Interactions of Divalent Cations with Single Calcium Channels from Rat Brain Synaptosomes

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ABSTRACT Voltage-dependent calcium channels from a rat brain membrane preparation ("synaptosomes") were incorporated into planar lipid bilayers. The effects of calcium, barium, strontium, manganese, and cadmium ions on the amplitudes and kinetics of single channel currents were examined. The order of single channel conductances was $g_{Ba} > g_{Ca} = g_{Sr} > g_{Mn}$, which was the inverse of the order of the mean channel open times: $T_{Mn} > T_{Ca} = T_{Sr} > T_{Ba}$. In contrast, the identity of the charge carrier had little or no effect on the mean closed times of the channel. Manganese, in the absence of other permeant ions, can pass through single channels (g_{Mn} = 4 pS). However, when added to a solution that contained another type of permeant divalent cation, manganese reduced the single channel current in a voltage-dependent manner. Cadmium, a potent blocker of macroscopic "ensemble" calcium currents in many preparations, reduced the current through an open channel in a manner consistent with Cd ions both not being measurably permeant and interacting with a single site. The permeant ions competed with cadmium for this site with the following order: Mn > Sr = Ca > Ba. These results are consistent with the existence of no less than one divalent cation binding site in the channel that regulates ion permeation.

INTRODUCTION

Calcium ions that enter many excitable cells through voltage-dependent channels affect many important physiological processes, including neurotransmitter release and muscle contraction (cf. Hagiwara and Byerly, 1981; Tsien, 1983). One important step in ion permeation through the calcium channel may be the association of Ca^{2+} with a binding site in the channel (cf. Hagiwara and Byerly, 1981). The existence of a binding site that regulates ion permeation has been supported by the observation that blocking ions such as cadmium reduce calcium currents in a manner consistent with them competing with the permeant ions (e.g., Ca^{2+}, Ba^{2+}, Sr^{2+}) for a single site. However, recent studies that have

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J. GEN. PHYSIOL. © The Rockefeller University Press · 0022-1295/86/02/0201/22$1.00 201
Volume 87 February 1986 201–222

examined the effects of mixtures of permeant ions on calcium channel currents suggest that two binding sites may regulate ion permeation and selectivity (Kostyuk et al., 1983; Hess and Tsien, 1984; Almers and McCleskey, 1984). Furthermore, the interpretation of the effects of permeant ions on calcium channel behavior has been complicated by evidence indicating that the gating of the calcium channel is affected by the ions passing through it (Siami and Kung, 1982; Deitmer, 1983; Cavalie et al., 1983; Nelson et al., 1984).

To characterize the dependence of single calcium channel kinetics and currents on permeant and blocking divalent cations, single calcium channels from rat brain synaptosomes were incorporated into planar lipid bilayers. These channels have been shown to be voltage dependent and to select for calcium, barium, and strontium over monovalent ions (Nelson et al., 1984) and magnesium (Nelson, 1985*a*).

In this study, it is shown that manganese ions can permeate single calcium channels from brain synaptosomes. In the presence of a more permeant divalent cation, manganese ions can reduce current through single channels. The nature of the permeant ion affected not only the magnitude of the single channel current, but it also affected the mean open time of the channel. When added to one side of a single channel, blocking ions such as cadmium reduced the single channel current in a dose-dependent manner. The results are consistent with the permeant and blocking ions interacting with the same binding site in the permeation pathway.

Preliminary results have been presented (Nelson, 1984*a*, *b*, 1985*b*).

METHODS

Planar Lipid Bilayers and Solutions

Planar lipid bilayers composed of phosphatidylethanolamine (33 mg/ml, bovine brain, Avanti Polar Lipids, Inc., Birmingham, AL) and phosphatidylserine (13–26 mg/ml, bovine brain, Avanti Polar Lipids, Inc.) in decane were painted across a 100–250-μm hole in a polystyrene or Lexan partition separating two identical solutions containing 250 mM of a divalent cation ($CaCl_2$, $BaCl_2$, $SrCl_2$, or $MnCl_2$), 7.5 mM HEPES, pH 7.0. The use of symmetric solutions minimized surface potential differences. High divalent cation concentrations were used for two reasons. (*a*) The single channel current is maximal at divalent cation concentrations of >150 mM (Nelson et al., 1984). This eliminates variation in the measured current that could result from small changes in the activity of the permeant ions at the channel's mouth. (*b*) With these concentrations of divalent cations, the bilayer membrane is saturated with divalent cations (McLaughlin et al., 1981). Cadmium ions were normally added from $CdCl_2$ concentrated stocks. Although the ion levels are expressed in concentrations, the activities of all ions are expected to be much lower than the concentrations (cf. Robinson and Stokes, 1959). The activity coefficients of the chloride salts of the four charge carriers (i.e., Ba, Ca, Sr, Mn) should be about the same. Therefore, the different effects of the four divalent ion types used here were not due to variations in ion activities. Cadmium forms complexes with chloride and has an activity coefficient lower than that of the permeant divalent cations (Robinson and Stokes, 1959).

Preparation of Membrane Vesicles

The median eminence was chosen as a source of Ca^{2+} channels because it is rich in nerve terminals and virtually devoid of neuronal cell bodies (cf. Moore and Johnston, 1982).

Nerve terminals are thought to have a high density of calcium channels. The membranes were prepared from 10–15 rat brain median eminences by a centrifugation procedure similar to that for preparing rat whole brain synaptosomes (Krueger et al., 1979; Nelson et al., 1983), with the omission of the sucrose gradient step. The synaptosome preparation has been shown to contain pinched-off resealed nerve terminals (Blaustein et al., 1977). These "resealed" nerve terminals are physiologically functional; they maintain resting potentials (Blaustein and Goldring, 1975), release neurotransmitters upon depolarization (cf. Nelson and Blaustein, 1982), and have functional sodium channels (Krueger and Blaustein, 1980) and calcium channels (Nachshen and Blaustein, 1980). The median eminences were obtained from freshly killed rats and were homogenized in 0.32 M sucrose (HEPES-buffered, pH 7.0, with 0.2% bovine serum albumin) at 900 rpm (12 strokes) in a glass-Teflon homogenizer. The homogenate was then centrifuged at 1,000 *g* for 10 min and the supernatant was retained. The pellet was washed in 0.32 M sucrose, homogenized, and centrifuged again at 1,000 *g* for 10 min. The pooled supernatants were centrifuged for 10 min at 1,000 *g* and the resulting pellet was discarded. The supernatant was then centrifuged at 12,000 *g* for 20 min and the supernatant was discarded. This final pellet was suspended in 0.4 M sucrose and frozen at −77°C in small aliquots (still in 0.4 M sucrose). This "synaptosome" preparation (~300 μg total protein) was stored in ~20 aliquots of 15 μg protein each in 100 μl of 0.4 M sucrose (~0.15 mg protein/ml) at −77°C. Identical results were obtained with both fresh and frozen material. The thawed "synaptosomes" in 0.4 M sucrose were sonicated (probe type, model 1510, Braun Instruments, Burlingame, CA) for 20 s before use. This membrane vesicle suspension was added to one side (defined as the *cis* side) of a preformed planar bilayer at a final concentration of 0.1–0.5 μg protein/ml solution.

