

# Influx of Calcium, Strontium, and Barium in Presynaptic Nerve Endings

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**ABSTRACT** Depolarization-induced (potassium-stimulated) influx of  $^{45}\text{Ca}$ ,  $^{85}\text{Sr}$ , and  $^{133}\text{Ba}$  was measured in synaptosomes prepared from rat brain. There are two phases of divalent cation entry, "fast" and "slow;" each phase is mediated by channels with distinctive characteristics. The fast channels inactivate (within 1 s) and are blocked by low concentrations ( $<1\ \mu\text{M}$ ) of La. The slow channels do not inactivate (within 10 s), and are blocked by high concentrations ( $>50\ \mu\text{M}$ ) of La. Divalent cation influx through both channels saturates with increasing concentrations of permeant divalent cation; in addition, each permeant divalent cation species competitively blocks the influx of other permeant species. These results are consistent with the presence of "binding sites" for divalent cations in the fast and slow channels. The Ca: Sr: Ba permeability ratio, determined by measuring the influx of all three species in triple-label experiments, was 6:3:2 for the fast channel and 6:3:1 for the slow channel. A simple model for ion selectivity, based on the presence of a binding site in the channel, could account well for slow and, to some extent, for fast, channel selectivity data.

## INTRODUCTION

When presynaptic nerve terminals are depolarized, there is an influx of Ca via voltage-regulated channels that are selective for divalent cations. The rise in cytoplasmic free Ca, caused by Ca entry, triggers the release of neurotransmitter (for reviews, see Katz, 1969, and Llinas, 1977). Sr, and to some extent Ba, like Ca, promotes the voltage-dependent release of transmitter from nerve terminals (Miledi, 1966; Dodge et al., 1969; McLachlan, 1977; Silinsky, 1978). This is consistent with the observation that Ca channels in many types of cells are permeable to Sr and Ba, as well as Ca ions (for reviews, see Reuter, 1973; Hagiwara, 1975; Hagiwara and Byerly, 1981).

Although Sr and Ba fluxes are "nonphysiological," a study of the movements of these ions is of interest because it may cast light on the mechanisms regulating Ca entry. There is direct evidence for the voltage-regulated entry of Ca, Sr, and Ba at the squid giant synapse (Katz and Miledi, 1969). However, little is known about the mechanism of divalent cation influx in

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vertebrate presynaptic nerve terminals because the small size of the terminals precludes direct electrical measurement of ionic flux. We have, therefore, characterized the influx of Ca, Sr, and Ba in a preparation of pinched-off presynaptic nerve endings (synaptosomes) by measuring radioisotope movements.

Synaptosomes retain many of the morphological and functional properties of intact neuronal tissue (Bradford, 1975; Blaustein et al., 1977). In particular, they are able to regulate Ca influx (Nachshen and Blaustein, 1980) and release transmitter in a Ca-dependent manner (Blaustein, 1975). The Ca entry in synaptosomes is, at least in part, voltage sensitive (Blaustein, 1975). We have identified two distinct phases of voltage-dependent Ca influx in synaptosomes isolated from rat brain (Nachshen and Blaustein, 1980). (a) There is a "fast" phase of Ca entry lasting ~1 s, which is abolished by prior depolarization and blocked by low (<1  $\mu\text{M}$ ) concentrations of La. (b) A second "slow" phase of Ca entry continues even after the synaptosomes are depolarized, for as long as 1 min; this phase is blocked by high (~0.1 mM) concentrations of La. It seems likely that the fast and slow phases of voltage-dependent Ca entry are mediated by separate channels that contribute to fast and slow phases of neurotransmitter release from presynaptic nerve endings (Drapeau and Blaustein, 1981).

In the present study we show that the fast and slow Ca channels are also able to regulate the entry of Sr and Ba. We describe the selectivity properties of these two pathways for the alkaline earth cations Ca, Sr, and Ba. A preliminary report of these findings has been published (Nachshen, 1981).

## METHODS

### *Preparation of Synaptosomes*

The preparation of synaptosomes from rat brains has been described in detail elsewhere (Krueger et al., 1979). In brief, a modification of the method of Hajos (1975) was used. 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 low-K solution containing: 145 mM NaCl; 5 mM KCl; 1 mM  $\text{MgCl}_2$ ; 0.02 mM  $\text{CaCl}_2$ ; 10 mM glucose; 10 mM Hepes, adjusted to pH 7.5 at 3°C with Tris. The diluted synaptosome suspension was centrifuged (13,000  $g$ ) for 10 min, and the pellet was resuspended in a low-K solution, pH adjusted to 7.5 at 30°C. The resuspended synaptosomes were gently agitated and warmed for 20 min at 30°C before proceeding with the experiments.

### *Measurement of Radioisotope Entry*

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  $\approx 400 \mu\text{l}$ ) contained 0.5-1  $\mu\text{Ci}$  of  $^{45}\text{Ca}$ ,  $^{85}\text{Sr}$ , or  $^{133}\text{Ba}$  (New England Nuclear, Boston, MA). The K concentration in the K-rich solution was increased by isosmotically substituting K for Na. In some experiments, low-K solutions with a reduced Na concentration were used and contained: 72.5 mM NaCl; 72.5 mM choline Cl; 5 mM KCl. In the corresponding K-rich solutions with reduced Na, the K concentration was increased by isosmotically

substituting K for choline. Divalent ion concentrations for the specific experiments are given in the Results section. In addition, all solutions contained glucose and pH buffers as indicated above.

Radioisotope entry was terminated by rapidly diluting the incubation media with 4.5 ml of ice-cold low-K solution containing 10 mM CaCl<sub>2</sub>, SrCl<sub>2</sub>, or BaCl<sub>2</sub>, or 1 mM LaCl<sub>3</sub>. No significant difference in the results was obtained with the various wash solutions. After quenching, the diluted suspensions were filtered on glass fiber filters (GF/A or GF/C; Whatman, Inc., Clifton, NJ), and the radioactivity retained on the filters was determined. Protein was measured by the method of Lowry et al. (1951).

#### *Fluorescence Measurements*

The voltage-sensitive fluorescent dye 3,3'-dipentyl 2,2'-oxacarbocyanine [diO-C<sub>5</sub>(3)] was used to follow changes in membrane potential (Blaustein and Goldring, 1975). Aliquots of synaptosome suspension containing ~0.5 mg protein in 50–100 μl of low-K solution were added to 2 ml of low-K solution with normal or reduced Na (see above), or K-rich solution. These solutions also contained diO-C<sub>5</sub>(3) (2.5 μM) and various amounts of divalent cation as specified in the text for the different experiments. The synaptosome suspension was then illuminated with light at a wavelength of 448 nm, and fluorescent emission was measured at 511 nm.

## RESULTS

### *Effects of Depolarization by Potassium on Sr and Ba Influx*

When synaptosomes are incubated in a low-K solution (see Methods) containing Ba, or Sr, there is a time-dependent influx of these ions (Fig. 1, ○). Because synaptosomes are permeable to K<sup>+</sup> and have a membrane potential ( $\cong -60$  mV; Blaustein and Goldring, 1975; Ramos et al., 1979) that is determined primarily by their K conductance, they can be depolarized by incubation in a K-rich solution (Blaustein and Goldring, 1975). Fig. 1 (●) shows that this depolarization greatly increases the influx of Sr and Ba, as well as the influx of Ca, as previously reported (Blaustein, 1975; Nachshen and Blaustein, 1980).

### *Effect of Na Replacement on the Influx of Sr and Ba*

The accumulation of Ca by synaptosomes is also increased when they are placed in a low-Na medium. This stimulated accumulation may involve a Na/Ca or Ca/Ca exchanger (Blaustein and Oborn, 1975). In solutions with half of the Na replaced by K, at least 20% of the K-stimulated Ca influx can be attributed to Na depletion, as opposed to depolarization per se, because this is the magnitude of the influx observed when half of the Na is replaced by an inert cation such as choline (Fig. 2; and see Nachshen and Blaustein, 1980). It was therefore important to determine whether a significant fraction of K-stimulated Sr and Ba influx might also be linked to Na removal.

