

Selectivity of the Ca Binding Site in Synaptosome Ca Channels

Inhibition of Ca Influx by Multivalent Metal Cations

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ABSTRACT K-stimulated (voltage-dependent) influx of ^{45}Ca was measured in synaptosomes (isolated presynaptic nerve terminals) from rat brain. Influx was terminated at 1 s with a rapid-filtration technique, so that most of the Ca uptake was mediated by inactivating ("fast") Ca channels (Nachshen, D. A., and Blaustein, M. P., 1980, *J. Gen. Physiol.*, 76:709-728). This influx was blocked by multivalent cations with half-inhibition constants (K_I) that clustered in three distinct groups: (a) $K_I > 1$ mM (Mg^{2+} , Sr^{2+} , and Ba^{2+}); (b) $K_I \approx 30$ -100 μM (Mn^{2+} , Co^{2+} , Ni^{2+} , Cu^{2+} , Zn^{2+} , and Hg^{2+}); (c) $K_I < 1$ μM (Cd^{2+} , Y^{3+} , La^{3+} and the trivalent lanthanides, and Pb^{2+}). Most of these ions had very little effect on synaptosome steady state membrane potential, which was monitored with a voltage-sensitive fluorescent dye, or on the voltage dependence of Ca influx, which was assessed by measuring voltage-dependent Ca uptake at two levels of depolarization. The blockers inhibited Ca influx by competing with Ca for the channel site that is involved in the transport of divalent cations. Onset of fast channel inhibition by Mg, Co, Ni, Cu, Zn, Cd, La, Hg, and Pb was rapid, occurring within 1 s; inhibition was similar after 1 s or 30 min of exposure to these ions. The inhibition produced by Co, Cu, Zn, Cd, La, and Pb could be substantially reversed within 1 s by removing the inhibitory cation. The relative efficacies of the lanthanides as fast channel blockers were compared; there was a decrease in inhibitory potency with decreasing ionic radius. A model of the Ca channel binding site is considered, in which inhibitory polyvalent cation selectivity is determined primarily by coulombic interactions between the binding site and the different cations. The site is envisaged as consisting of two anions (radius 1 Å) with a separation of 2 Å between them. Small cations are unable to bind effectively to both anions. The selectivity sequences predicted for the alkaline earth cations, lanthanides, and transition metals are in substantial agreement with the selectivity sequences observed for inhibition of the fast Ca channel.

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INTRODUCTION

Ca entry into a wide variety of cells is mediated by voltage-regulated channels that are selectively permeable to the alkaline earth cations Ca, Sr, and Ba (for recent reviews, see Hagiwara and Byerly, 1981; Edwards, 1982; Tsien, 1983). The exact mechanism of ion permeation is unknown, but one important step may be the association of Ca with a binding site in the channel. There is good evidence that in many types of cells, La and a variety of transition metals inhibit Ca transport by competing with Ca for this site. The factors controlling this competition are not, however, understood.

There is little direct information about the interaction of polyvalent cations with the Ca channels that are implicated in Ca-mediated transmitter release at central nervous system (CNS) presynaptic nerve terminals. One useful approach applied to the study of CNS presynaptic Ca channels involves the direct measurement of radiotracer ^{45}Ca entry into pinched-off nerve terminals (synaptosomes) isolated from rat brain (Blaustein, 1975; Nachshen and Blaustein, 1979*a*, *b*, 1980, 1982). Synaptosomes retain many of the functional and morphological properties of intact neuronal tissue (Bradford, 1975; Blaustein, 1975; Blaustein et al., 1977). In particular, they are able to regulate Ca influx and release neurotransmitters in a Ca-dependent manner (Blaustein, 1975).

There are two distinct components of voltage-dependent Ca influx. One component ("fast") lasts for 1–2 s and is abolished by prolonged depolarization and by low concentrations ($<1 \mu\text{M}$) of La. A second component ("slow") persists even after the synaptosomes are depolarized for as long as 1 min and is only blocked by high ($\sim 0.1 \text{ mM}$) concentrations of La. The two components are probably mediated by separate fast and slow Ca channels that are also permeable to Sr, Ba, and Mn (Nachshen and Blaustein, 1982; Drapeau and Nachshen, 1984) but not to Na (Krueger and Nachshen, 1980). The fast channels are of primary interest, because they are associated with voltage- and Ca-dependent dopamine release from striatal synaptosomes; the initial rate of this release is close to the rate evoked by nerve stimulation of intact tissue (Drapeau and Blaustein, 1983).

The relative efficacies of various metal cations as blockers of the fast Ca channel in synaptosomes may provide clues about the control of ionic selectivity. With this point in mind, I examined the effects of Mg^{2+} , Sr^{2+} , Ba^{2+} , Mn^{2+} , Co^{2+} , Ni^{2+} , Cu^{2+} , Zn^{2+} , Y^{3+} , Cd^{2+} , La^{3+} and the lanthanides, Hg^{2+} , and Pb^{2+} on Ca influx. These ions competitively inhibit Ca influx through the fast channels. The most potent blockers, with inhibition constants (K_i) of $<1 \mu\text{M}$ (Cd, Y, La and the lanthanides, and Pb), have ionic radii close to, or larger than, the ionic radius of Ca. For ions in chemical groups with similar properties (the alkaline earth cations; Y and La and the lanthanides; the divalent transition metals), there is a decrease in blocking potency with a decrease in ionic radius below that of Ca. A simple model of the Ca channel binding site that accounts for many of these findings is presented.

A preliminary report of some of these findings has been published (Nachshen, 1983).

METHODS

Preparation of Synaptosomes

A modification (Krueger et al., 1979) of the method of Hajos (1975) was employed for the preparation of synaptosomes from rat forebrains. Nerve terminal-enriched material, in a 0.8-M fraction from a sucrose gradient, was equilibrated by the gradual addition of 2–3 vol of Na solution containing (in mM): 145 NaCl, 5 KCl, 1 MgCl₂, 0.02 CaCl₂, 10 glucose, 10 HEPES, adjusted to pH 7.4 at 3°C with NaOH. The diluted synaptosome suspension was centrifuged (13,000 g) for 10 min, and the pellet was resuspended in Na solution, adjusted to pH 7.4 at 30°C. The resuspended synaptosomes were gently agitated for 20 min at 30°C before proceeding with the experiments.

Measurement of Ca Influx

Entry of radioisotope was determined by adding aliquots of the warmed synaptosome suspension to equal volumes of low-K or K-rich solution with tracer (see Nachshen and Blaustein, 1980). Typically, each sample (total = 360 μ l) contained 0.5–1 μ Ci of ⁴⁵Ca or ¹⁴¹Ce (New England Nuclear, Boston, MA). The final composition of the low-K solution was (mM): 72.5 NaCl, 72.5 choline Cl, 5 KCl. In the corresponding K-rich solution, the K concentration was increased by isosmotically replacing choline with K in order to maintain a constant external Na concentration and minimize the Na-dependent Ca influx (Blaustein and Oborn, 1975). Ca, Mg, glucose, and buffer concentrations were identical to those in the Na solution. The low-K and K-rich solutions with tracer also contained different metal chlorides, as indicated for the specific experiments. In experiments with CuCl₂, all of the HEPES buffer was replaced with Tris buffer, since CuCl₂ precipitated in solutions with HEPES buffer.

In a number of experiments, the synaptosomes were “predepolarized” before the addition of radiotracer (Nachshen and Blaustein, 1980) in order to eliminate Ca uptake via the fast channels. This was done by adding aliquots of the warmed synaptosome suspension to equal volumes of K-rich solution. After 10 s, an additional aliquot of solution with radiotracer and polyvalent cations was added, with the ionic composition adjusted so that incubation with tracer would occur in K-rich solution, as described above.

Radioisotope entry was terminated by rapidly diluting the incubation media with 4 ml of ice-cold Na solution containing 2 mM LaCl₃. After this, the diluted synaptosome suspensions were filtered (No. 25; Schleicher & Schuell, Inc., Keene, NH; or GF/A; Whatman Laboratory Products Inc., Clifton, NJ), and the filters were rinsed twice with 4-ml aliquots of La solution. The radioactivity retained by the filters was determined using standard liquid scintillation counting techniques. Protein was measured by the method of Lowry et al. (1951). The K-stimulated (depolarization-dependent) Ca influx was taken as influx in K-rich solution minus influx in low-K solution. All flux measurements were made in replicates of four or five, and the averaged values are given \pm the standard errors of the means. The values are corrected for radioisotope binding to the filters.

Fluorescence Measurements

The voltage-sensitive fluorescent dye 3,3'-dipentyl 2,2'-oxacarbocyanine [diO-C₅(3)] was used to determine changes in membrane potential (Blaustein and Goldring, 1975). Aliquots of synaptosome suspension (50 μ l) containing \sim 0.5 mg of protein in Na solution were added to 2 ml of low-K or K-rich solution with 2.5 μ M dye and various amounts of polyvalent cations as specified in the text. The synaptosome suspension was then illuminated with light at a wavelength of 448 nm, and fluorescent emission was measured at 511 nm after 1 min.

Chemicals

CuCl₂, HgCl₂, and ZnCl₂ were obtained from Sigma Chemical Co., St. Louis, MO; CdCl₂, CeCl₃, EuCl₃, PrCl₃, SmCl₃, TmCl₃, and YCl₃ were from Aldrich Chemical Co., Inc., Milwaukee, WI; DyCl₃, ErCl₃, GdCl₃, HoCl₃, LaCl₃, LuCl₃, NdCl₃, PbCl₂, and YbCl₃ were from Alfa Products, Danvers, MA; AlCl₃ was from J. T. Baker Chemical Co., Phillipsburg, NJ; GaCl₃, InCl₃, and TlCl₃ were from Pfaltz and Bauer, Inc., Stamford, CT. All these chemicals were of 99.9% purity or greater.

