Single Channel Measurements of the Calcium Release Channel from Skeletal Muscle Sarcoplasmic Reticulum

Activation by Ca^{2+} and ATP and Modulation by Mg^{2+}

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ABSTRACT A high-conductance (100 pS in 53 mM *trans* Ca^{2+}) Ca^{2+} channel was incorporated from heavy-density skeletal muscle sarcoplasmic reticulum (SR) fractions into planar lipid bilayers of the Mueller-Rudin type. *cis* Ca^{2+} in the range of 2–950 μM increased open probability (P_o) in single channel records without affecting open event lifetimes. Millimolar ATP was found to be as good as or better than Ca^{2+} in activation; however, both Ca^{2+} and ATP were required to fully activate the channel, i.e., to bring $P_o = 1$. Exponential fits to open and closed single channel lifetimes suggested that the channel may exist in many distinct states. Two open and two closed states were identified when the channel was activated by either Ca^{2+} or ATP alone or by Ca^{2+} plus nucleotide. Mg^{2+} was found to permeate the SR Ca channel in a *trans*-to-*cis* direction such that $iMg^{2+}/iCa^{2+} = 0.40$. *cis* Mg^{2+} was inhibitory and in single channel recordings produced an unresolvable flickering of Ca- and nucleotide-activated channels. At nanomolar *cis* Ca^{2+}, 4 μM Mg^{2+} completely inhibited nucleotide-activated channels. In the presence of 2 μM *cis* Ca^{2+}, the nucleotide-activated macroscopic Ba conductance was inhibited by *cis* Mg^{2+} with an IC_{50} equal to 1.5 mM.

INTRODUCTION

Skinned fiber and recent vesicle flux measurements have suggested that the sarcoplasmic reticulum (SR) contains a Ca release channel that permits Ca^{2+} efflux from the SR on a millisecond time scale (Endo, 1977; Stephenson, 1981; Fabiato, 1983; Martonosi, 1984; Nagasaki and Kasai, 1983; Ikemoto et al., 1985; Meissner et al., 1986). Ca^{2+} release rates visualized in isolated skeletal SR vesicles

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using rapid-flow and rapid-quench techniques have shown that the release channel is stimulated by Ca^{2+} and adenine nucleotides and inhibited by Mg^{2+} and ruthenium red (Nagasaki and Kasai, 1983; Ikemoto et al., 1985; Meissner et al., 1986). In a preliminary report (Smith et al., 1985), we showed that it was possible to incorporate this channel into planar lipid bilayers using the fusion technique of Miller and Racker. The SR Ca channel was identified on the basis of its selectivity for divalent cations, activation by adenine nucleotides, and inhibition by ruthenium red. Because of the presence of K^+-selective SR channels, only permeability ratios for the bi-ionic conditions Ca^{2+} or $Ba^{2+}/Tris^+$ or Ba^{2+}/Cs^+ could be determined. Ca^{2+} or $Ba^{2+}/Tris^+$ and Ba^{2+}/Cs^+ gave permeability ratios of 8.74 and 11.4, respectively. The adenine nucleotides ATP and AMP-PCP, in the presence of micromolar Ca^{2+}, were found to activate the channel by increasing the frequency and duration of channel opening. Nucleotides did not affect channel unit conductance. Complete channel activation, i.e., open probability near unity, was found to occur with 1–2 mM nucleotide and 2 μM free Ca^{2+}. Ruthenium red produced near-complete inhibition of nucleotide-activated channels at 1 μM by decreasing the frequency and duration of open events. The SR Ca channel was further distinguished from other SR channels by its rapid gating kinetics and high unit conductance (100 ± 4 pS in 53 mM Ca^{2+}, and 140 ± 30 pS in 53 mM Ba^{2+}) in Ca^{2+}- or Ba^{2+}-containing buffers.

In this report, we describe the basic single channel behavior for the high-conductance SR Ca channel in the presence of Ca^{2+}, Mg^{2+}, and adenine nucleotides. Both Ca^{2+} and adenine nucleotides were found to be activators of the channel in bilayers. Mg^{2+} was found to freely permeate the channel in a *trans*-to-*cis* direction and yet act as an efficient inhibitor of the channel when present in the *cis* bilayer chamber. A preliminary account of part of this work has been presented (Smith et al., 1986*a*).

METHODS

Isolation of SR Vesicles

Rabbit skeletal muscle SR was fractionated into heavy, intermediate, and light density vesicle fractions by differential and sucrose gradient centrifugation as described previously (Meissner, 1984). Briefly, heavy SR membranes containing the Ca^{2+} release channel were recovered from the 36–45% region of a sucrose gradient that contained rabbit skeletal membranes sedimenting at 2,600–35,000 *g*.

Planar Lipid Bilayer Methods

Mueller-Rudin planar bilayers containing phosphatidylethanolamine (25 mg/ml, bovine brain), phosphatidylserine (15 mg/ml, bovine brain), and diphytanoylphosphatidylcholine (10 mg/ml) (all from Avanti Polar Lipids, Inc., Birmingham, AL) in decane were painted across a 400-μm hole in a Lexan cup inserted into a cut-away PVC block (Smith et al., 1986*b*). In all experiments, the *cis* chamber is defined as the side to which SR vesicles are added; the opposite side is referred to as the *trans* chamber. In previous studies (Miller, 1978; Smith et al.,1985), SR vesicles were found to insert into the bilayer in an oriented fashion such that the *cis* chamber corresponded to the cytoplasmic space and the *trans* chamber was equivalent to the lumen of the SR. All additions of Ca^{2+}, Mg^{2+}, EGTA, or

adenine nucleotides were made to the *cis* chamber. Applied voltages are defined with respect to the *trans* chamber held at virtual ground and therefore agree with the normal cellular convention.

