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EFFECTS OF POTASSIUM, VERATRIDINE AND SCORPION VENOM ON CALCIUM ACCUMULATION AND TRANSMITTER RELEASE BY NERVE TERMINALS *IN VITRO*

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SUMMARY

1. ^{45}Ca uptake by pinched-off nerve terminals (synaptosomes) of rat brain incubated in standard physiological saline (including 132 mM-Na + 5 mM-K + 1·2 mM-Ca) at 30° C averages about 0·5 μmole Ca per g protein per minute. This may be equivalent to a Ca influx of about 0·03 p-mole/cm^2 sec.

2. The rate of ^{45}Ca uptake is increased when the concentration of K in the medium is increased above 15–20 mM, K replacing Na isosmotically. Maximum stimulation, a three- to six-fold increase in the rate of Ca uptake, occurs when $[K]_o$ is about 60 mM. The effect of increased $[K]_o$ is reversible.

3. The K-stimulated Ca uptake is associated primarily with the nerve terminal fraction of brain homogenates. The entering Ca is not accompanied by extracellular markers such as mannitol or inulin. Replacement of external chloride by methylsulphate or sulphate does not prevent the stimulation by K.

4. The effects of external K are quantitatively mimicked by Rb. Caesium also stimulates Ca uptake, but is only about one fifth as effective as K or Rb; Li is ineffective.

5. Two other depolarizing agents also stimulate Ca uptake by synaptosomes: veratridine ($7{\cdot}5 \times 10^{-6}$ to $7{\cdot}5 \times 10^{-5}$ M) and scorpion (*Leirus quinquestriatus*) venom ($6{\cdot}7 \times 10^{-7}$ to $6{\cdot}7 \times 10^{-6}$ g/ml.). The stimulatory effects of veratridine and scorpion venom, but not of increased [K] are blocked by 2×10^{-7} M tetrodotoxin.

6. Internal K also influences the rate of ^{45}Ca uptake by synaptosomes: lowering $[K]_i$ reduces the stimulatory effect of external K and veratridine.

7. Replacement of external Na by choline markedly inhibits the

response to veratridine, but has a much smaller effect on the response to increased $[K]_o$.

8. The Ca uptake mechanism has an apparent dissociation constant for Ca (K_{Ca}) of about 0·8 mM. Increasing $[K]_o$ increases the maximal rate of Ca uptake, but has no effect on K_{Ca}. The K-induced ^{45}Ca uptake is competitively inhibited by Mg^{2+}, Mn^{2+} and La^{3+}.

9. The release of acetylcholine and noradrenaline was also studied. Increasing $[K]_o$ stimulates external Ca-dependent acetylcholine release. Scorpion venom stimulates noradrenaline release from synaptosomes; this effect could be prevented by adding tetrodotoxin or removing external Ca.

10. These results indicate that synaptosomes may increase their permeability to Ca, accumulate Ca and release neural transmitter substances, when stimulated by depolarizing agents under appropriate physiological conditions.

INTRODUCTION

Recent studies have shown that presynaptic nerve ending (synaptosome) fractions prepared from brain homogenates retain many of the metabolic (Bradford, 1969, 1970; Bradford & Thomas, 1969), osmotic (Marchbanks, 1967; Keen & White, 1970, 1971) and alkali metal ion transport (Ling & Abdel-Latif, 1968; Bradford, 1969; Escueta & Appel, 1969; Blaustein & Wiesmann, 1970*a*) properties of more intact tissue preparations (and see Rodriguez de Lores Arnaiz & DeRobertis, 1972). The preceding article (Blaustein & Goldring, 1975) shows that synaptosomes may have membrane potentials which behave like K diffusion potentials. These findings indicate that the synaptosome surface membrane probably reseals when the ending is pinched off during homogenization. Synaptosome preparations may therefore be particularly suitable for investigating other aspects of nerve terminal function such as the mechanism of transmitter release at central nervous system synapses.

The electrophysiological studies of Katz & Miledi (1967*a*, 1969*a*, 1970, 1971) on the squid giant synapse provide convincing evidence that there is a depolarization-dependent calcium permeability increase at the presynaptic terminal. The subsequent entry of Ca ions is presumably involved in triggering the release of transmitter (Katz & Miledi, 1967*b*). Llinás, Blinks & Nicholson (1972) injected the Ca-detecting agent *aequorin* into the squid presynaptic terminal; they observed an increase in *aequorin* luminescence (indicative of increased intracellular ionized Ca^{2+}) upon depolarization of the presynaptic terminal – thereby providing additional support for this 'Calcium Hypothesis'. Furthermore, ^{45}Ca accumulation by rat superior cervical ganglia is enhanced by conditions which trigger

transmitter release, and pharmacological evidence indicates that most of the stimulation-induced uptake is confined to the presynaptic neurones (Blaustein, 1970). Recently, Miledi (1973) obtained direct evidence that intracellular injection of Ca in the presynaptic terminal triggers transmitter release at the squid giant synapse.

The present communication provides evidence that the depolarizing agents potassium, veratridine and scorpion venom stimulate Ca uptake and Ca-dependent transmitter release by rat brain synaptosomes. The effects of veratridine and scorpion venom, but not K, are blocked by tetrodotoxin. These data fit the 'Calcium Hypothesis', and indicate that the mechanism of transmitter release at central synapses is similar to that at peripheral synapses. The results emphasize the fact that synaptosomes behave in many ways like intact nerve endings.

Depolarization-induced Ca permeability increases are not limited exclusively to presynaptic nerve terminals; Ca conductance mechanisms with similar properties have been observed in a variety of tissues including vertebrate cardiac muscle, invertebrate muscle and squid axons (see reviews by Baker, 1972, and Reuter, 1973). The K-stimulated Ca uptake of synaptosomes also shares many of these properties, as will be noted below.

Preliminary reports of some of these findings have been published (Blaustein & Wiesmann, 1970*b*; Blaustein, Johnson & Needleman, 1972; Blaustein, Oborn, Goldring & Wiesmann, 1972; Blaustein, Blehm, Johnson, Needleman & Oborn, 1973).

METHODS

Solutions. The composition of many of the solutions used in these experiments was described in the preceding article (Blaustein & Goldring, 1975). Several additional solutions were used in the present study, however, and several of the standard solutions were occasionally modified. For example, the NaH_2PO_4 was omitted from the medium (such as Na + 5K) when $CaCl_2$ was increased above 1·2 mM, or when $MnCl_2$ or $LaCl_3$ was added to the medium.

In the methylsulphate solutions used for some experiments, all the NaCl and KCl (of the Na + 5K and 137 mM-K media) was replaced by Na- and K-methylsulphate, respectively. When Cl was replaced by SO_4^{2-}, the Na + 5K contained 2·5 mM-K_2SO_4, 43·5 mM-Na_2SO_4 and 155 mM sucrose (to keep both ionic strength and osmolarity constant); the K-rich saline contained 46 mM-K_2SO_4 and 155 mM sucrose. The total $CaCl_2$ concentration in the sulphate solutions was 8 mM, of which 1 mM was the calculated ionized concentration (cf. Hodgkin & Horowicz, 1959).

Two special solutions were also used in these experiments. EGTA (ethylene glycol-bis [β-aminoethyl ether] *N,N'*-tetracetic acid) 'stopping' solution (to chelate extrasynaptosomal Ca and halt Ca uptake) contained NaCl 120 mM, KCl 5 mM, and EGTA 30 mM, buffered to pH 7·6 (20° C) with Tris base. 'Na wash solution' (for washing synaptosomes at the end of Ca uptake studies) was similar in

composition to Na + 5K (see Blaustein & Goldring, 1975) except for the omission of NaH_2PO_4 and glucose; $CaCl_2$ was also omitted (Ca-free Na wash solution) when the synaptosomes were washed for net Ca determinations.

Preparation of presynaptic nerve endings. Synaptosomes, prepared from whole rat brain, were equilibrated with Ca-free Na + 5K, and then pre-incubated with Na + 5K (unless otherwise noted – see Results) for 12–15 min at 30° C, as described previously (Blaustein & Goldring, 1975).

Experimental procedures. Additional aliquots of solutions were added to the synaptosome suspensions, and a further incubation was carried out. In many instances these solutions contained radioactive tracers; ^{45}Ca was used for Ca influx studies. In several experiments, [^{3}H]mannitol or [^{14}C]inulin was used as an extracellular marker. The ionic composition of the added solution often differed from that of the pre-incubation medium, in that, for example, the extracellular K concentration was frequently elevated (by isosmotic substitution for Na; all solutions were isotonic). Drugs were also frequently added to the incubation solutions: veratridine was obtained from K & K Laboratories, Plainview, New York; scorpion (*Leiurus quinquestriatus*) venom was obtained from the Sigma Chemical Co., St Louis, Mo.; tetrodotoxin, grade A, was purchased from Calbiochem., San Diego, California. Details of the ionic composition and drug additions will be given in the Results section.

Ca content. In order to measure the Ca content of synaptosomes following incubation in Ca-containing solutions, it was first necessary to wash away the extrasynaptosomal Ca. The suspension (usually 1·5 ml.) was diluted with 1/3 vol. EGTA 'stopping' solution to immediately chelate the Ca in medium; then 3 vol. iced-cold Ca-free Na + 5K were added, and the suspension was centrifuged at 9000 *g* for 5 min at 3° C. The supernate was discarded and the pellet was rinsed with 8 ml. ice-cold Ca-free Na wash solution, and resuspended in another 8 ml. aliquot, and again centrifuged at 9000 *g*. The rinsing and washing procedure was repeated once more, and after a final rinse with Ca-free Na wash solution, the pellet was suspended in 2 ml. 0·1 % triton X-100. In most experiments the total Ca content of these samples was determined by atomic absorption spectroscopy in the presence of La (see Blaustein & Goldring, 1975). However, in a few instances when ^{45}Ca was present in the samples, the calcein fluorescence technique of Borle & Briggs (1968) was used to assay for Ca content in order to avoid dispersing ^{45}Ca into the atmosphere.

^{45}Ca uptake studies. Following equilibration with Na + 5K and centrifugation (see above) the synaptosome pellets were resuspended in 0·5 ml. Na + 5K, and preincubated for 15 min at 30° C. A 1·0 ml. aliquot of ^{45}Ca-containing incubation solution (for example, Na + 5K, or K-saline, or a mixture of the two) was then added to the suspension, to provide the desired K concentration and a ^{45}Ca specific activity of about 0·2 μc per μmole of $CaCl_2$. Alternatively, in some experiments, other cations (e.g. Li, Rb or Cs) or drugs (for example, tetrodotoxin and/or veratridine) were present in the ^{45}Ca incubation solutions. The suspensions were incubated for an additional period (usually 30 sec or 1 min) at 30° C. Additional information on incubation solutions and incubation conditions, including details of individual experiments will be given in the Results section.

At the end of the incubation period, ^{45}Ca uptake was rapidly terminated by the addition of 0·5 ml. ice-cold EGTA 'stopping' solution, followed by 6 ml. iced-cold Ca-free Na + 5K. The suspensions were then centrifuged at 9000 *g* for 5 min at 3° C and the supernatant solutions were decanted and discarded. The pellets were rinsed and washed as for the net Ca determinations, except that the wash solution used for the ^{45}Ca experiments contained 1·2 mM-Ca (unlabelled); the pellet was

usually washed and rinsed only once (see Results). The washed pellets were resuspended in 1·5 ml. 1 N-NaOH and digested at 60–70° C for 15 min. A 1·0 ml. aliquot was then transferred to a counting vial and neutralized with 1·5 ml. 0·67 N-HCl containing 2 mM-$CaCl_2$ as a carrier. After addition of 3 drops of H_2O_2 and 15 ml. Bray scintillation cocktail, the ^{45}Ca activity was determined by liquid scintillation counting. Samples of the ^{45}Ca incubation solutions were likewise diluted with NaOH, HCl, H_2O_2 and Bray solution, and counted in order to determine the specific activity of Ca. The remaining portion of the pellet digest was assayed for protein by the Lowry method.

Samples containing radioactive inulin or mannitol were handled in the same fashion as those containing ^{45}Ca, and were counted by liquid scintillation spectroscopy; [^{3}H]mannitol and ^{45}Ca or [^{14}C]inulin were measured simultaneously.

Acetylcholine release. Transmitter release was generally measured on samples of the crude mitochondrial fraction (P_2 of Gray & Whittaker, 1962). Since nearly all of the bound acetylcholine (ACh) and noradrenaline in this fraction is localized within synaptosomes (cf. Whittaker, 1965; DeRobertis, 1967), it was assumed that an increase in the concentrations of these transmitters in the supernatant solution would indicate release from the nerve terminals. Synaptosomes account for about 50–60 % of the protein in the P_2 fraction.