RESULTS

In order to investigate the factors that determine cation-fast Ca channel interaction, I examined the effects of 25 polyvalent metal cations on K-stimulated Ca influx. Nearly three-quarters of the K-stimulated Ca influx during a 1-s incubation is mediated by the fast Ca channels that inactivate with maintained depolarization (Nachshen and Blaustein, 1980). Figs. 1–3 show that a variety of divalent and trivalent cations (Fig. 1: Mg²⁺, Sr²⁺, Ba²⁺, Mn²⁺, Co²⁺, Ni²⁺, Cu²⁺, Zn²⁺, Y³⁺, Cd²⁺, Pb²⁺; Fig. 2A: Hg²⁺; Fig. 3: La³⁺, Ce³⁺, Pr³⁺, Nd³⁺, Sm³⁺, Eu³⁺, Gd³⁺, Dy³⁺, Ho³⁺, Er³⁺, Tm³⁺, Yb³⁺, Lu³⁺) reduce influx at 1 s in a concentration-dependent manner. The blockers cluster in three distinct groups (Fig. 1) with half-maximal inhibition of influx occurring at concentrations (*I*₅₀) of: (a) >1 mM for the weak blockers, Mg, Sr, and Ba; (b) ~50 μM for the first period transition metals and Hg; (c) <1 μM for the most potent blockers, Cd, Y, Pb, and the lanthanides.

How do the metal cations reduce the K-stimulated Ca influx measured at 1 s? A probable explanation is that they directly block Ca passage through the Ca channels (see below). Another possibility is that these ions alter either the transmembrane potential in the synaptosomes (approximately -60 mV; Blaustein and Goldring, 1975) or the negative surface potential (Nachshen and Blaustein, 1982), thereby diminishing Ca influx and the voltage-dependent activation of the presynaptic Ca channels (Nachshen and Blaustein, 1982). These different possibilities were tested for, as described below.

Block of K-stimulated Ca Influx Is Unrelated to Changes in Membrane Potential

Metal ion effects on membrane potential were ruled out as an explanation for the block of Ca uptake, in experiments with the voltage-sensitive fluorescent dye diO-C₅(3). Mn, Co, Ni, Zn, Cd, La, and Pb, at concentrations ≥*I*₅₀, had little or no effect on dye fluorescence in the absence of synaptosomes, on the baseline fluorescence of synaptosomes added to dye in low-K solution (not shown), or on the K-induced fluorescence signal (fluorescence in K-rich solution minus fluorescence in low-K solution; Δ*F*, Table I). Mg, Sr, and Ba were also without significant effect (Nachshen and Blaustein, 1982).

Cu and Hg had no effect on dye fluorescence in the absence of synaptosomes, but significantly reduced the K-induced fluorescence: Cu diminished the fluorescence signal of the synaptosomes in low-K and K-rich solutions, while Hg increased the fluorescence in the low-K solution. It seems unlikely, however, that these changes in the fluorescence signal underlie the observed decrease in K-stimulated Ca influx: 0.005 mM Cu reduced the K-induced fluorescence signal

(Table I) by 25%, but this concentration of Cu had no effect on K-stimulated Ca influx at 1 s (Fig. 1); 0.1 mM Hg completely abolished the K-induced fluorescence, but this concentration of Hg reduced K-stimulated Ca influx at 1 s by only one-half (Fig. 2). Thus, the effects of Cu and Hg on dye fluorescence are inconsistent with depolarization of the synaptosomes, and it is possible that there may be some specific interaction between these metals and the dye/synaptosome fluorescence signal.

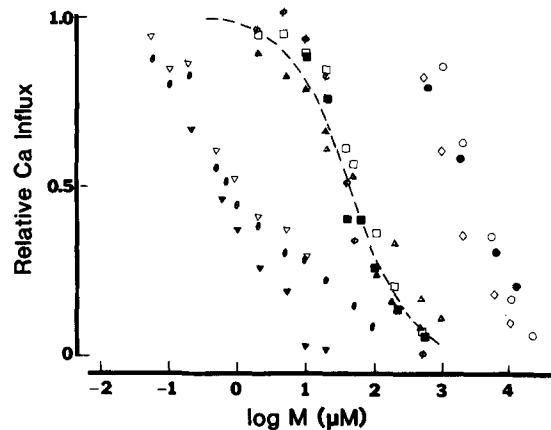


FIGURE 1. The effect of different metal ions on K-stimulated Ca influx. Influx of radiotracer Ca (0.02 mM) was measured at 1 s in low-K or K-rich solutions, containing Ba (\diamond), Sr (\bullet), Mg (\circ), Cu (ϕ), Mn (Δ), Zn (\blacktriangle), Co (\square), Ni (\blacksquare), Cd (∇), Y (θ), and Pb (\blacktriangledown). These metals were added as bivalent chlorides, except for the trivalent YCl_3 . The dashed line represents a curve drawn to the equation (Nachshen and Blaustein, 1982):

$$J_{Ca} = \frac{J_{Ca}^0}{1 + I/I_{50}}, \quad (1)$$

where J_{Ca} is the K-stimulated Ca influx in the presence of inhibitor, and J_{Ca}^0 is the K-stimulated Ca influx in the absence of inhibitor at a low concentration of external Ca. I is the inhibitory ion concentration, and I_{50} is the concentration of I at which influx is reduced by half. I_{50} has a value of 0.05 mM. All influx values have been normalized to a J_{Ca}^0 value of unity.

Block of K-stimulated Ca Influx Is Unrelated to Reduction in Surface Potential

Metal cation-surface charge interactions might effectively hyperpolarize the synaptosome membrane and reduce K-stimulated Ca influx. I tested for this possibility by comparing the blocking efficacies of the metal cations at two levels of depolarization, induced by incubating the synaptosomes with either 20 or 77.5 mM K (corresponding to membrane potentials of approximately -40 and 0 mV, respectively; Blaustein and Goldring, 1975; Nachshen and Blaustein, 1982). Because the dependence of Ca influx on external K (and membrane potential) is sigmoidal, a reduction in negative surface potential will have a

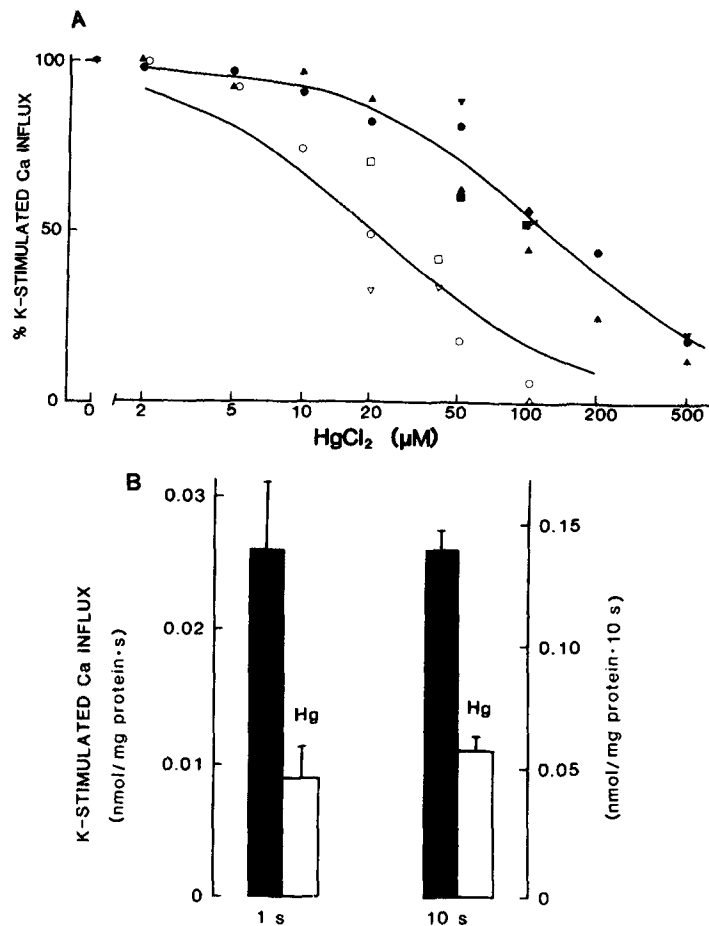


FIGURE 2. The effect of HgCl_2 on K-stimulated Ca influx. (A) Ca influx was measured at 1 s (solid symbols) and at 10 s in synaptosomes that had been predepolarized in K-rich solution for 10 s before the addition of radiotracer Ca (open symbols). Different symbols represent the results from different experiments. The results have been normalized to the Ca uptake obtained in the absence of Hg in each experiment (*). Standard errors for the different data points (not shown) ranged from 5 to 10% of the mean. The curves were drawn to Eq. 1, with I_{50} values of 0.12 mM (upper curve) or 0.02 mM (lower curve). (B) Synaptosomes were predepolarized for 10 s before the addition of Hg or radiotracer Ca. ^{45}Ca was then added with (open bars) or without (solid bar) 40 μM Hg; K-stimulated Ca influx was determined at 1 s (bars on left) and at 10 s (bars on right). Note the different scales for the two times.

greater relative effect on K-stimulated Ca influx at low concentrations of K (where the voltage dependence is steep) than it will at high concentrations of K (where the voltage dependence levels off; Nachshen and Blaustein, 1982). Table II shows the effect of several metal cations on K-stimulated Ca influx, induced

with either 20 or 77.5 mM K. Mg and, to a lesser extent, Cu were significantly more effective at the lower K concentration. Co, Ni, and Zn appeared more effective at the lower K concentration, but the difference in efficacies was not significant ($P > 0.1$). Mn, Y, Cd, and La were equally effective at both K concentrations. These results indicate that concentrations of most metal cations (including Sr and Ba; Nachshen and Blaustein, 1982) that significantly block K-stimulated Ca influx have little or no detectable effect on the voltage dependence of Ca influx and on surface potential. This result is not surprising, considering the low concentrations (<1 mM) of most metal cations that are required to block K-stimulated Ca influx (Figs. 1–3).