Sr and Ba influx was measured in control low-K solution (145 mM Na and 5 mM K), in low-K solution with reduced Na (72.5 mM Na, 72.5 mM choline, and 5 mM K), and in K-rich solution (72.5 mM Na and 77.5 mM K). Replacing Na with choline did not increase the influx of either Sr or Ba (Fig. 2), at either 1 s (not shown) or 10 s, with either high (>6 mM) or low (0.1 mM, not shown) divalent cation concentrations. Although no Na-dependent

Sr or Ba influx could be measured, in most subsequent experiments involving Ca, the Na concentration was kept constant by using low-K solutions with reduced Na (see Methods). This was done to facilitate comparison among K-stimulated Sr, Ba, and Ca fluxes. Experiments (not shown) with the voltage-

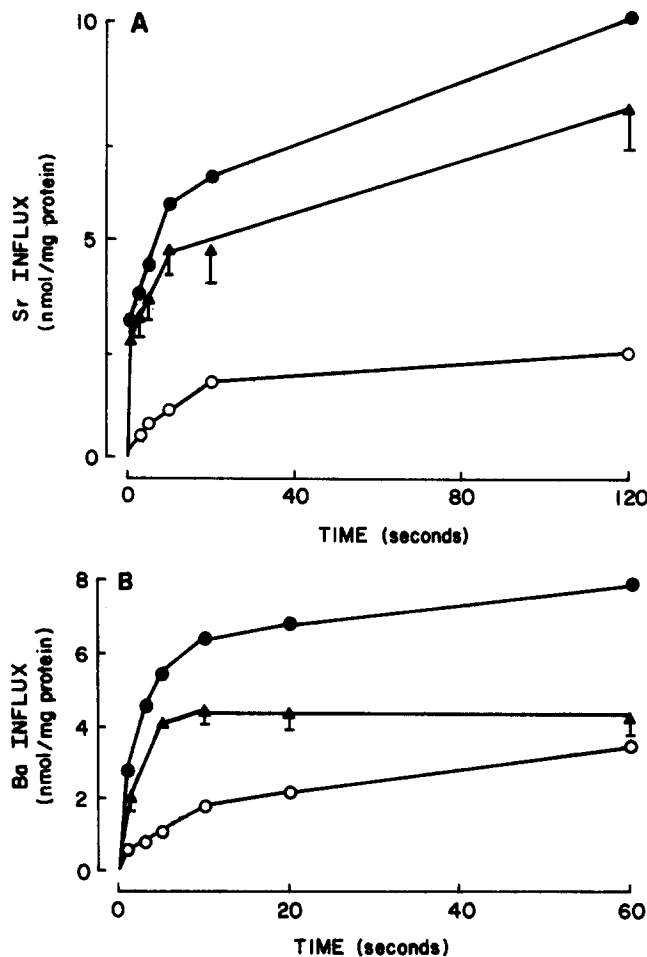


FIGURE 1. A. Time course of Sr influx. Incubation was in a low-K (○) or K-rich (●) solution. K-stimulated influx, the difference between uptake in K-rich and low-K solution, is also plotted (▲). All incubation solutions contained 2.4 mM Sr; 0.02 mM Ca; 1.0 mM Mg. B. Time course of Ba influx. Influx values are plotted as in A. The solutions had the same ion composition as above, except that Ba was substituted for Sr.

sensitive fluorescent dye diO-C<sub>5</sub>(3) indicated that replacement of half the Na with choline did not depolarize the synaptosomes.

#### *Alkaline Earth Cations and K-induced Fluorescence*

We evaluated the effects of the alkaline earth cations Ca, Sr, Ba, and Mg on synaptosome membrane potential by measuring changes in fluorescence with

the voltage-sensitive fluorescent dye diO-C<sub>5</sub>(3) (Blaustein and Goldring, 1975). None of the alkaline earth cations (20 mM) had any effect on baseline dye fluorescence in the absence of synaptosomes (not shown). Data from four experiments with synaptosomes are shown in Fig 3. Only Ba, a divalent cation that blocks K channels (Werman and Grundfest, 1961; Hagiwara et al., 1974; Hermann and Gorman, 1979), consistently reduced K-induced fluorescence (by ~20%) at a concentration of 5 mM. Sr, Mg, and Ca, at concentrations of 5 mM, had no significant effect. At higher concentrations (10–20 mM), Ca decreased fluorescence by 10–20%, whereas Sr and Mg were still without significant effect on K-induced fluorescence. Ni (1 mM) was also without significant effect, as was NaCl, when its concentration was increased from 72.5

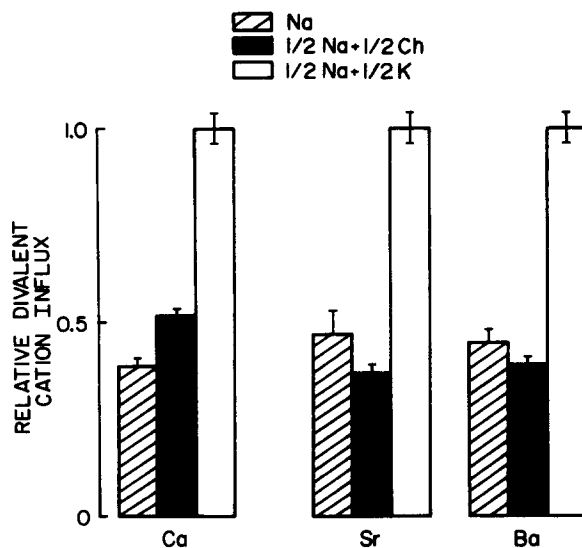


FIGURE 2. The effect of replacing Na with K or choline on the influx of Ca, Sr, and Ba. <sup>45</sup>Ca, <sup>85</sup>Sr, and <sup>133</sup>Ba fluxes are shown normalized to the influx in K-rich solution (open bar). The <sup>45</sup>Ca solutions contained 2 mM Ca; the <sup>85</sup>Sr solutions contained 0.02 mM Ca and 6 mM Sr; the <sup>133</sup>Ba solutions contained 0.02 mM Ca and 10 mM Ba. All solutions also contained 0.5 mM MgCl<sub>2</sub>. Incubation with tracer lasted 10 s.

to 102.5 mM in (hyperosmotic) K-rich solution (not shown). These observations indicate that in most of the experiments described below, changes in divalent cation concentration, or replacement of one divalent cation species by another, had little or no effect on K-induced fluorescence and membrane potential changes.

#### *Possible Electrostatic Effects of Alkaline Earth Cations*

Because we were investigating the effects of depolarization on the influx of divalent cations, it was important to evaluate the possible electrostatic interaction of these cations with negatively charged groups at the synaptosome membrane. Anionic groups produce a negative surface potential ( $\Delta\psi$ ) at the

boundaries of many biological membranes so that positive counterions are concentrated at the surface, whereas negative counterions are repelled. The negative surface potential also influences voltage-sensitive transport mechanisms in the membrane. Cations in solution, particularly divalent cations, electrostatically screen or "shield" the fixed negative charges, thereby reducing the surface potential and producing an apparent hyperpolarization of the membrane potential (Frankenhaeuser and Hodgkin, 1957). We therefore examined the effects of increasing divalent cation concentration on Ca influx,

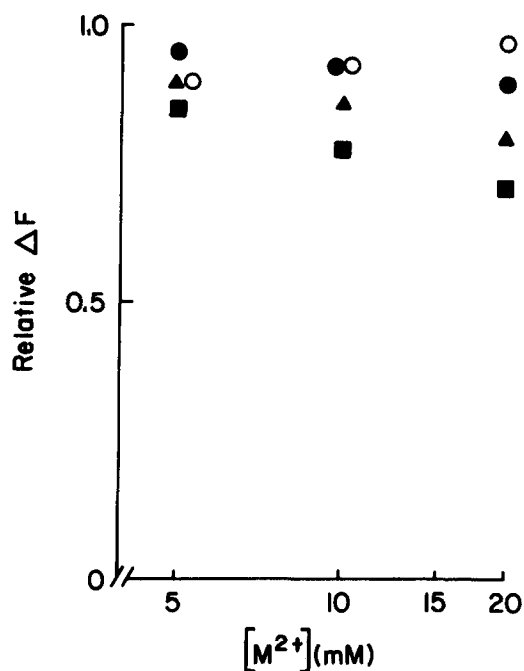


FIGURE 3. The effect of divalent cations on K-induced fluorescence. Fluorescence was measured in low K with reduced Na, or K-rich solutions (see Methods). The K-induced fluorescence change ( $\Delta F$ ) at different divalent cation concentrations is shown, normalized to  $\Delta F$  in control solution, containing 0.02 mM Ca and 0.5 mM Mg. Standard errors of the mean (not shown) ranged from 1–10%. ●, Mg; ▲, Ca; ○, Sr; ■, Ba.

at different levels of depolarization, established by using elevated concentrations of K.

