J. Physiol. (1975), **247**, *pp.* 657–686
With 9 text-figures
Printed in Great Britain

THE INFLUENCE OF SODIUM ON CALCIUM FLUXES IN PINCHED-OFF NERVE TERMINALS *IN VITRO*

BY M. P. BLAUSTEIN AND CAROL JEAN OBORN

From the Department of Physiology and Biophysics, Washington University School of Medicine, St Louis, Missouri 63110 U.S.A.

(*Received 17 July 1974*)

SUMMARY

1. The influence of internal and external Na concentrations on Ca movements have been measured in pinched-off presynaptic nerve terminals (synaptosomes). Ca uptake is enhanced when external Na (Na_o) is replaced by Li, choline or dextrose, in Na-loaded synaptosomes. Depletion of internal Na (Na_i) abolishes this stimulatory effect of external Na removal.

2. Ca uptake from Na-depleted media is proportional to $[Na]_i^2$, and averages about 1·5 μmole Ca/g synaptosome protein per minute when $[Na]_i$ is approximately 137 mM. This may correspond to a Ca influx of about 0·1 p-mole/cm^2 sec.

3. External Na is a competitive inhibitor of the Na_i-dependent Ca uptake. The interrelationship between $[Na]_o$, $[Ca]_o$ and Ca uptake indicate that two external Na ions may compete with one Ca at each uptake site.

4. The distribution of particles with Na_i-dependent Ca uptake activity parallels the distribution of synaptosomes in the preparative sucrose gradient. Thus, this Ca uptake activity is probably a property of the pinched-off nerve terminals *per se*, and not of the mitochondria which may contaminate the synaptosome fraction.

5. The Na_i-dependent Ca uptake mechanism requires an intact surface membrane, since synaptosomes subjected to osmotic lysis lose the ability to accumulate Ca by this route.

6. Ca efflux into Ca-free media is largely dependent upon the presence of external Na. The curve relating Ca efflux to $[Na]_o$ is sigmoid, and suggests that more than one external Na ion (perhaps 2 or 3) is needed to activate the efflux of each Ca ion.

7. The net Ca gain exhibited by Na-loaded synaptosomes incubated in Na-depleted media can be accounted for by the increased Ca uptake and decreased Ca loss observed under these conditions.

8. Treatment of synaptosomes with cyanide or 2,4-dinitrophenol decreases Ca uptake and enhances Ca efflux into Na-containing media. This results in a net loss of Ca from the terminals, even in the presence of external Ca.

9. In contrast to the Ca efflux from synaptosomes, the Ca efflux from brain mitochondria is not dependent upon external Na, and is reduced by succinate, a substrate which is known to fuel mitochondrial respiration.

10. The temperature coefficient (Q_{10}) of the Na_i-dependent Ca uptake is about 3.

11. The Na_i-dependent Ca uptake is reduced at low pH. The relationship between this Ca uptake and pH approximates a titration curve with a pK_a of about 5·6.

12. The data indicate that Ca transport in rat brain presynaptic terminals may involve a carrier-mediated Na–Ca exchange mechanism, and that some of the energy required for Ca extrusion may come from the Na electrochemical gradient across the surface membranes.

INTRODUCTION

It is well known that most cells, including neurones (e.g. Baker, 1972; Blaustein, 1974), are not completely impermeable to Ca. As shown by Hodgkin & Keynes (1957), squid axons gain a small amount of Ca during excitation. Furthermore, as discussed in the preceding article (Blaustein, 1975), an inward movement of Ca ions at nerve terminals may play a critical role in triggering neural transmitter release. In order to remain in steady Ca balance, the Ca which enters during activity must be extruded subsequently. The Nernst equation predicts that for cells with a membrane potential of the order of -60 mV (cytoplasm negative; cf. Blaustein & Goldring, 1975), and an extracellular Ca concentration of about 10^{-3} M, the intracellular Ca concentration at equilibrium should be about 10^{-1} M. Instead, the total Ca content of mammalian brain tissue, including synaptosomes (cf. Blaustein & Goldring, 1975), is only of the order of 10^{-3} mole/kg (Lolley, 1963; Tower, 1969). Moreover, much of the cellular Ca is apparently sequestrated in intracellular organelles such as mitochondria (Tower, 1968; Blaustein & Goldring, 1975). Although the intracellular ionized Ca^{2+} concentration ($[Ca^{2+}]_i$) of mammalian central neurones cannot be determined with certainty, evidence extrapolated from other preparations suggests that it may be rather low (perhaps 10^{-6} M or less): in squid giant axons, the axoplasmic $[Ca^{2+}]_i$ is only about 10^{-6} M or less (cf. Baker, 1972; Blaustein, 1974), and mitochondria from at least some mammalian tissues are apparently unable to carry out oxidative phosphorylation when the ambient (i.e. cytoplasmic) $[Ca^{2+}]$ exceeds about 10^{-6} M (cf. Lehninger, 1970).

Despite the gaps in our knowledge, the foregoing considerations clearly indicate that mammalian central neurones must be able to extrude Ca against a very large electrochemical gradient. The mechanism underlying this 'uphill' transport process is unknown. In squid axons (Blaustein & Hodgkin, 1969; Blaustein, Russell & De Weer, 1974) and crab neurones (Baker & Blaustein, 1968), there is evidence that Ca extrusion may involve an exchange for Na ions, and that the inward ('downhill') movement of Na may provide at least some of the energy for the uphill transport of Ca. Preliminary evidence (Blaustein & Wiesmann, 1970) indicated that a similar Na–Ca exchange mechanism may operate in pinched-off mammalian presynaptic nerve terminals. Kalix (1971) has reported that Ca efflux from mammalian peripheral nerve is dependent upon external Na. An external Na-dependent Ca efflux has also been observed in mammalian brain slices (Cooke & Robinson, 1971; Bull & Trevor, 1972; Stahl & Swanson, 1972), although there is uncertainty as to which cellular elements (neurones and/or glia) are responsible for this effect. In addition, there is now evidence that Na–Ca exchange may play an important role in Ca transport in a large variety of tissues including many types of muscle, secretory tissues, intestinal mucosa and renal epithelia (Blaustein, 1974).

Some of the kinetic properties of Na-dependent Ca fluxes, and the influence of metabolic poisons on Na–Ca exchange at presynaptic endings are discussed in the present report. The Na-dependent Ca fluxes appear to involve a mechanism which operates in parallel with, but independently of, the potential-dependent Ca permeability increase described in the preceding article (Blaustein, 1975).

METHODS

Solutions

The composition of most of the solutions used in these experiments has already been described (Blaustein & Goldring, 1975; Blaustein, 1975); unless otherwise noted, the Ca concentration in Ca-containing solutions was 1·2 mM. Not previously mentioned were the solutions used to test the effect of pH on ^{45}Ca uptake, and the 'mitochondrial medium'. The latter (cf. Bradford, 1969) contained (all in m-mole/l.): KCl, 140; KH_2PO_4, 40; $MgSO_4$, 6; and sucrose, 20; buffered to pH 7·2 (30° C) with tris base. Information about the addition of substrates (glucose or succinate) will be given in the Results section.

The acetylcholine and tris maleate buffer solutions (cf. Hutter & Warner, 1967) used to study the pH dependence of ^{45}Ca uptake contained (all in m-mole/l.): LiCl, 137; $MgCl_2$, 1·3; $CaCl_2$, 1·2; glucose, 5; acetylglycine, 10; and maleic acid, 10. They were titrated to the desired pH (between 4·0 and 8·0) with Tris base before being made up to volume.

Experimental procedures

Ca uptake. The preparation of synaptosomes (and mitochondria) from whole rat brain has already been described (Blaustein & Goldring, 1975), as have methods

for measuring ^{45}Ca uptake and net Ca accumulation (Blaustein, 1975). In nearly all of the Ca uptake experiments in the present study, however, the intracellular Na concentration was controlled by pre-incubating synaptosomes for 12–15 min at 30° C in mixtures of Ca-free K-free Na and Ca-free K-free Li (137 mM-Li); these solutions also contained 10^{-3} M ouabain to inhibit the Na–K exchange pump. Synaptosomes incubated with ^{42}K or ^{22}Na attain, respectively, a constant ^{42}K (Escueta & Appel, 1969) or ^{22}Na (Ling & Abdel-Latif, 1968), content within 5–10 min. It will therefore be assumed that after the 12–15 min pre-incubation the synaptosome Na concentration closely approximates that of the pre-incubation medium.

Following the pre-incubation, the synaptosome suspensions were centrifuged at 9000*g* for 5 min, at 3° C, and then re-suspended in appropriate Ca-containing solutions. For ^{45}Ca-uptake experiments, the synaptosome pellets were first re-suspended in unlabelled Ca-containing solutions, and diluted 10 sec later with ^{45}Ca-containing solutions of otherwise identical composition. The method of terminating Ca uptake with EGTA and removing extracellular Ca has already been described (Blaustein, 1975). Details of individual experiments will be given in the Results section.

Net Ca efflux. In some experiments, net Ca efflux was measured in the presence of external Ca; the standard incubation and washing protocol was similar to that used for net Ca uptake (Blaustein, 1975, and see above).

The effect of external Na on net Ca efflux was measured in the absence of external Ca. Synaptosomes from the sucrose gradient were equilibrated with Ca-free K-free Na or Ca-free K-free Li. The suspensions were centrifuged at 9000*g* for 5 min at 3° C, and resuspended in appropriate incubation solutions (see Results). Following incubation, the suspensions were again centrifuged, and the Ca content of both the pellets and the supernatant solutions was determined (cf. Blaustein & Goldring, 1975).

^{45}Ca efflux. In preliminary experiments synaptosomes were loaded with ^{45}Ca and then washed, as described for ^{45}Ca influx experiments (see above). The washed pellets were then resuspended in mixtures of K-free Na and K-free Li, or Ca-free K-free Na and Ca-free K-free Li. After a suitable incubation period (usually 5–10 min) at 30° C, the suspensions were centrifuged at 9000*g* for 5 min at 3° C; the pellet and supernatant solution were separated, and both were prepared for liquid scintillation counting (Blaustein, 1975).

An alternative protocol, which significantly shortened the duration of the experiments and improved statistical reliability was used for the ^{45}Ca efflux experiments to be described in detail below. The equilibrated synaptosomes from the sucrose gradient were suspended in a small aliquot (0·5–0·7 ml.) of Na + 5K (~ 2 mg protein/ml.) and pre-incubated for 15 min at 30° C. The terminals were then loaded with ^{45}Ca by adding an aliquot (0·4–0·7 ml.) of ^{45}Ca-containing isotonic K saline, or Na + 5K with scorpion (*Leiurus quinquestriatus*) venom 7×10^{-7} g/ml. or Na + 5K with $1{\cdot}5 \times 10^{-4}$ M veratridine (Blaustein, 1975). Ca uptake was terminated, after a further 1 or 2 min incubation at 30° C, by the rapid addition of 25–35 ml. Ca-free solution containing 1 mM-EGTA. Tetrodotoxin ($2{\cdot}5 \times 10^{-7}$ M) was also present in these solutions if scorpion venom or veratridine was used to stimulate Ca uptake (cf. Blaustein, 1975). Ca efflux did not appear to be influenced by the method of ^{45}Ca loading.