The *cis* chamber, inside the Lexan cup, contained 0.25 M choline Cl, 5 mM $CaCl_2$, 100 μM EGTA, 10 mM Tris HEPES, pH 7.4, and the *trans* chamber contained 50 mM choline Cl, 5 mM $CaCl_2$, 100 μM EGTA, 10 mM Tris HEPES, pH 7.4. Heavy SR vesicles in 0.3 M sucrose, 10 mM K Pipes (1,4-piperazinediethanesulfonic acid), pH 7.0, were added to the *cis* chamber and stirred (final protein concentration, 3 μg/ml). Shortly after vesicle addition, step-like vesicle-bilayer fusion events were observed. The resulting conductances were characteristic of an anion-conducting pathway with an equilibrium reversal potential of +25 mV. After vesicle-bilayer fusion, both chambers were perfused to remove any permeant anions and unfused vesicles. The *trans* chamber was perfused with 3 vol of 53 mM Ca^{2+} or Ba^{2+}/250 mM HEPES, pH 7.4, and the *cis* chamber was perfused with 3 vol 250 mM Tris/HEPES, pH 7.4. During the perfusion step, buffer was pumped into the bottom of each chamber via a small Tygon hose and simultaneously withdrawn through a hose positioned at the top of the chamber. The density of the HEPES perfusion buffer was greater than that of the choline Cl solution so that during perfusion the choline Cl was effectively displaced by the HEPES solutions. In control experiments designed to test the effectiveness of the perfusion, 6-carboxyl fluorescein fluorescence was monitored as a marker for the choline Cl buffer. After perfusion of 6-carboxyl fluorescein, fluorescence was decreased by >99%, which indicates a very efficient replacement by the HEPES perfusate.

Data Acquisition and Analysis

The current amplifier circuit for measuring single channels was built using an LF-157-AH operational amplifier (National Semiconductor, Santa Clara, CA) and a 10-GΩ feedback resistor (Smith et al., 1986*b*). Single channel fluctuations were recorded on a Racal (Saratoga, FL) FM tape recorder or digitized at 1 point/ms and transferred directly to a Bernoulli disk (Infax, GA) for storage, using an IBM XT computer. For kinetic analysis, most records were taken from FM tape, filtered at 300 Hz (−3-dB point from a low-pass eight-pole Bessel filter), and digitized at 1 point/ms. The duration of open and closed events was measured by placing two threshold detectors between the baseline and the open peak current. One detector (discr 1) was placed at 1 standard deviation (SD) from the mean baseline current and the second detector (discr 2) was placed at 1 SD from the mean single channel current. The initiation of an open event was defined as a transition that started from the baseline, crossed discr 1 and discr 2, and remained above discr 2 for two or more consecutive points. The termination of an open event was similarly defined as an event lying above discr 2 that crossed discr 2 and discr 1 and remained below discr 1 for two or more consecutive points. The two-threshold criterion (as opposed to a single threshold at the 50% open level) permitted a careful elimination of (*a*) fluctuations in baseline noise that would otherwise be counted as true events, and (*b*) true events that are attentuated by the filter setting and are thus erroneous. The use of the SD of the baseline and open channel noise distributions as threshold points was found to be a convenient way to compare data sets from recordings with different noise levels. By extrapolating the fitted exponentials to $t = 0$, we estimate that the fraction of short events (<2 ms) missed by our event-counting routines (or captured and discarded) was <20%. Open and closed duration histograms are given as the cumulative sum of the number of events of duration longer than time t, plotted as a function of t. That is, the fraction of events that, while being open or closed at $t = 0$, had not switched to the opposite state by

time t (Ehrenstein et al., 1974). Events of <2 ms were not included in the exponential fit of histograms. In all records, openings are presented as upward deflections.

RESULTS

The SR Ca release channel exhibits a complex kinetic profile when examined in planar lipid bilayers. Fig. 1 illustrates typical gating behavior observed when the channel is present in planar bilayers in the presence of: <10 nM *cis* Ca^{2+}, 2 μM

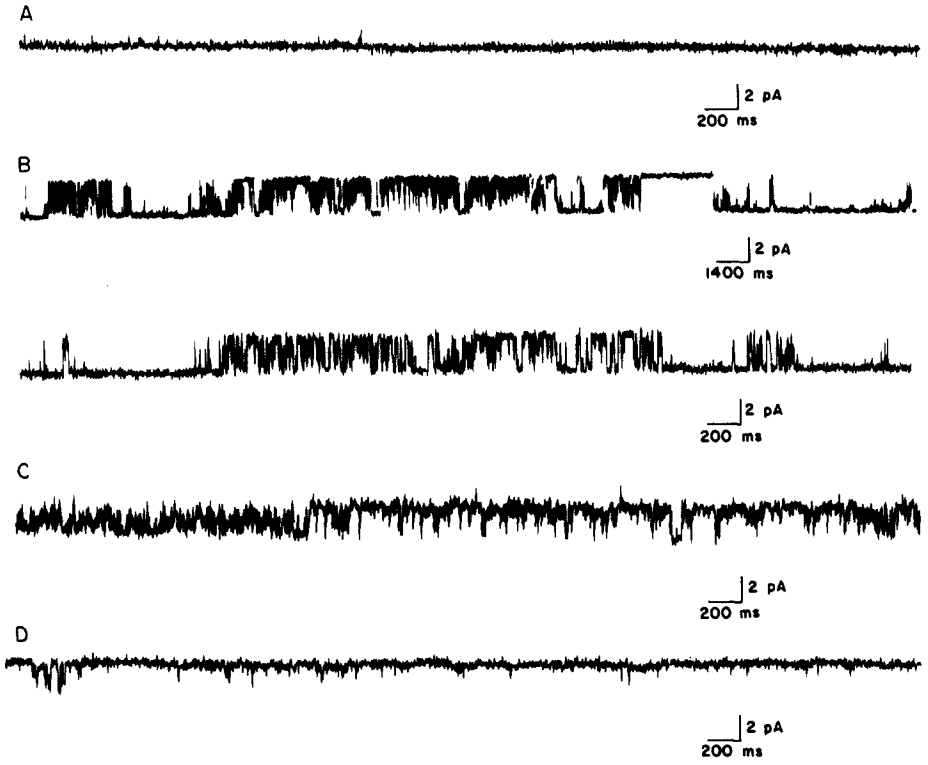

FIGURE 1. Single channel recordings of the SR Ca release channel. Recordings were made in the presence of 125 mM Tris/250 mM HEPES, pH 7.4, *cis*, and 53 mM $Ca(OH)_2$/250 mM HEPES, pH 7.4, *trans*. In addition, the *cis* chamber contained: (*A*) 0.1 mM Ca^{2+} plus 5.1 mM EGTA (<10 nM free Ca^{2+}), (*B*) 100 μM CaEGTA (2 μM free Ca^{2+}), (*C*) 10 mM ATP, 0.1 mM Ca^{2+}, and 5.1 mM EGTA, and (*D*) 100 μM CaEGTA and 1.75 mM ATP. The solid line in *D* indicates the closed current level. Records were taken at room temperature (20–22°C) and filtered at 300 Hz.