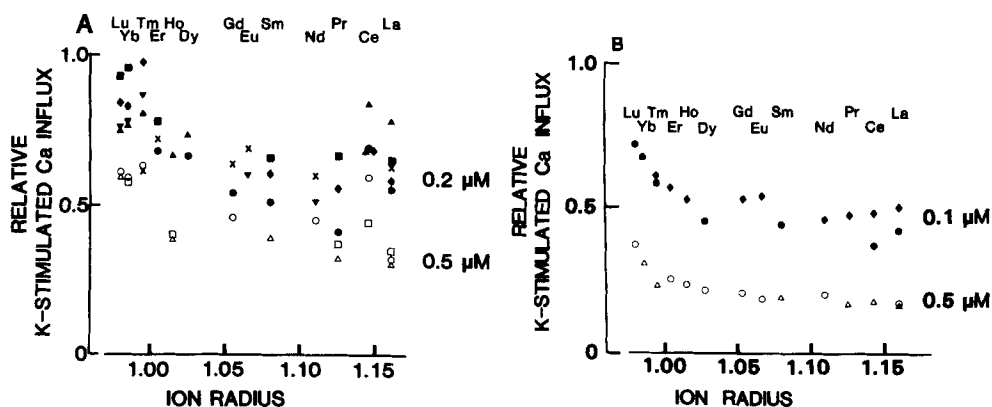


FIGURE 3. The relationship between ionic radius of the trivalent lanthanides and K-stimulated Ca influx. (A) Synaptosomes that had been incubated in control solution (pH 7.4) were mixed with low-K or K-rich solution (pH 7.4) containing radiotracer Ca and lanthanide ions; influx was measured for 1 s in the presence of 0.2 (solid symbols) or 0.5 μM (open symbols) lanthanide ion. Different symbols are from different experiments. The results are normalized to the influx values obtained without lanthanides in each experiment. Standard errors (not shown) ranged from 5 to 10% of the mean measurements. (B) Same as in A, except that the radiotracer- and lanthanide-containing solutions were adjusted to pH 6.3, so that the resultant mixture had a pH of 6.8. The ionic radii values (in angstroms) shown in this figure and used in subsequent calculations are from Shannon (1976), based on a coordination number of 8—the likely coordination number for lanthanides under most biological conditions (Moeller et al., 1965; Nieboer, 1975).

Effect of Metal Cations on Ca Influx through Noninactivating Ca Channels

Because some of the K-stimulated Ca influx measured at 1 s (~25%) is mediated by a distinctive population of noninactivating (slow) Ca channels (Nachshen and Blaustein, 1980), it was important to determine the effect of the various metal cations on these channels. This was done by measuring K-stimulated Ca influx in prepolarized synaptosomes (see Methods); prepolarization inactivates the fast Ca channels, and the remaining influx is mediated by the noninactivating (slow) Ca channel population. The effect of several metals on K-stimulated Ca

TABLE I
Effect of Multivalent Cations on K-induced Fluorescence

Ion concentration		I_{50}	ΔF
<i>mM</i>		<i>mM</i>	% of control
Mn	1.0	0.07	113
Co	0.2	0.06	82
Ni	0.2	0.04	100
Cu	0.005	0.04	73
Cu	0.05	—	77
Zn	0.08	0.03	96
Cd	1.0	0.001	100
La	0.1	0.0003	109
Hg	0.01	0.12	47
Hg	0.1	—	0
Pb	0.001	0.0004	97

Fluorescence was measured in low-K and K-rich solutions (see Methods) containing diO-C₅(3) and metal cations at concentrations that are indicated in the table. The K-induced fluorescence, ΔF (fluorescence signal in K-rich solution minus signal in low-K solution), in the presence of inhibitory ions is shown relative to the control ΔF , in the absence of inhibitory ions. Also included in the table are the I_{50} values (derived from Figs. 1-3) for metal cation block of K-stimulated Ca influx. In the absence of inhibitory cation, the fluorescence of synaptosomes in low-K solution was ~50% greater than the fluorescence of synaptosomes in low-K solution. The standard error of the control ΔF was ~5% of the mean.

TABLE II
Effect of Multivalent Cations on K-stimulated Ca Influx

Ion concentration	<i>mM</i>	K-stimulated Ca influx	
		20 mM K	77.5 mM K
		% of control	
Mg	3.5	23±4	48±4*
Mn	0.1	37±5	32±5
Co	0.05	39±7	54±6
Ni	0.04	26±9	38±6
Cu	0.04	33±5	55±3*
Zn	0.05	20±8	34±6
Y	0.0007	49±6	55±4
Cd	0.0007	54±13	51±4
La	0.0003	57±4	51±4

Ca influx at 1 s was determined in low-K or K-rich solutions containing either 20 or 77.5 mM K. These solutions also contained various metal cations at the concentrations indicated in the table. The influx values (means ± standard error, $n = 5$) obtained in the presence of inhibitory ions are normalized to control K-stimulated influx values obtained in the absence of inhibitory ions at the appropriate K concentration. Representative paired values from three different experiments are shown in the table. The results were compared using the Student's two-tailed t test.

* $P < 0.005$; for all the other pairs, $P > 0.1$.

influx at 10 s in prepolarized synaptosomes is shown in Tables III and IV. Y, Cd, Pb, and the lanthanides, as well as Ba, La, and Mn (see Nachshen and Blaustein, 1980), preferentially block the inactivating Ca channels, since they had less effect on Ca influx in prepolarized synaptosomes at concentrations close to, or greater than, their I_{50} values. Cu and Hg, as well as Sr (Nachshen and Blaustein, 1982), were more effective blockers of Ca influx after the synaptosomes were prepolarized. For the other ions tested (Mg, Co, Ni, and Zn), the levels of inhibition observed at 1 and 10 s with prepolarization were similar.

TABLE III
Effect of Multivalent Cations on K-stimulated Influx in Prepolarized Synaptosomes

Ion concentration	mM	K-stimulated Ca influx	
		1 s	10 s
		% of control	
Mg	3.5	53±3	62±4*
Sr	2.0	59±4	29±2
Ba	5.0	17±1	44±9‡
Mn	0.1	39±4	75±7‡
Co	0.05	45±5	56±6
Ni	0.05	43±3	32±8
Cu	0.04	68±3	31±7‡
Zn	0.03	49±5	53±5
Y	0.0007	49±6	86±7†
Cd	0.0007	49±7	92±7†
Hg	0.10	57±6	0±4‡
Pb	0.0004	58±4	90±2‡

Influx measurements were carried out as described in the text, in solutions containing inhibitory ions at the concentrations shown in the table. The K-stimulated influx values (means ± standard error, $n = 5$) have been normalized to the control K-stimulated influx values obtained in the absence of inhibitory ion. Paired values (from four different experiments) were compared using the Student's two-tailed t test. * $P > 0.05$; ‡ $P < 0.02$; † $P < 0.001$; † $P < 0.005$; all other P values were > 0.1 .

Because the efficacy of Hg in blocking K-stimulated influx appeared to be greater in prepolarized synaptosomes (Table III), its effects on Ca influx were examined in more detail. Fig. 2A shows that the I_{50} for block of Ca influx in prepolarized synaptosomes (open symbols) is $\sim 20 \mu\text{M}$, about five- to sixfold lower than the I_{50} for block of Ca influx at 1 s (solid symbols). One trivial explanation for this differential sensitivity might be that Hg progressively depolarizes, or adversely affects, the synaptosomes: thus, synaptosomes exposed to Hg for 10 s might have smaller Ca influx values than synaptosomes exposed to Hg for only 1 s. That this is not the case is shown in Fig. 2B. Synaptosomes were prepolarized for 10 s; K-stimulated Ca influx was then measured for either 1 or 10 s in the presence (open bars) or absence (solid bars) of $40 \mu\text{M}$ Hg. A similar

level of inhibition was observed at both times. These results suggest that preferential block of the slow Ca channels by Hg is not a result a progressive synaptosome deterioration in Hg-containing solution.

Time Dependence and Reversibility of Inhibition

Is the inhibition of K-stimulated Ca influx time dependent? It was essential to answer this question because in most experiments the synaptosomes were incubated with inhibitory ion for only 1 s, during the period in which radiotracer influx was determined. Synaptosomes were incubated in control solution with or without inhibitory ion (Fig. 4). After 30 min, aliquots of the suspensions were

TABLE IV
Effect of Lanthanides on K-stimulated Ca Influx in Predepolarized Synaptosomes

Ion	K-stimulated Ca influx % of control
La	68±10
Ce	90±38
Pr	82±12
Nd	83±13
Sm	96±12
Eu	94±9
Gd	89±24
Dy	91±23
Ho	ND
Er	ND
Tm	ND
Yb	100±10
Lu	104±14

Influx with radiotracer lasted for 10 s in solutions containing 50 μ M of the lanthanides shown in the table. Before incubation with radiotracer, the synaptosomes were incubated for 10 s in a K-rich solution without 45 Ca. The values (means \pm standard error, $n = 5$) are shown as percent of the control influx obtained in solutions without lanthanides. ND: not determined.

removed and added to low-K or K-rich solutions containing radiotracer Ca and inhibitory ion. K-stimulated Ca influx was then measured at 1 s. The level of influx obtained after 30 min in the presence of inhibitory ion (Fig. 4A, gray bars), was similar to (Mg, Co, Ni, La, Cd, Hg, Pb; $P > 0.1$, Student's t test), or only slightly less than (Cu, Zn; $P < 0.05$), the level of influx observed when the inhibitory ion was added for only 1 s, along with radiotracer Ca (open bars).

