Activation by Divalent Cations of a Ca^{2+}-activated K^+ Channel from Skeletal Muscle Membrane

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ABSTRACT Several divalent cations were studied as agonists of a Ca^{2+}-activated K^+ channel obtained from rat muscle membranes and incorporated into planar lipid bilayers. The effect of these agonists on single-channel currents was tested in the absence and in the presence of Ca^{2+}. Among the divalent cations that activate the channel, Ca^{2+} is the most effective, followed by Cd^{2+}, Sr^{2+}, Mn^{2+}, Fe^{2+}, and Co^{2+}. Mg^{2+}, Ni^{2+}, Ba^{2+}, Cu^{2+}, Zn^{2+}, Hg^{2+}, and Sn^{2+} are ineffective. The voltage dependence of channel activation is the same for all the divalent cations. The time-averaged probability of the open state is a sigmoidal function of the divalent cation concentration. The sigmoidal curves are described by a dissociation constant K and a Hill coefficient N. The values of these parameters, measured at 80 mV are: $N = 2.1$, $K = 4 \times 10^{-7}$ mM^N for Ca^{2+}; $N = 3.0$, $K = 0.02$ mM^N for Cd^{2+}; $N = 1.45$, $K = 0.63$ mM^N for Sr^{2+}; $N = 1.7$, $K = 0.94$ mM^N for Mn^{2+}; $N = 1.1$, $K = 3.0$ mM^N for Fe^{2+}; and $N = 1.1$ $K = 4.35$ mM^N for Co^{2+}. In the presence of Ca^{2+}, the divalent cations Cd^{2+}, Co^{2+}, Mn^{2+}, Ni^{2+}, and Mg^{2+} are able to increase the apparent affinity of the channel for Ca^{2+} and they increase the Hill coefficient in a concentration-dependent fashion. These divalent cations are only effective when added to the cytoplasmic side of the channel. We suggest that these divalent cations can bind to the channel, unmasking new Ca^{2+} sites.

INTRODUCTION

Several types of Ca^{2+}-modulated K^+ channels have been described in many types of cells and tissues (Latorre, 1986). All these channels are directly activated by cytoplasmic Ca^{2+}. Particular interest has been focused on a large-conductance, Ca^{2+}-activated K^+ channel since it combines an exquisite selectivity for K^+ and a large conductance. Although the ion-transport characteristics of this channel are under intense study (Blatz and Magleby, 1984; Yellen, 1984*a*, *b*; Cecchi et al., 1986; Eisenman et al., 1986; Cecchi et al., 1987), very little is known about the properties of the Ca^{2+}-binding sites of this protein. The scant results available suggest that of the divalent cations that activate the channel, Ca^{2+} is the most effective (Vergara, 1983; McManus and Magleby, 1984). Vergara (1983) showed that neither Mg^{2+} nor Ba^{2+}

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can activate the channel from rabbit skeletal muscle, and McManus and Magleby (1984) found that Sr^{2+} can replace Ca^{2+} in cultured rat muscle cells, but is ~200-fold less potent than Ca^{2+}. Regarding the characteristics of the Ca^{2+}-binding sites, Pallota (1985) showed that *N*-bromoacetamide removes a Ca^{2+}-dependent component of channel opening. Open probability in channels treated with *N*-bromoacetamide is no longer sensitive to internal Ca^{2+}. However, the remaining channel activity shows a voltage dependence similar to that found before treatment with the protein-modifying agent. The most simple explanation for these results is that the modification induced by *N*-bromoacetamide, a compound that cleaves peptide bonds on the COOH-terminal side of several amino acids, alters the Ca^{2+}-binding sites.

Gorman and Hermann (1979) studied the effects of electrophoretic injection of Ca^{2+} and other divalent cations on the K^+ current in *Aplysia* pacemaker neurons (see also Meech, 1976, 1980). They found that Ca^{2+} induces the largest increase in the K^+ conductance, as compared with Cd^{2+}, Sr^{2+}, Mn^{2+}, and Fe^{2+}. However, these results have to be viewed with caution. It is difficult to assess the internal divalent cation concentration, since there is insufficient information about the capacity of the cytoplasm to sequester the different divalent cations tested. Furthermore, Meech (1980) showed that Ba^{2+}, and probably other cations, cause release of intracellular Ca^{2+}. Finally, some divalent cations may increase the affinity of the channel for Ca^{2+} (Golowasch et al., 1986).

The large-conductance, Ca^{2+}-activated K^+ channel incorporated into planar lipid bilayer membranes (Latorre et al., 1982) offers an ideal system to study activation by a series of divalent cations. In this system, the divalent cation concentration can easily be controlled, avoiding the problems encountered in intact cells. In the present work, we show that, in the virtual absence of Ca^{2+} in the internal compartment, several divalent cations can activate the channel. In order of effectiveness, they are: $Cd^{2+} > Sr^{2+} > Mn^{2+} > Fe^{2+} > Co^{2+}$. None of these cations can activate the channel at low concentrations as does Ca^{2+} alone. When the divalent cations are added in the presence of Ca^{2+}, a potentiation of the Ca^{2+} activation is observed. This result suggests the existence of a modulatory site that controls Ca^{2+} activation. This site is only accessible from the cytoplasmic side of the channel and binds Mg^{2+}, Ni^{2+}, and probably other divalent cations. A preliminary report of part of this work has appeared in abstract form (Oberhauser et al., 1987).

METHODS

Planar Lipid Bilayers and Channel Incorporation

Planar lipid bilayers were formed from a lipid solution containing 12 mg of 1-palmitoyl,2-oleoyl phosphatidylethanolamine (POPE) and 3 mg of the analogous phosphatidylcholine (POPC) per milliliter of decane. This lipid solution was applied to a 100–250-μm hole in a plastic septum separating two identical solutions containing 300 mM KCl, 10 mM MOPS, pH 7.0. In order to incorporate the Ca^{2+}-activated K^+ channels, 5–15 μl of a transverse-tubule (T-tubule) membrane vesicle suspension was added to one side of the bilayer. T-tubule membrane vesicles were prepared from rat skeletal muscle as described by Moczydlowski and Latorre (1983*a*). The final protein concentration of the vesicle suspension was 10 mg/ml.

The single-channel current was measured by applying constant potentials ranging from −80 to +80 mV at 10-mV intervals during 1–2 min.

Channels incorporate into the bilayer with their cytoplasmic side facing the compartment to which the vesicles were added, and the Ca^{2+}-binding sites are exposed to this compartment. To determine the effectiveness of the different divalent cations as activators of the channel, it is necessary to maintain the Ca^{2+} concentration as low as possible. The contaminant Ca^{2+} concentration in the solutions, usually ~5 μM, was lowered to the nanomolar range by either precipitation with F^- or chelation with EGTA. Precipitation of the contaminant amounts of Ca^{2+} with F^- was used in the experiments with Cd^{2+}, Mn^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Hg^{2+}, Ni^{2+}, and Zn^{2+}, since the fluoride salts of these cations are soluble. The concentration of free Ca^{2+} expected for 300 mM KF is 0.1 nM. The actual free Ca^{2+} concentration was <3 nM, as measured with a Ca^{2+} electrode (Alvarez-Leefmans et al., 1981). Chelation of Ca^{2+} was used in experiments with Mg^{2+}, Sr^{2+}, and Ba^{2+}. To keep the Ca^{2+} concentration in the nanomolar range, the following protocol was used. After the incorporation of a single channel, the internal side was perfused with a solution containing 300 mM KCl, 200 μM EGTA, 10 mM MOPS, pH 7 (<10 nM free Ca^{2+}). Under these conditions, the Sr^{2+} or Ba^{2+} concentration was adjusted by adding the appropriate amounts of the chloride salt. At each concentration, a curve of the fraction of time the channel remains open (P_o) vs. V was taken. For each of the other divalent cations tested, the internal side was perfused with a solution containing the divalent cation and 300 mM KF, 10 mM MOPS, pH 7. The same protocol was followed in the case in which divalent cations were added on top of contaminant amounts of Ca^{2+}, but only KCl was used and EGTA was omitted.

