Biochimica et Biophysica Acta, 419 (1976) 295–308

BBA 77176

CARRIER-MEDIATED SODIUM-DEPENDENT AND CALCIUM-DEPENDENT CALCIUM EFFLUX FROM PINCHED-OFF PRESYNAPTIC NERVE TERMINALS (SYNAPTOSOMES) IN VITRO

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(Received July 7th, 1975)

SUMMARY

The influence of external cations on $^{45}Ca^{2+}$ efflux from Ca^{2+}-loaded synaptosomes has been examined The synaptosomes were pre-loaded with $^{45}Ca^{2+}$ by incubating the suspensions in potassium-rich media for 2 min The suspensions were then diluted into "efflux" media containing a "normal" (5 mM) K^+ concentration, the content of Na^+ and Ca^{2+} was varied, as noted below Efflux of $^{45}Ca^{2+}$ was measured for a 2-min period (except for "zero-time" samples), and was terminated by filtering the suspensions on 0 3 μm cellulose acetate filters $^{45}Ca^{2+}$ retained on the filters was determined by liquid scintillation spectroscopy The difference between the $^{45}Ca^{2+}$ in the "zero-time" samples (= "Ca^{2+} load") and in the samples incubated for 2 min was taken as the $^{45}Ca^{2+}$ efflux

$^{45}Ca^{2+}$ loss into Ca^{2+}-free efflux media containing ethyleneglycol-bis-(β-aminoethylether)-*N,N'*-tetraacetic acid (EGTA) was markedly influenced by the Na^+ concentration nearly 80 % of the $^{45}Ca^{2+}$ was lost from the synaptosomes if the media contained 132 mM Na^+, but only about 7 % was lost in 2 min if 97 % of the Na^+ was replaced mol-for-mol by choline In media containing 1 2 mM Ca^{2+} and 132 mM Na^+, the $^{45}Ca^{2+}$ uptake by synaptosomes previously loaded with $^{40}Ca^{2+}$ was significantly less than $^{45}Ca^{2+}$ loss from synaptosomes loaded with $^{45}Ca^{2+}$ Thus there was a net efflux of Ca^{2+} from the Ca^{2+}-loaded synaptosomes, this efflux was, presumably, Na^+ dependent

In media containing 1 2 mM $^{40}Ca^{2+}$ and only 4 mM Na^+, the $^{45}Ca^{2+}$ efflux from $^{45}Ca^{2+}$-loaded synaptosomes was significantly greater if most of the external Na^+ (128 mM) was replaced isomotically by Li^+ rather than by choline, guanidine or glucose This observation may be evidence for a Ca^{2+}-Ca^{2+} exchange which is promoted by Li^+ Both the Na^+-dependent and the Ca^{2+}-dependent Ca^{2+} effluxes were inhibited by Mn^{2+} The data are consistent with a Ca^{2+} carrier mechanism which can extrude Ca^{2+} in exchange for Na^+ or for Ca^{2+}, the latter being activated by Li^+ These properties bear a striking resemblance to those of a Ca^{2+} efflux

Abbreviation EGTA, ethyleneglycol-bis-(β-aminoethylether)-*N,N'*-tetraacetic acid

mechanism which has been characterized in squid axons This mechanism may therefore have evolved fairly early on in the history of the animal kingdom

INTRODUCTION

It has long been known that Ca^{2+} plays a critical role in the transmitter release process at presynaptic terminals [1] Although the precise role of Ca^{2+} is uncertain, there is now ample evidence that transmitter release is associated with entry of Ca^{2+} [2–5] and a rise in the concentration of internal ionized calcium ($[Ca^{2+}]_i$) [6] In order to remain in steady Ca^{2+} balance, the Ca^{2+} which enters the terminals during activity must be subsequently extruded The available data indicate that the $[Ca^{2+}]_i$ is normally considerably lower than $[Ca^{2+}]_o$ (the external Ca^{2+} concentration) in both invertebrate and vertebrate neurons, $[Ca^{2+}]_i$ may be of the order of 10^{-6} M or less [7, 8] Since the cytoplasm of resting neurons is negative, with respect to the external medium, this means that the Ca^{2+} must be extruded against a large electrochemical gradient