The EGTA-containing Ca-free solutions used for halting Ca uptake and measuring Ca efflux were Ca-free Na + 5K, Ca-free choline + 5K, Ca-free Li + 5K, Ca-free 264 mM sucrose + 5K, or mixtures of Ca-free Na + 5K and one of the Na-free solutions. The suspensions were maintained at 30° C. After varying periods of

time (see Results for details) 2 ml. aliquots were suction-filtered on to 25 mm-diameter glass fibre filters (Whatman GF/A) which were immediately washed 3 times with 12–15 ml. aliquots of ice-cold Ca-free Li + 5K or Ca-free choline + 5K; 'control' samples were filtered and washed immediately after terminating ^{45}Ca uptake. The entire filtration and wash procedure took less than 30 sec to complete. Preliminary experiments indicated that the glass-fibre filters retained more than 97 % of the synaptosome protein, but much less than 0·01 % of the supernatant solution ^{45}Ca; the solution flow rate through these filters was significantly more rapid than through 0·8 or 0·45 μmpore cellulose acetate filters with comparable amounts of synaptosome protein.

The washed filters were placed in liquid scintillation counting vials containing 1 ml. 0·1 % triton X-100 (Sigma Chemical Co., St Louis), and stored over-night. Bray scintillation cocktail (12 ml.) was then added, and the samples were counted in a liquid scintillation counter. Aliquots of the ^{45}Ca incubation solution were diluted with 0·1 % triton X-100 and counted to determine the specific activity of the ^{45}Ca taken up by the synaptosomes. The ^{45}Ca efflux was calculated as the difference between the ^{45}Ca content (per g protein) in the 'control' samples, and in the samples incubated in the EGTA solutions.

RESULTS

^{45}Ca uptake by synaptosomes; the effect of $[Na]_i$ and $[Na]_o$. The uptake of ^{45}Ca by synaptosomes, graphed as a function of time for incubation periods of up to 90 sec, is shown in Fig. 1. The conditions employed here differ significantly from those described in the preceding article (Blaustein, 1975), in that the synaptosomes for this study were pre-loaded with either Na or Li (Na-depleted), and incubated in media (generally K-free) containing Li or Na as the predominant cation. Fig. 1*A* indicates that the accumulation of Ca under all of these conditions is approximately linear with time for at least 90 sec. However, it is readily apparent that the rate of uptake is affected by changes in the internal and external Na concentrations ($[Na]_i$ and $[Na]_o$, respectively). The main observation is that, for Na-loaded synaptosomes, the rate of Ca uptake from Na-free media is considerably greater than from media in which Na is the predominant cation. In this instance Li was substituted for Na, but the effect is not due to the presence of Li in the incubation medium; rather, it is a consequence of the reduction of external Na, since similar results are obtained with other Na replacements such as dextrose or choline (Fig. 3 and Table 4).

A major fraction of this Ca accumulation is dependent upon internal Na irrespective of whether Na or Li is the predominant external cation. Thus, as shown in Fig. 1*A* (curves 3 and 4), when the synaptosomes are depleted of Na prior to incubation with ^{45}Ca, the rate of Ca uptake from the Na medium is reduced, and the extra Ca uptake due to removal of external Na is abolished. Although the Na-depleted synaptosomes for this experiment were prepared by equilibrating and pre-incubating them

with Ca-free K-free Li (cf. Table 1 in Blaustein & Goldring, 1975), the differences between the Na-loaded and the Li-loaded synaptosomes are apparently not a consequence of the Li, *per se*. Table 1 shows that synaptosomes pre-incubated in Na + 5K + ouabain, or in K-free Na, take up ^{45}Ca more rapidly than do synaptosomes pre-incubated with 5 mM-K

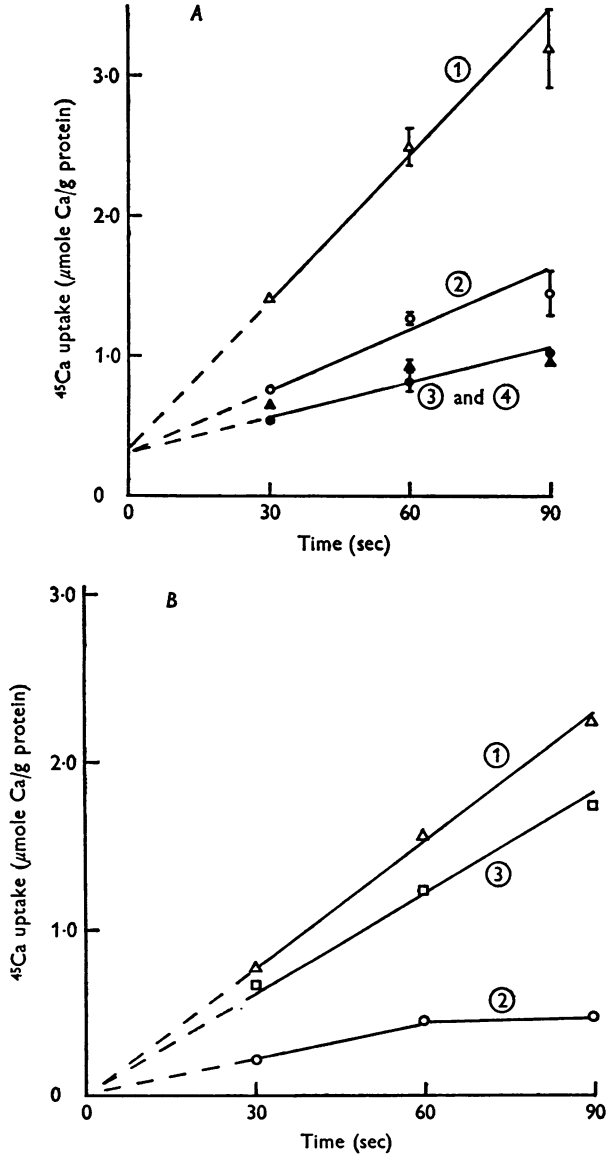

Fig. 1. For legend see facing page.

in the absence of ouabain. Pre-treatment with ouabain and/or K-free media should increase synaptosome $[Na]_i$ (Ling & Abdel-Latiff, 1968; Blaustein & Goldring, 1975). This suggests that an increase in internal Na tends to increase Ca entry in synaptosomes. With this in mind, the difference in Ca uptake between the Na-loaded and Li-loaded synaptosomes will be referred to as 'Na_i-dependent' Ca entry (Fig. 1*B*). The difference between the Ca uptake from Na-depleted media and from Na-rich (132 or 137 mM-Na) media (curve 3 of Fig. 1*B*) will be expressed as the 'low-Na stimulated' Ca influx (or, more specifically, the 'Li-stimulated' Ca influx if Li replaced external Na, as in Fig. 1).

Unfortunately, the effects of ouabain and/or K-free media (Table 1) are somewhat difficult to interpret. Although one effect of these treatments is, indeed, to increase $[Na]_i$, this occurs at the expense of a decrease in [K] and a fall in membrane potential (Blaustein & Goldring, 1975). Consequently, the increment in Ca uptake due to ouabain or K-free media might be accounted for by depolarization alone (cf. Blaustein, 1975). Depolarization cannot account for the difference in Ca uptake between Na-loaded and Li-loaded synaptosomes, however, since voltage-sensitive fluorochome studies (cf. Blaustein & Goldring, 1975) indicate that both groups of K-depleted synaptosomes may be depolarized. Thus, the Na_i-dependent Ca uptake probably occurs in the absence of a large change in membrane voltage, and can thereby be distinguished from the K-stimulated uptake described previously (Blaustein, 1975).

Further evidence that internal Li is not a specific inhibitor of Ca uptake may be inferred from the observation (data not shown) that synaptosomes pre-incubated in Ca-free K-free choline + 10^{-3} M ouabain or in Ca-free 137 mM-K do not exhibit a significant Li_o-stimulated Ca uptake.

The data in Table 2 provide more direct evidence that the 'internal' Na concentration of synaptosomes markedly influences ^{45}Ca uptake from Na-free media. Particularly noteworthy is the observation that the effects of pre-loading are at least partially reversible. Thus, replacing internal

---

Fig. 1, *A*, time course of ^{45}Ca uptake by synaptosomes as a function of internal and external Na concentration (Na replaced by Li). Curve 1 (Δ): synaptosomes pre-loaded with Na and incubated in K-free Li (with ^{45}Ca). Curve 2 (○): synaptosomes pre-loaded with Na and incubated in K-free Na. Curve 3 (●): synaptosomes incubated in K-free Na after pre-loading with Li. Curve 4 (▲): both pre-incubation and Ca incubation were in K-free Li. Each point is the mean of three determinations; the bars show the range of values where the range extends beyond the edges of the symbol. The lines were drawn by eye.

B, time course difference curves. Curve 1 (Δ) is the Na_i-dependent Ca uptake from K-free Li (curve 1 minus curve 4 of Fig. 1*A*). Curve 2 (○) depicts the Na_i-dependent Ca uptake from K-free Na (curve 2 minus curve 3 of Fig. 1*A*). Curve 3 (□), the Li-stimulated Ca uptake, is the difference between curves 1 and 2 of Fig. 1*A*.

TABLE 1. Effect of ouabain and K-free media on ^{45}Ca uptake by synaptosomes

Incubation medium*	Incubation time (min)	^{45}Ca uptake (μmole Ca/g protein per minute)		
		Total†	Increment due to ouabain or K-free media	P‡
Experiment 1				
Na + 5K	0·5	0·71 ± 0·01	—	—
Na + 5K + ouabain	0·5	0·82 ± 0·03	0·11	< 0·05
Experiment 2				
Na + 5K	6·0	0·21 ± 0·02	—	—
Na + 5K + ouabain	6·0	0·46 ± 0·04	0·25	< 0·005
Experiment 3				
Na + 5K	6·0	0·24 ± 0·01	—	—
K-free Na	6·0	0·30 ± 0·01	0·06	< 0·05
Experiment 4				
Na + 5K	2·0	0·88 ± 0·05	—	—
Na + 5K + ouabain	2·0	1·11 ± 0·03	0·23	< 0·01
K-free Na	2·0	1·10 ± 0·05	0·22	< 0·01
K-free Na + ouabain	2·0	1·08 ± 0·06	0·20	< 0·05
Li + 5K	2·0	0·50 ± 0·02	—	—
Li + 5K + ouabain	2·0	0·48 ± 0·02	−0·02	—

* Samples were equilibrated with this solution (Ca-free) and then pre-incubated in 0·5 ml. of the same (Ca-free) solution for 12 min at 30° C. Following pre-incubation, 0·5 ml. of the same solution containing 2·4 mM-Ca (unlabelled) was added, and 10 sec later 1·0 ml. of the same solution with 1·2 mM-Ca (labelled with ^{45}Ca) was added. Ouabain indicates the presence of 10^{-3} M ouabain in the pre-incubation and incubation solutions. At the end of the incubation period EGTA was added to stop ^{45}Ca uptake; the suspensions were then washed and prepared for counting (see Methods).

† Each value is the mean of three (Expts. 1–3) or four (Expt. 4) determinations ± S.E.

‡ Values of P based on Student's t test.

Li by Na during a second pre-incubation enhances Ca accumulation, while replacing internal Na by Li reduces Ca uptake.

The differential effect of 'internal' *vs.* 'external' Na also indicates that an 'osmotically intact' surface membrane is required for the Na_i-dependent and the low external Na-stimulated Ca fluxes. Previous results (Blaustein & Wiesmann, 1970 have shown that the Li-stimulated Ca accumulation is markedly reduced if the synaptosomes are exposed to very hypotonic solutions prior to incubation with ^{45}Ca. These observations further support the notion that when synaptosomes are treated 'physiologically', they tend to behave like tiny, intact 'cells'.