Moczydlowski and Latorre (1983*b*) found two problems that can affect the analysis of channel gating. First, there is a variation from channel to channel in the Ca^{2+} concentration dependence; second, at constant applied potential, there are spontaneous shifts in P_o. Because of these problems, comparisons between the activation curves induced by the different divalent cations were done in the same single-channel membrane and only membranes with a stable P_o were used. In two single-channel membranes, it was possible to test the complete series of divalent cations, and partial sequences were obtained in nine membranes.

Electrical Measurements and Data Analysis

The voltage-clamp circuit has been described in detail by Alvarez and Latorre (1978) and by Moczydlowski and Latorre (1983*b*). The current across the bilayer was measured with a low-noise current-to-voltage converter, filtered at 2 kHz with a two-pole Bessel low-pass active filter (FLTU-2, Datel, Canton, MA), and amplified and recorded continuously on an FM tape recorder (4D4714, Lockheed, Sarasota, FL) for later analysis. The membrane current was measured and voltages were applied via a pair of Ag/AgCl electrodes connected through 1 M KCl bridges. The electrophysiological convention is used, in which the external side of the channel is defined as zero potential. Membrane capacitance ranged between 150 and 300 pF and the experiments were conducted at 22 ±2°C.

The open-state probability, P_o, was measured as a function of voltage and divalent cation concentration. For single-channel membranes, the time average probability of the open state was obtained from digitized records (100 μs/point) as the time spent in the open-current level divided by the total time of the record, usually 60 s. The large-conductance, Ca^{2+}-activated K^+ channel exhibits rather complex kinetics. Long silent periods occur, especially at high Ca^{2+} concentrations and large voltages. These long closures are due to a slow blockade of the channel by divalent ions (Vergara and Latorre, 1983; Miller et al., 1987). Therefore, P_o was calculated excluding channel closures lasting >200 ms when these long events were evident. This ensures that measurements are related to activation of the channel and not to the slow blocking of the channels.

Chemicals

POPE and POPC were obtained from Avanti Polar Lipids, Inc., Birmingham, AL. Decane was obtained from Sigma Chemical Co., St. Louis, MO, KCl, $BaCl_2$-$2H_2O$, $CaCl_2$-$4H_2O$, $CdCl_2$, $CoCl_2$-$6H_2O$, $HgCl_2$, and $SrCl_2$-$2H_2O$ were obtained from Alfa Products, Danvers, MA. KF, $CuSO_4$-$5H_2O$, $FeSO_4$, $MgCl_2$-$4H_2O$, $Ni(NO_3)_2$-$6H_2O$, $Pb(NO_3)_2$, and $ZnCl_2$ were from E. Merck, Darmstadt, Federal Republic of Germany. $MnSO_4$-H_2O was from J. T. Baker Chemical Co., Phillipsburg, NJ. $EuCl_3$-$6H_2O$ and $TbCl_3$-$6H_2O$ were from Aldrich Chemical Co., Inc., Milwaukee, WI. All these chemicals were 99% purity or greater.

RESULTS

Divalent Cation Selectivity of Channel Activation

Fig. 1 shows single-channel records of the Ca^{2+}-activated K^+ channel from T-tubule membranes with different divalent cations added to the internal side, to a final concentration of 200 μM. All records are for the same channel, and the applied potential is 70 mV, with the exception of the one shown for Mn^{2+}, which was obtained at +80 mV. In the presence of Ca^{2+}, the channel remained open most of the time. When Ca^{2+} was removed, the channel remained closed and no openings were seen, even for periods lasting several minutes. From the different divalent cations tested, only those shown in Fig. 1 were able to increase the open-channel probability, in the virtual absence of Ca^{2+} (<3 nM). It is clear from Fig. 1 that the channel conductance in the presence of Cd^{2+} is smaller than in the presence of the other divalent cations. This decrease in conductance is caused by a fast channel blockade by Cd^{2+}, as discussed below. Mg^{2+}, Ni^{2+}, Zn^{2+} Cu^{2+}, Hg^{2+}, Pb^{2+}, and Ba^{2+} failed to activate the channel, even at concentrations as high as 1 mM. We have studied the effect of Mg^{2+} on channel activation in a wider range of concentrations (up to 50 mM) and voltages (±80 mV). Even at 50 mM Mg^{2+} and 80 mV, no channel activation was observed. In the absence of Ca^{2+}, trivalent cations like Eu^{3+} and Tb^{3+} also failed to activate the channel in the concentration range 60–200 μM. The lack of effect of these two lanthanide cations is interesting inasmuch as they have radii very similar to that of Ca^{2+} (~0.1 nm), and they bind strongly to troponin C (Leavis and Gergely, 1984).

In order to quantify the effects of divalent cations on channel activation, we have measured the equilibrium probability of residence in the open state, P_o, as a function of voltage and divalent cation concentration. P_o was obtained from records such as those shown in Fig. 1 lasting at least 1 min. Fig. 2 shows P_o vs. V curves for the several divalent cations able to activate the channel. The solid lines are the best fit for the experiment points to a Boltzmann distribution:

$$P_o(V) = \{1 + \exp[-nF(V - V_o)/RT]\}^{-1}, \quad (1)$$

where n is a constant, V_o is the voltage at which $P_o = 1/2$, V is the applied voltage, and F, R, and T have their usual meanings, Latorre et al. (1982) and Moczydlowski and Latorre (1983*b*) have shown previously that when Ca^{2+} is the channel agonist, the P_o vs. V curves are well described with a n of 2 and a V_o that is a function of the Ca^{2+} concentration. All the curves shown in Fig. 2 can be fitted with an n of ~2, which indicates that the voltage dependence of the channel is independent of the divalent

cation used as activator. Although the absolute value of V_0 at a given Ca^{2+} concentration is very variable from membrane to membrane, the displacement of V_0 as a function of Ca^{2+} concentration is well known (Moczydlowski and Latorre, 1983*b*). Therefore, we can calculate what Ca^{2+} concentration would give the P_0 vs. V curve found for each divalent cation as a measure of the relative activation potency. The