In a large variety of tissues [8], including squid axons [9] and mammalian peripheral nerve [10], Ca^{2+} efflux appears to depend, in part, upon the presence of Na^+ in the bathing medium Ca^{2+} efflux from $^{45}Ca^{2+}$-loaded brain slices is also dependent upon external Na^+ [11–13] These results have been taken as evidence that the Ca^{2+} efflux involves a carrier-mediated counterflow exchange of Na^+ for Ca^{2+} the movement of Na^+ into the cells, down its electrochemical gradient, may provide some of the energy for "uphill" Ca^{2+} extrusion [8, 9]

Isolated, pinched-off presynaptic terminals (synaptosomes) from brain homogenates retain considerably functional integrity [14] they have membrane potentials [15], and can accumulate Ca^{2+} and release transmitters when triggered by depolarizing agents [4, 5] As in the case of the other neural tissues mentioned above, the efflux of $^{45}Ca^{2+}$ from pre-loaded synaptosomes requires the presence of Na^+ in the bathing medium [16, 17] This suggests that as evolutionally divergent tissues as squid axons and mamalian central neurones may have very similar mechanisms for extruding Ca^{2+}

If a carrier mechanism is, indeed, involved in the exchange of Na^+ for Ca^{2+} it may also mediate the exchange ("exchange diffusion") [18] of Ca^{2+} for Ca^{2+} that is, under some circumstances Ca^{2+} efflux may depend upon external Ca^{2+} This appears to be the case in the squid axon, where a portion of the Ca^{2+} efflux is in fact dependent upon external Ca^{2+} [9] Furthermore, the Ca_o^{2+}-dependent Ca^{2+} efflux is promoted by Li^+ [19, 20] perhaps indicating that during Ca^{2+}-Ca^{2+} exchange the entering Ca^{2+} is accompanied by Li^+

In order to see how closely the Ca^{2+} carrier mechanism in mammalian nervous tissue resembles that of squid axons, we have examined the effects of external monovalent cations on the efflux of Ca^{2+} from $^{45}Ca^{2+}$-loaded synaptosomes in the presence and absence of external Ca^{2+} The results indicate that Li^+ is much more effective than choline or guanidinium in promoting Ca_o^{2+}-dependent Ca^{2+} efflux The data obtained in the presence of Na^+ are more difficult to interpret because, unlike these other monovalent cations, Na^+ promotes Ca^{2+} efflux even in the absence of external Ca^{2+} [16, 17]

MATERIALS AND METHODS

Preparation of synaptosomes Synaptosomes were prepared from whole rat brain homogenates by differential and sucrose density gradient centrifugation [21] The original Gray and Whittaker procedure [21] was altered slightly [22] to reduce the microsomal contamination in the crude mitochondrial pellet (P_2) the S_1 supernatant was centrifuged for only 25 min at 10 000 $\times g$ and 4 °C The resulting pellet (P_2) was re-suspended with a manual homogenizer in fresh 0 32 M sucrose (equal to the volume decanted) and again centrifuged for 25 min at 10 000 $\times g$ The pellet from this spin (P'_2) was re-suspended in 0 32 M sucrose, and the suspension layered on the discontinuous sucrose gradient [21] Following a 2-h centrifugation at 65 000 $\times g$ and 4 °C, the material at the 0 8–1 2 M sucrose interface ("synaptosomes") was removed and diluted with approx 10 volumes of ice-cold Ca^{2+}-free $Na^+ + 5\ K^+$* Aliquots of this suspension were centrifuged at 9000 $\times$ g for 4 min at 4 °C The supernatant solution was decanted, and the pellets were resuspended and incubated as described below

Solutions The composition of representative experimental solutions is shown in Table I In order to maintain isotonicity, intermediate monovalent cation concentrations were obtained by mixing appropriate solutions (e g $Na^+ + 5\ K^+$ and $Li^+ + 5\ K^+$, or $Li^+ + 5\ K^+$ and choline $+ 5\ K^+$) In a few instances $MnCl_2$ was added to some of the solutions (see Results)

Experimental procedures In order to return the synaptosomes to a physiological steady state, the pellets from the 9000 $\times g$ centrifugation (0 6–0 85 mg protein) were resuspended in 0 5 ml of $Na^+ + 5\ K^+$ and equilibrated for 12 min at 30 °C Calcium loading of the synaptosomes was initiated by the addition of 0 5 ml of