In a number of experiments (Fig. 4B), reversibility of the inhibition was also examined. Aliquots of synaptosomes incubated with inhibitory ion for 30 min were added to low-K or K-rich solution with radiotracer Ca but without inhibitory ion; K-stimulated Ca influx was measured at 1 s. The volumes of the low-K and K-rich solutions were adjusted so that a two- to fivefold dilution of inhibitory ion concentration was obtained (solid bars). Fig. 4B shows that when the concentration (micromolar) of Co, Cu, or Zn was reduced from 50 to 10 (solid bars), the

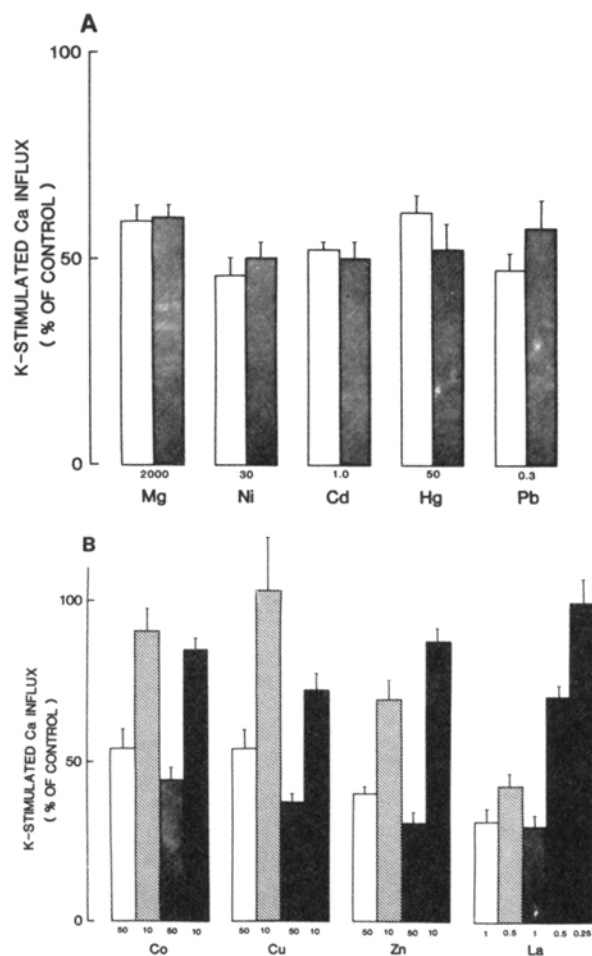


FIGURE 4. Time dependence and reversal of Ca channel inhibition. (A) Synaptosomes were incubated in control (145 mM Na and 5 mM K) solution, with (gray bars) inhibitory ion at the concentration (micromolar) indicated beneath the bars. After 30 min, aliquots of the synaptosome suspension were removed and added to low-K or K-rich solutions containing the same concentration of inhibitory ion, along with ^{45}Ca . Influx was terminated after 1 s, and K-stimulated influx was determined. Another batch of synaptosomes was incubated in control solution without inhibitory ion (open bars). After 30 min, aliquots of this suspension were removed and added to low-K or K-rich solution with inhibitory ion at the final concentration, which is indicated beneath the bars, along with ^{45}Ca ; K-stimulated Ca influx was determined at 1 s. All the results are normalized to the K-stimulated Ca influx that was obtained with synaptosomes that were not exposed to inhibitory ion. (B) As in A, except that some samples from the batch containing inhibitory ion were added to a large volume of low-K or K-rich solution without inhibitory ion (solid bars), so that the final concentration of inhibitor was reduced to the value shown beneath the solid bars. In addition, some samples from the batch of synaptosomes that were not exposed to inhibitory ion were added to low-K or K-rich solutions with inhibitory ion at the final concentration indicated beneath the bars with diagonal stripes.

level of Ca influx was similar to the level of influx observed in synaptosomes incubated with $10 \mu\text{M}$ inhibitory ion (bars with diagonal lines). The inhibition produced by Cd ($1 \mu\text{M}$) and Pb ($0.4 \mu\text{M}$) could similarly be reversed (not shown). Dilution of La (after 30 min) from 1 to $0.5 \mu\text{M}$ (solid bar) increased the level of influx above the level observed in synaptosomes incubated with $0.5 \mu\text{M}$ La (bar with diagonal stripes); dilution from 1 to $0.25 \mu\text{M}$ (solid bar) produced complete recovery of the influx level (compare with the effect of $0.2 \mu\text{M}$ La in Fig. 3A). This means that prolonged incubation of synaptosomes with low ($1 \mu\text{M}$) concentrations of La significantly enhances their ability to accumulate Ca. Further experimentation is required to elucidate the mechanism that underlies this effect. In any case, these experiments rule out the possibility that polyvalent cation inhibition of K-stimulated influx is caused by an irreversible process or by accelerated deterioration of the synaptosomes.

The rapid onset of inhibition, and the rapid reversal, suggest that the inhibitory metal cations gain access to the channel from the outside surface of the synaptosome plasma membrane. Possibly, these ions cannot block the channel from the inside; alternatively, the rate of passive uptake of these ions by the synaptosomes may be very low, or they may be rapidly sequestered and removed from the vicinity of the membrane's inner surface.

Metal Ions Compete with Ca for the Ca Channels

K-stimulated Ca influx through the Ca channels begins to saturate as the external Ca concentration is increased (Nachshen and Blaustein, 1980). This suggests that there is a binding site for Ca in the synaptosomal Ca channels (as in the Ca channels of many other preparations; see Hagiwara and Byerly, 1981). The alkaline earth cations Sr and Ba, which permeate through the fast Ca channels, are able to diminish Ca influx by competing with Ca for this site, and bind to this site in order to pass through the channels (Nachshen and Blaustein, 1982). Experiments were therefore performed to determine whether other metal ions diminish Ca influx by a similar mechanism. K-stimulated Ca influx was measured at 1 s at a variety of Ca concentrations in the presence or absence of several transition metals. Representative experiments with Hg ($100 \mu\text{M}$), La ($0.3 \mu\text{M}$), Ni ($40 \mu\text{M}$), and Cd ($0.5 \mu\text{M}$) are shown in Fig. 5. The influx values are plotted in reciprocal form vs. the reciprocal of the external Ca concentration. The straight lines joining the reciprocal data points are extrapolated to a common intercept at the y axis. The simplest interpretation of this result is that the metals do not alter the maximal Ca uptake, which was obtained with saturating concentrations of Ca, but that they increase the apparent half-saturation constant for Ca [$K_{M(\text{Ca})}$] by competing with Ca. Similar results were obtained with the other metals as well. From these experiments it is possible to calculate the inhibition constant, K_I , for the metal cation-binding site interaction (see the legend to Fig. 5). Table V summarizes the calculated K_I values. For most of the ions tested, K_I and I_{50} values are in excellent agreement. This means that competitive inhibition with Ca accounts for the block of K-stimulated Ca influx at 1 s. The two ions whose K_I and I_{50} values do not correspond are Mg and Hg. The finding that

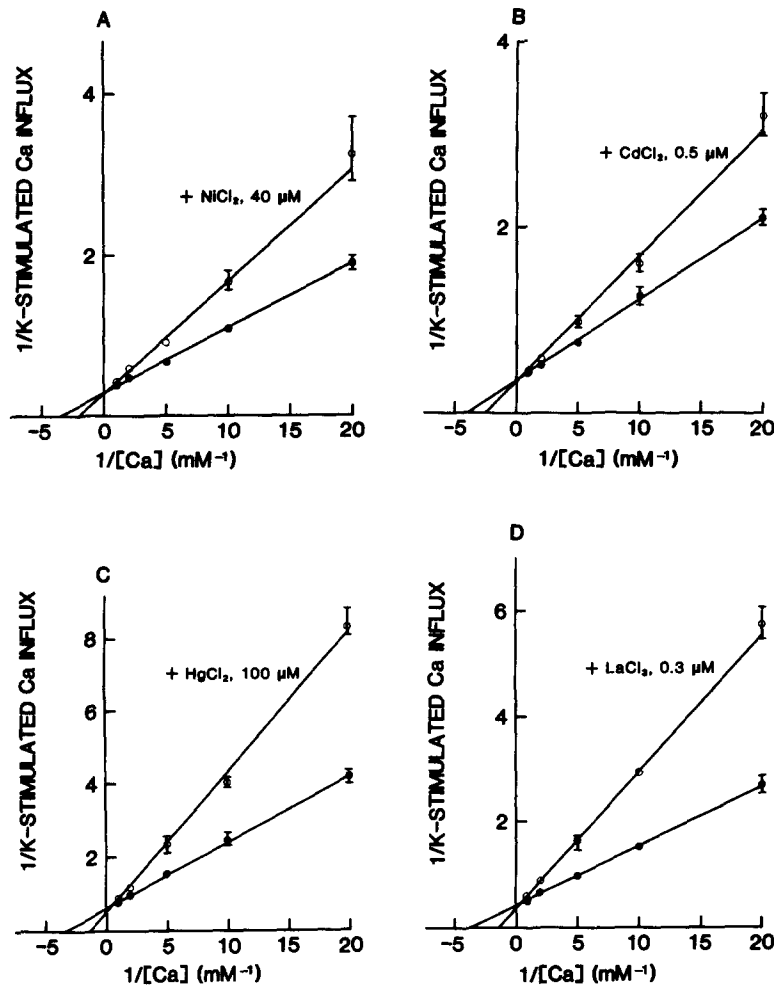


FIGURE 5. The effect of Ca concentration on K-stimulated Ca influx in the presence (open symbols) or absence (solid symbols) of inhibitory ions. Influx (nanomoles per milligram times seconds) was measured at 1 s in solutions containing 0.05–1 mM Ca without or with: (A) Ni, 40 μM ; (B) Cd, 0.5 μM ; (C) Hg, 100 μM ; (D) La, 0.3 μM . The data are plotted in double-reciprocal form. The straight lines joining the data points have been fitted by eye. The half-saturation constant for Ca, K_M , is the (negative) reciprocal of the intercept of the line joining the solid symbols with the x axis. The inhibition constant for the inhibitory ions, K_I , was calculated from the equation:

$$K_I = \frac{I}{(K_M'/K_M) - 1}, \quad (2)$$

where K_M' is the (negative) reciprocal of the intercept of the line joining the open symbols with the x axis, and I is the inhibitory ion concentration. The K_I values obtained for Ni, Cd, La, and Hg are given in Table V.

