J. Physiol. (1985), **361**, *pp.* 251–268
With 11 *text-figures*
Printed in Great Britain

THE EARLY TIME COURSE OF POTASSIUM-STIMULATED CALCIUM UPTAKE IN PRESYNAPTIC NERVE TERMINALS ISOLATED FROM RAT BRAIN

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(*Received 13 June 1984*)

SUMMARY

1. K-stimulated (voltage-dependent) ^{45}Ca uptake in rat brain synaptosomes was measured at times ranging from 0·1 to 10 s, in experiments that employed a rapid-mixing device to initiate and terminate radiotracer uptake. The rapid mixing did not disrupt the functional integrity of the synaptosomes, as judged by their ability to take up Ca.

2. In solutions containing a low (0·02 mM) concentration of Ca, the rate of K-stimulated Ca uptake measured after 0–0·12 s of depolarization was 8 times greater than that measured after 5–10 s of depolarization. The decline in rate of K-stimulated Ca uptake was not due to tracer backflux from the synaptosomes, nor to Ca loading of the nerve terminals, since it also occurred after synaptosomes were depolarized in solutions without Ca. It is suggested that this decline in rate of Ca uptake after depolarization was due to inactivation of voltage-dependent Ca channels in the nerve terminals. This inactivation appeared to be voltage rather than Ca dependent.

3. The extent to which K-stimulated Ca uptake declined after depolarization in high-K solution depended on the K concentration that was used to depolarize the synaptosomes. Whereas pre-incubation in solution with one-half of the Na replaced by K significantly reduced subsequent K-stimulated Ca uptake, pre-incubation in non-depolarizing solution, with one-half of the Na replaced by choline, had no significant effect on subsequent K-stimulated Ca uptake.

4. In solutions containing a high (0·5–2 mM) concentration of Ca, the rate of K-stimulated Ca uptake measured after 0–0·12 s was 40 times greater than that measured after 5–10 s. High Ca accelerated the rate at which K-stimulated Ca uptake declined with prolonged depolarization. The effect was mimicked by high (10 mM) concentrations of Sr, but not of Ba. The accelerated rate of decline observed with high Ca could be either a direct effect of Ca on the Ca channels or, more probably, an indirect effect of Ca loading on the nerve terminals.

5. The apparent efficacy of several Ca-channel blockers (Ni, La and verapamil) in reducing K-stimulated Ca uptake was enhanced when the synaptosomes were

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depolarized in the presence of inhibitory agents for brief (< 1 s) intervals before K-stimulated Ca uptake was measured.

INTRODUCTION

The pivotal role of Ca in stimulating transmitter release from presynaptic nerve endings has been well established by studies showing that Ca entry into the nerve terminals acts as a trigger for neurosecretion (see reviews by Katz, 1969; Llinas, 1977). Relatively little is known, however, about the factors that control Ca uptake in mammalian presynaptic nerve terminals, because their small size makes them difficult to study by conventional electrophysiological techniques. This problem has been partially surmounted by the use of an *in vitro* preparation, consisting of isolated, pinched-off presynaptic nerve endings (synaptosomes) that retain many of the morphological, biochemical and functional properties of intact neuronal tissue (Bradford, 1975; Blaustein, Kendrick, Fried & Ratzlaff, 1977).

Radiotracer flux studies, employing ^{45}Ca and other radioisotopes, have proven particularly useful in demonstrating that Ca uptake in synaptosomes is linked to the release of neurotransmitter (Blaustein, 1975) and in showing that this uptake is regulated by voltage-dependent Ca channels (Nachshen & Blaustein, 1980, 1982). These tracer-flux studies are, however, severely constrained by poor time resolution, typically in the order of seconds or tens of seconds; this time scale exceeds by many orders of magnitude the time scale for several important physiological events, such as the nerve-terminal action potential that evokes transmitter release. Also, sizeable amounts of Ca are taken up by the synaptosomes after only a few seconds of depolarization, and this Ca loading may alter the properties of the nerve terminals.

With these difficulties in mind, I have used a rapid-mixing apparatus to study the time course of K-stimulated (voltage-dependent) Ca uptake in synaptosomes, at times ranging from 0·1 to 10 s. Results obtained with the rapid mixer indicated that the rate of K-stimulated Ca uptake diminished rapidly ('inactivated') after the synaptosomes had been depolarized for less than 1 s. Inactivation of Ca uptake occurred even when the synaptosomes were depolarized in solutions without Ca. These results are consistent with the notion that Ca-channel inactivation in synaptosomes is voltage dependent. At the same time, increasing the external Ca concentration from 0·02 to 0·5 mM accelerated the rate of inactivation by a factor of ~ 4. This apparent increase in inactivation may be due either to a Ca-dependent component of Ca-channel inactivation, or to the Ca loading of the synaptosomes.

A novel finding is that the apparent efficacy of several Ca-channel blockers (Ni, La and verapamil) in reducing K-stimulated Ca uptake was enhanced when the synaptosomes were depolarized in the presence of the inhibitor for brief (< 1 s) intervals.

A preliminary report of some of these findings has been published (Nachshen, 1982).

METHODS

Preparation of synaptosomes

Synaptosomes were prepared from rat forebrains by a modification (Krueger, Ratzlaff, Stricharz & Blaustein, 1979) of the method of Hajos (1975). In brief, the 0·8 M (nerve-terminal enriched) fraction of a sucrose gradient was equilibrated by the gradual addition of ice-cold Na solution that contained (mM): NaCl, 145; KCl, 5; $MgCl_2$, 2; glucose, 10; *N*-2 hydroxyethylpiperazine-*N'*-2-ethane sulphonic acid (HEPES), 10; pH adjusted to 7·4 with NaOH. The diluted synaptosome suspension was warmed at 30 °C for 20 min before proceeding with the experiments.