cis Ca^{2+}, <10 nM *cis* Ca^{2+} plus 10 mM ATP, and 2 μM *cis* Ca^{2+} plus 1.75 mM ATP, in each case with 53 mM *trans* Ca^{2+} as the current carrier. At <10 nM *cis* Ca^{2+}, the channel is virtually silent. The current trace in Fig. 1*A* is a typical recording at <10 nM free *cis* Ca^{2+} and shows no clearly resolvable open channel events. With 2 μM Ca^{2+} in the *cis* chamber, the channel was much more active. Open events were frequent and of variable duration. Both short and long events

were seen either separately or grouped into bursts of activity sometimes lasting several seconds. In single channel recordings, the open probability, P_o, can vary from one experiment to the next over a range as large as 0.005–0.55. However, in most experiments, the P_o was usually in the range of 0.02–0.15 for single channel membranes. The current trace in Fig. 1*B* in the presence of 2 μM *cis* Ca^{2+} displayed a P_o of 0.39 at 0 mV with no observable double open events even at more positive voltages, where P_o is generally higher. Fig. 1*C* illustrates the single channel behavior recorded in the presence of <10 nM Ca^{2+} plus 10 mM *cis* ATP. This buffer condition results in a partially activated state with a P_o of 0.6. Fig. 1*D* shows a current trace taken in the presence of 2 μM Ca^{2+} and 1.75 mM *cis* ATP. Under these conditions, the channel is almost exclusively found in

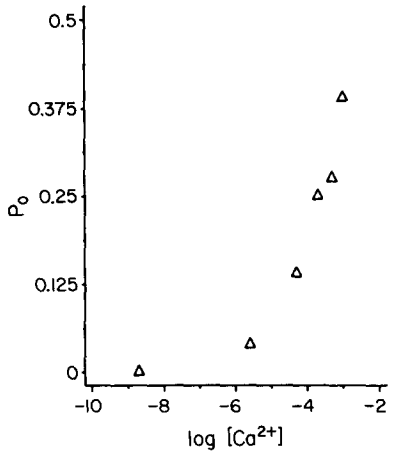

FIGURE 2. Effect of *cis* Ca^{2+} on channel open time. P_o at different *cis* [Ca^{2+}] was determined from single channel recordings made in 100 μM EGTA, 125 mM Tris (base)/250 mM HEPES, pH 7.4, *cis*, and 53 mM $Ca(OH)_2$/250 mM HEPES, pH 7.4, *trans*. The entire plot is constructed from recordings made during one experiment.

the open state, with brief closed transitions occurring only infrequently. The P_o in this trace was determined to be >0.99. This single channel behavior is comparable to the situation in passively loaded SR vesicles, where optimal $^{45}Ca^{2+}$ flux rates have also been obtained at micromolar Ca^{2+} and millimolar adenine nucleotide (Meissner et al., 1986).

The SR Ca release channel in bilayers is activated by increasing *cis* Ca^{2+} from nanomolar to micromolar concentrations. In Fig. 2, a plot of single channel open probability (P_o) vs. *cis* Ca^{2+} concentration shows that when *cis* Ca^{2+} was raised from <10 nM to 2 μM, P_o increased from 0 to 0.04. P_o increased a total of 10-fold when *cis* Ca^{2+} was raised from 2 to 950 μM. Beyond 950 μM *cis* Ca^{2+}, a decrease in P_o was observed. Fig. 3 shows open and closed time histograms that were constructed from single channel current records similar to those shown in Fig. 1. Fig. 3, *A* and *B*, shows the open and closed event distributions for a channel at two *cis* Ca^{2+} concentrations. In *A*, an open event histogram was

constructed from current traces recorded in the presence of 2 μM *cis* Ca^{2+}. P_o in this instance was 0.039. The open event distribution was fitted to the sum of a double-exponential function with $\tau_1 = 6.5$ ms and $\tau_2 = 35$ ms. When the *cis* Ca^{2+} concentration was increased to 200 μM in *A*, the resulting histogram could be fitted with the time constants $\tau_1 = 6.5$ ms and $\tau_2 = 32$ ms. However, P_o at this