Incorporation of Channels

Using the procedure described above, channels spontaneously incorporated into preformed planar lipid bilayers. When many channels were incorporated into a bilayer, the protein concentration was reduced accordingly in order to reduce the probability of ion channel incorporation. As shown by Nelson et al. (1984), the channels described in this report exhibit characteristic constant unitary conductances and voltage-dependent kinetics. These properties were used to identify incorporated calcium channels. In the absence of high divalent cations, a variety of K^+ channels (Nelson and Reinhardt, 1984) and a batrachotoxin-activated, saxitoxin-sensitive sodium channel (cf. Krueger et al., 1983) have been incorporated from this preparation into planar lipid bilayers. However, thus far, in divalent cation solutions, the calcium channel presented here was the primary channel type observed. Occasionally, a high-conductance (~400 pS) anion-selective channel (cf. Blatz and Magleby, 1983) was seen in divalent cation ion salt solutions. The incorporation of calcium channels does not appear to depend on the nature of the divalent cation (Ca^{2+}, Ba^{2+}, Sr^{2+}, or Mn^{2+}) in the *cis* side, and does not depend on the presence of an osmotic gradient (cf. Cohen et al., 1982). In addition, negatively charged phospholipids in the bilayer do not appear to play a critical role in channel incorporation. Similar observations have been made for the incorporation of saxitoxin-sensitive Na^+ channels from rat brain into planar lipid bilayers (Worley, 1984). However, for the incorporation of both Ca^{2+} channels and Na^+ channels from brain, the presence of higher ionic strength solutions (e.g., having >100 mM $CaCl_2$ or 150 mM NaCl in the *cis* side) seemed to improve the probability of incorporating a functional channel. Furthermore, after incorporation of ion channels, perfusion of the *cis* side with a vesicle-free solution did not prevent further incorporation, which suggests that perfusion cannot remove vesicles that are in close approximation to the bilayer membrane (see Fig. 5) (cf. Cohen et al., 1982). Therefore, the *cis* or *trans* side of the bilayer was perfused only when the ionic composition needed

to be changed. These channels preferentially (>90%) incorporated into the bilayer with the orientation shown in the figures, i.e., *cis*-positive potentials caused the channels to open. Single channels that incorporated with the opposite orientation could be easily identified by their characteristic voltage dependence (cf. Nelson et al., 1984).

Electrical Recording

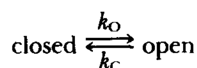
The side of the bilayer to which brain membrane vesicles were added was defined as the *cis* side and the opposite side of the bilayer was defined as the *trans* side. The voltages indicated in all experiments are *cis* minus *trans*, with the *trans* side held at virtual ground. The current across the bilayer was measured and command voltages were applied to the solutions via a pair of Ag/AgCl electrodes (Krueger et al., 1983; French et al., 1984; Nelson et al., 1984). The cutoff frequency (−3 dB) of the current-to-voltage converter (10^9 Ω feedback resistor) was 600 Hz. Ionic current records were recorded continuously on an FM tape recorder (Store 4, Racal, Sarasota, FL). Experiments were conducted at room temperature (20–24°C).

The conductance of the phospholipid bilayer before the addition of biological material was <10 pS. For analysis, the experiments were played back through an eight-pole Bessel filter (902 LPF 1B, Frequency Devices, Haverhill, MA) at 50 Hz (i.e., open and closed times <5 s could not be determined). For the open and closed time analysis, only fluctuations that reached >90% open or closed were used.

As inferred from their voltage dependence, these calcium channels have incorporated into the bilayer oriented with the extracellular ends of the channels facing the *trans* side and their cytoplasmic ends facing the *cis* side. The voltages reported here would then correspond to the usual cellular convention (inside minus outside). Therefore, the upward current deflections shown in the figures correspond to outward currents (positive charges moving from the *cis* side to the *trans* side, i.e., from inside to outside) (cf. Lee and Tsien, 1984).

Analysis

For the open time analysis, experiments with a low number of channels (one to three) were used. The open times were determined (by hand) from single-level fluctuations that terminated in a closure; simultaneous openings were rare and were not counted. If a conductance level corresponds to a single, time-homogeneous Markovian kinetic state (e.g., Colquhoun and Hawkes, 1981), then the lifetime in any single state should be exponentially distributed. Both the open times and closed times longer than ~10 ms could be fit by single exponentials. Because of the limited time resolution of the experiments, the fit of open and closed time distributions by single exponentials should be taken as a minimal model (see below). With this model, there are two states of the channel:



$$\text{closed} \underset{k_C}{\overset{k_O}{\rightleftharpoons}} \text{open}$$

where k_C is the rate constant for the transition from the open state to the closed state, and k_O is the rate constant for the transition from the closed state to the open state. The probability distribution function (PDF) of the lifetimes of the open state should then be equal to:

$$\text{PDF}_O = \exp(-k_C t), \qquad (1)$$

where the mean lifetime of the open state is equal to $1/k_C$ and t is the time after the channel opened. The PDF for the closed time intervals should be equal to:

$$\text{PDF}_C = \exp-(k_O)(N)t, \qquad (2)$$

where the mean lifetime of the closed state of an individual channel is equal to $1/k_O$, t is the time after a channel closed, and N is the number of functional channels in the membrane.

It should be emphasized that a two-state model is clearly a simplification. As can be seen in Figs. 1*A*, 3*A*, and 8, the open state is punctuated by unresolved, brief fluctuations (<10 ms) toward the closed state. These brief closures cannot be fully resolved because of the large bilayer membrane current noise (~1 pA rms noise at 500 Hz) and the small single channel currents (<1 pA). These fast fluctuations suggest that the synaptosomal calcium channel possesses a second closed state (i.e., two closed states and an open state) (cf. Reuter et al., 1982; Brown et al., 1982; Fenwick et al., 1982; Hagiwara and Ohmori, 1983; Cavalie et al., 1983).

RESULTS

Ion Dependence of the Single Channel Conductances and Open Times

Voltage-dependent channels were identified as "calcium" channels because they select for divalent cations over monovalent cations, anions, and magnesium (see below), and permit either Ba^{2+}, Sr^{2+}, and Ca^{2+} to permeate (Nelson et al., 1984; Nelson, 1985*c*). Fig. 1*A* shows that in addition to barium and strontium ions, manganese can also move through open single calcium channels from synaptosomes incorporated into planar lipid bilayers (see also Fig. 5*A*). Fig. 1*B* shows that with equimolar concentrations of Ba, Sr, and Mn, the following order of single channel conductances was observed: Ba (7.5 pS) > Sr (6 pS) > Mn (4 pS). The single channel conductance with calcium as the charge carrier was the same as that with strontium (see Nelson et al., 1984). Over the voltage range examined, the current carried by these ions through single channels was approximately proportional to the applied voltage (i.e., the single channel conductances were voltage independent).

The species of ion carrying charge through a single channel affected not only the single channel conductance, but it also affected the channel's open times. For example, at +100 mV, channels with Ba^{2+} as the charge carrier had a shorter mean open time than when Ca^{2+} or Sr^{2+} was the charge carrier (Nelson et al., 1984). This study further extends this observation of Nelson et al. (1984) by showing that Mn^{2+}, which has the lowest permeation rate of the ions studied (Fig. 1*B*), has the longest mean open time (Fig. 1*C*). At +120 mV, the mean open times with Ba^{2+}, Sr^{2+}, and Mn^{2+} were 266, 501, and 1,067 ms, respectively (Fig. 1*C*). Thus, the sequence of single channel conductances and closing rate constants [= 1/(mean open times)] for the permeant ions is the same: $Ba^{2+} > Sr^{2+} = Ca^{2+} > Mn^{2+}$. Fig. 1*C* shows that open time distributions for the different current carriers can be fit by single exponentials.