Pellets from the 1 hr centrifugation at 17,000 *g* (= 'P_2') were resuspended in 0·32 M sucrose (~ 4 mg protein/ml. sucrose), and diluted with 7 vol. ice-cold Ca-free Na + 5K. The suspensions were centrifuged (9000 *g* for 3 min at 3° C), and the supernatant solution was decanted and discarded. The pellets (~ 2·5 mg protein) were re-suspended in 0·7 ml. Ca-free Na + 5K + 50 mM eserine, and pre-incubated for 10 min at 30° C. An aliquot (0·7 ml.) of either Na + 5K ± 2·4 mM-Ca or 137 mM-K ± 2·4 mM-Ca was then added to each suspension; these solutions all contained 50 μM eserine, to prevent the hydrolysis of free ACh. Following a further 10 min incubation at 30° C, the suspensions were centrifuged at 9000 *g* for 3 min at 3° C. The supernatant solutions were decanted, diluted with 1·0 ml. Na + 5K + eserine, and stored at − 14° C until assayed (within 2–3 days) for ACh.

The pellets were suspended in 3 ml. H_2O containing 50 μM eserine and sufficient HCl to lower the pH to 4. One ml. was frozen and saved for protein assay (by the Lowry method); the remaining 2 ml. were covered and placed in a boiling water bath for 10 min. They were then neutralized to pH 7 with 1 N-NaOH, and centrifuged at 15,000 *g* for 10 min at 3° C. These supernatants (pellet extracts) were also decanted and stored at − 14° C for future ACh assay; the sediment was discarded.

The guinea-pig ileum preparation of Blaber & Cuthbert (1961) was used to bio-assay for ACh. The ileal segments were attached to a muscle tension transducer and contractions were recorded on a Physiograph (E & M Instrument Co., Houston, Texas). At the end of each experiment, atropine was added to the ileum bath (final concentration = 10^{-6} M) to be certain that the contractions induced by the test solutions were prevented by this ACh blocking agent. This was taken as presumptive evidence that the ileum contractions were a consequence of ACh in the test solutions.

Noradrenaline release studies. The synaptosome (and crude mitochondrial fraction) incubation methods for measuring noradrenaline release have been described previously (Blaustein, Johnson & Needleman, 1972; and see Results). The noradrenaline content of supernatant solutions and pellet extracts was assayed fluorometrically after absorption on to, and elution from alumina, and after oxidation by the trihydroxyindole method (Chang, 1964; and see Blaustein *et al.* 1972).

RESULTS

Ca accumulation by synaptosomes

Effect of external K on Ca uptake; time course of Ca uptake. Synaptosomes are known to contain large stores of transmitter substances (e.g. Whittaker, 1965; De Robertis, 1967), and recent evidence (Blaustein & Goldring, 1975) indicates that they may have resting membrane potentials. It therefore appeared worth while to determine whether or not synaptosomes treated with depolarizing agents such as K, could take up Ca and release transmitter substances in accordance with the 'Calcium Hypothesis' (Katz & Miledi, 1967*b*).

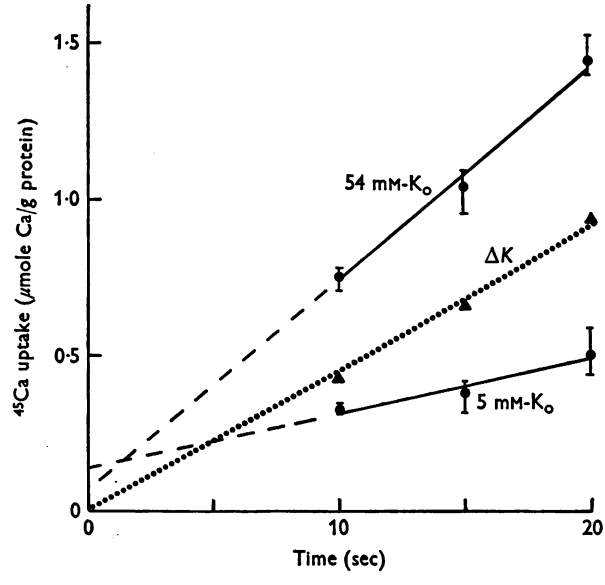

Fig. 1. Early time course of ^{45}Ca uptake by synaptosomes incubated in media containing 5 mM or 54 mM-K. The K-stimulated Ca uptake is indicated by the dotted lines. The abscissa indicates the period of incubation with ^{45}Ca. The $[Ca]_0$ was 1·2 mM in all pre-incubation and incubation solutions. Two washes, in contrast to the usual single wash, were employed in this experiment in order to reduce the extrasynaptomal ^{45}Ca (compare with Fig. 2*A* and see text). Each symbol indicates the mean of three determinations; the bars show the range of the individual values.

Fig. 1–4 illustrate the finding that partial replacement of external Na by K stimulates ^{45}Ca uptake and induces a net gain of Ca by synaptosomes; the effect is observed when as little as 10–15 m-mole (per litre) of Na are replaced by K (Fig. 3). When 49 m-mole Na are replaced by K, the rate of ^{45}Ca accumulation by synaptosomes (indicated by the slopes

of the regression lines) is increased about three- to fourfold (Fig. 1); this increased rate of uptake was observed with incubations as short as 2 sec.

In K-rich media the rate of ^{45}Ca uptake increased with increasing external K concentration, up to $[K]_o$ = 60 mM (see Fig. 3*A*, *B*), and was linear with time for the first 2–3 min. Thereafter, the ^{45}Ca uptake rate from the K-rich solutions gradually slowed to the rate of uptake from the 5 mM-K medium (Fig. 2*A*).

The early (2–3 min) increase in the rate of Ca uptake from K-rich media did not appear to be accompanied by an equally large increase in Ca efflux, since the net Ca content of the synaptosomes increased at about the same rate as did the ^{45}Ca uptake under these conditions (Fig. 2*B*). Furthermore, the magnitude of the K-stimulated ^{45}Ca uptake was approximately equal to the net Ca gain.

The late (after 2–3 min) decline in the rate of ^{45}Ca uptake from K-rich media (Fig. 2*A*) and the levelling off of the net Ca gain (Fig. 2*B*) might indicate either a delayed reduction ('inactivation') of the Ca permeability increase induced by K (cf. Katz & Miledi, 1971; Baker, Meves & Ridgeway, 1973*b*), or an increased efflux (Ca–Ca exchange) which becomes manifest as the concentration of Ca within the terminals increases. Evidence favouring the latter alternative is shown in Fig. 2*A*: the triangles indicate the amount of ^{45}Ca accumulated by synaptosomes incubated for 4, 6 or 10 min, respectively, in 50 mM-K media when ^{45}Ca was present only during the last 1 min of incubation. Assuming that the ^{45}Ca in the synaptosome pellet due to incomplete extra-synaptosomal washout and to 'non-specific' binding (see below) was identical to that for synaptosomes incubated with ^{45}Ca from the time the K-rich solution was first introduced (about 1·0 μmole Ca/g protein – extrapolated from the upper curve), the broken lines indicate the rate of ^{45}Ca influx after various incubation periods in K-rich solution. Note that the slopes of these broken lines are considerably steeper than the slope of the upper curve at the corresponding times, implying that the permeability to Ca is still elevated.

The slopes of the interrupted lines (Fig. 2*A*) do decrease with time, however, which may indicate a slow inactivation of the K-stimulated Ca permeability increase following prolonged depolarization. If this inactivation follows a single-exponential time course, the data of Fig. 2*A* indicate that it has a time constant of the order of 15–20 min.

The curves of ^{45}Ca uptake versus time do not extrapolate to zero uptake at zero time; however, the uptake curves from Na + 5K and from K-rich media do extrapolate to about the same zero-time values (Figs. 1, 2*A*). Although some of the initial 'uptake' might be due to 'non-specific' binding to nerve terminals and contaminating particles (see below), the fact that the magnitude of this early 'uptake' is greatly reduced by washing the pellets twice (Fig. 1, as compared to Fig. 2*A*) suggests that it is due primarily to residual ^{45}Ca from the incubation medium which has been incompletely washed out. These observations indicate that, at the end of a single wash, the amount of extra-synaptosomal ^{45}Ca remaining in the pellet was equivalent to about 0·1–0·2 % of the ^{45}Ca initially present in the incubation medium. Similar values for residual incubation medium are obtained when the impermeable anion, sulphate (labelled with ^{35}S), is used as an extra-synaptosomal marker (M. P. Blaustein, unpublished data).

Since the synaptosome pellets were usually washed only once, the residual extra-synaptosomal ^{45}Ca may be expected to account for about 90–95% of the ^{45}Ca in the pellets of synaptosomes incubated in Na + 5K (e.g. see Figs. 2*A*, 9 and 10). Under these circumstances the difference between the measured uptake from

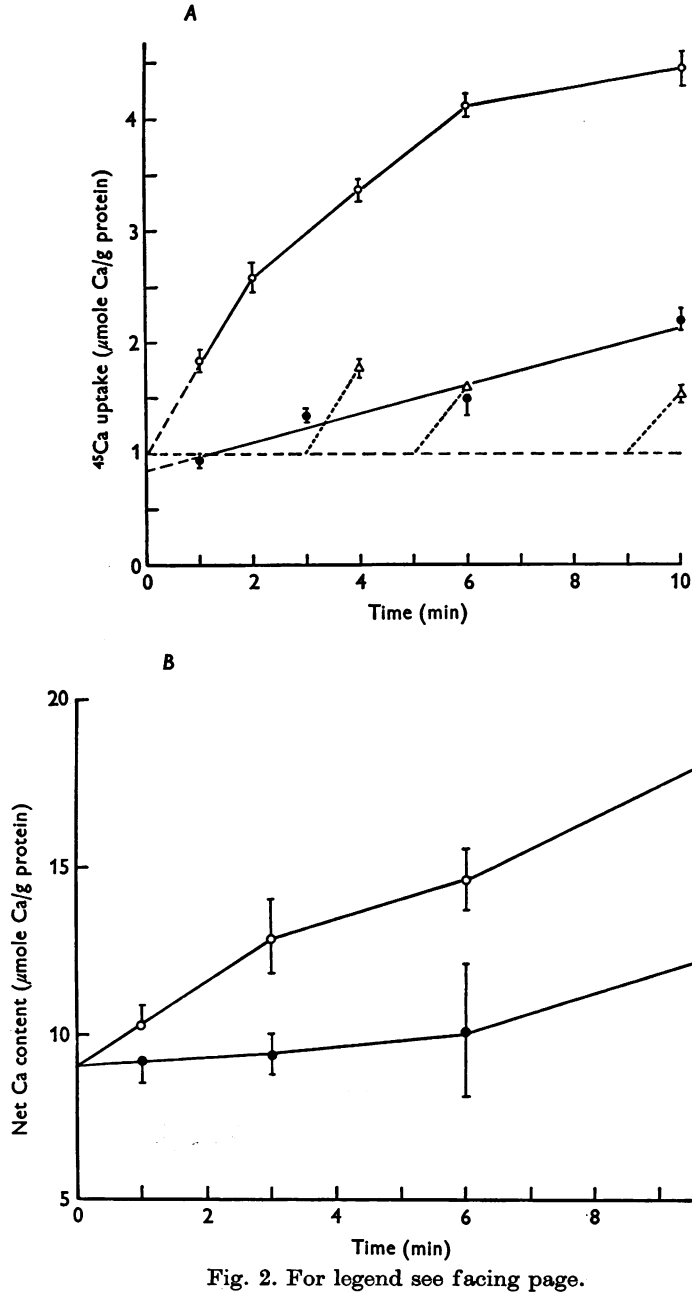

Fig. 2. For legend see facing page.

K-rich media and from Na + 5K – that is, the 'K-stimulated Ca uptake' (cf. the 'ΔK' curve of Fig. 1) – should be an adequate and useful measure of the rate of ^{45}Ca uptake from the K-rich media.

The data on Ca uptake kinetics have been obtained from experiments in which incubation with ^{45}Ca solutions lasted for 60 sec or less. Thus, these data all refer to the early, linear portions of the ^{45}Ca uptake curves (Figs. 1, 2*A*, *B*).

Identification of particles exhibiting K-stimulated Ca-accumulating activity. When the crude mitochondrial fraction ('P_2') of rat brain homogenates is subjected to discontinuous sucrose density gradient centrifugation, most of the material exhibiting K-stimulated Ca-accumulating activity is found at the 0·8–1·2 M sucrose interface (Table 1). This position in the sucrose gradient corresponds to the location of most of the pinched-off nerve terminals ('synaptic bodies': Gray & Whittaker, 1962). The fact that some activity is also observed at the 0·32–0·8 M sucrose interface and in the pellet (Table 1) is consistent with previous observations that the separation is not complete and that some nerve terminals contaminate these fractions (Table 1, column 8). Nevertheless, the relative distribution of the K-stimulated Ca uptake activity in the three gradient fractions is much more closely correlated with the distribution of morphologically intact nerve endings than it is with the fragmented membranes and free mitochondria also present in the gradient (Table 1; and cf. Michaelson & Whittaker, 1963). Furthermore, since 'free' mitochondria are also known to accumulate Ca, it is important to note that this energy-linked process is not influenced by the relative concentrations of Na and K in the medium (e.g. Drahota & Lehninger, 1965; Carafoli, Gamble, Rossi & Lehninger, 1967; Lazarewicz, Haljamäe & Hamberger, 1974). These data

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Fig. 2. *A*, time course of ^{45}Ca uptake by synaptosomes from 5 mM-K (●) and 50 mM-K (○) media. Conditions were similar to those of Fig. 1, except that incubation with ^{45}Ca was more prolonged as indicated on the abscissa. Also shown are data (△) for the 'unidirectional influx' of ^{45}Ca after long incubations in unlabelled 50 mM-K media (containing 1·2 mM-Ca). For these samples, the K concentration of the medium was raised from 5 to 50 mM at 'zero' time; an aliquot of 50 mM-K medium containing ^{45}Ca was added at 3, 5 or 9 min, and ^{45}Ca uptake was terminated 1 min later with EGTA. Each point on the graph is the mean of three determinations; bars indicated ± 1 S.E.

