiac and skeletal channels appears to be similar. $Ca^{2+}$ as the sole ligand is unable to activate fully the channels and increases the open probability (Po) of both types of channels by increasing the frequency of channel opening. However, the open lifetimes of SR $Ca^{2+}$-release channels are very brief; 70–90% of events are too short to be resolved, and 90% of resolved events are close to the minimum resolvable duration. Hence, the resolution of the single-channel events is an important factor limiting the correct description of SR $Ca^{2+}$-release channel gating. Experimental evidence indicates that, unlike skeletal muscle, the mechanism for excitation-contraction (EC) coupling in cardiac muscle is $Ca^{2+}$-induced $Ca^{2+}$ release (Fabiato, 1985; Cannell et al., 1987; Nabauer et al., 1989; Beuckelmann and Wier, 1988; Bers, 1991). It is vital, therefore, to obtain an accurate description of cytosolic $Ca^{2+}$ activation of the cardiac SR $Ca^{2+}$-release channel. In this study we have investigated the mechanisms underlying $Ca^{2+}$ activation of the cardiac SR $Ca^{2+}$-release channel using a monovalent cation ($Cs^+$ in the native channel; $K^+$ in the purified channel) as the permeant ion. $K^+$ and $Cs^+$ exhibit similar behavior in the cardiac $Ca^{2+}$-release channel in terms of affinity, permeability, and conductance (Tinker et al., 1992b). The use of $Cs^+$ as the permeant ion in the native channel is possible because of the low conductance of this ion in the SR $K^+$ channel (Cukierman, et al., 1985). The larger conductance obtained using $K^+$(730 pS; Lindsay and Williams, 1991) or $Cs^+$ (460 pS; Sitsapesan and Williams, 1994) rather than $Ca^{2+}$ (90 pS; Ashley and Williams, 1990), as the conducting ion significantly improves the resolution of the single-channel events and therefore should provide more information on channel gating kinetics.

Using a monovalent cation as the permeant ion also allows us to investigate other aspects of channel gating. We have demonstrated that the open probability (Po) of the cardiac SR $Ca^{2+}$-release channel is dependent on the holding potential (Sitsapesan and Williams, 1994), and this phenomenon is more easily detected under symmetrical ionic conditions. We have therefore investigated whether $Ca^{2+}$ activation of the channel is modified by voltage. It is often assumed that the $Ca^{2+}$ flowing through the channel from the luminal to the cytosolic channel face, both during EC coupling in the intact cell and when channels are isolated and incorporated into bilayers, has access to the cytosolic activation site (Stern and Lakatta, 1992; Stern, 1992). This assumption has important consequences for EC coupling; however, we have experimental evidence to the contrary (Sitsapesan and Williams, 1994). In the early study of $Ca^{2+}$ activation of the cardiac SR $Ca^{2+}$-release channel (Ashley and Williams, 1990), $Ca^{2+}$ was used as the permeant ion. Under these conditions, $Ca^{2+}$ did not increase the duration of channel opening. It has been suggested that Ashley and Williams (1990) did not observe an increase in the duration of open lifetimes with increasing cytosolic [$Ca^{2+}$], because the high level of $Ca^{2+}$ flowing

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through the open channel from the luminal side (50 mM luminal $Ca^{2+}$) swamped the activation site (Stern and Lakatta, 1992; Stern, 1992). By using a monovalent cation as the permeant ion we can test this assumption by ensuring a very low [$Ca^{2+}$] flowing through the channel.

The purification of both cardiac and skeletal release channels has led to the publication of reports describing many variable effects of cytosolic $Ca^{2+}$ (e.g., Smith et al., 1988; Ma et al., 1988). Such effects may reflect damage to the channels during the purification procedure. However, where experiments are performed only on those channels that close completely at subactivating cytosolic [$Ca^{2+}$] and exhibit no subconductance states, the gating of the purified skeletal channel appears to be very similar to that of the native channel (Shomer et al., 1993). No report on the gating of the purified cardiac SR $Ca^{2+}$-release channel has been published except in abstract form (Sitsapesan et al., 1992), and therefore we have examined in detail the mechanisms of $Ca^{2+}$ activation of the purified channel using $K^+$ as the permeant ion.

Due to the improved resolution provided by the use of a monovalent cation as the permeant ion, the results of our experiments have yielded further information about $Ca^{2+}$ activation of the cardiac SR $Ca^{2+}$-release channel. Importantly, we have demonstrated that the purified channel exhibits gating characteristics similar to the native channel, indicating that our method of purification does not damage the channels. Our study provides a steady-state gating scheme for the cardiac channel, which can be used as the basis for further work investigating the physiological and pharmacological modification of cardiac SR $Ca^{2+}$-release channel gating.

## MATERIALS AND METHODS

### Preparation of SR membrane vesicles

SR membrane vesicles were prepared from sheep hearts obtained from a local abattoir as previously described (Sitsapesan et al., 1991). Membrane vesicles were frozen rapidly in liquid nitrogen and stored at −80°C.

### Purification of the $Ca^{2+}$-release channel

Ryanodine binds specifically and with high affinity to $Ca^{2+}$-release channels. This property and the availability of [$^3$H] ryanodine has made possible the purification of the ryanodine receptor and the demonstration that this protein is the SR $Ca^{2+}$-release channel (Lindsay and Williams, 1991). The ryanodine receptor was solubilized with 3-((3-cholamidopropyl)-dimethylammonio)-1-propane sulphonate (CHAPS) as previously described by Lindsay and Williams (1991). 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 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 h on ice, unsolubilized material was sedimented by centrifugation for 45 min at 36,000 rpm in a Sorvall T-875 rotor (Newtown, CT).

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 that 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 (Lindsay and Williams, 1991) 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 clamped at various potentials relative to ground using Ag-AgCl electrodes and bridges containing 2% agar in 3 M LiCl. Current flow through the bilayer was measured using an operational amplifier as a current-voltage converter (Miller, 1982).

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 either the above $Cs^+$ solution or 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. After 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 $K^+$ or 250 mM $Cs^+$ for recording from purified ryanodine receptors. The native SR $Ca^{2+}$-release channel incorporates into the membrane in a fixed orientation such that the *cis* chamber always corresponds to the cytosolic side and the *trans* chamber to the luminal side of the channel. The free [$Ca^{2+}$] and pH of all solutions were determined at 23°C using a calcium electrode (93–20, Orion Research, Boston, MA) and Ross-type pH electrode (81–55, Orion) as described previously (Sitsapesan et al., 1991). Cytosolic or *cis* $Ca^{2+}$ was increased by additions of $CaCl_2$. Subnanomolar $Ca^{2+}$ concentrations were obtained by additions of EGTA and the free [$Ca^{2+}$] calculated using the computer program EQCAL (Biosoft, Cambridge, UK).