One possible explanation for the effect of the permeant ion on the single channel open time is that these ions affect the voltage dependence of the channel. Fig. 2 shows that the closing rate constant (1/mean open time) decreases e-fold for a 30-mV depolarization, regardless of the nature of the permeant ion. Although the permeant ion did not appear to affect the voltage dependence of the closing rate constants (i.e., the slopes of $1/T_o$ vs. voltage plots), it did cause a shift in this plot. This shift could be interpreted as an effect on the internal free energy of closing, i.e., the free energy of channel closing at 0 mV (cf. Miller and Rosenberg, 1979).

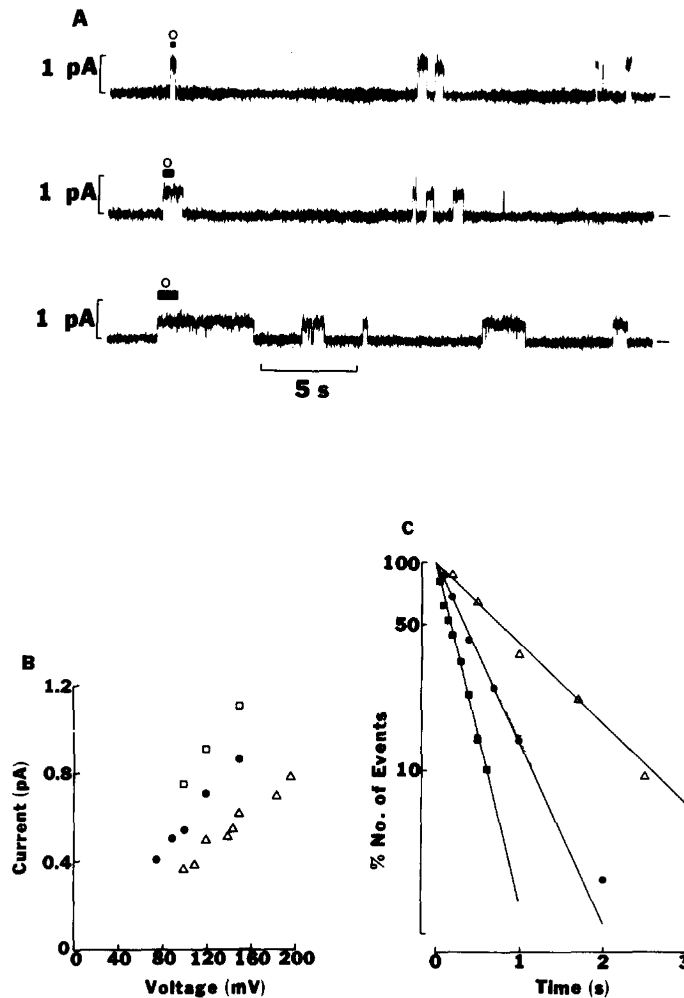


FIGURE 1. Ion dependence of the single channel currents and mean open times. (*A*) Original record of current fluctuations in symmetric concentrations of Ba^{2+} (top record), Sr^{2+} (middle record), and Mn^{2+} (bottom record). The holding potential was +120 mV, and the solid bars at the right indicate the current level at which all channels were closed. The filled rectangles above the first current step in each trace indicates the mean open time (0) for each current carrier. The mean open times (0) were determined from the slopes of the open time distributions seen in *C*. (*B*) Current-voltage relationship of a single channel with Ba^{2+} (□), Sr^{2+} (●), and Mn^{2+} (△). The divalent cation equilibrium potential and the zero-current potential were at 0 mV. Single channel conductances for Ba^{2+}, Sr^{2+}, and Mn^{2+} were 7.5, 6.0, and 4.1 pS, respectively (±0.2 pS, $n > 50$). (*C*) Effects of Ba^{2+} (■), Sr^{2+} (●), and Mn^{2+} (△) on the distribution of single channel open times at +120 mV. The total number of events and the slopes and correlation coefficients of the linear regression lines through the log-transformed data were: (Ba^{2+}) 102, $-3.76\ s^{-1}$, −0.998; (Sr^{2+}) 36, $-1.995\ s^{-1}$, −0.999; (Mn^{2+}) 221, $-0.937\ s^{-1}$, −0.998.

Interactions of Mixtures of Permeant Ions: Mn^{2+} Blocks

Fig. 1 shows that in the absence of other permeant ions, manganese can pass through these single channels. Fig. 3 shows that when added to a solution that contains another species of permeant ion (e.g., Sr), Mn^{2+} can block. The addition of 25 mM Mn^{2+} to the *cis* side reduced the single channel current at +100 mV by 27% (Fig. 3, *A* and *B*). The reduction in single channel current by Mn^{2+} was more pronounced at +50 mV (46%) (Fig. 3*C*). Therefore, the "block" by Mn^{2+}

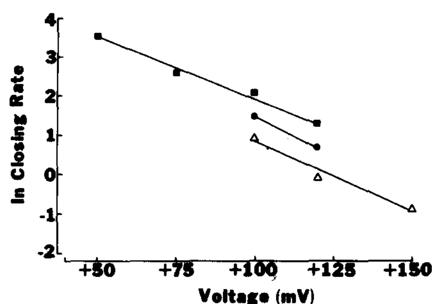


FIGURE 2. Effect of voltage on the log transform of the closing rate constants. The closing rate constants were determined from the open time distributions (e.g., Fig. 1*C*; also see Eqs. 1 and 2). In the case of a single energy barrier, the voltage dependence of the rate constants will be given by:

$$k_O = k_{O0.5}\exp(m)(q)(F/RT)(V_m - V_{0.5});$$

$$k_C = k_{C0.5}\exp-(q)(1 - m)(F/RT)(V_m - V_{0.5}),$$

where k_O is the opening rate constant, k_C is the closing rate constant, $k_{O0.5}$ and $k_{C0.5}$ are the rate constants at $V_{0.5}$ and are equal, and m is the fraction of the membrane potential that influences the transition from the closed state to the open state at the peak of the energy barrier. Because the voltage dependences of $\ln k_C$ and $\ln k_O$ (not shown; however, see Fig. 3*C* of Nelson et al., 1984) were about equal, this suggests that m is ~0.5 and that q (the apparent "gating" charge) is ~1.7. In other words, the closing and opening rate constants changed e-fold for ~30 mV depolarization. ■, Ba^{2+}; ●, Sr^{2+}; △, Mn^{2+}.

appears to be voltage dependent, with less positive voltages favoring block. In addition, the amplitudes of the single channel currents in this Mn^{2+}/Sr^{2+} mixture at +100 mV were similar to that in Mn^{2+} alone (cf. Fig. 1). Furthermore, Mn^{2+} not only reduced the single channel conductance with Sr^{2+} as the charge carrier, but it also increased the mean open time (Fig. 4*A*). This result suggests that the effects of the permeant ions on the amplitudes of the single channel currents and mean open times may be related (see Discussion).

