

Some Properties of Potassium-stimulated Calcium Influx in Presynaptic Nerve Endings

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ABSTRACT Potassium-stimulated ^{45}Ca entry into rat brain synaptosomes was measured at times ranging from 1 to 60 s. The K-rich solutions were used to depolarize the synaptosomes. Backflux of ^{45}Ca from the synaptosomes was negligible during the first 10–20 s of incubation. An initial (“fast”) phase of K-stimulated Ca entry, lasting from 1 to 2 s was observed. This phase was inhibited by low concentrations of La ($K_I \approx 0.3 \mu\text{M}$). It was also abolished (“inactivated”) by incubating the synaptosomes in depolarizing solutions (containing veratridine, gramicidin, or elevated $[\text{K}]_o$) before the addition of ^{45}Ca . An additional long lasting (“slow”) phase of K-stimulated Ca entry was also detected. This “slow” Ca entry was much less sensitive to La ($K_I > 100 \mu\text{M}$) and was not affected by depolarizing the synaptosomes before the addition of ^{45}Ca . The rate of influx during the fast phase was about four times the rate of Ca influx during the slow phase. Neither the fast nor slow phase of Ca entry was sensitive to tetrodotoxin ($10 \mu\text{M}$), a potent blocker of Na channels, but both phases were inhibited by Ni, Mn, Mg, and other agents that block Ca channels. The data are consistent with the presence of two distinct populations of voltage-regulated, divalent cation-selective pathways for Ca entry in presynaptic brain nerve endings.

INTRODUCTION

Voltage-regulated pathways for divalent cations (Ca channels) control the entry of calcium in many cells (see reviews by Reuter [1973], Baker and Glitsch [1975], and Hagiwara [1975]). These Ca channels share some characteristics: they are blocked by polyvalent cations (e.g., La, Ni, Co, and Mg) but are not affected by tetrodotoxin (TTX), a selective inhibitor of Na channels (Narahashi, 1974). Ca-conductance pathways have been most rigorously studied in cells large enough to be impaled with microelectrodes or injected with Ca-sensitive dyes. Ca entry into nerve endings is of special interest because it triggers transmitter release (Katz and Miledi, 1965 and 1969; Katz, 1969; Llinas et al., 1972; Blaustein, 1975); however, the small dimensions of mammalian presynaptic terminals preclude the use of impalement or injection methods. We have attempted to partially circumvent this problem by measuring ^{45}Ca fluxes in a preparation of pinched-off presynaptic nerve endings (synaptosomes) from rat brain. Synaptosomes retain many functional prop-

erties of intact neuronal tissue (Bradford, 1975; Blaustein et al., 1977); in particular, they release transmitter after a depolarization-induced Ca influx (Blaustein, 1975). Some of the pathways that regulate Ca fluxes in synaptosomes have already been described (Blaustein, 1975; Blaustein and Oborn, 1975; Blaustein and Ector, 1976; Nachshen and Blaustein, 1979 *a* and 1979 *b*).

We now report that there are two distinct phases of depolarization-stimulated Ca entry in synaptosomes; a "fast" phase, mediated by pathways that close (inactivate) after 1 s, and a "slow" phase, mediated by pathways that do not inactivate during long lasting depolarizations (1–2 min). These two pathways differ by 100- to 1,000-fold in their sensitivities to block by La, but share many properties characteristic of Ca channels in other systems. Preliminary reports of some of these findings have appeared in abstract form (Nachshen, 1979 and 1980).

MATERIALS AND METHODS

Preparation of Synaptosomes

Synaptosomes were prepared from rat brains by a modification (Krueger et al., 1979) of the method of Hajos (1975). The nerve terminal-enriched material in the 0.8 M sucrose fraction from the sucrose gradient was equilibrated by the gradual addition of 2.5–3.0 vol of ice-cold standard (low-K) solution. This solution contained (mM): NaCl, 145; KCl, 5; MgCl₂, 1; glucose, 10; CaCl₂, 0.02; and *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (HEPES), 10, adjusted to pH 7.5 at 25°C with tris(hydroxymethyl)aminomethane (Tris). In some experiments either Tris-maleate or 2, (*N*-morpholino)ethane sulfonic acid-HEPES buffer was substituted for the Tris-HEPES in the various solutions; no differences in the results were observed.

The diluted synaptosome suspension was centrifuged (13,000 *g*) at 4°C for 10 min, and the pellet was resuspended in low-K solution. The resuspended synaptosomes were gently agitated and warmed for 20 min at 30°C before the experiments were continued.

Measurement of ⁴⁵Ca Entry

⁴⁵Ca entry was determined by adding aliquots of the warmed synaptosome suspension to equal volumes (100–200 μ l) of low-K or K-rich solution with tracer. The K concentration in the K-rich solution was adjusted by isosmotically substituting K for Na; in some experiments either Li or choline, instead of K, was substituted for Na. Most solutions contained only 0.02 mM CaCl₂. When the divalent ion concentration was increased by more than 2 mM, choline chloride was added to the control solutions to compensate for osmotic pressure differences. ⁴⁵Ca entry was terminated by rapidly diluting the incubation media (~0.4 ml vol) with 4.5 ml of ice-cold low-K solution containing 2.0 mM CaCl₂. For short test incubations with ⁴⁵Ca (1–3 s), the pipettings were timed with a metronome. After ⁴⁵Ca entry had been quenched, the diluted suspensions were filtered by suction through glass-fiber filters (GF/A, 2.4 cm in diameter, Whatman, Inc., Clifton, N. J.), and the filters were then washed with two more 4.5-ml aliquots of ice-cold low-K solution. The washed filters were placed in vials containing 10 ml of scintillation cocktail, and the ⁴⁵Ca content of the trapped material was measured by liquid scintillation spectrometry. Protein was determined by the method of Lowry et al. (1951). Extra entry of ⁴⁵Ca (K-stimulated, choline-

stimulated, or Li-stimulated) was calculated as the difference between ^{45}Ca uptake from the low-K solution and from the appropriate stimulating solution. In each experiment, means and standard errors (SE) of the means were calculated from three to five replicate samples for every experimental condition. In the text, "uptake" is used to indicate net ^{45}Ca accumulation; "influx" is used to indicate that the entry is unidirectional.