TABLE I

COMPOSITION OF REPRESENTATIVE SOLUTIONS

In addition to the constituents listed in the table, all solutions also contained, in mmol/l $MgCl_2$, 1 3, NaH_2PO_4, 1 2, glucose, 10, Tris base, 20 The solutions were buffered to pH 7 65 at 25 °C by titration with maleic acid All solutes were reagent grade, or highest grade available Glass-distilled water was used for all solutions The salt concentrations are expressed as mmol/l

Solution	NaCl	LiCl	KCl	$CaCl_2$	EGTA
$Na^+ + 5\ K^+ + Ca^{2+}$ *	132	0	5	1 2	0
$Na^+ + 5\ K^+$ (0 Ca^{2+}) **	132	0	5	0	0
Ca^{2+}-free $Na^+ + 5\ K^+$ *	132	0	5	0	0 5
137 mM KCl saline + Ca^{2+}	0	0	137	1 2	0
$Li^+ + 5\ K^+ + Ca^{2+}$	0	132	5	1 2	0
Ca^{2+}-free $Li^+ + 5\ K^+$	0	132	5	0	0 5

* In some solutions, all of the NaCl was replaced by an equimolar amount of choline chloride (= choline $+ 5\ K^+ + Ca^{2+}$ or Ca^{2+}-free choline $+ 5\ K^+$) or guanidine HCl (= guanidine $+ 5\ K^+ +$ Ca^{2+} or Ca^{2+}-free guanidine $+ 5\ K^+$) In glucose $+ 5\ K^+ + Ca^{2+}$ and Ca^{2+}-free glucose $+ 5\ K^+$, the 132 mM NaCl was replaced by 264 mM glucose

** The Ca^{2+} content of this solution, measured by atomic absorption spectroscopy, was 3–4 μM

* See Table I

137 mM potassium chloride/saline [5] containing $^{45}Ca^{2+}$ (specific activity $\simeq 2\ \mu Ci$ per $\mu mol\ Ca^{2+}$ in the final 1 0 ml suspension) After 2 min of incubation at 30 C with $^{45}Ca^{2+}$, 0 3 or 0 4 ml of well-stirred synaptosome suspension was pipetted into an erlenmeyer flask containing 10 ml of an appropriate efflux solution (see Table I and Results)* In some instances the solution was Ca^{2+}-free choline+5 K^+, these samples were mixed and immediately filtered to determine the amount of ^{45}Ca loading All other samples were mixed with solutions at 30 °C, and were incubated for 2 min and then filtered as noted previously [17], Ca^{2+} efflux is linear for about 2 min

The synaptosome suspensions were vacuum filtered on pre-washed 0 3 μm pore diameter Gelman or Millipore cellulose acetate filters Each filter was then rapidly washed with 15 ml of ice-cold choline+5 K^+, trials with protein-free solutions indicated that after one 15-ml wash only about 0 5–1 % of the counts remaining on the protein-containing filters could be attributed to extra-synaptosomal $^{45}Ca^{2+}$

The washed filters were transferred to liquid scintillation counting vials, 10 ml of toluene/Triton X-100 scintillation cocktail [23] was added, and the samples were counted in a Packard Tricarb liquid scintillation counter Small aliquots of the $^{45}Ca^{2+}$ incubation solutions were also counted in order to compute the $^{45}Ca^{2+}$ specific activity in the loading solutions The protein content of synaptosome pellets from each experiment was determined on an auto-analyzer by the method of Lowry et al [24], bovine serum albumin was employed as a standard

Calcium efflux was computed as the difference between the $^{45}Ca^{2+}$ remaining in the synaptosomes (i e on the filters) after the 2-min efflux period and the $^{45}Ca^{2+}$ content immediately after loading