Ion Dependence of Channel Closed Times

The addition of Mn^{2+} to an Sr^{2+}-containing solution caused a decrease in the single channel conductance and the closing rate constant (Figs. 3 and 4*A*). In

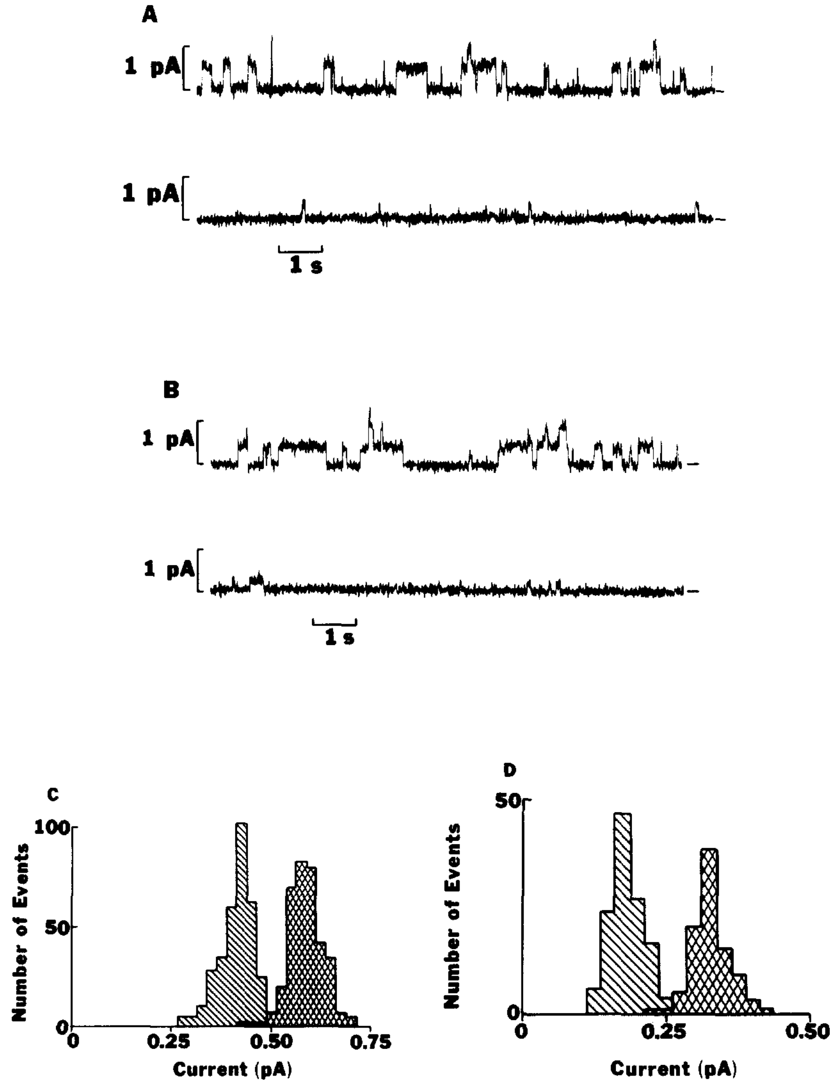


FIGURE 3. Reduction of single channel currents by Mn^{2+}. (*A*) Original record at +50 (upper trace) and +100 (lower trace) mV with Sr^{2+} as the charge carrier. (*B*) Original record at +50 (lower trace) and +100 (upper trace) mV after the addition of 25 mM Mn^{2+} to the *cis* side. (*C* and *D*) Effect of Mn on the single channel currents at +100 (*C*) and +50 (*D*) mV. At +100 mV, the mean single channel currents in Sr^{2+} alone (■) and in Sr^{2+} plus Mn^{2+} (▧) were 0.57 ± 0.06 pA (SD, n = 142) and 0.41 ± 0.05 pA (SD, n = 136), respectively. At +50 mV, the mean single channel currents in Sr^{2+} alone (■) and in Sr^{2+} plus Mn^{2+} (▧) were 0.33 ± 0.04 pA (SD, n = 109) and 0.18 ± 0.04 pA (SD, n = 123), respectively. Single channel current amplitudes were measured for the middle of the baseline current noise to the middle of the open channel current noise. For this analysis, at +100 and +50 mV, the records were filtered at 50 and 20 Hz, respectively. At 20 Hz, a change in current of ~0.05 pA could be detected.

contrast, Fig. 4*B* shows that Mn^{2+} did not significantly affect the mean closed time, which suggests that the opening rate constant is not substantially influenced by the ionic composition of the bathing solution. Furthermore, if the effect of Mn^{2+} on the open times was mediated by a simple surface charge effect, then one would expect that the channel's closed times would decrease because they are also voltage sensitive (cf. Nelson et al., 1984).

Determination of the Real Closed Time

The measured mean closed time is equal to the real closed time of an individual channel divided by the number of functional channels in the membrane (see Eq. 2). There are two approaches that can be used to determine the real closed time.

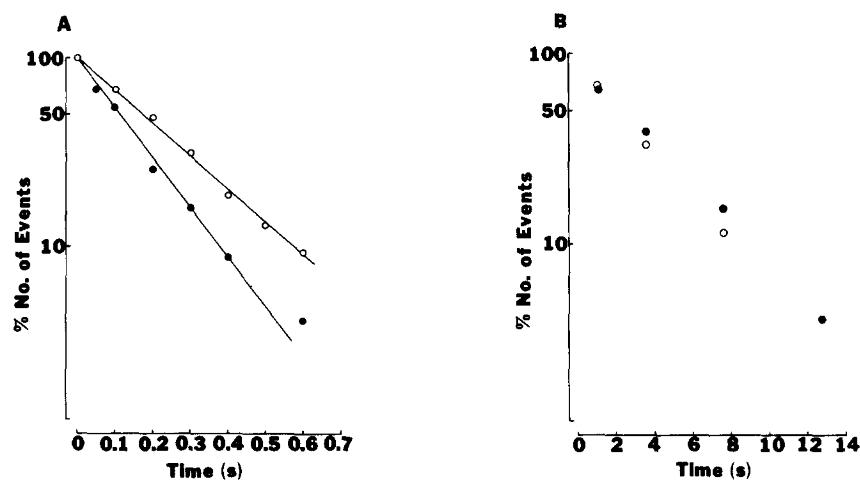


FIGURE 4. Effect of Mn^{2+} plus Sr^{2+} on channel open and closed times. (*A*) Open time distributions at +100 mV for Sr^{2+} (●) and Sr^{2+} plus Mn^{2+} (○). The total number of events and the slopes and correlation coefficients of the linear regression lines through the log-transformed data were: (Sr^{2+}) 125, −5.0 s^{-1}, −0.998; (Sr^{2+} plus Mn^{2+}) 86, −3.3 s^{-1}, −0.995. (*B*) Closed time distribution at +100 mV for Sr^{2+} (●) and Sr^{2+} plus Mn^{2+} (○). The mean closed times under both conditions were 3.4 s.