B, net Ca content of synaptosomes incubated in Na + 5K + 1·2 mM-Ca or 50 mM-K + 1·2 mM-Ca for varying periods of time. Conditions were identical to those used for the experiment of *A*. Net Ca content was assayed by the spectrophotofluorometric method of Borle & Briggs (1968). Each point on the graph is the mean of three determinations; bars indicate ± 1 S.E.

TABLE 1. Distribution of K-stimulated ^{45}Ca uptake activity in the discontinuous sucrose density gradient

Sucrose gradient region (and predominant particle morphology)	Gradient*	$[K]_o$ in incubation medium (mM)	^{45}Ca uptake (μmole Ca/g protein per minute)		Protein in layer (mg)	Relative 'specific' distribution	
			Total	K-stimulated		K-stimulated ^{45}Ca uptake†	Synaptosomes‡
0·32–0·8 M interface	1	5	0·62	.	.	.	.
(fragmented membranes)	1	60	0·99	0·37	8·54	0·25	0·38
	2	5	0·61	.	.	.	.
	2	60	1·07	0·46	8·86	0·30	.
0·8–1·2 M interface	1	5	0·90	.	.	.	.
(synaptosomes)	1	60	3·28	2·38	15·59	1·59	1·50
	2	5	0·89	.	.	.	.
	2	60	3·30	2·41	15·95	1·59	.
Pellet (mitochondria)	1	5	0·29	.	.	.	.
	1	60	0·74	0·45	4·08	0·30	0·57
	2	5	0·29	.	.	.	.
	2	60	0·70	0·41	4·47	0·27	.

* Data are from two gradients prepared from a single crude mitochondrial (P_2) fraction (cf. Gray & Whittaker, 1962). Each gradient contained the P_2 material derived from about 1·3 rat brains. Each ^{45}Ca uptake value (total) is the mean of two determinations; no individual value deviated from the mean by more than 5%.

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

‡ Calculated from Fig. 2 of Michaelson & Whittaker (1963) as:

Relative 'specific' distribution of synaptosomes

$$= \frac{\text{fraction of recovered nitrogen associated with synaptosomes in layer}}{\text{fraction of recovered nitrogen associated with synaptosomes in entire gradient}} \div \frac{\text{protein in layer}}{\text{total protein}}.$$

TABLE 2. Effect of hypotonic shock on ^{45}Ca uptake and retention by synaptosomes incubated in 5 mM-K and K-rich media

Conditions*	^{45}Ca content of synaptosomes (μmole Ca/g protein per minute)		% Decrease of ΔK§
	Total†	ΔK‡	
Experiment 1			
Control, 5K	0·80 ± 0·04	.	.
Control, 54 mM-K	2·22 ± 0·02	1·42	.
Pre-incubation lysis, 5K	0·32 ± 0·01	.	.
Pre-incubation lysis, 54K	0·48 ± 0·10	0·16	89
Experiment 2			
Control, 5K	0·91 ± 0·05	.	.
Control, 60K	2·16 ± 0·05	1·25	.
Pre-incubation lysis, 5K	0·55 ± 0·03	.	.
Pre-incubation lysis, 60K	0·75 ± 0·03	0·20	84
Post-incubation lysis, 5K	1·16 ± 0·05	.	.
Post-incubation lysis, 60K	1·90 ± 0·14	0·74	41

* Synaptosomes from the sucrose gradient were equilibrated with ice-cold Ca-free Na + 5K ('controls' and 'post-incubation lysis' groups) or with the same solution diluted 1:50 with distilled water ('pre-incubation lysis' groups). All samples were pre-incubated with 3 ml. (Expt. 1) or 0·75 ml. (Expt. 2) Na + 5K for 12 min at 30° C; 2 ml. (Expt. 1) or 0·5 ml. (Expt. 2) ^{45}Ca-containing Na + 5K (5K samples) or 137 mM-K saline (54 or 60 K samples) were then added, and the suspensions were incubated for an additional 3 min at 30° C. ^{45}Ca uptake was terminated with EGTA. The suspensions were centrifuged and washed in the usual fashion (see Methods) except that the 'post-incubation lysis' pellets were washed once with a 1:50 dilution (with distilled water) of the standard wash solution. In all instances, lysed tissue samples were centrifuged at 15,000 *g*, rather than 9000 *g*, to ensure sedimenting the smaller lighter particles which remained in the suspensions.

† Each value is the mean of three (Expt. 1) or 4 (Expt. 2) determinations ± S.E.

‡ Increment in Ca uptake due to increased $[K]_o$.

§ Percentage decrease in ΔK due to lysis.

imply that the K-stimulated Ca uptake is most likely associated with the presynaptic endings *per se*, rather than with other organelles which may contaminate the synaptosome fraction.

Is the Ca uptake the result of a leaky membrane or synaptosome swelling? The data in Table 2 indicate that an intact synaptosome surface membrane is probably necessary for the K-stimulated Ca uptake, because the stimulation is markedly reduced following exposure to hypotonic media. However, once taken up by the synaptosomes, Ca is stored in a compartment which is relatively insensitive to osmotic shock (possibly intrasynaptosomal mitochondria, cf. Blaustein & Goldring, 1975) since a large fraction of the previously accumulated Ca is retained in the particulate

material following a post-incubation exposure to hypotonic media (Table 2, Expt. 2).

Evidence that K-rich solutions do not render synaptosomes irreversibly leaky to Ca is given in Table 3. These data show that synaptosomes previously exposed to K-rich media take up Ca from K-rich or K-poor solutions at nearly the same rate as do synaptosomes not previously exposed to the K-rich solutions.

TABLE 3. Reversibility of the effects of increased $[K]_o$

Experiment 1*

Time (min) ...	0–3	3–10	10–25	25–28	^{45}Ca uptake (μmole Ca/g protein per minute)		De-crease†
Temp (° C) ...	22	4	30	30			
Conditions	$[K]_o$	$[K]_o$ (m-mole/l.)	$[K]_o$	$[K]_o$	Total	ΔK	(%)
5K–5K	5	5	5	5	1·02 ± 0·03	.	.
5K–58K	5	5	5	58	2·31 ± 0·03	1·29	.
54K–5K	54	10	5	5	1·11 ± 0·03	.	.
54K–58K	54	10	5	58	2·34 ± 0·06	1·23	5

Experiment 2‡

Time (min) ...	0–12	12–13	13–17	17–18			
Temp (° C) ...	30	30	30	30			
Conditions	$[K]_o$	$[K]_o$ (m-mole/l.)	$[K]_o$	$[K]_o$			
5K–9K	5	5	10	9	1·70 ± 0·09	.	.
5K–41K	5	5	10	41	3·62 ± 0·17	1·92	.
49K–9K	5	49	10	9	1·69 ± 0·07	.	.
49K–41K	5	49	10	41	2·97 ± 0·15	1·28	33

* In this experiment the synaptosomes were centrifuged in the 3–10 min time period, and were re-suspended in fresh solution at 10 min. All solutions were Ca-free except the last solution (during the 25–28 min period); this solution also contained ^{45}Ca and had a final Ca concentration of 1·2 mM. Each Ca uptake value is the mean of three determinations ± S.E.

† Due to previous exposure to elevated $[K]_o$.

‡ All solutions for this experiment contained 1·2 mM-Ca; only the last solution (during the 17–18 min period) contained ^{45}Ca. Samples were not centrifuged after the start of the incubation; K concentrations were changed by addition of K-rich or K-poor solution only. Each ^{45}Ca uptake value is the mean of four determinations ± S.E. For both experiments, the sum of Na + K in the medium was always 137 mM.

If synaptosomes are relatively permeable to chloride (but see Blaustein & Goldring, 1975), they might be expected to swell when external NaCl is replaced by KCl (cf. Keen & White, 1971). The K-stimulated Ca uptake observed in Cl-containing solutions might then conceivably represent a passive redistribution of Ca due to bulk flow of water and solutes into

the synaptosomes. However, this explanation seems unlikely for two reasons: in the first place, synaptosomes are relatively impermeable to Ca (Keen & White, 1970). Secondly, as shown in Table 4, replacement of most of the external Cl by the impermeant anions, sulphate (Keen & White, 1970, 1971) and methylsulphate (Hutter & Noble, 1960), does not abolish the K-stimulated Ca uptake.

TABLE 4. Effect of anions on K-stimulated and scorpion venom-stimulated ^{45}Ca uptake by synaptosomes

Incubation medium*	Anion	^{45}Ca uptake (μmole Ca/g protein per minute) Total†	Δ‡
Na+5K	Cl^-	1·01 ± 0·01	.
64 mM-K	Cl^-	3·29 ± 0·14	2·28
Na+5K+s.v.+TTX	Cl^-	0·92 ± 0·04	.
Na+5K+s.v.	Cl^-	2·65 ± 0·11	1·73
Na+5K	$CH_3SO_4^-$	1·04 ± 0·02	.
64 mM-K	$CH_3SO_4^-$	2·54 ± 0·12	1·50
Na+5K+s.v.+TTX	$CH_3SO_4^-$	0·95 ± 0·04	.
Na+5K+s.v.	$CH_3SO_4^-$	1·63 ± 0·06	0·68
Na+5K	SO_4^{2-}	6·51 ± 0·31	.
64 mM-K	SO_4^{2-}	10·64 ± 0·73	4·13
Na+5K+s.v.+TTX	SO_4^{2-}	6·35 ± 0·15	.
Na+5K+s.v.	SO_4^{2-}	10·42 ± 0·79	4·07

* Synaptosomes were pre-incubated at 30° C for 15 min in 0·5 ml. of Na+5K media containing the Na and K salts of the anions indicated in column 2; some samples also contained 0·2 μM tetrodotoxin (+TTX). Isotonic solutions (1·0 ml.) containing ^{45}Ca were then added; as indicated, some of these solutions contained sufficient K to raise the K concentration to 64 mM, or sufficient scorpion venom (+s.v.) to bring the final concentration to $6{\cdot}7 \times 10^{-6}$ g/ml. Incubation with ^{45}Ca at 30° C lasted 1 min.

† The Ca concentration in the chloride and methylsulphate solutions was 1·2 mM; in the sulphate solutions the total Ca concentration was 8 mM of which 1 mM was calculated to be ionized (see Hodgkin & Horowicz, 1959). All values are the mean of three determination ± S.E. of mean.

‡ The K-stimulated ^{45}Ca uptake (scorpion venom absent) or the tetrodotoxin-sensitive ^{45}Ca uptake (scorpion venom present).

The absolute values for ^{45}Ca uptake from the sulphate solutions given in Table 4 are open to question, since the concentration of the ionized Ca is uncertain. The rather large values for ^{45}Ca uptake might be accounted for if the concentration of ionized Ca in the medium (calculated to be 1 mM, from the solubility product data of Brink, 1954) was underestimated (cf. Fig. 7 for the relationship between $[Ca]_o$ and K-stimulated Ca uptake). Nevertheless, regardless of the absolute fluxes, Table 4 clearly shows that Ca uptake is significantly stimulated by increasing $[K]_o$ or by adding scorpion venom to the medium (in the absence of tetrodotoxin – see page 640), whether Cl^-, SO_4^{2-} or $CH_3SO_4^-$ is the predominant external anion.

TABLE 5. Effects of K and scorpion venom on the uptake of Ca, mannitol and inulin by synaptosomes

Conditions*	^{45}Ca uptake (μmole Ca/g protein per minute)			[^{3}H]mannitol uptake (μmole/g protein per minute)			[^{14}C]inulin uptake (as ml. ECF/g protein per minute)**		
	Total†	Δ‡	P§	Total†	Δ‡	P§	Total†	Δ	P§
Experiment 1									
Na + 5K	0·64 ± 0·02	.	.	1·37 ± 0·03	.	.	.	.	.
60 mM-K	2·45 ± 0·25	1·81	< 0·005	1·50 ± 0·08	0·13	> 0·1	.	.	.
Na + 5K	.	.	.	1·15 ± 0·04	.	.	2·18 ± 0·13	.	.
60 mM-K	.	.	.	1·47 ± 0·08	0·32	< 0·05	2·17 ± 0·05	−0·01	> 0·1
Experiment 2		.							
Na + 5K	0·71 ± 0·01	.	.	1·34 ± 0·01	.	.	.	.	.
60 mM-K	2·68 ± 0·08	1·97	< 0·001	1·57 ± 0·06	0·17	< 0·05	.	.	.
Na + 5K + s.v. + TTX	0·55 ± 0·02	.	.	0·71 ± 0·02	.	.	.	.	.
Na + 5K + s.v.	1·07 ± 0·03	0·52	< 0·001	0·78 ± 0·02	0·07	< 0·1	.	.	.