RESULTS

External Na^+-dependent Ca^{2+} efflux

As reported previously [17], when Ca^{2+} is omitted from the efflux medium (to minimize Ca^{2+}-Ca^{2+} exchange), the loss of isotope from $^{45}Ca^{2+}$-loaded synaptosomes is largely dependent upon the presence of Na^+ in the bathing medium Similar results were obtained in the present study as shown in Table II, on the average, 79 % of the $^{45}Ca^{2+}$ was lost from $^{45}Ca^{2+}$-loaded synaptosomes incubated in Ca^{2+}-free Na^+ – 5 K^+ for 2 min at 30 °C However, if the external Na^+ concentration was reduced to 4 mM by isosmotic substitution of choline, only 7 % of the radioactive label was lost during a 2-min incubation (line 6), the $^{45}Ca^{2+}$ efflux was only slightly greater (12–22 %) if LiCl (line 4), guanidine HCl (line 8) or glucose (line 10) was the NaCl replacement

The relationship between external Na^+ concentration and $^{45}Ca^{2+}$ efflux into Ca^{2+}-free media is illustrated in Fig 1A The data were obtained with mixtures of Ca^{2+}-free Na^++5 K^+ and Ca^{2+}-free choline+5 K^+ There is very little Ca^{2+} efflux into solutions containing about 4 mM Na^+ At slightly higher external Na^+ concentrations, Ca^{2+} efflux rises steeply, it then appears to level off as the external Na^+ concentration is increased still further Although, as noted earlier [17], this curve

* Because of this dilution procedure the minimal Na^+ concentration during efflux in the nominally Na^+-free solutions was 4 mM

TABLE II

EFFECTS OF EXTERNAL MONOVALENT CATIONS AND CALCIUM ON $^{45}Ca^{2+}$ EFFLUX FROM SYNAPTOSOMES

Composition of efflux solutions*			Percent of $^{45}Ca^{2+}$ load lost per 2 min**	ΔCa^{2+}***	P†	ΔLi^{+}-choline††	P†
Monovalent cations	1 2 mM $CaCl_2$	0 5 mM EGTA					
$Na^{+}+5\ K^{+}$	+	0	70 5±2 3(6)	−8 6± 2 7	< 0 01		
$Na^{+}+5\ K^{+}$	0	+	79 1±1 4(7)				
$Li^{+}+5\ K^{+}$	+	0	52 7±2 8(12)	33 1± 5 4	< 0 001	27 1±5 1	< 0 001
$Li^{+}+5\ K^{+}$	0	+	19 6±4 6(7)			12 7±5 5	< 0 05
Choline+5 K^{+}	+	0	25 6±4 3(9)	18 7± 5 3	< 0 01		
Choline+5 K^{+}	0	+	6 9±3 1(7)				
Guanidine+5 K^{+}	+	0	39 ±7 (1)	17 ±11	> 0 05		
Guanidine+5 K^{+}	0	+	22 ±8 (1)				
Glucose+5 K^{+}	+	0	28 ±4 (1)	16 ± 7	> 0 05		
Glucose+5 K^{+}	0	+	12 ±6 (1)				

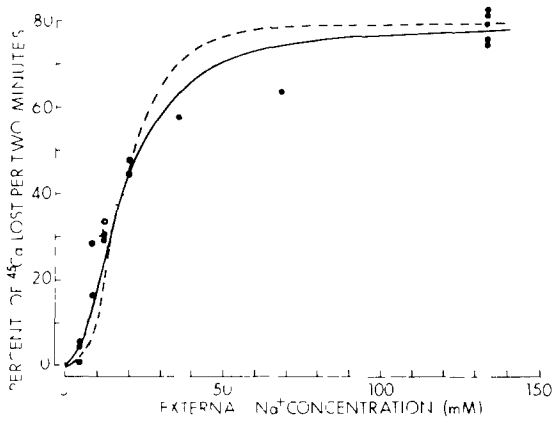
* See Table I

** The mean $^{45}Ca^{2+}$ load for these experiments was 7 72±0 52 μmol Ca^{2+}/g protein, the data are from a total of 18 experiments The number of experiments in which the efflux for each condition was determined is given in parentheses, three measurements of efflux were made in each experiment For the glucose and guanidine, the values shown refer to the means ±S E for these three measurements In all other experiments the values shown refer to the average of the means from each experiment ±S E

*** Decrement (−) or increment in Ca^{2+} efflux due to the addition of 1 2 mM $CaCl_2$ and deletion of EGTA

† Confidence limits for the ΔCa^{2+} and ΔLi^{+}-choline values determined by the student's t-test