### Data acquisition and analysis

Single-channel recordings were displayed on an oscilloscope and recorded on Digital Audio Tape. For analysis, data were low-pass filtered at 4 kHz and digitized at 8 kHz. Channel Po and the lifetimes of open and closed events were monitored by 50% threshold analysis. Statistical significance of the difference between mean values of Po and mean lifetime duration were assessed using Student's t-test with $P < 0.05$ being regarded as significant. Under the above conditions, lifetimes with durations of <0.2 ms were not fully resolved and were therefore excluded from the fitting procedure. Lifetimes accumulated from approximately 3-min 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 (Colquhoun and Sigworth, 1983). A missed-events correction was applied as described by Colquhoun and Sigworth (1983). A likelihood ratio test was used to compare fits to up to six exponentials (Blatz and Magleby, 1986) by testing twice the difference in $\log_e$ (likelihood) against the $\chi$-squared distribution at the 1% level.

Autocorrelation analysis was carried out on sequential lifetime files to assess the correlation of successive dwell times (Labarca et al., 1985; Kerry et al., 1987). For the following sequence of dwell times $y_1, y_2, \cdots, y_n$, mean $y$, the autocorrelation coefficient ($r_k$) for the $k$th event was estimated by

$$r_k = \frac{\sum_{i=1}^{n-k} (y_i - \bar{y})(y_{i+k} - \bar{y})}{\sum_{i=1}^{n} (y_i - \bar{y})^2} \quad (1)$$

The 95% confidence limits were set at $-n^{-1} \pm 2n^{-0.5}$.

To analyze the bursting behavior of the channel the interval between bursts was defined and calculated according to the method of Magleby and Pallotta (1983) with slight modification to allow for up to five closed states.

Single-channel current amplitudes were measured from digitized data using manually controlled cursors. Channel Po values were obtained from 3 min of steady-state recording with the exception of Po values used for the Po-versus-voltage relationship, where recordings of 30 s were used.

## Materials

All solutions were prepared using MilliQ deionized water (Millipore, Harrow, UK). [$^3$H] ryanodine was obtained from New England Nuclear (Boston, MA). Aqueous counting scintillant was purchased from Canberra Packard (Pangbourne, Berkshire, UK).

## RESULTS

### Orientation of the purified cardiac SR $Ca^{2+}$-release channel in the bilayer

Native SR $Ca^{2+}$-release channels always incorporate into the bilayer with the cytosolic channel face on the *cis* side of the bilayer (Ashley and Williams, 1990). However, with the purified channel, other investigators have suggested that the channel protein may incorporate in mixed orientations (Smith et al., 1988). To investigate the cytosolic $Ca^{2+}$ activation of the purified channel it is crucial to be certain of the orientation of the channels in the bilayer. Changes in both cytosolic and luminal [$Ca^{2+}$] are reported to modify Po (Ma et al., 1988; Fill et al., 1990), and therefore $Ca^{2+}$ activation is not a reliable test of the orientation of the channel. Lindsay et al. (1991) and Tinker et al. (1992a) have demonstrated that the purified cardiac $Ca^{2+}$-release channel is only blocked by tetraethyl ammonium (TEA) from the *cis* side of the bilayer, never from the *trans* side. These experiments demonstrate that the purified channel does incorporate into the bilayer in a fixed orientation but do not tell us whether this is the same orientation in which the native $Ca^{2+}$-release channel fuses with the bilayer. Using $Cs^+$ as the permeant ion we now find that the native $Ca^{2+}$-release channel is also blocked only from the *cis* side. Fig. 1 *i* illustrates current fluctuations through typical native channels in symmetrical 250 mM $Cs^+$. 20 mM TEA, when added to the *trans* chamber has no effect on single-channel conductance, whereas a reduced conductance at positive potentials is observed when TEA (20 mM) is added to the *cis* chamber. The corresponding current-voltage relationships are shown in Fig. 1 *ii*. This experiment was repeated twice. We are therefore certain that the native and the purified channel incorporate into the bilayer in the same fixed orientation, the *cis* side corresponding to the cytosolic channel face and the *trans* side to the luminal face.

### Basic gating model for native $Ca^{2+}$-activated channels where a monovalent cation is the permeant ion

The luminal [$Ca^{2+}$] was maintained at 10 $\mu$M throughout all the experiments and steady-state lifetime and Po measurements were performed over 3-min periods at + and −40 mV. The presence of $Cl^-$ channels in the SR membrane prevents the routine use of $Cl^-$ as the anion in the recording solution. To record current flow through the $Ca^{2+}$-release channel in

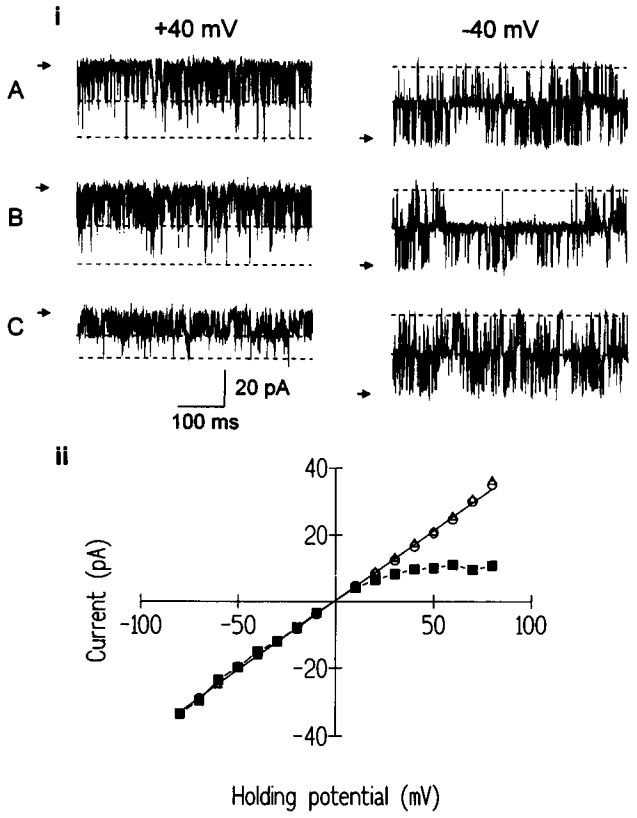



FIGURE 1 The effect of TEA in the native channel in symmetrical 250 mM CsPIPES solutions. (*i*) Single-channel fluctuations from an experiment where at least two channels have incorporated into the bilayer. The arrows indicate the zero current level, and the dotted lines indicate the open channel levels. The control situation is shown in (*A*) where the channels are activated by 10 $\mu$M cytosolic $Ca^{2+}$. TEA (20 mM) was then added to the *trans* chamber (*B*) with no effect on single-channel conductance. After the subsequent addition of TEA (20 mM) to the *cis* chamber (*c*) a reduction in the single-channel current amplitude can be observed at +40 mV. (*ii*) Current-voltage relationship of the native channels in symmetrical 250 mM CsPIPES in the absence of TEA (○), after 20 mM *trans* TEA (△) and after 20 mM *cis* TEA (■).