$K_{I(\text{Mg})} > I_{50(\text{Mg})}$ is consistent with Mg reducing Ca influx both by competing with Ca for the channel sites and by screening fixed negative charges near the channel. The K_I value of Mg, 5.7 mM, agrees well with the I_{50} value of ~6 mM obtained for Mg block of Ca influx after correcting for surface charge effects (see Nachshen and Blaustein, 1982, Fig. 4B). The finding that $K_{I(\text{Hg})} < I_{50(\text{Hg})}$ may be explained by the observation that Hg preferentially blocks slow Ca channels: with Hg concentrations of <0.1 mM, most of the block observed at 1 s may be due to inhibition of the slow channels ($I_{50} \approx 0.02$ mM; Fig. 2).

The $K_{M(\text{Ca})}$ values derived from the experiments testing for competitive inhibition ranged from 0.25 to 0.45 mM (mean: 0.30 ± 0.02 , $n = 9$), in good agreement with previously reported $K_{M(\text{Ca})}$ values (0.32 ± 0.04 ; Nachshen and Blaustein, 1982).

TABLE V
Comparison of K_I and I_{50} Values for Inhibitors of K-stimulated Ca Influx

Ion	Radius Å	I_{50} mM	K_I mM
Mg	0.72	3.0	5.7
Sr	1.18	2.6	2.0
Ba	1.35	1.5	ND
Mn	0.83	0.07	0.05
Co	0.75	0.06	0.06
Ni	0.69	0.04	0.05
Cu	0.73	0.04	0.03
Zn	0.74	0.03	0.04
Y	1.02	0.001	0.0007
Cd	0.95	0.001	0.0006
La	1.16	0.0003	0.0002
Hg	1.02	0.12	0.06
Pb	1.19	0.0004	0.0004

K_I values were calculated from the results of experiments like those shown in Fig. 5, using Eq. 2 (see legend to Fig. 5). I_{50} values are taken from the experiments shown in Figs. 1-3. The table also gives ionic radius values for different inhibitory ions, taken from Shannon (1976), assuming a coordination number of 6 for divalent cations, and a coordination number of 8 for Y and the lanthanides (Moeller et al., 1965; Nieboer, 1975).

Effect of Lanthanide Ionic Radius on K-stimulated Ca Influx

Because all of the trivalent lanthanide cations are similar to Ca in many of their chemical and physical properties (Martin and Richardson, 1979), but decrease in ionic radius with increasing atomic number, they are useful as probes of Ca binding sites in biological membranes (Dos Remedios, 1981). I therefore compared the relative efficacies of the lanthanides as blockers of the fast Ca channel. K-stimulated Ca influx was measured at 1 s in solutions buffered to pH 7.4 (see Methods), containing either 0.2 or 0.5 μM of the lanthanides (Fig. 3A). The results indicated a general trend for less effective inhibition with decreasing ionic radius.

Lanthanide ions undergo hydrolysis at alkaline pH, with pK_a 's (Perrin and Dempsey, 1979) ranging from 9.0 (La) to 7.9 (Lu). Since there is little hydrolysis

at neutral or slightly acid pH (Morris, 1976), the experiments were repeated using slightly acid solutions (pH 6.8, PIPES buffer, 10 mM). In the acidified solutions, the trend for less effective inhibition with decreasing ionic radius was clearer. Lanthanide ions with a radius (Shannon, 1976) of $\sim 1 \text{ \AA}$ or more were similar in blocking efficacy; ions with a radius of $< 1 \text{ \AA}$ were less effective blockers. It was also noted that the lanthanide ions were more effective at the acidic pH than they were at pH 7.4 (compare Figs. 3A [0.2 μM] and 3B [0.1 μM]). This was contrary to expectations, because H^+ competes with Ca for the channel (Nachshen and Blaustein, 1979a); at a lower pH there should be less lanthanide ion-binding site interaction. Presumably, the lessened hydrolysis of the lanthanide ions at low pH more than offsets competition for the binding sites.

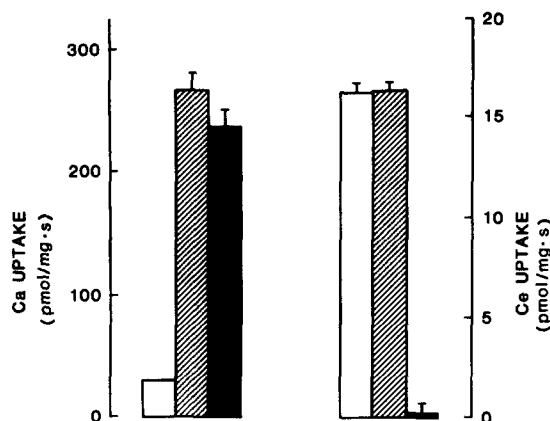


FIGURE 6. The effect of replacing Na with K on Ca and Ce uptake. Synaptosomes were incubated for 1 s in solutions containing 145 mM Na and 5 mM K (open bars), or 72.5 mM Na and 77.5 mM K (hatched bars), along with 0.02 mM Ca and 0.15 μM Ce, as well as radiotracer (^{45}Ca , bars on left; ^{141}Ce , bars on right). After 1 s, uptake was stopped by diluting the samples with a large volume of ice-cold solution, containing (mM): 150 choline Cl, 10 EGTA, 15 MgCl_2 , 10 HEPES, pH 7.4, at 3°C . The samples were filtered and rinsed with two aliquots of ice-cold Na solution with La, as described in Methods. This modified filtration procedure was adopted to minimize Ce binding to the tissue and filters. The solid bars show the K-stimulated uptake, which is equal to the uptake in K-rich solution minus the uptake in low-K solution. Note the different scales for the ^{45}Ca and ^{141}Ce uptake values.

In view of the fact that the lanthanides have ionic radii similar to that of Ca (Shannon, 1976), I attempted to measure lanthanide permeation through the fast channels. Synaptosomes were incubated for 1 s in low-K or K-rich solutions (pH 7.4) containing either ^{45}Ca (20 μM) alone, ^{45}Ca (20 μM) and Ce (0.15 μM), or Ca (20 μM) and ^{141}Ce (0.15 μM). The extent of radiotracer uptake is shown in Fig. 6. No K-stimulated Ce influx was observed. Similar results were seen in three more experiments. Because of the substantial baseline accumulation of radiotracer cerium (possibly due to tissue binding), a small amount of K-stimulated Ce influx might not be detected. Given the magnitude of the standard errors, the Ce/Ca influx ratio, normalized for the differences in external ion concentration, could be as large as, but no larger than, 1:4.

Group IIIA Metals

In a number of experiments, the effects of AlCl_3 , GaCl_3 , InCl_3 , and TlCl_3 were tested. These metal chlorides did not reduce K-stimulated Ca influx at concentrations of $\leq 200 \mu\text{M}$. This result is ambiguous, because the chemistry of these compounds in solution is complex and they are readily hydrolyzed. At higher concentrations ($>200 \mu\text{M}$), influx was reduced, but this may have been a result of acidification of the medium. At these concentrations, a visible precipitate was formed.

DISCUSSION

The purpose of this study was to examine the effects of polyvalent metal cations on Ca influx through the fast Ca channels in synaptosomes in order to characterize the factors that control channel selectivity. The fast channel is of particular interest, because it is involved in the release of neurotransmitter from synaptosomes at near-physiological rates (Drapeau and Blaustein, 1983). 25 of the 29 metal cations that were tested blocked the K-stimulated (voltage-dependent) Ca influx in a dose-dependent manner. The block was rapid in onset and reversible, and it could not be attributed to a change in membrane potential or surface potential. Inhibition of Ca influx was relieved by increasing the external Ca concentration, in a manner suggesting that the inhibitory ions competed with Ca for a binding site in the channel. This site has been implicated in divalent cation permeation (Nachshen and Blaustein, 1982).