Determination of Ca uptake

1. Hand-pipetting technique. Aliquots of the synaptosome suspension (typically 180 μl, containing 0·5 mg protein) were ejected into tubes containing equal volumes of low- or high-K solution with ^{45}Ca (0·5–1 μCi). The final composition of the low-K solution was (mM): NaCl, 72·5; choline chloride, 72·5; KCl, 5. The K concentration of the high-K solution was increased by isosmotically replacing choline with K, to control for the effects of Na reduction on Ca influx (Blaustein & Oborn, 1975). All solutions contained Mg, glucose and buffer, as indicated above. Ca concentrations for specific experiments are given in the Results section. The tubes with radioisotope were vigorously vortexed during the addition of synaptosome suspension, to ensure effective mixing. After timed intervals of 1–10 s, 4 ml ice-cold Na solution containing 4 mM-$LaCl_3$ (La quench solution) was added to the tubes to halt Ca uptake and efflux (Jansson, Gripenberg, Harkonen & Korpijoki, 1977; Nachshen & Blaustein, 1980). In some experiments, a choline+ethylene glycol-bis (β-aminoethyl ether) *N,N'*-tetracetic acid (EGTA) quench solution, consisting of 150 mM-choline chloride, 1 mM-EGTA, and 10 mM-HEPES (Ph 7·4, 3 °C) was used. This solution was as effective as the La quench solution in halting Ca uptake and efflux, and it also reduced ^{45}Ca binding to the synaptosomes (M. P. Blaustein, D. A. Nachshen, E. Rasgado-Flores & S. Sanchez-Armass, unpublished observations). After the synaptosome suspensions were diluted with quench solution, they were filtered (Schleicher & Schuell no. 25 glass fibre filters). The filters were rinsed twice with 4 ml aliquots of La quench solution, and radioactivity retained by the filters was measured using standard liquid scintillation spectroscopy techniques. Protein was measured by the method of Lowry, Rosebrough, Farr & Randall (1951).

K-stimulated (depolarization-dependent) Ca uptake was calculated as: uptake in high-K solution minus uptake in low-K solution.

In several experiments the synaptosomes were depolarized in high-K solution prior to the addition of radiotracer (pre-depolarized). Typically, a small aliquot of warmed synaptosome suspension was mixed with an equal volume of high-K solution without radiotracer, and an additional aliquot of high-K solution with radiotracer was added after a predetermined interval. Ca uptake in low-K solution was determined in a similar manner: a small aliquot of warmed synaptosome suspension was mixed with an equal volume of Na solution without radiotracer, and an additional aliquot of low-K solution with radiotracer was added after a predetermined interval. ^{45}Ca uptake was then measured, as described above.

2. Rapid-mixing technique. These experiments were carried out with a Durrum D-133 Multimixing system (Fig. 1), whose performance in the millisecond time scale was initially checked by measuring the rate of alkaline hydrolysis of 2,4-dinitrophenol acetate (Verjovski-Almeida, Kurzmack & Inesi, 1978). The performance of the instrument during experiments was monitored by connecting the pneumatic actuator to a velocity transducer whose output led to a storage oscilloscope. Flow rates through the apparatus were calculated for each sample that was collected. The rapid mixer was used to measure the time course of Ca uptake, and to determine the effect of varying pre-depolarization intervals on subsequent Ca uptake.

In order to measure the time course of Ca uptake, one drive syringe (syringe A, Fig. 1) was filled with 2·5 ml synaptosome suspension, and a second drive syringe (syringe B, Fig. 1) was filled with 2·5 ml ^{45}Ca-labelled low- or high-K solution. The contents of syringes A and B were mixed, and the resultant mixture (C, Fig. 1) was advanced into a delay line by the movement of the pneumatic actuator. After an electronically timed delay interval to allow for tracer uptake by the synaptosomes, the mixture was further advanced and mixed with La quench solution (D, Fig. 1). The quenched mixture (E) was collected and filtered. The filters were rinsed with La quench solutions and the retained radioactivity was measured as described above.

In order to determine the effect of varying pre-depolarization intervals on Ca uptake, syringe A was filled with 2·5 ml synaptosome suspension and syringe B was filled with 2·5 ml Na or high-K solution *without radiotracer*. The contents of the syringes were mixed and advanced into the delay line. After a predetermined delay interval (pre-depolarization interval), the resultant mixture (C) was mixed with tracer-containing solution (D). The reaction mixture (E) was collected, and held in the syringe for a 2 s interval to allow for tracer uptake. The contents of the syringe were then mixed manually with a large volume of choline + EGTA quench solution. The diluted suspension was filtered, and Ca uptake was determined as described above.

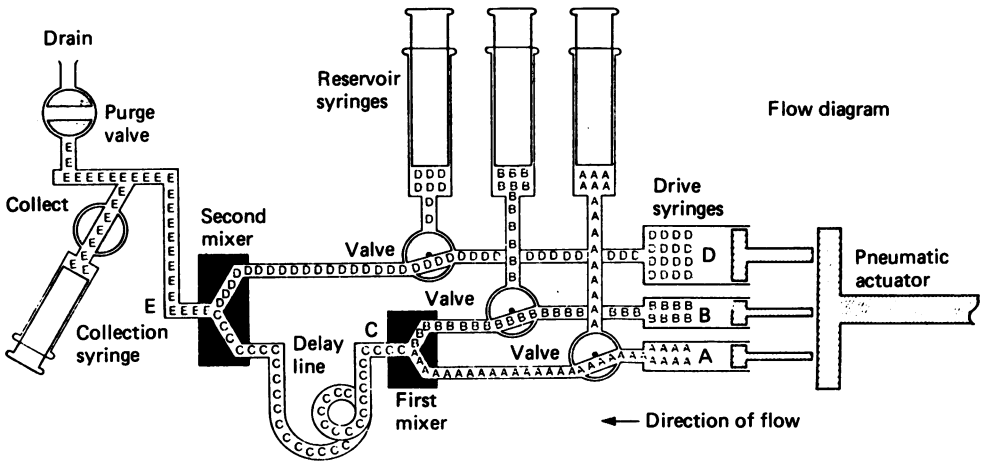

Fig. 1. Schematic representation of the rapid-mixing apparatus. The drive syringes A, B, and D are filled with reactants via the reservoir syringes. The pneumatic actuator moves the drive-syringe plungers simultaneously, advancing the reactants. Reactants A and B are mixed in the first mixer, and form C, which flows through a delay line. The travel of the plungers is stopped for a predetermined delay interval and then started again, so that C and D are mixed in the second mixer. The mixture of C and D (E), is allowed to discharge through the drain valve until the previous sample is purged from the lines, and is then directed into the collection syringe. Further details are given in the text.

All estimates of Ca uptake are based on three or more replicate samples. These replicates were staggered with respect to time, in order to obviate the effect of ageing on the synaptosomes. The values that are given have been corrected for radiotracer binding to the filters.