The upper curve in Fig. 4A summarizes results from 17 experiments in which K was varied from 5 to 77.5 mM, at low divalent ion concentration (0.2 mM Ca and 0.5 mM Mg). K-stimulated Ca influx is also plotted as a function of membrane depolarization (upper abscissa calibration), determined in separate experiments, with the voltage-sensitive dye diO-C<sub>5</sub>(3). The absolute membrane potential in 5 mM K is not known, but has been estimated to be about -60 mV (Blaustein and Goldring, 1975). Ca influx is a nonlinear

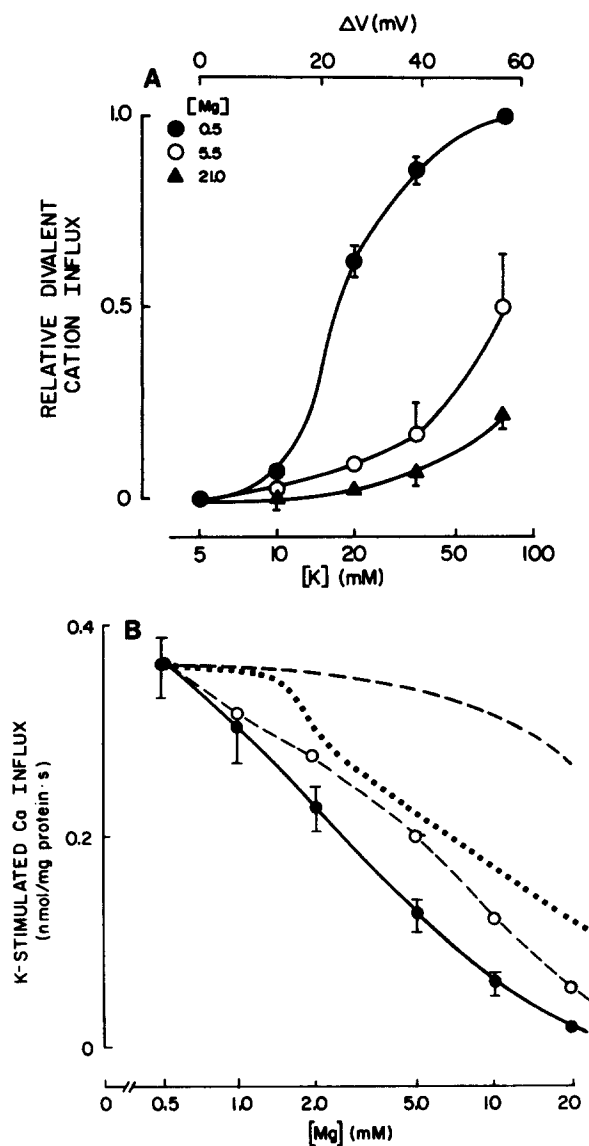


FIGURE 4. A. The effect of varying potassium concentration on K-stimulated Ca influx in the presence of 0.5 (●), 5.5 (○), and 21 (▲) mM Mg. All solutions contained 0.2 mM Ca. Incubations lasted 1 s. The upper abscissa shows the changes in membrane potential with increasing [K], determined in separate experiments with the voltage-sensitive dye diO-C<sub>5</sub>(3). B. The effect of increasing Mg concentrations on K-stimulated Ca influx. Isotonicity was maintained by adding sucrose (60 mM) to the 0.5 mM Mg solutions. Mg was then increased at the expense of sucrose. All solutions contained 0.02 mM Ca. The dashed line indicates the expected reduction in Ca influx, caused by electrostatic effects on the K dependence of uptake. The total inhibition due to screening is indicated by the dotted line (see text). The filled symbols are experimental values. The open symbols are these same values corrected for screening, according to the factor indicated by the dotted line. The solid line joining the data points has no theoretical significance.

function of  $\log K$  (and membrane potential): there is little increase in Ca influx when K is raised from 5 to 10 mM, but as K is raised to 35 mM, influx increases sharply, and at higher K concentrations it levels off.

Increasing the Mg ion concentration changed the shape of the curve relating Ca influx to K concentration. When Mg was raised to 5 mM (three experiments), the increase in Ca influx with increasing K was more gradual; in addition, no leveling off of the curve was observed. With 21 mM Mg (two experiments), the curve was even less steep, and a high K concentration (20–35 mM) was required to elicit a significant increase in Ca influx. These observations are consistent with a surface charge model: high concentrations of Mg produce an apparent hyperpolarization of the synaptosome membrane potential and shift the curve relating Ca influx to K (and voltage) to the right. When Mg was increased from 0.5 to 5 mM, the apparent voltage shift was at least 10–15 mV, and when Mg was increased from 0.5 to 21 mM, the apparent shift was at least 20 mV.

Taking the shift produced by Mg as an upper limit for electrostatic interaction (see below), it is possible to compute an upper limit for surface charge density ( $\sigma$ ) (McLaughlin et al., 1971). In five experiments  $\sigma$  was calculated to be 1 charge/76 Å<sup>2</sup> (range: 1/33–1/110). This value is very close to estimates of  $\sigma$  at the nerve terminal of the frog neuromuscular junction (1 charge/75 Å<sup>2</sup>, Madden and Van Der Kloot, 1978; 1 charge/154 Å<sup>2</sup>, Muller and Finkelstein, 1974).

Other divalent cations were also tested at various K concentrations. The addition of 5 mM Sr or Ba shifted the control Ca influx curves by  $\leq 5$  mV, whereas 10 mM Sr shifted the curves by 5–10 mV. An increase in Ca from 0.2 to 2 mM produced a consistent, but statistically insignificant, shift to the right. At higher concentrations of Ca, Sr, or Ba, reliable estimates of shift could not be made. The fact that not all the divalent cations are equivalent in shifting the K curve may indicate that there is binding of some of these ions to the fixed charges (e.g., Blaustein and Goldman, 1968; Hille et al., 1975). Another possibility is that some of the divalent cations modify the K dependence of Ca influx by actions unrelated to their electrostatic effects.

