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The Gating of the Sheep Skeletal Sarcoplasmic Reticulum Ca^{2+}-release Channel is Regulated by Luminal Ca^{2+}

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Abstract. The effects of changes in luminal [Ca^{2+}] have been investigated in sheep skeletal sarcoplasmic reticulum (SR) Ca^{2+}-release channels after activation of the channels by different ligands from the cytosolic side of the channel. Native heavy SR membrane vesicles were incorporated into planar phospholipid bilayers under voltage-clamp conditions. Experiments were carried out in symmetrical 250 mM Cs^+. Lifetime analysis indicates that channels activated solely by cytosolic Ca^{2+} exhibit at least two open and five closed states. The open events are very brief and are close to the minimum resolvable duration. When channels are activated solely by cytosolic Ca^{2+}, luminal Ca^{2+} does not appear to exert any regulatory effect. The P_o and duration of the open and closed lifetimes are unchanged. However, if channels are activated by ATP alone or by ATP plus cytosolic Ca^{2+}, increases in luminal [Ca^{2+}] produce marked increases in P_o and in the duration of the open lifetimes. Our results demonstrate that maximum activation of the skeletal SR Ca^{2+}-release channel by ATP cannot be obtained in the absence of millimolar luminal [Ca^{2+}].

Key words: Sarcoplasmic reticulum — Skeletal muscle — Calcium-release channel — Ryanodine receptor — Channel gating

Introduction

We have established that the gating of the sheep cardiac sarcoplasmic reticulum (SR) Ca^{2+}-release channel can be modified by changing the luminal [Ca^{2+}] [18]. Importantly, the effects of luminal Ca^{2+} are dependent on the mechanism of cytosolic channel activation. The gating and open probability (P_o) of cardiac channels, which are activated by cytosolic Ca^{2+} as the sole ligand, appear to be unaffected by changes in luminal [Ca^{2+}]. In contrast, the P_o and the duration of open lifetimes of channels activated by the phosphodiesterase inhibitor, sulmazole, in the absence of activating cytosolic Ca^{2+}, are markedly increased with elevated luminal [Ca^{2+}]. These results indicate that Ca^{2+}, flowing through the channel from the lumen of the SR, is unlikely to have access to the activation site (at least in the absence of a restricted space around the channel). However, in the presence of an agonist other than Ca^{2+}, large changes in P_o may occur with changes in luminal Ca^{2+} [18]. This profound effect may have important consequences for excitation-contraction coupling (EC-coupling) in cardiac muscle.

The effects of changes in luminal [Ca^{2+}] on the gating of skeletal SR Ca^{2+}-release channels has not been examined in such detail. The mechanism for EC-coupling appears to be different in cardiac and skeletal muscle. Ca^{2+}-induced Ca^{2+}-release (CICR) is thought to be the mechanism in cardiac cells (for review *see* [3]) whereas in skeletal muscle a mechanical interaction between the voltage sensor (dihydropyridine receptor) and the Ca^{2+}-release channel may occur (for review *see* [13, 14]). Differences in gating of skeletal and cardiac channels have been reported [5] although unfortunately channels from the same species were not compared. Such differences are not unexpected given that only 66% homology exists between the primary sequences for cardiac and skeletal ryanodine receptors [12]. We have therefore investigated the effects of changing the luminal [Ca^{2+}] on the sheep skeletal SR Ca^{2+}-release channel when activated by cytosolic Ca^{2+} alone or by another ligand. Smith et al. [20] reported that ATP could activate the rabbit skeletal SR Ca^{2+}-release channel both in the presence and absence of activating levels of cytosolic Ca^{2+}. We have investigated the dependence of the sheep skeletal channel on luminal [Ca^{2+}] when activated under the

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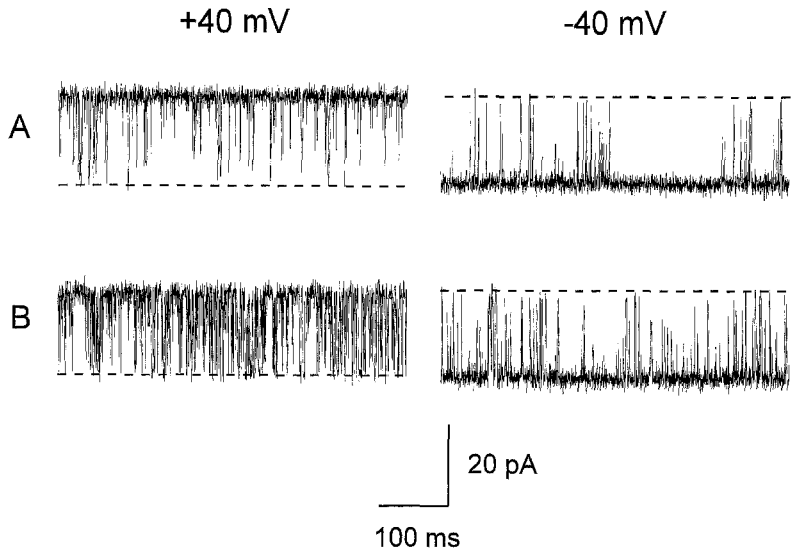

Fig. 1. Current fluctuations through a single skeletal SR Ca^{2+}-release channel recorded in symmetrical 250 mM Cs^+ solutions and held at ±40 mV. The broken lines indicate the open channel levels. In *A* the cytosolic and luminal free [Ca^{2+}] was 5 μM and the P_o was 0.022 and 0.012 at +40 mV and –40 mV respectively. In *B* the cytosolic free [Ca^{2+}] was increased to 15 μM and P_o increased to 0.35 and 0.07 at +40 mV and –40 mV respectively.

following conditions: (i) by cytosolic Ca^{2+} only, (ii) by ATP only and (iii) by ATP in the presence of activating cytosolic Ca^{2+}.

Our results demonstrate that luminal Ca^{2+} regulates the gating of channels activated solely by ATP or ATP plus cytosolic Ca^{2+} but has no significant effect on channels activated by cytosolic Ca^{2+} alone. We suggest that conformational changes resulting from the binding of cytosolic agonists determine the ability of luminal Ca^{2+} to increase P_o. The effects of luminal Ca^{2+} appear to be mediated by specific binding sites on the luminal face of the channel and not through screening of surface charge, binding of Ca^{2+} at a site in the pore or interaction with the cytosolic Ca^{2+} activation site.

Materials and Methods

Preparation of SR Membrane Vesicles

SR membrane vesicles were prepared from sheep skeletal muscle obtained from a local farm as previously described for cardiac SR vesicles [16]. Membrane vesicles were frozen rapidly in liquid nitrogen and stored at –80°C.

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 [10].

Bilayers were formed in solutions containing 250 mM CsOH and 140 mM PIPES, pH 7.2. 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. SR vesicles 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 the solution containing 250 mM Cs^+. In experiments where 50 mM luminal Ba^{2+} was used as the permeant ion in the absence of Cs^+, the *cis* and *trans* recording solutions were (in mM): 250 HEPES, 125 Tris, pH 7.2 and 250 glutamic acid, 10 HEPES, pH to 7.2 with $Ba(OH)_2$ respectively. The native SR Ca^{2+}-release channels incorporate into the membrane in a fixed orientation such that the *cis* chamber corresponds to the cytosolic side and the *trans* chamber to the luminal side of the channel (*see* Results). The free [Ca^{2+}] and pH of all solutions were determined at 23°C using a calcium electrode (Orion 93–20) and Ross-type pH electrode (Orion 81–55) as described previously [16]. Cytosolic or luminal 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 (DAT). To obtain maximum information about the open and closed lifetime distributions when recording in symmetrical 250 mM Cs^+, data were low-pass filtered at 4 kHz and digitized at 8 kHz using an AT-based system (Intracel, Cambridge, UK). Under these conditions, lifetimes with durations of less than 0.2 msec were not fully resolved and were therefore excluded from the fitting procedure. In the majority of experiments however, a divalent cation was added to the luminal side of the channel thereby reducing the single channel conductance and therefore the signal-to-noise ratio. To compare the gating of the channel in the presence of different luminal divalent cation concentrations the data were filtered at 2 kHz and digitized at 4 kHz. At this level of filtering, the minimum resolvable event duration was 0.35 msec. Channel P_o and the lifetimes of open and closed events were monitored by 50% threshold analysis. Lifetimes accumulated from approximately 3 min of steady-state recordings were stored in sequential files and displayed in noncumulative histograms. Lifetime analysis was performed only when a single channel was present in a bilayer. To test for a single channel, millimolar cytosolic ATP and millimolar luminal Ca^{2+} were added to fully open any channels in the bilayer. Individual lifetimes were fitted to a probability density function (pdf) using the method of maximum likelihood