"Predepolarization" Protocol

In some experiments synaptosome suspensions were incubated in K-rich solution before incubation with ^{45}Ca ("predepolarized") by adding an aliquot (100–200 μl) of synaptosome suspension to an equal volume of K-rich solution, with Na and K concentrations adjusted to give the required K concentration. After a specified time (see Results), 90% of the suspension was withdrawn and added to K-rich solution with ^{45}Ca . Although prolonged incubation in K-rich solution did not irreversibly change the Ca-influx pathways (see Results), we suspected that after predepolarization synaptosomes might not repolarize instantaneously to a precise control level in low-K solution. Therefore, control ^{45}Ca uptake in these experiments was estimated by measuring ^{45}Ca uptake from low-K solution into control synaptosomes. Stimulated Ca uptake was then calculated as: (uptake from K-rich solution [after predepolarization]) minus (uptake from low-K solution [without predepolarization]).

RESULTS

Time-Course of K-Stimulated Ca Uptake

When synaptosomes are incubated in control (low-K) solution, containing 145 mM Na and 5 mM K, there is a time-dependent ^{45}Ca uptake (Fig. 1 *A* and *B*). This rate of uptake is greatly increased (3- to 10-fold, depending upon the experimental conditions) when the synaptosomes are incubated in a K-rich (depolarizing) solution (Blaustein and Goldring, 1975; Fig. 1 *A*). Preliminary data suggest that at least part of the Ca uptake in low-K solution is mediated via pathways distinguishable from those that mediate K-stimulated Ca entry: concentrations of Ni, Ba, and Mg that greatly reduce K-stimulated Ca entry have little effect on accumulation from low-K solution (data not shown). Further research is required to elucidate the properties of the system(s) that govern Ca uptake by synaptosomes in control solution; this report focuses primarily on the properties of K-stimulated ^{45}Ca uptake.

The time-course of K-stimulated Ca uptake between 3 and 40 s is illustrated in Fig. 1 *A*. By 3 s (the time of the first measurement) there was a substantial K-stimulated Ca uptake (Fig. 1 *A*). During the next 10–20 s the rate of uptake was almost constant. At longer times, the rate declined and approached zero. Indeed, in other experiments (data not shown), we observed that little additional ^{45}Ca was accumulated when incubation in K-rich medium was extended from 1 to 2 min.

The time-course of Ca uptake during the initial 10 s of incubation with ^{45}Ca was examined in more detail. Data from a typical experiment are shown in Fig. 1 *B*. Between 1 and 10 s, K-stimulated Ca uptake (\blacktriangle) proceeded at a near-constant rate. This phase of uptake will be referred to as the "slow" phase. The linearity of uptake during the "slow" phase suggests that stimu-

lated Ca uptake measured between 0 and 10 s is predominantly unidirectional. However, the regression line for the time-course of K-stimulated Ca uptake did not extrapolate to the origin; neither were the data points adequately fit by a simple exponential function. Substantial K-stimulated Ca accumulation,

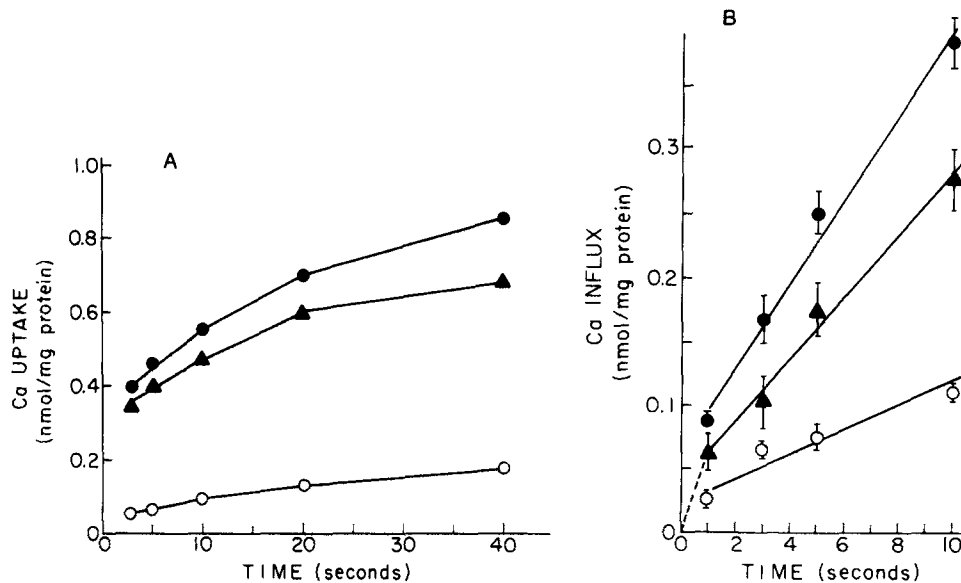


FIGURE 1 *A* and *B*. The time-course of Ca uptake in synaptosomes. In this and subsequent figures standard error bars are drawn unless they fall within the symbols. ^{45}Ca uptake was measured in low-K (145 mM Na + 5 mM K, ○) and K-rich (73 mM Na + 77 mM K, ●) test solutions, and K-stimulated Ca uptake (▲) was taken as the difference (see Methods).

measured after only 1 s, will be called the “fast” phase of Ca influx. In 20 experiments we found that the K-stimulated Ca influx during the fast phase was 3.8 ± 0.4 (mean and SE)-fold greater than the rate of stimulated influx averaged over the next 9 s. The initial rate of Ca influx (at times <1 s) cannot be adequately resolved with present methods; therefore, a ratio of $\sim 4:1$ is a lower limit. The decrease in rate of Ca influx, measured after 1 s, will be called “inactivation”; it will be shown that this inactivation probably reflects closing of some of the pathways through which Ca enters.

K Dependence of Ca Influx

The K dependence of Ca influx for 1-s test incubations is shown in Fig. 2 (●). There was only a small increase in Ca influx when $[\text{K}]_0$ was raised from 5 to 25 mM. As $[\text{K}]_0$ was raised above this level, entry increased sharply, and continued to increase up to at least 77.5 mM K. Similar results were observed for 10-s test incubations (Fig. 2, ○).