* Synaptosomes (0·6 mg protein in Expt. 1; 1·3 mg protein in Expt. 2) were pre-incubated for 12 min at 30° C in 0·7 ml. Na + 5K. An 0·5 ml. aliquot of Na + 5K or 137 mM-K, containing 12 mM-[^{3}H]mannitol and either ^{45}Ca or [^{14}C]inulin, was then added; as indicated in Expt. 2, some of the added Na + 5K solutions also contained 0·02 mg scorpion venom per ml. (= s.v.), without or with 10^{-6} M tetrodotoxin (= TTX). Synaptosomes were incubated in the labelled media for 30 sec.

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

‡ Note that the final mannitol concentration in the incubation medium was 5 mM – as compared to a Ca concentration of 1·2 mM.

§ The Student's *t*-test was used to calculate the significance of the difference between the uptake from Na + 5K (or Na + 5K + s.v. + TTX) and 60 mM-K (or Na + 5K + s.v.).

** Inulin 'uptake' was calculated as the volume of labelled incubation solution (= ml. ECF) which contained the same number of ^{14}C counts as the synaptosome pellet.

Could 'reverse exocytosis' account for the K-stimulated Ca uptake? There is now considerable evidence that, following transmitter release at the neuromuscular junction, vesicle membrane is retrieved to the interior of the terminals (Ceccarelli, Hurlbut & Mauro, 1973; Heuser & Reese, 1973). In the neurohypophysis, secretory activity is associated with the uptake of extracellular fluid (Nordmann, Dreifuss, Baker, Ravazzola, Malaisse-Lagae & Orci, 1974). Therefore, to eliminate the possibility that the Ca uptake by the terminals results from incorporation of bulk extracellular fluid (by pinocytosis or 'reverse exocytosis') in the interior of re-forming vesicles, the uptake of ^{45}Ca was compared with the uptake of [^{3}H]mannitol (measured simultaneously) and [^{14}C]inulin (measured in companion synaptosome samples). The results (Table 5) show that there is very little stimulation of mannitol or inulin uptake by K. Incorporation of bulk external medium could, at most, account for only about 2% of the K-stimulated Ca uptake.

The conclusion from the experiments of Tables 2–5 is that the K-induced increment in Ca uptake is the consequence of an increase in Ca permeability.

The data in Table 5 do not necessarily rule out the possibility that the synaptosomes release transmitters (see page 646) by an exocytotic process. As shown by Heuser & Reese (1973) during periods of rapid stimulation, synaptic vesicle membrane may be transiently incorporated into surface membrane. Subsequently, during recovery, some of the surface membrane (presumably the original vesicle membrane) may pinch-off and re-enter the cytoplasm as 'coated vesicles', carrying with them some engulfed extracellular fluid. The latter processes may not be observed in the synaptosome experiments because the temperature is rapidly reduced and the extracellular isotope is diluted at the time that the incubation is terminated.

Cation specificity of stimulated Ca uptake and the effects of cation concentration. Fig. 3*A* illustrates the effect of replacing varying amounts of external Na by K on the rate of ^{45}Ca accumulation by synaptosomes. When K is used as the Na substitute, an increase above about 15 or 20 mM-K stimulates the rate of Ca uptake by synaptosomes. This effect continues to increase with increasing $[K]_o$, up to about 60 mM-K, at which point the curve usually reaches a plateau. The increase in ^{45}Ca uptake is paralleled by a net gain of Ca, as shown in Fig. 3*B*.

The effects of other alkali metal ions have also been tested; when a Na is replaced by Rb, the results are virtually indistinguishable from those observed with increased $[K]_o$ (Fig. 4). Cs is much less effective and, compared to the action of K and Rb, about 4–5 times as much Na must be replaced by Cs in order to stimulate Ca uptake to the same degree. Li is even less effective; 50–75% of the Na must be replaced by Li before any stimulatory effect is observed. As discussed elsewhere (Blaustein & Oborn, 1975), the increased Ca uptake from Li media is probably due

primarily to removal of Na, rather than to a specific effect of Li; when choline or dextrose is used as a Na replacement, the effect is indistinguishable from that observed with Li. The sequence of relative effectiveness of the alkali metal ions in stimulating Ca uptake, K $\simeq$ Rb > Cs $\gg$ Li,

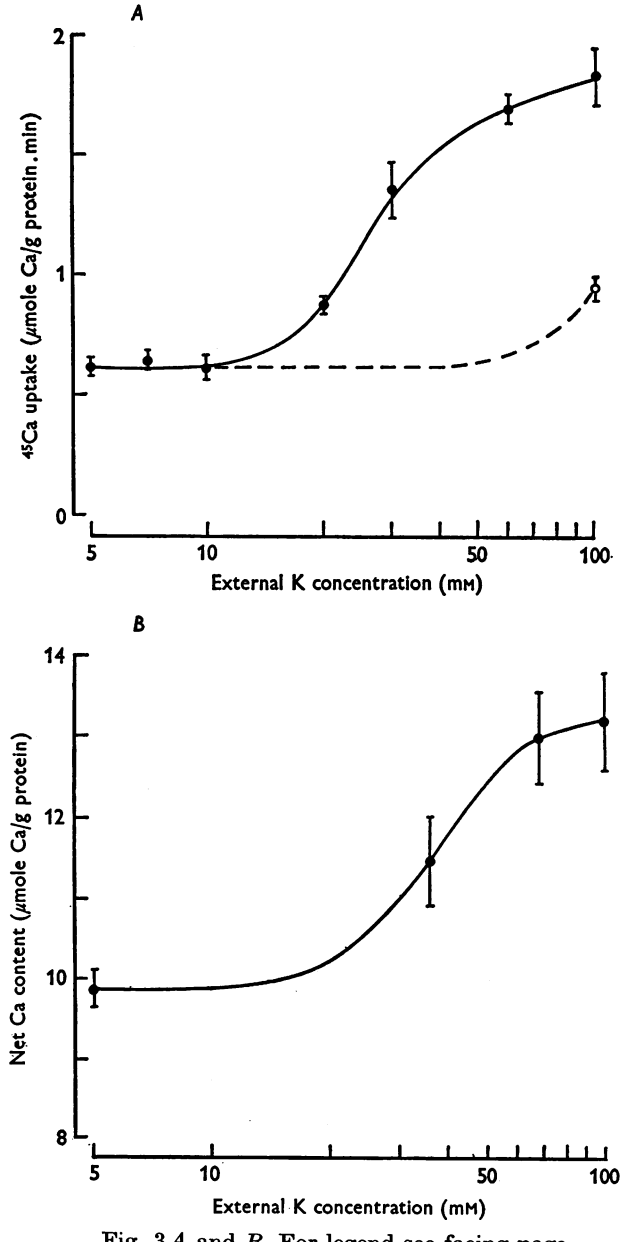

Fig. 3*A* and *B*. For legend see facing page.

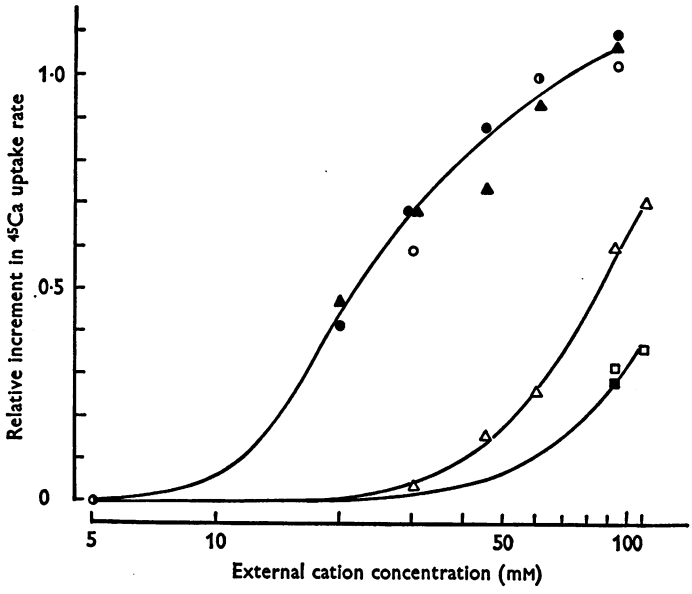

Fig. 4. Relative increment of ^{45}Ca uptake rate graphed as a function of external cation concentration for several different alkali metal ions. Data from two experiments (filled vs. open symbols) are shown. The ^{45}Ca uptake from Na + 5K (0·75 and 0·67 μmole Ca/g protein per minute, in Expts. 1 and 2, respectively) has been subtracted, and the increments on substituting K (○, ●), Rb (▲), Cs (△) or Li (□, ■) isosmotically for Na are compared to the increment in Na + 60 mM-K (3·16 and 3·01 μmole Ca/g protein per minute, in Expts. 1 and 2, respectively). All solutions contained at least 5 mM-K; the alkali metal cation concentration (excluding Na) of the incubation solution given by the abscissa is the sum of $5 + x$, where x represents the concentration of the substituted cation. The protocol for these experiments was similar to that described in the caption to Figs. 1 and 2*A*: all samples were incubated with ^{45}Ca solution for 30 sec. Each symbol indicates the difference between the means of three determinations; the curves were drawn by eye.

Fig. 3. *A*, effect of external K concentration on rate of ^{45}Ca accumulation. The ^{45}Ca-labelled incubation solutions contained mixtures of Na + 5K and 137 mM-K ($[Ca]_o$ = 1·2 mM) to give the indicated K concentration (●). Some samples (○) were incubated in ^{45}Ca-labelled 5 mM-K + 38 mM-Na + 94 mM-Li + 1·2 mM-Ca. Incubation with ^{45}Ca was for 2 min at 30° C. Each point is the mean of three determinations ± S.E.

B, effect of external K concentration on net Ca content of synaptosomes. Experimental protocol was similar to that used for the experiment of *A*, except that no radioactive tracers were used and incubation was for 4 min at 30° C. Ca content was determined by atomic absorption spectrophotometry. Each point is the mean of four determinations; bars indicate ± 1 S.E.

parallels their relative effectiveness in depolarizing nerve and muscle (Shanes, 1958; Sjodin, 1959; Adrian & Slayman, 1966) and, presumably, synaptosomes (Blaustein & Goldring, 1975).

Although in most experiments, such as those of Figs. 3 and 4, other alkali metal ions were used to replace Na on a mole-for-mole basis, the Na ion, *per se*, probably does not play a role in the K-stimulated Ca uptake. As shown in Table 6, when a choline-based solution (choline + 5K) is used as the reference medium, isosmotic replacement of choline by K also stimulates ^{45}Ca uptake.

TABLE 6. Effect of external monovalent cations on rate of ^{45}Ca uptake by synaptosomes

Monovalent cation concentration in ^{45}Ca incubation solution (m-mole/l.)*			^{45}Ca uptake (μmole Ca/g protein per minute)		Decrease‡ (%)
$[Na]_o$	$[Choline]_o$	$[K]_o$	Total	ΔK†	
132	0	5	0·41 ± 0·03	.	.
87	0	50	1·14 ± 0·10	0·73	.
54	0	83	1·35 ± 0·06	0·94	.
0	132	5	0·50 ± 0·01	.	.
0	87	50	0·96 ± 0·02	0·46	37
0	54	83	1·06 ± 0·02	0·56	40

* Synaptosomes were pre-incubated for 12 min at 30° C in Na-free, Ca-free 137 mM-K medium. After centrifugation and removal of the supernantant solution the pellets were re-suspended in 1·7 ml. aliquots of ^{45}Ca incubation solutions (1·2 mM-Ca), with the monovalent cation concentrations indicated. Incubation with ^{45}Ca lasted 1 min at 30° C. Each total ^{45}Ca uptake value is the mean of three determinations ± S.E.

† Increment in Ca uptake due to increased $[K]_o$.

‡ Percentage decrease of ΔK due to removal of external Na.