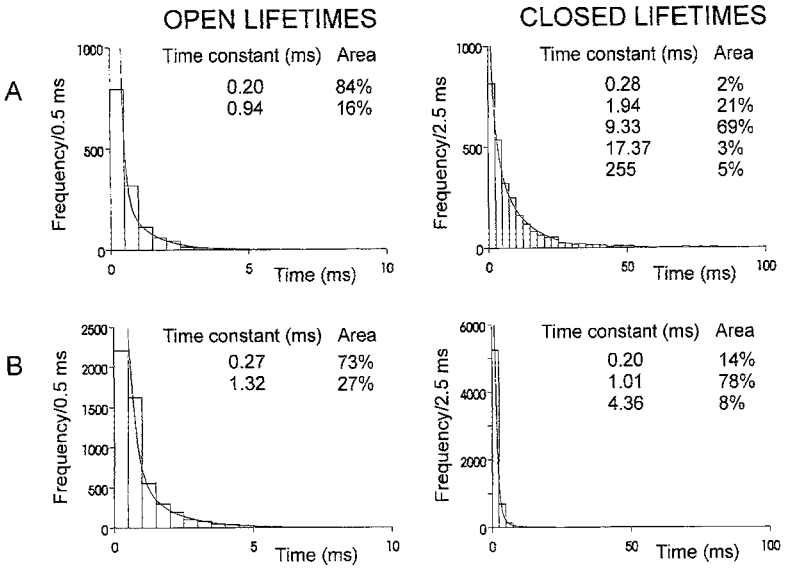

Fig. 2. Open and closed lifetime distributions and probability density functions (pdf) from the channel illustrated in Fig. 1 at the holding potential of +40 mV. The cytosolic free $[Ca^{2+}]$ is 5 μM in *A* and 15 μM in *B*. The open and closed time constants and percentage areas obtained from the best fit to the data 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: $f(t) = a_1(1/\tau_1)\exp(-t/\tau_1) + \ldots\ldots + a_n(1/\tau_n)\exp(-t/\tau_n)$ with areas a and time constants τ. At −40 mV the best fit to the open and closed lifetime distributions is given by: 73(1/0.27)exp(−*t*/0.27) + 27(1/1.32)exp(−*t*/1.32) and 13(1/0.38)exp(−*t*/0.38) + 15(1/1.42)exp(−*t*/1.42) + 55(1/4.32)exp(−*t*/4.32) + 13(1/8.72)exp(−*t*/8.72) + 4(1/85.25)exp(−*t*/85.25) respectively at 5 μM cytosolic Ca^{2+} and by 85(1/0.19)exp(−*t*/0.19) + 15(1/0.94)exp(−*t*//0.94) and 47(1/1.03)exp(−*t*/1.03) + 52(1/5.25)exp(−*t*/5.25) + 1(1/159)exp(−*t*/159) respectively at 15 μM cytosolic Ca^{2+}.

[6]. A missed events correction was applied as described by Colquhoun & Sigworth [6]. A likelihood ratio test was used to compare fits to up to six exponentials [4] by testing twice the difference in $\log_e$ (likelihood) against the chi-squared distribution at the 1% level. Channel P_o values were obtained from three min of steady-state recording. When more than one channel was incorporated into the bilayer, nP_o was calculated according to the formula: $nP_o = [O_{t1} + 2(O_{t2}) + 3(O_{t3}) \ldots\ldots + n(O_{tn})]/T$ where O_{t1} = time in the first open channel level, O_{t2} = time in the second open channel level, T = total recording time and n = maximum number of channels recorded in the bilayer. Single-channel current amplitudes were measured from digitized data using manually controlled cursors.

Results

Cytosolic Ca^{2+}-activated Channels

Mechanism of Cytosolic Ca^{2+} Activation

The gating of the sheep skeletal SR Ca^{2+}-release channel when activated solely by cytosolic Ca^{2+} is characterized by very brief opening events which appear similar to those of the sheep cardiac channel [2, 19]. Figure 1 illustrates the current fluctuations through a channel recorded in symmetrical 250 mM CsPIPES at ±40 mV. P_o is very low in 5 μ*M* Ca^{2+} (*A*) and at −40 mV the P_o was significantly lower than at +40 mV (0.010 ± 0.004 at +40 mV, 0.002 ± 0.001 at −40 mV; $n = 7$, SEM). Increasing the cytosolic Ca^{2+} to 15 μM (*B*) increased P_o to 0.034 ± 0.013 at +40 mV and 0.021 ± 0.008 at −40 mV ($n = 7$, SEM). Thus, although the voltage dependence of the skeletal channel has not been investigated over the range of voltages that has been tested in the sheep cardiac channel [18], the skeletal channel does exhibit voltage dependence of P_o which at ±40 mV is similar to that of the cardiac channel. Analysis of the open and closed lifetimes indicates that the increase in P_o observed when the cytosolic $[Ca^{2+}]$ is raised is due to an increase in the frequency of channel opening; no increase in the duration of the open lifetimes can be detected. The mean open lifetimes of the skeletal channel in 5 μM cytosolic Ca^{2+} at +40 and −40 mV (when the data are filtered at 2 kHz) are 0.46 ± 0.07 and 0.41 ± 0.06 msec respectively (n = 6; SEM) compared with 0.54 ± 0.09 and 0.52 ± 0.08 msec (n = 7; SEM) at 15 μM cytosolic Ca^{2+}. Figure 2 shows the distribution of the open and closed lifetimes of the channel illustrated in Fig. 1 at 5 μM cytosolic Ca^{2+} (A) and after P_o has been increased by increasing the cytosolic $[Ca^{2+}]$ to 15 μM (*B*). If the data were filtered at 4 kHz and the P_o is low, the best fit of the pdf is obtained with five exponentials to the closed lifetime distribution and two exponentials to the open lifetime distribution thus indicating at least two open and five closed states. If the data is filtered at 2 kHz or less, thereby increasing the minimum resolvable event duration from 0.2 to 0.35 msec, normally only three or four rather than five closed states can be distinguished even at low P_o values (for example *see* Fig. 4 legend). When P_o is increased by increasing cytosolic $[Ca^{2+}]$, the best fit to the open lifetime distribution is still obtained with two exponentials, however, the duration of the closed lifetime exponentials is reduced and only four or three exponentials can be detected. Figures 1 and 2 show data from a channel that exhibited the maximum increase in P_o that we observed when cytosolic $[Ca^{2+}]$ was increased from 5 to 15 μM and demonstrates that even under such conditions very little effect on the open lifetime distribution could be detected.