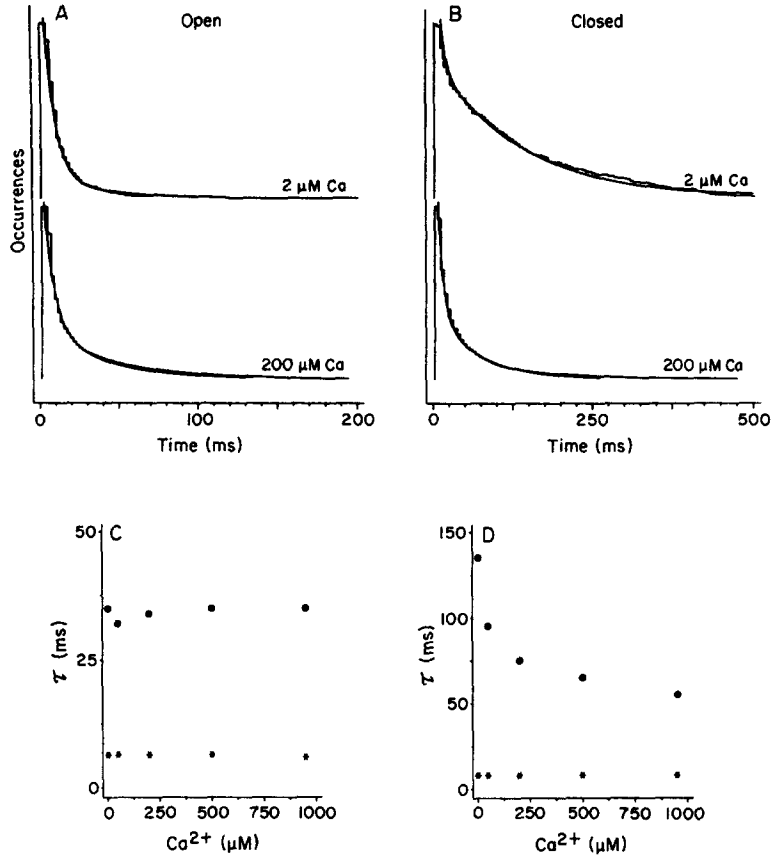

FIGURE 3. Effect of *cis* Ca^{2+} on open and closed event lifetimes. The cumulative event histograms in *A* and *B* were constructed from single channel records made in the presence of 125 mM Tris (base)/250 mM HEPES, pH 7.4, *cis*, and 53 mM $Ca(OH)_2$/250 mM HEPES, pH 7.4, *trans*, and either 2 or 200 μM free *cis* Ca^{2+}. τ values from exponential fits are plotted in *C* (open) and *D* (closed) vs. *cis* Ca^{2+} concentration.

higher Ca^{2+} was found to be 0.25. This increase in P_o was due to an increase in the total number of open events and not to an increase in the open state lifetimes. In *B*, the closed time histograms at 2 and 200 μM *cis* Ca^{2+} were fitted to double-exponential sums as in *A*. In each case, the shorter lifetime was fitted by $\tau_1 = 8$ ms; however, the longer τ_2 changed from 135 ms at 2 μM Ca^{2+} to 75 ms at 200 μM Ca^{2+}. The resulting time constants from *A* and *B* are plotted vs. *cis* Ca^{2+} concentration in *C* and *D*. In *C*, the open time τ_1 and τ_2 do not vary significantly

over the range of 2–950 μM Ca^{2+}, whereas P_o increased from 0.039 to 0.39 over the same concentration range (see Fig. 2). In Fig. 3*D*, the short closed lifetime remained constant at 8 ms over the entire Ca^{2+} activation range, whereas the longer closed τ varied in a manner dependent on the *cis* Ca^{2+} concentration. The decrease in one or more of the closed state τ values was expected to account for the observed increase in P_o during activation. However, the lack of any dependence of the open state on Ca^{2+} concentration was an unexpected finding. This is different from the model proposed for the Ca^{2+}-activated K^+ channel, in which the open state lifetime is directly dependent on activating Ca^{2+} concentration (Barrett et al., 1982; Moczydlowski and Latorre, 1983). Another dissimilar aspect of Ca^{2+} activation of the SR Ca channel is that P_o never reaches a value of 1 in the absence of adenine nucleotide.

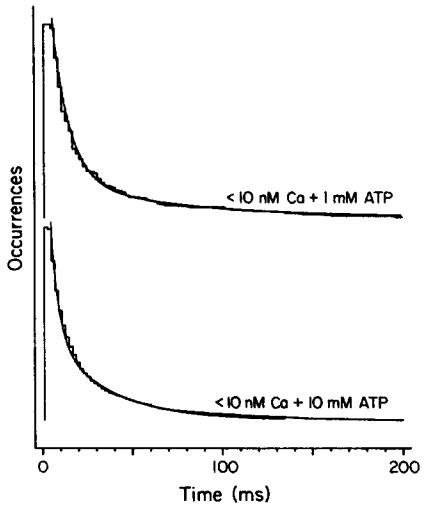

FIGURE 4. Effect of ATP on open lifetimes at low *cis* Ca^{2+}. Histograms were made as in Fig. 3, in the presence of 0.1 mM Ca^{2+} plus 5.1 mM EGTA (<10 nM free Ca^{2+}) and 1 or 10 mM ATP.

As shown in Fig. 1, ATP in the absence or presence of Ca^{2+} appears to be an efficient activator of the SR Ca release channel. Fig. 4 shows open time histograms made from single channel experiments recorded at <10 nM *cis* Ca^{2+} with 1 or 10 mM ATP present in the *cis* buffer. The exponential fit at 1 mM ATP gave τ_1 (open) = 10 ms and τ_2 (open) = 60 ms. In the case of 10 mM ATP, the fit gave τ_1 = 11 ms and τ_2 = 80 ms. P_o in this experiment increased from 0.10 at 1 mM to 0.62 at 10 mM ATP. This indicates that ATP in the absence of Ca^{2+} can activate the channel to an extent similar to or greater than Ca^{2+} alone. However, ATP alone is not sufficient to maximally activate the channel. A P_o of 1 was not obtained even at 10 mM ATP, a concentration that is well above saturation in vesicle flux experiments (Meissner et al., 1986). Adenine nucleotides in the presence of micromolar Ca^{2+} increase the amount of time the channel spends in the open state (Fig. 1). This effect on P_o is brought about by increasing the frequency and duration of open channel events. In Fig. 5, *A* and *B*, open and

closed time histograms were constructed from single channel recordings taken in the presence of 0 and 1.75 mM ATP at a constant 2 μM *cis* Ca^{2+}. The histograms were fitted as in Fig. 3 and the resulting τ values were plotted vs. ATP concentration in *C* and *D*. ATP in the presence of micromolar Ca^{2+} affects both the long open and closed states. The short open and closed τ values are largely unaffected by ATP. Table I summarizes the effects of Ca^{2+} and ATP on the open and closed event lifetimes.