RESULTS

The time course of K-stimulated Ca uptake

There was a time-dependent uptake of Ca in synaptosomes that were incubated in a low-K solution, with one-half of the Na replaced by choline (Fig. 2, ○). This uptake was greatly increased (Blaustein, 1975; Nachshen & Blaustein, 1980) when the synaptosomes were depolarized (Blaustein & Goldring, 1975) by replacing one-half of the Na with K (Fig. 2, ●) instead of with choline. The extra, K-stimulated, Ca uptake (uptake in high-K minus uptake in low-K solution; Fig. 2, ▲) has been found to be mediated by voltage-dependent Ca channels (Blaustein, 1975; Nachshen & Blaustein, 1980, 1982). The values obtained for K-stimulated Ca uptake with the rapid mixer at times of 1–10 s are similar to previously reported values (Nachshen

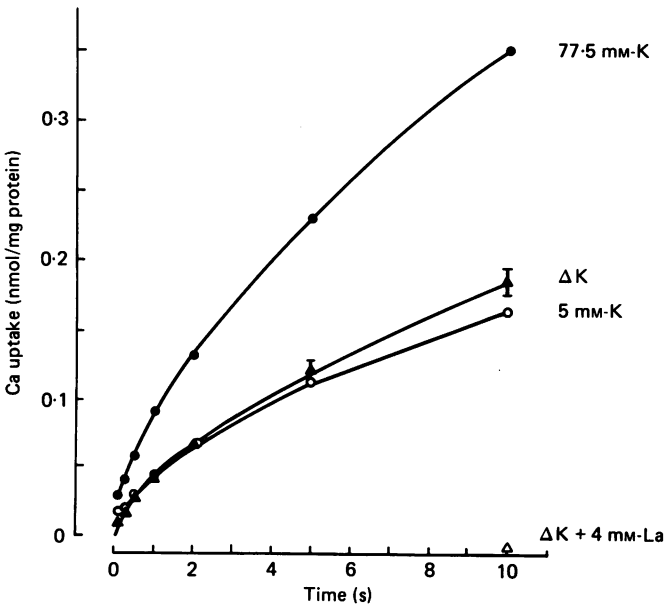

Fig. 2. The time course of Ca uptake in synaptosomes. The rapid mixer was used to initiate and terminate ^{45}Ca uptake. ^{45}Ca uptake was measured in low-K (○) and high-K (●) solutions. ▲, K-stimulated Ca uptake, the difference between uptake in high-K and low-K solution, △, K-stimulated Ca uptake obtained in solutions containing 4 mM-La. All solutions contained 0·02 mM-Ca. In this and subsequent Figures, standard error bars are drawn unless they fall within the symbols.

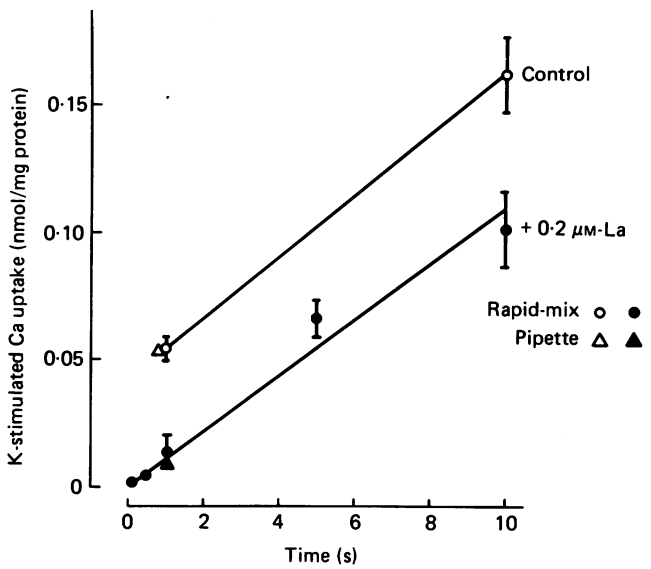

Fig. 3. The effect of 0·2 μM-La on K-stimulated Ca uptake. Synaptosomes were mixed with solutions containing 0·02 mM-Ca, with (filled symbols) or without (open symbols) La. Uptake was initiated and terminated either by rapid mixing (circles), or by hand-pipetting (triangles).

& Blaustein, 1980) obtained in experiments employing the hand-pipetting technique (see Methods). The two techniques were compared directly in several experiments, one of which is shown in Fig. 3 (also see Fig. 8). There was no significant difference in K-stimulated Ca uptake determined using the rapid mixer (Fig. 3, ○) and by the hand-pipetting method (Fig. 3, △). Thus, the rapid-mixing procedure did not disrupt the synaptosomes, as judged by their ability to take up Ca in a voltage-dependent manner.

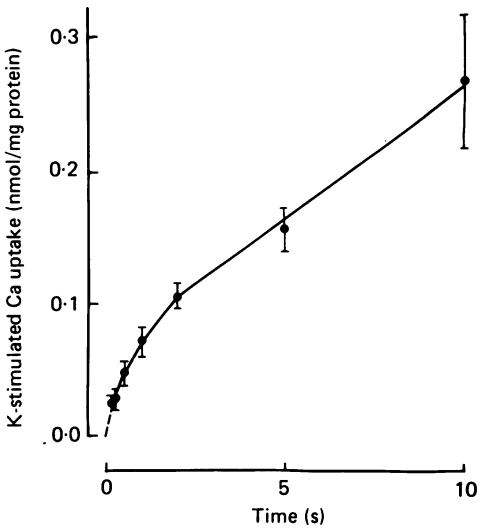

Fig. 4. The average time course of K-stimulated Ca uptake with 0·02 mM-Ca. This Figure summarizes results from nine experiments with the rapid mixer; synaptosomes were incubated in 0·02 mM-Ca, and K-stimulated Ca uptake was determined at times ranging from 0·1 to 10 s.

Previous studies of K-stimulated Ca uptake in synaptosomes (Nachshen & Blaustein, 1980) have shown a component of K-stimulated Ca uptake at early times ($\sim$ 1 s), that is blocked by very low concentrations of La (< 1 μM): a second component of K-stimulated Ca uptake, prominent after prolonged depolarization, is blocked by La only at high (> 100 μM) concentrations. Results with the rapid mixer confirmed this finding. Low concentrations of La (0·2 μM) reduced K-stimulated Ca uptake at 1 s by nearly three-quarters, but had no effect on the rate of K-stimulated Ca uptake that was measured after 1–10 s (Fig. 3, filled symbols). High concentrations of La (4 mM; Fig. 2, △) effectively blocked all K-stimulated Ca uptake (also see Fig. 7), even after 10 s of depolarization. These results are a further indication that the properties of the synaptosomes are unchanged by rapid mixing.