These results indicate that the mechanisms of Ca^{2+}-activation in the sheep skeletal and sheep cardiac channel are essentially the same [2, 19]. One difference between

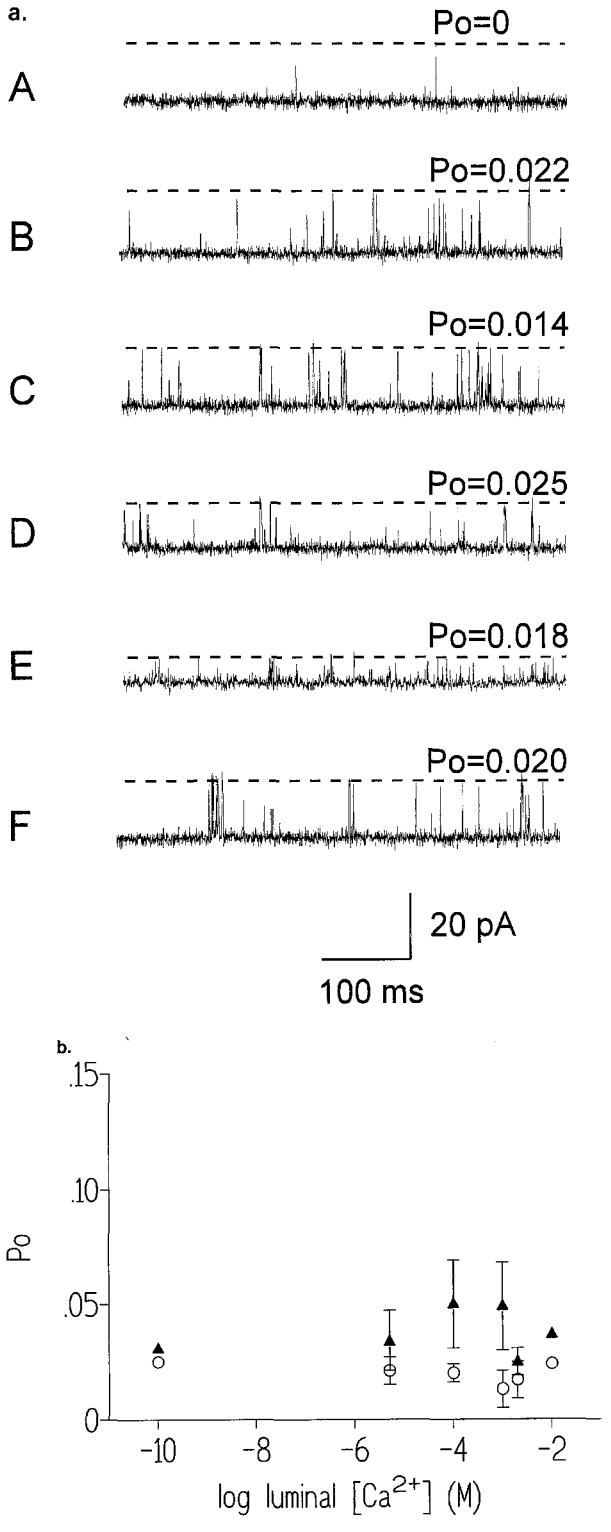

Fig. 3. In A, the effects of changing the luminal $[Ca^{2+}]$ of a representative channel activated by cytosolic Ca^{2+} only are shown. The holding potential is –40 mV. The broken lines indicate the open channel level and P_o levels are indicated above each trace. In *A*, the cytosolic and luminal $[Ca^{2+}]$ was 5 μM and P_o was virtually zero. After increasing the cytosolic free $[Ca^{2+}]$ to 15 μM an increase in P_o was observed (*B*). The luminal $[Ca^{2+}]$ was changed sequentially to 100 μM (C), 1 mM (*D*), and 10 mM (*E*) by adding $CaCl_2$, and approximately 60 pM (*F*) after reperfusion of the *trans* chamber and addition of 12 mM EGTA. In B the effects of luminal Ca^{2+} on the P_o of seven channels activated solely by cytosolic Ca^{2+} at +40 mV (triangles) and –40 mV (circles) are summarized. Each point is the mean of at least 3 values. Standard error bars are shown when $n \geq 4$.

the two isoforms that we did observe was in the duration of the open lifetimes. When the data are filtered at 2 kHz, the mean open lifetime of the skeletal channel activated solely by cytosolic Ca^{2+} is 0.54 ± 0.09 msec at +40 mV. In comparison, the mean open lifetime of the cardiac channel when filtered at 2.5 kHz but under otherwise identical conditions is 0.96 ± 0.21 msec [18]. In the cardiac channel, if the resolution of the single channel events is good enough to filter the data at 2 kHz or greater, three open states can be detected. The time constant of the third exponential is generally approximately 2–4 msec [18, 19]. However, in the skeletal channel, even when the resolution is good enough to filter the data at 4 kHz, only 2 exponentials can be detected, with the time constant of the second exponential rarely greater than 1.5 msec.

Effects of Changes in Luminal $[Ca^{2+}]$

Figure 3*a* illustrates the effect of increasing the luminal $[Ca^{2+}]$ on a representative skeletal SR Ca^{2+}-release channel which has been activated by 15 μM cytosolic Ca^{2+} alone. No significant change in P_o or open lifetime duration could be detected at either +40 or –40 mV over a large range of luminal $[Ca^{2+}]$ (pM to 10 mM). This is demonstrated for a number of channels in Fig. 3*b*.

We conducted similar experiments to investigate if luminal addition of the divalent cation Ba^{2+} had any effect on the P_o of the skeletal channel. Our results indicate that although the conductance of the channel is reduced as the relative divalent/monovalent cation ratio is increased, the gating of the channel is virtually unaffected. This is clearly demonstrated in Fig. 4 where the gating behavior of a typical channel recorded in symmetrical 250 mM Cs^+ solutions and activated solely by cytosolic Ca^{2+} (15 μM) is shown before (*A*) and after addition of 1 mM luminal Ba^{2+} (*B*) and compared with a channel recorded with 50 mM luminal Ba^{2+} in the absence of Cs^+ (*C*). The open and closed lifetime distributions for each $[Ba^{2+}]$ are described in the figure legend. Note the predominance of brief open events in each case. All current fluctuations were filtered at 2 kHz. When channels were activated solely by cytosolic Ca^{2+}, irrespective of the luminal Ba^{2+}/Cs^+ ratio, two open and three to four closed states were observed. Fluctuations in P_o result from variations in the frequency of channel opening. Thus, changing the permeant ion from a divalent to a monovalent cation does not appear to alter the