(*a*) One approach is to determine the number of channels in the membrane. This approach requires that the number of channels in the membrane not change during the course of the measurements and that sufficiently long records be taken when the channel has a reasonably high probability of being open (i.e., $P > 0.3$). This approach was used to determine that there were two functional channels in the membrane of the Mn^{2+} experiment shown in Fig. 1 (Mn^{2+} was the charge carrier and the real mean closed time was 23.3 s at +100 mV). In this experiment, the number of channels did not change over a 1-h period (i.e., the apparent mean closed times at +100, +120, and +150 mV did not change) and it was possible to depolarize the membrane to +200 mV ($P > 0.6$) to verify the number of functional channels. However, this approach is often difficult to

implement because the number of channels in the bilayer often increases with time (i.e., Fig. 5). (*b*) An alternative approach is to determine how the apparent mean closed time decreases as channels incorporate or become functional. Fig. 5 illustrates such an experiment, where it was possible to monitor the incorporation of calcium channels by determining how the apparent mean closed times

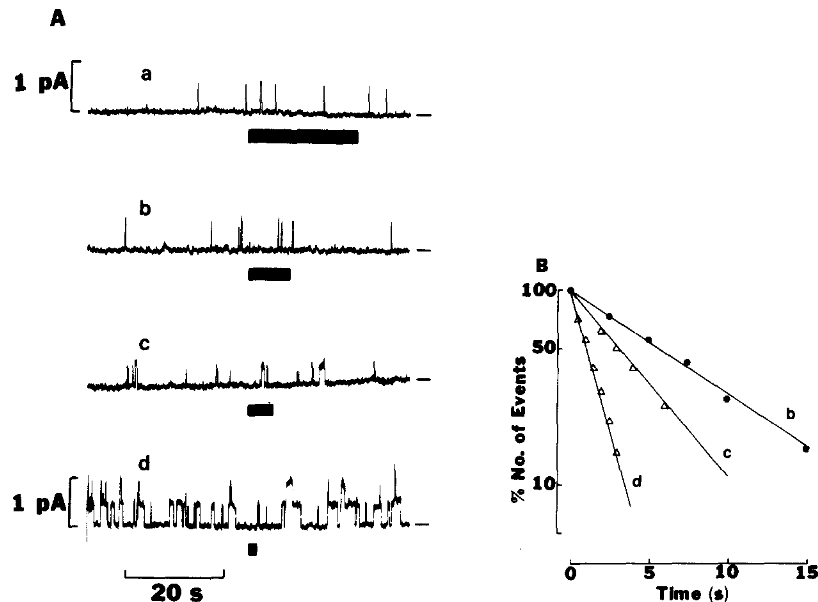


FIGURE 5. Effect of ion composition and number of channels in the membrane on the apparent mean closed times. (*A*) Original record of the time course of incorporation of channels into the bilayer. The top record (trace *a*) shows current fluctuations shortly after the detection of the first channel at +100 mV with Sr^{2+} as the charge carrier. The apparent mean closed time (denoted by the solid rectangular bar) and mean current remained constant for the first 16 min of the experiment. At 16 min after the detection of the first event, there was an approximately threefold increase in the mean current and a threefold decrease in the apparent mean closed time, which suggests that the number of functional channels in the bilayer had increased by a factor of 3 (trace *b*). The mean closed time or mean current level did not change for next 15 min. After ~32 min, the $SrCl_2$ was exchanged for $MnCl_2$ (trace *c*) (note changes in single channel currents and open times). After replacing Sr^{2+} with Mn^{2+}, the apparent mean closed time decreased from 8 to 5 s (*B*). After 60 min, the mean closed time decreased to ~1 s (trace *d*; panel *A*). (*B*) Closed time distributions from traces *b–d* in panel *A*. The total number of events and the slopes (mean closed time) of the log-transformed data were: (Sr^{2+}, *b*) 104, −0.1232 s^{-1} (8.12 s); (Mn^{2+}, *c*) 23, −0.2155 s^{-1} (4.64 s); (Mn^{2+}, *d*) 129, −0.6631 s^{-1} (1.5 s).

decreased with time. In this experiment, the number of channels in the bilayer appeared to increase from 1 to 15 over 70 min (i.e., the apparent mean closed time decreased 15-fold).

Fig. 5, *A* and *B*, shows that when Sr^{2+} was exchanged for Mn^{2+}, the apparent mean closed time decreased from 8.1 to 4.6 s. Therefore, after the solution change, there were five functional channels in the bilayer, i.e., the real mean

closed time with Mn^{2+} (23.3 s from the experiment in Fig. 1) divided by the apparent mean closed time (4.6 s). In the experiment shown in Fig. 5, at the beginning of the experiment, the apparent mean closed time (21.6 s) with Sr^{2+} as the charge carrier was similar to the real closed time with Mn^{2+}. These results suggest that there was one functional channel in the bilayer at the beginning of the experiment in Fig. 5, and then two more channels incorporated into the bilayer. Sr^{2+} was then replaced by Mn^{2+} and the number of channels increased from 3 to 5 and eventually to 15. The real opening rate of the channel at this

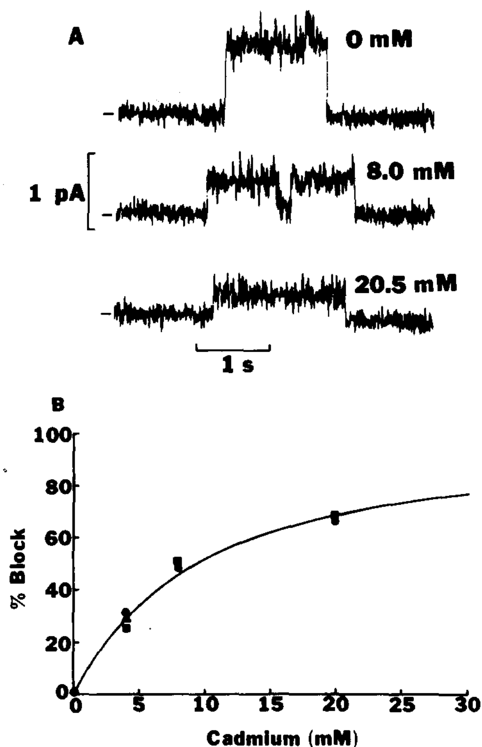


FIGURE 6. Cd^{2+} block at +150 mV. (*A*) Original record. The solid lines to the left of each record indicate the current level where all channels are closed. Cd^{2+} was added directly to the *cis* side from a 1-M stock solution. (*B*) Cd^{2+} concentration vs. percent block at three different voltages. Percent block is the percent reduction of the single channel currents, with 50% block being one-half the single current in the absence of Cd^{2+} and 100% block being zero-current flow through the channel. The curve was derived from Eq. 3. ●, +150 mV; ■, +120 mV; ▲, +100 mV.

voltage was therefore ~0.04 s^{-1} and was not dependent on the nature of the charge carrier.

Reduction of Single Channel Currents by Cd^{2+}

Cadmium ions are potent blockers of calcium currents in a variety of preparations (see Hagiwara and Byerly, 1981). Fig. 6 shows that when cadmium is added to the *cis* side of a bilayer containing a calcium channel, the single channel current amplitude is reduced. Fig. 6*B* shows that the relationship between the cadmium

concentration and the percent reduction of the single channel current can be fit with a rectangular hyperbola (Eq. 3), with $K_{0.5}$ being the concentration (9.3 mM) of cadmium that reduces the single channel current by 50% and the maximal block ($Block_{max}$) (100%) being the percent of the current reduced by infinite concentrations of Cd^{2+} (see below and Discussion).

$$\text{Block} = (\text{Block}_{max})/(1 + K_{0.5}/Cd^{2+}). \quad (3)$$

Furthermore, the efficacy of block was not substantially affected by voltages between +100 and +150 mV (Fig. 6*B*).