The time course of K-stimulated Ca uptake in synaptosomes was determined initially in solutions with low (0·02 μM) concentrations of Ca, so as to minimize possible effects of Ca loading on the nerve terminals. In these solutions the rate of K-stimulated Ca uptake was high at first, and declined rapidly during the course of a 10 s depolarization (Fig. 2, ▲). Similar results were seen in eight additional experiments; the average time course of K-stimulated Ca uptake (nine experiments) is shown in Fig. 4. The average rate (nmol/mg protein . s) of K-stimulated Ca uptake

measured over the earliest (0–0·14 s) interval, 0·17 ± 0·04, was 8 times greater than the average rate measured over the latest (5–10 s) interval, 0·02 ± 0·01. Thus, even under conditions where there was little Ca loading of the nerve terminals, the rate of K-stimulated Ca uptake declined precipitously after a few seconds of depolarization.

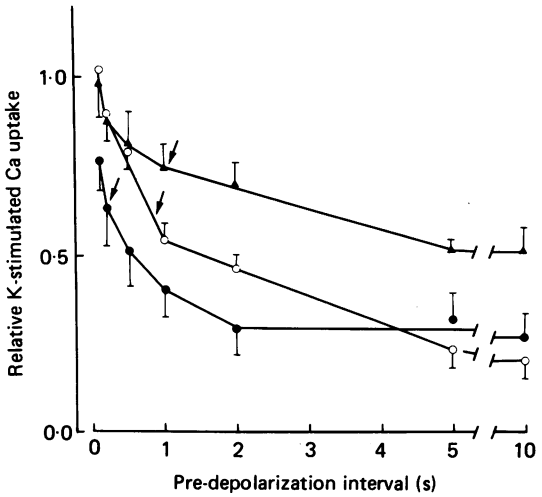

Fig. 5. The effect of Ca and pre-depolarization on K-stimulated Ca uptake. Synaptosomes were depolarized with the rapid mixer for varying time intervals before the addition of radiotracer (pre-depolarized). After the pre-depolarization intervals indicated in the Figure, radiotracer Ca was added and uptake was allowed to proceed for 2 s before the addition of choline + EGTA quench solution. The K-stimulated uptake values have been normalized to the uptake obtained after 2 s in non-pre-depolarized synaptosomes (pre-depolarization interval = 0 s). The arrows indicate the half-times for inactivation, i.e. the pre-depolarization intervals at which K-stimulated Ca uptake was half-way between the initial and steady-state levels. ▲, pre-depolarization in solution with no added Ca and 20 μM-EGTA; uptake in 30 μM-Ca and 10 μM-EGTA. ○, pre-depolarization in 40 μM-Ca and 20 μM-EGTA; uptake in 30 μM-Ca and 10 μM-EGTA. ●, pre-depolarization in 0·5 mM-Ca; uptake in 0·25 mM-Ca and 0·2 mM-EGTA. Each data point represents the mean (± S.E. of mean) of three to five experiments.

The effect of pre-depolarization on K-stimulated Ca uptake

The decline in rate of K-stimulated Ca uptake with increasing times of depolarization is not due to backflux of radiotracer from the synaptosomes. This was demonstrated (Nachshen & Blaustein, 1980) by depolarizing synaptosomes in solutions without radiotracer, and observing that the subsequent rate of K-stimulated Ca uptake was substantially reduced (inactivated). I have been able to study this inactivation process more carefully, by using the rapid mixer to control the duration of the depolarization interval prior to the addition of radiotracer (pre-depolarization interval). Fig. 5 (○) shows that when synaptosomes were pre-depolarized in a solution with low concentrations (0·02 mM) of unlabelled Ca for periods of 0·1–10 s, the rate of K-stimulated Ca uptake measured over subsequent 2 s intervals was significantly diminished. The rate of decline was steep at first and gradually levelled off. K-stimulated Ca uptake reached a nearly steady level after 5–10 s. The half-time

for inactivation (time required for K-stimulated Ca uptake to reach a level half-way between the initial and steady-state values; indicated by the arrow in Fig. 5) was slightly less than 1 s in solutions containing 0·02 mM-Ca.

Inactivation of K-stimulated Ca uptake could also be induced when there was no Ca loading of the synaptosomes. When Ca was omitted from the pre-depolarization solution, and EGTA (20 μM) was added to remove contaminant Ca (< 10 μM;

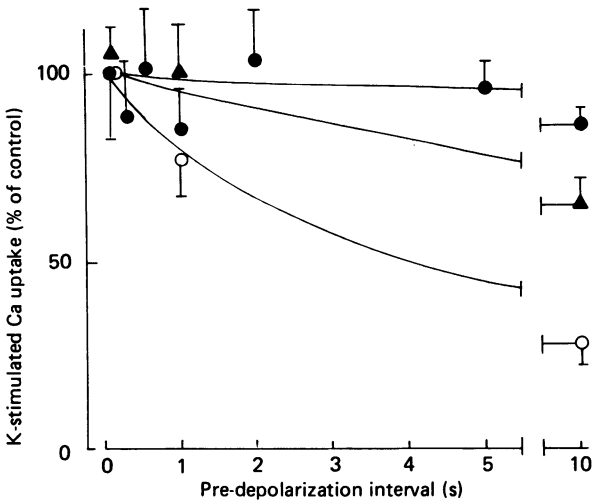

Fig. 6. The effect of different K concentrations in the pre-depolarization solution on K-stimulated Ca uptake. Synaptosomes were pre-depolarized in solutions without radiotracer, containing 137·5 mM-Na + 12·5 mM-K (●); 130 mM-Na + 20 mM-K (▲); or 72·5 mM-Na + 77·5 mM-K (○). K-stimulated Ca uptake, after pre-incubation without radiotracer, lasted for 2 s, in solution containing 72·5 mM-Na + 77·5 mM-K. Pre-depolarization solutions contained 0 Ca + 20 μM-EGTA, and the uptake solutions contained 30 μM-Ca + 10 μM-EGTA. The rapid mixer was used to control the pre-depolarization interval, and to initiate ^{45}Ca uptake.

determined by atomic absorption spectroscopy) there was still a significant reduction in the subsequent rate of K-stimulated Ca uptake (Fig. 5, ▲). Ca removal did, however, diminish the level of inactivation that was attained after 10 s of pre-depolarization; also, the half-time for inactivation was increased slightly to a value of 1–2 s.

Since one-half of the Na was replaced by K in the pre-depolarization solutions, it was important to establish whether lowering the external Na concentration could, in itself, induce inactivation of subsequent Ca uptake. Synaptosomes were equilibrated in Na solution without Ca (+20 μM-EGTA) and transferred to solutions with one-half of the Na replaced by choline or K. Aliquots of these suspensions were removed after intervals of 3–10 s, and K-stimulated Ca uptake was determined. Whereas pre-incubation in solution with one-half of the Na replaced by K (i.e. pre-depolarization) significantly reduced K-stimulated uptake, pre-incubation in solution with one-half of the Na replaced by choline had no significant effect on subsequent K-stimulated uptake (not shown).