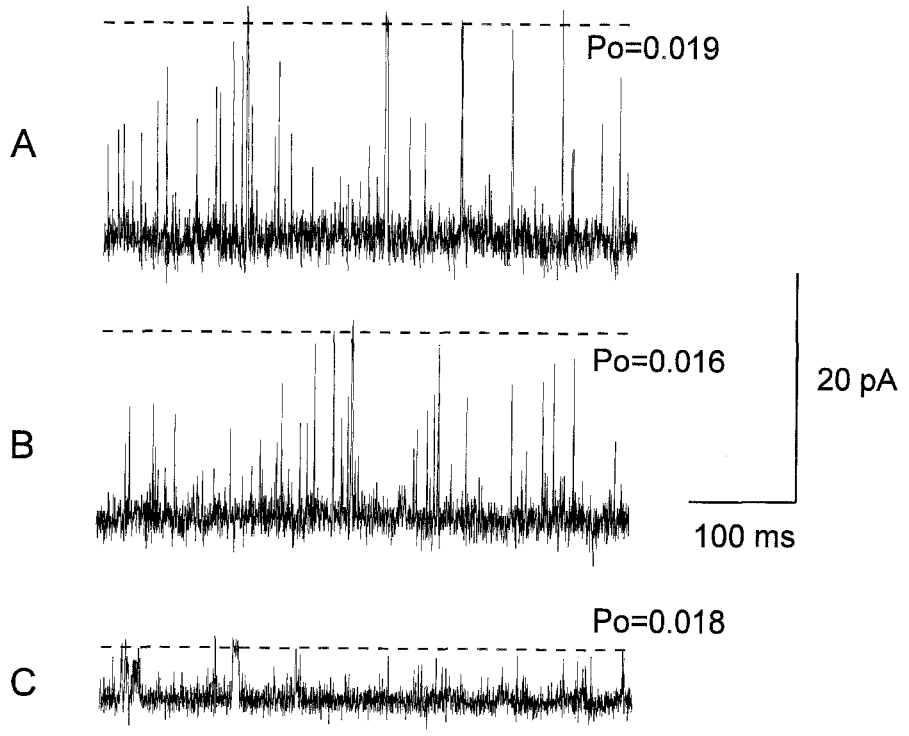

Fig. 4. Representative current fluctuations through single skeletal channels with various luminal [Ba^{2+}]. Channels were activated by 10–15 μM cytosolic Ca^{2+}. The broken lines indicate the open channel levels. P_o values are shown above each trace. All data were filtered at 2 kHz and digitized at 4 kHz in order to make direct comparisons. In *A*, recordings were made at the holding potential of –40 mV in symmetrical 250 mM Cs^+ with no added Ba^{2+}. Data from the same channel are shown in *B* after addition of 1 mM luminal Ba^{2+} (holding potential still –40 mV). The recording shown in *C* is taken from a separate channel with approximately 50 mM luminal Ba^{2+} as the permeant ion. The *cis* chamber contained 250 mM HEPES, 125 Tris, pH 7.2 and the *trans* chamber contained 250 mM glutamic acid, 10 HEPES, pH to 7.2 with $Ba(OH)_2$. Fits to the open lifetime distributions are best described by: (*A*) 90(1/0.35)exp (–*t*/0.35) + 10(1/0.97)exp(–*t*/0.97); (*B*) 91(1/0.42)exp(–*t*/0.42) + 9(1/0.9) exp(–*t*/0.9); (*C*) 96(1/0.35)exp(–*t*/0.35) + 4(1/1.17)exp(–*t*/1.17). Fits to the closed lifetime distributions are best described by: (*A*) 9(1/0.39)exp (–*t*/0.39) + 41(1/4.8)exp(–*t*/4.8) + 50(1/34)exp(–*t*/34); (*B*) 87(1/4.41)exp (–*t*/4.41) + 5.5(1/39)exp(–*t*/39) + 7(1/318)exp(–*t*/318); (*C*) 10(1/0.78) exp(–*t*/0.78) + 76(1/15)exp(–*t*/15) + 14(1/50)exp(–*t*/50).

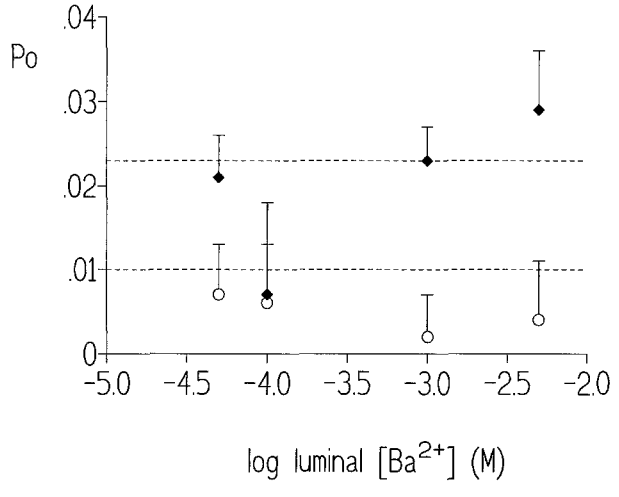

Fig. 5. A graph of the relationship between P_o and the luminal [Ba^{2+}] for 4 single channels activated solely by cytosolic Ca^{2+} (15 μM) at +40 mV (diamonds) and –40 mV (circles). Error bars are SEM. The upper and lower broken lines indicate the mean P_o at +40 mV and –40 mV respectively in the absence of added luminal Ba^{2+}. The P_o before adding luminal Ba^{2+} was 0.023 ± 0.006 at +40 mV and 0.010 ± 0.004 at –40 mV (SEM, n = 4).

basic gating characteristics of the Ca^{2+}-activated sheep skeletal SR Ca^{2+}-release channel. A similar observation has been made for the gating of the cardiac channel [18, 19]. Figure 5 illustrates that luminal Ba^{2+} had no significant effect on P_o over a large range of concentrations (25 μM to 10 mM).

Channels Activated by ATP at Subactivating Cytosolic [Ca^{2+}]

If the cytosolic [Ca^{2+}] is reduced to picomolar levels, the P_o of the sheep skeletal SR Ca^{2+}-release channel is reduced to zero. In the presence of 5 μM luminal [Ca^{2+}], addition of ATP can then activate the channel. This effect is demonstrated in Fig. 6. Interestingly, the voltage dependence of the ATP-dependent openings is different from that of the Ca^{2+}-activated openings (Fig. 6*B*). The P_o of channels activated solely by 1 mM ATP (with 5 μM luminal Ca^{2+}) is 0.020 ± 0.006 at +40 mV compared with 0.079 ± 0.02 (n = 8; SEM) at –40 mV. P_o is greater at negative voltages and the open lifetimes are longer (0.39

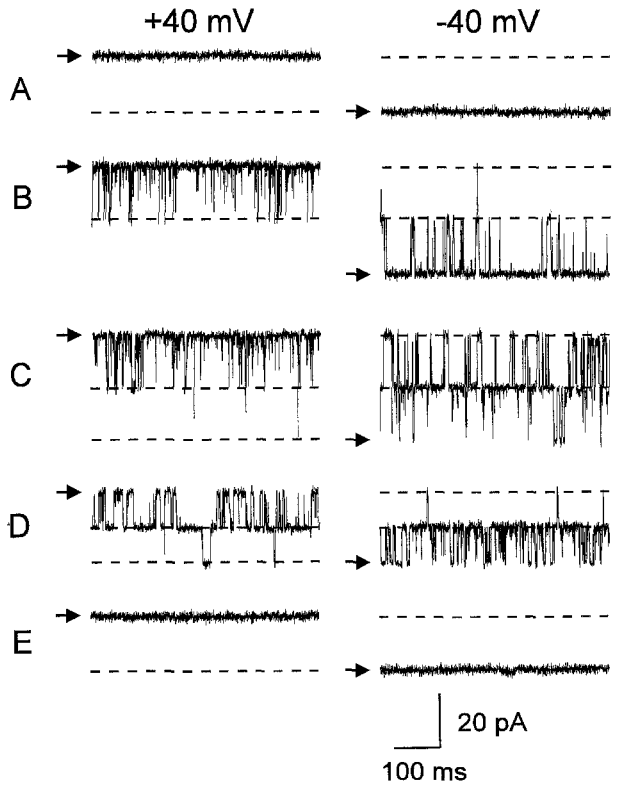

Fig. 6. The effects of increasing the luminal $[Ca^{2+}]$ on the gating of channels activated by ATP in the absence of activating cytosolic $[Ca^{2+}]$. Recordings were obtained in symmetrical 250 mM Cs^+ at holding potentials of ±40 mV. The broken lines indicate the open channel levels and the arrows indicate the zero current level. At least two channels are present in the bilayer. In *A* the luminal $[Ca^{2+}]$ is 5 μM and the cytosolic $[Ca^{2+}]$ has been lowered to approximately 60 pM after addition of 12 mM EGTA; $P_o = 0$. Addition of 1 mM ATP induces brief openings at +40 mV and slightly longer openings at −40 mV (*B*). Increasing luminal Ca^{2+} to 100 μM (*C*) and 2 mM (*D*) further increases the P_o and the duration of channel openings. Reperfusion of the *trans* chamber and addition of 12 mM EGTA lowers the luminal $[Ca^{2+}]$ to approximately 60 pM and abolishes all channel openings (*E*).

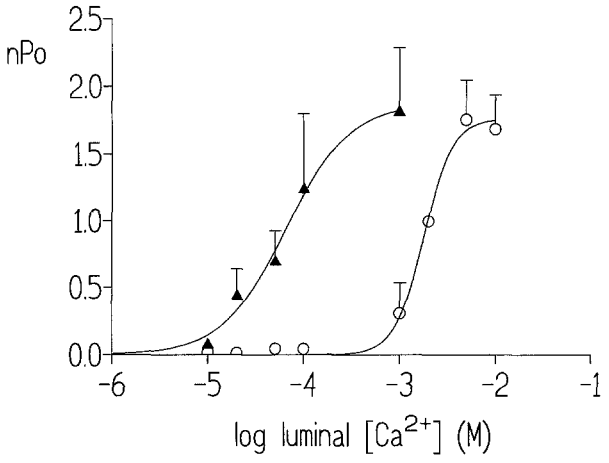

Fig. 7. The relationship between nP_o and luminal $[Ca^{2+}]$ for channels activated solely by ATP (1 mM) at +40 mV (circles) and −40 mV (triangles). Data from 8 experiments are shown. Error bars indicate SEM. Picomolar levels of luminal Ca^{2+} are not shown but P_o is zero at both ±40 mV (n = 3). nP_o values are normalized to the value at +40 mV and 2 mM luminal Ca^{2+}.