TABLE 2. Effects of internal Na and of ouabain on synaptosome ^{45}Ca uptake from K-free Li

Pre-incubation medium*		Total ^{45}Ca uptake†	Na_i-dependent Ca uptake‡
First pre-incubation	Second pre-incubation	(μmole Ca/g protein per minute)	
K-free Li	—	0·56 ± 0·02	—
K-free Li + ouabain	—	0·56 ± 0·02	—
K-free Li + ouabain	K-free Li + ouabain	0·44 ± 0·01	—
K-free Li + ouabain	K-free Na + ouabain	1·58 ± 0·06	1·02
K-free Na	—	1·90 ± 0·06	1·34
K-free Na + ouabain	—	2·03 ± 0·06	1·47
K-free Na + ouabain	K-free Na + ouabain	2·27 ± 0·07	1·75
K-free Na + ouabain	K-free Li + ouabain	0·94 ± 0·03	0·36

* All pre-incubation solutions were Ca-free, and, where indicated, contained 10^{-3} M ouabain. Each pre-incubation period at 30° C lasted 12 min. Synaptosome suspensions were centrifuged at the end of the first pre-incubation period and the supernatant solutions were discarded. Some of the pellets were then resuspended in 2·0 ml. of the second pre-incubation medium, and agitated for 12 min more in a 30° C water-bath. Then these samples were centrifuged and the supernatants discarded. After either the first or second pre-incubation (where indicated) the pellets were suspended in 0·5 ml. K-free Li containing 1·2 mM-Ca (unlabelled); 10 sec later, 1·0 ml. of a similar solution labelled with ^{45}Ca was added, and the suspensions were incubated for one minute at 30° C.

† Each value is the mean of three determinations ± S.E.

‡ Relative to Ca uptake by synaptosomes pre-incubated in K-free Li.

Two additional features relating to the time course of Ca accumulation by synaptosomes deserve consideration: the uptake at 'zero-time', and the uptake during prolonged incubations. When the lines in Fig. 1*A* are extrapolated to 'zero-time', they all converge to approximately the same point, a value of 'Ca uptake' which is significantly greater than zero. This effect is largely accounted for by the residual extrasynaptosomal ^{45}Ca (cf. Blaustein, 1975) which has not been completely washed out. Although the magnitude of this residual extrasynaptosomal ^{45}Ca was not determined by extrapolation in each experiment, it should be the same in all samples incubated in media containing identical Ca concentrations and ^{45}Ca specific activities. Thus, the subtractions used to determine the Na_i-dependent Ca uptake or low Na_o-stimulated Ca uptake should eliminate this residual ^{45}Ca from further consideration.

With regard to the problem of prolonged incubations, evidence presented in a previous communication (Blaustein & Wiesmann, 1970) indicated that with incubations longer than 3–4 min, ^{45}Ca accumulation appeared to saturate under conditions of elevated internal and reduced external Na; the uptake from Na-rich media was linear with time for at least 10–12 min. The apparent saturation occurring in the Na-depleted media was attributed to possible overloading of intra-synaptosomal storage sites (e.g. mitochondria) due to the large net accumulation of Ca by synaptosomes loaded with Na and exposed to Na-free media (see below). An alternative explanation is that prolonged incubation in Na-poor media leads to a marked reduction of $[Na]_i$, which also tends to reduce the entry of Ca

(Fig. 1). Whatever the explanation, these saturation effects have not been further investigated, and will be ignored in the discussion which follows. Their possible contribution to the observations described below has been minimized since most of the data (including all of the kinetic data) were obtained during the period of linear uptake (i.e. with incubations of 30–60 sec).

Distribution of Na_i-dependent ^{45}Ca uptake activity in the sucrose gradient. Table 3 shows that the Na_i-dependent Ca uptake activity and the nerve terminals are distributed in parallel fashion in the three layers of the discontinuous sucrose gradient, with a peak at the 0·8–1·2 M sucrose interface. The Na_i-dependent Ca uptake, like the K-stimulated Ca uptake (Blaustein, 1975) and the Li-stimulated Ca uptake (Blaustein & Wiesmann, 1970), therefore appears to be a property of the nerve terminals *per se*, and not of the 'free' mitochondria which contaminate the preparation. This is consistent with previous observations that Ca accumulation by mitochondria is not influenced by the Na/K ratio in the medium (Lazarewicz, Haljamäe & Hamberger, 1974) and does not require alkali metal ions (Carafoli, Gamble, Rossi & Lehninger, 1967). Additional evidence that the particles located at the 0·8–1·2 M sucrose interface (predominantly synaptosomes) and in the pellet (predominantly mitochondria) metabolize Ca in different ways will be presented below (p. 671).

Effect of $[Na]_o$ on Ca influx. The influence of mixtures of Na and Li on Ca uptake is illustrated by the data in Figs. 2, 3, and 5. As mentioned above, replacement of external Na by Li has little effect on Li-loaded synaptosomes (Fig. 1); Na-loaded synaptosomes have a somewhat higher rate of Ca uptake from the Na-rich medium, and the accumulation increases as external Na is progressively replaced by Li (Fig. 2). The precise shape of the Na_i-dependent Ca uptake curve at the low-Na end was somewhat variable from preparation to preparation: although the curve usually increased monotonically as Na was decreased, in several instances the Na_i-dependent Ca uptake from 17 mM-Na + 120 mM-Li was slightly greater than from 137 mM-Li (see Table 4).

Replacement of Na_o by choline or dextrose has an effect comparable to that seen with Li; the Ca uptake curves for the three substitutions are shown in Fig. 3. In fact, complete replacement of Na_o by two different substitutes, when tested in a single experiment, generally induced quantitatively similar Na_i-dependent Ca uptakes (Table 4).

Also shown in Fig. 3 is the effect on Ca uptake of replacing Na_o by K (Blaustein, 1975). Although this curve was obtained on synaptosomes with a relatively low $[Na]_i$, the condition required for a large K-stimulated Ca uptake, it is noteworthy that the K effect appears to saturate at an external Na concentration at which the Na_i-dependent uptake is only about half-maximal.

TABLE 3. Distribution of Na_i-dependent Ca^{45} uptake activity in the discontinuous sucrose gradient

Sucrose gradient region* (and predominant particle morphology)	Pre-incubation medium†	^{45}Ca uptake (μmole Ca/g protein per minute)		Protein in layer (mg)	Relative 'specific' distribution	
		Total‡	Na_i-dependent		Na_i-dependent ^{45}Ca uptake§	Synaptosomes‖
0·32–0·8 M interface (fragmented membranes)	Ca-free Li	0·50 ± 0·01	—	—	—	—
	Ca-free Na	1·23 ± 0·07	0·73	16·4	0·64	0·38
0·8–1·2 M interface (synaptosomes)	Ca-free Li	0·71 ± 0·07	—	—	—	—
	Ca-free Na	2·33 ± 0·11	1·62	34·6	1·40	1·50
Pellet (mitochondria)	Ca-free Li	0·46 ± 0·02	—	—	—	—
	Ca-free Na	0·73 ± 0·04	0·27	10·4	0·23	0·57

* Two gradients were prepared from a single crude mitochondria (P_2) fraction. The comparable fractions from the two gradients were combined for the ^{45}Ca uptake experiment.

† The pre-incubation media also were K-free, and contained 10^{-3} M ouabain.

‡ The tissue samples (containing about 1·5 mg protein) were pre-incubated with 2·0 ml. solution for 15 min at 30° C. After centrifugation the supernatant solution was discharged, 0·5 ml. K-free Li + 1·2 mM-Ca was added, followed 10 sec later by 1·0 ml. K-free Li + 1·2 mM-Ca containing ^{45}Ca. The suspensions were incubated for 1 min at 30° C and ^{45}Ca uptake was terminated with EGTA. Each value shown is the mean of four determinations ± S.E.

§ Relative 'specific' distribution of Na_i-dependent ^{45}Ca uptake $= \dfrac{Na_i\text{-dependent Ca uptake in layer}}{Na_i\text{-dependent Ca uptake in entire gradient}} \div \dfrac{\text{protein in layer}}{\text{total protein}}$.

‖ Calculated from Fig. 2 of Michaelson & Whittaker (1963; see Blaustein, 1975).

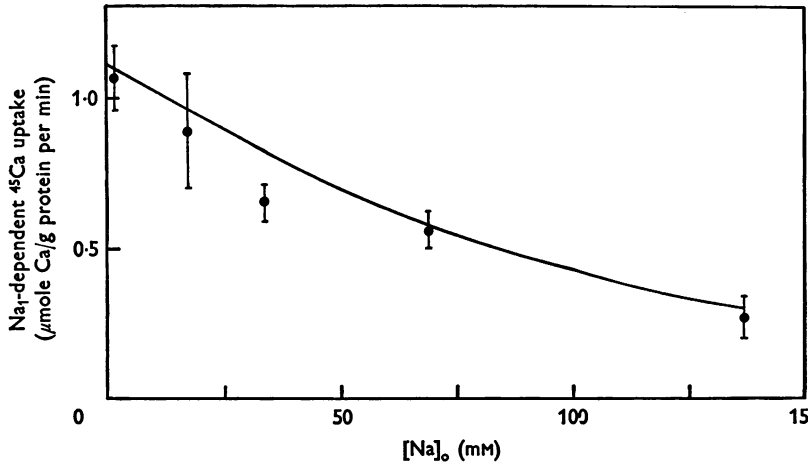

Fig. 2. Effect of replacing external Na by Li on the Na_i-dependent ^{45}Ca uptake by synaptosomes. Synaptosomes were pre-loaded with either Na or Li, and then incubated in mixtures of ^{45}Ca-containing K-free Na and K-free Li; the Na concentration of the mixture is given on the abscissa. The Na_i-dependent uptake (cf. Fig. 1*B* and text) is given on the ordinate. Each point is the mean value from five different experiments; the bars indicate the S.E. On the assumption that 2 external Na^+ ions compete with 1 Ca^{2+} (cf. Fig. 7*A*, *B* and text), the data have been fitted to the equation (cf. Baker & Blaustein, 1968):

$$M_{in}^{Ca} = \frac{\overset{*}{M}_{in}^{Ca}}{1+(K_{Ca}/[Ca]_o)(1+[Na]_o/\bar{K}_{Na})^2},$$

where $\overset{*}{M}_{in}^{Ca}$ is the maximal Na_i-dependent Ca influx, with a value of 1·3 μmole Ca/g protein per minute. The influx at any $[Na]_o$ and $[Ca]_o$ is given by M_{in}^{Ca}. The dissociation constant for Ca_o, $\bar{K}_{Ca}$, is equal to 0·2 mM; $\bar{K}_{Na}$, the mean apparent inhibitory constant for Na_o, has a value of 40 mM.

Effect of $[Na]_o$ on Ca efflux and the net movement of Ca. Calcium efflux from synaptosomes also is influenced by external Na. In the absence of external Ca, the Ca efflux decreases significantly when most of the external NaCl is replaced by LiCl, choline Cl, sucrose, dextrose or tetramethyl-ammonium Cl. Fig. 4*A* illustrates the data from an experiment on the time course of ^{45}Ca efflux. With the medium containing primarily LiCl or sucrose, the rate constant for ^{45}Ca efflux was about 0·09 min^{-1}. When Na was the predominant external cation, the rate constant for ^{45}Ca loss was about 0·4 min^{-1} for the first 2 min, but the rate of Ca loss then slowed to about the rate observed in the absence of external Na. These data may indicate that two different mechanisms are involved in Ca efflux, only the more rapid being Na_o-dependent.