The amount of inactivation that is induced by pre-incubation in high-K solution depends on the K concentration used to depolarize the synaptosomes during the pre-depolarization interval. When synaptosomes were pre-depolarized for as long as 10 s in 12·5 mM-K (Fig. 6, ●), there was no significant inactivation. When the K concentration was increased to 20 mM, there was no significant inactivation at 0·1 or 1 s, but it was significant after 10 s (Fig. 6, ▲). Further experiments are required to determine whether altering the membrane potential affects the rate of inactivation or the steady-state level of inactivation that is attained after prolonged depolarization.

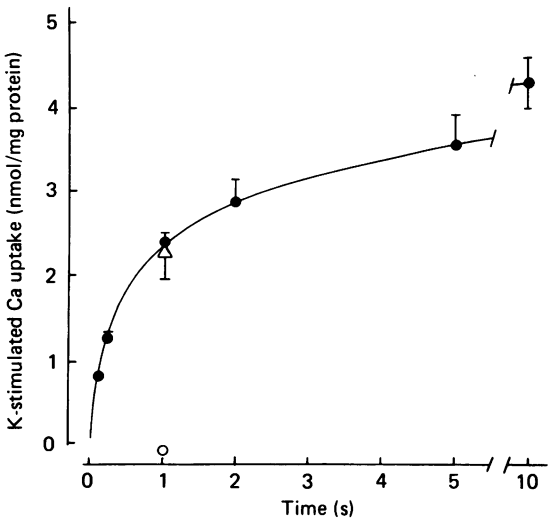

Fig. 7. The time course of K-stimulated Ca uptake in the presence of 0·5 mM-Ca. Data points obtained using the rapid mixer are indicated by the circles. △, K-stimulated Ca uptake at 1 s, determined using the hand-pipetting technique in the same experiment. ○, Ca uptake measured at 1 s in solutions containing 4 mM-La, using the rapid mixer.

The effect of high Ca concentration on the time course and the inactivation of K-stimulated Ca uptake

Although the results in the preceding section indicate that inactivation of K-stimulated Ca uptake can proceed in the absence of Ca, they also show that Ca removal does affect the inactivation process (Fig. 5, compare ▲ and ○). The time course of K-stimulated Ca uptake and the time course of inactivation were therefore examined in solutions containing high (0·5–2 mM) concentrations of Ca.

The effect of high Ca on the time course of K-stimulated Ca uptake is shown in Fig. 7. The most pronounced effect of high Ca was to enhance the decline in rate of K-stimulated Ca uptake during prolonged depolarizations. In three different experiments the rate of uptake measured over the earliest (0–0·12 s) interval was 36–44 times greater than that measured over the latest (5–10 s) interval. In accordance with this finding, when synaptosomes were pre-depolarized in solutions containing 0·5 mM-Ca, the half-time for inactivation of K-stimulated Ca uptake (measured in solutions containing 0·05 mM-Ca; Fig. 5, ●) was reduced to 0·25 s from a value of 1 s.

Does Ca loading of the synaptosomes without depolarization also induce inactivation of K-stimulated Ca uptake? I attempted to answer this question by incubating synaptosomes in low-Na solution with 0·5 mM-Ca for 3 min prior to assaying K-stimulated Ca uptake. Under these incubation conditions, 2·5 nmol Ca/mg protein was taken up, an amount equivalent to the K-stimulated Ca uptake after 2 s in high-Ca solution (Fig. 7). The extra Ca uptake in low-Na solution is presumably mediated by a Na–Ca exchanger in the presynaptic nerve terminals (Blaustein & Oborn, 1975). Pre-incubation in low-Na, high-Ca medium did not significantly reduce subsequent K-stimulated Ca uptake (not shown). This result is consistent with the notion that Ca-loading of the nerve terminals in the absence of depolarization does not induce inactivation of K-stimulated Ca uptake, and that there is little Ca-dependent inactivation (Brehm, Eckert & Tillotson, 1980; Eckert & Tillotson, 1981) of the presynaptic Ca channels. However, it must be pointed out that a Ca load taken up during a brief 1–2 s interval may be handled quite differently by the nerve terminal than an equivalent Ca load that is taken up over the course of 3 min, and may affect K-stimulated Ca uptake to a much greater degree.

The effect of Ba and Sr on the time course of K-stimulated Ca uptake

Since Sr and Ba permeate through the Ca channels in synaptosomes (Nachshen & Blaustein, 1982), I have investigated whether these ions might mimic the effect of high-Ca concentrations on the time course of K-stimulated Ca uptake.

The addition of 10 mM-Ba to solutions containing 0·02 mM-Ca reduced K-stimulated Ca uptake at 1 s from a control value of $0{\cdot}06 \pm 0{\cdot}01$ nmol/mg protein (eight experiments) to a value of $0{\cdot}02 \pm 0{\cdot}003$ nmol/mg protein (five experiments). K-stimulated ^{133}Ba uptake at 1 s, measured in separate experiments employing the hand-pipetting technique (see Nachshen & Blaustein, 1982), averaged $4{\cdot}3 \pm 1{\cdot}0$ ($n = 4$) nmol/mg protein. Despite the sizeable uptake of Ba, and the reduction in Ca uptake, the shape of the curve relating uptake to time was not significantly altered (Fig. 8). The rate of K-stimulated Ca uptake in solutions containing 10 mM-Ba was $\sim$ 10 times lower at 5–10 s than it was at 0–0·1 s. This decline in rate of uptake is similar to the 8-fold decline in rate of K-stimulated Ca uptake that was measured in low-Ca solutions without Ba.

In solutions containing 10 mM-Sr, K-stimulated Ca uptake was reduced by two-thirds, and the K-stimulated ^{85}Sr uptake at 1 s was $6{\cdot}2 \pm 0{\cdot}5$ nmol/mg protein ($n = 3$). Unlike Ba, however, Sr drastically altered the time course of K-stimulated Ca uptake (Fig. 9). The rate of Ca uptake measured over the latest (5–10 s) interval was 60 times smaller than the rate of Ca uptake measured over the earliest (0–0·12 s) interval. Thus Sr, but not Ba, mimics the effects of Ca (continuous line, Fig. 9), and accelerates the decline in rate of K-stimulated Ca uptake that occurs with prolonged depolarization.