K-stimulated ^{45}Ca influx dependent upon lowering the external concentra-

tion of Na (cf. Blaustein [1975] and Blaustein and Oborn [1975]) was estimated by measuring Ca influx after part of the Na had been replaced with choline, instead of K, in the test incubation solutions. The influx obtained by subtracting the choline-stimulated from the total K-stimulated Ca influx presumably represents the depolarization-dependent component of the K-stimulated influx. It was found that choline-stimulated Ca influx was an almost constant fraction of K-stimulated Ca influx at either 1 or 10 s, with both high (2 mM) and low $[Ca]_0$ (0.02 mM) (Fig. 3). Furthermore, in most experiments < 15% of the K-stimulated Ca influx was found to depend upon reducing $[Na]_0$ rather than raising $[K]_0$. Therefore, correcting for this fraction of influx, with $[K]_0 = 77.5$ mM, did not significantly modify the results.

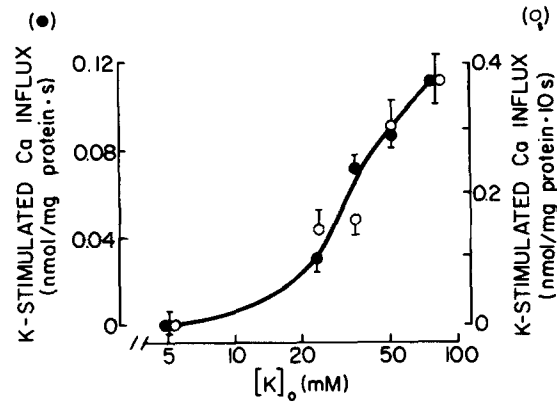


FIGURE 2. The effect of varying $[K]_0$ on K-stimulated Ca influx. 1-s (●) and 10-s (○) test incubation with ^{45}Ca . The ordinates are scaled to the K-stimulated uptake value with 77.5 mM K.

A slow, but not a fast phase of ^{45}Ca influx was observed in synaptosomes depolarized with veratridine, batrachotoxin, or gramicidin, in low-K solutions with tracer (cf. Krueger et al. [1977]). We cannot, therefore, rigorously exclude the possibility that the fast phase of Ca influx is activated specifically by elevated levels of K rather than by depolarization. A more likely explanation of the result described above, however, is that the rate at which veratridine, batrachotoxin, and gramicidin depolarize synaptosomes is slow by comparison with the rate of inactivation.¹

K Dependence of Inactivation

In many types of cells there are voltage-dependent processes that decrease the conductance of pathways carrying inward Ca current (I_{Ca}) (Geduldig and

¹ In recent experiments (unpublished) we have obtained evidence for "surface charge" effects on K-stimulated Ca influx during the fast phase. At high concentrations (<10 mM) of Mg the relationship between Ca influx and $[K]_0$ (cf. Fig. 6) is shifted in a hyperpolarizing direction. This is consistent with divalent cations screening fixed negative charges in the vicinity of a Ca entry pathway that is voltage stimulated rather than K stimulated.

Gruener, 1970; Baker et al., 1973 *b*; Standen, 1975; Oertel et al., 1977; Reuter and Scholz, 1977; Adams and Gage, 1979). To determine whether inactivation of Ca entry into synaptosomes might also be voltage dependent, we measured ^{45}Ca influx in synaptosomes that had been incubated in K-rich solution for 10–15 s before the addition of tracer (predepolarized; see Methods). As illustrated in Fig. 4 (\blacktriangle), preincubation in K-rich solution abolished the initial fast phase of K-stimulated Ca influx. The data points fell on a straight line that extrapolated to the origin, indicating that ^{45}Ca movement was unidirectional (i.e., backflux was negligible). The rate of influx was similar to the rate

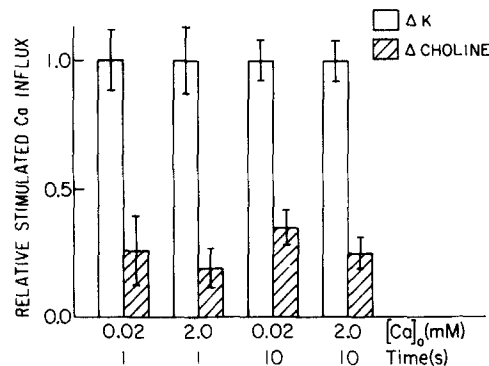


FIGURE 3. The effects of Ca and time on K- and choline-induced Ca influx. Influx of ^{45}Ca was measured in low-K solution or in solutions with 75 mM NaCl replaced isosmotically by either K (high K) or choline (high choline). The open bars represent normalized K-stimulated Ca influx (high K minus low K), and the hatched bars represent normalized choline-stimulated Ca influx (high choline minus low K) during 1- or 10-s test incubations, with low (0.02 mM) and high (2.0 mM) Ca (as indicated in the figure). K-stimulated Ca uptake values (nmol/mg protein·s) were (mean \pm SEM): 1 s, low Ca: 0.06 ± 0.01 ; 1 s, high Ca: 1.23 ± 0.16 ; 10 s, low Ca: 0.03 ± 0.002 ; and 10 s, high Ca: 0.38 ± 0.03 .

of accumulation during the slow phase of Ca entry in synaptosomes that were not predepolarized (Fig. 4, \bullet). This is evidence that K-stimulated ^{45}Ca movement during the slow phase was also unidirectional, and that backflux of ^{45}Ca was probably negligible for at least 10 s. The fact that K-stimulated ^{45}Ca movement in synaptosomes remains unidirectional for at least 10 s may, at first, seem surprising; substantial backflux of ^{22}Na is detected after 1–2 s (Krueger and Blaustein, 1980). The likely explanation is that the intraterminal ionized Ca concentration is maintained at a very low level ($\sim 10^{-7}$ M) and that Ca entering the synaptosomes is rapidly sequestered by intraterminal organelles (Blaustein et al., 1977; Blaustein et al., 1980).

K-stimulated Ca influx in control, “nonpredepolarized” synaptosomes (Fig. 4) at 1 s was four to five times greater than K-stimulated Ca influx in predepolarized synaptosomes (\blacktriangle) at 1 s. This is in accord with the ratio of

influxes during fast and slow phases, calculated above as $\sim 4:1$, as might be expected if, after ~ 1 s in K-rich solution, the inactivation process reached a steady state.