The effect of internal K concentration on K-stimulated Ca uptake. The concentration of internal K ($[K]_i$), as well as external K ($[K]_o$), influences K-stimulated Ca uptake. Internal K has been altered in one of several ways: as indicated previously (Blaustein & Goldring, 1975), poisoning with ouabain causes synaptosomes to lose K and gain Na, especially if K is omitted from the external medium. A second method of depleting internal K is to pre-incubate terminals in Na-free, K-free Li media; this should also reduce the Na content of the synaptosomes. Alternatively, the terminals can be pre-incubated in 137 mM-K saline, in which case their $[K]_i$ should approach $[K]_o$ (or even surpass it, if the Donnan effect of the internal anion is taken into account). Fig. 5 illustrates the effects of increasing and decreasing $[K]_i$ on the K-stimulated Ca uptake. Note

that when $[K]_i$ is reduced, the curve of Ca uptake versus $[K]_o$ is shifted to the left on the $[K]_o$ axis (and the magnitude of the K-stimulated uptake is decreased); conversely, the curve is shifted in the direction of increasing $[K]_o$ when $[K]_i$ is increased. These are the kinds of effects to be expected if the Ca uptake is linked to the synaptosome transmembrane potential, and if the potential is determined primarily by the $[K]_o/[K]_i$

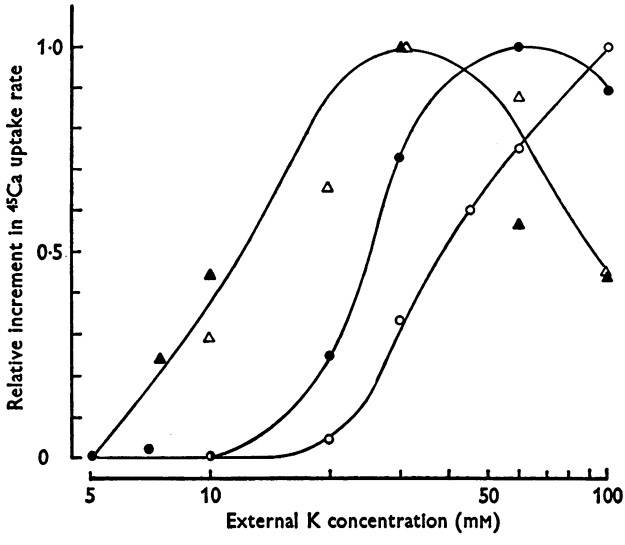

Fig. 5. Effect of external K concentration on the relative increment in ^{45}Ca uptake rate for synaptosomes with different internal K concentrations. The four symbols represent different synaptosome preparations and indicate the composition of the pre-incubation solutions employed in the respective experiments: ●, standard Na+5K salt solution; ▲, Na+5K+1 mM ouabain; △, Li+5K+1 mM ouabain; ○, Na-free 137 mM-K+1 mM ouabain. All samples were pre-incubated for 15 min at 30° C. Suspensions preincubated in 137 mM-K medium (○) or K-free Li (△) were centrifuged and the pre-incubation medium decanted and discarded (see Blaustein & Oborn, 1975), before the ^{45}Ca solutions were added; in all other instances the ^{45}Ca solutions were added directly to the pre-incubation suspensions to provide the final K concentration indicated on the graph. In the experiment denoted by the open triangles, the ^{45}Ca solutions added were mixtures of Na-free 137 mM-K and Na-free 137 mM-Li. The ^{45}Ca uptake from Na+5K (Li+5K in the case of the open triangles) has been subtracted from all data points. The ^{45}Ca uptake from 37 mM-Na+5 mM-K+95 mM-Li has also been subtracted from the ^{45}Ca uptake from 37 mM-Na+100 mM-K media, to compensate for the displacement of external Na, which has a significant effect when $[Na]_o$ is reduced below about 70 mM (see text and Blaustein & Oborn, 1975). The difference (K-stimulated ^{45}Ca uptake) curves are graphed relative to the maximal increment observed in each preparation. The maximum K-stimulated ^{45}Ca uptake values for the respective preparations were: ▲, 0·35; △, 0·56; ●, 1·48; and ○, 1·01 μmole Ca/g protein per minute.

ratio (Blaustein & Goldring, 1975) as in most nerve cells where the resting K permeability greatly exceeds the permeability to other ions.

In the experiments of Fig. 5 the synaptosome K concentrations were not measured. However, if it is assumed that the synaptosomes pre-incubated in 137 mM-K saline (Fig. 5, open circles) did equilibrate approximately with the external environment, these synaptosomes should have had a $[K]_i$ of about 137 mM. If the synaptosome membrane behaves like a perfect K electrode (Blaustein & Goldring, 1975) which obeys the Nernst equation:

$$V_M \simeq E_K = \frac{RT}{F} \ln \frac{[K]_o}{[K]_i},$$

the internal K concentrations for the synaptosomes used to obtain the other curves of Fig. 5 can be calculated with the additional assumption that the 'threshold' (voltage) for increasing Ca permeability remains constant when $[K]_i$ is changed. The $[K]_i$ for the 'normal' curve (solid circles) of Fig. 5, calculated in this way, is about 95 mM, as compared to values of 100–150 mM estimated from other types of data (Blaustein & Goldring, 1975). Similar calculations suggest that $[K]_i$ may be about 40 mM in the Na-loaded (filled triangles) and Li-loaded (open triangles) synaptosomes.

Kinetics of K-stimulated Ca uptake. Fig. 6, which illustrates the effect of temperature on ^{45}Ca uptake, shows that the Q_{10} for the K-stimulated Ca uptake is about 1·4 for the temperature range between 4 and 30° C. This value, which corresponds to an activation energy of about 2·6 kcal/mole, is low enough to be associated with a physical process such as diffusion through a water-filled pore. It is significantly lower than the Q_{10} of 3 found for the Na_i-dependent Ca accumulation by synaptosomes (Blaustein & Oborn, 1975).

According to Fig. 6, the ^{45}Ca uptake from Na + 5K has a Q_{10} of about 1·1; however, with a 1 min incubation about 90% of the apparent ^{45}Ca uptake from this medium is due to trapped medium (see above). This value is therefore not a true estimate of the Q_{10} for the uptake from Na + 5K which should be based on the change in the slope of the time course curves (cf. Figs. 1 and 2*A*).

When the effect of external Ca concentration ($[Ca]_o$) was tested on the rate of Ca uptake at different external K concentrations, the maximal rate of uptake appeared to be a function of $[K]_o$. This is illustrated in Fig. 7 where the reciprocal of the rate of Ca uptake is graphed as a function of the reciprocal of $[Ca]_o$; data are shown for four different K concentrations. The data points for each $[K]_o$ provide fairly good fits to straight lines, and all of the lines extrapolate to approximately the same point on the ordinate, $-1{\cdot}25\ mM^{-1}$. This may indicate that the Ca uptake process is half-saturated when $[Ca]_o$ is about 0·8 mM, and that the affinity is not influenced by $[K]_o$.

The results of Fig. 7 may be interpreted in terms of a voltage-dependent increase in Ca permeability (Katz & Miledi, 1967*a*, 1969*a*, 1970), if increasing $[K]_o$ does

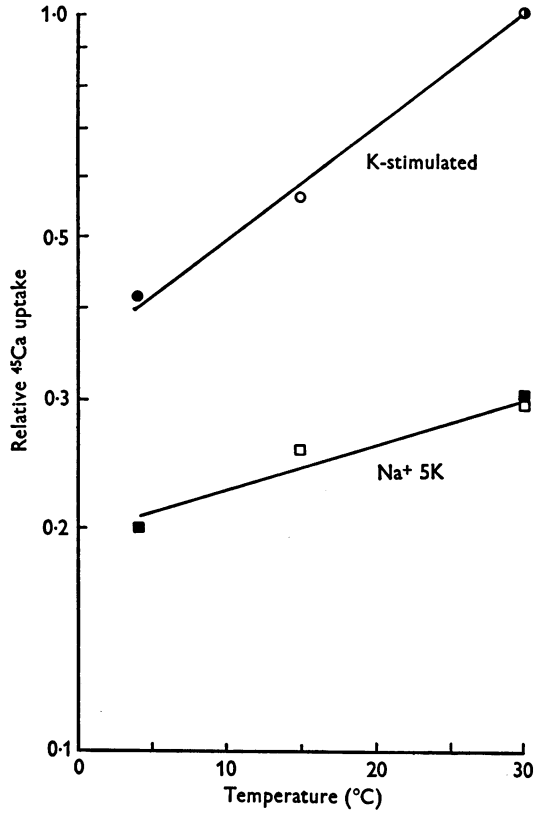

Fig. 6. Effect of temperature on K-stimulated ^{45}Ca uptake. The data are from two experiments (open *vs.* filled symbols), and are scaled to a value of 1·0 for the K-stimulated uptake from 54 mM-K at 30° C. Actual values for the K-stimulated uptake at 30° C in the two experiments were: 1·11 (○) and 0·89 (●) μmole Ca/g protein per minute, respectively. Uptake from Na + 5K at 30° C was 0·33 (□) and 0·26 (■) μmole Ca/g protein per minute, respectively. Samples represented by filled symbols were incubated for 2·5 min, those represented by open symbols were incubated with ^{45}Ca for 3 min. In each experiment three samples were tested for each condition, and the data in the graph represent the mean values (Na + 5K) or difference of the means (K-stimulated).

indeed depolarize pinched-off presynaptic nerve endings (Blaustein & Goldring, 1975). According to this view, depolarization due to increased $[K]_o$ would tend to open membrane channels which are selectively permeable to Ca^{2+} ions. With sufficient depolarization ($[K]_o \geqslant 60$ mM), virtually all the channels would be open (provided 'inactivation' is minimal – see page 623). At lower K concentrations the fraction of channels open at any instant would be a function of $[K]_o$; this would account for the fact that the extrapolated lines cross the ordinate ($[Ca]_o^{-1} = 0$) at different places.

The 'apparent linearity' of the curves in Fig. 7 may be somewhat misleading if

the relationship between Ca permeability (or Ca conductance) and membrane potential (see Fig. 12) is significantly influenced by the external divalent cation concentration. In squid axons, for example, the late (tetrodotoxin-insensitive) Ca entry at low $[Ca]_o$ exceeds that at high $[Ca]_o$, with small depolarizations; the reverse is true with large depolarizations (Baker, Hodgkin & Ridgeway, 1971; but see Katz & Miledi, 1970). The data from the experiments of Fig. 7 may hint at a Ca conductance shift in the depolarizing direction with increasing $[Ca]_o$ (cf. Fig. 12) since Ca uptake falls off more rapidly at $[Ca]_o$ = 6 mM than at $[Ca]_o$ = 0·4 or 1·2 mM, when $[K]_o$ is reduced from 60 to 25 mM. However, resolution of this problem will require experiments with $[K]_o$ in the range of 10–25 mM, where the relationship between log $[K]_o$ (or membrane potential) and log Ca conductance is very steep.

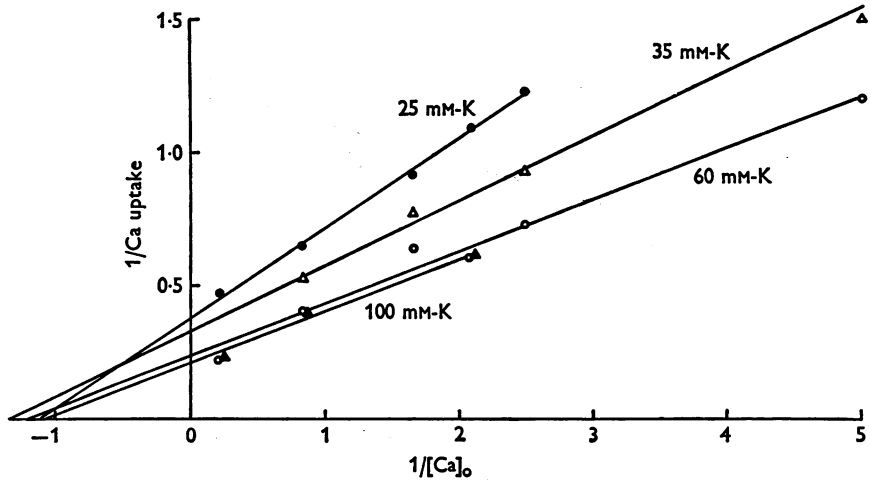

Fig. 7. Reciprocal of K-stimulated ^{45}Ca uptake (μmole Ca/g protein per minute) graphed as a function of the reciprocal of the Ca concentration (mM) in the incubation medium. Data from two different synaptosome preparations are indicated by the filled *vs.* open symbols. Suspensions were pre-incubated for 12 min at 30° C in Na + 5K + 0·2 mM-Ca (●, ▲), or in Na + 5K + 0·48 mM-Ca (○, △). Aliquots of media containing ^{45}Ca and sufficient Ca and K to bring the final concentrations to the desired levels (25 mM-K, ●; 35 mM-K, △; 60 mM-K, ○; 100 mM-K, ▲; were then added, and the suspensions were incubated for 30 sec. Three samples were tested for each condition ($[K]_o$ and $[Ca]_o$) in each experiment. The mean ^{45}Ca uptake from Na + 5K + the appropriate $[Ca]_o$ has been subtracted from the mean ^{45}Ca uptake at elevated $[K]_o$. The regression lines were obtained by the method of least squares.

Dodge & Rahamimoff (1969) examined the effect of $[Ca]_o$ on transmitter release at the frog neuromuscular junction. Their calculations indicated that the release process had a relative affinity for external Ca of about 1·1 mM (range = 0·5–2·0 mM-Ca). The similarity between this value and the half-saturation constant for Ca uptake obtained from Fig. 7 (especially at high $[K]_o$ where conductance shifts may not be a problem) may be more than fortuitous if the Ca entry step was rate limiting in the experiments of Dodge and Rahamimoff (also see Katz & Miledi, 1970).