†† Increment in Ca^{2+} efflux due to replacement of choline by Li^{+}, either in the presence or absence of $CaCl_2$



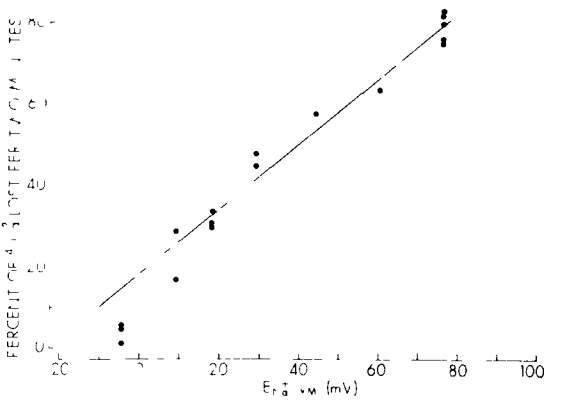


Fig 1 (A) Effect of external Na^+ on $^{45}Ca^{2+}$ efflux into Ca^{2+}-free solutions Na^+ was replaced isosmotically by choline The ordinate shows the percent of the $^{45}Ca^{2+}$ load lost during a 2-min efflux period Data from six synaptosome preparations from the current study are shown Each point on the graph represents the mean of three Ca^{2+} efflux determinations from one synaptosome preparation, two to four different external Na^+ concentrations were tested in each experiment The curves were calculated from the equation [17]

$$M_{Ca^{2+}} = \frac{M^*_{Ca^{2+}}}{1 + \left(\frac{\bar{K}_{Na^+}}{[Na^+]_0}\right)^n}$$

where $M_{Ca^{2+}}$ is the Ca^{2+} efflux to any external Na^+ concentration ($[Na^+]_0$) The maximal Ca^{2+} efflux, $M^*_{Ca^{2+}}$, has a value of 80 % of the Ca^{2+} load lost per 2 min, $\bar{K}_{Na^+}$ is the apparent mean half-saturation constant for external Na^+, with a value of 18 mM The exponent, n, has a value of 1 ( ) 2 (———) or 3 (- - -) (B) Calcium efflux from synaptosomes graphed as a function of the electrochemical gradient for sodium ($E_{Na^+} - V_{memb}$, in millivolts) The data are from the same experiments as A The average membrane potential, V_{memb}, of synaptosomes in the efflux solutions was assumed to be −45 mV [15] The calculation of the sodium equilibrium potential, E_{Na^+} (= 60 log $[Na^+]_o$ $[Na^+]_i$), was based on the assumption that the internal Na^+ concentration, $[Na^+]_i$, was 40 mM The line was drawn by eye

appears to be sigmoid, it is difficult to be certain of the precise relationship between the Na^+ concentration and the Ca^{2+} efflux A Hill plot of these data (log $[M_{Ca^{2+}}/(M_{Ca^{2+}}{}^* - M_{Ca^{2+}})]$ versus log $[Na^+]_0$, cf caption to Fig 1A) had a least-squares slope (Hill coefficient) of 1 8±0 2 The data may therefore be consistent with a mechanism that requires more than one (perhaps two) external Na^+ to activate the efflux of one Ca^{2+}, perhaps by Na^+-Ca^{2+} exchange [17] If this is indeed the case, and if energy derived directly from the Na^+ electrochemical gradient can be used to extrude Ca^{2+} against its electrochemical gradient [8, 9], then it would be of interest to examine the relationship between the Na^+ electrochemical gradient and the Ca^{2+} efflux Consequently, Ca^{2+} efflux data from Fig 1A have been graphed as a function of the calculated Na^+ electrochemical gradient in Fig 1B In these calculations the resting membrane potentials (V_{memb}) was assumed to be −45 mV [15], irrespective of the concentration of Na^+ or choline in the efflux solution If choline is significantly less permeable than Na^+ [15], the V_{memb} would be more negative at lower external Na^+ concentrations, so that the points at small $E_{Na^+} - V_{memb}$ values would be shifted to the right For the calculations of the sodium equilibrium potential (E_{Na^+}), the internal Na^+ concentration ($[Na^+]_i$) was assumed to be a constant 40 mM Smaller values of $[Na^+]_i$, or a positive correlation between $[Na^+]_i$ and $[Na^+]_0$ (the external Na^+ concentration), due to inward leak of Na^+, would also tend to make the curve steeper at low $E_{Na^+} - V_{memb}$ values The correlation between Ca^{2+} efflux and the Na^+ electrochemical gradient is readily apparent (Fig 1B), and is not markedly affected by the precise values chosen for V_{memb} and $[Na^+]_i$ However, the foregoing considerations indicate that there may be significant deviation from linearity especially at low values of E_{Na^+}