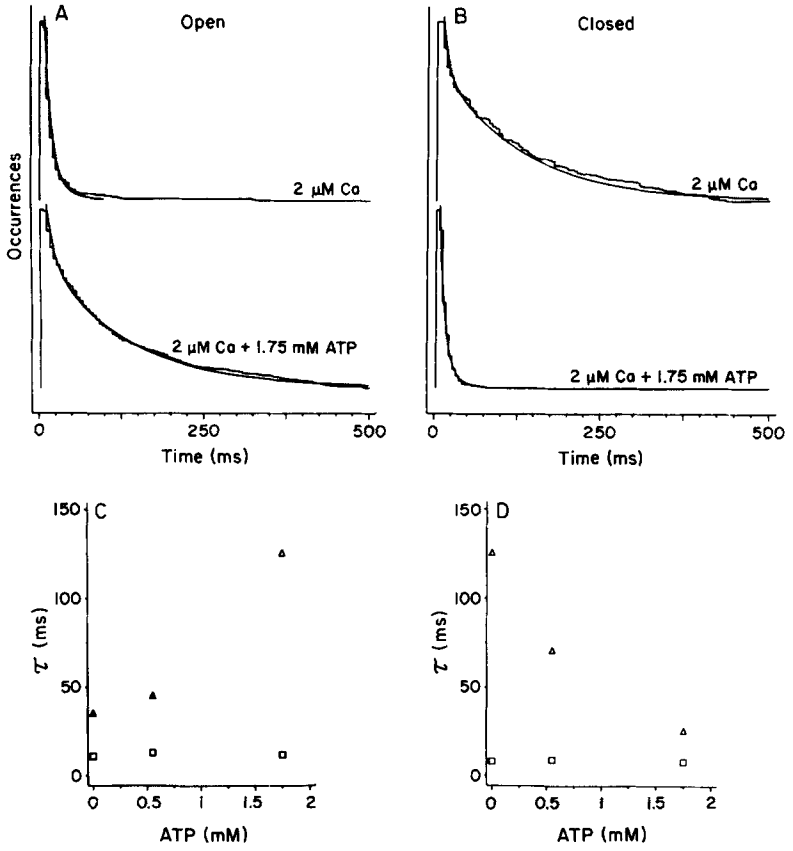

FIGURE 5. Effect of ATP on open and closed lifetimes at micromolar *cis* Ca^{2+}. Histograms were made as in Fig. 3, but at constant 2 μM *cis* Ca^{2+} with 0 mM *cis* ATP (top) or 1.75 mM *cis* ATP (bottom). τ values from exponential fits at 0, 0.55, and 1.75 mM *cis* ATP are plotted vs. [ATP] in *C* (open) and *D* (closed).

Mg^{2+} plays an important role in the modulation of Ca^{2+} currents through the SR Ca release channel. Ca^{2+} release from passively loaded SR vesicles is inhibited by external Mg^{2+} with a half-maximal inhibitory concentration near 100 μM (Meissner et al., 1986). No effect of intravesicular Mg^{2+} on the Ca^{2+} efflux rate was observed when vesicles were passively loaded with $^{45}Ca^{2+}$ in solutions containing millimolar Mg^{2+} (Meissner et al., 1986). In planar lipid bilayers, the SR Ca release channel was inhibited by *cis* (external) Mg^{2+}, whereas no inhibition

TABLE I

Effects of Ca^{2+} and ATP on Single Channel Lifetimes

		Open			Closed		
Ca^{2+}	ATP	τ_1	τ_2	N_{τ_2}/N_{τ_2}	τ_1	τ_2	N_{τ_2}/N_{τ_2}
M	*M*		*ms*			*ms*	
2×10^{-6}		6.5	35	—	8	135	0.9
5×10^{-5}		6.5	32	3.2	8	95	2.1
2×10^{-4}		6.5	34	2.7	8	75	2.2
5×10^{-4}		6.5	35	2.2	8	65	2.4
9.5×10^{-4}		6	35	2.5	8	55	3.7
$<1 \times 10^{-8}$	1×10^{-3}	10	65	5.2	11	130	0.8
$<1 \times 10^{-8}$	1×10^{-2}	11	80	2.4	12	45	3.0
2×10^{-6}	5.5×10^{-4}	13	45	2.2	9	70	2.0
2×10^{-6}	1.75×10^{-3}	12	125	0.3	8	25	9.0

was seen to occur with *trans* Mg^{2+}. In fact, the SR Ca release channel conducted substantial Mg^{2+} current when Mg^{2+} was present in the *trans* bilayer buffer. This finding was anticipated in light of a recent report (Nagasaki and Kasai, 1984), which indicated that Ca^{2+}-induced Mg^{2+} flux from SR vesicles could be measured with chlortetracycline fluorescence stopped-flow techniques. Fig. 6 compares the single channel conductance obtained with 53 mM *trans* Ca^{2+} to that observed in the presence of 125 mM *trans* Mg^{2+}. The slope conductance calculated for 53 mM *trans* Ca^{2+} was 96 ± 4 pS, whereas that for 125 Mg^{2+} was 39 ± 3 pS. The conductance ratio $Mg^{2+}/Ca^{2+} = 0.40$ is surprising when one considers that Mg^{2+} currents through other Ca^{2+} channels (Almers and Palade, 1981) are so small that single channel fluctuations caused by Mg^{2+} currents have not been detected (Brown et al., 1984; Hess et al., 1984; Affolter and Coronado, 1985).

The effects of *cis* Mg^{2+} on single channel and macroscopic currents are described in Figs. 7 and 8. Fig. 7 shows single channel recordings of an SR Ca channel recorded with 53 mM *trans* Ca^{2+} as the conducting divalent cation. In

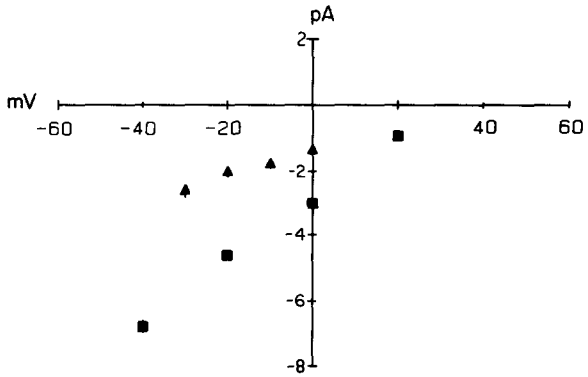

FIGURE 6. Comparison of single channel current voltage relationship with Mg^{2+} or Ca^{2+} as the current carrier. Single channel recordings were made with 100 μM CaEGTA (2 μM free Ca^{2+}), 125 mM Tris (base)/250 mM HEPES, pH 7.4, *cis*, and with either 53 mM $Ca(OH)_2$/250 HEPES, pH 7.4 (■), or 250 mM Mg/gluconate (hemi-Mg^{2+} salt), 10 mM Mg/HEPES, pH 7.4, *trans* (▲).