The relationship between the external Na concentration and Ca efflux

is shown in Fig. 4*B*. These are data from three experiments in which efflux into mixtures of Ca-free Na + 5K and Ca-free choline + 5K was measured after 2 min incubation. The sigmoid shape of the curve may indicate that the efflux of one Ca^{2+} ion is activated by more than a single Na^+ – perhaps 2 or 3, as suggested for barnacle muscle, or squid axons (Blaustein *et al.* 1974). Assuming that the Ca efflux involves an Na–Ca exchange mechanism, this may mean that 2 or 3 Na^+ ions enter the terminals in exchange for each exciting Ca^{2+}.

TABLE 4. ^{45}Ca accumulation of synaptosomes incubated in Na-Li and dextrose-Li mixtures

Incubation medium*	^{45}Ca uptake (μmole Ca/g protein per minute)†		
	Na-loaded synaptosomes‡	Li-loaded synaptosomes§	Na_i-dependent ^{45}Ca uptake‖
K-free Na	1·08	0·80	0·28
$\frac{1}{8}$ Na–$\frac{7}{8}$ Li	2·58	1·30	1·28
K-free Li	2·53	1·37	1·16
K-free dextrose	2·35	1·22	1·13
$\frac{7}{8}$ Dextrose–$\frac{1}{8}$ Li	2·85	1·20	1·65
$\frac{3}{4}$ Dextrose–$\frac{1}{4}$ Li	2·58	1·30	1·28

* Synaptosomes were incubated for 30 sec at 30° C, in K-free Na, K-free Li, K-free dextrose, or mixtures of two of these solutions, as indicated. All incubation solutions contained 1·2 mM-Ca.

† Each value is the mean of two determinations. All individual values were within ± 5 % of the respective means.

‡ Pre-incubated for 10 min at 30° C in K-free, Ca-free Na with 1 mM ouabain.

§ Pre-incubated for 10 min at 30° C in K-free, Ca-free Li with 1 mM ouabain.

‖ Uptake by Na-loaded synaptosomes minus uptake by Li-loaded synaptosomes.

The combination of increased Ca influx and decreased Ca efflux in Na-depleted media suggests that the Ca content of synaptosomes should increase if they are incubated in Na-depleted media containing Ca. Indeed, the data of Fig. 5 confirm this prediction: Ca content is significantly increased ($P < 0{\cdot}005$) in Na-loaded synaptosomes incubated in K-free Li, but not in a solution containing 34 mM-Na and 103 mM-Li.

The relation between $[Na]_i$ and Ca uptake. The quantitative relationship between $[Na]_i$ and ^{45}Ca uptake is illustrated in Fig. 6. For these experiments synaptosomes were pre-incubated with various mixtures of Ca-free K-free Na and Ca-free K-free Li (the Na concentration is shown on the abscissa) in the presence of 1 mM ouabain for 15 min at 30° C. During this period the synaptosome monovalent cation content may be expected to reach a new steady level approximating the monovalent cation composition

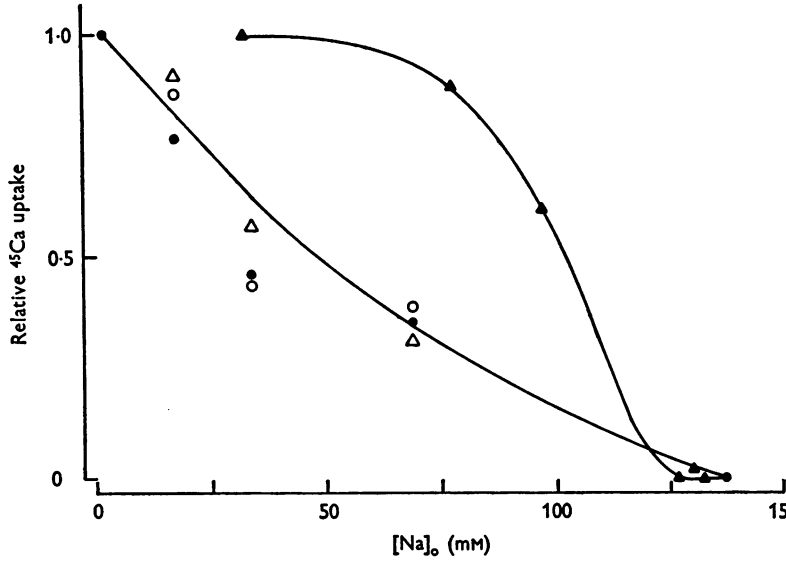

Fig. 3. Effect of replacing external Na by Li, choline or dextrose on ^{45}Ca accumulation. Ordinate shows the relative increase in the 'low Na-stimulated' Ca uptake; the external Na concentration is indicated on the abscissa. The Na-Li data (●) are the mean values from five experiments (see Fig. 2); the mean Na_i-dependent ^{45}Ca uptake from K-free Li was $1{\cdot}06 \pm 0{\cdot}11$ μmole Ca/g protein per minute. The open circles are data from a single experiment in which choline replaced Na; the Na_i-dependent ^{45}Ca uptake from K-free choline was 2·01 μmole Ca/g protein per minute. The Na-dextrose curve (Δ) shows the mean values from two experiments; the mean Na_i-dependent ^{45}Ca uptake from K-free dextrose was 1·75 μmole Ca/g protein per minute. For all experiments shown, the mean Na_i-dependent ^{45}Ca uptake from K-free Na was $0{\cdot}27 \pm 0{\cdot}07$ μmole Ca/g protein per minute. The lower curve is based on the equation given in the caption to Fig. 2.

For comparison, the K-stimulated Ca uptake data from Fig. 3*A* of Blaustein (1975) are also shown (▲). In this case, the synaptosomes were pre-incubated in Na + 5K, and were not pre-loaded with Na. The K-stimulated ^{45}Ca uptake at 100 mM-$[K]_o$ (i.e. uptake at 100 mM-K minus uptake at 5 mM-K) was 1·21 μmole Ca/g protein per minute.

of the bathing medium (see Methods). Following centrifugation, the pre-incubation medium was discarded and the terminals were incubated in either K-free Li or K-free Na to measure ^{45}Ca uptake. Ca entry is graphed as a function of the Na concentration of the pre-incubation solution in Fig. 6*A*. The same data, re-graphed in Fig. 6*B*, clearly indicate that the Na_i-dependent Ca uptake from both K-free Li and K-free Na is directly proportional to the square of the internal Na concentration, although the uptake from the Na-containing medium appears to saturate at elevated $[Na]_i$. The latter effect, which was also observed in the two other experi-

ments in which it was tested for, is strikingly similar to the effect of $[Na]_i$ on Ca uptake in cardiac muscle (Glitsch, Reuter & Scholtz, 1970, Fig. 3).

An unexpected feature of these experiments was the apparent lack of saturation of Ca uptake from K-free Li, as $[Na]_i$ was increased. In view of the interactions between external Na and Ca on the transport mechanism (see below), the effect of altering $[Ca]_o$ was also examined. The preliminary experiments indicate that Ca uptake from K-free Li is directly proportional to $[Na]_i^2$, and does not appear to saturate at $[Na]_i \simeq 137$ mM, when $[Ca]_o$ is as low as 0·04 mM, or as high as 7·6 mM.

Effect of $[Ca]_o$ on the rate of Ca accumulation. The foregoing results show that Ca influx is enhanced by raising the internal Na concentration, and efflux is promoted by external Na. On the other hand, raising external Na antagonizes Ca influx; this suggests that Na and Ca ions may compete for the same sites on the membrane. In order to explore this possibility further, Ca uptake was measured at several different external Na and Ca concentrations. The mean values for the Na_i-independent Ca influx from these experiments is graphed in Fig. 7*A*. A double reciprocal (Lineweaver–Burke) plot of these data (1/Ca uptake *vs.* 1/Ca concentration) is shown in Fig. 7*B*. Despite the scatter, especially at the very low Ca concentrations where there may be uncertainty as to the precise Ca concentration, the experimental data provide a reasonable fit to the theoretical curves expected on the basis of a competition between two Na^+ ions and one Ca^{2+} ion (see caption to Fig. 2). The calculated value for the apparent Ca dissociation constant in Na-free solution (K_{Ca}) is 0·2 mM-Ca.

Influence of metabolism on Ca fluxes in synaptosomes and mitochondria. The data on the distribution of the Na_i-dependent and Li-stimulated Ca fluxes in the sucrose gradient (p. 666, above, and Blaustein & Wiesmann, 1970) provide evidence that the Ca fluxes under discussion are not simply a result of contamination of the synaptosome layer by 'free' mitochondria. Nevertheless, since electron micrographs indicated that most synaptosomes contain mitochondria, and since mitochondria are known to store Ca (e.g. Lehninger, 1970; Carafoli & Rossi, 1971), a comparison of the properties of Ca fluxes in synaptosomes and in mitochondria seems worth while. The data in Table 5, from two different sucrose gradient preparations, compare tissue fragments obtained from the 0·8–1·2 M sucrose interface (synaptosomes) and from the mitochondrial pellet (cf. Bradford, 1969, regarding respiration of mitochondria and synaptosomes). In the first experiment (*A*), both synaptosomes and mitochondria were handled in the manner routinely used to study net Ca efflux from synaptosomes. Removal of Na from the Ca-free medium, while markedly reducing Ca efflux from synaptosomes, has little if any influence on the

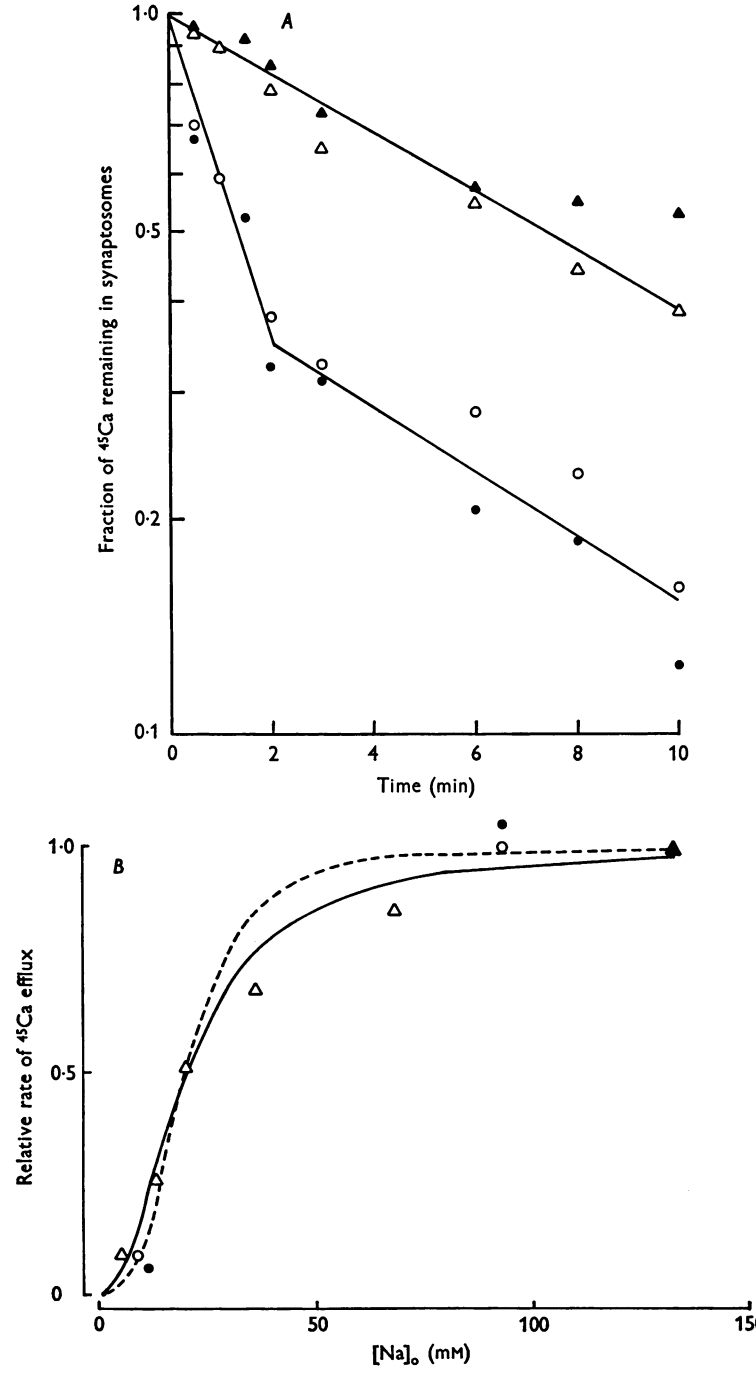

Fig. 4. For legend see facing page.

very rapid loss of Ca from mitochondria (cf. Drahota & Lehninger, 1965); the slight decreases observed might be explicable on the basis of contamination of the mitochondrial pellet with synaptosomes (cf. Table 3). Poisoning by cyanide stimulates Ca efflux from synaptosomes, presumably as a consequence of interruption of intrasynaptosome–mitochondrial electron transport. Similar results were obtained in one other experiment (data not shown) when 2,4-dinitrophenol (DNP) was used to uncouple oxidative phosphorylation.