Inhibition of Ca uptake by polyvalent cations. A number of polyvalent cations, including Mg^{2+} (del Castillo & Engbaek, 1954; del Castillo & Katz, 1954), Mn^{2+} (Katz & Miledi, 1969*b*; Heuser & Miledi, 1971; Kajimoto & Kirpekar, 1972; Meiri & Rahamimoff, 1972) and La^{3+} (Miledi, 1971; Kajimoto & Kirpekar, 1972) are known to inhibit the release of neural transmitter substances. These cations also interfere with depolarization-induced inward currents carried by Ca ions at nerve

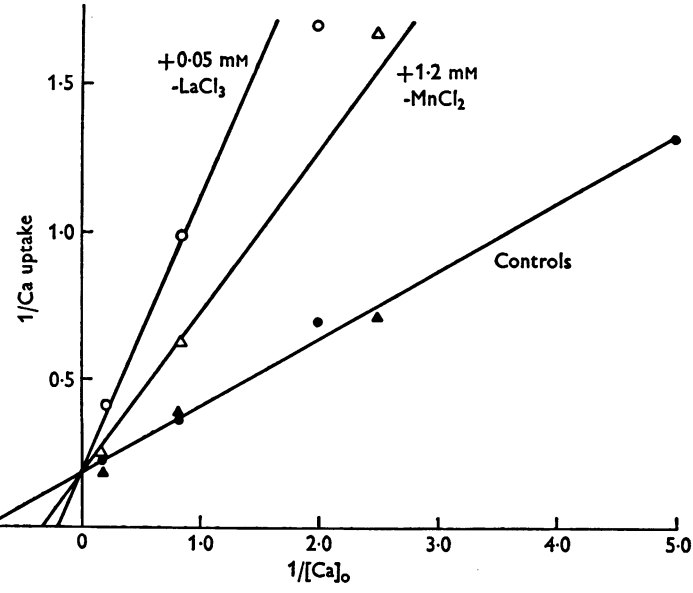

Fig. 8. Effects of Mn^{2+} and La^{3+} on K-stimulated ^{45}Ca uptake by synaptosomes. Pre-incubation and ^{45}Ca-incubation procedures were similar to those described in the caption to Fig. 7, except that some synaptosomes were pre-incubated and incubated in media containing 0·05 mM-$LaCl_3$ (○) or 1·2 mM-$MnCl_2$ (△). Each symbol represents the difference between the mean uptake from 60 mM-K (three determinations) and Na + 5K (three determinations). The data are from two experiments, represented by circles and triangles, respectively, and are graphed as double-reciprocals (Lineweaver–Burke plot). The regression line for the K-stimulated uptake in the absence of inhibitors (●, ▲) was determined by the method of least squares. The lines in the Figure fit the equation (cf. Jenkinson, 1957; Dodge & Rahamimoff, 1967):

$$v_{Ca} = \frac{V_{Ca}}{1 + \frac{K_{Ca}}{K_I}\frac{[I]_o}{[Ca]_o} + \frac{K_{Ca}}{[Ca]_o}},$$

where v_{Ca} is the K-stimulated Ca uptake at any $[Ca]_o$ and V_{Ca} is the maximal K-stimulated Ca uptake with a value of 5·2 μmole Ca/g.min. The apparent half-saturation constant for Ca, K_{Ca}, was 1·25 mM. The apparent half-saturation constants for the inhibitor cations (I), K_{La} and K_{Mn} were, respectively, 0·012 mM-La and 1·0 mM-Mn.

endings (Miledi, 1971), in squid axons (Baker, Meves & Ridgeway, 1973*a*) and in muscle fibres (Hagiwara & Nakajima, 1966); Mg enters squid axons during electrical activity, perhaps via the 'late Ca conductance' pathway (Baker & Crawford, 1972). On the assumption that the K-stimulated Ca uptake of synaptosomes may be a reflexion of an inward Ca current, the effects of these blocking cations on synaptosomes was examined.

Mg, Mn and La were all found to inhibit the K-stimulated Ca uptake. The results of one Mn experiment and one La experiment are shown in Fig. 8. In each case, Ca uptake was measured at several different Ca concentrations, either in the presence of inhibitor or in its absence; only a single inhibitor concentration was used in the experiments shown here (multiple inhibitor concentrations were tested in several other experiments). The data are given in the Figure as double reciprocals (Lineweaver–Burke plots). The straight lines were drawn on the assumption that Mn and La are competitive inhibitors of Ca entry. The Mg^{2+} data (not illustrated) indicate that this ion is about one tenth as effective as Mn.

Effects of scorpion venom and veratridine on synaptosome Ca accumulation. The ability of veratridine to depolarize certain tissue (including synaptosomes) by increasing Na permeability (P_{Na}), and the blockage of this effect by tetrodotoxin, was discussed in the preceding article (Blaustein & Goldring, 1975). Like veratridine, the venom of certain scorpions depolarizes cells with an Na-action potential (Adam & Weiss, 1959; Narahashi, Shapiro, Deguchi, Scuka & Wang, 1972) by blocking inactivation of the Na conductance mechanism (Koppenhöfer & Schmidt, 1968), thereby increasing P_{Na}. The depolarizing action of scorpion venom is also prevented or reversed by tetrodotoxin (T. Narahashi, personal communication). It is apparent that the mechanism of depolarization induced by veratridine and scorpion venom is different from the mechanism of K-induced depolarization (which simply involves alteration of the K diffusion potential). The effects of veratridine and scorpion venom on synaptosome Ca uptake were therefore examined in hope of providing independent, corroborative evidence that the increment in Ca uptake is a consequence of depolarization.

When either veratridine ($7{\cdot}5 \times 10^{-6}$ to $7{\cdot}5 \times 10^{-5}$ M; Fig. 9) or scorpion venom (*Leiurus quinquestriatus* venom, $6{\cdot}7 \times 10^{-7}$ to $6{\cdot}7 \times 10^{-6}$ g/ml.; Fig. 10) is added to the standard salt solution, ^{45}Ca uptake is markedly stimulated; the stimulatory effects are completely prevented if 2×10^{-7} M tetrodotoxin is also present in the incubation medium (Fig. 10 and Tables 4, 5, 7, 9 and 10). Contrariwise, the K-stimulated Ca uptake is unaffected or slightly enhanced by tetrodotoxin (Table 7). This differential effect of

tetrodotoxin on the K-stimulated and on the veratridine- and scorpion-venom-stimulated Ca accumulations clearly implies that different mechanisms are involved in the actions of K on the one hand, and veratridine and scorpion venom on the other. The sensitivity of the veratridine and scorpion venom effects to tetrodotoxin indicates that they may significantly increase P_{Na} in synaptosomes (cf. Blaustein & Goldring, 1975), as in intact tissues.

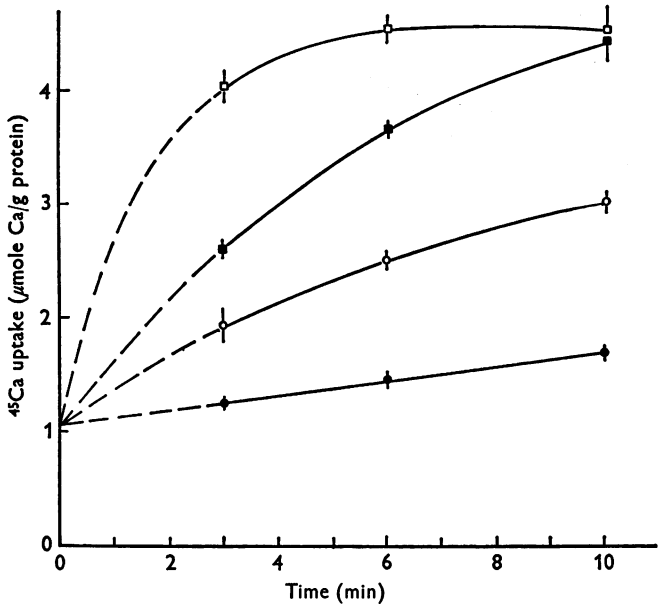

Fig. 9. Effect of veratridine on ^{45}Ca accumulation by synaptosomes. Synaptosomes were pre-incubated in Na + 5K + 1·2 mM-Ca for 15 min at 30° C. An 0·5 ml. aliquot of Na + 5K + 1·2 mM ^{45}Ca medium was then added. This solution contained no veratridine (●), or sufficient veratridine to bring the final concentration in the suspension medium to $7{\cdot}5 \times 10^{-6}$ M (○), 3×10^{-5} M (■) or $7{\cdot}5 \times 10^{-5}$ M (□). EGTA was added (see Methods) to terminate ^{45}Ca uptake at the times indicated on the graph. Each symbol represents the mean of three determinations; the bars indicate ± 1 S.E.

The veratridine-stimulated Ca uptake, like the K-stimulated Ca uptake (see page 628), is at least partially reversible. If, as shown in Table 7, tetrodotoxin is added after 1 min of exposure to veratridine, but before incubation with ^{45}Ca, the ^{45}Ca uptake is significantly reduced.

The incomplete recovery might in part be related to an increase in synaptosome $[Na]_i$ which may accompany the large increase in P_{Na}, since increasing $[Na]_i$ tends to drive Ca into synaptosomes (Blaustein & Oborn, 1975). To minimize the change in $[Na]_i$, most of the scorpion venom and

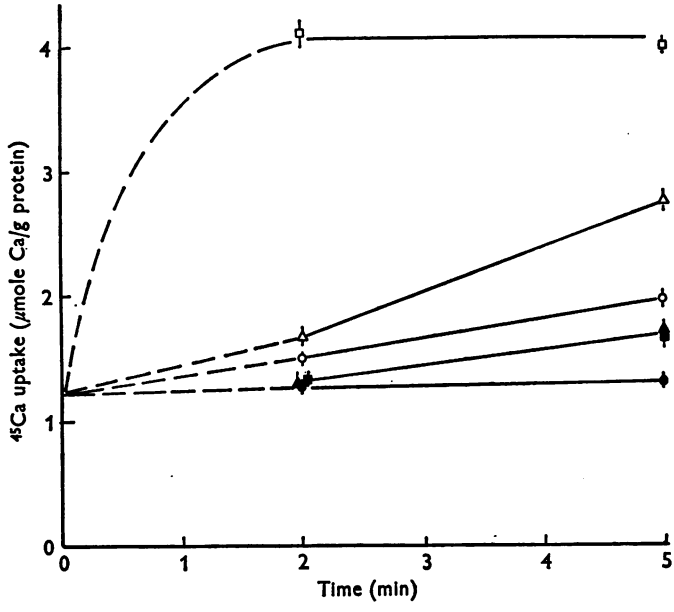

Fig. 10. Effect of scorpion venom and tetrodotoxin on ^{45}Ca accumulation by synaptosomes. Experimental procedures were similar to those described in the caption to Fig. 9. Scorpion venom and/or tetrodotoxin were present only during the ^{45}Ca incubation; filled symbols refer to samples incubated in the presence of 2×10^{-7} M tetrodotoxin. The scorpion venom concentration in the incubation medium was either $6{\cdot}7 \times 10^{-7}$ g/ml. (△, ▲) or $6{\cdot}7 \times 10^{-6}$ g/ml. (□, ■). Controls (○, ●) were incubated without scorpion venom. Each symbol represents the mean of three determinations; the bars indicate ± 1 S.E.

TABLE 7. Reversibility of veratridine-stimulated ^{45}Ca uptake

Time of addition of (sec)*					^{45}Ca uptake (μmole Ca/g protein per minute)	
TTX	Ver	TTX	^{45}Ca	EGTA	Total†	Δ‡
0	10	—	20	50	1·36 ± 0·06	.
—	0	—	70	100	2·50 ± 0·10	1·14
—	0	60	70	100	2·02 ± 0·13§	0·66

* Following a 12 min pre-incubation at 30° C. (0) indicates addition at zero-time; (–) indicates tetrodotoxin omitted. The total volume of all suspensions during the ^{45}Ca incubation was 2·0 ml. TTX = 2×10^{-7} M tetrodotoxin. Ver = $7{\cdot}5 \times 10^{-5}$ M veratridine.

† Each entry is the mean of five determinations ± S.E.

‡ Increment in ^{45}Ca uptake above the uptake measured when tetrodotoxin was added before veratridine.

§ Significantly lower than the Ca uptake without the 1 min veratridine pre-incubation; $P < 0{\cdot}05$.

veratridine experiments involved only 30–60 sec exposures to these agents. Moreover, the maximum rate of Ca uptake from Na-rich media by Na-loaded synaptosomes, about 0·2–0·4 μmole/g protein per minute (Blaustein & Oborn, 1975), cannot account for the entire veratridine or scorpion venom effect.