CsCl solutions, it is necessary to fuse the $Ca^{2+}$ channels in the absence of $Cl^-$ channels or use asymmetric solutions and hold the membrane at the $Cl^-$ reversal potential. It is therefore more practical to replace $Cl^-$ in the recording solution with an anion impermeant in the SR $Cl^-$ channel. However, the effect of changing the anion on channel gating behavior is not known. We have therefore compared the gating of single SR $Ca^{2+}$-release channels in symmetrical 250 mM CsCl (in those experiments where no $Cl^-$ channels were incorporated) with that of channels in symmetrical 250 mM CsPIPES. The Po of channels held at +40 mV and activated by 10 $\mu$M cytosolic $Ca^{2+}$ was not significantly different in CsCl (0.054 ± 0.026, mean ± SE; $n$ = 4) compared with CsPIPES solutions (0.025 ± 0.014, mean ± SE; $n$ = 4). As reported for channels where $Ca^{2+}$ was the permeant ion, the mean open lifetime events were very brief; 0.60 ± 0.02 ms (mean ± SE) in CsPIPES compared with 0.65 ± 0.6 ms (mean ± SE, $n$ = 4) in CsCl. Lifetime analysis demonstrates that at 10 $\mu$M cytosolic $Ca^{2+}$, the open lifetime distribution

is best fitted by three exponentials for channels recorded either in CsCl or CsPIPES indicating that there are at least three open states. Most of the open events occur to the two shortest time constants. Table 1 compares the time constants and respective areas for the best fits to the data. In contrast, the closed lifetime distribution is very sensitive to Po. At very low Po values, the best fit to the data is obtained with five exponentials. As Po is increased four or three exponentials may provide the best fit. This is true for channels recorded both in CsCl and CsPIPES. Fig. 2 illustrates the relationship between Po and the number of exponentials providing the best fit to the closed lifetime distribution. Probability density functions, obtained by the method of maximum likelihood, were calculated according to the equation, $f(t) = a_1(1/\tau_1)\exp(-t/\tau_1) + \cdots + a_n(1/\tau_n)\exp(-t/\tau_n)$. A typical example of the PDF fitted to the closed lifetime distribution of a native channel in 250 mM CsCl and activated by 10 $\mu$M cytosolic $Ca^{2+}$ is:

$$f(t) = 0.09(1/0.20)\exp(-t/0.20) + 0.23(1/0.88)\exp(-t/0.88) + 0.44(1/4.19)\exp(-t/4.19) + 0.18(1/10.91)\exp(-t/10.91) + 0.06(1/66.0)\exp(-t/66.0)$$

The above PDF can be compared with a representative example of the closed time constants and respective areas of a channel recorded in 250 mM CsPipes at +40 mV with 10 $\mu$M free cytosolic $Ca^{2+}$ (see Table 3). These results indicate that replacing the $Cl^-$ in the recording solution has no observable effect on open probability or on the basic gating behavior of the channel.

## Basic gating model for purified SR $Ca^{2+}$-release channels

The Po and gating of the purified channel was investigated both in symmetrical 250 mM $Cs^+$ and in symmetrical 210 mM KCl with 10 $\mu$M cytosolic and luminal $Ca^{2+}$. The Po of purified channels in symmetrical $K^+$ and $Cs^+$ solutions was 0.031 ± 0.026 (mean ± SE, $n$ = 7) and 0.023 ± 0.006 (mean ± SE, $n$ = 4), respectively. The mean open lifetime was 0.83 ± 0.19 ms (mean ± SE, $n$ = 7) in symmetrical $K^+$ and 0.46 ± 0.25 ms (mean ± SE, $n$ = 4) in symmetrical $Cs^+$. Therefore, under these conditions no significant difference was observed by changing the permeant ion from $Cs^+$ to $K^+$.

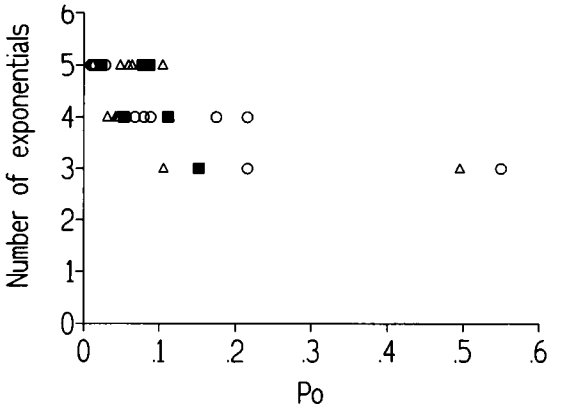



FIGURE 2 The relationship between the number of exponentials that provide the best fit to the closed lifetime distribution and the open probability of the channel when activated solely by cytosolic $Ca^{2+}$. The closed lifetimes were obtained from six native channels in CsPIPES (○), four native channels in CsCl (■), and four purified channels in KCl solutions (△).

The Po and mean open lifetime of the purified channel was also not significantly different from that of native channels (described in the previous section). As for the native channel, the open lifetime distribution was best fitted by three exponentials (Table 1), and the number of exponentials fitting the closed lifetime distribution depended on Po (Fig. 2) when either $K^+$ or $Cs^+$ was the permeant ion. At very low Po values five exponentials provided the best fit to the data. Therefore, our results indicate that the gating of the purified channel is essentially identical to that of the native channel under conditions where a monovalent cation is the permeant ion.