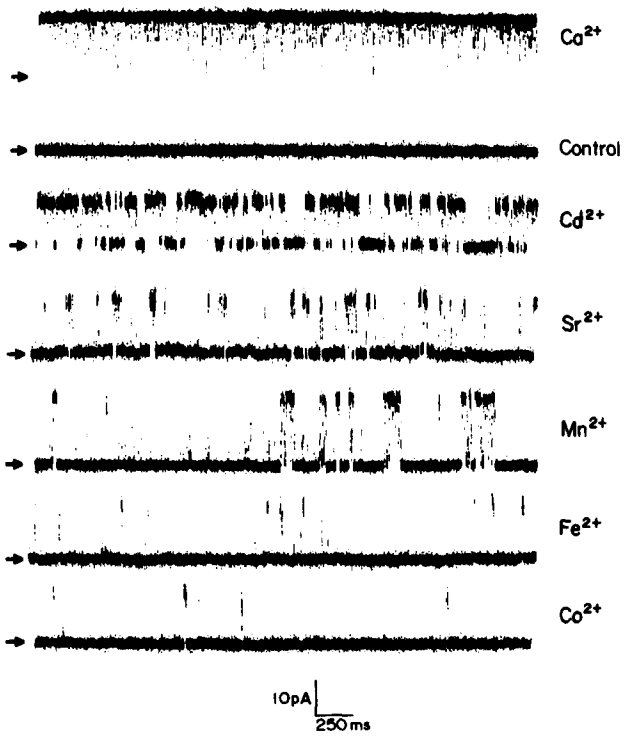

FIGURE 1. Ca^{2+}-activated K^+ channel current fluctuations in the presence of various divalent cations. The record shown for Ca^{2+} was obtained in symmetrical 300 mM KCl, 10 mM MOPS, pH 7, and an internal Ca^{2+} concentration of 200 μM. The internal side was subsequently perfused with a solution containing 300 mM KCl, 10 mM MOPS, pH 7, and 200 μM EGTA ($[Ca^{2+}] \approx 3$ nM) and the control record was taken. Under these conditions, Sr^{2+} was added to the internal side to a final concentration of 200 μM and channel current fluctuations were recorded. The channel current records for the other divalent cations were obtained by successive perfusions of the internal side with a solution containing 300 mM KF, 10 mM MOPS, pH 7, and subsequent addition of the different X^{2+} to a final concentration of 200 μM. All records are from the same single-channel membrane and were taken at 70 mV, with the exception of that for Mn^{2+} (80 mV). Arrows indicate the closed state.

ratio of this equivalent Ca^{2+} concentration to the actual X^{2+} concentration is: $Ca^{2+}:Cd^{2+}:Sr^{2+}:Mn^{2+}:Fe^{2+}:Co^{2+} = 1:1.2 \times 10^{-2}:5.5 \times 10^{-3}:1.2 \times 10^{-3}:8.9 \times 10^{-4}:4.5 \times 10^{-4}$.

As stated in the Methods, because of the variability of the Ca^{2+} concentration dependence from channel to channel, it is desirable to test all the different divalent

cations in the same single-channel membrane. The long recording periods and the multiple solutions changes required make these experiments difficult. Nonetheless, we obtained the complete divalent cation selectivity sequence in only two different channels, and with other nine different channels we obtained partial sequences that are consistent with the results shown in Fig. 2 (Table I).

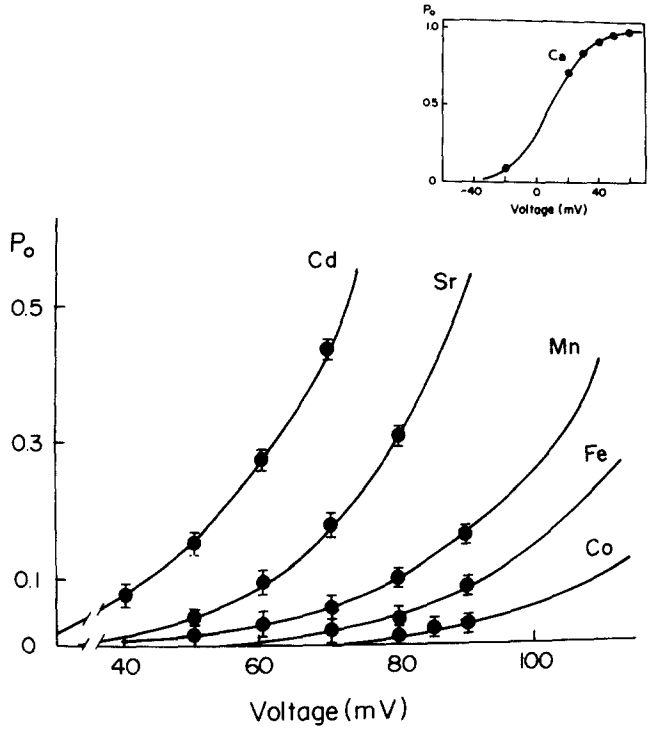

FIGURE 2. P_o vs. voltage curves obtained in the presence of various divalent cations. The time-averaged (1 min) probability of residence in P_o was measured as the fraction of time in the open state at various voltages and at a fixed $[X^{2+}]$ = 200 μM. The solid lines are the best fit to the data using Eq. 1 and the following parameters: Ca^{2+} (inset): n = 2, V_o = 8 mV; Cd^{2+}: n = 1.9, V_o = 74 mV; Sr^{2+}: n = 2, V_o = 91 mV; Mn^{2+}: n = 1.8, V_o = 120 mV; Fe^{2+}: n =1.8, V_o = 126 mV; Co^{2+}: n =2, V_o = 140 mV. Data were obtained in symmetrical 300 mM KCl, 10 mM MOPS, pH 7 (Ca^{2+} and Sr^{2+}) or 300 mM KF (other divalent cations; see Fig. 1).

Divalent Cation Concentration Dependence

More information about the mechanisms of interaction of the divalent cations with the Ca^{2+}-activated K^+ channel from T-tubule membranes can be obtained by plotting P_o vs. $[X^{2+}]$. Fig. 3 shows such curves for the different divalent cations able to activate the channel. It is clear from Fig. 3 that at any given divalent cation concentration the sequence for channel activation is the same to the one determined from Fig. 2. The solid curves in Fig. 3 are drawn according to the relationship

$$P_o([X^{2+}]) = [X^{2+}]^N/(K + [X^{2+}]^N), \quad (2)$$

where N is the Hill coefficient, which measures the apparent number of divalent cations involved in the channel activation. In a system displaying multiligand binding

equilibria, N is the lower limit of the number of sites involved in the reaction (Adair, 1925) and K is the overall dissociation constant. Moczydlowski and Latorre (1983*b*) found N's ranging from 1.2 to 2 with Ca^{2+} used as activator; more recently, Golowash et al. (1986) found Hill coefficients averaging 2 (range, 1.7–2.4) for the same channel. Furthermore, Golowash et al. (1986) showed that N is essentially voltage independent. In the present work, we found an N for Ca^{2+} binding to the channel of 1.3–2.4. For the other divalent cations, we found that the Hill coefficients ranged from 1 for Fe^{2+} to 3.0 for Cd^{3+} (for more detail, see legend to Fig. 3). From Eq. 2, we have calculated the $[X^{2+}]$ at which $P_o = 1/2$ as another measure of activation effectiveness. Using this criterion, the relative activation with respect to Ca^{2+} is:

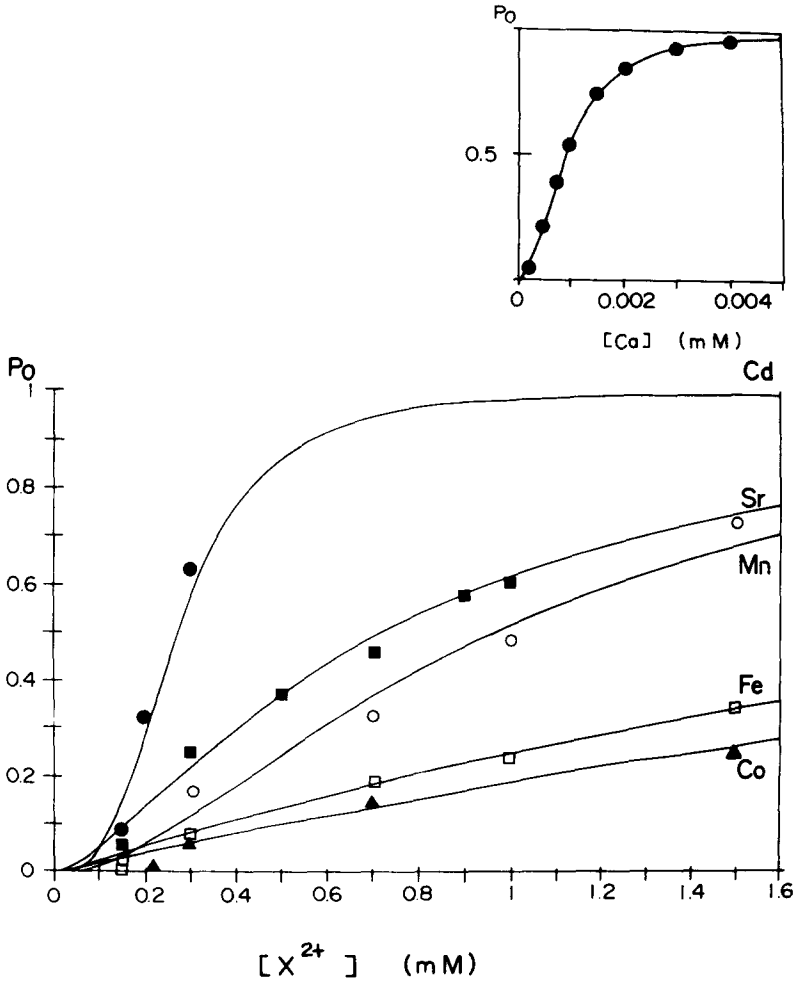

FIGURE 3. P_o vs. divalent cation concentration curves. P_o was measured at various $[X^{2+}]$ and at a fixed voltage (80 mV). The inset shows the Ca activation curve. The solid lines are the best fit to the data using Eq. 2 with the following parameters: Ca^{2+}: $N = 2.1$, $K = 4 \times 10^{-7}$ mM^N; Cd^{2+}: $N = 3.0$, $K = 0.02$ mM^N; Sr^{2+}: $N = 1.45$, $K = 0.63$ mM^N; Mn^{2+}: $N = 1.7$, $K = 0.94$ mM^N; Fe^{2+}: $N = 1.1$, $K = 3.0$ mM^N; Co^{2+}: $N = 1.1$, $K = 4.35$ mM^N. All data were obtained from the same single-channel.

TABLE I

Divalent Cation Activation Sequence

Ca^{2+}	Cd^{2+}	Sr^{2+}	Mn^{2+}	Fe^{2+}	Co^{2+}	*N*
>0.99	0.70	0.31	0.10	0.04	0.01	2
>0.99	0.68	0.28	—	—	—	1
>0.99	0.70	—	0.10	—	—	3
>0.99	—	—	—	0.05	0.02	2
>0.99	—	0.25	—	—	0.02	1
>0.99	—	—	0.14	—	0.01	2

This table summarizes the P_o values obtained at 80 mV in the presence of 200 μM of the divalent cation. *N* is the number of channels in which a particular set of ions was studied.

Ca^{2+}:Cd^{2+}:Sr^{2+}:Mn^{2+}:Fe^{2+}:Co^{2+} = 1:2.8 × 10^{-2}:6.2 × 10^{-3}:4.7 × 10^{-3}:1.6 × 10^{-3}:1.2 × 10^{-3}. These values are in reasonable agreement with those obtained from the P_o vs. *V* data and indicate that channel activation is very specific for Ca^{2+}.

Divalent Cations Enhance Channel Activation by Ca^{2+}

We have tested the activation of the channel by divalent cations in the presence of 1 μM Ca^{2+}. Typical single-channel records are shown in Fig. 4. In these experiments, the divalent cation concentration was adjusted to raise P_o to a value close to 0.5. Two features of these records are worth mentioning: first, a cation like Ni^{2+}, unable to activate the channel in the absence of internal Ca^{2+}, greatly increases the probability of opening when Ca^{2+} is present; second, cations like Mn^{2+} and Cd^{2+} also increased P_o to values greater than those expected from the results obtained in the absence of Ca^{2+}. For the sake of comparison, 20 μM Cd^{2+} increased P_o to a value comparable to that obtained with a Ca^{2+} concentration of 10 μM. Therefore, under this condition, Cd^{2+} is almost as potent as a channel activator as Ca^{2+} (see also Fig. 5). This effect cannot be due to Ca^{2+} contaminating our divalent cation solutions, since atomic absorption analysis indicates that <1 μM Ca^{2+} is introduced to the

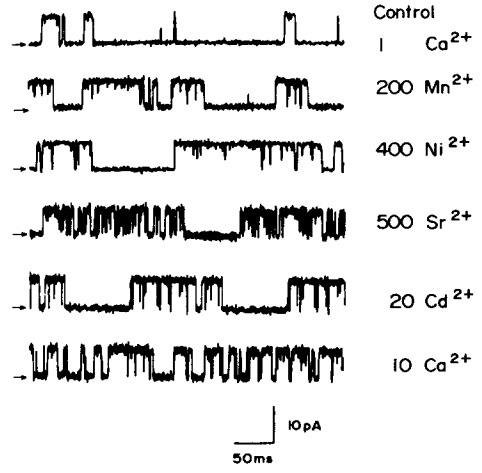

FIGURE 4. Activation by divalent cations in the presence of Ca^{2+}. The experiment was performed in a single channel as in Fig. 1, but with perfusion of the internal side successively with a solution containing 300 mM KCl, 10 mM MOPS, pH 7, and 1 μM Ca^{2+} (control). The concentration of different divalent cations (micromolar) is indicated to the right of the figure and was chosen to get P_o near 0.5. P_o values for the control, Mn^{2+}, Ni^{2+}, Sr^{2+}, Cd^{2+}, and Ca^{2+} were 0.14, 0.45, 0.56, 0.52, 0.46, and 0.57, respectively. *V* = 40 mV. Arrows indicate the closed state.

internal solution when the different divalent cations are added. This amount of Ca^{2+} would have increased the P_o value only marginally. Mg^{2+} has the same behavior as Ni^{2+}, but its effect on P_o is noticeable at much larger concentrations (>1 mM) (Golowash et al., 1986).