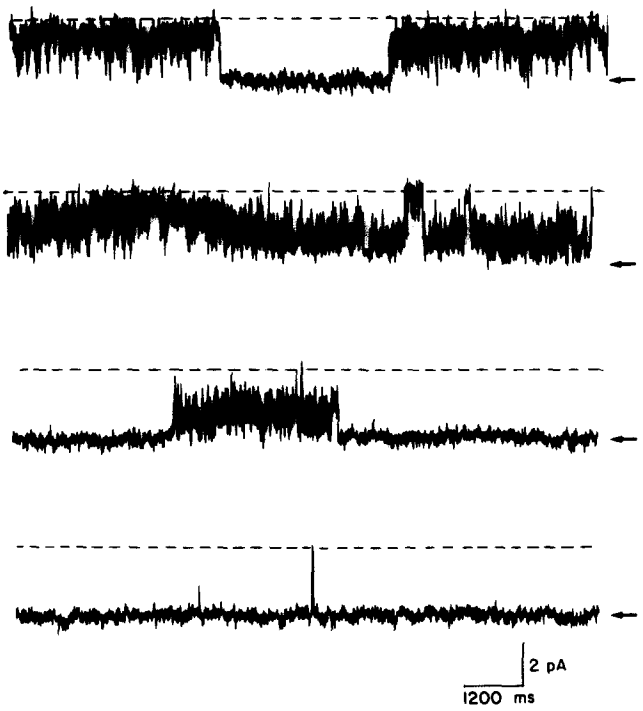

FIGURE 7. Effect of Mg^{2+} on single SR Ca channels in the presence of nanomolar *cis* Ca^{2+}. Single channel records were made in Tris (*cis*)/Ca (*trans*) HEPES, pH 7.4, with 0.1 mM Ca^{2+}, 3.7 mM EGTA (<10 nM free *cis* Ca^{2+}), and 1.84 mM ATP added to the *cis* chamber (upper trace). The lower traces were recorded at 12-s intervals after addition of 0.120 mM *cis* Mg^{2+}. The arrows indicate baseline current and the dashed lines indicate open channel current.

the first trace, the channel was partially activated in the presence of 1.84 mM ATP and <10 nM *cis* free Ca^{2+}. When 0.120 mM Mg^{2+} was added *cis* (4 μM free Mg^{2+}) (second trace), the channel began to flicker so rapidly that individual transitions were almost impossible to resolve. There were some open events that

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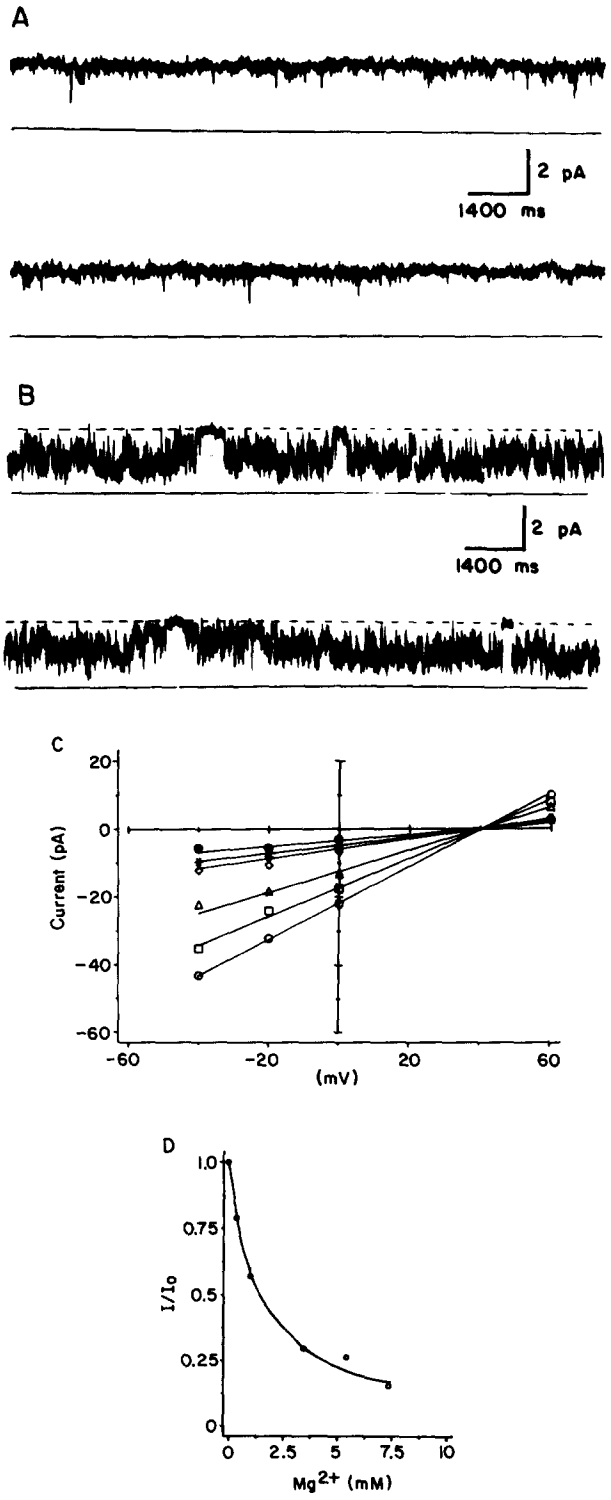
FIGURE 8. (*opposite*) Effect of *cis* Mg^{2+} on single channel and macroscopic currents in the presence of micromolar *cis* Ca^{2+}. (*A* and *B*) Single channel records made in Tris/Ca HEPES, pH 7.4, plus 100 μM CaEGTA (2 μM free Ca^{2+}), *cis*, and 0.55 mM AMP-PCP (*A*) or 0.55 mM AMP-PCP plus 0.56 mM Mg^{2+} (as gluconate salt) (*B*). The solid lines indicate the baseline current and the dashed lines indicate the open channel current. (*C*) Macroscopic current-voltage relationship made in 100 μM CaEGTA, 3.6 mM AMP-PCP, 125 mM Tris (base)/250 mM HEPES, pH 7.4, *cis*, and 50 mM $Ba(OH)_2$/250 mM HEPES, pH 7.4, *trans*. Total *cis* Mg^{2+} was: 0 (○), 3.4 (□), 4.4 (△), 6.97 (◇), 8.97 (*), and 11 mM (●). Slopes from linear regression of the data at each Mg^{2+} gave 0.541 (○), 0.429 (□), 0.310 (△), 0.162 (◇), 0.143 (*), and 0.083 (●). (*D*) Mg^{2+} inhibition fit to a single-site titration curve of the form $I/I_o = (1 + cis\ Mg^{2+}/K_i)^{-1}$ with K_i = 1.5 mM. Free Mg^{2+} was calculated using ATP binding constants given in Fabiato (1981).