Screening of surface charge by Mg may underlie a substantial portion of this divalent cation's inhibitory effects on Ca influx (see Muller and Finkelstein, 1974). The inhibition due to screening was evaluated by using the Graham equation (see McLaughlin et al., 1971) to calculate  $\Delta\psi$  as a function of the external Mg concentration for a given surface charge density of 1 charge/76 Å<sup>2</sup>. A decrease in surface potential with increasing concentrations of Mg effectively hyperpolarizes the membrane. In a K-rich solution ( $K_0 = 77.5$  mM), the potential across the membrane is therefore diminished from a control value of  $\Delta V$  to a new value of  $\Delta V - \Delta\psi$ . Ca influx is accordingly reduced. The control curve in Fig. 4A (Mg = 0.5 mM) was used to estimate this reduction, shown by the upper dashed line in Fig. 4B. In addition, as the surface charges are screened, Ca ions are electrostatically repelled. The surface concentration of Ca is thereby diminished by a factor of  $\exp(\Delta\psi/29)$ . The dotted line in Fig. 4B shows how Ca influx is reduced by both of these



screening effects, as the external Mg ion concentration is increased. Fig. 4B also shows data from an experiment in which the K-stimulated Ca influx was measured at different concentrations of Mg (●). Half-maximal inhibition occurred at a concentration of ~3 mM. The open symbols are data points that have been corrected for the screening; the corrected curve presumably reflects specific block of the Ca channels by Mg. After the correction, half-maximal inhibition occurred at a concentration of ~6 mM. It is evident, therefore, that both screening of fixed negative charges and specific block of the Ca channel are important in determining divalent cation influx.

#### *Two Phases of K-stimulated Divalent Ion Influx*

The time course of K-stimulated (K-rich minus low-K) Sr or Ba influx, like the time course of K-stimulated Ca influx (Nachshen and Blaustein, 1980), is biphasic (Fig. 1, ▲). There is an initial "burst" of cation accumulation, lasting for 2–3 s. After this "fast" phase, K-stimulated entry proceeds at a much slower rate.

The finding that influx diminishes or levels off rapidly is open to several interpretations. One possibility is that isotopic equilibrium is approached after a relatively short time. Another possible explanation is that the channels mediating Sr or Ba influx during the fast phase are inactivated by depolarization. The latter explanation seems likely because we have previously shown that there are inactivating channels for Ca entry in synaptosomes (Nachshen and Blaustein, 1980). Ca influx through these inactivating channels can be abolished if the synaptosomes are incubated in a K-rich solution before the addition of radiotracer Ca ("predepolarized"). To test for inactivation, the synaptosomes were therefore depolarized before the addition of Ba or Sr. If net accumulation is limited *only* by tracer backflux, predepolarization should have no effect on the time course of K-stimulated influx after tracer is added. Fig. 5 shows, however, that predepolarization drastically reduced the initial rates of Sr and Ba influx. After predepolarization, the time course of K-stimulated Sr and Ba influx was approximately linear, and extrapolated to the origin, as shown by the lower curves in Fig. 5A and B. This suggests that the channels mediating the fast phase of Sr and Ba entry, like the channels mediating the fast phase of Ca entry, inactivate.

After the fast Ca channels inactivate, Ca influx occurs through noninactivating slow channels (Nachshen and Blaustein, 1980). The results in Fig. 5 suggest that Sr and Ba also move through these channels. The two phases of K-stimulated Ca, Sr, and Ba influx were therefore examined separately. The fast phase was studied by measuring influx after very brief (1-s) incubations with tracer. The slow phase was studied by preincubating the synaptosomes for 10 s in a high-K solution containing 1 mM Mg, 0.02 mM Ca, and no tracer, so that the fast channels would be inactivated. This was followed by a 5- or 10-s incubation with tracer and various concentrations of the divalent cation under study.

In earlier experiments we had found that La selectively inhibited the fast component of Ca influx (Nachshen and Blaustein, 1980). The effect of La on

the fast and slow phases of K-stimulated Ca, Sr, and Ba influx is shown in Fig. 6. As can be seen, 20  $\mu$ M La was far more effective as a blocker during the fast phase than during the slow phase of K-stimulated influx. These

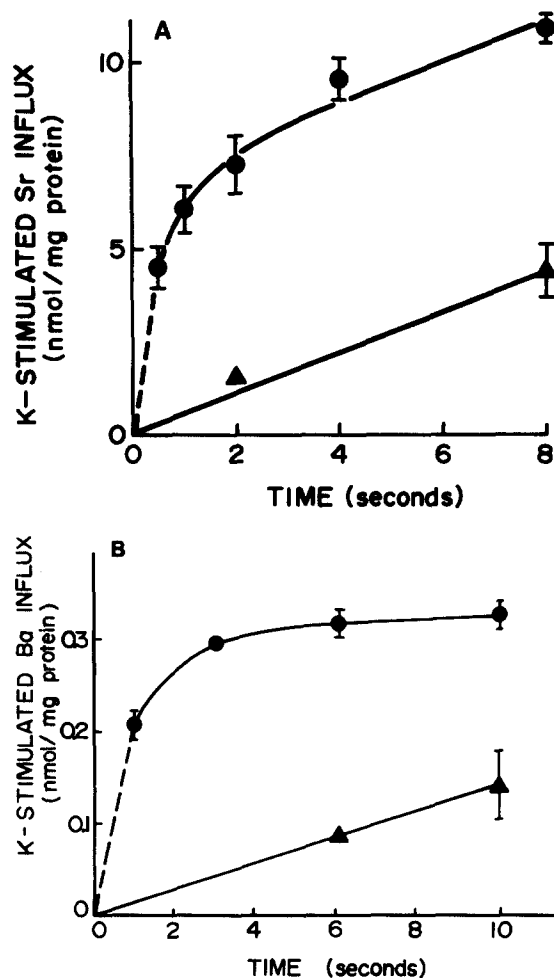


FIGURE 5. The effect of preincubation in K-rich solution on K-stimulated influx. Synaptosomes were preincubated for 10 s in a standard low-K (●) or K-rich (▲) solution, containing 0.02 mM Ca and 0.5 mM Mg. Test incubation with isotope was then initiated. During test incubation the solutions contained either 2 mM Sr (A), or 0.12 mM Ba (B). K-stimulated influx values were obtained by subtracting the flux obtained at low K from the flux obtained at high K.

findings support the idea that separate transport mechanisms mediate the fast and slow phases of K-stimulated Ca, Sr, and Ba influx.

The rate of Sr entry in prepolarized synaptosomes resembled the rate of Sr entry in control synaptosomes measured between 3 and 8 s (upper curve,

Fig. 5A). The rate of Ba entry in prepolarized synaptosomes was significantly *greater* than the rate of Ba entry in control synaptosomes measured after 3 s.

A possible explanation for this result is that Ba, but not Sr, rapidly approaches isotopic equilibrium (in a few seconds). This could happen if Sr, like Ca, were effectively sequestered by intraterminal organelles and Ba were only slowly sequestered. It is known that the mitochondria and the sarcoplasmic reticulum in many cell types take up Ca and Sr, but not Ba, at significant rates (see Lehninger, 1970; Nagai et al., 1965). There is, however, little information about the selectivity of intraterminal organelles for divalent

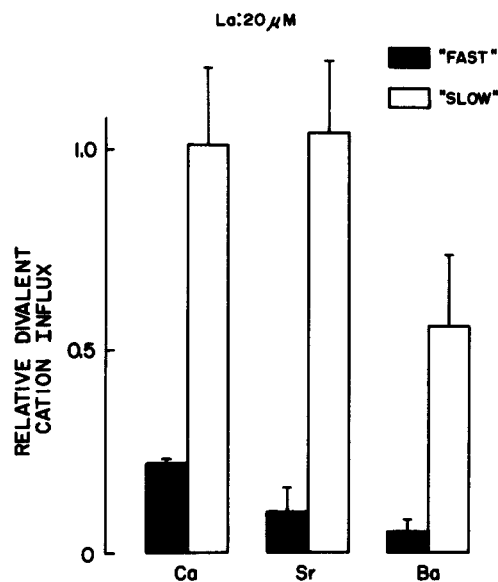


FIGURE 6. The effect of La ( $20 \mu\text{M}$ ) on the fast and slow phases of divalent cation influx. Influx measurements for the fast and slow phases were carried out as described in the text. The  $^{45}\text{Ca}$ -containing solutions were made up with 0.02 mM Ca and 0.05 mM Mg. The  $^{86}\text{Sr}$  and  $^{133}\text{Ba}$  solutions contained, in addition, 0.1 mM Sr and 0.1 mM Ba.

cations. Work along these lines is proceeding in our laboratories. Whatever the final explanation for the rapid leveling off of Ba influx in non-prepolarized synaptosomes, in prepolarized synaptosomes the K-stimulated influx of all three divalent cations was nearly linear for times up to 10 s. This means that measurements made in that time span are good approximations of initial rates of ion entry through the slow channel.