Net Ca^{2+} loss from Ca^{2+}-loaded synaptosomes

The magnitude of the Ca^{2+} efflux from $^{45}Ca^{2+}$-loaded synaptosomes into Na^+-containing solutions, about 2 5–3 μmol Ca^{2+}/g protein per min (Table II), is considerably greater than the resting Ca^{2+} influx of about 1 μmol Ca^{2+}/g protein per min (ref 5 and unpublished data of Ector, A C and Fried, R C) This suggests that the Ca^{2+} efflux, even into Ca^{2+}-containing solutions, may involve a net loss of Ca^{2+} from the synaptosomes In order to verify this possibility, both Ca^{2+} efflux and Ca^{2+} influx were examined in comparably treated synaptosomes from a single preparation (Table III) Some synaptosomes were loaded with $^{45}Ca^{2+}$ in the manner routinely employed for all other efflux experiments Aliquots of these suspensions were then added to $Na^+ + 5\ K^+$ containing non-radioactive $CaCl_2$, and the efflux of $^{45}Ca^{2+}$ was measured (Table III, line 1) The K^+-rich Ca^{2+}-loading solutions used for other groups of synaptosomes contained only $^{40}Ca^{2+}$, after Ca^{2+} loading, 0 4-ml aliquots of these synaptosome suspensions were added to flasks containing 10 ml $Na^+ + 5\ K^+$ labelled with $^{45}Ca^{2+}$ so that the Ca^{2+} uptake could be measured (Table III, line 2) The results (Table III) show that in this experiment the total Ca^{2+} efflux was about 9 2 μmol Ca^{2+}/g protein per 2 min, while Ca^{2+} influx was only about 1 6 μmol Ca^{2+}/g protein per 2 min Thus, there was a net Ca^{2+} loss of about 7 6 μmol Ca^{2+}/g protein per 2 min It seems clear, from the data in Fig 1 and Table II, that this Ca^{2+} efflux must be externally Na^+ dependent, since Ca^{2+} efflux declines significantly when external Na^+ is replaced even in the presence of external Ca^{2+} This Na^+-dependent Ca^{2+} efflux may involve an exchange of Na^+ for Ca^{2+}, thereby implying that Na^+-Ca^{2+} exchange plays a critical role in net Ca^{2+} extrusion

TABLE III

COMPARISON OF Ca^{2+} EFFLUX AND Ca^{2+} INFLUX IN SYNAPTOSOMES AFTER CA^{2+} LOADING

Ca^{2+} loading solution (first 2 min incubation)	Ca^{2+} efflux or influx solution (second 2 min incubation)	$^{45}Ca^{2+}$ efflux (−) or influx (+)* during second 2 min incubation (μmol Ca^{2+}/g protein per 2 min)	Net Ca^{2+} efflux
67 mM Na^+ + 71 mM K^+ + 1.2 mM $^{45}CaCl_2$	Na^+ + 5 K^+ + 1.2 mM $^{40}CaCl_2$	−9.22 ± 0.12**	−7.64 ± 0.68
67 mM Na^+ + 71 mM K^+ + 1.2 mM $^{40}CaCl_2$	Na^+ + 5 K^+ − 1.2 mM $^{45}CaCl_2$	+ 1.58 ± 0.67	