In Fig. 5, we show plots of P_o vs. V for several divalent cations able to activate the channel added to a final concentration of 300 μM, in addition to 3 μM Ca^{2+}. All the curves show the same voltage dependence and, when the V_o's (Eq. 1) are compared, the effectiveness of the different cations in activating the channel follows the sequence: $Ca^{2+} > Cd^{2+} > Mn^{2+} > Fe^{2+} > Co^{2+} > Ni^{2+} > Sr^{2+} > Mg^{2+}$. This sequence is obtained at an internal Ca^{2+} concentration between 3 and 10 μM, but, at higher Ca^{2+} concentrations, the positions of Fe^{2+} and Co^{2+} are reversed (see below). Under these conditions, cations like Ba^{2+}, Cu^{2+}, Zn^{2+}, and Sn^{2+} are not able to activate the channel. However, it is difficult to assess the effect of Ba^{2+} in activating the channel because it acts as a blocker at micromolar concentrations (Vergara

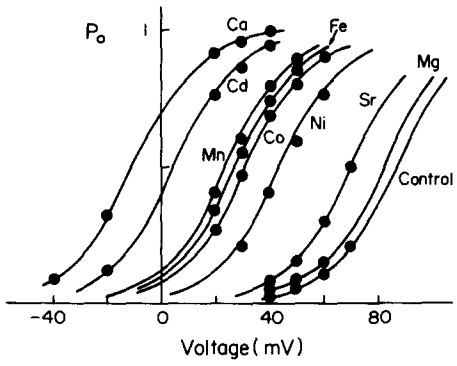

FIGURE 5. Shifts of the P_o vs. V curves induced by divalent cations in the presence of Ca^{2+}. The solid lines are the best fit to the data using Eq. 1. Parameters as follows: control (3 μM Ca^{2+}): n = 2.0, V_o = 88 mV; Mg^{2+}: n = 2.0, V_o = 85 mV; Sr^{2+}: n = 2.0, V_o = 71 mV; Ni^{2+}: n = 2.0, V_o = 44 mV; Co^{2+}: n = 1.9; V_o = 32 mV; Fe^{2+}: n = 2.0, V_o = 28 mV; Mn^{2+}: n = 2.0, V_o = 24 mV; Cd^{2+}: n = 1.9, V_o = 10 mV; Ca^{2+}: n = 2, V_o = −22 mV. Symmetrical 150 mM KCl, 10 mM MOPS, pH 7. In all cases, the concentration of divalent cation on the internal side was 300 μM.

and Latorre, 1983; Miller et al., 1987). Sr^{2+}, Pb^{2+}, and Cd^{2+} also block the channel, causing long channel closures.

In conclusion, all the divalent cations shown in Fig. 5 are more effective as channel activators when Ca^{2+} is present in the internal solution. Most notably, Ni^{2+} and Mg^{2+} activate the channel in the presence of contaminant amounts of Ca^{2+}, in spite of the fact that they do not activate in the absence of Ca^{2+}. Actually, Ni^{2+} under these conditions is more effective than Sr^{2+}, a cation that activates the channel in the absence of Ca^{2+}.

Divalent Cations and Molecularity of Activation

Golowasch et al. (1986) found that Mg^{2+} enhances the sigmoidicity of the Ca^{2+}-activation curve; i.e., it increases the Hill coefficient (Eq. 2). In the presence of 10 mM internal Mg^{2+}, N was 4.5, compared with a control value of ~2. Fig. 6 and Table II show the effect of the different divalent cations on the Ca^{2+}-activation

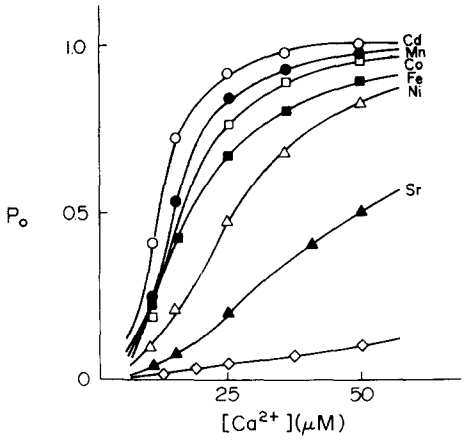

FIGURE 6. Ca^{2+} activation curves in the presence of various divalent cations. The solid lines are the best fit to the data using Eq. 2. The Hill coefficients and apparent dissociation constants for Ca^{2+} in the presence of other divalent cations are: Cd: $N = 3.1$, $K = 6.7 \times 10^{-7}$ mM^N; Mn: $N = 3$, $K = 3.0 \times 10^{-6}$ mM^N; Co: $N = 2.8$, $K = 1.1 \times 10^{-5}$ mM^N; Fe: $N = 2.0$, $K = 3.3 \times 10^{-4}$ mM^N; Ni: $N = 2.4$, $K = 1.6 \times 10^{-4}$ mM^N; Sr: $N = 2.0$, $K = 2.5 \times 10^{-3}$ mM^N; control (◇) with Ca^{2+} at the indicated concentration as the only divalent species present: $N = 2.0$, $K = 2.2 \times 10^{-2}$ mM^N $V = 30$ mV. The internal solution contained 150 mM KCl, 10 mM MOPS, pH 7, and $[X^{2+}] = 150$ μM. Mg^{2+} at this concentration does not have any appreciable effect on the Ca^{2+} activation curve.

curve. Clearly, all the divalent cations shown in Fig. 6 can increase the apparent affinity of the channel for Ca^{2+}. Cd^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, and Mg^{2+} also increase the Hill coefficient.

Ni^{2+} has the same effect described by Golowasch et al. (1986) for Mg^{2+}. Ni^{2+} is not a channel activator by itself, but potentiates the Ca^{2+} ability to increase P_o. Fig 7*A* shows single-channel current records taken in the absence and at two different concentrations of Ni^{2+}. Ni^{2+} increases the P_o almost to 1 when the internal Ni^{2+} concentration is 1 mM. Fig. 7 *B* shows that Ni^{2+} increases the Hill coefficient for the Ca^{2+}-activation curve in a concentration-dependent fashion. The Hill coefficient is 2.4 at 200 μM Ni^{2+} and 3.1 at 500 μM Ni^{2+}. Thus, Mg^{2+} and Ni^{2+} share the same property of enhancing Ca^{2+} activation without a direct interaction with the Ca^{2+}-

TABLE II

Hill Coefficients for Ca^{2+} Activation in the Presence of Various Divalent Cations

	Divalent cation concentration (mM)			
X^{2+}	0.15	0.5	5.0	10.0
Cd^{2+}	3.1	—	—	—
Sr^{2+}	2.0	2.0	—	—
Mn^{2+}	3.0	4.1	—	—
Co^{2+}	2.9	3.6	—	—
Ni^{2+}	2.4	3.1	—	—
Mg^{2+}	2.0	2.0	4.0	5.8

Hill coefficients were calculated by fitting the P_o values obtained at different Ca^{2+} concentrations and at the indicated $[X^{2+}]$ to Eq. 2. Data were obtained at +30 mV. Note that the average Hill coefficient in the presence of Ca^{2+} alone is 2.

binding sites. Ni^{2+} shows clear effects on the Hill coefficients at concentrations that are 10-fold lower than those needed to promote the same effects with Mg^{2+}. Furthermore, Ni^{2+} only increases the Hill coefficient when added to the internal side. Given the present results, it appears that Ni^{2+} and Mg^{2+} fall in the class of "allo-