#### *Effect of Varying External Ca, Sr, and Ba on K-stimulated Divalent Ion Influx*

The effect of increasing Sr on K-stimulated Sr influx, during the fast and slow phases, is shown in Fig. 7 (●). Influx began to saturate with higher concentrations of Sr. Partial saturation was noticeable at Sr concentrations that had

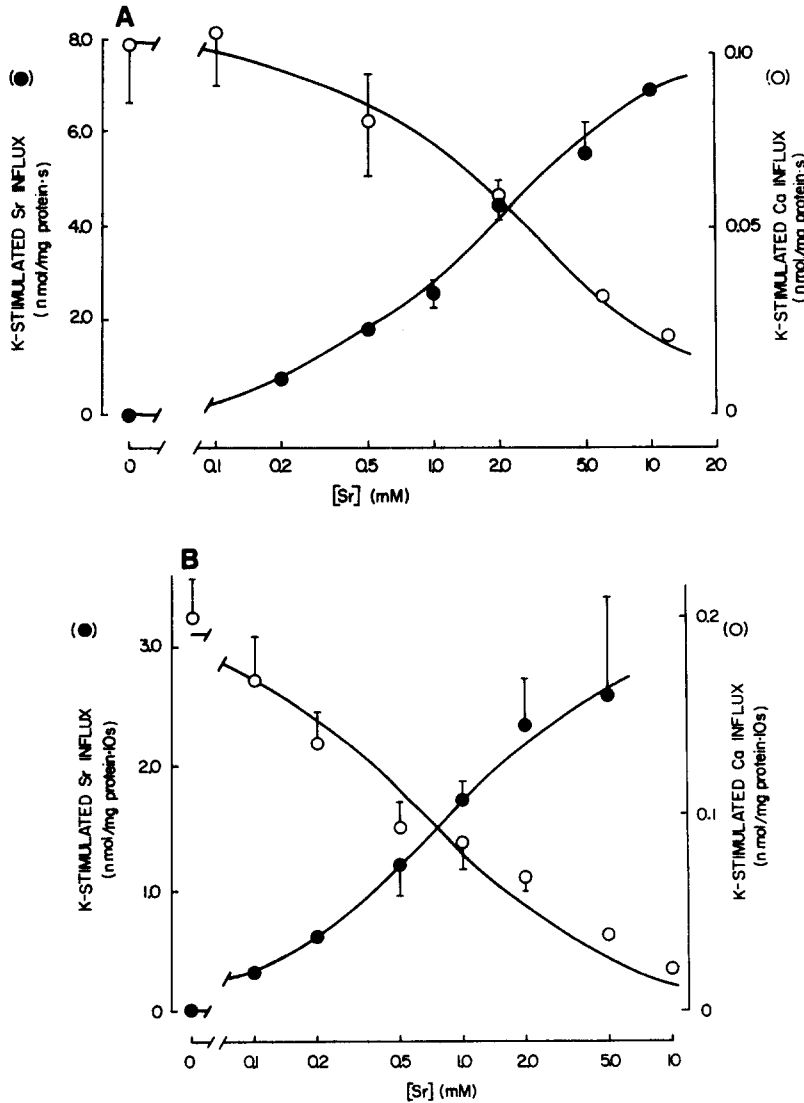


FIGURE 7. The effect of increasing Sr concentration on K-stimulated  $^{86}\text{Sr}$  (●) or  $^{45}\text{Ca}$  (○) influx. All solutions contained 0.02 mM and 0.5 mM Mg. The data points in this figure (and Fig. 8) were fit by the method of least squares, using Eq. 1 (solid symbols), or Eq. 2 (open symbols). A. Incubation lasted 1 s.  $K_{m(\text{Sr})}$  was calculated as 1.9 mM, and  $K_{I(\text{Sr})}$  was calculated as 2.6 mM before correcting for screening. After correcting, the data were replotted, and  $K_m$ ,  $K_I$ , and  $J_{\text{max}}$  values were recalculated (see text). After correction,  $K_{m(\text{Sr})}$  was 3.5 mM, and  $K_{I(\text{Sr})}$  was 6.0 mM. B. Incubation with tracer lasted 10 s, after the synaptosomes had been depolarized for 10 s in the absence of strontium (see text).  $K_{m(\text{Sr})} = 0.8$  mM, and  $K_{I(\text{Sr})} = 0.7$  mM before correction. After correction (see above),  $K_{m(\text{Sr})} = 1.9$  mM, and  $K_{I(\text{Sr})} = 1.0$  mM.

little effect on the voltage dependence of Ca influx (i.e., surface charge effects) or on K-induced depolarization (see previous sections). K-stimulated Ba influx during the fast and slow phases also began to saturate at high Ba concentrations (Fig. 8). Again, partial saturation was seen at Ba concentrations that had little effect on surface charge or on K-induced depolarization. Thus, the saturation is not simply a consequence of surface charge screening or of a reduction in the magnitude of K-induced depolarization. Saturation would be expected for a channel with a binding site for the transported ion (see Hille, 1975). Therefore, our results suggest that there is a saturable binding site at the divalent cation channels of synaptosomes, as there is at the Ca channels of other preparations (Hagiwara, 1975; Akaike et al., 1978).

The relationship between influx and external ion concentration for a saturating channel may, in the *simplest* case (e.g., with no voltage-dependent binding to the site), be described by the following equation:

$$J_X = \frac{J_{\max(X)}}{1 + \frac{K_{m(X)}}{[X]}} \quad (1)$$

This resembles a Michaelis-Menten equation, and contains two parameters that reflect the permeability properties of the system:  $J_{\max(X)}$ , the maximal rate of influx at high concentrations of  $X$ , and  $K_{m(X)}$ , the concentration of  $X$  at which the influx ( $J_X$ ) is half-maximal.

$K_{m(X)}$  and  $J_{\max(X)}$  values for Sr and Ba, during fast and slow phases of influx, were calculated from experiments like those shown in Figs. 7 and 8, after correcting for surface charge effects, by applying a least-squares best-fit from Eq. 1 to the corrected data points, plotted in double-reciprocal (Lineweaver-Burke) fashion. The average values obtained with and without correction are listed in Table I along with average  $K_m$  and  $J_{\max}$  values for Ca, from experiments in which Ca influx was measured when the Ca concentration was varied between 0.02 and 1 mM Ca (cf. Nachshen and Blaustein, 1980, Fig. 8). In this Ca concentration range, the correction factor is negligible.

Table I shows that the mean corrected  $K_{m(Sr)}$  values at 1 s (fast), and at 10 s, after prepolarization (slow), were significantly different. This is consistent with the notion that the fast and slow phases of influx are mediated by separate transport mechanisms.