Permeant Ions Affect Cd^{2+} Block

In synaptosomes (Nachshen, 1984) as in other preparations (cf. Hagiwara et al., 1974), competition between the permeant ions and the blocking ions determines

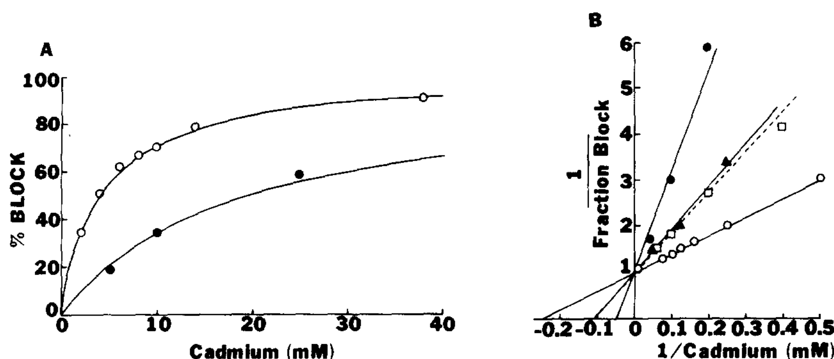


FIGURE 7. Effects of permeant ion on Cd^{2+} block. (*A*) Cd^{2+} concentration vs. percent block with Ba^{2+} (○) and Mn^{2+} (●) as the charge carriers at +120 mV. (*B*) Effect of the permeant ions on the apparent dissociation constant, $K_{0.5}$, for cadmium. Reciprocal plot of Cd^{2+} concentration vs. fraction block with Mn^{2+} (●), Sr^{2+} (▲), Ca^{2+} (□), and Ba^{2+} (○) as the charge carriers. The $K_{0.5}$ values for block as determined from the best-fit linear regression lines through the data points were 4.1 (Ba^{2+}), 9.3 (Ca^{2+} and Sr^{2+}), and 21.6 (Mn^{2+}) mM.

the rate of ion movement through the channel. To test this proposal on the single channel level, the effects on Cd^{2+} on single channel currents carried by Ba^{2+}, Ca^{2+}, Sr^{2+} and Mn^{2+} were tested (Fig. 7). In all cases, Cd^{2+} reduced the single channel current in a dose-dependent manner. However, as illustrated in the double-reciprocal plots of Cd^{2+} vs. block (Fig. 7*B*), the concentration of Cd^{2+} needed for 50% block ($K_{0.5}$) depended on the nature of the permeant ion, with $K_{0.5(Mn)} > K_{0.5(Ca)} = K_{0.5(Sr)} > K_{0.5(Ba)}$. Furthermore, in high concentrations of Cd^{2+}, the single channel current was <1% of the current in the absence of Cd^{2+}, which indicates that in the presence of Ca^{2+}, Sr^{2+}, Ba^{2+}, or Mn^{2+}, Cd ions cannot permeate rapidly enough to support a measurable single channel current. These results suggest that a binding site accessible from the *cis* side regulates the block by inorganic ions.

Sidedness of Block by Cd^{2+}

Fig. 8 shows that Cd^{2+} on the *cis* side was more effective in blocking ionic current from the *cis* side to the *trans* side. Cd^{2+} (24 mM), when added to the *trans* side, resulted in a 26% reduction in single channel current. The same concentration of Cd^{2+}, when added to the *cis* side, resulted in a greater reduction (71%) in single channel current. The reason for the differences in the efficacy of block is not yet clear. A lower apparent affinity for block by Cd^{2+} on the *trans* side could be explained by the following possibilities: (*a*) the block by Cd^{2+} is voltage dependent; (*b*) voltage-dependent binding of a permeant ion in the channel reduces the affinity of Cd^{2+}; (*c*) the channel has two different Cd^{2+} binding sites, one accessible from the *cis* side and the other accessible from the *trans* side.

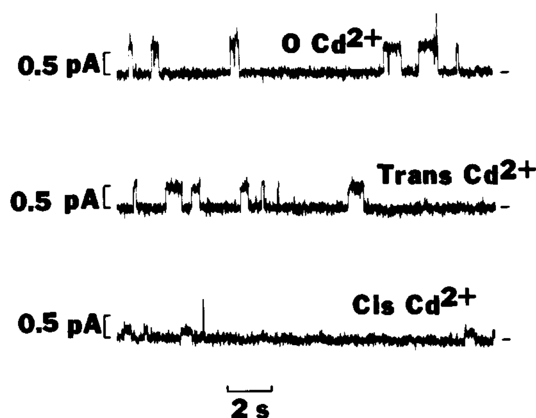


FIGURE 8. Sidedness of Cd^{2+} block. At +100 mV, with Sr^{2+} as the charge carrier, the single channel current was 0.62 pA ($n > 50$). After the addition of $CdCl_2$ (24 mM) to the *cis* side, the mean single channel current was 0.19 pA ($n > 50$). To restore the original single channel current, the *cis* side was then perfused with 0-Cd^{2+} solution. $CdCl_2$ (final concentration, 24 mM) was then added to the *trans* side and the single channel current was reduced to 0.46 pA ($n > 50$).

Effects of La, Mg, Na, and K Ions on the Single Channel Currents

The efficacy of block depended not only on the nature of permeant ion species but also on the nature of the blocking ion. La^{3+} was found to be the most potent inorganic blocker, with a $K_{0.5}$ of ~150 μM (with Sr^{2+}) (Nelson et al., 1984). Fig. 3 shows that Mn^{2+} is a less potent blocker than both La^{3+} and Cd^{2+}. Mg ions did not affect divalent cation movement through these Ca channels. When *cis* $CaCl_2$ solution was replaced with a solution containing 3 mM $CaCl_2$ and 247 mM $MgCl_2$, the resulting single channel current-voltage relationship extrapolated to a potential (+35 mV) that was close to the Ca equilibrium potential (E_{Ca}, +55 mV). By contrast, the Mg equilibrium potential (E_{Mg}) was nominally negative infinity and the Cl equilibrium potential (E_{Cl}) was 0 mV. This result indicates that these channels select for Ca^{2+} over Mg^{2+} and Cl^-. Furthermore, Mg ions did not affect the single channel conductance (5.1 pS) or the mean open times (250 ms, at

+100 mV). Since the channels are selecting for Ca^{2+} over Mg^{2+}, these results suggest that with 3 mM Ca^{2+} on the *cis* side, the channels were saturated with respect to the charge carrier. Saturation of the Ca^{2+} channel at such concentrations is in accord with the information obtained from intact synaptosomes (Nachshen and Blaustein, 1982). These results suggest that Mg^{2+}, even in high concentrations, does not block the channel.

Furthermore, high concentrations (200 mM) of Na and K ions did not reduce the single channel currents. For example, the single channel conductance with Ca^{2+} as the charge carrier was 5.3 ± 0.4 pS and, in the presence of the same concentration of Ca^{2+} (150 mM) plus 200 mM K, the single channel conductance was 5.4 ± 0.4 pS. The order of potency of the inorganic ions, tested thus far, in reducing current flow through this Ca^{2+} channel was: $La^{3+} > Cd^{2+} > Mn^{2+} \gg Mg^{2+}, Na^{+}, K^{+}$.

DISCUSSION

Comparison with Other Ca^{2+} Channels

The properties of synaptosomal Ca^{2+} channels from rat brain have been extensively characterized by investigating the properties of divalent cation isotopic influx that has been stimulated by depolarizing with high external K^+ (Nachshen and Blaustein, 1979, 1980, 1982; Drapeau and Nachshen, 1984; Nachshen, 1984). The synaptosomal Ca^{2+} channels as characterized by isotopic flux techniques share the following properties with the synaptosomal Ca^{2+} channels incorporated into planar lipid bilayers. (*a*) Ba^{2+}, Ca^{2+}, Sr^{2+}, and Mn^{2+} pass through both types of the channels with the same order of maximum flux rates. (*b*) Mn^{2+} can both permeate and block. (*c*) Inorganic blocking ions, when added to one side of the membrane, compete with the permeant ions for a single site. (*d*) The order of apparent affinities of the permeant ions for the site is the same: Mn > Ca > Sr > Ba. (*e*) The order of apparent affinities of the blocking ions for this site is also the same: La > Cd > Mn ≫ K, Na. (*f*) In both systems, the channels are inhibited by micromolar concentrations of the organic blockers D-600 and verapamil (Nelson, 1985*c*) and the channels do not completely inactivate.