Identity of the Fast Ca Channel

There appear to be three categories of inhibitory polyvalent cations: (a) ions that block only in the millimolar concentration range (Sr, Ba, and Mg; Nachshen and Blaustein, 1982); (b) ions that are effective at a concentration range of 30–100 μM (Mn, Co, Ni, Cu, and Zn); and (c) ions that act at very low concentrations of $\leq 1 \mu\text{M}$ (Cd, Y, Pb, and the lanthanides). It is striking that many of the metal cations that block Ca influx through the fast channels in synaptosomes also block voltage- and Ca-dependent transmitter release at the neuromuscular junction (see Ginsborg and Jenkinson, 1979). For some of these ions (e.g., Mg: Jenkinson, 1957; Co: Weakly, 1973; Pb: Manalis and Cooper, 1973; Cd: Satoh et al., 1982), the block of release can be relieved by increasing the external Ca concentration, which is consistent with an effect on the presynaptic Ca channels. Although it is not possible to compare directly the blocking efficacies of polyvalent cations in synaptosomes and at the neuromuscular junction, the general order of inhibitory potency appears similar: (a) Mg (Jenkinson, 1957), Sr (Meiri and Rahamimoff, 1971), and Ba (Silinsky, 1978) are weak antagonists of transmitter release, acting at millimolar concentrations; (b) Mn (Meiri and Rahamimoff, 1972), Co (Weakly, 1973), Ni, and Zn (Benoit and Mambriani, 1970) are more effective, acting at concentrations of 0.1–0.5 mM; (c) Y, Pb, Cd, and the lanthanides (Bowen, 1972; Toda, 1976; Satoh et al., 1982; Metral et al., 1978; Manalis and Cooper, 1973) are the most potent, acting at concentrations of ≤ 0.02 mM. Thus, the fast Ca channels in synaptosomes may be similar to the Ca channels that mediate transmitter release at the neuromuscular junction. This view is supported by

evidence that both K-stimulated Ca influx in synaptosomes and K- and Ca-dependent transmitter release at the neuromuscular junction are relatively insensitive to the organic Ca channel antagonists verapamil and D-600 (Nachshen and Blaustein, 1979b).

Evaluation of Ca Channel Inhibition by Polyvalent Cations

Competition between Ca and the inhibitory cations occurred at the site that is involved in the transport of the permeant ions Ca, Sr, Ba (Nachshen and Blaustein, 1982), and Mn (Drapeau and Nachshen, 1984). The inhibition constant, K_I , provides a measure of the inhibitory cation-binding site interaction. It is difficult to assign exact values to K_I , because the precise free ion concentration of some of the metals in solution (e.g., CdCl_2) is unknown. In addition, there is a slow Ca channel population that contributes to the flux measurements at 1 s: ions that preferentially interact with the fast channels (Mn, Cd, Y, and the lanthanides) will have their K_I 's overestimated, whereas ions that preferentially interact with the slow channels (Hg and Cu) will have their K_I 's underestimated. Nonetheless, a simple calculation, based on the apparent concentration dependence of block and on the fact that only about one-quarter to one-third of the influx at 1 s is mediated by the slow channels (Nachshen and Blaustein, 1980), indicates that the factor of error caused by a heterogeneous channel population cannot be larger than 2–3. There also may be surface charge or membrane potential effects that lead to overestimation of some K_I values, but the experimental data indicate that for most ions these effects are minimal. For Mg, the only ion that clearly affects the voltage dependence of Ca uptake (Table II), K_I is increased by a factor of 2 after correcting for surface charge effects (from ~3 to ~6 mM; Nachshen and Blaustein, 1982). For other ions, the correction factor is likely to be much smaller.

A Model for Inhibitory Cation Selectivity

The results of this study indicate that multivalent metal cations block Ca uptake by competing with Ca for a site at the Ca channel. To what extent can a simple coulombic model, based only on electrostatic interactions between the multivalent cations and a putative binding site, account for the observed inhibitory ion selectivity sequence? Models of this type have been successfully applied to explain the binding selectivity of glass electrodes, artificial ionophores, and biological membranes toward alkali metal and alkaline earth cations (Eisenman, 1969; Diamond and Wright, 1969).

Eisenman (1961) recognized that equilibrium selectivity among the group IA cations could be described as a balance between the energies of ion-water interaction (i.e., the energies of hydration) and the energies of ion-binding site interaction. For interactions of a coulombic nature, the primary variable controlling cationic selectivity is the field strength of the anion, represented in a coulomb model by the equivalent anionic radius, r_x . A similar theory has been developed to predict alkaline earth cation sequences (see review by Diamond and Wright, 1969). The simplest type of binding site, consisting of only a single monovalent or divalent cation, does not, however, give rise to the observed sequence of alkaline earth cation binding to the Ca channel, $\text{Ca} > \text{Ba} \geq \text{Sr} > \text{Mg}$,

at any value of r_x . In order to obtain the observed selectivity sequence, it is necessary to model the binding site as consisting of at least two anions, with a spacing of 1.45–2.25 Å between them (see Appendix and Table VI).

Another clue to the possible structure of the Ca channel binding site is provided by the relative inhibitory efficacies of the different lanthanide cations (Fig. 3). Lanthanide cations of diameters ranging from 2.04 to 2.32 Å are similar in efficacy, but there is a significant decrease in efficacy with lanthanide ions of a smaller diameter. This result might also be explained if the binding site consisted of two anions with a spacing of ~ 2.0 Å between them: small lanthanide ions would have less favorable coulombic interactions with the site than would larger lanthanide cations (see Appendix) because they would not be able to simultaneously contact both of the anions.

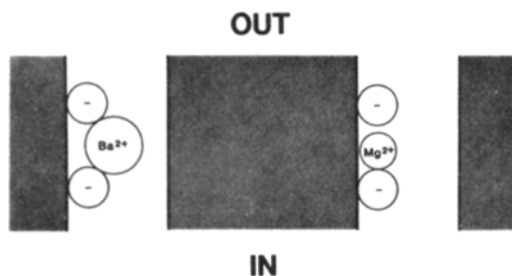


FIGURE 7. Hypothetical model of the Ca channel binding site in synaptosomes. The binding site is envisaged as consisting of two spherical anions, of ionic radius 1.0 Å, and of charge -1 , with a space between the anions (D) just large enough to accommodate cations of a diameter = 2.04 Å. Small cations (e.g., Mg^{2+} , ionic diameter = 1.44 Å) cannot contact both anionic groups (channel on right); larger cations (e.g., Ba^{2+} , ionic diameter = 2.70 Å) are in contact with both anionic groups (channel on left). The narrowest distance between the anions and the opposing channel wall can be no less than the diameter of the largest known permeant cation, i.e., Ba^{2+} . No assumptions are made about the distance of the binding site within the membrane (represented by the shaded gray areas).

A hypothetical model of the Ca channel binding site that incorporates these features is shown in Fig. 7. The binding site is modeled as two spherical anions, each with a charge of -1 . The spacing between the anions, D , is 2.04 Å. This arrangement gives the experimentally observed sequence of inhibition for alkaline earth and lanthanide cations (Fig. 8, *A* and *B*, broken lines). Although the negative sites are represented as fully charged spheres (e.g., carboxylic oxygens), it should be stressed that a similar treatment can be made for a model with negative centers that are dipoles (Eisenman, 1969).

The model predicts the selectivity sequence for divalent, nonalkaline earth cations as well (Fig. 8*C*, broken line). The predicted sequence (see Appendix), $Pb > Cd > Mn > Zn, Co, Ni, Cu$, is similar to the selectivity sequence for block of the fast Ca channel ($Pb > Cd > Mn, Zn, Co, Ni, Cu$; Table V), except for the

position of Mn. A discrepancy of this nature is not surprising, because the model neglects all noncoulombic interactions, which are particularly significant for the transition and heavy metals. Indeed, the substantial success of the model in predicting the selectivity sequence of the Ca channel for these metals suggests that purely coulombic interactions are a primary determinant of inhibitory ion selectivity.

The model also predicts $\text{Ca}^{2+}/\text{Na}^{+}$ selectivity, as expected for closely spaced anion sites (see Diamond and Wright, 1969). Although there is no estimate of K_1 available for Na, this prediction is consistent with the observation that the permeability for Na relative to Ca at the fast Ca channel is small (Krueger and Nachshen, 1980; also see below). Another prediction, that the binding site should have a high affinity for H ions, has been experimentally verified: the fast channel is blocked in a competitive manner as the external pH is reduced to values of ≤ 6 (Nachshen and Blaustein, 1979a).

In considering selectivity among different chemical subgroups (e.g., lanthanide ions vs. alkaline earth cations, or nonalkaline earth vs. alkaline earth divalent cations), noncoulombic interactions must clearly be taken into account. In general, transition metals and heavy metals have "soft" outer electron shells, electron orbital asymmetry, and the ability to polarize donor groups at the binding site. These factors could increase the energies of interaction for transition metals, as compared with alkaline earth cations of a similar radius. For the lanthanides and Y, in addition, the effect of an extra positive charge must be considered; trivalent ions may concentrate at the mouth of the channel or in the channel because of negative surface charge and voltage gradients across the membrane.