The effect of depolarization on K-stimulated Ca uptake in the presence of Ca-channel blockers

K-stimulated Ca uptake in synaptosomes is reduced by a variety of Ca-channel blockers (Hagiwara & Byerly, 1981), including the organic Ca antagonists, transition metals and lanthanides (Blaustein, 1975; Nachshen & Blaustein, 1979, 1980;

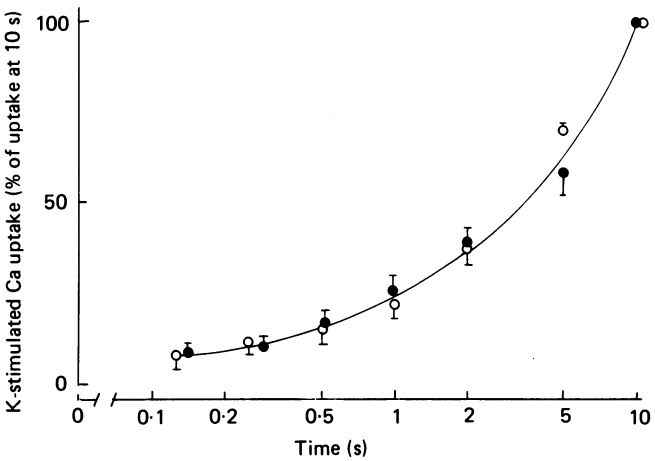

Fig. 8. The time course of K-stimulated Ca uptake in the presence (five experiments, ●), and absence (nine experiments, ○) of 10 mM-Ba. All solutions contained 0·02 mM-Ca. The results have been normalized to the uptake measured at 10 s, and are shown on a semilogarithmic scale to facilitate comparison between two sets of data points. Values for K-stimulated Ca uptake in the absence of Ba are replotted from Fig. 4*A*. All experiments were carried out using the rapid-mixing technique.

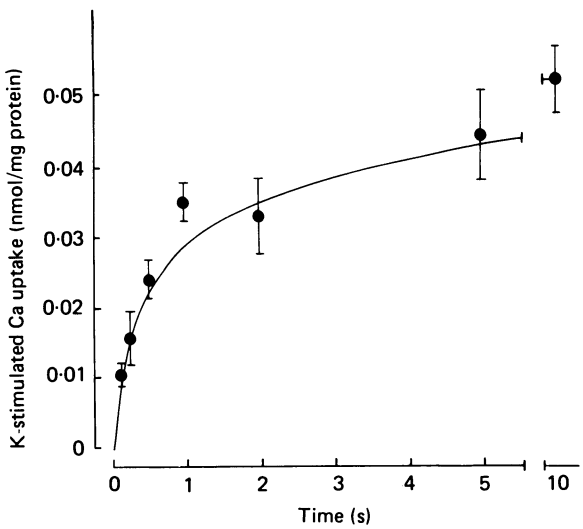

Fig. 9. The time course of K-stimulated Ca uptake in the presence of 10 mM-Sr and 0·02 mM-Ca. The line indicates the normalized time course of K-stimulated Ca (0·5 mM) uptake in the absence of Sr, drawn from Fig. 7, and normalized to the uptake measured at 10 s.

Nachshen, 1984*b*). Since it has been suggested that some Ca-channel blockers bind preferentially to open or to inactivated Ca channels (Lee & Tsien, 1983), it was of some interest to determine whether pre-depolarization of the nerve terminals in the presence of inhibitory agents might alter subsequent block of K-stimulated Ca uptake.

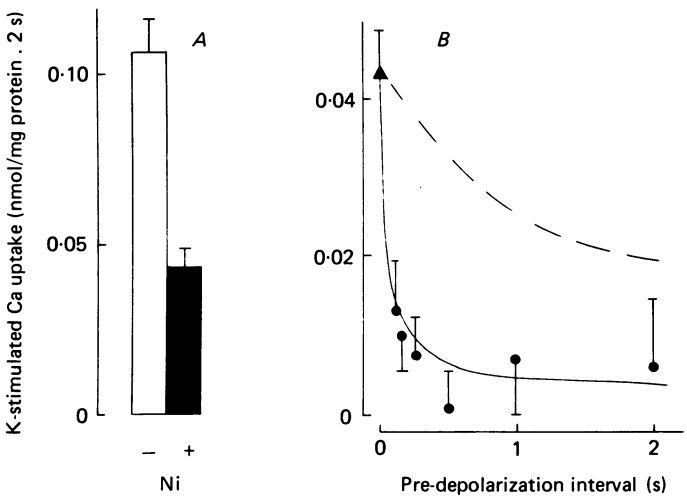

Fig. 10. The effect of Ni (40 μM) on K-stimulated Ca uptake, with and without pre-depolarization. *A*, synaptosomes were resuspended in Na solution, and mixed with low- or high-K solutions containing radiotracer, with (filled bar) or without (open bar) Ni. Uptake was terminated after 2 s. *B*, synaptosomes were pre-depolarized in the presence of Ni (●), for times ranging from 0·1 to 2 s. After the pre-depolarization interval, the suspensions were mixed with solutions containing radiotracer and Ni, and K-stimulated Ca uptake at 2 s was measured. ▲, K-stimulated Ca uptake in the presence of Ni, for non-pre-depolarized synaptosomes (pre-depolarization interval = 0), i.e. the same value as indicated by the filled bar. The dashed line indicates the time course of inactivation after pre-depolarization in 0·02 mM-Ca, replotted from the open circles in Fig. 5 and scaled to the K-stimulated Ca uptake in the presence of Ni without pre-depolarization. Uptake was determined using the rapid mixer. *A* and *B* are from the same experiment.

Results from an experiment with the transition metal, Ni, are shown in Fig. 10. When non-pre-depolarized synaptosomes were mixed with solutions containing ^{45}Ca and Ni (40 μM), K-stimulated Ca uptake was reduced by ~ 60 % (Fig. 10, filled bar) relative to the control K-stimulated Ca uptake (Fig. 10, open bar). Block of K-stimulated Ca uptake by Ni was not enhanced when synaptosomes were resuspended in non-depolarizing Na solution with Ni for 30 min before K-stimulated Ca uptake was measured (Fig. 4*A* in Nachshen (1984*b*)). However, when synaptosomes were pre-depolarized in the presence of Ni, for times as brief as 0·1 s, subsequent K-stimulated Ca uptake in the presence of Ni was almost completely abolished (Fig. 10). The effect of different intervals of pre-depolarization in the presence of Ni on subsequent K-stimulated Ca uptake is indicated by the filled circles in Fig. 10; the apparent half-time for inactivation in the presence of Ni is less than 0·1 s. The dashed line in Fig. 10 shows the expected time course of inactivation in solutions containing