Although the properties of single calcium channels in the bilayer and those of "intact" synaptosomes are similar, it would be desirable to compare the properties of single channels in the intact tissue and in the bilayer. Because of the small size of the synaptosomes (<1 μm diam) (Blaustein et al., 1977), it has not been possible to measure single channel currents by means of the patch-clamp technique. However, single calcium channels in some other tissues have been studied with the patch-clamp technique (Reuter et al., 1982; Brown et al., 1982, 1984; Fenwick et al., 1982; Hagiwara and Ohmori, 1983; Cavalie et al., 1983). In addition, Ca^{2+} channels from *Paramecium cilia* have been incorporated into planar lipid bilayers (Ehrlich et al., 1984). The conductances of single Ca^{2+} channels with Ba^{2+} as the charge carrier (usually ~100 mM) have ranged from ~2 (Ehrlich et al., 1984) to 25 pS (Reuter et al., 1982). The conductances of single Ca^{2+} channels from rat clonal pituitary cells (Hagiwara and Ohmori, 1983) and from

rat brain synaptosomes (Nelson et al., 1984) were similar (7–9 pS with Ba^{2+} as the charge carrier). Ba ions permeate single Ca^{2+} channels more rapidly than Ca ions (Lux and Nagy, 1981; Cavalie et al., 1983; Nelson et al., 1984). Although the ability of Sr ions to permeate calcium channels can be considered one of the criteria for the identification of a calcium channel (Hagiwara and Byerly, 1981; Nelson, 1985*a*), this property has not been extensively investigated at the single channel level in other tissues. With the exception of cardiac muscle, the effects of inorganic calcium channel blockers have not been investigated at the single channel level in other preparations. In cardiac muscle, cadmium ions caused discrete interruptions in current flow through single channels, which is consistent with the block mechanism presented below (Hess et al., 1985).

Single calcium channel activity has been difficult to measure in excised patches (cf. Fenwick et al., 1982; Cavalie et al., 1983; but see Carbone and Lux, 1984; Nilius et al., 1985; Reuter, 1985). One possible explanation for the viability of calcium channels in the bilayer is that certain calcium channel types require the presence of intracellular constituents such as phosphorylating enzyme systems for an appreciable opening probability (Reuter, 1983; Cachelin et al., 1983; Bean et al., 1984). In the absence of such regulation, the curve of the probability of the channel being open is shifted toward more positive potentials. Therefore, at physiological membrane potentials, such a nonphosphorylated calcium channel would appear "nonfunctional." However, with the bilayer method, a very wide voltage range can be explored (−200 to +200 mV) and the new activation curve for the channel can be found. In fact, the activation curve for the calcium channel reported here is shifted toward more positive potentials (Nelson, 1985*a*). Therefore, this channel would appear nonfunctional at 0 mV. Another explanation for the viability of single calcium channels in the bilayer is that some types of calcium channels may be more stable than others. For example, in squid axons (DiPolo et al., 1983) and in barnacle muscle cells (Keynes et al., 1973), calcium channels can survive extensive perfusion of the internal compartment, whereas calcium channels in other preparations seem to be somewhat labile (cf. Lee and Tsien, 1984). Thus, in a cell membrane that contains multiple types of calcium channels (Nachshen and Blaustein, 1980; Nowycky et al., 1985), it is possible that one type of channel may be more stable than another (cf. Armstrong and Matteson, 1984; Nilius et al., 1985).

Ion Movement Through Single Ca Channels from Brain Synaptosomes

To move through a calcium channel, a divalent cation must bind and dissociate with a site(s) in the permeation pathway (cf. Hagiwara and Byerly, 1981). Blocking ions such as lanthanum, cobalt, cadmium, and nickel appear to exert their effects by binding to this site with high affinity. Although it has been generally assumed that the blocking site is the same as the permeation site, studies on the block by cadmium of calcium currents and sodium currents through calcium channels in frog skeletal muscle have suggested that this may not be the case (Almers and McCleskey, 1984).

In this paper, evidence for the mechanism of inhibition of ion movement through calcium channels by the inorganic blocking ions is presented. The block

by cadmium can be explained by a mechanism whereby one cadmium ion binds to a single site in the open state. When a cadmium ion is bound to this site, other more permeant ions cannot pass through the channel and cadmium itself leaves the site too slowly to support a measurable current. If an equilibrium exists between the free Cd^{2+} and cadmium bound to this site, then the open channel should fluctuate between conducting and nonconducting states. However, if the lifetimes of the blocked and unblocked states are too brief (<3 ms) to be resolved by the recording system, then, in the presence of cadmium, the apparent open channel current corresponds to a time-averaged value determined by rapid blocking and unblocking events. Increasing concentrations of Cd^{2+} should then reduce this time-averaged conductance in a single-site binding scheme (see Eq. 3) (cf. Woodhull, 1973), as was observed.

The concentration of cadmium needed to reduce the single channel current by 50% ($K_{0.5}$) depended on the nature of the permeant ion, which suggests that permeant ions and the blocking ions interact at the same site. For a single-site scheme, the $K_{0.5}$ for the cadmium will be inversely related to the dissociation constant of the permeant ion [K_m(M)] for the site [Eq. 4, assuming that the concentration of permeant ion, M, is much greater than K_m(M)] (cf. Hagiwara et al., 1974; Nachshen and Blaustein, 1982; Byerly et al., 1985). This assumption is supported by the observation that lowering the concentration of permeant ion from 250 to 100 mM did not affect the single channel conductance (Nelson et al., 1984).

$$K_{0.5} = [K_m(B)][M]/[K_m(M)]; \tag{4}$$

$$K_m = k_{off}/k_{on}. \tag{5}$$

K_m(B) is the dissociation constant of the blocking ion, and k_{off} and k_{on} are the off rate and on rate constants for an ion that binds to this site. The $K_{0.5}$ depended on the nature of the charge carrier, with $K_{0.5}(Mn) > K_{0.5}(Ca, Sr) > K_{0.5}(Ba)$ (Fig. 7). Because the concentration of the permeant ions was the same in each experiment, the effect of the permeant ion on $K_{0.5}$ should reflect differences in the dissociation constants of the permeant ions for the site, with the order of dissociation constants of the permeant ions being opposite to that of the half-block constant of cadmium [i.e., $K_m(Ba) > K_m(Ca) = K_m(Sr) > K_m(Mn)$]. This order of dissociation constants of the permeant divalent cations is the same as the order of the single channel conductances of the four current carriers (Fig. 1). The parallel sequence between the dissociation constants and the single channel currents is consistent with the rate of ion movement through a channel (i.e., the single channel current) approximating the off rate constant of the permeant ion from the site. Thus, if the on rate constants for the four permeant ions are similar, then the sequence of dissociation constants should be the same as the sequence of single channel currents or off rate constants (Eq. 5).