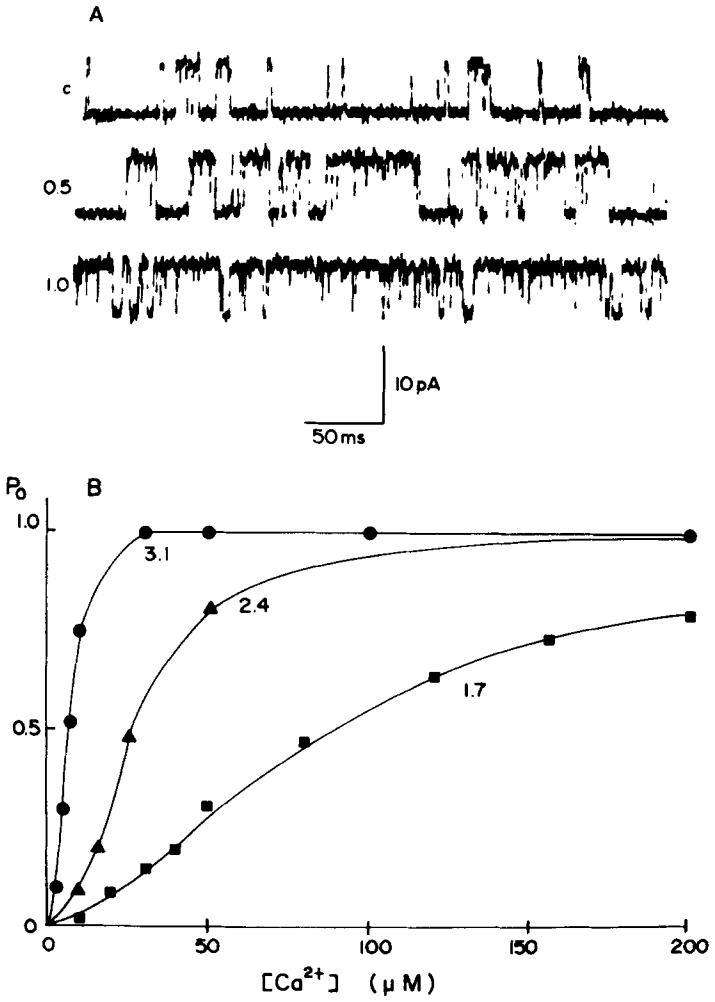

FIGURE 7. Effect of Ni^{2+} on the Ca^{2+}-activation curve. (*A*) Records of single Ca^{2+}-activated K^+ channels were taken in the absence and in the presence of Ni^{2+} at the concentration shown to the left of the records (millimolar). The Ca^{2+} concentration was 25 μM. All traces were recorded at 30 mV and taken in the same single-channel membranes. (*B*) Ca^{2+}-activation curves measured at 30 mV in the absence (■) and in the presence of 0.5 mM (▲) or 1 mM (●) Ni^{2+}. The solid curves are drawn according to Eq. 2 with the Hill coefficients shown.

steric effectors." In other words, Ni^{2+} is a divalent cation that does not compete with or substitute for Ca^{2+}, but enhances the cooperativity of Ca^{2+} activation.

The property of increasing the Hill coefficient is not only a property of Ni^{2+} and Mg^{2+}. The fits to Eq. 2 of the data presented in Fig. 6 indicate that Cd^{2+}, Mn^{2+}, and

Co^{2+} are also able to increase the Hill coefficient for Ca^{2+} activation. However, the interpretation of these results is not as straightforward as for the cations that do not activate the channel in the absence of Ca^{2+}. Cd^{2+}, Mn^{2+}, and Co^{2+} can substitute for Ca^{2+} and we therefore expect competition effects between these ions and Ca^{2+} for the Ca^{2+} sites. Despite these reservations, the large changes in the Hill coefficients promoted by Mn^{2+} and Co^{2+} suggest that these divalent cations have some properties in common with both Ni^{2+} and Mg^{2+} and with Ca^{2+}.

Multivalent Cations and Channel Blockade

Ca^{2+}, at millimolar concentrations, is able to alter the current-voltage curve for the open channel in a voltage-dependent manner. Our results indicate that this phenomenon is due to a fast blocking that is intensified at positive voltages (Moczydlowski and Latorre, 1983*b*; Eisenman et al., 1986). This fast type of blockade is induced by most of the divalent cations and trivalent cations we have studied in the present work. For the purpose of comparison of the blockade characteristics induced by the different cations, we have fitted the experimental data with the following equation (Woodhull, 1973; Coronado and Miller, 1979)

$$\langle i \rangle = i_o / \{1 + [B]/K_d(0) \exp(z\delta FV/RT)\}, \quad (3)$$

where $\langle i \rangle$ is the average current obtained in the presence of the blocker, i_o is the current in the absence of the blocker, $[B]$ is the blocker concentration, $K_d(0)$ is the apparent dissociation constant of the blocking reaction at zero voltage, z is the ion valence, and δ is the fractional electrical distance at which the blocking site is located. Table III shows the values for $K_d(0)$ and $z\delta$ obtained for the different dival-

TABLE III

Parameters for the Fast Blockade Induced by Multivalent Cations

Cation	$K_d(0)$	$z\delta$	$[X^{2+}]$
	mM		*mM*
Tb^{3+}, Eu^{3+}	0.045	0.48	0.06–0.20
Pb^{2+}	0.40	0.34	0.06–0.18
Cu^{2+}	0.60	0.30	0.10
Hg^{2+}	0.74	0.31	0.06
Ca^{2+}	1.80	0.40	1.0–5.0
Mn^{2+}	4.0	0.35	1.0–3.0
Zn^{2+}	5.1	0.30	0.70
Fe^{2+}	8.4	0.42	0.20–3.0
Mg^{2+}	25.0	0.35	2.0–50
Sr^{2+}	90.0	0.40	0.2–7.0

Solutions on both sides of the membrane contained 150 mM KCl, 10 mM MOPS, pH 7. The additional multivalent cation was added to the internal side only. The parameters $z\delta$ and $K_d(0)$ were obtained by fitting the current-voltage relationships for the open channel obtained in the presence of the various multivalent cations to Eq. 3. The last column shows the cation concentration range at which the current-voltage relationships for the channel current fluctuations were obtained. When the effect of cations other than Ca^{2+} were studied, the Ca^{2+} concentration was always <50 μM.

ent and trivalent cations tested. The potency of binding of the blocking site follows the sequence: Tb^{3+}, $Eu^{3+} > Pb^{2+} > Cd^{2+} > Cu^{2+} > Hg^{2+} > Ca^{2+} > Mn^{2+} > Zn^{2+} > Fe^{2+} > Mg^{2+} > Sr^{2+}$. This sequence is different from the one obtained for channel activation. We did not observe any channel conductance decrease in the presence of Co^{2+} (7 mM), Ni^{2+} (1.5 mM), or Ba^{2+} (10 μM). The mean value of δ for the different divalent cations is 0.18 ± 0.03 and that for trivalent cations is 0.16, which suggests that both divalent and trivalent cations are interacting with the same site.

We have also observed in the presence of internal Sr^{2+}, Pb^{2+}, or Cd^{2+} long periods during which the channel remains quiescent. Preliminary results indicate that these quiescent periods are due to a channel blockade similar to that induced by Ca^{2+} and Ba^{2+} (Vergara and Latorre, 1983). Furthermore, cations like Cu^{2+}, Zn^{2+}, and Hg^{2+} decrease the mean number of active channels when added to the internal side, but not when added to the external side at the same concentration (data not shown). The effects of Hg^{2+} and Zn^{2+} become irreversible if the concentration of the divalent cation is higher than 50 μM Hg^{2+} or 600 μM Zn^{2+}. The channel can be protected from the deleterious effect of internal Zn^{2+} if the Ca^{2+} concentration on the internal side is raised. The effect of Hg^{2+} is independent of the internal Ca^{2+} concentration. Zn^{2+} probably binds to the Ca^{2+} sites without activating, and Hg^{2+} modifies channel activity by interacting with disulfide bonds present in the protein and required for channel gating.