## Cytosolic $Ca^{2+}$ activation

### *Effect on open probability*

Figs. 3 and 4 illustrate the effects of increasing the cytosolic [$Ca^{2+}$] on the gating of a typical purified cardiac SR $Ca^{2+}$-release channel and a native channel, respectively. In all cases, at approximately 100 pM $Ca^{2+}$, no opening events were observed. Increases in Po were observed between 10 $\mu$M and 100 $\mu$M cytosolic $Ca^{2+}$ in both purified and native channels. The figures illustrate the characteristic brief opening events of both the native and purified channels. We would like to emphasize the variability between channels that we observed. The concentration of $Ca^{2+}$ required to maximally activate the channels varied between 20 and 100 $\mu$M $Ca^{2+}$. Such variability is a common phenomenon in single

**TABLE 1 Open lifetime parameters of native channels recorded in 250 mM CsPIPES ($n$ = 4) and 250 mM CsCl ($n$ = 4) and purified channels in 210 mM KCl ($n$ = 4) activated by 10 μM cytosolic $Ca^{2+}$**

| | $\tau_1$ | Area | $\tau_2$ | Area | $\tau_3$ | Area |
|---|---|---|---|---|---|---|
| CsPIPES | .20 ± .01 | 43 ± 5 | .58 ± .08 | 50 ± 2 | 1.88 ± .49 | 7 ± 5 |
| CsCl | .21 ± .02 | 64 ± 9 | .82 ± .23 | 26 ± 8 | 2.1 ± .42 | 10 ± 4 |
| Purified channel | .26 ± .04 | 65 ± 28 | 1.07 ± .32 | 31 ± 12 | 3.25 ± .76 | 4 ± 3 |

Time constants ($\tau_1$, $\tau_2$, $\tau_3$) and percentage areas were obtained from maximum likelihood fitting of the data. Mean values ± SE are shown.

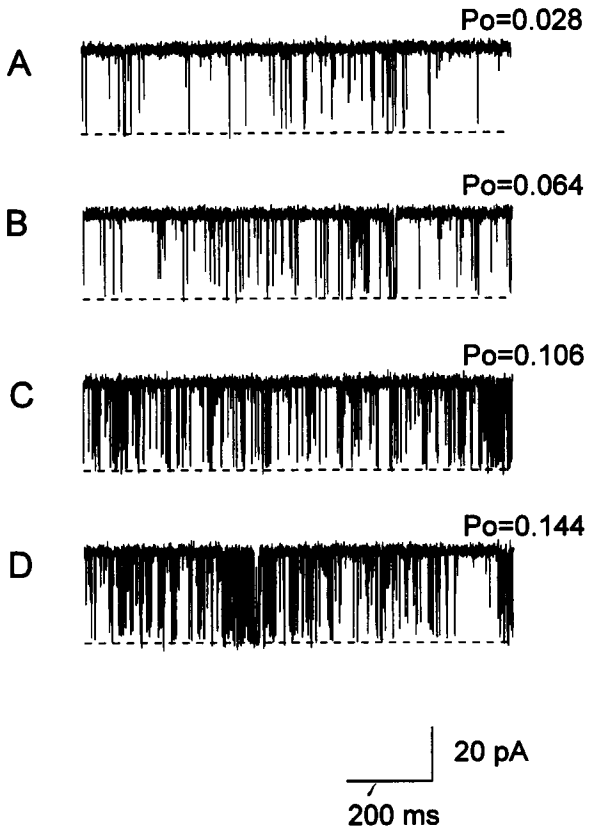



FIGURE 3 Cytosolic $Ca^{2+}$ activation of a typical purified $Ca^{2+}$-release channel recorded in symmetrical 210 mM KCl and voltage clamped at +40 mV. The dotted lines indicate the open channel level. Luminal [$Ca^{2+}$] was maintained at 10 $\mu$M, and the cytosolic free [$Ca^{2+}$] was increased sequentially by addition of $CaCl_2$ from 10 $\mu$M (*A*) to 20 $\mu$M (*B*), 40 $\mu$M (*C*), and 80 $\mu$M (*D*).

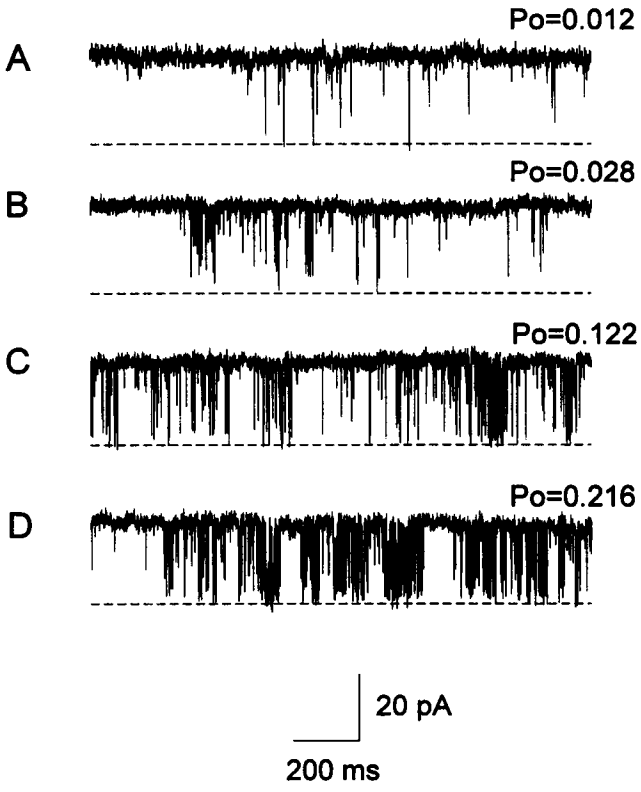



FIGURE 4 Current fluctuations through a single native $Ca^{2+}$-release channel recorded from a single representative experiment in symmetrical 250 mM $Cs^+$ and voltage clamped at +40 mV are shown. The dotted lines indicate the open channel level. As with the purified channel, the luminal [$Ca^{2+}$] was maintained at 10 $\mu$M, and the cytosolic free [$Ca^{2+}$] was increased from 10 $\mu$M (*A*) to 20 $\mu$M (*B*), 50 $\mu$M (*C*), and 100 $\mu$M (*D*). Fluctuations in the baseline noise result from the open and closing of SR $K^+$ channels, which incorporated into the bilayer together with the $Ca^{2+}$-release channel.

channel behavior (e.g., see McManus and Magleby, 1991). In skeletal SR $Ca^{2+}$-release channels a similar wide variability in $Ca^{2+}$-activated channel Po values was observed (Shomer et al., 1993). In experiments where $Ca^{2+}$ was used as the permeant ion, Ashley and Williams (1990) reported that $Ca^{2+}$ alone was unable to fully activate the native cardiac SR $Ca^{2+}$-release channel, and Po was rarely elevated above 0.5. We find the same behavior in native and purified channels using $Cs^+$ and $K^+$ as permeant ions. We also find no significant difference in the maximum Po levels observed with native and purified channels. The maximum Po at +40 mV for the native channels in CsPIPES was 0.306 ± 0.074; $EC_{50}$ = 43.9 ± 8.9 $\mu$M $Ca^{2+}$; (mean ± SE, $n$ = 6) and for the purified channels in KCl was 0.187 ± 0.081; $EC_{50}$ = 30.2 ± 9.0 $\mu$M $Ca^{2+}$ (mean ± SE, $n$ = 6). The corresponding Hill coefficients for $Ca^{2+}$ activation also varied from channel to channel (range 2.4 to 3.95; 3.13 ± 0.29, mean ± SE). These results indicate that the interaction of a minimum of three to four $Ca^{2+}$ ions is required for maximal activation of the channels under our experimental conditions.