The K dependence of inactivation was examined by preincubating synaptosomes (see Methods) in tracer-free solution with various $[K]_0$ (Fig. 5). After 10 s, K-stimulated ^{45}Ca influx was measured for 1 s in solutions containing 77.5 mM KCl as described in Methods. The relationship between K-stimulated Ca influx and $[K]_0$ in the preincubation solutions is shown in Fig. 5. Inactivation of Ca influx was detected as $[K]_0$ was increased above 10 mM,

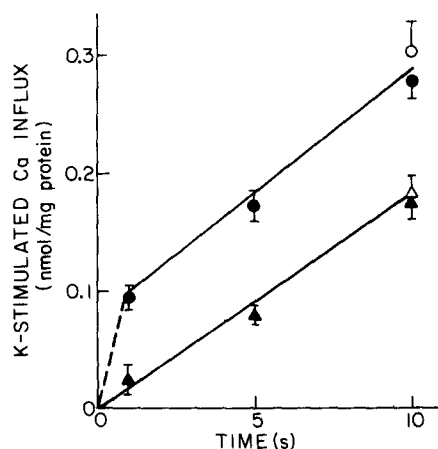


FIGURE 4. The effect of preincubation in high-K solution on K-stimulated Ca influx. Synaptosomes were preincubated for 15 s in a standard low-K (●) or a high-K (▲) solution. Test incubation was then initiated, and K-stimulated Ca influx was determined. Open symbols indicate that the test incubation solutions contained $10 \mu\text{M}$ TTX.

and appeared to be almost maximal, amounting in this experiment to $\sim 50\%$, with 50 mM K. In twelve experiments, preincubation with 77.5 mM K inactivated the K-stimulated Ca influx by $64 \pm 3\%$ (mean \pm SE).

Prolonged incubation in K-rich solution did not appear to irreversibly change the Ca-influx pathways. In control experiments, synaptosomes were preincubated for 3 min in either low-K or K-rich solutions. The synaptosomes were then centrifuged (for 6 min at 13,000 *g*) and resuspended in low-K solution. After 20 min (see Methods), test uptake with ^{45}Ca was initiated. K-stimulated Ca influx was normal in magnitude, and the time-course of uptake was biphasic, despite the prior exposure to K-rich solution (data not shown).

There was no decline in the rate of (slow phase) K-stimulated ^{45}Ca influx during incubation in K-rich solution for times up to 1 min when tracer ^{45}Ca uptake was assayed only for brief (3-s) periods (Fig. 6). This indicates that the declining rate of Ca influx after 10–20 s in K-rich solution (Fig. 1 A, ●) is not caused by inactivation but by ^{45}Ca backflux from the synaptosomes.

It was important to determine whether inactivation of the Ca influx was indeed voltage dependent, or whether it was a K-specific effect caused by replacing NaCl with KCl. Before the addition of tracer, synaptosomes in low-K solution were depolarized by veratradine (Blaustein and Goldring, 1975) and batrachotoxin (Krueger and Blaustein, 1980), drugs that open Na channels (Ohta et al., 1973; Albuquerque and Daly, 1976) or by gramicidin D (Blaustein and Goldring, 1975), an antibiotic that forms monovalent cation channels in membranes (Podleski and Changeux, 1969). After a 10- to 30-s preincubation with or without depolarizing agent, synaptosomes were added to low-K or K-rich solutions containing ^{45}Ca , and influx was assayed for 1–10 s. No fast phase of K-stimulated Ca influx could be observed after

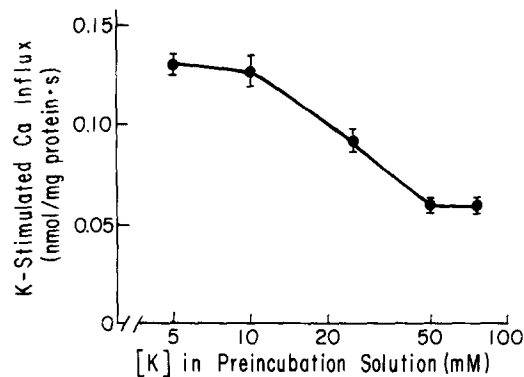


FIGURE 5. The effect of varying $[\text{K}]_0$ during prepolarization on K-stimulated Ca influx. Synaptosomes were preincubated for 10 s in solutions with various amounts of K isosmotically replacing Na. After 10 s, a 1-s test ^{45}Ca influx was initiated by adding the preincubated synaptosomes to test solutions, with the alkali metal cations adjusted to give a final concentration of (mM) KCl, 77.5 and NaCl, 72.5.

preincubation with any of the agents described, but a slow phase of K-stimulated Ca influx of a magnitude comparable to that found in K-predepolarized synaptosomes was measured.

The conductance pathways for Ca in some cells are inactivated after Ca entry (Brehm and Eckert, 1978; Tillotson, 1979) or an increase in $[\text{Ca}^{2+}]_{\text{in}}$ (Akaike et al., 1978 *b*). We therefore examined the effects on inactivation of Ca entry during prepolarization (Fig. 7). Synaptosomes were preincubated in K-rich or low-K solutions with or without free Ca. After 15 s, K-stimulated ^{45}Ca influx was measured for 1 s (see Methods). The Ca-free preincubation solutions contained $\sim 1 \mu\text{M}$ total Ca, as measured by atomic absorption spectroscopy, and $50 \mu\text{M}$ EGTA. Fig. 7 shows that prepolarization, with Ca and without Ca, inactivates K-stimulated Ca influx to the same extent. This does not rule out the possibility that inactivation may be controlled by Ca release from intraterminal stores after stimulation (cf. Erulkar and Rahamimoff [1978]); however, depolarization of the synaptosomes, rather than Ca entry, appears to trigger the inactivation.

We could find no evidence that the inactivation was modified by Ca loading of the synaptosomes. When synaptosomes were prepolarized in 0.02 mM Ca-containing medium, inactivation after 10 s and 60 s was similar (data not shown). Inactivation was also similar for synaptosomes prepolarized (for 10 s) with 0.02 or 2 mM Ca in the external solution.

Fast and Slow Phases of Ca Influx Share Several Characteristics

Besides similar dependencies on $[K]_0$ (Fig. 2), fast and slow phases of K-stimulated Ca influx have several other characteristics in common.