A
2 pA
1400 ms
B
2 pA
1400 ms
C
Current (pA)
20
0
-20
-40
-60
-60
-20
20
60
(mV)
D
I/I₀
1.0
0.75
0.5
0.25
0
0
2.5
5
7.5
10
Mg2+ (mM)

were long enough in duration to be fully resolved, and from these we can tell that the single channel conductance had not been appreciably decreased. By 16 s after *cis* Mg^{2+} addition (third trace), channel openings appeared as widely separated bursts of activity. Within these bursts, the transitions were for the most part poorly resolved, resulting in an apparent attenuation of the single channel conductance. As in the second trace, however, some fully resolved events could be recorded. 30 s after Mg^{2+} addition (fourth trace), inhibition was essentially complete, with channel openings occurring only infrequently.

Fig. 8, *A* and *B*, shows the effect of *cis* Mg^{2+} on nucleotide-activated single channel currents recorded in the presence of 2 μM *cis* Ca^{2+}. In *A*, a channel was fully activated ($P_o > 0.99$) in the presence of 2 μM Ca^{2+} and 0.55 mM *cis* AMP-PCP. In *B*, 0.56 mM Mg^{2+} (170 μM free Mg^{2+}) was added to the *cis* chamber, which resulted in an unresolvable flickering of the channel. Most events were too brief to be resolved; however, as in Fig. 7, a few openings were long enough to be recorded with the full single channel current. Since resolution at the single channel level was poor, multichannel experiments were performed to determine an IC_{50} for *cis* Mg^{2+} inhibition. In Fig. 8*C*, a macroscopic Ba^{2+} current from Ca release channels in the presence of 3.6 mM AMP-PCP and 100 μM CaEGTA (2 μM free Ca^{2+}) was inhibited by repeated additions of Mg^{2+} to the *cis* buffer. Slope conductances from linear regression of the data points at each Mg^{2+} were normalized to the value at zero Mg^{2+} and plotted as θ, the fraction of the current remaining, vs. free Mg^{2+} (determined using binding constants for MgATP from Fabiato, 1981). The resulting plot was fit to a single-site titration curve of the form $\theta = I/I_o = (1 + Mg^{2+}/K_i)^{-1}$, where $K_i = 1.5$ mM. This 50% inhibitory concentration may be a slight overestimation caused by the uncertainty in the actual degree of complexation between Mg^{2+}, Ca^{2+}, and the ATP analogue. Nevertheless, we feel that at this point it is important to emphasize the modulatory role that Mg^{2+} exerts on the Ca release channel in vitro.

DISCUSSION

Ca^{2+} and adenine nucleotide activation of the skeletal SR Ca release channel are important clues to understanding the mechanism of excitation-contraction coupling in muscle. Chemical messengers and voltage activation of the SR Ca^{2+} release channel have been considered by many to be the mechanisms whereby t-tubule depolarization causes Ca^{2+} release (Endo, 1977; Martonosi, 1984). However, several studies have suggested that the SR membrane is incapable of sustaining transmembrane potentials of more than a few millivolts (Meissner, 1983). Also, single channel measurements of SR Ca release channels have demonstrated only a weak voltage dependence for channels in artificial membranes (Smith et al., 1985). Various substances have been proposed as chemical messengers in excitation-contraction coupling. In the recent literature, inositol 1,4,5-trisphosphate (IP_3) has received much attention as a possible messenger in t-tubule/SR coupling (Hirata et al., 1984; Somlyo, 1985; Vergara et al., 1985; Volpe et al., 1985). We have been unable to demonstrate activation of SR Ca channels by IP_3 in both bilayer and vesicle flux experiments (Smith, J. S., R. Coronado, and G. Meissner, unpublished results; Rousseau et al., 1986). On the

other hand, Ca^{2+}-induced opening of SR Ca channels, i.e., Ca^{2+}-induced Ca^{2+} release, has been demonstrated repeatedly and reproducibly in skinned fibers (Stephenson, 1981; Endo, 1977; Fabiato, 1983), isolated SR vesicles (Kirino et al., 1983; Nagasaki and Kasai, 1983; Meissner, 1984; Meissner et al., 1986), and now in planar bilayers. In light of these findings, the effects of Ca^{2+} must be considered relevant to the process of excitation-contraction coupling in muscle.

The high-conductance SR Ca channel activates when *cis* Ca^{2+} is increased from nanomolar to micromolar concentrations (Fig. 2). Activation in bilayers continues with repeated increases in *cis* Ca^{2+} until ~1 mM free Ca^{2+} is present in the *cis* chamber. This behavior is different from that observed in vesicle flux and skinned fiber experiments, which indicate that inactivation of radioisotope flux or tension development occurs in the 50–100-μM range (Fabiato, 1985; Meissner et al., 1986). In addition, the behavior of the channel in the bilayers differed from the expected behavior in muscle in that Ca^{2+}-stimulated channel openings did not inactivate with time. These differences may be due to the time-invariant *cis* and *trans* Ca^{2+} concentrations in the bilayer experiments as well as the large *trans* Ca^{2+} driving force (53 mM), which may prevent inactivation by *cis* Ca^{2+} at some low-affinity inhibitory site(s).