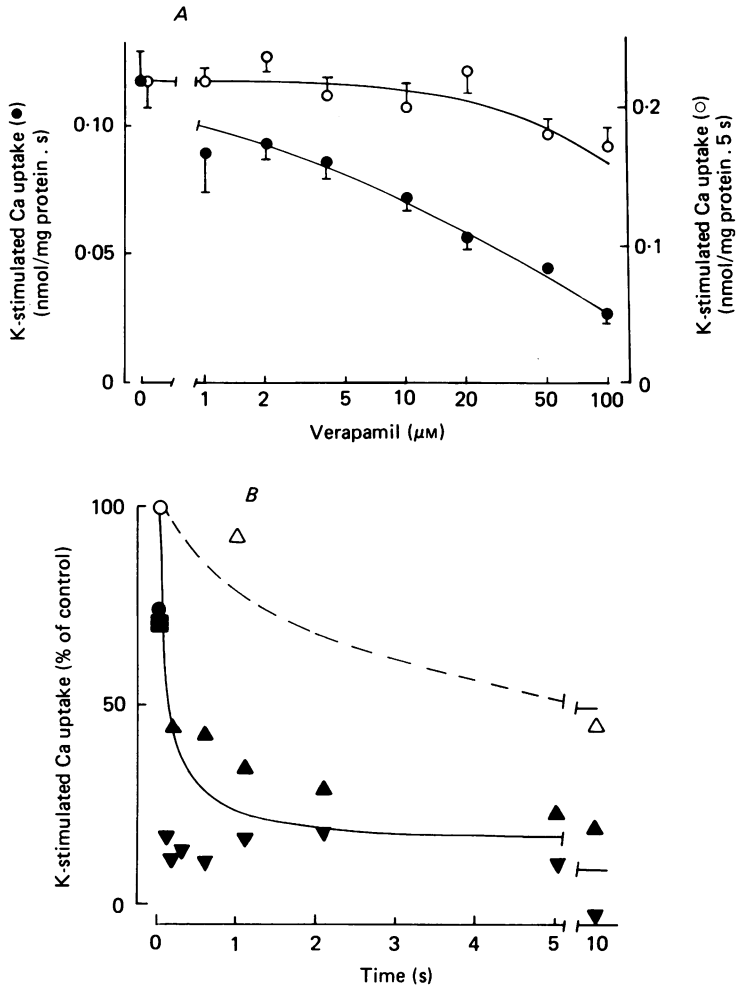

Fig. 11. The effect of verapamil on K-stimulated Ca uptake, with and without pre-depolarization. *A*, synaptosomes were resuspended in Na solutions and mixed for 1 s with low- or high-K solutions containing radiotracer and verapamil (●, ordinate on the left). Alternatively, the synaptosomes were mixed with high-K solution *without* radiotracer or verapamil; after 10 s pre-depolarization, solutions containing radiotracer and verapamil were added, and K-stimulated Ca uptake at 5 s was measured (○, ordinate on the right). These experiments were done using the hand-pipetting technique. All solutions contained 0·02 mM-Ca. *B*, the effect of pre-depolarization in the presence of verapamil (10 μM) on K-stimulated Ca uptake. Synaptosomes were pre-depolarized for varying intervals in the presence (▲, ▼) or absence (△) of verapamil, and K-stimulated Ca uptake was measured at 2 s after pre-depolarization. Alternatively, the synaptosomes were mixed with non-depolarizing solution without verapamil or radiotracer; solutions containing radiotracer with (●, ■) or without (○) verapamil were added, and K-stimulated Ca uptake was measured at 2 s. Results from two different experiments (▲ △ ●; ▼ ■) are shown, normalized to the K-stimulated Ca uptake obtained in the absence of either pre-depolarization or verapamil (pre-depolarization interval = 0). Mixing was controlled by the rapid-mixer. Pre-depolarization and pre-incubation solutions contained 0 Ca + 20 μM-EGTA. Uptake solutions contained 30 μM-Ca EGTA. The dashed line indicates the time course of inactivation expected for synaptosomes in 0 Ca + 20 μM-EGTA, replotted from Figure 5. Error bars have been omitted for the sake of clarity

0·02 mM-Ca, replotted from Fig. 4, and scaled to the K-stimulated Ca uptake measured in the presence of Ni, without pre-depolarization (Fig. 10, ▲).

Similar results were obtained with 0·2 M-La (not shown), and with the organic Ca antagonist, verapamil (Fig. 11). Fig. 11*A* (filled symbols) shows that when synaptosomes in Na solution were mixed with solutions containing verapamil and ^{45}Ca, there was a concentration-dependent block of K-stimulated Ca uptake at 1 s; half-maximal inhibition occurred at verapamil concentrations of 10–20 μM. In addition, if the synaptosomes were depolarized for 10 s in solutions *without* verapamil or radiotracer, subsequent K-stimulated Ca uptake was only slightly reduced by verapamil, even at concentrations as high as 100 μM (Fig. 11*A*, ○). However, if synaptosomes were pre-depolarized *in the presence* of 10 μM-verapamil, for times as brief as 0·1 s, subsequent K-stimulated Ca uptake in the presence of verapamil was almost completely abolished (Fig. 11*B*, filled symbols).

DISCUSSION

The experiments described in this report show that K-stimulated (voltage-dependent; Nachshen & Blaustein, 1980, 1982) Ca uptake in synaptosomes can be accurately measured after a time as short as 0·1 s. Reliable measurement of Ca uptake in the interval immediately following depolarization of the nerve terminals is important, because this Ca uptake is likely to be involved in neurotransmitter release. Studies of [^{3}H]dopamine release from striatal synaptosomes (Drapeau & Blaustein, 1983) indicate that the rate of release during the initial second of depolarization is 7 times higher than the rate of release averaged over the next 9 s, and approaches physiological rates of release. In addition, neurotransmitter release is well correlated with Ca entry during the initial second but not after longer times. An important goal of future studies is to develop methods for measuring both Ca uptake and transmitter release in synaptosomes after similar, more physiologically relevant, brief intervals after depolarization.

The rate of voltage-dependent Ca uptake in synaptosomes is ~ 6 nmol/mg protein . s during the initial 0·1 s of depolarization, when the external solution contains 0·5–2·0 mM-Ca. This rate of Ca uptake is ~ 4 times greater than previous estimates that are based on measurements made at 1 s (Nachshen & Blaustein, 1980). Assuming that the internal volume of the synaptosomes is ~ 4 μl/mg protein (Blaustein, 1975), the amount of Ca that would be taken up by the synaptosomes during a 1 ms depolarization (the duration of the nerve-terminal action potential), would be sufficient to raise the internal Ca concentration from a resting value of -10^{-7} M (Nachshen, 1984*a*) to a value above 10^{-6} M if the Ca load were distributed homogeneously in the nerve terminal cytoplasm. Interestingly, the exocytosis of catecholamine from leaky bovine adrenal medullary cells has been found to be half-maximal at Ca concentrations of ~ 10^{-6} M (Knight & Baker, 1982).