We have previously reported (Nachshen and Blaustein, 1979 *a*) that the rate of K-stimulated Ca influx in synaptosomes saturates with increasing Ca concentrations, for 10-s incubations with ^{45}Ca . The Ca concentration dependence of K-stimulated influx was examined, for 1-s (Fig. 8, ●) incubations

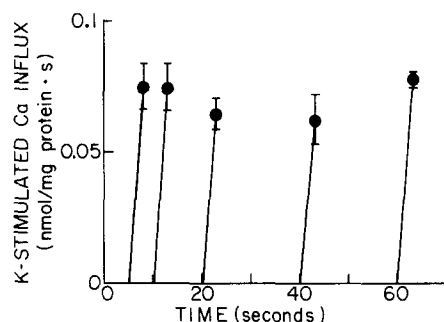


FIGURE 6. The effect of varying prepolarization time on K-stimulated Ca influx. Points on the abscissa indicate the time at which prepolarization was terminated and test incubation initiated by the addition of depolarized synaptosomes to test solutions with ^{45}Ca for 3 s.

without prepolarization (fast phase) and for 10-s (Fig. 8, ○) incubations after prepolarization (slow phase) of the synaptosomes. The data for both fast and slow components of influx were well fit by curves generated by a Michaelis-Menten-type equation (see legend to Fig. 8), with a K_{Ca} of 0.2 mM. This type of equation may be used to describe fluxes mediated either by carriers or by channels with saturable binding sites (Hille, 1975).

K-stimulated Ca influx at 1 s and 10 s was reduced as the external pH was lowered from 7.5 to 5.8 (data not shown; see Nachshen and Blaustein [1979 *a*]); both fast and slow pathways for K-stimulated Ca entry appear to be very pH sensitive. Ni blocked both the fast (Fig. 9, ●) and slow (Fig. 9, ○) K-stimulated influxes to about the same extent (K_I 's [dissociation constant of inhibitory cation I] $\approx 30 \mu\text{M}$). Mg and Ba were far less potent blockers than Ni and also were not very specific for either fast or slow phases (Fig. 10). Similarly, the organic "Ca antagonist," D-600 (Fleckenstein, 1977), inhibited the two phases to a comparable extent (data not shown; cf. Nachshen and Blaustein, [1979 *b*]). Tetrodotoxin (TTX), a potent and highly selective blocker of Na channels, at a concentration of 10 μM , had no effect on either phase (Fig. 4, open symbols).

The time-course of K-stimulated Ca influx was measured at incubation temperatures of 30° and 7°C (data not shown). The Q_{10} for K-stimulated Ca influx was ~ 2 , for both fast and slow phases. We could not, therefore, use temperature sensitivity to separate the two phases of Ca entry.

Fast and Slow Phases of K-stimulated Ca Influx are Mediated by Different Pathways

As noted above, many agents (Ni, Ba, Mg, D-600, H^+) blocked both fast and slow phases of Ca influx at similar concentrations; however, La and Mn were more selective.

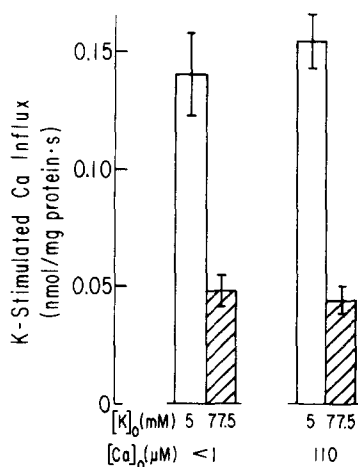


FIGURE 7. The effect of Ca removal during preincubation on K-stimulated Ca influx. Preincubation in either standard (open bars) or K-rich (crosshatched bars) solutions lasted 10 s. During test incubation with tracer, all solutions contained 50 μM Ca and 12 μM EGTA. The K and approximate ionized Ca concentrations during preincubation are indicated below the bars.

The effect of La on K-stimulated Ca influx at 1 s, and at 10 s after prepolarization, is illustrated in Fig 11. During the fast phase of uptake (at 1 s; filled symbols), a La concentration of $\sim 0.5 \mu M$ was sufficient to reduce influx by $\sim 50\%$; increasing the La concentration to $\sim 2 \mu M$ blocked 75% of this stimulated Ca influx. Increasing the La concentration further, however, had little effect until concentrations $> 50 \mu M$ were reached. Thus, there appear to be two components of Ca influx at 1 s: the first is blocked by very low concentrations of La ($< 2 \mu M$), and the second (residual) component is only affected by La concentrations $> 50 \mu M$. When prepolarized synaptosomes were incubated with ^{45}Ca and La for 10 s (Fig. 11, open symbols), concentrations of 50–200 μM were required to inhibit ^{45}Ca influx by $\sim 50\%$. This range overlaps that required to inhibit the second component of Ca influx in nonprepolarized synaptosomes (Fig. 11, filled symbols). La block of the fast

(1-s) component of influx appeared to be bimolecular, i.e., it fitted a Michaelis-Menten-type equation after correction for the La-resistant component. La block of the slow phase, however, deviated sharply from bimolecular kinetics; as seen in Fig. 11 (open symbols), the inhibition of Ca influx was very steeply dependent on $[La]_0$ in the range of 10^{-4} to 10^{-3} M.

The observations described above show that the fast and slow phases of Ca

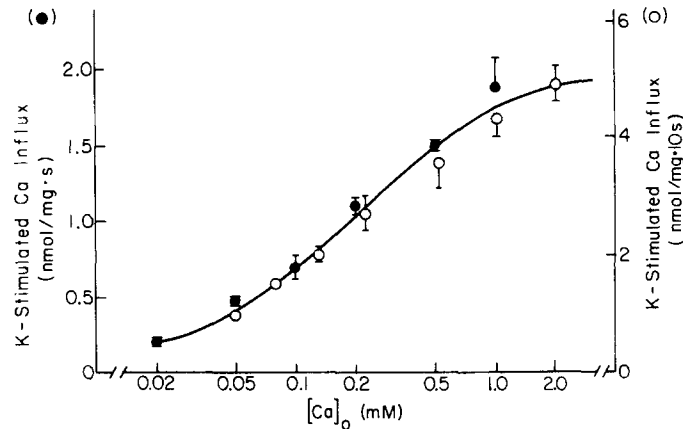


FIGURE 8. Effect of varying $[Ca]_0$ on K-stimulated Ca uptake. Results from experiments measuring influx for 1 s (●), or 10 s after prepolarization of the synaptosomes (○) are shown. Data points were scaled to maximal influx values for Ca, derived by fitting each set of data by the method of least squares to an equation of the form

$$J_M = \frac{J_{Max(M)}}{1 + K_M/[M]} \quad (1)$$

where J_M is the K-stimulated influx of the permeant ion M (=Ca), K_M is M's apparent Michaelis-Menten constant, and $J_{Max(M)}$ is J_M 's maximal value at the concentration of M, $[M]$, $\gg K_M$. K_{Ca} at 1 s was 0.21 mM, and K_{Ca} at 10 s, after prepolarization, was 0.18 mM. The curve shown in Fig. 10 was derived by fitting both sets of normalized data points to Eq. 1. Ca influx values in low (5 mM)-K solution were (nmol/mg protein·s): 0.019 and 0.018 with 0.05 mM Ca and 0.24 and 0.11 with 1 mM Ca, at 1 and 10 s, respectively.