Experiment *A* of Table 5 also shows that cyanide has an insignificant effect on the large loss of Ca from 'free' mitochondria (the slight stimulation might, perhaps, be due to contamination with synaptosomes). Depletion of normal mitochondrial substrates (pyruvate and Krebs Cycle intermediates;

Fig. 4. *A*, time course of ^{45}Ca efflux from synaptosomes. Four synaptosome pellets (approximately 20 mg protein) were each suspended in 0·7 ml. Na + 5K and pre-incubated for 15 min at 30° C. A 0·7 ml. aliquot of the same solution, but including ^{45}Ca and $1{\cdot}5 \times 10^{-4}$ M veratridine (to stimulate Ca uptake; cf. Blaustein, 1975), was then added to each suspension, and incubation was continued for another 2 min. Ca uptake was then terminated by diluting the suspensions with 29 ml. Ca-free Na + 5K + 1 mM EGTA + $2{\cdot}5 \times 10^{-7}$ M tetrodotoxin (○, ●), Ca-free Li + 5K + EGTA + TTX (△) or Ca-free 264 mM sucrose + 5K + EGTA + TTX (▲). The suspensions were maintained at 30° C and were agitated by bubbling O_2 through the flasks. At the times indicated on the graph, 2·0 ml. aliquots of the suspensions were filtered through glass fibre filters. The lines were drawn by eye.

B, effect of external Na concentration ($[Na]_o$) on the relative rate of ^{45}Ca efflux. Efflux into Na + 5K (132 mM-Na) is taken as 1·0. The triangles refer to an experiment in which synaptosomes were loaded with ^{45}Ca by treatment with scorpion venom (see Methods, and Blaustein, 1975); each symbol is the mean of four efflux determinations. The open and filled circles refer to two experiments in which a K-rich solution (50 mM-K) was used to load synaptosomes with ^{45}Ca; each symbol is the mean of three efflux determinations. The data were obtained by subtracting the amount of ^{45}Ca remaining in synaptosomes after a 2 min incubation in an efflux solution with the Na concentration shown, from the ^{45}Ca content of synaptosomes determined immediately after ^{45}Ca loading (see Methods and Fig. 4*A* caption). The curves fit the equation:

$$M_o^{Ca} = \frac{\overset{*}{M}_o^{Ca}}{1 + (\bar{K}_{Na}/[Na]_o)^n},$$

where $\overset{*}{M}_o^{Ca}$ is the maximum rate of ^{45}Ca efflux and M_o^{Ca} is the efflux at any $[Na]_o$. $\bar{K}_{Na}$ is the apparent half-saturation constant for Na, with a value of 20 mM-Na. The exponent, n has a value of 2 (continuous curve) or 3 (interrupted curve). The mean (± S.E.) rate of ^{45}Ca efflux into Na + 5K (132 mM-Na) for the three experiments was $1{\cdot}59 \pm 0{\cdot}16$ μmole Ca/g protein per minute.

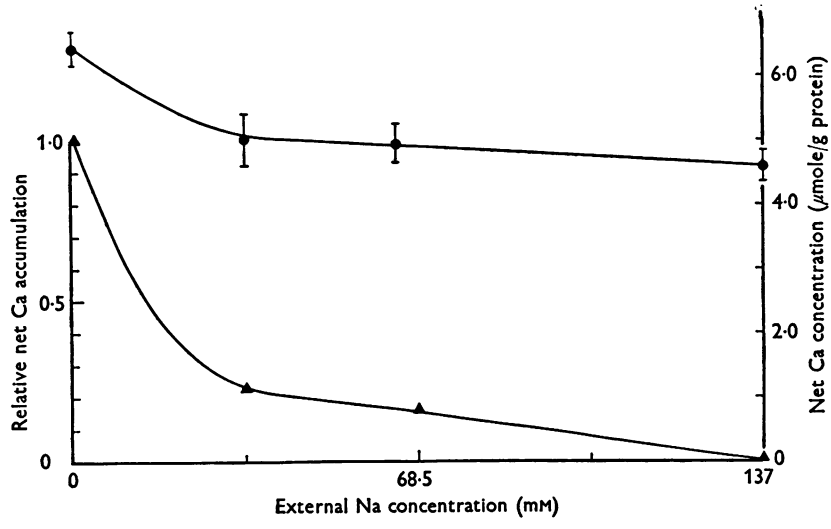

Fig. 5. Effect of replacing external Na by Li on synaptosome Ca concentration and net Ca uptake. The right-hand ordinate scale gives the total Ca concentration. Each point (●) indicates the mean (bars are ± S.E.) of four determinations of total Ca for an experiment in which synaptosomes were pre-loaded in Ca-free K-free Na, and then incubated for 5 min at 30° C in mixtures of K-free Na and K-free Li containing 1·2 mM-$CaCl_2$. The relative net increment in synaptosome Ca content due to partial or complete replacement of Na by Li is indicated by the triangles and left-hand ordinate scale. The latter data are the mean values from three experiments in which the synaptosomes were incubated in the presence of 1·2 mM-$CaCl_2$ at 30° C for 3 or 5 min. For these experiments, the mean Ca concentration in K-free Na was 5·63 ± 0·27 μmole/g protein; the net increase in Ca due to complete replacement of external Na by Li was 0·47 ± 0·07 μmole/g protein per minute. Both curves were drawn by eye.

Fig. 6*A*, effect of internal Na concentration on ^{45}Ca uptake from K-free Na (●) and from K-free Li (○). The Li_o and Na_o curves were obtained with different synaptosome preparations; the Ca uptake from K-free Li, by Na-loaded and by Li-loaded synaptosomes, respectively, was virtually identical for the two preparations. Each point is the mean of three determinations. For these experiments, the synaptosomes were pre-incubated for 15 min in mixtures of Ca-free K-free Na and Ca-free K-free Li; the Na concentrations of these mixtures are indicated on the abscissa.

B, effect of internal Na concentration on the Na_i-dependent ^{45}Ca uptake. The data are from the same experiments as in Fig. 6*A*, with the ^{45}Ca uptake by Li-loaded synaptosomes subtracted from both curves. Open circles: Na_i-dependent Ca uptake from K-free Li. Filled circles: Na_i-dependent Ca uptake from K-free Na. Note that in this graph the Ca accumulation is shown as a function of the square of the internal Na concentration. The lines were drawn by eye.

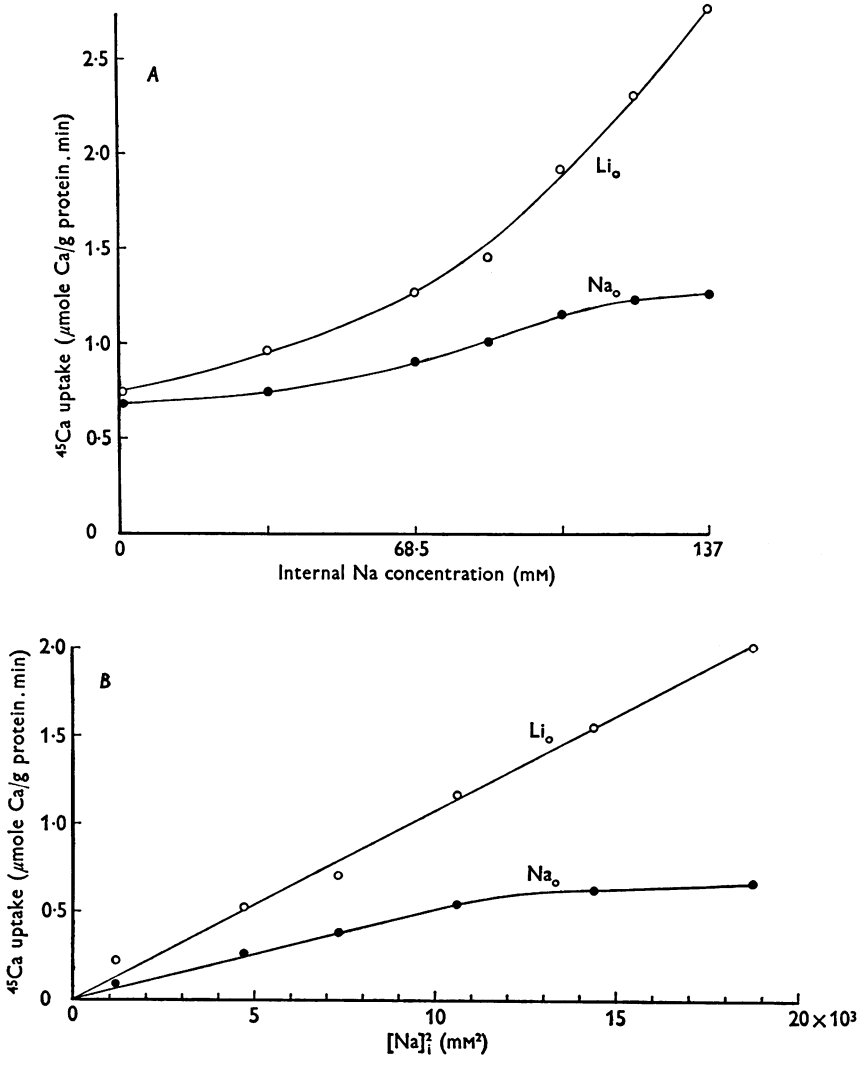

Fig. 6. For legend see facing page.

cf. Bradford, 1969) could account for this large Ca efflux. This view is supported by the observation that addition to the medium of an appropriate substrate (succinate; cf. Hamberger, Blomstrand & Lehninger, 1970), as in Expt *B*, significantly reduces the loss of Ca from the mitochondria. In this experiment, both the synaptosomes and the mitochondria were incubated in mitochondrial medium (see Methods). Note that neither the absence nor presence of metabolic substrate (glucose or succinate) influences Ca efflux from synaptosomes. This lack of effect