Resolution of K-stimulated Ca uptake at times < 0·1 s is limited at present by the substantial binding of Ca to the synaptosomes. Although Ca binding can be reduced by using a choline + EGTA quench solution (M. P. Blaustein, D. A. Nachshen, E. Rasgado-Flores & S. Sanchez-Armass, unpublished observations), the ^{45}Ca reaction mixture must be diluted with a large volume of this quench solution to block Ca

efflux effectively. With the present configuration of the rapid-mixing device this is only possible with a delay of 1–2 s for sample collection and handling. However, it should be possible to improve the time resolution to the limits of the capabilities of the instrument (20–30 ms) by modifying the rapid mixer.

The most notable feature of the time course of K-stimulated Ca uptake is the rapid decline in rate of uptake after depolarization for as short a time as 0·1 s. This decline is not caused by tracer efflux or by Ca loading of the synaptosomes, and might reflect inactivation of the Ca channels in the nerve terminals. Ca-channel inactivation has been described in many different preparations (see reviews by Hagiwara & Byerly, 1981; Tsien, 1983). In some cells the inactivation is Ca dependent (Brehm *et al.* 1980; Eckert & Tillotson, 1981; Ashcroft & Stanfield, 1981), whereas in other cells the inactivation is voltage rather than Ca dependent (Fox, 1981; Cota, Nicola Siri & Stefani, 1983). Results obtained by depolarizing synaptosomes in Ca-free solution show clearly that inactivation can occur in the absence of external Ca. It is unlikely that the inactivation in Ca-free solution is triggered by the release of internal Ca from intraterminal stores: when synaptosomes are depolarized in solutions without Ca there is no increase in the free intraterminal Ca concentration (D. A. Nachshen, unpublished observations), as measured by the fluorescent Ca indicator, Quin 2 (Tsien, Pozzan & Rink, 1982). Thus, Ca-channel inactivation in synaptosomes appears to be primarily voltage rather than Ca dependent.

However, high concentrations of external Ca do accelerate the rate of decline of K-stimulated Ca uptake. There is evidence that both voltage-dependent and Ca-dependent inactivation are significant in some cells (see Tsien, 1983), and it is possible that Ca does affect the kinetics of Ca-channel inactivation in synaptosomes. However, other explanations for the effect of high Ca on K-stimulated Ca uptake cannot be ruled out: Ca loading of the nerve terminal might reduce the electrochemical driving force for Ca entry, limit the capacity of the nerve terminals to take up Ca, or increase the rate of Ca efflux.

Some evidence against a direct effect of Ca on the Ca channels is provided by experiments with Ba and Sr (Figs. 8 and 9). These divalent ions are known to reduce Ca-dependent inactivation of Ca channels when they are the current carriers (see Tsien, 1983). None the less, Sr and Ba did not prevent the decline in rate of K-stimulated Ca uptake with prolonged depolarization. Indeed, Sr mimicked the effect of Ca, and accelerated the decline in rate of Ca uptake (compare Figs. 7 and 9). It is interesting to note that Sr, unlike Ba, utilizes the Na-dependent Ca extrusion mechanism (Sanchez-Armass, Nachshen & Blaustein, 1982) and is readily taken up by the non-mitochondrial (ATP-dependent) Ca buffering system (Rasgado-Flores, Nachshen & Blaustein, 1982) in synaptosomes. Further studies of the effects of Sr and Ba on intraterminal Ca metabolism may be useful for determining whether Ca extrusion and sequestration can modify the early time course of K-stimulated Ca uptake.

K-stimulated Ca uptake was not completely abolished after prolonged depolarization in high-K solution (Fig. 4; also see Nachshen & Blaustein, 1980). One explanation of this result (Nachshen & Blaustein, 1980) is that there are two populations of Ca channels in synaptosomes, only one of which inactivates. In support of this hypothesis, the inactivating and the non-inactivating components of K-stimulated

Ca uptake were observed to have different sensitivities to a variety of Ca-channel blockers, including La and Mn. The results described in this report, show, however, that there may be a complicated interaction between blockers and nerve-terminal depolarization, even after a time as short as 0·1 s after depolarization of the nerve terminals. Thus, it is possible that the Ca channels in brain nerve endings undergo transition among several substates (e.g. closed, open and inactivated), with differing affinities for verapamil, La and other inhibitory ions.

An unexpected finding in these studies is that inhibition of K-stimulated Ca uptake by Ni, La and verapamil was enhanced in synaptosomes that were pre-depolarized for brief (< 1 s) intervals in the presence of these Ca-channel blockers. Results obtained with verapamil are particularly striking, because previous studies have shown that the organic Ca antagonists, effective blockers of Ca channels in heart and smooth muscle (Fleckenstein, 1977), are weak blockers of Ca channels in presynaptic nerve terminals (Hauesler, 1972; Van der Kloot & Kita, 1975; Gotgilf & Magazanik, 1977; Nachshen & Blaustein, 1979), and act only at high (> 10 μM) concentrations. However, Ca channels in brain synaptosomes are sensitive to block by the organic Ca antagonist verapamil, at concentration < 10 μM, if the nerve terminals are depolarized for a short time in the presence of the drug before measuring uptake. The implication of this finding is that differential sensitivity to verapamil, and possibly other organic Ca antagonists, may in part be a consequence of differences in the voltage dependence of either drug–channel interaction or of the Ca channel itself. The increased inhibition that was observed when synaptosomes were pre-depolarized in the presence of blockers could be caused either by an increase in the rate of Ca-channel inactivation, or by an increase in the affinity of the Ca channels for these blockers. In either case, this potentiation of blocking efficacy is transient, because inhibition of Ca uptake is not enhanced and is even reduced when these blockers are added after pre-depolarization for 10 s (e.g. Fig. 11*A*; also see Nachshen & Blaustein, 1980). Additional investigation is necessary to determine the mechanism of enhanced Ca-channel inhibition.

I thank Professor M. P. Blaustein for his encouragement and helpful comments. Expert technical assistance was provided by Mr G. Walker. The work was supported by U.S. Public Health Service Grants NS 16461 and 20464 to D. Nachshen, and NS 16106 to M. P. Blaustein.

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