The Po of the cardiac SR $Ca^{2+}$-release channel is dependent on the holding potential (Sitsapesan and Williams, 1994). When activated by cytosolic $Ca^{2+}$ alone the Po is greatest at positive potentials. To establish if the mechanism of $Ca^{2+}$ activation is different at positive and negative voltages we have compared Po values and open and closed lifetimes at +40 and −40 mV. Fig. 5 illustrates the relationship between Po and cytosolic [$Ca^{2+}$] for a purified channel at +40 and −40 mV and demonstrates that at all [$Ca^{2+}$] investigated, the Po is lower at the negative voltage.

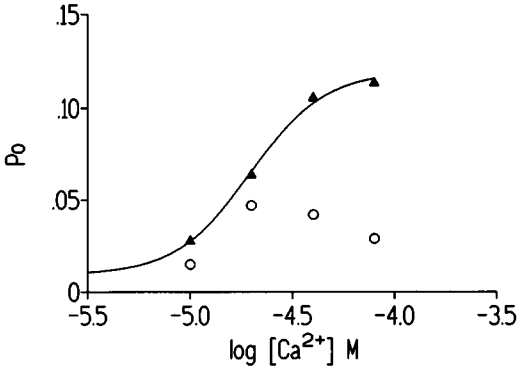



FIGURE 5 The relationship between Po and the free activating cytosolic [$Ca^{2+}$] for a representative purified $Ca^{2+}$-release channel. Po was measured in 210 mM symmetrical KCl at +40 mV (▲) and −40 mV (○).

**TABLE 2 Open lifetime parameters of native channels in 250 mM CsPIPES ($n$ = 6) and purified channels in 210 mM KCl ($n$ = 4) when maximally activated by cytosolic $Ca^{2+}$**

| | $\tau_1$ | Area | $\tau_2$ | Area | $\tau_3$ | Area |
|---|---|---|---|---|---|---|
| Native channel | .35 ± .09 | 36 ± 5 | 1.38 ± .49 | 56 ± 4 | 4.48 ± 1.69 | 8 ± 2 |
| Purified channel | 0.43 ± .18 | 71 ± 7 | 1.78 ± 1.0 | 27 ± 6 | 5.57 ± 2.7 | 2 ± 0.42 |

Time constants ($\tau_1$, $\tau_2$, $\tau_3$) and percentage areas obtained from maximum likelihood fitting of the data. Mean values ± SE are shown.

## Lifetime analysis

How do changes in cytosolic $Ca^{2+}$ modulate Po? The mean open lifetime at maximal activation was 1.64 ± 0.53 ms for purified channels and 1.18 ± 0.36 ms (mean ± SE, $n$ = 6) for native channels, which suggests a slight increase in duration compared with that at 10 $\mu$M $Ca^{2+}$. However, this is not a significant effect. Lifetime analysis indicates that the predominant mechanism for the $Ca^{2+}$-induced increase in Po is an increase in the frequency of openings. Table 2 details the average time constants and areas obtained at the maximum Po occurring with cytosolic $Ca^{2+}$ activation for purified and native channels. When compared with the open lifetimes at 10 $\mu$M $Ca^{2+}$ when Po is low (Table 1), it is clear that no significant change in the open lifetime distribution has occurred. However, although the percentage of events occurring to each time constant is not altered by increasing the $[Ca^{2+}]$, there may be a trend toward a small increase in the duration of the open events, particularly those of the longest time constant. This may only be obvious as Po approaches 0.5. However, Po was only elevated to such high levels in 3 of 12 experiments and therefore at present we cannot draw any firm conclusions about possible increases in open lifetime durations at high Po values. Fig. 6 illustrates the effect of cytosolic $Ca^{2+}$ on the open and closed lifetime distribution of a purified channel. In general, very little effect on open lifetimes is observed. However, we wanted to show an extreme example where Po is elevated to 0.496 to illustrate the maximum increase in open lifetime duration that we observe. Clearly, the predominant cause of the increase in Po is the decrease in the duration of closed lifetimes. As the $[Ca^{2+}]$ is increased, a progressive decrease in the duration of all the closed time constants occurs (Fig. 6) until only three or four closed time constants can be detected.

Table 3 gives the open and closed lifetime constants for a typical native channel in symmetrical CsPIPES at ± 40 mV and demonstrates that the mechanism for $Ca^{2+}$ activation is not altered by voltage. At comparable Po values the same number of open and closed states were detected with a similar proportion of events occurring to each state irrespective of voltage. Hence at positive and negative voltages $Ca^{2+}$ increases Po by increasing the frequency of channel opening with little effect on the duration of open states.

Previous work on the native cardiac SR $Ca^{2+}$-release channel using 50 mM luminal $Ca^{2+}$ solutions indicated only two open states and three closed states at all cytosolic-activating $[Ca^{2+}]$ (Ashley and Williams, 1990). The duration of open