± 0.09 msec at +40 mV; 1.57 ± 0.58 msec at −40 mV). A similar change in the voltage-dependence of P_o was observed in the cardiac channel when activated by sulmazole at subactivating cytosolic $[Ca^{2+}]$ [18]. Increasing the luminal $[Ca^{2+}]$ to millimolar concentrations causes marked increases in the P_o both at positive and negative voltages (Fig. 6*D;* note the increase in duration of channel openings). However, the increase in P_o occurs over a lower range of $[Ca^{2+}]$ at −40 mV than at +40 mV. The relationship between P_o and luminal $[Ca^{2+}]$ for channels activated solely by ATP can be observed in Fig. 7. The luminal Ca^{2+} EC_{50} value for channels held at −40 mV is 64 μM, while for channels held at +40 mV it is 1.8 mM (n = 7). Hill slopes at −40 and +40 mV are 1.6 and 2.8 respectively indicting that more than one Ca^{2+} ion must bind for maximal activation of the channel. If the luminal $[Ca^{2+}]$ of channels activated by ATP at subactivating cytosolic $[Ca^{2+}]$ is reduced to picomolar levels the P_o becomes zero (n = 3) at ±40 mV (demonstrated in Fig. 6*E*).

The characteristic effect of increasing the luminal $[Ca^{2+}]$ of channels activated solely by ATP is a lengthening of the open lifetime durations. This can be observed in the channel traces (Fig. 6) and also upon inspection of the lifetime distributions. Figure 8 illustrates how long the open lifetimes are in comparison with channels activated solely by cytosolic Ca^{2+} (*cf.* Fig. 2).

We investigated if luminal Ba^{2+} could substitute for Ca^{2+} and increase the P_o of channels activated by ATP in the absence of activating cytosolic Ca^{2+}. Additions of Ba^{2+} in concentrations up to 10 mM (*B, C,* and *D*) are unable to significantly increase P_o at either positive or negative voltages (Fig. 9). Subsequent addition of millimolar luminal Ca^{2+} (*E*) increased P_o and the duration of open lifetimes. This experiment was repeated three times.

Similar increases in P_o and duration of open lifetimes were obtained when Ca^{2+}-glutamate was added to the *trans* chamber rather than $CaCl_2$ (n = 3) (*data not shown*).

Orientation of Skeletal Channels in the Bilayer

Can the effects of luminal Ca^{2+} described above be due to channels which have incorporated into the bilayer with the cytosolic channel side facing the *trans* chamber? The different gating kinetics of channels activated solely by Ca^{2+} or solely by ATP indicate that this probably is not so. However, we have tested this possibility by using the channel blocker, tetraethyl ammonium (TEA). In the

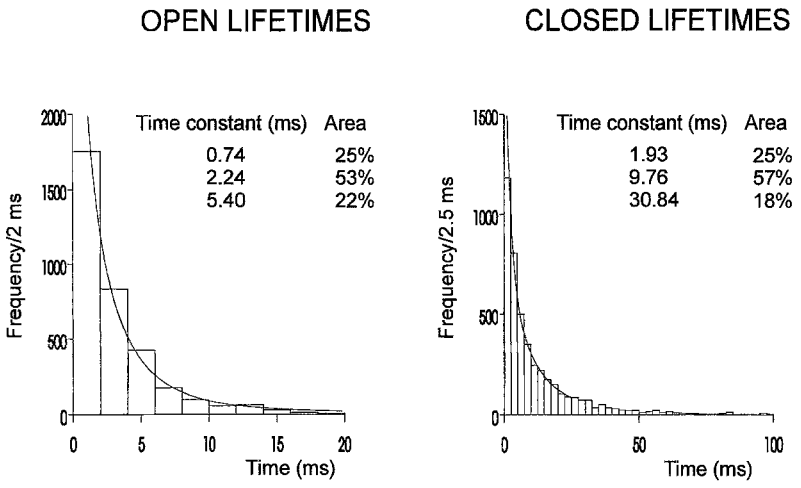

Fig. 8. Open and closed lifetime distributions and pdfs from a typical single channel activated solely by ATP (1 mM) in the presence of 1 mM luminal Ca^{2+}. Holding potential = −40 mV. Data were filtered at 2 kHz and digitized at 4 kHz. Time constants and percentage areas are shown.

cardiac channel, we used TEA to demonstrate that both the native and purified channels always incorporate into the bilayer with the cytosolic channel side facing the *cis* chamber [19]. TEA will only block the channels from the *cis* chamber and has no action when added to the *trans* chamber [19, 22]. As for the cardiac channel, we found that TEA (20 mM) blocked Cs^+ current through skeletal channels when added to the *cis* chamber but had no effect when added to the *trans* chamber (n = 3) indicating that channels incorporate into the bilayer in a fixed orientation. Figure 10 illustrates current fluctuations through channels activated by ATP where millimolar luminal Ca^{2+} has increased P_o (*A*). Reperfusion of the *trans* chamber with CsPIPES solution (free [Ca^{2+}] 5 μM) lowers P_o and increases conductance due to removal of the higher affinity but lower conductance divalent cation (*B*). Addition of TEA (20 mM) to the *trans* chamber has no effect on conductance (*C*). However, addition of 20 mM TEA to the *cis* chamber causes block of channel current at +40 mM (*D*). Figure 11 demonstrates the current-voltage relationship of the skeletal channels before and after *trans* and *cis* addition of TEA (20 mM). These experiments indicate that the effects of luminal Ca^{2+} that we observe are not simply the result of channels incorporating into the bilayer in different orientations.

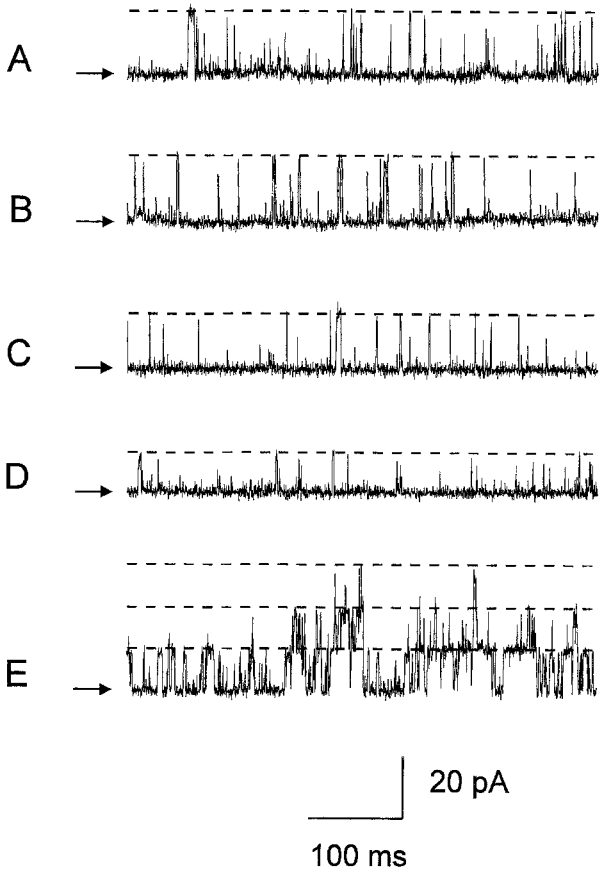

Fig. 9. Effect of luminal Ba^{2+} on channels activated by ATP (1 mM) at subactivating [Ca^{2+}] (60 pM). The holding potential is −40 mV. The broken lines indicate the open channel levels and the arrows indicate the zero current level. In *A*, the luminal [Ca^{2+}] is 5 μM and this is enough to cause a few long openings. Adding luminal Ba^{2+}; 100 μM (*B*), 2 mM (*C*), 10 mM (*D*) does not significantly alter P_o or the duration of channel openings. Subsequent addition of 2 mM luminal Ca^{2+} results in a large increase in P_o (*E*).