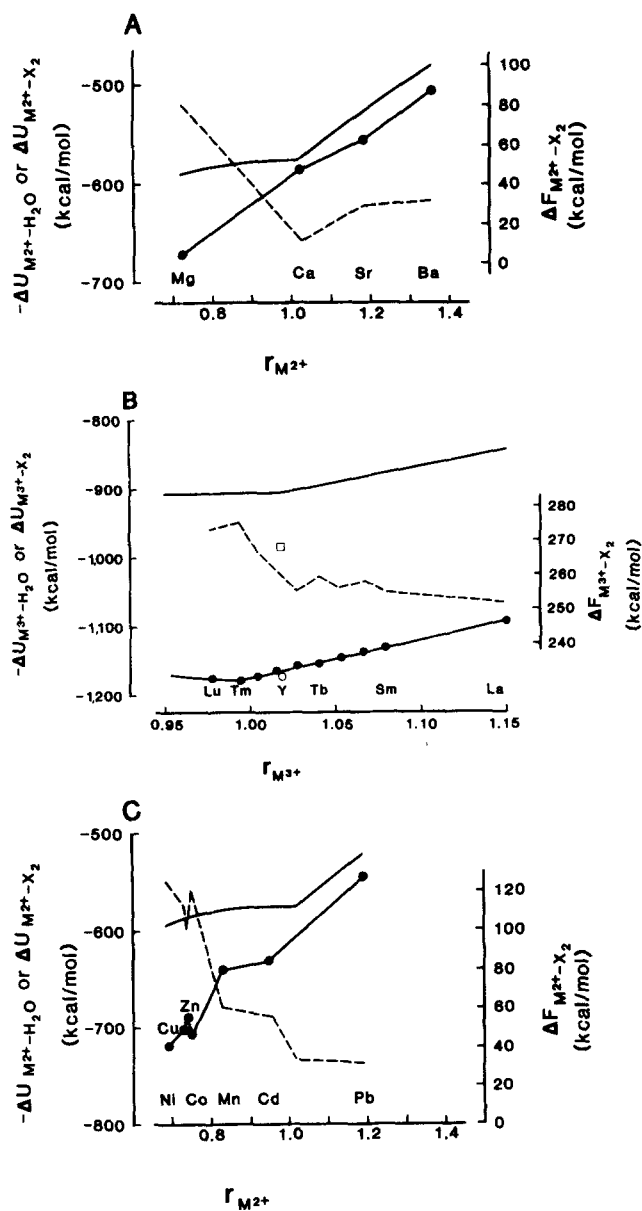
The model explains, however, why all potent competitive Ca channel blockers, both divalent (Cd, Pb) and trivalent (the lanthanides and Y), have ionic radii close to or larger than the ionic radius of Ca. Ions with a smaller radius are not able to coordinate effectively with both of the donor groups at the binding site. Some metal ion chelators, such as EGTA, may achieve Ca/Mg selectivity in a similar manner (Tsien, 1980).

The model shown in Fig. 7 is sequential: cations bind first to one anion and then to the other as they enter the channel. An alternative, equally plausible, model can be constructed with two anions near each other, at the same depth within the channel. If the binding is sequential, however, the selectivity sequences may be voltage dependent. At extreme voltages, cation binding to one anion will be facilitated, thereby increasing the distance between the cation and the second anion. This is equivalent to increasing the interanion spacing (D). The lanthanide cation selectivity sequence should be particularly sensitive to this effect, and it would be interesting to test this model by measuring lanthanide ion efficacies at several levels of synaptosome membrane depolarization.

Ca Channel Heterogeneity

Polyvalent cations inhibit Ca influx through Ca channels in almost all preparations (for reviews see Hagiwara and Byerly, 1981; Edwards, 1982). It is notable, however, that the inhibitory sequences differ from one type of cell to another. In barnacle muscle, for example, $\text{La} > \text{Co} > \text{Ni}$ (Hagiwara and Takahashi, 1967),

whereas in *Helix* neuron, $Ni > La > Co$ (Akaike et al., 1978). The calculations presented in the Appendix explicitly show that small shifts ($<0.5 \text{ \AA}$) in the spacing between two negative charges can profoundly alter polyvalent cation selectivity sequences (Table VI) and hence binding site occupancy. Slight shifts of this nature may underlie variations in polyvalent cation inhibitory sequences in different Ca channels.



Ca Channel Permeability

Is there any relationship between the strength of an ion's interaction with the binding site and the rate of its passage through the fast Ca channel? The mechanism for the transport of Ca, Sr, Ba (Nachshen and Blaustein, 1982), and Mn (Drapeau and Nachshen, 1984) involves ion binding to the Ca channel sites, so that influx saturates when all of the sites are occupied; for Mn, Sr, and Ba, half-saturation occurs at an ion concentration, K_M , that is equal to the half-inhibition constant for block of Ca influx, K_I . The permeability of these ions (P_M) relative to that of Ca (P_{Ca}) is given by the equation (Nachshen and Blaustein, 1982):

$$P_M/P_{Ca} = [J_{\max(M)} \times K_{M(Ca)}] / [J_{\max(Ca)} \times K_{M(M)}], \quad (3)$$

where J_{\max} is the maximal rate of ion influx, which occurs at high ion concentrations, when all of the binding sites are occupied. Sr and Ba, which are weak channel blockers, have maximal flux rates that are much larger than the maximal flux rate of Ca (five- and sixfold, respectively; Nachshen and Blaustein, 1982), while the intermediate channel blocker Mn has a maximal flux rate that is 1/10 as large as that of Ca (Drapeau and Nachshen, 1984). Since voltage-dependent Ce influx could not be detected, its maximal flux rate is unknown. However, assuming that the upper limit for Ce/Ca permeability is 1:4 (see Results above) and that the mechanism for Ce transport is the same as the mechanism for the transport of Sr, Ba, and Mn [i.e., $K_{M(Ce)} = K_{I(Ce)} = I_{50(Ce)}$], a value for the upper limit of $J_{\max(Ce)}$ can be obtained by rearrangement of Eq. 3 [$J_{\max(Ca)} = 2.7$ nmol/mg·s and $K_{M(Ca)} = 0.3$ mM; Nachshen and Blaustein, 1982], 0.5 pmol/mg·s. This value is $<1/1,000$ the J_{\max} value for Ca. Thus, there may be a relation between K_M and J_{\max} ; the same factors that favor the strong interaction of polyvalent cations with donor groups at the channel binding site may also limit the rate of ion passage through the channel after binding has taken place. At the same time, Eq. 3 suggests that ions with large K_M values (Mg, and perhaps Na) have low relative permeabilities. Presumably, the occupancy of the channel by these ions, relative to the occupancy by Ca, is very low.

FIGURE 8. (*opposite*) Free energies of dehydration ($-\Delta U_{M^z-H_2O}$; ●), binding ($\Delta U_{M^z-X_2}$; —), and exchange ($\Delta F_{M^z-X_2}$; ---) for the reaction $M^z(H_2O)_n + 2X^- = M^{z-2}X_2 + nH_2O$, as a function of cation radius (in angstroms). z is the valence of the cation M, and n is the number of water molecules that must be removed to dehydrate M^z . The circles (left to right) indicate experimental hydration energies for the following cations. (A) Mg^{2+} , Ca^{2+} , Sr^{2+} , Ba^{2+} (hydration energy values from Marcus and Kertes, 1969). (B) Lu^{3+} , Tm^{3+} , Er^{3+} , Ho^{3+} , Y^{3+} (open circle), Dy^{3+} , Tb^{3+} , Gd^{3+} , Eu^{3+} , Sm^{3+} , and La^{3+} . The hydration energies of La^{3+} and Y^{3+} are from Marcus and Kertes (1969), Table IB1. Hydration energies for the other lanthanide cations have been calculated from heats of sublimation, ionization potentials, and standard energies of formation compiled by Dean (1979). $\Delta F_{M^{3+}-X_2}$ for Y^{3+} is indicated by the open square. (C) Ni^{2+} , Cu^{2+} , Zn^{2+} , Co^{2+} , Mn^{2+} , Cd^{2+} , Pb^{2+} (hydration energy values are from Marcus and Kertes [1969], Table IB1). All ionic radius values are from Shannon (1976).

APPENDIX

A sufficient condition for binding site selectivity among cations is for the interaction energy between the site and the cation to have a different dependence on cation radius than the interaction energy of the cation with water (Eisenman, 1961). The particular selectivity sequence that arises at the site depends on its field strength, which, in a coulombic model, can be represented by its effective anion radius (r_x).

For monovalent cations, the distance between the anions (D) has no effect on the number of selectivity sequences that arise as r_x is varied (Eisenman, 1961). It has not, however, been recognized that the effect of intersite spacing on divalent cation selectivity sequences is critical. If D is small, so that the divalent cation contacts two anions, the internal energy (kilocalories per mole) of divalent cation (M^{2+})–anion X interaction, $\Delta U_{M^{2+}-X_2}$, can be calculated from Coulomb's law as:

$$\Delta U_{M^{2+}-X_2} = \frac{-4(332)}{r_{M^{2+}} + r_x} + \frac{332}{D + 2r_x}, \quad (4)$$

where $r_{M^{2+}}$ and r_x are the ionic radii of cation and anion, respectively. If, however, D is large, so that the divalent cation is in direct contact with only one of the anions (see Truesdell and Christ, 1967, Eqs. 11–29), the appropriate equation is:

$$\Delta U_{M^{2+}-X_2} = \frac{-2(332)}{r_{M^{2+}} + r_x} - \frac{2(332)}{D + r_x - r_{M^{2+}}} + \frac{332}{D + 2r_x}. \quad (5)$$

The selectivity of an anionic site for two cations depends on the differences in strength of coulombic interaction between the cations and the site (derived from Eqs. 4 and 5) and between the cations and water. Thus, the selectivity of the anion pair for M^{2+} relative to Ca^{2+} , $\Delta F_{M^{2+}-Ca^{2+}}$, given by the free energy (kilocalories per mole) for the exchange $CaX_2 + M^{2+} = MX_2 + Ca^{2+}$, is:

$$\Delta F_{M^{2+}-Ca^{2+}} = (\Delta U_{M^{2+}-H_2O} - \Delta U_{M^{2+}-X}) - (\Delta U_{Ca^{2+}-H_2O} - \Delta U_{Ca^{2+}-X}), \quad (6)$$

where $\Delta U_{M^{2+}-H_2O}$ and $\Delta U_{Ca^{2+}-H_2O}$ are the free energies of hydration (Marcus and Kertes, 1969) for M^{2+} and Ca^{2+} , respectively. Fig. 9 shows plots of $F_{M^{2+}-Ca^{2+}}$ vs. r_x , the anion radius, for the alkaline earth cations, at three different values of D (angstroms): A, 1.4; B, 2.04; C, 2.26. The different series of selectivity sequences (I–VII) that are derived from these plots are listed in Table VI (columns A–C). This table also shows (column D) the sequences that are predicted when the binding site consists of two anions with an infinite space between them or with no space between them (i.e., a divalent anion). Only sequences I and VII are retained for all values of D . Thus, the predicted selectivity sequences for the alkaline earth cations depend both on anion radius and interanion spacing.