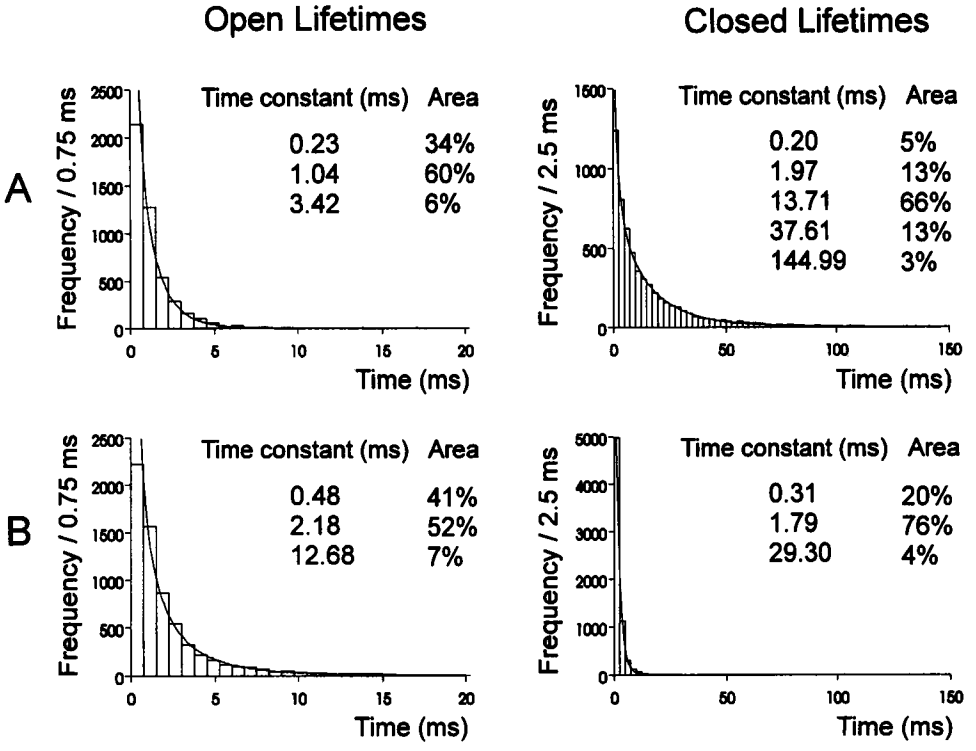



FIGURE 6 Lifetime distributions and PDFs from a purified channel when activated by 10 $\mu$M (Po = 0.048) ($A$) and 20 $\mu$M cytosolic $Ca^{2+}$ (Po = 0.496) ($B$). Open and closed time constants and percentage areas are shown. Lifetimes were determined as described in Materials and Methods. PDFs were fitted by the method of maximum likelihood according to the equation: $f(t) = a_1(1/\tau_1)\exp(-t/\tau_1) + \cdots + a_n(1/\tau_n)\exp(-t/\tau_n)$ with areas $a$ and time constants $\tau$.

**TABLE 3 Comparison of lifetime parameters from a channel held at ± 40 mV**

| | | Open lifetime distribution | | Closed lifetime distribution | |
|---|---|---|---|---|---|
| Holding potential | Po | Time constant (ms) | Area (%) | Time constant (ms) | Area (%) |
| +40 mV | | | | | |
| 10 μM $Ca^{2+}$ | .009 | 0.21 | 32 | 0.22 | 8 |
| | | 0.53 | 50 | 0.48 | 3 |
| | | 1.48 | 18 | 8.02 | 26 |
| | | | | 51.08 | 53 |
| | | | | 307 | 10 |
| 50 μM $Ca^{2+}$ | 0.056 | 0.22 | 59 | 0.22 | 1 |
| | | 0.76 | 30 | 0.69 | 36 |
| | | 1.77 | 11 | 10.93 | 59 |
| | | | | 157 | 4 |
| 100 μM $Ca^{2+}$ | 0.080 | 0.181 | 46 | 0.40 | 19 |
| | | 0.5 | 48 | 2.46 | 42 |
| | | 1.114 | 6 | 7.99 | 36 |
| | | | | 61.83 | 3 |
| −40 mV | | | | | |
| 10 μM $Ca^{2+}$ | 0.001 | Too few events for analysis | | | |
| 50 μM $Ca^{2+}$ | 0.015 | 0.23 | 50 | 0.19 | 8 |
| | | 0.55 | 44 | 1.56 | 6 |
| | | 1.23 | 6 | 3.21 | 18 |
| | | | | 21.4 | 60 |
| | | | | 156 | 8 |
| 100 μM $Ca^{2+}$ | 0.07 | 0.20 | 51 | 0.41 | 30 |
| | | 0.38 | 40 | 1.84 | 30 |
| | | 1.11 | 9 | 10.7 | 36 |
| | | | | 48.8 | 4 |

lifetimes was not increased with increasing Po. Therefore it was concluded that cytosolic $Ca^{2+}$ increased Po solely by increasing the frequency of channel opening. In the present study we find slightly different results. Are these dissimilarities caused by using $Cs^+$ or $K^+$ rather than $Ca^{2+}$ as the conducting ion or are they simply the result of improved resolution? In high-$Ca^{2+}$ solutions the poor signal-to-noise ratio necessitates a high level of filtering (1 kHz), and events shorter than 1 ms cannot be properly resolved (Sitsapesan and Williams, 1990). In $Cs^+$ or $K^+$ solutions the improved resolution allows events as short as 0.2 ms to be fully resolved. Therefore, we have filtered at 4 and 1 kHz data obtained from the channels recorded in $Cs^+$ and $K^+$ solutions and compared the subsequent lifetime analysis. The results of this analysis from a representative channel are displayed in Table 4. When the data obtained at 10 μM [$Ca^{2+}$] were filtered at 4 kHz, lifetime analysis indicated at least three open and five closed states. Filtering at 1 kHz we found that two exponentials represent the best fit to the open lifetime distribution. More than 90% of the events occurred to the shortest open state in each case, and no increase in the duration of open lifetimes was detected. Only three exponentials to the closed lifetime distributions could be identified at 10 μM [$Ca^{2+}$], and the time constants to all three were reduced at 50 μM [$Ca^{2+}$]. These results are identical to those observed by Ashley and Williams (1990) and Sitsapesan and Williams (1990) when the $Ca^{2+}$-release channels were recorded in high luminal $Ca^{2+}$ solutions and data were filtered at ≤1 kHz. We therefore suggest that the differences observed when $Cs^+$ or $K^+$ replace $Ca^{2+}$ as the permeant ion are the result of improved resolution rather than the introduction of different gating kinetics. The results of Sitsapesan and Williams (1994) confirm this explanation. Using $K^+$ or $Cs^+$ as the permeant ion, the Po and lifetime characteristics of both native and purified channels, when activated solely by cytosolic $Ca^{2+}$, are unchanged by increasing the luminal $Ca^{2+}$ to millimolar concentrations.

## Burst analysis

We have used the method of Magleby and Pallotta (1983) to calculate the interval between bursts and investigated the effects of increasing the cytosolic [$Ca^{2+}$] on the bursting behavior of the purified channel. As the Po of the channels was increased by increasing the [$Ca^{2+}$], we observed a decrease in the interburst interval. For example, at 10 μM $Ca^{2+}$ (average Po = 0.022) the interburst interval was 69.1 ± 16.7 ms ($n$ = 4) compared with 14.6 ± 3.74 ms ($n$ = 4) at the maximal channel activation occurring with $Ca^{2+}$ alone (Po = 0.187). In contrast, no significant change in the mean burst length (32.8 ± 13 ms at 10 μM $Ca^{2+}$; 28.0 ± 8.9 ms at maximal activation) or the mean number of openings/burst (5.2 ± 1.3 at 10 μM $Ca^{2+}$; 7.02 ± 1.5 at maximal activation, $n$ = 4) occurred. At 10 μM $Ca^{2+}$, the percentage of bursts consisting of a single opening was 34.6 ± 5.5%, and this value was unchanged when channels were maximally acti-