When tested as a function of $[Ca]_o$, the veratridine-stimulated Ca uptake saturated at elevated $[Ca]_o$. The apparent K_{Ca} for this uptake was about 0·8–1 mM, similar to the value obtained for the K-stimulated Ca uptake (see page 636).

Some other similarities between the K-stimulated Ca uptake and the scorpion venom- or veratridine-stimulated Ca uptake were also observed. As shown in Tables 4 and 5 respectively, the action of scorpion venom was not dependent upon the presence of a permeable anion in the medium, nor did it have much of an effect upon mannitol uptake. Recently, pentobarbitol (200–300 μM) was found to inhibit the uptake of Ca triggered by K and by veratridine (Blaustein & Ector, 1975).

TABLE 8. Effects of external K concentration, veratridine and tetrodotoxin on ^{45}Ca uptake by synaptosomes

			^{45}Ca uptake (μmole Ca/g protein per minute)		
$[K]_o$	Veratridine ($7{\cdot}5 \times 10^{-5}$ M)	Tetrodotoxin (2×10^{-7} M)	Total*	ΔK†	ΔVer‡
5	−	−	0·76 ± 0·03	.	.
5	+	−	1·52 ± 0·04	.	0·76
5	−	+	0·75 ± 0·02	.	.
5	+	+	0·77 ± 0·01	.	0·02
40	−	−	1·66 ± 0·07	0·90	.
40	+	−	2·25 ± 0·14	0·73	0·59
40	−	+	1·76 ± 0·13	1·01	.
40	+	+	1·77 ± 0·04	1·00	0·01
90	−	−	3·15 ± 0·11	2·39	.
90	+	−	2·97 ± 0·10	1·45	−0·18
90	−	+	3·48 ± 0·07	2·73	.
90	+	+	3·42 ± 0·10	2·65	−0·06

* Synaptosomes were incubated with ^{45}Ca solutions for 2 min at 30° C. Each value is the mean of three determinations ± S.E.

† Increment in ^{45}Ca uptake due to increase of $[K]_o$.

‡ Increment in ^{45}Ca uptake, at a given $[K]_o$, due to addition of veratridine, either in the presence or absence of tetrodotoxin.

When synaptosomes are treated with veratridine in the presence of increased $[K]_o$, the veratridine-induced increment in Ca uptake (ΔVer, see Table 8) is either reduced (at intermediate $[K]_o$) or abolished (at high $[K]_o$). The data in Table 8 are consistent with the idea that the

increased Ca uptake is a consequence of synaptosome 'depolarization'. Thus, in partially depolarized synaptosomes ($[K]_o$ = 40 mM, for example) the amount of depolarization induced by veratridine should be reduced (compared to the effect seen with synaptosomes in Na + 5K). If the synaptosomes are sufficiently depolarized ($[K]_o$ = 90 mM, for example), veratridine should have only a small effect on membrane potential (cf. Blaustein & Goldring, 1975) and virtually no effect on Ca uptake (cf. Figs. 3*A* and 12, which may provide information about the relationship between membrane potential and Ca uptake if the conclusions of Blaustein & Goldring, 1975, are valid).

TABLE 9. Effect of ouabain on veratridine-stimulated ^{45}Ca uptake by synaptosomes

Ouabain (10^{-3} M)	Veratridine ($7{\cdot}5 \times 10^{-5}$ M)	Tetrodotoxin (2×10^{-7} M)	^{45}Ca uptake (μmole Ca/g protein per minute)	
			Total*	ΔVer†
−	−	−	0·95 ± 0·02	.
−	+	−	2·04 ± 0·05	1·09
−	+	+	0·86 ± 0·01	−0·09
+	−	−	1·16 ± 0·06	.
+	+	−	1·41 ± 0·05‡	0·25
+	+	+	0·95 ± 0·02	−0·21

* Synaptosomes were pre-incubated in 0·5 ml. Ca-free Na + 5K or Ca-free, K-free Na with 10^{-3} M ouabain (see column 1) at 30° C. After 15 min, 0·5 ml. Na + 5K + 2·4 mM-Ca, with veratridine or tetrodotoxin (as indicated in columns 2 and 3) was added. Ten sec later, 0·5 ml. Na ± 5K + 1·2 mM-Ca (with veratridine or tetrodotoxin in appropriate samples) labelled with ^{45}Ca, was added. ^{45}Ca uptake was terminated with EGTA after a 1 min incubation at 30° C. Each value is the mean of three determinations ± S.E.

† Increment due to veratridine or to veratridine + tetrodotoxin.

‡ Significantly less than uptake without ouabain ($P < 0{\cdot}05$).

The data in Table 9 provide additional evidence that the veratridine- (and, presumably, scorpion-venom-) induced increase in Ca uptake requires a large [K] gradient in the normal direction ($[K]_o \ll [K]_i$), and perhaps a low $[Na]_i$. This experiment shows that ouabain, which decreases synaptosome $[K]_i$ (cf. Blaustein & Goldring, 1975) and increases $[Na]_i$ (Ling & Abdel-Latiff, 1968), greatly reduces the veratridine-triggered increment in Ca uptake. Ouabain also decreases the K-stimulated Ca uptake (see Fig. 5 caption).

Not only must $[Na]_i$ be low, but, as illustrated in Table 10, $[Na]_o$ must be high (i.e. there must be a large, inwardly directed Na concentration gradient) in order for veratridine to stimulate Ca uptake. This observation

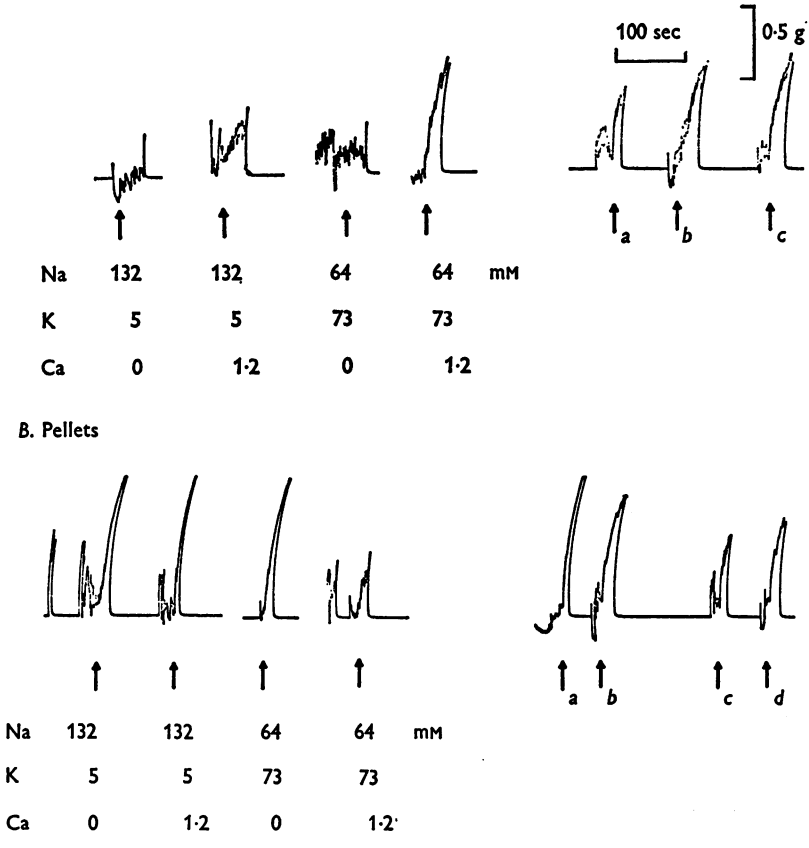

Fig. 11. Acetylcholine assay: guinea-pig ileum tension recordings. The ileal segment was incubated in a 4·9 ml. bath at 37° C, under 1·0 g resting tension; 100 μl. volumes of acetylcholine standards (right-hand records) or test solutions (left-hand records) were added to the ileal bath solution. The time of addition is indicated by the arrow below each tension record. *A*, synaptosomes were incubated for 10 min at 30° C in solutions having the Na, K and Ca composition shown below the tension records. When added to the ileal bath, 100 μl. of the supernatant solutions affected tension in the manner shown. The contractions induced by ACh standards 5×10^{-10}, 6×10^{-10} and 8×10^{-10} g/l. (*a*, *b* and *c*, respectively) are also indicated. *B*, after incubation and centrifugation, acetylcholine was extracted from the synaptosome pellets (see Methods). Aliquots (100 μl.) of these extracts elicited the tension changes shown; the Na, K and Ca composition of the solution in which the synaptosomes were incubated is shown below the respective tension record. The records at the right show the tension developed in response to ACh standards 5×10^{-9}, 2×10^{-9}, 8×10^{-10} and 10^{-9} g/l. (*a–d*, respectively). The 100 sec and 0·5 g calibrations refer to all the records. Further details of the experiment are given in the text.

adds support to the hypothesis that veratridine stimulates Ca uptake by increasing synaptosome P_{Na} – a permeability change which should depolarize synaptosomes if $[Na]_o \gg [Na]_i$ (cf. Blaustein & Goldring, 1975). By comparison, replacement of external Na by choline has a much smaller effect on the K-stimulated Ca uptake (Table 6), and is consistent with the evidence that depolarization of synaptosomes by increasing $[K]_o$ is not dependent upon $[Na]_o$ (Blaustein & Goldring, 1975).

Transmitter release

Acetylcholine release. The foregoing experiments, and those described in the preceding article (Blaustein & Goldring, 1975) are consistent with the idea that depolarization of presynaptic terminals causes an increase in Ca permeability and, consequently, an inward flow of Ca^{2+} ions (due to the inwardly directed Ca electrochemical gradient – see Blaustein & Oborn, 1975). According to the 'Calcium Hypothesis', these are the preliminary steps in the sequence of events which normally results in neural transmitter release (Katz, 1969; and see Douglas, 1968). Since rat brain synaptosomes contain high concentrations of transmitter sub-

TABLE 10. Effect of external Na on veratridine-stimulated ^{45}Ca uptake by synaptosomes

Incubation*	^{45}Ca uptake (μmole/g protein per minute)		Decrease in Δ TTX (%)
	Total†	ΔTTX‡	
Experiment 1			
Na + veratridine	1·91 ± 0·07	1·11	.
Na + veratridine + TTX	0·80 ± 0·03	.	.
Choline + veratridine	0·76 ± 0·03	0·24	78
Choline + veratridine + TTX	0·52 ± 0·03	.	.
Experiment 2			
Na + veratridine	1·62 ± 0·05	0·92	.
Na + veratridine + TTX	0·70 ± 0·02	.	.
Choline + veratridine	0·59 ± 0·01	0·14	85
Choline + veratridine + TTX	0·45 ± 0·03	.	.

* Veratridine = $7{\cdot}5 \times 10^{-5}$ M; TTX = 2×10^{-7} M tetrodotoxin.

† Synaptosomes were pre-incubated for 15 min in Na-free, Ca-free 137 mM-K medium at 30° C. Following centrifugation (5 min at 9000 *g*), the supernatants were decanted and the pellets were re-suspended in either 1·0 ml. Na + 5K + 1·2 Ca or 132 mM choline + 5K + 1·2 Ca. Ten sec later a 0·5 ml. aliquot of the same solution labelled with ^{45}Ca was added; this solution also contained veratridine (final concentration was $7{\cdot}5 \times 10^{-5}$ M) and, where indicated, TTX (final concentration was 2×10^{-7} M). ^{45}Ca uptake was terminated after 1 min by the addition of EGTA. Each value is the mean of three (Expt. 2) or four (Expt. 1) determinations ± S.E.

‡ TTX-sensitive ^{45}Ca uptake.

stances including acetylcholine and noradrenaline (e.g. Whittaker, 1965; DeRobertis, 1967), a logical next step in exploring the functional integrity of the synaptosomes was to determine whether or not appropriate physiological stimuli could trigger transmitter release.

Fig. 11 shows ACh bio-assay data from an experiment in which ACh release from a 'crude mitochondrial (P_2) fraction' was measured. The results clearly indicate that, in the presence of Ca, increasing $[K]_o$ triggers ACh release, since the tissue concentration is greatly reduced and a

TABLE 11. Effect of scorpion venom on noradrenaline (NA) release from crude mitochondrial fraction

	Noradrenaline release (ng NA/mg protein)	
Conditions*	Total†	Δ‡
Experiment 1		
Pre-incubation, only	0·35 ± 0·05	.
Na + 5K	0·66 ± 0·09	0·31
Na + 5K + s.v.	1·10 ± 0·08	0·75
Na + 5K + s.v. + TTX	0·73 ± 0·11	0·38
Ca-free Na + 5K + s.v.	0·48 ± 0·06	0·13
Experiment 2		
Pre-incubation, only	0·60 ± 0·05	.
Na + 5K	1·05 ± 0·09	0·45
Na + 5K + s.v.	1·45 ± 0·10	0·85
Na + 5K + s.v. + TTX	1·01 ± 0·10	0·41
Ca-free Na + 5K	1·09 ± 0·10	0·49

* Tissue samples (~ 1·5 mg protein) from the crude mitochondrial fraction were suspended in 0·5 ml. Ca-free Na + 5K containing cocaine, 0·1 mg/ml. The suspensions were pre-incubated for 12 min at 30° C. Some samples were diluted with 0·5 ml. Ca-free Na + 5K and immediately centrifuged for assay (pre-incubation only). The remaining suspensions were diluted with 0·5 ml. Na + 5K + 2·4 mM Ca ± scorpion venom (s.v., 12·5 μg/ml.) ± tetrodotoxin (TTX, 4×10^{-7} M) and incubated for an additional 10 min at 30° C. The suspensions were then centrifuged at 10,000 *g* for 5 min at 3° C. The supernatant solution was decanted and prepared for noradrenaline assay (Blaustein, Johnson & Needleman, 1972); the protein content of the pellet was determined by the Lowry method.