#### *Competitive Interactions between Ca, Sr, and Ba*

We obtained additional evidence showing that Ca, Sr, and Ba move through the same channels in experiments with Ni, an ion that inhibits both fast and slow phases of K-stimulated Ca influx at similar concentrations (Nachshen and Blaustein, 1980). K-stimulated influx of Sr, Ba, and Ca was measured in solutions containing 0.1 mM Sr, 0.1 mM Ba, and 0.02 mM Ca, along with tracer (either  $^{45}\text{Ca}$ ,  $^{85}\text{Sr}$ , or  $^{133}\text{Ba}$ ; Fig. 9), in the absence (open bars) or presence (hatched bars) of Ni. Ni, at a concentration of 40  $\mu\text{M}$ , blocked the K-stimulated influx of all three divalent cations to the same extent during the

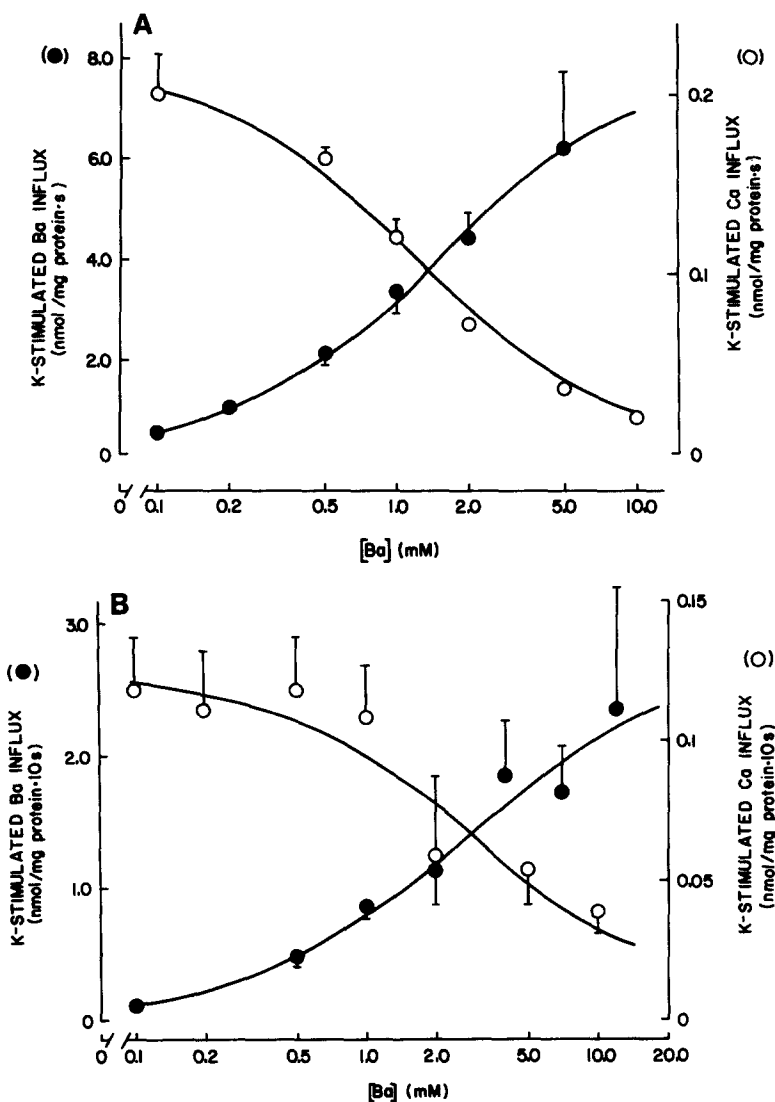


FIGURE 8. The effect of increasing Ba concentration on K-stimulated Ba or Ca influx. The protocol used was the same as that used in the experiment shown in Fig. 7, except that  $^{133}\text{Ba}$  influx (●) and  $^{46}\text{Ca}$  influx (○) were measured with increasing concentrations of Ba. A. 1-s incubation.  $K_{\text{m}(\text{Ba})} = 1.5$  mM;  $K_{\text{I}(\text{Ba})} = 1.2$  mM before correction;  $K_{\text{m}(\text{Ba})} = 4.2$  mM and  $K_{\text{I}(\text{Ba})} = 2$  mM after correction for screening (see legend to Fig. 7A). B. 10-s incubation, after depolarization.  $K_{\text{m}(\text{Ba})} = 2.4$  mM and  $K_{\text{I}(\text{Ba})} = 3.4$  mM before correction.  $K_{\text{m}(\text{Ba})} = 6.5$  mM, and  $K_{\text{I}(\text{Ba})} = 10$  mM after correction for screening (see legend to Fig. 7A).

slow phase. Similar results were observed during the fast phase (not shown). The simplest explanation of these results is that Ca, Sr, and Ba are transported via the same pathways.

Not only do Ca, Sr, and Ba use the same fast and slow uptake mechanisms,

TABLE I  
 $K_m$ ,  $J_{max}$ , AND  $P_{Ca}/P_X$  VALUES FOR THE "FAST" AND "SLOW" PHASES OF  
 K-STIMULATED INFLUX

| Ion | Number of experiments | Fast phase  |                  |              | Number of experiments | Slow phase  |                  |              |
|-----|-----------------------|-------------|------------------|--------------|-----------------------|-------------|------------------|--------------|
|     |                       | $K_m$       | $J_{max}$        | $P_{Ca}/P_X$ |                       | $K_M$       | $J_{max}$        | $P_{Ca}/P_X$ |
|     |                       | <i>mM</i>   | <i>nmol/mg·s</i> |              |                       | <i>mM</i>   | <i>nmol/mg·s</i> |              |
| Ca  | 5                     | (0.32±0.04) | (2.7±0.4)        | (1)          | 3                     | (0.43±0.05) | (0.26±0.06)      | (1)          |
|     |                       | 0.32±0.04*  | 2.7±0.4          | 1            |                       | 0.43±0.05*  | 0.26±0.06        | 1            |
| Sr  | 3                     | (2.5±0.4)   | (8.3±0.5)        | (2.5)        | 3                     | (0.9±0.2)   | (0.33±0.05)      | (1.6)        |
|     |                       | 4.5±1.1‡    | 12.7±2.0         | 3.0          |                       | 1.5±0.5‡    | 0.48±0.1         | 1.9          |
| Ba  | 4                     | (1.1±0.2)   | (6.6±1.2)        | (1.4)        | 4                     | (1.9±0.4)   | (0.23±0.08)      | (5.0)        |
|     |                       | 4.2±0.4*    | 17.6±2.8         | 2.0          |                       | 7.9±2.6*    | 1.24±0.5         | 3.9          |

$K_m$  and  $J_{max}$  values ( $\pm$  SEM) for the two phases of K-stimulated entry were calculated from experiments like those shown in Figs. 7 and 8. The values, uncorrected for the effects of divalent cations on surface charge, are shown in parentheses. Values derived from corrected data (see Fig. 4B, and text) are shown beneath.  $P_{Ca}/P_X$  values are calculated from the  $K_m$  and  $J_{max}$  values in the table, using Eq. 4B.

\*  $K_m$  values for fast and slow phases are not significantly different ( $P > 0.2$ ).

‡  $K_m$  values for fast and slow phases are significantly different ( $P < 0.05$ ).

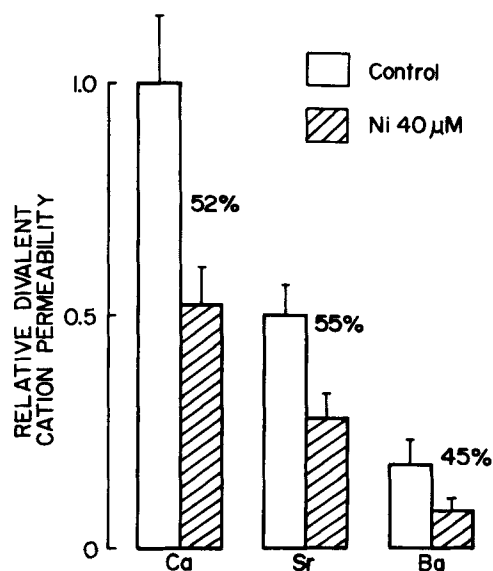


FIGURE 9. The effect of 40  $\mu$ M Ni on K-stimulated influx of divalent cations during the slow phase. In addition to the divalent ion concentrations listed in the text, all solution contained 0.5 mM Mg. The results have been normalized by dividing the influx of each divalent cation by its external concentration (see Eq. 3).

but, as might be expected, the different divalent cations interact with each other. Thus, Sr (Fig. 7, O) and Ba (Fig. 8, O) block the K-stimulated influx of Ca during the fast and slow phases. Conversely, Ca inhibits the K-stimulated influx of Sr and Ba during the fast phase (Fig. 10) and of Sr during the slow phase (data not shown). Ca block of Ba influx during the slow phase could

not be measured accurately because of the small size of the Ba flux (see Figs. 1B and 2B).