Activation by either Ca^{2+} or ATP can be demonstrated with similar four-state kinetics consisting of two open and two closed states. The presence of more than four distinct states is almost certain; however, our bilayer frequency resolution limits the number of states that can be satisfactorily identified. Activating Ca^{2+} causes the channel to open more frequently but does not increase the duration of open events (see Figs. 2 and 3 and Table I). In Fig. 3, it was shown that Ca^{2+} affected only the duration of the longer closed state. Neither the two open state τ values nor the short closed τ was changed by increasing *cis* Ca^{2+}. Therefore, we propose that Ca^{2+} causes activation by interacting with the channel in an inactive closed state. The Ca^{2+}-activated closed channel either opens or reverts to a Ca^{2+}-unbound closed state (Fig. 9*A*). The frequency of channel opening increases with increased Ca^{2+} because of more frequent interaction (binding) with the activating site(s). Saturation of P_o at values less than unity may be due to the presence of multiple closed states. According to the scheme, Ca^{2+} interacts with only the inactive closed state and promotes transitions away from that closed state into other open or closed states. Saturation of activation will occur when the Ca^{2+}-affected closed state becomes insignificant compared with the other molecular states. At saturation, the Ca^{2+}-activated channel would gate according to the apparent rates of transition between Ca^{2+}-bound open and closed states.

We consider channel activation by adenine nucleotides to proceed through a similar mechanism. ATP in the absence of Ca^{2+} increases the frequency of open events and decreases the duration of closed events fit with the longer τ_2 (Fig. 4 and Table I). ATP seems to promote longer open event lifetimes than Ca^{2+}; however, as with Ca^{2+}, ATP affects primarily the long closed state τ_2. In the above scheme, we consider that ATP causes activation by interacting with the closed channel at a site distinct from the Ca^{2+} activation site and that the ATP-activated state is distinct from the Ca^{2+}-activated state.

ATP and Ca^{2+} together produce a synergism of activation that increases the

duration of open events and allows the channel to remain open nearly 100% of the time. We consider this to be evidence for a unique set of open states produced by the interaction of both activating ligands with sites on the channel. Again, we consider the Ca^{2+}-plus-nucleotide open channel to be a molecular species distinct from either of the single ligand-activated states. In light of these assumptions, we propose that a minimum model for activation by Ca^{2+}, adenine nucleotide, or both ligands must consider at least six ligand-activated closed states and six ligand-activated open states (Fig. 9*B*). The binding of one or more ligands to an inactive, non–ligand-bound channel promotes transitions away from that state. When only one activating ligand is present, saturation of activation at values less

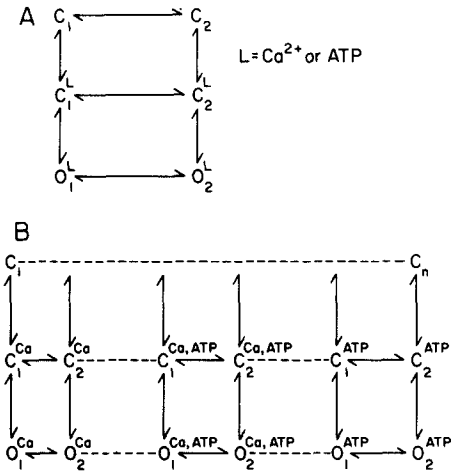


FIGURE 9. Model for SR Ca channel activation by Ca^{2+} and ATP. (*A*) Model for activation by one ligand. C_1 and C_2 represent inactive closed states. C_1^L and C_2^L are ligand-activated closed states. O_1^L and O_2^L are ligand-activated open states. L can be either Ca^{2+} or ATP. (*B*) Model for activation by both Ca^{2+} and ATP. $C_1 \ldots C_n$ represent inactive closed states. The dashed lines indicate the possible presence of additional states.

than unity occurs when the rate of transition away from the inactive closed state(s) becomes great enough to allow the channel to gate according to the apparent rate constants governing the transitions between ligand-activated closed and open states. Transitions between single-ligand–activated and double-ligand–activated states may occur either from inactive closed states or ligand-activated states. With saturating amounts of Ca^{2+} and ATP present, rates of transition into open states are accelerated to a point where transitions back into closed states are rare.

The question of how Mg^{2+} fits into the reaction scheme is much more difficult to approach because of the multiplicity of possible binding sites that may be present on the channel. Mg^{2+} is undoubtedly an important modulator of SR Ca release channel activity in vivo, although its precise role is at present uncertain. It has been proposed that Mg^{2+} together with nucleotide increases the cooperative interactions leading to channel opening (Meissner et al., 1986). We have

demonstrated in bilayers a negative modulatory effect of Mg^{2+} on the Ca release channel, but the mechanism of this interaction and its significance to channel function have yet to be explained. Several authors have suggested that Mg^{2+} competes with Ca^{2+} for the Ca^{2+} activation site(s) (Nagasaki and Kasai, 1983; Kirino et al., 1983; Meissner et al., 1986). The experiments described in Fig. 7 strongly suggest such a scenario. 4 μM Mg^{2+} at <10 nM *cis* Ca^{2+} completely inhibited the nucleotide-activated channel, whereas 1.5 mM Mg^{2+} was required to inhibit 50% of the nucleotide-activated channel conductance at 2 μM *cis* Ca^{2+}. At micromolar *cis* Ca^{2+}, another mode of action would be ion blockade by Mg^{2+} at some site near the conduction pathway (Yamamoto and Kasai, 1982). The finding that Mg^{2+} is significantly permeant, although less so than Ca^{2+}, also suggests that *cis* Mg^{2+} might interact near the conduction pathway when present at millimolar concentrations. To better define the precise interactions of Mg^{2+} with the Ca release channel, it will be necessary to improve bandwidth resolution at the single channel level or to resort to noise analysis for answers.

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