Effects of Luminal [Ca^{2+}] on Channels Activated by Cytosolic Ca^{2+} and ATP

Figure 12 illustrates the effect of luminal Ca^{2+} on a typical channel activated by 1 mM ATP in the presence of 1 μM free cytosolic Ca^{2+}. Clearly, P_o is low with 5 μM luminal Ca^{2+} but increasing the luminal [Ca^{2+}] results in full activation of the channel at ±40 mV. With 5 μM luminal Ca^{2+}, P_o is 0.199 ± 0.09 at −40 mV and 0.208 ± 0.12 at +40 mV (n = 5; SEM). Lowering the luminal [Ca^{2+}] to picomolar levels does not reduce the P_o to zero as it does for channels activated solely by ATP. At approximately 56 pM luminal Ca^{2+}, P_o is 0.177 ± 0.14 at −40 mV and 0.175 ± 0.24 at +40 mV. It can be seen that in the presence of millimolar luminal Ca^{2+} the channel is

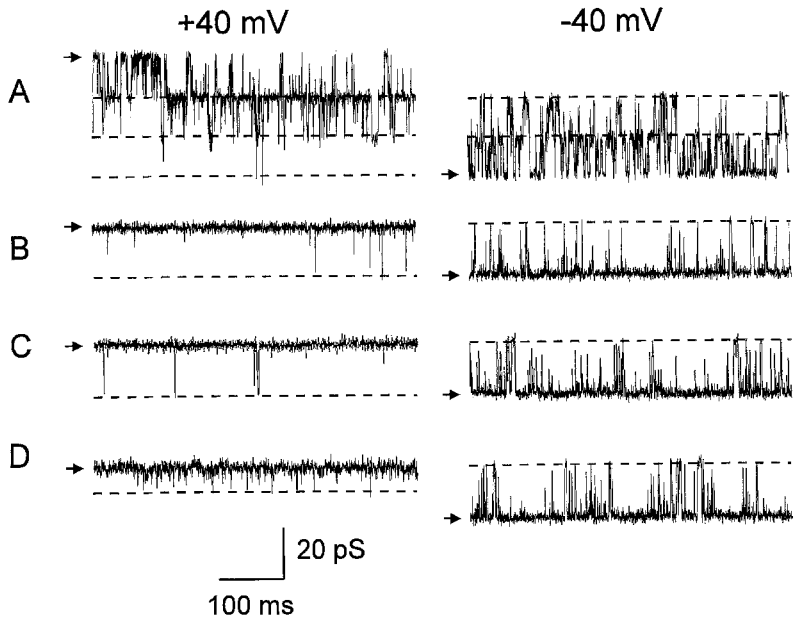

Fig. 10. Block of current by TEA only when added to the *cis* chamber demonstrates that the increase in P_o caused by increasing luminal [Ca^{2+}] is not due to channels incorporating into the bilayer in different orientations. Recordings were made in symmetrical 250 mM Cs^+. The broken lines indicate the open channel levels and the arrows indicate the zero current level. In *A*, channels are activated solely by ATP (1 mM) and the presence of 2 mM luminal Ca^{2+} has resulted in the characteristic long open events at both positive and negative voltages and a decrease in single channel conductance. In *B* the *trans* chamber has been reperfused with 250 mM Cs^+ and the lowering of luminal Ca^{2+} to 5 μM has increased single channel conductance and lowered P_o. In *C*, 20 mM TEA has been added to the *trans* chamber. No block of current can be detected. After adding 20 mM TEA to the *cis* chamber, block of current is observed at +40 mV (*D*).

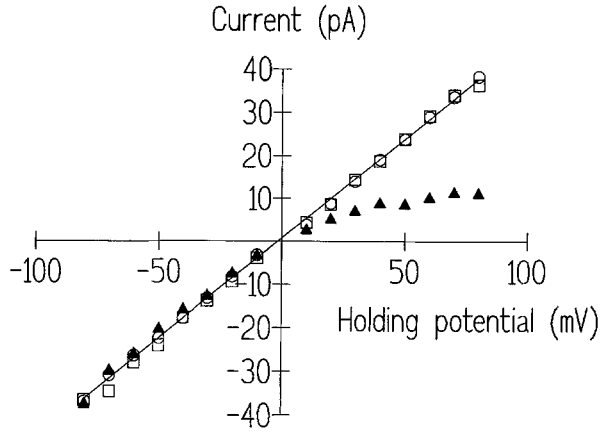

Fig. 11. TEA blocks the skeletal SR Ca^{2+}-release channel only when added to the *cis* chamber. Control current-voltage relationship in symmetrical 250 mM Cs^+ (circles), after addition of 20 mM *trans* TEA (squares), and after addition of 20 mM *cis* TEA (triangles). The conductance of the channel in symmetrical 250 mM Cs^+ is 457 ± 12 pS (SEM; n = 5).

almost fully open at both positive and negative voltages (*C*). Such a high P_o cannot be achieved with ATP in the absence of cytosolic Ca^{2+} irrespective of the luminal [Ca^{2+}]. Unlike channels activated by cytosolic Ca^{2+} alone or channels activated by ATP alone, there does not appear to be any difference in P_o at ±40 mV. In fact, the P_o-luminal Ca^{2+} relationship is virtually superimposable at ±40 mV. This is illustrated in Fig. 13. The EC_{50} values at +40 mV and −40 mV are 107 μM and 89 μM (n = 4) respectively. In the absence of many points on the activation curve, we tentatively suggest that only one luminal Ca^{2+} ion must bind for full activation of the channel (The Hill slopes are 0.75 at +40 mV and 0.68 at −40 mV).

Discussion

We have used the sheep skeletal SR Ca^{2+}-release channel for these experiments as it allows a direct comparison under identical experimental conditions with our work on the gating and conductance of the cardiac channel, and eliminates possible problems arising from the comparison of channels from different species. In many ways, the two isoforms are very similar. The conductance of the channels in 250 mM CsPIPES are almost identical (457 ± 12 pS skeletal; 462 ± 4 pS cardiac, [18]) and the mechanisms of the cytosolic Ca^{2+}-activation appear to be similar [2, 19]. The results of the present study also demonstrate that luminal Ca^{2+} regulates gating similarly in both isoforms of the channel. As with the cardiac channel, we find that luminal Ca^{2+} does not modify the gating of skeletal SR Ca^{2+}-release channels activated solely by cytosolic Ca^{2+}. However, in the presence of a second ligand (ATP) or when the channels are activated by ATP alone, luminal [Ca^{2+}] regulates both the P_o and duration of open lifetimes. We find that the long opening events resulting from channel activation with ATP only (and which are associated with cytosolic Ca^{2+}-independent activation of SR Ca^{2+}-release channels at both the ATP and caffeine sites [17, 18, 27]) require the binding of luminal Ca^{2+}.