* Each value is the mean ± S.E. of five determinations

** The $^{45}Ca^{2+}$ load for the efflux samples was 12.15 ± 0.54 μmol Ca^{2+}/g protein

External Ca^{2+}-dependent Ca^{2+} efflux

The squid giant axon is particularly well-suited for ion flux studies, and extensive investigation of the Ca^{2+} efflux in this preparation indicates that an exchange of Na^+ for Ca^{2+} is probably involved [9, 19, 20] Under some circumstances, the same transport mechanism appears to mediate an exchange of Ca^{2+}-for-Ca^{2+}, this type of exchange is particularly prominent when Li^+ is the predominant external monovalent cation [19, 20] To see if a similar Ca^{2+}-Ca^{2+} exchange occurs in synaptosomes, the external Ca^{2+}-dependent Ca^{2+} efflux was examined as a function of the monovalent cation composition of the efflux solution The results of these experiments are given in Table II and Fig 2 The main observation is that the extra efflux of $^{45}Ca^{2+}$, when 1 2 mM $^{40}CaCl_2$ is added to the efflux solution (ΔCa^+ in Table II) is considerably greater in the presence of 132 mM LiCl, than with choline chloride, guanidine HCl or glucose present In Ca^{2+}-free media, replacement of external choline by Li^+ was much less effective in stimulating Ca^{2+} efflux (see ΔLi^+-choline column in Table II) In the two experiments in which all four solutions ($Li^+ \pm Ca^{2+}$ and choline $\pm Ca^{2+}$) were tested simultaneously, the $^{45}Ca^{2+}$ loss increased from 11 8±7 1 ($n = 6$) to 15 5±4 3 ($n = 6$) percent when Li^+ replaced choline in the absence of Ca^{2+}, and from 24 8±6 4 ($n = 6$) to 45 5±4 6 ($n = 6$) percent in the presence of external Ca^{2+} (n is the number of determinations, the $^{45}Ca^{2+}$ load averaged 7 52±0 41 μmol Ca^{2+}/g protein for these two experiments) These results are consistent with the idea that, as in squid axons, Ca^{2+}-Ca^{2+} exchange is activated by Li^+

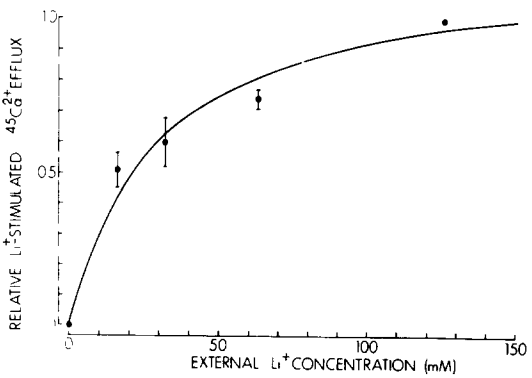


Fig 2 The relative Li^+-stimulated $^{45}Ca^{2+}$ efflux from synaptosomes graphed as a function of the Li^+ concentration in the efflux solution The efflux solutions were mixtures of $Li^+ + 5\ K^+$ and choline + 5 K^+, they all contained 1 2 mM $CaCl_2$ The data are the averaged values (±S E) from four experiments, the two end-points and two of the three intermediate points were obtained (in triplicate samples) in each experiment The mean 2-min efflux into choline + 5 K^+ (i e 0 mM Li^+) was 22±4 % of the $^{45}Ca^{2+}$ load, and 56±5 % into $Li^+ + 5\ K^+$, the mean $^{45}Ca^{2+}$ load for these experiments was 6 70±1 22 μmol Ca^{2+}/g protein The curve was calculated from the equation

$$M_{Ca^{2+}} = \frac{M^*_{Ca^{2+}}}{1 + \frac{K_{Li^+}}{[Li^+]_o}}$$

where $M_{Ca^{2+}}$ is the relative Li^+-stimulated Ca^{2+} efflux at any external Li^+ concentration ($[Li^+]_o$) The apparent maximal Li^+-stimulated Ca^{2+} efflux, $M^*_{Ca^{2+}}$, had a value of 1 2 (i e 20 % greater than the efflux into $Li^+ + 5\ K^+$), and the apparent half-saturation constant for Li^+, K_{Li^+}, was 30 mM

The Li^+ concentration dependence of the $^{45}Ca^{2+}$ efflux into Ca^{2+}-containing solutions is illustrated in Fig 2 The efflux solutions consisted of mixtures of Li^+ + 5 K^+ and choline+5 K^+ in these experiments The curve of Fig 2 shows that the Li^+-stimulated component of the $^{45}Ca^{2+}$ efflux appears to saturate, with a half-saturation value (K_{Li+}) of about 20–30 mM Li^+