influx differ by 100- to 1,000-fold in their sensitivity to block by La; this is evidence for two separate pathways mediating K-stimulated Ca entry. One pathway, predominant during the first second of incubation, is La sensitive and inactivates rapidly in K-rich solution. The other pathway, prominent at longer times, is La insensitive and does not inactivate in K-rich solution. This is shown in Fig. 12: La (20 μ M) greatly reduced K-stimulated Ca influx in nonprepolarized synaptosomes (closed symbols) but had little effect on K-stimulated Ca influx in prepolarized synaptosomes (open symbols); the time-course of Ca influx was very similar in nonprepolarized synaptosomes with La (\blacktriangle) and in prepolarized synaptosomes with (\triangle) or without (\circ) La.

Fig. 13 shows that Mn was also a more selective blocker of the fast phase of Ca influx (measured at 1 s) than of the slow phase; i.e., the K_I for the block of the fast phase was about one-third to one-fifth the K_I for block of the slow phase. This is additional evidence that two different pathways mediate K-stimulated Ca influx in synaptosomes.

DISCUSSION

Tracer flux studies have been useful for investigating ion transport processes, particularly in biological systems that are too small to be studied electrophys-

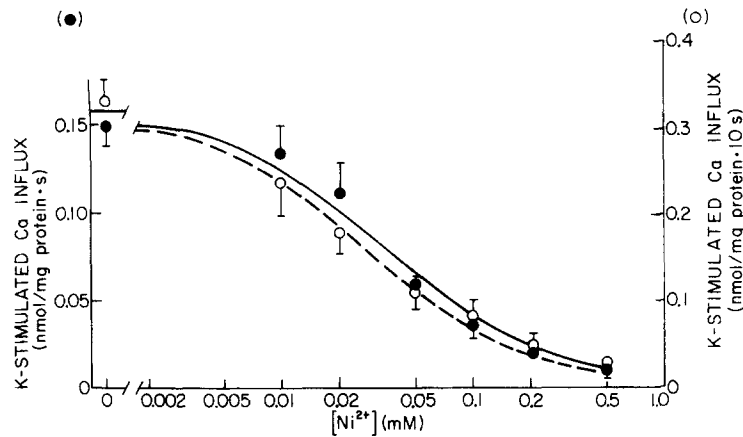


FIGURE 9. The effect of Ni on K-stimulated Ca influx at 1 s, without prepolarization (●), and at 10 s, after prepolarization for 15 s (○). The curves drawn through the data points for K-stimulated Ca influx (J_{Ca}) were fit by the method of least squares to the equation

$$J_{Ca} = \frac{J_{Ca}^0}{1 + [I]/K_I} \quad (2)$$

where $I = Ni$, and $J_{Ca}^0 = J_{Ca}$ in the absence of I . K_{Ni} , the apparent inhibitory constant (of Ni) was found to be 0.037 mM at 1 s without prepolarization, and 0.027 mM at 10 s, after prepolarization.

iologically. The present report provides an example of the use of tracer fluxes for the investigation of the electrophysiological properties of nerve terminals.

In this study, we have obtained evidence that there are two voltage-regulated pathways for Ca entry in presynaptic terminals. The fast phase of K-stimulated Ca influx in synaptosomes has not previously been described. Although several researchers have, in fact, shown data suggesting a fast phase (for example, see Fig. 2, Sohn and Ferrendelli [1975]; Fig. 2, Wonnacott et al. [1978]), they have not commented on this observation. Other studies on Ca entry in synaptosomes have noted but have not had sufficient time resolution to describe accurately the initial time-course of Ca entry (Janson et al., 1977), or have involved inefficient methods for quenching ⁴⁵Ca influx (e.g., centrif-

ugation [Blaustein, 1975]). By using a rapid-filtration technique, we have been able to show (a) that there are distinct fast and slow phases of K-stimulated, and presumably depolarization-regulated, Ca entry in synaptosomes, and (b) that a voltage-dependent inactivation process limits this Ca entry.

There are several problems associated with the electrophysiological study of

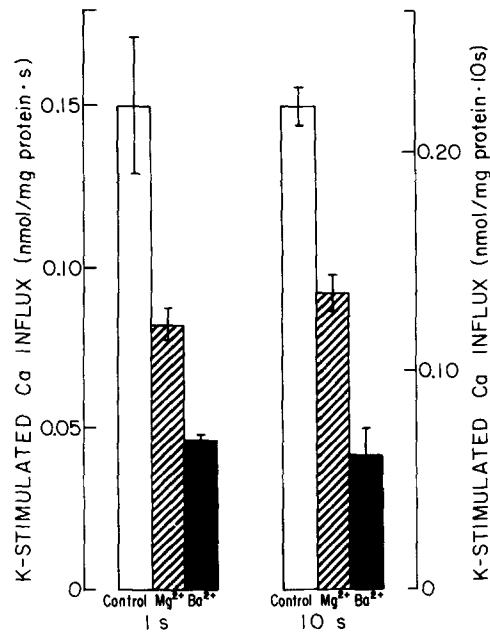


FIGURE 10. The effect of Ba and Mg on K-stimulated Ca influx. Control solutions contained (open bars) 0.5 mM MgCl₂ + 4.5 mM choline chloride, high Mg solutions (crosshatched bars) contained 3.5 mM MgCl₂, and barium solutions (solid bars) contained 3 mM BaCl₂ and 0.5 mM MgCl₂, in addition to the usual concentrations of NaCl, KCl, CaCl₂, glucose, and buffer. ⁴⁵Ca influx values (mean ± SE) from low K were (nmol/mg protein): 0.012 ± 0.001 and 0.065 ± 0.005 in control solutions, 0.013 ± 0.001 and 0.055 in high-Mg solutions, and 0.013 ± 0.001 and 0.083 ± 0.004 in Ba solutions, at 1 s and at 10 s, respectively.