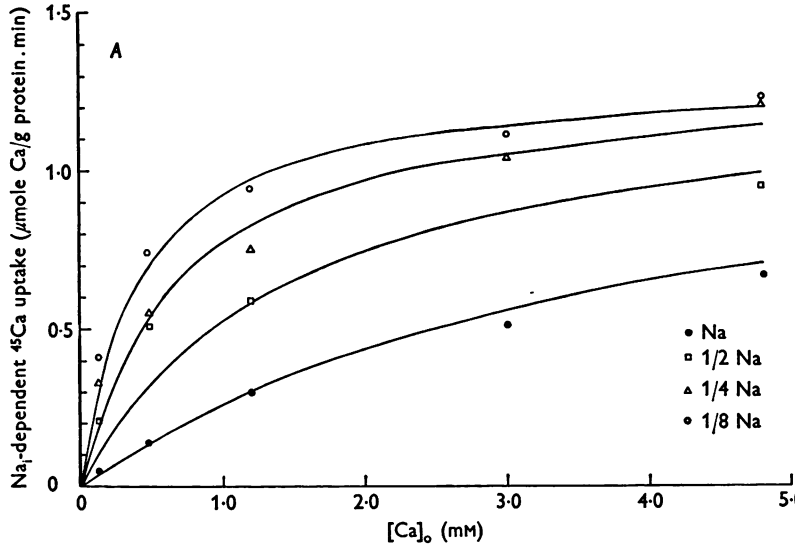

Fig. 7*A*, effect of external Ca concentration on the rate of the Na_i-dependent ^{45}Ca uptake from various K-free Na and K-free Li mixtures. The data are the mean values for six experiments; in most experiments only one or two of the external Na concentrations were tested. The filled circles indicate the uptake from K-free Na; squares, half of the external Na replaced by Li; triangles, $\frac{1}{4}$ Na–$\frac{3}{4}$ Li; and open circles, $\frac{1}{8}$ Na–$\frac{7}{8}$ Li.

B, data from Fig. 7*A* plotted as the reciprocal of the ^{45}Ca uptake (1/μmole per/g protein.min) versus the reciprocal of the external Ca concentration (1/mM). Symbols denoting the various external Na concentrations are the same as for Fig. 7*A*. The curves in Figs. 7*A* and *B* were calculated from the equation and constants given in the caption to Fig. 2.

is probably due to the fact that synaptosomes have sufficient stores of substrates to maintain metabolism for 15–30 min in the absence of an exogenous supply (cf. Bradford, 1969). These observations help to distinguish between the properties of free mitochondria and of synaptosomes obtained from the same sucrose gradient (see also Fig. 2, Table 3, and Blaustein, 1975).

Cyanide also influences Ca uptake by synaptosomes: as shown in Table 6, Ca influx from Na-containing media is significantly inhibited. On the other hand, uptake from K-free Li (by Na-loaded synaptosomes) is significantly increased by the addition of cyanide and iodoacetate.

The effects of the metabolic inhibitors on Ca influx and efflux in Na-containing media indicate that these poisons should induce a net loss of Ca from synaptosomes incubated in the standard (Na + 5K) medium containing Ca. Indeed, as shown by the data in Table 7, cyanide and, especially, DNP, do cause a net loss of Ca from synaptosomes in this medium. While

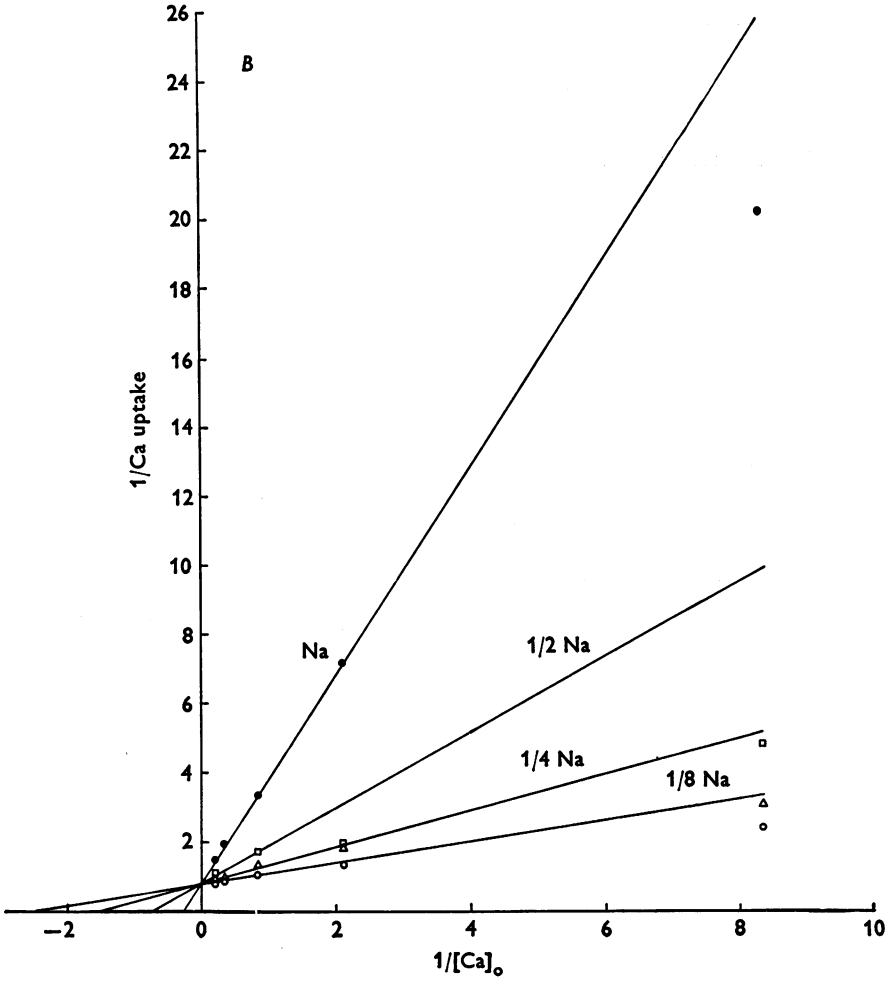

Fig. 7*B*. For legend see facing page.

this might mean that Ca extrusion from nerve terminals does not necessarily require the direct intervention of high-energy metabolic intermediates (such as ATP), a sudden increase in free $[Ca^{2+}]_i$ perhaps as a consequence of release from mitochondria (see above), could also lead to a short-term net loss of Ca.

Effects of temperature and pH on Ca influx. The data in Fig. 8 illustrate the effect of temperature on the Na_i-dependent Ca uptake. In the range between 10 and 30° C, this uptake has a Q_{10} of about 3, which corresponds to an activation energy of about 18 kcal/mole. The Q_{10} for the Na_i-dependent Ca uptake is markedly different from the

TABLE 5. Comparison of factors affecting Ca efflux from synaptosomes and mitochondria

A, effects of external Na and of cyanide*

Equilibrated only ...	Synaptosomes 4·18 ± 0·10 μmole Ca/g protein		Mitochondria 19·61 ± 0·78 μmole Ca/g protein	
Incubation medium	Net Ca efflux (μmole Ca/g protein per 15 minutes)	% lost	Net Ca efflux (μmole Ca/g protein per 15 minutes)	% lost
Ca-free Na + 5K	1·33 ± 0·06	31·6	14·67 ± 0·34	74·8
Ca-free Na + 5K + 2 mM-CN	2·16 ± 0·20†	51·7	17·20 ± 0·62	87·7
Ca-free Li + 5K	0·06 ± 0·07†	1·4	13·37 ± 1·06	68·2
Ca-free Li + 5K + 2 mM-CN	0·53 ± 0·13†	12·7	13·57 ± 0·68	69·2

B, effect of metabolic substrate‡

Equilibrated only ...	Synaptosomes 3·36 ± 0·06 μmole Ca/g protein		Mitochondria 11·76 ± 1·11 μmole Ca/g protein	
Metabolic substrate added	Net Ca efflux (μmole Ca/g protein per 15 minutes)	% lost	Net Ca efflux (μmole Ca/g protein per 15 minutes)	% lost
None	0·95 ± 0·11	28·3	4·45 ± 0·17	37·9
10 mM glucose	0·89 ± 0·04	26·5	4·13 ± 0·30	35·2
10 mM succinate	0·89 ± 0·04	26·5	1·99 ± 0·18§	16·9

* Mitochondria and synaptosomes (from the same sucrose gradient) were equilibrated with Ca-free Na + 5K. After centrifugation the supernatant solutions were discarded and the tissue samples were re-suspended in the media listed in column 1. Samples were incubated for 15 min at 30° C. The amount of Ca remaining in the tissue, and the amount appearing in the incubation medium were both determined; all the paired Ca efflux values (from tissue and supernate) agreed to within ± 5 %. Each value in columns 2 and 4 is the mean of the eight determinations (on four tissue samples) ± S.E.

† In both Na and Li media, 2 mM-CN significantly stimulated Ca efflux ($P < 0·005$). The Ca efflux into the Li solutions was significantly less than into the Na solutions ($P < 0·005$).

‡ Mitochondria and synaptosomes (from the same sucrose gradient) were equilibrated with substrate-free mitochondrial medium (see Methods) and incubated in the same solution containing the metabolic substrate shown in column 1. The suspensions were incubated for 15 min at 30° C. Each value in columns 2 and 4 is the mean Ca efflux ± S.E. of three paired (three supernatant solution and three pellet) determinations.

§ Significantly less Ca efflux than with glucose or no substrate ($P < 0·005$ by Student's *t* test).

TABLE 6. Effect of metabolic poisons on ^{45}Ca uptake by synaptosomes

Incubation medium*	Incubation time (min)	^{45}Ca uptake† (μmole Ca/g protein per minute)	Increment (+) or decrement (−) due to poison	P‡
Experiment 1				
Na + 5K	6	0·242 ± 0·003	—	—
Na + 5K + CN	6	0·206 ± 0·005	−0·032	< 0·005
Na + 5K	12	0·218 ± 0·016	—	—
Na + 5K + CN	12	0·174 ± 0·005	−0·044	< 0·01
Experiment 2				
K-free Na	3	0·74 ± 0·03	—	—
K-free Na + CN + IAA	3	0·54 ± 0·02	−0·20	< 0·05
K-free Li	3	1·41 ± 0·03	—	—
K-free Li + CN + IAA	3	1·79 ± 0·10	+0·38	> 0·1

* Synaptosomes were equilibrated with Ca-free Na + 5K (Expt. 1) or Ca-free K-free Na (Expt. 2). CN = 2 mM-NaCN; IAA = 2 mM-Na iodoacetate.

† For Expt. 1 each value is the mean of four determinations, and for Expt. 2, the mean of three determinations ± S.E.

‡ Significance of the difference between uptake in the absence and in the presence of the metabolic poisons (Student's t test).

TABLE 7. Effect of metabolic poisons on net calcium content of synaptosomes

Incubation medium*	Net Ca content† (μmole Ca/g protein)	Net Ca loss (−) or gain (+) during incubation
Na + 5K (pre-incubation only)	6·86 ± 0·22	—
Na + 5K	7·20 ± 0·27	+0·34
Na + 5K + 2 mM-CN	6·47 ± 0·19	−0·39‡
Na + 5K + 1 mM-DNP	5·46 ± 0·12	−1·40§

* Synaptosomes were equilibrated with Ca-free Na + 5K. They were pre-incubated in Na + 5K (containing 1·2 mM-Ca) for 12 min at 30° C. Additional Na + 5K with or without cyanide (CN) or 2,4-dinitrophenol (DNP) was then added and the suspensions were incubated for 10 min more. EGTA and Ca-free Na + 5K was then added, and the synaptosomes were centrifuged and washed once with Ca-free Li + 5K. EGTA was added to the first group (pre-incubated only) immediately following the second Na + 5K addition.