The dissimilar gating kinetics, P_o-voltage relationships and effects of luminal Ca^{2+} on channels activated by (i) cytosolic Ca^{2+} alone, (ii) ATP alone, and (iii) ATP

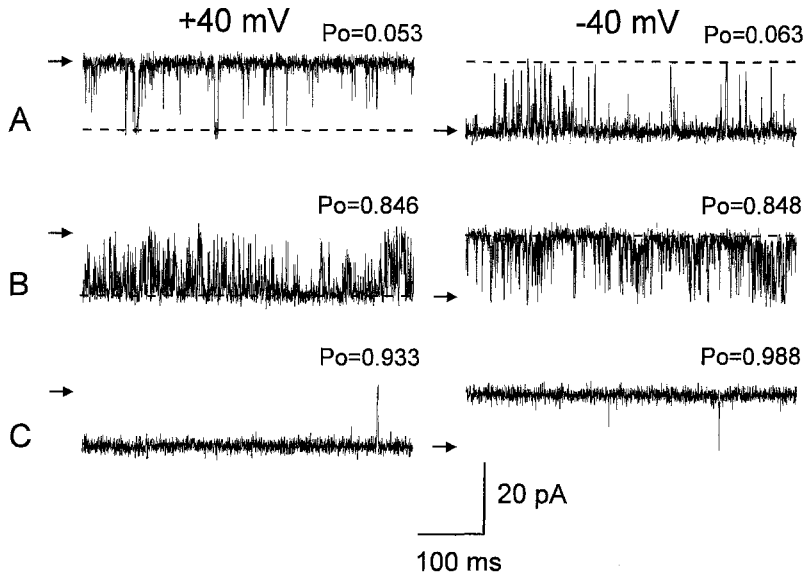

Fig. 12. An illustration of the effect of luminal Ca^{2+} on a single representative skeletal SR Ca^{2+}-release channel activated by ATP plus cytosolic Ca^{2+} together. The arrows indicate the closed channel level. P_o values are given above each trace. In *A*, the channel is activated by 1 mM ATP and 1 μM free cytosolic Ca^{2+} and the luminal $[Ca^{2+}]$ is 5 μM. In *B*, luminal-free $[Ca^{2+}]$ is 100 μM and a marked increase in P_o occurs (note the rapid gating). In *C* luminal $[Ca^{2+}]$ is increased to 1 mM and the channel is almost fully open at both voltages.

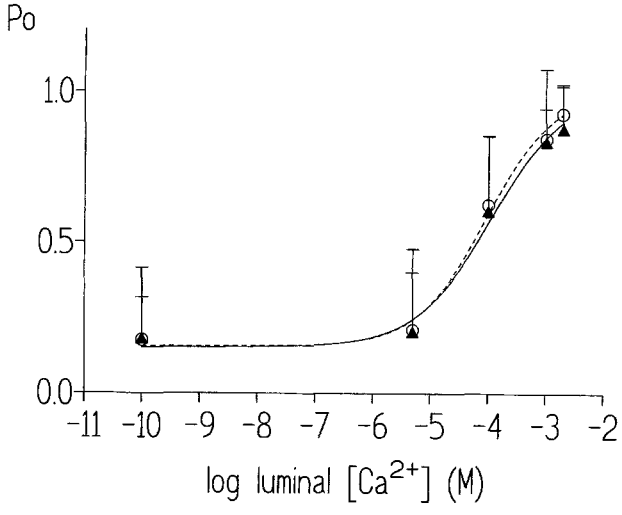

Fig. 13. The relationship between P_o and luminal $[Ca^{2+}]$ for channels activated by 1 μM cytosolic Ca^{2+} plus ATP (1 mM) at +40 mV (circles) and −40 mV (triangles). The data from five single channels are shown. Error bars indicate SD. At picomolar luminal Ca^{2+} levels P_o does not decline to zero.

plus cytosolic Ca^{2+} suggest that three distinct modes of gating are operating.

Channels Activated by Cytosolic Ca^{2+} Only

We have found that a cytosolic Ca^{2+}-induced increase in the P_o of skeletal channels is associated with an increase in the frequency of channel opening. No increase in the duration of the open events could be detected. Thus, Ca^{2+} must be binding only to a closed channel state or states to increase P_o. This mechanism for Ca^{2+}-activation was also reported for rabbit skeletal channels where Ca^{2+} rather than Cs^+ was the permeant ion [20]. This is also the mechanism for Ca^{2+}-activation of the sheep cardiac channel with either Ca^{2+} or Cs^+ as the permeant ion. Lifetime analysis suggests that for the Ca^{2+}-activated skeletal channel there are at least five closed states and at least two open states. Under identical conditions, at least five closed states and three open states can be detected in the sheep cardiac channel [19]. The mean open lifetime of skeletal channels under similar recording conditions is approximately 0.5 msec compared with approximately 1 msec in cardiac channels (*see* Results) and it appears that it is the long open state that is absent in skeletal channels. The openings of the skeletal channels therefore appear to be slightly shorter than those of the cardiac channel.

Increasing the luminal $[Ca^{2+}]$ to 10 mM or reducing it to picomolar levels appeared to have no effect on the P_o or the open and closed lifetime durations of skeletal channels at +40 or −40 mV when activated solely by cytosolic Ca^{2+}. We reported a similar effect in cardiac channels [18]. This result suggests that the Ca^{2+} flowing through the channel in the bilayer does not have access to the cytosolic Ca^{2+}-activation site.

Addition of luminal Ba^{2+} in concentrations up to 10 mM also has no significant effect on the open probability of the skeletal SR Ca^{2+}-release channel. The larger conductance of the channel in Ba^{2+} solutions compared to Ca^{2+} solutions provided a way of testing if the gating of the channel with a monovalent cation as the permeant ion is different from that with a divalent cation as the current carrier. We find no evidence to suggest that the basic gating behavior of the Ca^{2+}-activated skeletal channel can be modified simply by changing the permeant ion from a divalent to a monovalent cation (at least under the high ionic conditions of these experiments). When the

channels are activated solely by cytosolic Ca^{2+}, it appears that using Cs^+ rather than Ca^{2+} or Ba^{2+} as the permeant ion merely increases conductance and thereby improves the resolution of the lifetimes. As the mean open lifetime of the skeletal channel is very brief (close to 0.5 msec when the filtering is 2 kHz) most of the open lifetimes are unresolved if Ca^{2+} is the conducting ion (*unpublished observations*).

Channels Activated by ATP Only

The important aspects of this mechanism of channel gating can be summarized as follows: Channels activated by ATP in the absence of activating cytosolic Ca^{2+} (or sulmazole in the absence of activating Ca^{2+} [18]) have an absolute requirement for the presence of micromolar or higher concentrations of luminal Ca^{2+}. P_o is zero at picomolar luminal Ca^{2+}. The P_o-voltage relationship is altered by the luminal $[Ca^{2+}]$ and a lower luminal $[Ca^{2+}]$ is required to increase P_o at negative voltages. The Hill slopes indicate that more than one luminal Ca^{2+} must bind for maximal activation. As the luminal $[Ca^{2+}]$ is increased both the frequency and the duration of open events is increased.

Experiments on the cardiac channel at +40 mV and 10 μM luminal Ca^{2+} indicate that sulmazole activation in the absence of activating cytosolic Ca^{2+} results in an increase in the frequency of channel opening without an increase in duration of open lifetimes and therefore sulmazole binds to the closed channel state [18]. In the present study, the effect of ATP in the absence of cytosolic Ca^{2+} is similar. Only brief openings are observed at +40 mV with contaminant levels of luminal Ca^{2+} indicating that under such conditions the effects of ATP result from an interaction of this ligand with a closed channel state. Increases in open lifetime duration appear to occur only when the luminal $[Ca^{2+}]$ is increased and therefore it appears that it is the binding of luminal Ca^{2+} to an open channel state that results in the lengthening of open lifetimes rather than the binding of an agonist (to the Ca^{2+}, caffeine or adenine nucleotide binding site) at the cytosolic channel side to an open channel state.

Channels Activated by ATP Plus Cytosolic Ca^{2+}

Channels activated by cytosolic Ca^{2+} plus ATP are dependent on the luminal $[Ca^{2+}]$ for full activation but openings can still be obtained in the absence of luminal Ca^{2+} (unlike channels which are activated by ATP alone). In the presence of sufficient luminal Ca^{2+}, a P_o of 1 can be obtained with channels activated by cytosolic Ca^{2+} plus ATP. In contrast, full activation of the channel cannot be achieved with ATP or cytosolic Ca^{2+} as the sole ligand regardless of the luminal $[Ca^{2+}]$ [20]. The Hill slopes indicate that only one luminal Ca^{2+} is required for full activation of channels activated by ATP plus cytosolic Ca^{2+}. There appears to be no dependence of P_o on voltage. This could be a characteristic of such channel activation or could simply be the result of particular concentrations of ATP and/or cytosolic Ca^{2+} at which the voltage dependence of Ca^{2+}-activation and the voltage dependence of ATP activation cancel each other out.