**TABLE 4 Effect of changing the level of filtering on the observed open and closed lifetime distributions**

| $Ca^{2+}$ ($\mu$M) | Po | Filter frequency (kHz) | Open lifetime distribution | | Closed lifetime distribution | |
|---|---|---|---|---|---|---|
| | | | Time constant (ms) | Area (%) | Time constant (ms) | Area (%) |
| 10 | 0.012 | 4 | 0.18 | 38 | 0.20 | 9 |
| | | | 0.38 | 57 | 2.51 | 8 |
| | | | 2.81 | 5 | 5.12 | 20 |
| | | | | | 37.4 | 51 |
| | | | | | 174 | 12 |
| | | 1 | 1.10 | 94 | 7.74 | 30 |
| | | | 8.88 | 6 | 82.8 | 51 |
| | | | | | 366 | 19 |
| 50 | 0.122 | 4 | 0.25 | 40 | 0.23 | 8 |
| | | | 0.78 | 54 | 1.1 | 23 |
| | | | 3.58 | 6 | 2.83 | 11 |
| | | | | | 6.95 | 50 |
| | | | | | 25.1 | 8.0 |
| | | 1 | 1.41 | 97 | 3.87 | 30 |
| | | | 8.7 | 3 | 12.2 | 57 |
| | | | | | 37.8 | 13 |

The same single-channel data were filtered at 4 kHz (digitized at 8 kHz) and at 1 kHz (digitized at 2 kHz).

vated by $Ca^{2+}$ (34.7 ± 4.7%) as was the number of openings/burst when unit openings were excluded (7.19 ± 1.6 at 10 $\mu$M $Ca^{2+}$; 9.92 ± 1.7 at maximal activation). No significant increase in the mean open time within bursts could be detected. This was 0.454 ± 0.058 ms (0.313 ± 0.031 ms for unit bursts) at 10 $\mu$M $Ca^{2+}$ and 0.596 ± 0.095 ms (0.358 ± 0.049 ms for unit bursts) when maximally activated. These values are close to the minimum resolvable duration, and therefore any real change in mean open time may be too small to be detected. Similarly, the mean open time of bursts consisting of a single opening appears to be no different from the mean open time within a burst consisting of two or more openings. These results confirm other lifetime analysis suggesting that the primary mechanism for $Ca^{2+}$ activation is an increase in the frequency of channel opening. $Ca^{2+}$ binds to closed channel states, and there is no evidence that the opening to any one particular open state is more $Ca^{2+}$ dependent than another.

## Autocorrelation analysis

Correlations between successive open or closed events may occur if there is more than one pathway between open and closed states. The number of pathways linking the open and closed states is equal to the sum of the geometrically decaying components describing the autocorrelation function (ACF) +1. Autocorrelation analysis revealed significant positive ACFs with correlations at small lag values with single-channel events obtained from both native and purified channels. A representative example of the correlation is illustrated in Fig. 7. No attempt was made to resolve the number of components to the ACF. Previous autocorrelation analysis performed on data obtained from the native $Ca^{2+}$-release channel under conditions where $Ca^{2+}$ was the permeant ion yielded similar results (Ashley and Williams, 1990; Sitsapesan et al., 1991).

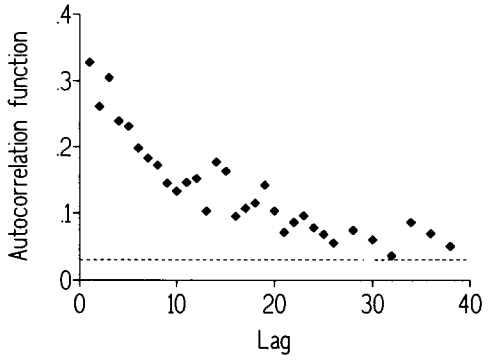



FIGURE 7 Closed lifetime autocorrelation function for up to every 40th closing (lag = 1–40) from a purified channel activated by 50 $\mu$M cytosolic $Ca^{2+}$. Events shorter than 0.2 ms were excluded from the analysis. The dotted line indicates the 95% confidence limit for zero correlation.

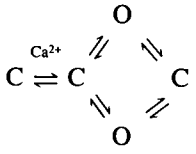
## DISCUSSION

We already know that the conductance of the purified sheep cardiac SR $Ca^{2+}$-release channel is identical to that of the native channel when compared in $Ca^{2+}$ or $Cs^+$ solutions (Lindsay and Williams, 1991; Williams, 1992). What is clear from the present study is that the gating of the purified channel appears to be no different from that of the native channel, and sensitivity to activating $Ca^{2+}$ is unchanged. In addition, we now know the fixed orientation with which the purified channel incorporates into bilayers. We have also demonstrated that replacing the $Cl^-$ content of the recording solutions with PIPES does not alter channel gating characteristics. Similarly, no difference in gating of the SR $Ca^{2+}$-release channel can be distinguished by using $K^+$ rather than $Cs^+$ as the permeant ion.

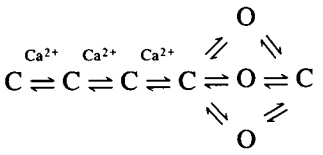
For both native and purified channels the relationship between Po and [$Ca^{2+}$] is extremely steep under the conditions

of our experiments. Hill coefficients obtained from data from both native and purified channels range from 2.4 to 3.95 indicating that the binding of at least three to four $Ca^{2+}$ ions is required for maximal activation. However, the relationship between Po and cytosolic [$Ca^{2+}$] reported by Ashley and Williams (1990) indicates that only one $Ca^{2+}$ ion may bind to the channel for maximal activation. This difference may be explained by the improved resolution of the single-channel openings in those experiments where a monovalent cation is used as the conducting ion. Both in $Ca^{2+}$ solutions and $K^+$ or $Cs^+$ solutions the open probability of the channel is fairly low when activated by cytosolic $Ca^{2+}$. Channel openings are very brief with a mean open lifetime of less than 1 ms. With $Ca^{2+}$ as the permeant ion and the large surface area of bilayers (300 $\mu$m diameter) used by Ashley and Williams (1990), only events with durations >2 ms could be resolved. Therefore, a steep rise in the number of channel openings may be masked by the level of noise and appear to be a more gradual effect. As the resolution is improved the true relationship between open probability and [$Ca^{2+}$] should become more apparent. The maximum Po achieved with cytosolic $Ca^{2+}$ as the sole ligand is similar irrespective of the permeant ion and luminal [$Ca^{2+}$] (cf. Ashley and Williams, 1990).