† Each value is the mean of four determinations ± S.E.

‡ $P < 0·01$ when compared to synaptosomes incubated in Na + 5K without inhibitor.

§ $P < 0·001$ when compared to synaptosomes incubated in Na + 5K without inhibitor (Student's t test).

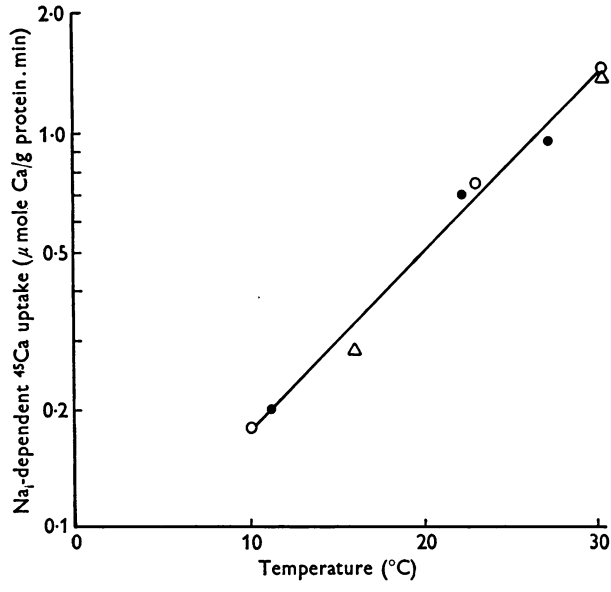

Fig. 8. Effect of temperature on the internal Na-dependent Ca efflux. Synaptosomes were pre-loaded with Na or Li, and incubated for 1 min (△, ▲) or 30 sec (○) in ^{45}Ca-containing K-free Li, at the temperatures indicated on the abscissa. The ^{45}Ca uptake by the Li-loaded terminals has been subtracted; each symbol refers to a different experiment and indicates the difference between the means of two (●) or three (○, △) determinations. The line was drawn by eye.

Q_{10} for the K-stimulated Ca uptake (about 1·4; Blaustein, 1975), and provides an important means of distinguishing between these two mechanisms of Ca entry (see Discussion). In one preliminary experiment, the Ca efflux into K-free Na, tested at 5, 20 and 30° C, was found to have a Q_{10} of 2·5.

Ca entry is also influenced by the pH of the bathing medium, as shown in Fig. 9. The Na_i-dependent Ca influx data fit a titration curve with an apparent pK_a of about 5·6. This type of result could be obtained if a titratable group (e.g. imidazolium; cf. White, Handler, Smith & Stetten, 1954) were located at or near the Ca-binding site on the presumed Ca carrier (see Discussion and Blaustein, 1974). In addition, titration of acidic groups on the membrane, which are not directly associated with the presumed carrier, may reduce the surface charge. This in turn would reduce the Ca^{2+} concentration at the membrane surface, with respect to the concentration in bulk solution, thereby tending to decrease influx.

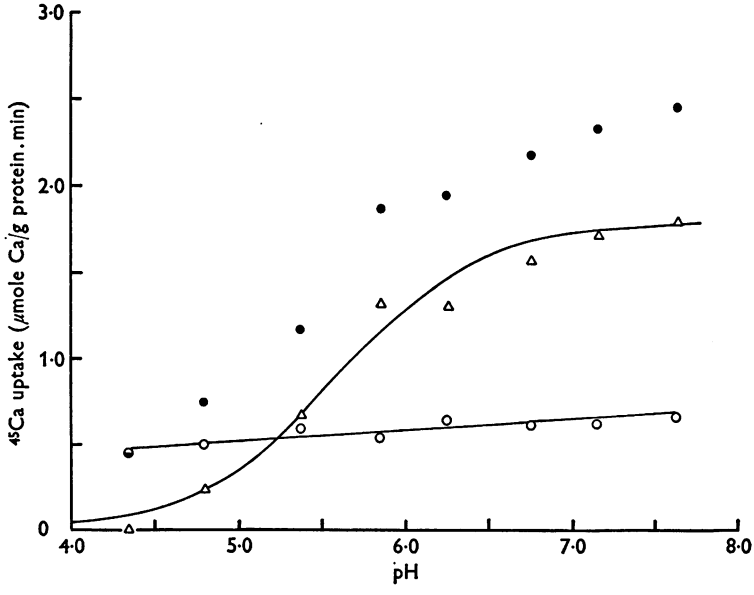

Fig. 9. The influence of external pH on ^{45}Ca uptake by Li-loaded (○) and Na-loaded (●) synaptosomes. The difference curve (the Na_i-dependent Ca uptake) is indicated by the triangles. The synaptosomes were incubated with ^{45}Ca for 1 min in K-free Li buffered with acetylglycine and Tris maleate (see Methods). Each circle represents the mean of three determinations. The sigmoid curve is the titration curve of a weak acid with a pK_a of 5·6.

DISCUSSION

The data reported here provide further evidence of the integrity of synaptosome surface membranes. The main observation is that in presynaptic nerve terminals, as in other nervous tissue preparations, both invertebrate (Baker & Blaustein, 1968; Baker, Blaustein, Hodgkin & Steinhardt, 1969; Blaustein & Hodgkin, 1969, Blaustein *et al.* 1974) and vertebrate (e.g. Kalix, 1971; Bull & Trevor, 1972; Stahl & Swanson, 1972), the influx and efflux of Ca may be coupled, in part, to the movement of Na in the opposite direction. Although it has not previously been possible to localize the site of the apparent Na–Ca exchange in mammalian brain slice preparations (e.g. Stahl & Swanson, 1972; Bull & Trevor, 1972), the results of the present study clearly indicate that Na–Ca exchange does occur in the neural elements. The data show that the Na-dependent Ca fluxes are a property of 're-sealed' nerve endings: Na-dependent Ca fluxes are not observed in mitochondria or in disrupted terminals.

Comparison between Na-dependent Ca-uptake and 'depolarization-induced' Ca uptake. The preceding article (Blaustein, 1975) described experiments

which indicate that synaptosomes accumulate Ca when treated with depolarizing agents. The agents used in that study were known to either increase Na permeability and presumably, $[Na]_i$ (e.g. veratridine and scorpion venom), or decrease $[Na]_o$ (since Na_o was partially replaced by K or Rb). It is therefore important to know whether or not 'depolarization-induced' Ca entry is also a manifestation of Na–Ca exchange. This possibility seems rather unlikely for several reasons. In the first place, the condition which markedly enhances the Na_i-dependent Ca uptake, namely, a high $[Na]_i$, significantly inhibits the K-stimulated Ca uptake (see Fig. 5 of Blaustein, 1975). The maximal Na_i-dependent Ca uptake, observed with Na-loaded synaptosomes incubated in Na-containing media, about 0·5 μmole Ca/g protein per minute on the average, is only about one fourth the maximal rate of the K-stimulated Ca uptake (with $[Na]_o$ = 77 mM). Since both the Na-loaded and the Li-loaded synaptosomes are probably depolarized (on the basis of tests with a voltage sensitive fluorochrome – see Blaustein & Goldring, 1975), the Na_i-dependent Ca uptake cannot be attributed to a conductance change caused by a large change in membrane potential.

Even more important, are the differences in kinetics. A maximum K-stimulated Ca uptake is observed when about half the external Na is replaced by K, whereas replacement of Na by Li, choline or dextrose induces a peak Ca uptake when most or all of the external Na is removed (Fig. 3). The K-stimulated Ca uptake shows an apparent affinity for Ca of about 1 mM, and this value is relatively unaffected when $[K]_o$ is increased from 25 to 100 mM, and $[Na]_o$ is concomitantly reduced from 112 to 37 mM (cf. Fig. 7 of Blaustein, 1975). By way of contrast, as indicated by the data in Fig. 7*B* (above), the Na_i-dependent Ca uptake shows an apparent affinity for Ca (K'_{Ca}) which varies as $[Na]_o$ is altered: K'_{Ca} may be about 2–3 mM at an $[Na]_o$ of 112 mM, but only about 0·7 mM at an $[Na]_o$ of 37 mM.

There is also a considerable difference in the temperature coefficients of the two Ca uptake mechanisms: the Na_i-dependent Ca uptake has a Q_{10} of about 3 (Fig. 8), while the Q_{10} for the K-stimulated Ca uptake is only about 1·4 (Blaustein, 1975).

Finally, we have recently obtained pharmacological evidence for two different mechanisms (Blaustein & Ector, 1975): low concentrations (0·2–0·3 mM) of pentobarbitone significantly inhibit (by about 50%) the K-stimulated Ca uptake, but have a negligible effect ($\sim$ 10% inhibition) on the Na_i-dependent or Li_o-stimulated Ca uptake.

In sum, the foregoing considerations suggest strongly that the two mechanisms of Ca uptake by synaptosomes, K-stimulated uptake and Na_i-dependent uptake, are indeed different. Furthermore, the Na_i-

dependent mechanism may be expected to permit only a very limited entry of Ca under normal physiological circumstances (high $[Na]_o$ and low $[Na]_i$). One possibility is that the Na–Ca exchange mechanism may be involved primarily in Ca extrusion, as has been proposed for squid axons (Blaustein & Hodgkin, 1969; Blaustein *et al.* 1974) and other tissues (cf. Blaustein, 1974).

Possible role of Na–Ca exchange in 'active' Ca extrusion from nerve terminals. As noted in the Introduction, the Ca which apparently enters presynaptic terminals during depolarization must subsequently be extruded against a large electrochemical gradient. The data described in this report show that Ca efflux into a Ca-free medium is largely dependent upon external Na. The Ca efflux mechanism could operate in a manner analogous to the sodium pump (which extrudes Na in exchange for K at the expense of ATP): Ca could be extruded in exchange for Na, at the expense of ATP hydrolysis. A straightforward mechanism of this kind seems unlikely, however, since some of the data presented above show that Na_o-dependent Ca efflux proceeds quite readily in metabolically poisoned synaptosomes. Of course, since the intracellular concentration of ionized Ca^{2+} is likely to be increased dramatically under these circumstances, due to Ca release from mitochondria and perhaps other stores, the increased Ca efflux might mean that the rise in $[Ca^{2+}]_i$ is more than sufficient to compensate for the reduction in fuel supply (e.g. ATP). However, it should be noted that, unlike the Na pump situation, in the case of coupling between Na entry and Ca exit, Na would move down its electrochemical gradient, and could thereby provide at least some of the energy necessary to extrude Ca. This type of mechanism has been proposed for the squid axon (Blaustein & Hodgkin, 1969), and recent experiments with internally dialysed, metabolically poisoned squid axons (DiPolo, 1973, Blaustein *et al.* 1974) indicate that the Na_o-dependent Ca extrusion mechanism can operate in the virtual absence of ATP. However, these findings do not necessarily rule out a possible role for ATP either in altering the cation affinities of the presumed Na–Ca exchange carrier (cf. Baker & Glitsch, 1973), or in fuelling an independent and parallel Ca transport mechanism, perhaps similar to the one which operates in erythrocytes (Schatzmann & Vincenzi, 1969).