The next step was to determine whether or not this interaction is competitive; i.e., do all three divalent cations bind to the same site at the channel? The Michaelis-Menten model for competitive binding predicts that the influx of one permeant ion species,  $Y$ , in the presence of an inhibitory ion,  $X$ , is given by:

$$J_Y = \frac{J_{\max(Y)}[Y]/K_m(Y)}{1 + [Y]/K_m(Y) + [X]/K_I(X)} \quad (2A)$$

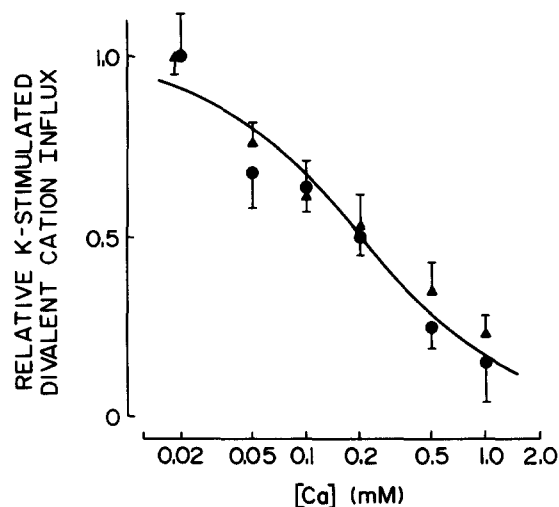


FIGURE 10. The effect of Ca on K-stimulated Sr and Ba influx during the fast phase. K-stimulated Sr (●) or Ba (▲) influx was measured at 1 s. Solutions contained either 0.1 mM Sr or Ba, as well as 0.5 mM Mg. The data points have been normalized to the influx values obtained in solution with 0.02 mM Ca. The curve is a fit derived from Eq. 2 to the normalized data points, with a  $K_{I(\text{Ca})}$  value of 0.2 mM.

$J$ ,  $J_{\max}$ , and  $K_m$  have the same meaning as in Eq. 1, and  $K_{I(X)}$  is the inhibitory constant of ion  $X$ . If the concentration of  $Y$  is low with respect to  $K_m(Y)$ , and held constant, then Eq. 2A can be rewritten as:

$$J_Y = \frac{J_Y^*}{1 + [X]/K_{I(X)}} \quad (2B)$$

where  $J_Y^*$  is  $Y$ 's influx when  $[Y]$  is very low, in the absence of  $X$ . If  $X$  and  $Y$  compete for the same channel site, and if both ions are transported through the channel, then  $K_{I(X)}$  must equal  $K_m(X)$ , as defined in Eq. 1.

$K_I$  values for Sr block of Ca influx, Ba block of Ca influx, and Ca block of Sr and Ba influx, were calculated from the data in Figs. 7, 8, and 10, using best fits generated by Eq. 2B. The uncorrected  $K_I$  values given in the figure legends corresponded closely to the uncorrected  $K_m$  values. After correcting



for surface charge, the  $K_I$  values corresponded to the  $K_m$  values given in Table I, as predicted by the Michaelis-Menten model for competitive interaction.

In some experiments we tested for competitive binding by measuring the influx of one divalent cation at various concentrations, in the presence of low and high concentrations of a second divalent cation. Fig. 11 shows the results of a representative experiment, graphed in a double reciprocal (Lineweaver-Burke) plot. K-stimulated Sr influx (fast) was measured at varying Sr concentrations, in solutions that contained either 0.02 or 0.4 mM Ca. The double reciprocal plot of the data points are fit by straight lines that meet at a common intercept at the Y axis. This indicates that the interaction between Ca and Sr is competitive. Similar experiments (not shown) performed with Sr and Ca during the slow phase, and with Ca and Ba during fast and slow

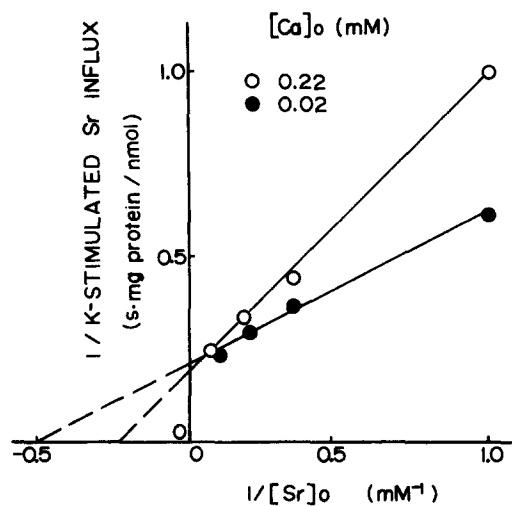


FIGURE 11. Effect of Sr concentration on  $^{86}\text{Sr}$  influx with 0.02 (●) and 0.22 (○) mM Ca. Data are plotted in double reciprocal form. Measurements were at 1 s. The  $K_{m(\text{Sr})}$  value obtained by extrapolating the line joining the solid points to the intercept with the X axis was 2 mM (uncorrected). The  $K_{I(\text{Ca})}$  value obtained by extrapolation after correcting for competition with Sr was 0.22 mM.

phases, confirmed that all three divalent cations compete with each other for both the fast and slow pathways.

#### *Selectivity of the Fast and Slow Pathways*

Tracer studies permit direct determination of ion permeabilities in a system that contains a mixture of different permeant ion species, because the movement of each radioisotope can be independently ascertained. In any given solution containing  $X$  and  $Y$ , ions with identical charge, the permeability of ion  $X$  ( $P_X$ ) relative to the permeability of ion  $Y$  ( $P_Y$ ) is:

$$\frac{P_X}{P_Y} = \frac{J_X}{J_Y} \cdot \frac{[Y]}{[X]} \quad (3)$$

This relative permeability is, a priori, fixed only for a specific set of ionic conditions; for different permeability mechanisms, it may, or may not, depend on  $[X]$  and  $[Y]$ .

A triple-label technique was used to determine the relative permeabilities of Ca, Sr, and Ba in solutions containing all three divalent cations. Results from a typical experiment are shown in Fig. 9. In some experiments, permeability was determined in the presence of high Sr and Ba concentrations. The permeability ratios obtained in these experiments are summarized in Table II. During both the fast and slow phases,  $P_{Ca} > P_{Sr} > P_{Ba}$ . Although the  $P_{Ca}/P_{Sr}$  ratio was similar during both phases,  $P_{Ca}/P_{Ba}$  differed by a factor of two. This is another indication that the fast and slow phases of K-stimulated influx are mediated by distinct channels, with slightly different selectivities for the divalent cations.

TABLE II  
DIRECT DETERMINATIONS OF  $P_{Ca}/P_X$  VALUES

| Experiment | Divalent ion concentration |      | Fast phase      |                 | Slow phase      |                 |
|------------|----------------------------|------|-----------------|-----------------|-----------------|-----------------|
|            | Sr                         | Ba   | $P_{Ca}/P_{Sr}$ | $P_{Ca}/P_{Ba}$ | $P_{Ca}/P_{Sr}$ | $P_{Ca}/P_{Ba}$ |
|            | <i>mM</i>                  |      |                 |                 |                 |                 |
| 1          | 0.1                        | 0.1  | 2.5             | 3.7             | —               | —               |
| 2          | —                          | 0.1  | —               | 3.3             | —               | —               |
| 2          | —                          | 10.0 | —               | 3.2             | —               | —               |
| 3          | 0.12                       | —    | 2.3             | —               | —               | —               |
| 3          | 12.0                       | —    | 2.6             | —               | —               | —               |
| 4          | 0.1                        | 0.1  | —               | —               | 2.0             | 5.6             |
| 5          | 0.12                       | —    | —               | —               | 2.7             | —               |
| 5          | 12.0                       | —    | —               | —               | 2.3             | —               |
| 6          | 0.1                        | 0.1  | —               | —               | 1.6             | 5.0             |

All solutions contained, in addition to Sr and/or Ba, 0.02 mM Ca and 0.5–1.0 mM Mg.