How Does Luminal Ca^{2+} Increase P_o?

Our experiments with TEA (Figs. 7 and 11) demonstrate that the regulation of channel open probability with additions of Ca^{2+} to the *trans* side result from additions of Ca^{2+} to the luminal channel side and cannot be explained by incorporation of the channels with the cytosolic side facing the *trans* chamber.

Luminal Ca^{2+} increases the P_o of channels activated solely by ATP or by cytosolic Ca^{2+} plus ATP but appears to have no effect on channels activated solely by cytosolic Ca^{2+}. This result excludes the possibility that luminal Ca^{2+} flowing through the channel has direct access to the cytosolic Ca^{2+}-activation site/s. Therefore the binding of luminal Ca^{2+} to the cytosolic activation site cannot explain the increase in P_o observed by increasing the luminal $[Ca^{2+}]$ of channels activated by ATP only or by cytosolic Ca^{2+} plus ATP.

Why does luminal Ca^{2+} have no effect on channels activated solely by cytosolic Ca^{2+} but can increase the P_o and duration of open lifetimes of channels activated solely by ATP (or sulmazole, [18])? One interpretation of the data would involve a scheme in which the binding of ATP (or sulmazole) results in a conformational change in the channel which exposes the luminal Ca^{2+} binding site/s. At picomolar levels of luminal Ca^{2+}, the ATP- (or sulmazole) bound channel cannot open (unless activating levels of cytosolic Ca^{2+} are present). Therefore, luminal Ca^{2+} must bind to an ATP-bound closed channel state to induce the channel to open. The Hill slopes for the P_o-luminal Ca^{2+} relationship indicate that at least two Ca^{2+} bind for maximal activation. From the gating behavior of the channels described above it would appear that for channels activated by ATP alone, luminal Ca^{2+} must bind to both an open and a closed channel state for maximal activation. As luminal Ca^{2+} has no effect on channels activated by cytosolic Ca^{2+} alone this would suggest that luminal Ca^{2+} either does not have access to its luminal binding site when the channel is activated by cytosolic Ca^{2+} in the absence of another ligand, or that the binding of cytosolic Ca^{2+} exerts an inhibitory effect on the tendency of luminal Ca^{2+} to increase the duration of open lifetimes.

Channels activated by (i) cytosolic Ca^{2+} alone (ii) cytosolic Ca^{2+} plus ATP and (iii) ATP alone exhibit different P_o-voltage relationships. Moreover, the P_o-

voltage relationships of (i) and (ii) above are independent of the luminal [Ca^{2+}] while the P_o-voltage relationship of (iii) appears to be modified by changes in luminal [Ca^{2+}]. Therefore, the binding of different cytosolic agonists may confer conformational changes such that the voltage sensor detects voltage differently.

Is the regulation of P_o by luminal Ca^{2+} the result of binding to specific Ca^{2+}-binding sites or can the effect be explained by a change in surface potential caused by a screening of negative charges on the luminal side of the bilayer by Ca^{2+}? In fact a change in surface charge near the luminal mouth of the dog cardiac SR Ca^{2+}-release channel has been suggested to mediate changes in conduction [25]. However, in our experiments, we find that Ba^{2+} cannot substitute for Ca^{2+} and therefore we believe that a change in surface potential cannot explain the luminal Ca^{2+}-dependence of P_o. We therefore hypothesize that luminal Ca^{2+} binds to specific binding sites accessible from the luminal side of the channel. Does luminal Ca^{2+} bind within the conduction pathway to cause an increase in P_o? We believe that this is unlikely as the effects on P_o are observed at luminal [Ca^{2+}] far lower than are required to observe a change in conductance. As the effects of luminal Ca^{2+} cannot be reproduced with Ba^{2+} (which also binds within the conduction pathway; [24]) in concentrations up to 10 mM this would confirm this hypothesis. In addition, when the channel is activated by cytosolic Ca^{2+} plus ATP, the P_o-luminal Ca^{2+} relationship is identical at positive and negative voltages. This would not be expected for a binding site within the conduction pathway. Therefore the most probable location for the Ca^{2+}-binding sites is on the luminal face of the channel outside the conduction pathway (voltage drop).

Voltage Dependence of Channels Activated by ATP Only

The voltage-dependence of channels activated by ATP at subactivating cytosolic [Ca^{2+}] appears to be modified by changing the luminal [Ca^{2+}]. There are a number of possible explanations for this effect: (i) Luminal Ca^{2+} may bind either directly to the voltage sensor or to a site regulating the voltage sensor resulting in a shift in the voltage-dependence of the channel. (ii) There may be screening of surface charge by luminal Ca^{2+}, although we believe that this explanation can be discounted as Ba^{2+} does not substitute for Ca^{2+}. (iii) Another possible explanation is one of three hypotheses first suggested by Moczydlowski & Latorre [11] to explain the voltage-dependence of Ca^{2+}-activated K^+ channels. In this scheme, the binding of Ca^{2+} is not itself voltage-dependent but the Ca^{2+}-binding site is coupled to a dipole in the membrane which, when exposed to an electric field, alters the affinity of the binding site for Ca^{2+}. Further experiments are required before we can fully understand the complex mechanisms involved in the apparent modification of voltage-dependence by luminal Ca^{2+}.

Physiological Significance

What are the implications of the results of this study for EC coupling? In skeletal muscle, it is thought that depolarization of the T-tubules directly opens the SR Ca^{2+}-release channels allowing Ca^{2+} to be released from the SR to the contractile proteins [1, 14]. Presumably the resultant P_o of the activated release channels will depend (among other factors) on the levels of modulatory cytosolic ligands such as Ca^{2+}, ATP, Mg^{2+}, Ca^{2+}-binding proteins, etc. The results of the present study indicate that the synergistic effect of cytosolic ATP and Ca^{2+} on channel P_o is highly dependent upon the free [Ca^{2+}] at the luminal channel face. Our experiments indicate that as the level of luminal Ca^{2+} available for release declines, not only will the luminal to cytosolic concentration gradient for Ca^{2+} be reduced (thereby reducing the single-channel conductance and hence the quantity of Ca^{2+} leaving the SR with each opening [23]), but also P_o will be decreased. In skeletal muscle SR, free intraluminal [Ca^{2+}] as high as 2.6 mM [9] or 3.6 mM [26] have been reported. The precise role of calsequestrin in regulating the free intraluminal [Ca^{2+}] is not fully understood and some controversy exists as to whether a conformational change in calsequestrin occurs prior to Ca^{2+} release [8, 26]. Therefore the exact free [Ca^{2+}] in the region of the Ca^{2+}-release channel luminal binding sites is not known. However, the effects of luminal Ca^{2+} on open probability occur within the range of free [Ca^{2+}] calculated to be present in skeletal muscle SR lumen and therefore may represent a physiological mechanism whereby the luminal [Ca^{2+}] controls P_o.

By its very nature, Ca^{2+}-induced Ca^{2+}-release should display positive feedback and models of EC-coupling involving this process are unstable unless they include mechanisms whereby release is inactivated [7, 21]. It has therefore been suggested that a mechanism must exist to inactivate SR Ca^{2+}-release channel during EC-coupling. In skeletal muscle, one possible mechanism is inactivation of the channels by high cytosolic [Ca^{2+}] [5, 15, 20]. Our results indicate another possible mechanism of inactivation. As the [Ca^{2+}] at the luminal channel face is reduced, the P_o is markedly lowered. In addition, the results of Tinker et al. [23] demonstrate that a reduction in Ca^{2+} current will also occur (even if P_o remains constant) due to a decrease in channel conductance as Ca^{2+} activity in the lumen is reduced. We therefore suggest that the level of luminal Ca^{2+} may play a vital role in regulating the amount of Ca^{2+} flowing from the SR to the cytoplasm and may represent a mechanism of "inactivation" of SR Ca^{2+} current.

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