To obtain an estimate of the K_m of a permeant ion, the on rate constant is assumed to be diffusion-limited or $\sim 10^9$ M^{-1} s^{-1} (cf. Almers and McCleskey, 1984). Without information to the contrary, this assumption is reasonable because: (*a*) the on rate constant for calcium ions to block sodium currents through

single calcium channels was close to diffusion-limited (Lansman et al., 1985); (*b*) the block by cadmium was not voltage dependent over a 50-mV range, which suggests that the site may be close to the aqueous phase on the *cis* side (Fig. 6); (*c*) the on rate constants of calcium onto other calcium binding proteins also appear to be diffusion-limited (e.g., troponin C; Potter and Johnson, 1982). If the primary pathway for an ion to leave the site is into the *trans* side, then the single channel current should closely approximate the off rate constant for an ion from the binding site (at +120 mV, k_{off} for barium ions should be ~2.7×10^6 s^{-1}). Therefore, the dissociation constant for barium would then be ~2.7 mM (Eq. 5). This value for the K_m is similar to the values that were reported for synaptosomal calcium channels as measured by barium isotopic flux (Nachshen and Blaustein, 1982) or for other calcium channels (Hagiwara and Byerly, 1981; but see Hess and Tsien, 1984; Almers and McCleskey, 1984).

Furthermore, from the ratio of $K_{0.5}$ with different current carriers, a ratio of dissociation constants for the permeant ions can be obtained [Eq. 6, M and K_m(B) are constant].

$$K_{0.5}(\mathrm{Ca, Sr})/K_{0.5}(\mathrm{Ba}) = K_m(\mathrm{Ba})/K_m(\mathrm{Ca, Sr}). \tag{6}$$

Using K_m(Ba) = 2.7 mM and Eq. 6, the dissociation constants of strontium, calcium, manganese, cadmium, and lanthanum have been estimated to be 1.2 mM, 1.2 mM, 0.5 mM, 44 μM, and 0.7 μM, respectively. The differences in the dissociation constants of the four permeant ions are greater than predicted from the differences in single channel currents, which suggests that the unitary currents are not a completely accurate measure of the off rate constants or that permeant ions have different on rate constants. Furthermore, if the channel has two divalent cation binding sites that exhibit ion-ion repulsion, then the true dissociation constants will be less than those calculated above (Hess and Tsien, 1984; Almers and McCleskey, 1984).

Using the calculated K_m for cadmium (44 μM) and assuming that the on rate constant is also diffusion-limited, the off rate constant of cadmium from the site can be calculated to be ~4.4×10^4 s^{-1} (Eq. 5). If cadmium ions could move from the binding site to the *trans* side, this k_{off} would correspond to a current of ~0.015 pA. A cadmium current of this magnitude is below the limit of detection of the recording system (~0.1 pA). This k_{off} is in accord with the observation that cadmium ions cannot support a measurable single channel current (Fig. 7). From the k_{off} of cadmium, an estimate for the mean blocked time (T_{block}) can be determined:

$$T_{block} = 1/k_{off}. \tag{7}$$

The calculated mean block time (23 μs) is clearly far below the experimental resolution (3 ms).

The effects of cadmium ions on single channel currents support the existence of one binding site in the channel. However, the effects of manganese ions on single channel currents carried by a more permeant ion (e.g., Sr^{2+}) suggest a more complicated picture. With the ratio K_m(Sr)/K_m(Mn) being ~2.4/1 and the

single channel conductances for Sr and Mn being 6 and 4 pS, respectively, the single-site scheme presented above predicts that in the presence of 250 mM Sr^{2+}, concentrations of manganese >1 M would be needed to reduce the single channel conductance to a level close to that of Mn alone. Surprisingly, at +100 mV, 25 mM Mn^{2+} reduced the single channel conductance to the level supported by Mn^{2+} alone (4 pS). This concentration of Mn^{2+} would have been predicted to reduce the single channel conductance from ~6 to 5.6 pS or by 0.04 pA at +100 mV. Furthermore, the block was more pronounced at +50 mV and the single channel conductance level appeared to be less than that in Mn alone. These results cannot be easily explained by a simple one-site model of the channel. However, currents through a channel that contains two binding sites could be affected by Mn ions in such a manner (cf. Hess and Tsien, 1984; Almers and McCleskey, 1984).

Ion Dependence of the Single Channel Open Times

The identity of the charge carrier has been shown to affect the kinetics of single calcium currents in ventricular myocytes (Cavalie et al., 1983) and macroscopic calcium channel currents (Siami and Kung, 1982; Deitmer, 1983). As determined from noise analysis, a similar relationship has been found between the single channel conductances and the closing rate constants when different alkali cations carry current through the acetylcholine receptor channel (Ascher et al., 1978; Marchais and Marty, 1979; Gage and Van Helden, 1979). In addition, a similar conclusion has been obtained from comparisons of Rb^+ and K^+ movement through squid axon K^+ channels (Swenson and Armstrong, 1981) and single K^+ channels from skeletal muscle (Spruce et al., 1984).

One possible explanation for the ion dependence of the open times is that the binding of a permeant ion to a site in the channel affects another conformational transition, i.e., channel closing. This is supported by the observation that the order of the single channel currents and closing rate constants is the same, i.e., Ba > Ca = Sr > Mn. In fact, there appeared to be a parallel change in the dissociation constants and the closing rate constants on changing the permeant ion. For example, the ratio K_m(Ba)/K_m(Ca, Sr) and the ratio of closing rate constants, k_c(Ba)/k_c(Ca, Sr), were 2.2 and 1.9, respectively. Furthermore, the ratios K_m(Ba)/K_m(Mn) and k_c(Ba)/k_c(Mn) were 5.4 and 4.0, respectively. These observations suggest that there is a relationship between the free energy of binding of a permeant ion to a site in the channel and the energy barrier that the channel has to overcome in order to close.

Another possible explanation for the effects of the permeant ion on open times and single channel currents is that the calcium channel fluctuates rapidly between two different conducting states, each having a different lifetime. These conducting states could represent a blocked and unblocked configuration of the channel (Neher and Steinbach, 1978) or two different conducting conformations of the channel (Läuger, 1984). As shown in Fig. 1, the open state of the calcium channel is punctuated by very brief current deflections toward the closed state, which supports the existence of another state of the channel. The measured single channel current and mean open time would be an average of the time spent at

the two conductance levels. The permeant ion could affect the single channel current level and the mean open time by affecting the distribution between the two conducting conformations. Furthermore, the effects of the permeant ions on the open times of calcium channels raise the interesting possibility that the open times are also concentration dependent (Läuger, 1984). It may be possible to distinguish between these possibilities by incorporating calcium channels into much smaller bilayers, thus enabling higher-resolution recordings.

In conclusion, the work presented in this report supports the existence of at least one binding site in the channel that regulates ion permeation and affects the channel's kinetics. The inorganic calcium channel blockers appear to exert their effects by competing with the permeant ions for this site.

Special thanks go to B. K. Krueger and R. J. French for making this work possible. I thank J. Lederer, J. F. Worley III, and M. Cannell for comments on the manuscript, and J. F. Worley and R. Reinhardt for help during some of the manganese experiments.
This work was supported by the American Heart Association, National (84–879), Maryland, and Greater Miami, Inc. Chapters, and contract DAMD17-82-C-2188 from the U.S. Army Medical Research and Development Command (to B. K. Krueger and R. J. French).

Original version received 4 June 1985 and accepted version received 23 October 1985.

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