Ca inactivation in excitable membranes. Inward Ca current (via Ca channels) is often masked by outward K current; and Ca influx may itself trigger an increased K-conductance (Lew, 1970; Meech, 1972; Krnjevic et al., 1978). Optimal Ca-current separation necessitates the use of pharmacological agents, such as tetraethylammonium ions or aminopyridines, and/or the replacement of permeant K by other (impermeant) ions (e.g., Cs). These procedures may, in some cases, modify Na conductance (e.g., Cs [Ebert and Goldman, 1976;

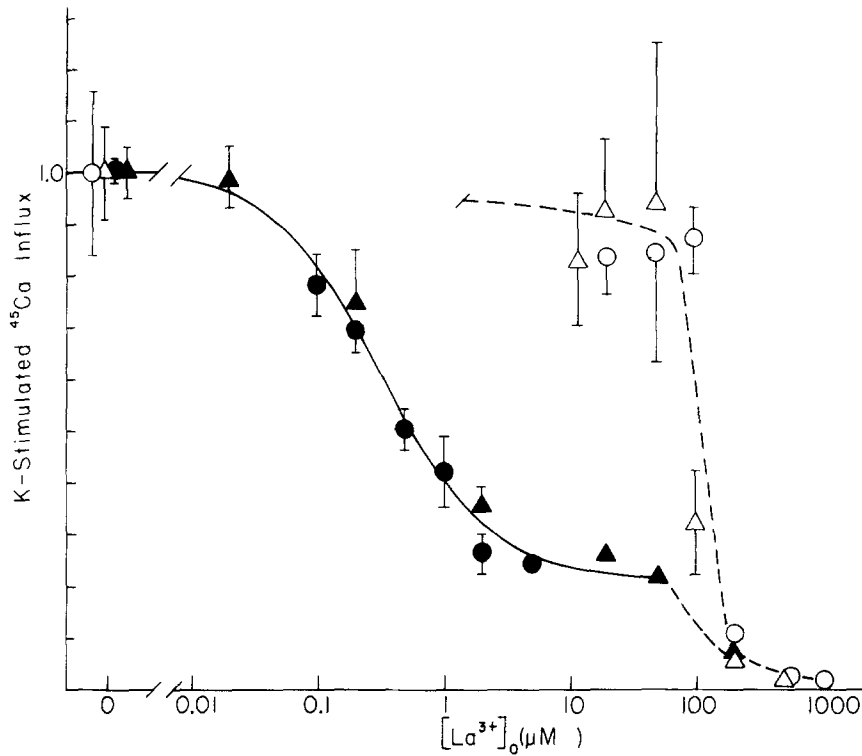


FIGURE 11. The effects of La on fast and slow phases of K-stimulated Ca influx. Data from four experiments (indicated, respectively, by the four symbols), normalized to K-stimulated Ca influx in the absence of La, are shown. (●,▲) Preincubation in Na + 5 K, 1-s test uptake. (○,△) Preincubation in high K, 10-s test uptake. The curve joining the data points for the fast phase (solid line; ●,▲) was fit (by the method of least squares) to the equation

$$J_{Ca} = \left(\frac{J_{Ca}^{\circ}}{1 + [I]/K_I} + 0.2 J_{Ca} \right) \times 0.83, \quad (3)$$

where $I = La$, J_{Ca} is K-stimulated Ca influx, J_{Ca}° is J_{Ca} in the absence of inhibitory cations, $[I]$ is the concentration of La, and K_I is La's apparent dissociation constant. In this figure, J_{Ca}° was arbitrarily set at unity. K_{La} was found to have a value of $0.3 \mu M$. The dashed curves were drawn by eye, and have no theoretical significance. The various J_{Ca}° measurements were: $0.26 \text{ nmol/mg protein} \cdot \text{s}$; $0.11 \text{ nmol/mg protein} \cdot \text{s}$; $0.13 \text{ nmol/mg protein} \cdot 10 \text{ s}$; and $0.21 \text{ nmol/mg protein} \cdot 10 \text{ s}$.

Schauf & Bullock, 1978]), and it might not be surprising if they were found to modify Ca conductance to some extent as well.

These problems can be partially circumvented by directly monitoring Ca uptake with optical probes. Both aequorin (Baker et al., 1973 *b*) and arsenazo III (Ahmed and Connor, 1979) have been employed to study Ca channel inactivation. However, it has been pointed out (Baker et al., 1973 *b*) that

intracellular Ca probes alone cannot distinguish between Ca entry and redistribution. The tracer-flux studies we describe provide, therefore, a convenient method for focusing directly on Ca influx, and the data supplement electrophysiological studies of Ca channels in other systems. At present, our methods are limited in their ability to resolve the time-course of the fast phase of Ca influx. It may be possible, however, to increase time resolution considerably using rapid-mixing techniques (Verjovski-Almeida et al., 1978).

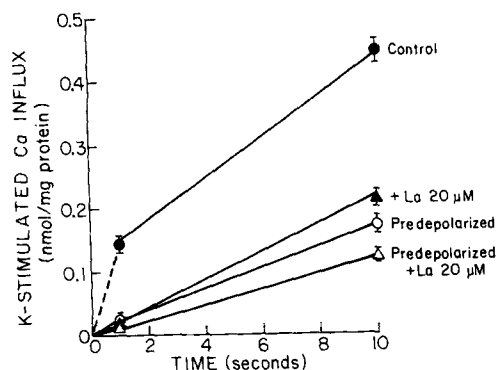


FIGURE 12. Effect of La on the time-course of K-stimulated Ca influx. (●,▲) Preincubation in low-K solution. (○,△) Preincubation in K-rich solution. (▲,△) + 20 μ M La in the incubation solution. Ca influx values in low (5 mM)-K solutions were (in nmol/mg protein): 0.016 and 0.072 without La, and 0.024 and 0.081 in the presence of La, at 1 and 10 s, respectively.

Multipathway vs. Multistate Models

At 1 s, during the fast phase, the predominant pathways for Ca entry are La sensitive; after predepolarization, the sensitivity of the pathways for Ca entry to block by La decreases 100- to 1,000-fold. These findings might be explained in two different ways. One possibility is that fast and slow phases of influx are each mediated by distinct pathways. The fast pathways undergo transition among several states (e.g., closed, open, and inactivated), with only one state (open) permitting Ca entry. For the slow pathways, only two states could be distinguished under the present experimental conditions (i.e., closed and open). Fast and slow pathways are similar in many respects (e.g., in their sensitivity to pH, Ni, D-600, Mg, and Ba), but have different affinities for La and Mn.