Ashley and Williams (1990) used $Ca^{2+}$ as the permeant ion and with the limited resolution of their experiments detected two open states and three closed states. $Ca^{2+}$ appeared to increase Po solely by increasing the frequency of channel opening. The channel could not be maximally opened with $Ca^{2+}$ alone, and autocorrelation analysis indicated a cyclical gating scheme. They therefore proposed the following gating scheme for the $Ca^{2+}$-release channel activated by cytosolic $Ca^{2+}$:




Improving the resolution of the open and closed lifetimes by using $Cs^+$ or $K^+$ as the permeant ion has demonstrated that the scheme is more complicated and clearly requires modification. There are at least five closed and three open states, and the slope of the Po − [$Ca^{2+}$] relationship indicates that a minimum of three or four rather than one $Ca^{2+}$ must bind for maximal activation. Previous experiments where the native cardiac SR $Ca^{2+}$-release channel was cooled to 10°C or lower had already indicated that more than two open states may exist (Sitsapesan et al., 1991). The principal mechanism for the increase in Po is an increase in frequency of openings. As the Po is progressively increased by $Ca^{2+}$, the duration of all the closed states is reduced, and eventually only three or four closed states can be detected. Autocorrelation analysis, although it confirmed the presence of a cyclical gating scheme, did not further clarify exactly how many pathways link the open and closed states. Therefore the simplest modification of the above scheme would be:




Sequential binding of $Ca^{2+}$ to the first three closed states would be required before the channel could open. This model would account for the fact that full activation cannot be achieved by cytosolic $Ca^{2+}$ alone and that when $Ca^{2+}$ activates the channel to a Po of up to approximately 0.3, an increase in the frequency of openings occurs with little change in the duration and proportion of openings to each time constant.

More difficult to explain is the possible trend toward an increase in the duration of openings when Po approaches 0.5. We could speculate that a $Ca^{2+}$-induced elevation in Po always leads to a slight increase in duration of the open states, but the level of resolution and the lack of sufficient numbers of the longer events prevents the detection of a significant increase or the presence of more than three open states. An increase in the duration of open lifetimes would indicate a $Ca^{2+}$-dependent pathway between two open states, and the above kinetic model would have to be modified to account for $Ca^{2+}$ binding to an open state and possible openings to a fourth open state from one of the longer of the three resolved open states. Obviously, this would have to be proved experimentally.

Ashley and Williams (1990) did not observe any $Ca^{2+}$-induced increase in the duration of open lifetimes. However, this would be expected given that they could not resolve events shorter than 2 ms. Under the experimental conditions of Ashley and Williams (1990), 70–90% of events are shorter than 2 ms and therefore are excluded from lifetime analysis (compared with 20–50% stripping of events in the present study). The results reported here suggest that, for steady-state gating, any increase in the duration of open lifetimes resulting from $Ca^{2+}$ activation would be from a value of 0.6 ms or less to a value close to 1 ms. To determine with any accuracy the degree to which an increase in open lifetime duration contributes to the increase in Po observed with $Ca^{2+}$ activation, one requires an even further increase in resolution. Another possible way of answering this question would be to cool the channels. This increases the duration of the single-channel events, and in particular the open events (Sitsapesan et al., 1991). Increasing the mean open time to well above the minimum resolvable time should allow a more accurate determination of open lifetime duration and should resolve the question of whether cytosolic $Ca^{2+}$ as a sole ligand can increase the duration of the open lifetimes.

In addition to obtaining a more detailed understanding of the mechanisms of $Ca^{2+}$ activation by using monovalent cations rather than $Ca^{2+}$ as the permeant ion, we have also demonstrated that the gating of the channel is essentially unaltered by changing the permeant ion (at the relatively high permeant ion concentrations used in this study) (Table 4).

These observations have important implications for EC coupling. This work confirms other experimental evidence that luminal $Ca^{2+}$ does not have direct access to the cytosolic $Ca^{2+}$ activation site (Sitsapesan and Williams, 1994). The experiments of Ashley and Williams (1990) were conducted with 50 mM luminal $Ca^{2+}$, and they measured current flow in the luminal to cytosolic direction. If the luminal $Ca^{2+}$ flow contributed to the $[Ca^{2+}]$ at the cytosolic activation site, then one would not expect to observe the $Ca^{2+}$ activation reported by Ashley and Williams (1990) with micromolar and submicromolar cytosolic $Ca^{2+}$. The activation site would simply be swamped by the $Ca^{2+}$ flowing through the open channel and near maximal Po would be obtained even at low micromolar cytosolic $[Ca^{2+}]$. One would also expect the Po-$[Ca^{2+}]$ relationship obtained using 50 mM luminal $Ca^{2+}$ to be different from that obtained using 10 $\mu$M luminal $Ca^{2+}$. However, the activation of the cardiac SR $Ca^{2+}$-release channel occurs over the same cytosolic $[Ca^{2+}]$ range with either $Ca^{2+}$ or monovalent cations as charge carrier, and maximum Po attained is unchanged. Stern (1992) has argued that open lifetimes do not change in these experiments because the activation site is swamped by $Ca^{2+}$ when the channel is open. We do not agree with this hypothesis. In the experiments reported here, luminal $Ca^{2+}$ was maintained at 10 $\mu$M. The open lifetimes observed with 10 $\mu$M luminal $Ca^{2+}$ and 50 mM luminal $Ca^{2+}$ at different cytosolic $[Ca^{2+}]$ are identical when the data are filtered at the same level and no change in the duration of open lifetimes can be detected. These results suggest that the luminal $Ca^{2+}$ flowing through the channel does not affect either open lifetime duration or Po when the channel is activated solely by cytosolic $Ca^{2+}$; presumably it does not have access to the cytosolic $Ca^{2+}$ activation site.

In summary, we have investigated the mechanisms involved in cytosolic $Ca^{2+}$ activation of the purified cardiac SR $Ca^{2+}$-release channel to determine if the gating of this channel is modified during the purification procedure. We have used the native channel as a standard and with the improved resolution of single-channel events resulting from the use of monovalent cations rather than $Ca^{2+}$ as the permeant ion we have extended our knowledge of the gating and cytosolic $Ca^{2+}$ activation of the $Ca^{2+}$-release channel. We have demonstrated that the $Ca^{2+}$ sensitivity and channel gating kinetics are unchanged using our method of purification. In the absence of SR $K^+$ and $Cl^-$ channels, recording current fluctuations through functional purified SR $Ca^{2+}$-release channels can now be achieved under physiological ionic conditions. Consequently, the present study provides a framework for further experiments aimed at elucidation of the role of the cardiac SR $Ca^{2+}$-release channel in EC coupling.

We would like to thank Dr. Allan Lindsay for the preparation of the purified cardiac $Ca^{2+}$-release channels and for the computer programming that enabled us to test for four or more exponential fits to the lifetime distribution and display of lifetime distributions and PDFs. We would also like to thank Dr. Andrew Tinker for preparation of the purified cardiac $Ca^{2+}$-release channel and for helpful discussion. The burst analysis was carried out using a single-channel analysis program written by John Dempster, Department of Physiology and Pharmacology, University of Strathclyde. This work was supported by the British Heart Foundation.

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