DISCUSSION

Ca^{2+}-binding Sites and Ionic Radius

The effectiveness of divalent cations in activating the Ca^{2+}-activated K^+ channel of muscle membrane follows the sequence $Ca^{2+} > Cd^{2+} > Sr^{2+} > Mn^{2+} > Fe^{2+} > Co^{2+}$. Mg^{2+}, Ni^{2+}, Zn^{2+}, Hg^{2+}, Pb^{2+}, and Ba^{2+} do not activate the channel. Fig. 8 shows a plot of the log of the relative ability of divalent cations, X^{2+}, in increasing P_o vs. crystal ionic radius, r_x. In Fig. 8, [Ca] is the Ca^{2+} concentration necessary to obtain the same P_o vs. V curve obtained with a given divalent cation concentration. The activation sequence we found differs somewhat from the one found for a Ca^{2+}-activated K^+ conductance in *Aplysia* neurons (Meech, 1976, 1980; Gorman and Hermann, 1979). The sequence reported by Gormann and Hermann (1979) is: $Ca^{2+} > Cd^{2+} > Hg^{2+} > Sr^{2+} > Mn^{2+} > Fe^{2+}$. Meech (1976) found that, besides these ions, Pb^{2+} is also able to activate the K^+ conductance in *Aplysia* neurons. Pb^{2+} is also able to activate the Ca^{2+}-activated K^+ channel of red blood cells (Grygorzyk and Schwarz, 1983). Ca^{2+}-activated K^+ channels from muscle membrane, on the other hand, are inhibited by Hg^{2+} and Pb^{2+} and activated by Co^{2+}. However, the results obtained by Meech (1976) and Gormann and Hermann (1979) must be viewed with caution inasmuch as the actual free Ca^{2+} concentration in the cell before and after injection of the different divalent cations is unknown.

The ability to activate the channel seems to be based on cation size; only cations with radii >0.072 (Co^{2+}) or <0.113 nm (Sr^{2+}) are able to activate the channel. However, size alone does not completely determine the ability of a given cation to activate the channel studied here. Cd^{2+}, with a diameter almost identical to that of Ca^{2+}, is ~100-fold less potent as a channel activator than the latter cation. The chemical

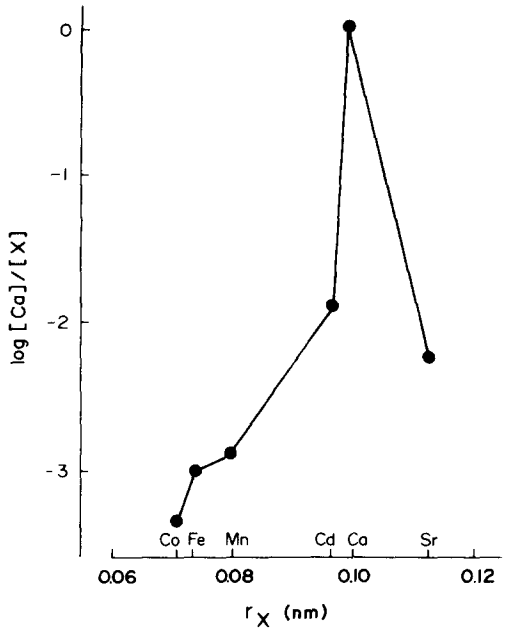

FIGURE 8. The relative potency of the divalent cations as activators of the channel is presented as the log([Ca]/[X]). [X] is the divalent cation concentration (200 μM for all cations); [Ca] is the Ca^{2+} concentration necessary to obtain the same V_o (Eq. 1) that is observed at 200 μM for any other divalent cation. The abscissa, r_x, is the Pauling ionic radius.

nature of the cation probably plays an important role in determining the agonist capacity of divalent cations. Thus, Ca^{2+} and Cd^{2+} differ appreciably in their coordination chemistry. Ca^{2+} forms ionic bonds with oxygen ligands (e.g., carboxyl and carbonyl groups), whereas Cd^{2+} is expected to have a higher affinity for nitrogen and sulfur (Williams, 1977). The importance of the chemical properties of the cation is also shown by the results obtained with trivalent cations. Both Tb^{3+} and Eu^{3+} failed to activate the channel, despite the fact that they have radii very similar to that of Ca^{2+}.

Comparison with Ca^{2+}-binding Proteins

The divalent selectivity sequence for activation we found follows the same order as those found for Ca^{2+}-binding proteins such as calmodulin and troponin C (with the exception of Pb^{2+} in the case of troponin). However, parvalbumin binds Cd^{2+} more strongly than Ca^{2+} (Table IV). In the absence of detailed structural information about the Ca^{2+}-binding sites of the Ca^{2+}-activated K^+ channel, any comparison with other molecules able to bind Ca^{2+} selectively must be viewed with caution. We also

TABLE IV

Comparison of Ca^{2+}-activated K^+ Channel with other Ca^{2+}-binding Proteins

Protein	Divalent cation sequence	Reference
K(Ca) channel	Ca > Ca > Sr > Mn > Fe > Co ≫ Mg, Ni, Cu, Zn, Hg, Pb, Ba	This article
Troponin C	Ca > Cd > Sr > Pb > Mn ≫ Mg, Ni, Zn, Co, Ba	Fuchs (1974)
Calmodulin	Ca > Cd > Hg > Sr > Mn > Zn > Pb > Co > Mg, Ni, Ba	Chao et al. (1984)
Parvalbumin	Cd > Ca > Sr > Mg	Cave et al. (1979)

point out that we are measuring the ability of a certain divalent cation to increase the probability of opening. We are not measuring divalent cation binding directly. It may well be that of the several cations unable to activate the channel, some of them bind to the activating sites, but are unable to promote the conformational change that leads to channel opening. Competition experiments between Ca^{2+} and the other divalent cations can be useful in answering the question of which cations actually bind to the sites. However, this type of experiment is difficult because of the capacity of some divalent cations to allosterically activate the channel and of some others to block the channel. Evidence that some of the divalent cations that do not activate interfere with Ca^{2+} binding is given by the results obtained with Pb^{2+} (data not shown). Pb^{2+} is able to induce a slow blockade and a decrease in P_o. The decrease in P_o suggests that Ca^{2+} and Pb^{2+} are competing for the same site(s).

Our data suggest that the divalent cations that are able to activate the Ca^{2+}-activated K^+ channel in the absence of Ca^{2+} do so by binding to the same site as Ca^{2+}. We stated above that Pb^{2+} appears to bind but is unable to activate the channel. If this is the case, the divalent cations able to bind to the Ca^{2+}-activated K^+ channel are the same as those able to bind to troponin C (Fuchs, 1971, 1974; for a review, see Leavis and Gergely, 1984) and calmodulin (Chao et al., 1984). Therefore, it is possible that in regard to Ca^{2+}-binding sites, all these proteins have a common ancestor.

Ca^{2+}-binding Sites and Electrostatic Models

Eisenman's (1962) ion selectivity theory has been extended to include divalent cations (for a review, see Diamond and Wright, 1969). A model for divalent cation–binding sites was proposed by Truesdell and Christ (1967) and used by Nachsen (1984) to account for the ion selectivity of synaptosomal Ca^{2+} channels. The model is based on purely coulombic interactions between the anions forming the binding site and the divalent cation. The binding site is viewed as consisting of two anions, each with a radius r_s separated by a distance D. The selectivity sequence for the alkali earth cations we obtained here (Fig. 8) for the Ca^{2+}-activated K^+ channel is explained if the site is considered to be formed by two anions with charge -1 and setting $r_s = 0.025$–0.1 nm with $D = 0.200$–0.214 nm. As found experimentally, ion size appears to be an important factor determining the ability to interact with the site. For example, Mg^{2+}, with a radius of 0.066 nm is too small to interact with both anions simultaneously, and Ba^{2+} is too large to fit well in the site. Ca^{2+}, with a radius of 0.1 nm, makes a perfect fit, followed by Cd^{2+} (0.097 nm), Sr^{2+} (0.113 nm), and Mn^{2+} (0.08 nm). These predictions cannot be obtained if D approaches zero (a divalent anion) or infinity (a monovalent anion). Anions forming the binding site are not an absolute requirement of the model. The same approach can be followed if, instead of representing the negative sites as fully charge spheres, one chooses a model in which the negative centers are parts of dipoles (Eisenman, 1962).