A second possibility is that fast and slow phases of Ca entry may *both* be mediated by one type of pathway that undergoes transition among several substates (closed, open, and inactivated) with different affinities for La. The persistent slow phase of Ca influx after predepolarization would then be explained by a "window" in the steady-state activation and inactivation relationships. Although there is no compelling evidence, we favor the multipathway model, because it is less restrictive and involves fewer ad hoc

assumptions. Of interest in this context is preliminary evidence for two distinct Ca-channel populations in cardiac Purkinje fibers (Seigelbaum, 1978) and in brain stem neurons (Yarom and Llinas, 1979).

Identity of the Pathways Mediating K-stimulated Ca Entry in Synaptosomes

The rate of K-stimulated Ca influx increases significantly at K concentrations of 10–20 mM. This is equivalent to a synaptosome depolarization of ~10–20

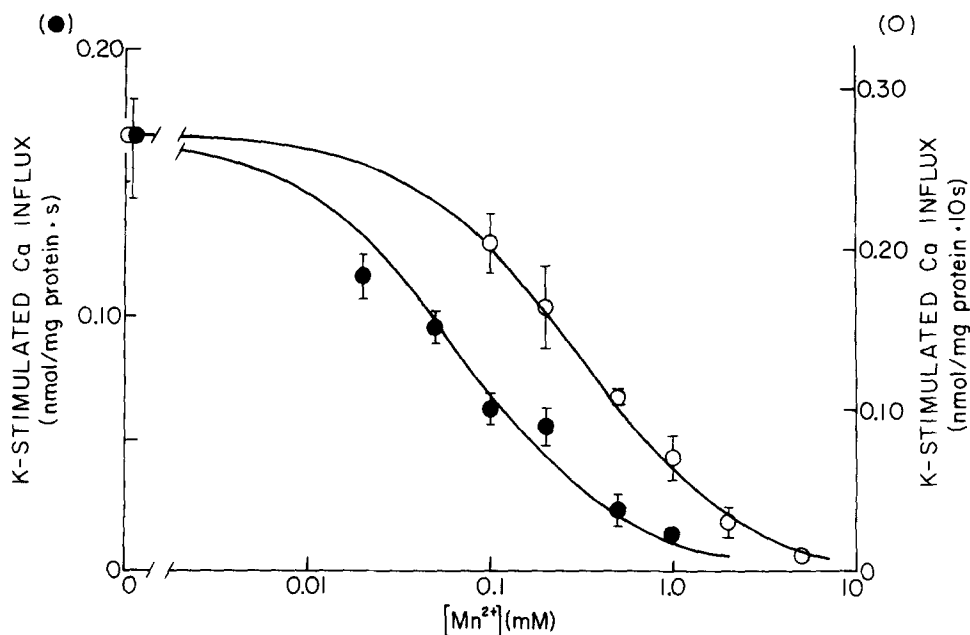


FIGURE 13. The effect of Mn on the fast and slow phases of Ca influx. Results from two experiments (● = 1-s, ○ = 10-s influx after prepolarization) are shown, normalized to measurements of K-stimulated Ca influx in the absence of Mn. Curves were fit to the data points (by the method of least squares) using an equation of the form given in the legend to Fig. 2, where $I = Mn$; J_{Ca}^o , J_{Ca} , and K_I have the same meaning as in Fig. 9. K_{Mn} at 1 s was 0.07 mM; K_{Mn} at 10 s was 0.30 mM.

mV above the resting potential (≈ -60 mV, Blaustein and Goldring [1975]; cf. Ramos et al. [1979]). This membrane potential, ~ -40 mV, is close to the level for Ca-channel activation in many cells (for examples, see Hagiwara [1975], Reuter and Scholz [1977], and Akaike et al. [1978 b]).

In other respects as well, the pathways for K-stimulated Ca influx in synaptosomes resemble Ca channels in a number of well-studied systems. (a) These pathways, like Ca channels, are not sensitive to TTX (Fig. 4 A), but they are sensitive to block by low concentrations of La, Ni, Mn, and D-600, and by higher concentrations of Mg and Ba (cf. Baker et al. [1973 a],

Hagiwara [1975], and Akaike et al. [1978 *a* and 1978 *b*]). (*b*) The pathways for K-stimulated Ca entry in synaptosomes are extremely sensitive to changes in the extracellular pH, having apparent pK_a 's in the range of 6.5–7.5 (see Nachshen and Blaustein [1979 *a*]). Ca channels in other preparations are also very pH sensitive (Vogel and Sperelakis, 1977; Spitzer, 1979), and are blocked at external H^+ concentrations that have little effect on conductance of Na channels (pK_a , 5–6) and K channels (pK_a , 4–5) (see Hille, 1975). (*c*) In addition, like Ca channels in other cells (for examples, see Reuter [1973] and Hagiwara [1975]), the K-stimulated pathways in synaptosomes are selective for divalent cations: they are permeable to Ba and Sr (Nachshen, 1980), but not to Na (Krueger and Nachshen, 1980). (*d*) Finally, the observation that a voltage-regulated inactivation process limits Ca entry in nerve endings parallels the observation that I_{Ca} inactivates as a function of time and voltage in many cells (Geduldig and Gruener, 1970; Baker et al., 1973 *b*; Standen, 1975; Reuter and Scholz, 1977; Oertel et al., 1977; Akaike et al., 1978 *b*; Moolenaar and Spector, 1979). We cannot rigorously exclude the possibility that either fast or slow pathways for K-stimulated Ca influx in synaptosomes are, in fact, carriers. In view of the similarities between the K-stimulated Ca influx described here and I_{Ca} in many other systems, however, we believe that there may be two distinct Ca-channel populations in presynaptic nerve terminals. An alternative possibility is that the fast phase of Ca entry is mediated by channels, whereas the slow phase represents carrier-mediated Ca entry.

The physiological roles of the fast and slow pathways remain to be determined. It is tempting to speculate that one type of pathway or channel (fast) could modulate primarily the phasic release of transmitter after an action potential. The other type of carrier or channel could modulate transmitter release after longer lasting depolarization of presynaptic nerve endings (for example, as a consequence of tetanic stimulation or of presynaptic activity at axo-axonic synapses). The processes mediated by the fast and slow Ca pathways could, perhaps, be separated by using low concentrations of La.

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