In the squid axon, the kinetics of the Na dependence and the influence of the membrane potential on the Na_o-dependent Ca efflux indicate that the Na and electrical gradients may be able to provide sufficient energy to maintain a Ca gradient ($[Ca^{2+}]_o/[Ca^{2+}]_i$) of about 10^4 (Blaustein, 1974). Unfortunately, the available kinetic data from the synaptosomes are insufficient to permit similarly detailed conclusions about the stoichiometry of Na–Ca exchange in this preparation. The sigmoid shape of the curve

relating Ca efflux to $[Na]_o$ (Fig. 4*B*) suggests that two or more Na^+ ions may enter in exchange for each exciting Ca^{2+}. The square-law relationship between $[Na]_i$ and Ca influx (Fig. 6*B*) is also consistent with a 2-for-1 exchange. With a Na gradient ($[Na]_o/[Na]_i$) of about 10, and a stoichiometry of 2 Na^+-for-1 Ca^{2+}, the Na–Ca exchange mechanism could only provide sufficient energy to maintain a Ca gradient of 10^2 (Blaustein & Hodgkin, 1969). However, as indicated in the Introduction, the transmembrane Ca gradient may be of the order of 10^3, or perhaps even 10^4.

With a stoichiometry of 3 Na^+-for-1 Ca^{2+} (which may be compatible with the data of Fig. 4*B*), the energy available from the Na gradient could, on thermodynamic grounds provide a Ca gradient of about 10^3. In the squid axon, one complete cycle of the Ca carrier mechanism may bring about the exit of one Ca^{2+} and the entry of three Na^+ (including one uncompensated positive charge; Blaustein *et al.* 1974). Although this mechanism could, theoretically, support a Ca gradient of about 10^4 (Blaustein & Hodgkin, 1969; Blaustein *et al.* 1974), the synaptosome data presented above do not necessarily favour this more complex model.

Recently obtained data (M. P. Blaustein, unpublished) indicate that Ca efflux from ^{45}Ca-loaded synaptosomes is promoted by external Ca if Li, but not choline, is the predominant external monovalent cation. These results are similar to those reported for squid axons (Blaustein, 1974; Blaustein *et al.* 1974), and may provide support for the latter model.

The data available thus far do, however, justify the conclusion that an Na–Ca exchange mechanism is probably involved in Ca transport in rat brain presynaptic terminals. Many of its properties are similar to those of the squid axon Ca transport mechanism, and the Na gradient probably provides at least some of the energy for Ca extrusion against the electrochemical gradient. It seems likely that a mobile carrier mechanism (cf. Wilbrandt & Rosenberg, 1961) is involved in the Na-dependent Ca fluxes in synaptosomes because there is evidence for counter-transport (of Na in exchange for Ca), and for Ca–Ca exchange diffusion (since external Ca can promote ^{45}Ca efflux; Blaustein & Wiesmann, 1970).

Some quantitative aspects of Na–Ca exchange. In the preceding article (Blaustein, 1975), the resting Ca influx for synaptosomes incubated in Na + 5K was reported to be about 0·5 μmole/g protein per minute, or about 0·03 p-mole/cm^2 sec. If the maximum Na_o-dependent Ca efflux, for ^{45}Ca-loaded synaptosomes, is about 1·5 μmole/g protein per minute (cf. Fig. 4*B*), then net Ca efflux into Na-containing solutions may be about 1·0 μmole/g protein per minute, or about 0·06 p-mole/cm^2 sec. Assuming that a maximal depolarization-induced Ca uptake (about 6 times resting level; Blaustein, 1975) occurs during physiological stimu-

lation, and assuming that the Ca conductance increase, following each action potential in the terminal, is maintained for about 2 msec (but cf. Katz & Miledi, 1968), the net gain of Ca will be about 0·004 p-mole/cm^2 per impulse. Under these circumstances, net Ca loss through Na–Ca exchange might be able to keep pace with the Ca entry when the stimulation rate is as high as 150 impulses per sec (although other mechanisms may also be responsible for rapid regulation of $[Ca^{2+}]_i$; cf. Almaes, Meiri, Rahamimoff & Rahamimoff, 1974). Despite the rather tenuous assumptions required for the foregoing calculations, it seems satisfactory that the available data do not rule out a possible role for Na–Ca exchange in the maintenance of neuronal Ca homoeostasis.

Some of the preliminary experiments in this study were performed by Dr W. P. Wiesmann. We are grateful to Dr N. C. Kendrick for helpful suggestions regarding the manuscript. The project was supported by N.I.H. grant NS-08442.

REFERENCES

ALMAES, E., MEIRI, U., RAHAMIMOFF, H. & RAHAMIMOFF, R. (1974). Possible role of mitochondria in transmitter release. *J. Physiol.* **241**, 30–31*P*.

BAKER, P. F. (1972). Transport and metabolism of calcium ions in nerve. *Prog. Biophys. molec. Biol.* **24**, 177–223.

BAKER, P. F. & BLAUSTEIN, M. P. (1968). Sodium-dependent uptake of calcium by crab nerve. *Biochim. biophys. Acta* **150**, 167–170.

BAKER, P. F., BLAUSTEIN, M. P., HODGKIN, A. L. & STEINHARDT, R. A. (1969). The influence of calcium on sodium efflux in squid axons. *J. Physiol.* **200**, 431–458.

BAKER, P. F., BLAUSTEIN, M. P., KEYNES, R. D., MANIL, J., SHAW, T. I. & STEINHARDT, R. A. (1969). The ouabain-sensitive fluxes of sodium and potassium in squid giant axons. *J. Physiol.* **200**, 459–496.

BAKER, P. F. & GLITSCH, H. G. (1973). Does metabolic energy participate directly in the Na^+-dependent extrusion of Ca^{2+} ions from squid giant axons? *J. Physiol.* **233**, 44–46*P*.

BLAUSTEIN, M. P. (1974). The interrelationship between sodium and calcium fluxes across cell membranes. *Rev. Physiol. Biochem. Pharmac.* **70**, 33–82.

BLAUSTEIN, M. P. (1975). Effects of potassium, veratridine and scorpion venom on calcium accumulation and transmitter release by nerve terminals *in vitro*. *J. Physiol.* **247**, 617–655.

BLAUSTEIN, M. P. & ECTOR, A. C. (1975). Barbiturate inhibition of calcium uptake by depolarized nerve terminals *in vitro*. *Molec. Pharmacol.* (in the Press).

BLAUSTEIN, M. P. & GOLDRING, J. M. (1975). Membrane potentials in pinched-off presynaptic nerve terminals monitored with a fluorescent probe: evidence that synaptosomes have potassium diffusion potentials. *J. Physiol.* **247**, 589–615.

BLAUSTEIN, M. P. & HODGKIN, A. L. (1969). The effect of cyanide on the efflux of calcium from squid axons. *J. Physiol.* **200**, 497–527.

BLAUSTEIN, M. P., RUSSELL, J. M. & DE WEER, P. (1974). Calcium efflux from internally-dialyzed squid axons: the influence of external and internal cations. *J. Supramolec. Struct.* (in the Press).

BLAUSTEIN, M. P. & WIESMANN, W. P. (1970). Effect of sodium ions on calcium movements in isolated synaptic terminals. *Proc. natn. Acad. Sci. U.S.A.* **66**, 664–671.

BRADFORD, H. F. (1969). Respiration *in vitro* of synaptosomes from mammalian cerebral cortex. *J. Neurochem.* **16**, 675–684.

BULL, R. J. & TREVOR, A. J. (1972). Sodium and the flux of calcium ions in electrically-stimulated cerebral tissue. *J. Neurochem.* **19**, 1011–1022.

CARAFOLI, E., GAMBLE, R. L., ROSSI, C. S. & LEHNINGER, A. L. (1967). Super-stoichiometric ratios between ion movements and electron transport in rat liver mitochondra. *J. biol. Chem.* **242**, 1196–1204.

CARAFOLI, E. & ROSSI, C. S. (1971). Calcium transport in mitochondria. *Adv. Cytopharmacol.* **1**, 209–227.

COOKE, W. J. & ROBINSON, J. D. (1971). Factors influencing calcium movements in rat brain slices. *Am. J. Physiol.* **221**, 218–225.

DIPOLO, R. (1973). Calcium efflux from internally dialyzed squid axons. *J. gen. Physiol.* **62**, 575–589.

DRAHOTA, Z. & LEHNINGER, A. L. (1965). Movements of H^+, K^+ and Na^+ during energy-dependent uptake and retention of Ca^{++} in rat liver mitochondria. *Biochem. biophys. Res. Commun.* **19**, 351–356.

ESCUETA, A. V. & APPEL, S. H. (1969). Biochemical studies of synaptosomes *in vitro*. II. Potassium transport. *Biochemistry N.Y.* **8**, 725–733.

GLITSCH, H. G., REUTER, H. & SCHOLZ, H. (1970). The effect of internal sodium concentration on calcium fluxes in isolated guinea-pig auricles. *J. Physiol.* **209**, 25–43.

HAMBERGER, A., BLOMSTRAND, C. & LEHNINGER, A. L. (1970). Comparative studies on mitochondria isolated from neuron-enriched and glia-enriched fractions of rabbit and beef brain. *J. cell Biol.* **45**, 221–234.

HODGKIN, A. L. & KEYNES, R. D. (1957). Movements of labelled calcium in squid giant axons. *J. Physiol.* **138**, 253–281.

HUTTER, O. F. & WARNER, A. E. (1967). The pH sensitivity of the chloride conductance of frog skeletal muscle. *J. Physiol.* **189**, 403–425.

KALIX, P. (1971). Uptake and release of calcium in rabbit vagus nerve. *Pflügers Arch. ges. Physiol.* **326**, 1–14.

KATZ, B. & MILEDI, R. (1968). The role of calcium in neuromuscular facilitation. *J. Physiol.* **195**, 481–492.

LAZAROWICZ, J. W., HALJAMÄE, H. & HAMBERGER, A. (1974). Calcium metabolism in isolated brain cells and subcellular fractions. *J. Neurochem.* **22**, 33–45.

LEHNINGER, A. L. (1970). Mitochondria and calcium ion transport. *Biochem. J.* **119**, 129–138.

LING, C-M. & ABDEL-LATIF, A. A. (1968). Studies on sodium transport in rat brain nerve-ending particles. *J. Neurochem.* **15**, 721–729.

LOLLEY, R. N. (1963). The calcium content of isolated cerebral tissues and their steady-state exchange of calcium. *J. Neurochem.* **10**, 665–676.

MICHAELSON, I. A. & WHITTAKER, V. P. (1963). The subcellular localization of 5-hydroxytryptamine in guinea pig brain. *Biochem. Pharmac.* **12**, 203–211.

SCHATZMANN, H. J. & VINCENZI, F. F. (1969). Calcium movements across the membrane of human red cells. *J. Physiol.* **201**, 369–395.

STAHL, W. L. & SWANSON, P. D. (1972). Calcium movements in brain slices in low sodium or calcium media. *J. Neurochem.* **19**, 2395–2407.

TOWER, D. B. (1968). Ouabain and the distribution of calcium and magnesium in cerebral tissues *in vitro*. *Expl Brain Res.* **6**, 273–283.

TOWER, D. B. (1969). Inorganic constituents. *Handbook of Neurochemistry*, vol. 1, ed. LAJTHA, A., pp. 1–24. New York: Plenum Press.

WHITE, A., HANDLER, P., SMITH, E. L. & STETTEN, DEW., Jr. (1954). *Principles of Biochemistry*, p. 148. New York: McGraw Hill Book Co., Inc.

WILLBRANDT, W. & ROSENBERG, T. (1961). The concept of carrier transport and its corollaries in pharmacology. *Pharmac. Rev.* **13**, 109–183.