Are the values of r_s and D reasonable for a Ca^{2+}-binding site? Einspahr and Bugg (1978) have reviewed a large number of crystal structures of Ca^{2+} complexes and concluded that the most common ligands at the Ca^{2+}-binding site on proteins are the peptide carbonyl groups and the carboxyl groups from glutamic and aspartic acid residues. The average distance for Ca^{2+} contacts with carboxyl-oxygen atoms is

~0.235 nm for sixfold coordination, and a similar distance is found for the contacts of Ca^{2+} with carbonyl-oxygen atoms. These data indicate that if the Ca^{2+}-binding sites in the Ca^{2+}-activated K^+ channel are structured as postulated by Einspahr and Bugg (1978), the site diameter is of the order of 0.4 nm, compared with $D = 0.2$ nm obtained from the electrostatic model. This difference probably results from the assumption that the binding site is composed of only two anions. However, it is possible, by increasing D, to construct a more realistic model with a larger number of negative charges. The value found for r_s (0.025–0.1) is reasonable inasmuch as the carbon-oxygen distance is 0.07 nm (Pauling, 1967).

Regulation of Channel Activation by Divalent Cations

In the Results, we described the effects of Mg^{2+} and Ni^{2+}. These cations do not activate in the absence of Ca^{2+}, but they dramatically enhance Ca^{2+} activation. The effect of Mg^{2+} on the Ca^{2+}-activated K^+ channel has been studied previously (Golowash et al., 1986). We have confirmed and extended these results to other divalent cations. At least three different mechanisms can be postulated to account for the results obtained for Ni^{2+} and Mg^{2+} in the presence of Ca^{2+}: (*a*) an increase in the site affinity for Ca^{2+}; (*b*) an increase in the apparent molecularity for activation; and (*c*) a change in the surface potential. Our results show that these divalent cations induce an increase in the apparent affinity and an increase in the Hill coefficient for Ca^{2+} activation. We do not think that a surface charge phenomenon plays an important role here. A change in the surface charge density in or near the channel does not change the Hill coefficients; it would only shift the activation curve along the Ca^{2+} concentration axis. As a control, we added Mg^{2+} or Ni^{2+} to the external side and found a slight shift to the right of the Ca^{2+}-activation curve, with no change in the Hill coefficient.

Golowash et al. (1986) have proposed that Mg^{2+} reveals Ca^{2+} sites already present in the channel-forming protein in the absence of Mg^{2+}. We think the same conclusion can be applied to Ni^{2+} and possibly to other divalent cations as well. In this regard, the Hill coefficient is Mg^{2+} or Ni^{2+} concentration dependent. The Hill coefficient increases as the concentration of these divalent cations is increased on the cytoplasmic side of the channel. These results can be explained by assuming that the channel has modulatory sites able to bind Mg^{2+} or Ni^{2+}. The modulatory site would be different from the Ca^{2+}-binding sites and would induce the necessary conformational change in the channel-forming protein to expose new Ca^{2+}-binding sites (a minimum of six at 10 mM Mg^{2+}). Increasing the concentration of Mg^{2+} or Ni^{2+} would simply increase the probability that the modulator sites are occupied.

A Hill coefficient of 6 implies binding of a minimum of six Ca ions for complete activation of the channel. This implies in turn that the kinetic scheme of Ca^{2+} activation compatible with the data should contain at least six closed and six open states. By adjusting the equilibrium constants for Ca^{2+} binding to appropriate values it is possible to generate P_o vs. $[Ca^{2+}]$ curves like those obtained in the presence of Ni^{2+} (Fig. 7 *B*) or Mg^{2+} (Golowash et al., 1986). The fact that at least six Ca^{2+}-binding sites are apparent in the presence of Mg^{2+} is in agreement with the kinetic studies of McManus and Magleby (1985). They found that at least six closed and three open states are necessary to fit their kinetic data. Preliminary kinetic data obtained

in planar bilayers taken at a 2.5-kHz bandwidth indicate that Mg^{2+}induces the appearance of new closed and open states. This finding is consistent with the increase in the Hill number for Ca^{2+} activation when Mg^{2+} is added to the internal side.

Divalent Cation Affinity of the Modulator Site(s)

The divalent cation affinity of the modulator site can be inferred from the increase in the Hill coefficient for Ca^{2+} activation at a given divalent cation concentration. We assume here that the larger the Hill coefficient induced by the divalent cation at that concentration, the higher the affinity for the modulator site. We further assume that the Hill coefficient increases upon binding of the divalent cation to the modulator site. The sequence is then (Table II): $Cd^{2+} > Mn^{2+} > Co^{2+} > Ni^{2+} > Mg^{2+} \gg Sr^{2+}, Ca^{2+}$. Taking into account only the alkali earth ions, the sequence corresponds to a high-field-strength sequence in which the ion with the smallest crystal radius is prefered. This sequence is very different from the one obtained for the Ca^{2+}-binding sites: $Ca^{2+} > Sr^{2+} \gg Mg^{2+}, Ba^{2+}$. We note here that some divalent cations bind only to the modulator site (Mg^{2+}, Ni^{2+}) and others to the Ca^{2+} sites and to the modulator site (e.g., Cd^{2+}, Co^{2+}, Mn^{2+}). The case of Sr^{2+} is worth mentioning here. Sr^{2+} is able to increase the apparent affinity of Ca^{2+} activation, but not the Hill coefficient. Therefore, it is not clear at present whether this divalent cation binds to any extent to the modulator site.

Mg^{2+}-binding Sites in Ca^{2+}-binding Proteins

It is well known that troponin C and the parvalbumins, but not calmodulin, bind Mg^{2+} (Leavis and Gergely, 1984; Klee et al., 1980). Troponin C has two high-affinity sites that bind Mg^{2+} and Ca^{2+} and two lower-affinity sites that only bind Ca^{2+} and probably two Mg^{2+}-specific sites (Potter and Gergely, 1975). In troponin C, there is no evidence of positive cooperativity regarding Ca^{2+} binding induced by Mg^{2+}. Indeed, Mg^{2+} lowers the affinity of two of the four Ca^{2+}-binding sites in troponin C by straight competition (Potter and Gergely, 1975) and the same statement is valid for parvalbumin (Cox et al., 1977). However, Mg^{2+} enhances positive cooperativity in the dimeric crayfish muscle Ca^{2+}-binding protein. This protein contains six Ca^{2+}-binding sites, and in the absence of Ca^{2+}, it binds four Mg ions. Mg^{2+} also induces positive cooperativity in sandworm muscle Ca^{2+}-binding protein. In this case, Ca^{2+} binding becomes strongly cooperative at physiological levels of Mg^{2+} (Cox et al., 1977). Thus, the existence of a regulatory Mg^{2+} site in the large-conductance, Ca^{2+}-activated K^+ channel makes this ion pathway more closely related to other Ca^{2+}-binding proteins. In the muscle cell, this would allow large changes of channel activation by much smaller fluctuations of the internal free Ca^{2+} concentration.

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