† Noradrenaline (NA) content of the supernatant solutions. Each value is the mean of four determinations ± S.E.

‡ Increment in NA release during 10 min incubation.

significant amount of ACh appears in the incubation solution. In quantitative terms, the tissue concentration was originally about 300–400 n-mole ACh/g protein, and about half of this ACh was released during a 10 min incubation in Ca-containing 73 mM-K saline. If correction is made for the fact that only about half of the protein in the crude mitochondrial

fraction is synaptosomal protein (see Table 1), these observations are in close agreement with the findings of de Belleroche & Bradford (1973).

Noradrenaline release. Synaptosome noradrenaline release, which is somewhat easier to measure because of the availability of a sensitive fluorescence assay, is also triggered by K-rich media in the presence of Ca (Blaustein, Johnson & Needleman, 1972). Veratridine, likewise, induces a Ca-dependent release of noradrenaline; this release, but not the K-induced release, can be blocked by tetrodotoxin (Blaustein, Johnson & Needleman, 1972). As shown in Table 11, the actions of scorpion venom mimic those of veratridine: this venom triggers a Ca-dependent release of noradrenaline from synaptosomes which is blocked by tetrodotoxin. The venom of another scorpion (*Tityus serrulatus*) has been shown to stimulate Ca-dependent tetrodotoxin inhibitable-ACh secretion from rat brain slices (Gomez, Dai & Diniz, 1973).

The fact that conditions which stimulate Ca uptake by synaptosomes also trigger transmitter (noradrenaline and ACh) provides strong circumstantial evidence that Ca entry may trigger transmitter secretion in these isolated endings by mechanisms similar to those of intact nerve terminals (cf. Katz, 1969).

DISCUSSION

The physiological competence of synaptosomes. The main thrust of these experiments has been to examine some of the physiological properties of pinched-off nerve endings. It should now be apparent that the isolated endings appear to retain a considerable degree of functional integrity. Perhaps most important, from the physiological viewpoint, is the evidence that synaptosomes exhibit some of the special properties of intact nerve terminals: depolarizing agents reversibly increase the Ca permeability of synaptosomes, and the ensuing Ca entry can be correlated with the release of neural transmitter substances. This sequence of events (depolarization, increased Ca permeability, Ca entry, and transmitter release) has been well documented at peripheral nerve terminals (cf. Katz, 1969). The data presented here indicate that this sequence is also involved in the transmitter release process at mammalian central endings.

Some quantitative aspects of synaptosome function: problems of interpretation. Several serious shortcomings must be considered before attempting to evaluate, in quantitative terms, some of the data obtained in the present study. One important problem stems from the fact that a very heterogeneous population of terminals (classified on the basis of transmitter species) was employed for these experiments. There is, at present, no evidence to indicate that the Ca entry mechanism in cholinergic and noradrenergic endings, for example, are identical. However,

in view of the apparently widespread distribution of such mechanisms in secretory tissues (e.g. Douglas, 1968; Katz, 1969; Rubin, 1970), the simplest hypothesis is that most of these voltage-sensitive Ca entry mechanisms are indeed similar, if not identical. The similarity between the apparent affinity for Ca obtained in the present experiments (p. 636) and the Ca affinity at frog neuromuscular junction nerve endings (Dodge & Rahamimoff, 1967) may be evidence for the uniformity of the Ca entry mechanisms. Nevertheless, the synaptosome data reported here can only serve as a measure of the average activity of the synaptosome population until appropriate information becomes available for individual classes of terminals.

A second problem arises from the fact that the synaptosome preparations, while greatly enriched with nerve endings, also contain significant numbers of contaminating particles (free mitochondria, myelin and fragmented membranes – usually in the form of apparently empty vesicles). Michaelson & Whittaker (1963) estimated that about 70% of the protein (nitrogen), in synaptosome preparations is associated with morphologically intact nerve terminals. Furthermore, we must also consider the possibility that some of the 'morphologically intact' terminals may not be 'functionally intact'.

If these reservations are kept in mind, a quantitative evaluation of some of the synaptosome data may provide useful information about the physiology of mammalian C.N.S. presynaptic terminals – information which is not yet available from other sources.

The resting Ca influx in synaptosomes. In those experiments in which correction could be made for ^{45}Ca in the extra-synaptosomal space (e.g. Figs. 1, 2*A*, Table 5), the rate of ^{45}Ca uptake from Na + 5K averaged 0·5 μmole Ca/g protein per minute. Several assumptions are required to convert this value to a synaptosome Ca influx. In the first place it will be assumed that all of the Ca uptake is into synaptosomes, since glucose will not directly fuel Ca uptake by contaminating mitochondria (cf. Blaustein & Oborn, 1975).

Synaptosome surface area can be calculated from diameter and total volume (occluded water space) measurements. The aqueous space which is inaccessible to [^{35}S]sulphate or [^{14}C]inulin (M. P. Blaustein, unpublished data; and see Blaustein & Goldring, 1975) averaged 4·5 ml./g protein in synaptosomes incubated in physiological saline. Although about 30% of the protein is non-synaptosomal, it will be (arbitrarily) assumed that about two thirds of this non-synaptosomal protein may nevertheless be associated with particles (e.g. free mitochondria and 'microsomes') which exclude sulphate and inulin. Thus, the 'corrected' intrasynaptosomal space may be about 3·5 ml./g protein.

Nerve endings incubated in (approximately) 300 m-osmole/kg solutions have diameters of 0·6–0·7 μm (e.g. Gray & Whittaker, 1962; and M. P. Blaustein, unpublished data). Such 'average' measurements, taken from electron micrographs, may slightly underestimate the particle size (cf. Clementi, Whittaker & Sheridan, 1966); a mean diameter of 0·75 μm has therefore been used for the calculation of synaptosome volume and surface area, along with the simplifying assumption that synaptosomes are spherical. A spherical particle with a diameter of 0·75 μm has a surface area of $1{\cdot}8 \times 10^{-8}$ cm^2 and a volume of $2{\cdot}2 \times 10^{-13}$ cm^3. Thus, about 16×10^{12} synaptosomes would have a total volume of 3·5 ml. (the total intrasynaptosomal volume per g protein), and a total surface area of about 3×10^5 cm^2. The average Ca uptake from Na + 5K, 0·5 μmole/g protein per min, may therefore be equivalent to a synaptosome Ca influx of about 0·03 p-mole/cm^2.sec. This value may be compared with the Ca influx in (1) resting squid axons ($[Ca]_o$ = 10·7 mM), 0·08 p-mole/cm^2.sec (Hodgkin & Keynes, 1957); (2) HeLa cells ($[Ca]_o$ = 1·3 mM), 0·055 p-mole/cm^2.sec (Borle, 1969); and (3) frog muscle fibres ($[Ca]_o$ = 1·0 mM), 0·26 p-mole/cm^2.sec (Curtis, 1966), although the latter value does not take into account the surface area of the transverse tubules which are exposed to the external medium.

The K-stimulated Ca uptake. When the external K concentration is increased from 5 to about 60 mM the Ca uptake increases by about 2 μmole/g protein per min, which corresponds to a Ca influx of about 0·12 p-mole/cm^2.sec. If this increment in Ca uptake is the consequence of a specific voltage-dependent increase in Ca conductance (g_{Ca}), and the Ca^{2+} enters as a net flow of current, the current density of this Ca influx would be about 0·024 μA/cm^2 if the Ca conductance mechanism is distributed uniformly over the surface of each nerve terminal. This is about twice the minimal Ca current required to release about 250 quanta of ACh at a frog neuromuscular junction, following the arrival of a nerve impulse (Katz & Miledi, 1969*a*) and would require that half of the entering Ca ions be directly involved in the transmitter release process. If, however, the voltage-sensitive Ca channels are confined primarily to the synaptic region (cf. Katz & Miledi, 1969*a*), which may occupy only 5–10 % of the nerve terminal surface area, the Ca current density in this region may then be about 0·2–0·5 μA/cm^2 in the 'depolarized' synaptosomes.

An estimate of the relationship between membrane potential and the Ca conductance change may also be made by using some of the data given in the preceding (Blaustein & Goldring, 1975) and subsequent (Blaustein & Oborn, 1975) articles. The effect of $[K]_o$ on synaptosome membrane potential may be readily calculated, if it is assumed that

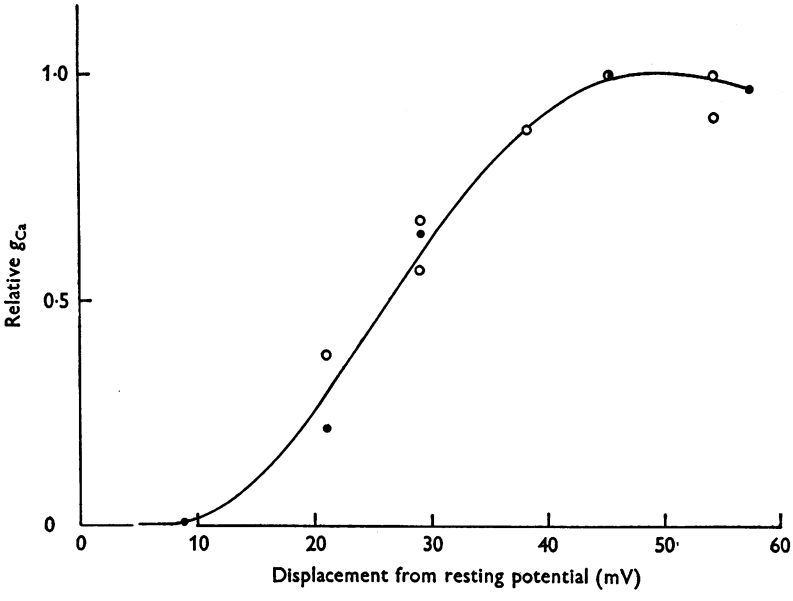

Fig. 12. Effect of synaptosome membrane potential on Ca conductance. The abscissa shows the displacement of the membrane potential from its resting value, as calculated from eqn. (8). The ordinate shows the increment in g_{Ca} relative to that in 60 mM-K (1·0, at 45 mV). The relative g_{Ca} values were Ca calculated from the data in Fig. 3*A* (●) and Fig. 4 (○), using eqn. (8), with the assumption that V_{Ca} was +100 mV and the synaptosome resting potential (in Na+5K) was −60 mV. A K-stimulated Ca uptake (in 60 mM-K) of 2 μmole/g protein per minute corresponds to a g_{Ca} of about 0·2 μmho/cm^2 if the voltage-sensitive Ca channels are distributed uniformly over the synaptosome surface, and to about 2–4 μmho/cm^2, if the Ca channels are confined to the synaptic region.

$[K]_I$ and $[Na]_I$ do not change instantaneously when K is substituted for external Na. As discussed previously (Blaustein & Goldring, 1975), the increment in synaptosome membrane potential (ΔV_m in mV), when $[K]_o$ is increased, should be given by:

$$\Delta V_m = 60\{\log([K]'_o + 0{\cdot}05[Na]'_o) - \log([K]_o + 0{\cdot}05[Na]_o)\}, \quad (1)$$

where $[K]_o$ and $[Na]_o$ are 5 and 132 mM, respectively, and $[K]'_o$ and $[Na]'_o$ are the altered values of these ion concentrations; 0·05 is the resting K/Na permeability ratio (Blaustein & Goldring, 1975). The Ca conductance (g_{Ca}) can then be calculated from the K-stimulated Ca influx ($\Delta_K M_{in}^{Ca}$) data,

$$g_{Ca} = \frac{\Delta_K M_{in}^{Ca} \times F}{V_m - V_{Ca}} = \frac{\Delta_K I_{Ca}}{V_m - V_{Ca}}, \quad (2)$$

with the help of two additional approximations: (1) that the synaptosome

resting potential in Na + 5K is about −60 mV (cf. Blaustein & Goldring, 1975) and (2) that the Ca equilibrium potential, V_{Ca}, is about +100 mV (cf. Blaustein & Oborn, 1975). In eqn. (2) F is Faraday's number and $\Delta_K I_{Ca}$ is the K-stimulated Ca current.

Fig. 12 shows the relationship between the Ca conductance and the displacement from the resting membrane potential, calculated from eqns. (1) and (2).

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