Relative permeabilities for Ca, Sr, and Ba may also be computed indirectly, by measuring K-stimulated influx of each ion, at a variety of divalent cation concentrations (see Figs. 7 and 8). If  $K_{I(X)} = K_{m(X)}$ , and  $K_{I(Y)} = K_{m(Y)}$ , expressions for  $J_X$ , or  $J_Y$  (see Eq. 2A), can be combined, to give:

$$\frac{J_X}{J_Y} = \frac{J_{\max(X)} K_{m(Y)} [X]}{J_{\max(Y)} K_{m(X)} [Y]} \quad (4A)$$

This can be rewritten, using Eq. 3, as:

$$\frac{P_X}{P_Y} = \frac{J_{\max(X)} K_{m(Y)}}{J_{\max(Y)} K_{m(X)}} \quad (4B)$$

Thus, if Eq. 2A adequately describes the concentration dependence of divalent cation influx, it should be possible to determine permeability ratios from estimates of  $J_{\max}$  and  $K_m$  with Eq. 4B.

Permeability ratios for the fast and slow phases computed from the appropriate  $K_m$  and  $J_{max}$  values are summarized in Table I. These model-dependent estimates of selectivity are in substantial agreement with the direct determinations of selectivity (Table II) for the slow phase.

In addition, Eq. 4B suggests that the permeability ratio should be independent of divalent ion concentration. That this is so is shown in Table II.

#### DISCUSSION

The tracers  $^{85}\text{Sr}$  and  $^{133}\text{Ba}$ , along with  $^{45}\text{Ca}$ , were used to study some of the pathways that regulate divalent cation entry in presynaptic nerve endings. We observed that there are two voltage-dependent pathways for the entry of these cations, and that all three divalent cations compete for both pathways or channels. The channels can be separated on the basis of their selectivity properties, as well as on the basis of their sensitivity to La and prolonged depolarization. Like Ca channels in many systems, both types of channels permit the passage of Ca, Sr, and Ba, but not Na (Krueger and Nachshen, 1980); also, they are blocked by a number of transition metals and by La, but not by tetrodotoxin (Nachshen and Blaustein, 1980).

It is unclear whether these channels are carriers or pores. One line of evidence, however, suggests that the Na-dependent exchange mechanism in synaptosomes, studied by Blaustein and Oborn (1975), is not involved in the influx of Sr and Ba. Reduction of external Na activated the exchanger and stimulated Ca influx; lowering Na did not, however, stimulate the influx of Sr and Ba (Fig. 2). K-induced depolarization, on the other hand, increased the entry of all three divalent cations. Thus, the K-activated and the Na-dependent pathways have different properties.

In general, selectivity of an ion channel is specified by its relative permeability for various ions. This relative permeability can be calculated from electrophysiological determinations of the reversal potential when the current is carried by different ion species. In the case of the Ca channel, however, the asymmetry of Ca distribution on both sides of the membrane makes accurate determination of the reversal potential difficult (see Hagiwara and Byerly, 1981, for a discussion of this point). In addition, inward current through the Ca channel is often masked by outward K current; this problem is compounded in selectivity studies because different divalent cations influence the K conductance to various degrees (Werman and Grundfest, 1961; Hagiwara et al., 1974; Hermann and Gorman, 1979).

Relative permeability can, however, be directly determined from measurements of radiotracer influx. We have used this approach to measure the selectivity of the fast and slow Ca channels in presynaptic nerve endings. For both types of channel, we find that  $P_{\text{Ca}} > P_{\text{Sr}} > P_{\text{Ba}}$ . This sequence is in agreement with that calculated for Ca channels in tunicate and sea urchin eggs from a modified Goldman-Hodgkin-Katz current equation (Okamoto et al., 1977), and for Ca channels in *Aplysia* neurones, calculated from apparent shifts in current reversal potential (Adams and Gage, 1980).

We further characterized the divalent cation channels in synaptosomes by

measuring influx at a variety of divalent cation concentrations. Data for the individual divalent cations, and for the interaction among the different divalent cations, appear to fit a simple model. The model is based on the hypothesis that the channel has a binding site to which the divalent cation must bind before it crosses the membrane. The model predicts that, at low ion concentrations, influx will depend both on the binding affinity of the ion for this site and on the rate of ion movement across the membrane after binding (i.e., the mobility). At high ion concentrations, only mobility will determine influx, because the sites are presumably saturated. This type of model has been used to explain some of the features of Ca channel selectivity in barnacle muscle (Hagiwara, 1975) and snail neurones (Akaike et al., 1978), and is equivalent, in its simplest form, to a two-barrier/single-well model (see Hille, 1975). According to this interpretation, the parameter  $J_{\max}$  in Eq. 1 reflects the ion mobility. For both fast and slow channels,  $J_{\max(\text{Ba})} > J_{\max(\text{Sr})} > J_{\max(\text{Ca})}$ , as has been found in barnacle muscle (see Hagiwara, 1975). Interestingly, this corresponds to the sequence of ionic mobility in aqueous solution for the alkali earth cations. The parameter  $K_m$  is more complex, because it relates both to the rate constants for association and dissociation from the site, as well as to the mobility (Hille, 1975). The binding site, or energy well, may consist of either charged groups, or of dipoles. The presence of anionic sites is suggested by the pH sensitivity of K-stimulated Ca influx (Nachshen and Blaustein, 1979). The apparent permeability sequence,  $\text{Ca} > \text{Sr} > \text{Ba}$ , is also that expected for a channel with an anionic, high field strength binding site (Diamond and Wright, 1969).

As we have shown in the Results section, permeability ratios for the slow phase are fairly well predicted by the parameters  $J_{\max}$  and  $K_m$  for Ca, Sr, and Ba. Both the model and the direct measurements of slow channel permeability indicate that  $P_{\text{Ca}} > P_{\text{Sr}} > P_{\text{Ba}}$ . Thus, the data relating ion influx to changes in the external divalent ion concentration are consistent with a simple competitive-binding model for transport via the slow channel. For the fast channel, a discrepancy was noted, in that the model-dependent prediction was  $P_{\text{Sr}} < P_{\text{Ba}}$ , whereas the direct measurements showed that  $P_{\text{Ba}} < P_{\text{Sr}}$ . However, in view of the possible errors entailed in the computation of  $K_m$  and  $J_{\max}$  (because of surface charge effects)—particularly in the case of Ba, an ion that appears to approach isotopic equilibrium very rapidly—this discrepancy is probably not significant. Strengthening this view, and consistent with the model, we found that Ba and Sr did not modify channel selectivity:  $P_{\text{Ca}}/P_X$  values were similar at both high and low concentrations of Sr and Ba (Table II), as well as in the presence, or absence, of an inhibitory divalent cation (Fig. 9).

One alternative simple explanation for the discrepancy that cannot as yet be ruled out is that the different divalent cation species modify fast channel kinetics. If, for example, Ba were to slow the rate of inactivation of the fast pathway,  $J_{\max(\text{Ba})}$  would be overestimated in comparison with  $J_{\max(\text{Ca})}$ . This would lead to an underestimate of  $P_{\text{Ca}}/P_{\text{Ba}}$  as determined by the parameters  $J_{\max}$  and  $K_m$ , from Eq. 4B. Ba and Sr actions on Ca channel kinetics would not be surprising, because these ions seem to slow Ca channel inactivation in

other systems (Brehm and Eckert, 1978; Tillotson, 1979; Eckert and Tillotson, 1981). A direct attack on this problem would require accurate resolution of the initial rate of divalent cation influx, so that the inactivation rate constant could be determined. This may be feasible, using rapid-mixing techniques. Studies along this line are currently being undertaken in our laboratory.

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