Inspection of Table VI also shows that the Ca channel selectivity sequence, $Ca > Ba \geq Sr > Mg$, is not expected at a binding site that consists of either one monovalent or divalent anion (column D). Also, the observed selectivity sequence can only be expected to occur when D lies between 1.45 and 2.25 Å.

Fig. 8A shows a plot of $\Delta U_{M^{2+}-X_2}$, the internal energy of cation–binding site interaction, vs. $r_{M^{2+}}$ for divalent cations, derived from Eqs. 4 and 5. Also shown is a plot of $-\Delta U_{M^{2+}-H_2O}$ minus hydration energy vs. $r_{M^{2+}}$ for the alkaline earth cations. The difference curve, $\Delta F_{M^{2+}-X_2} = \Delta U_{M^{2+}-H_2O} - \Delta U_{M^{2+}-X_2}$, shown in the same figure, indicates binding selectivity: greater selectivity for M^{2+} corresponds to smaller values for $\Delta F_{M^{2+}-X_2}$, as defined in Eq. 6. A similar set of calculations for the trivalent cations is plotted in Fig. 8B. Inspection of Fig. 8B makes it clear that small changes in D substantially alter the predicted lanthanide selectivity sequence: if $D > 2.28$ Å (the diameter of Ce^{3+}), there will

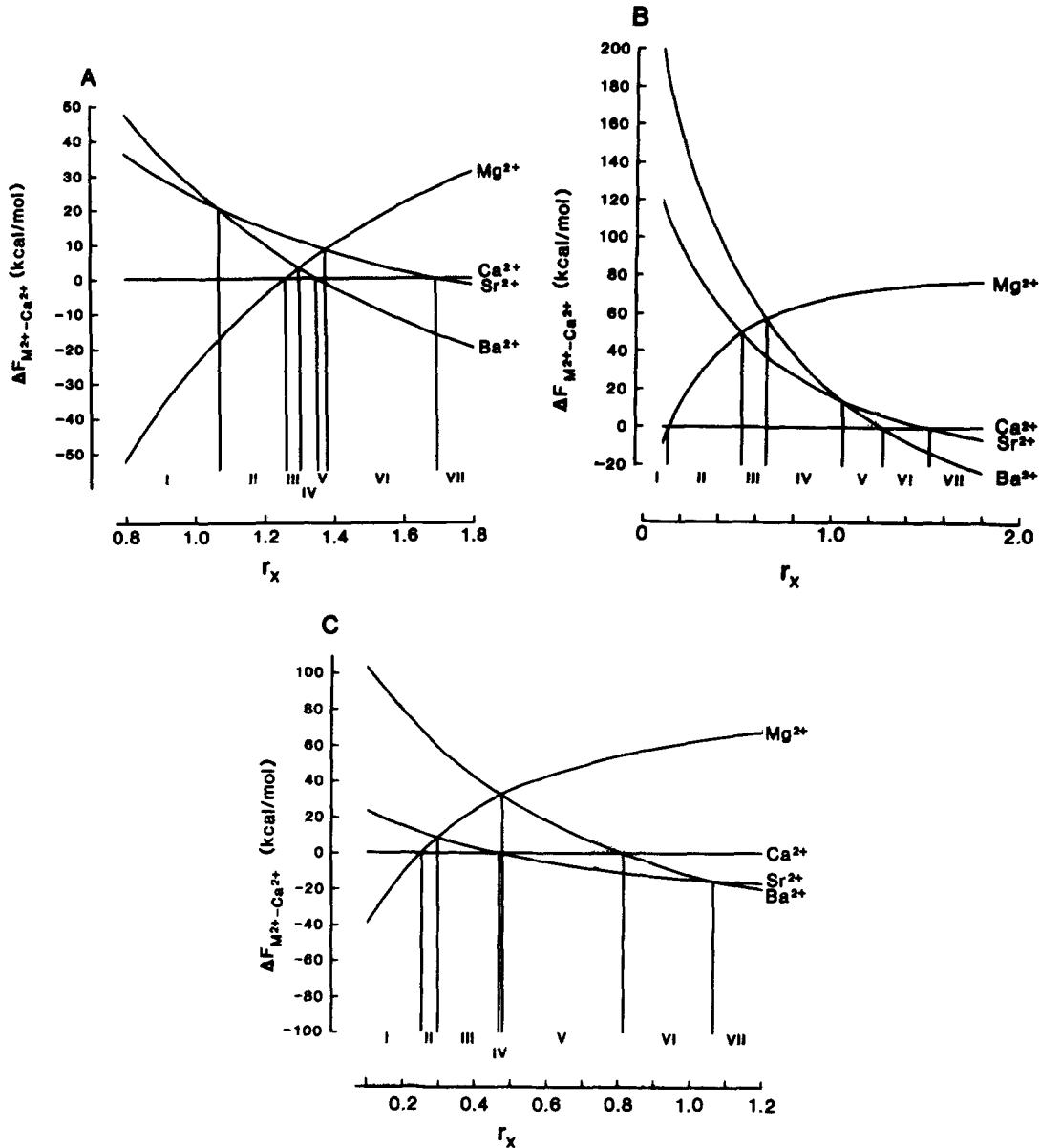


FIGURE 9. Free energies of reaction, $\Delta F_{M^{2+}-Ca^{2+}}$, for the exchange $CaX_2 + M^{2+} = MX_2 + Ca^{2+}$, as a function of ionic radius, r_x (in angstroms), of the anion. The free energies were calculated according to Eqs. 4-6, using the following values (kilocalories per mole) for $U_{M^{2+}-H_2O}$ (Marcus and Kertes, 1969, Table IB1): Mg^{2+} , 669.6; Ca^{2+} , 586.7; Sr^{2+} , 555.8; Ba^{2+} , 514.1. (A) $D = 1.4 \text{ \AA}$; (B) $D = 2.04 \text{ \AA}$; (C) $D = 2.26 \text{ \AA}$. The roman numerals beneath each set of isotherms denote selectivity sequences (I-VII) for the different ranges of r_x that are demarcated by the vertical lines. The ionic radius values (shown in Table V) used in the calculations are taken from Shannon (1976).

be a shallow and monotonic decrease in binding with decreasing ionic radius; if $D < 1.95$ Å (the diameter of Lu^{3+}), there will be a steep and monotonic decrease. A nonmonotonic decrease will only be obtained at intermediate values of D , and the value of D determines the point of inflection. Similar sets of plots for the divalent, nonalkaline earth cations are presented in Fig. 8C (Hg^{2+} has been excluded, because HgCl_2 undergoes negligible dissociation in aqueous solution).

It may be noted that the energies of exchange shown in Fig. 8 are far greater than those extracted from the experimental data; $\Delta F_{M^{2+}-Ca^{2+}}$ should be equal to $\ln(K_1/K_{Ca})$. The magnitude (but not the order) of selectivity depends, however, on the degree of binding site hydration (Eisenman, 1961, 1975), and cation-binding site interactions may occur in a partially hydrated milieu. Indeed, the necessity for a partially hydrated milieu is also indicated by the magnitude of the electrostatic repulsion that the two anions would experience in a medium of low dielectric strength. In a medium with a dielectric constant

TABLE VI
Selectivity Sequence Predictions for Alkaline Earth Cation Binding

	(A) $D = 1.4$ Å	(B) $D = 2.04$ Å	(C) $D = 2.26$ Å	(D) $D \rightarrow 0,$ $D \rightarrow \infty$
(I)	Mg>Ca>Sr>Ba	Mg>Ca>Sr>Ba	Mg>Ca>Sr>Ba	Mg>Ca>Sr>Ba
(II)	Mg>Ca>Ba>Sr	Ca>Mg>Sr>Ba	Ca>Mg>Sr>Ba	Ca>Mg>Sr>Ba
(III)	Ca>Mg>Ba>Sr	Ca>Sr>Mg>Ba	Ca>Sr>Mg>Ba	Ca>Mg>Ba>Sr
(IV)	Ca>Ba>Mg>Sr	Ca>Sr>Ba>Mg	Sr>Ca>Mg>Ba	Ca>Ba>Mg>Sr
(V)	Ba>Ca>Mg>Sr	Ca>Ba>Sr>Mg	Sr>Ca>Ba>Mg	Ba>Ca>Mg>Sr
(VI)	Ba>Ca>Sr>Mg	Ba>Ca>Sr>Mg	Sr>Ba>Ca>Mg	Ba>Ca>Sr>Mg
(VII)	Ba>Sr>Ca>Mg	Ba>Sr>Ca>Mg	Ba>Sr>Ca>Mg	Ba>Sr>Ca>Mg

The table shows selectivity sequences predicted for alkaline earth cation binding to two centers of negative charge, with a space between the charges large enough to accommodate cations of a diameter $< D$. The different sets of sequences (A–C) correspond to the three sets of isotherms (A–C) shown in Fig. 7. Sequences I–VII arise with increasing values of anion radius. Only sequences I and VII are constant for all values of D . Column D: the set of sequences arising for two anions at infinite or zero spacing corresponds to the set of sequences calculated by Diamond and Wright (1969).

of ≤ 12 , the potential at a distance of 2–3 Å from a negative charge would be > 500 mV, an unreasonably high value. The potential would only be ~ 60 mV, however, in a medium with a dielectric constant of 80, which is close to that of water.

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