In the presence of NaCl, the addition of $CaCl_2$ to the efflux solution generally inhibited $^{45}Ca^{2+}$ efflux slightly (Table II) The latter finding is different from observations made on squid axons, where the addition of Ca^{2+} to Na^+-containing external medium stimulates Ca^{2+} efflux [9, 19, 20] If a carrier-mediated Ca^{2+} transport mechanism is involved, the data in Table II may indicate that under the conditions of these experiments in synaptosomes, Na^+-loaded carriers tend to cycle inward more rapidly than do Ca^{2+}-loaded carriers

The influence of manganese on $^{45}Ca^{2+}$ efflux

In squid axons (Blaustein, unpublished data) and barnacle muscle fibers [25], the external Na^+-dependent Ca^{2+} efflux is inhibited by Mn^{2+} To see if Mn^{2+} also inhibits Ca^{2+} extrusion from synaptosomes, the effects of this ion were tested on the Li^+-stimulated (external Ca^{2+}-dependent) Ca^{2+} efflux, and on the Na^+-dependent Ca^{2+} efflux The results are given in Table IV Experiments 1 and 2 show that 2 4 mM Mn^{2+} reduced the Ca^{2+} efflux into Li^+ + 5 K^+ by about 33 %, and 5 0 mM Mn^{2+} by about 55 % This presumably reflects the inhibition of Ca^{2+}-Ca^{2+} exchange, since most of the Ca^{2+} efflux into Li^+-containing solutions is Ca^{2+} dependent (Table II)

Experiments 3 and 4 show the effects of Mn^{2+} on $^{45}Ca^{2+}$ efflux into Na^+-containing solutions In both experiments, the efflux was significantly inhibited by 5 0 mM Mn^{2+}, but only slightly so by 2 4 mM Mn^{2+} The interpretation of experiment 3 is somewhat ambiguous because the efflux solutions contained 1 2 mM $^{40}CaCl_2$, so that it is difficult to tell whether the Mn^{2+} inhibited Na^+-Ca^{2+} or Ca^{2+}-Ca^{2+} exchange Since the addition of ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*′-tetraacetic acid (EGTA) to the efflux solutions would have chelated Mn^{2+} as well as Ca^{2+}, it could not be added in these experiments Therefore, in experiment 4, aliquots of the incubation suspension (containing $^{45}Ca^{2+}$) were diluted 40-fold into the efflux solutions which contained neither added $^{40}CaCl_2$ nor EGTA Thus the specific activity of the Ca^{2+} in the efflux solutions was about the same as that in the incubation solutions, although the $CaCl_2$ concentration was reduced from 1 2 mM to about 32 μM* Nevertheless, about 68 % of the $^{45}Ca^{2+}$ was lost from the synaptosomes during a 2-min efflux period in 66 mM Na^+ + 66 mM choline + 5 K^+ This net loss of Ca^{2+} is apparently Na^+-Ca^{2+} exchange and not Ca^{2+}-Ca^{2+} exchange The observation that Mn^{2+} also inhibited the Ca^{2+} efflux in this experiment is therefore consistent with the idea that it inhibits Na^+-Ca^{2+} exchange

The Mn^{2+} experiments also provide evidence that the Na^+-dependent and Li^+-stimulated losses of $^{45}Ca^{2+}$ from synaptosomes are not simply manifestations of desorption of Ca^{2+} from superficial binding sites If this were the case, the addition of more divalent cation (Mn^{2+}) would have been expected to increase, rather than decrease the loss of $^{45}Ca^{2+}$ from the synaptosomes

* Measured by atomic absorption spectroscopy

TABLE IV

EFFECT OF Mn^{2+} ON Na_o^+-DEPENDENT AND Li_o^+-STIMULATED (Ca_o^{2+}-DEPENDENT) Ca^{2+} EFFLUX FROM SYNAPTOSOMES

Efflux solution	$MnCl_2$ (mM)	$^{45}Ca^{2+}$ efflux (μmol Ca^{2+}/g protein per 2 min)*	Percent of $^{45}Ca^{2+}$ load lost	ΔMn^{2+}** (%)	P***
Experiment 1 ($^{45}Ca^{2+}$ load = 9 10±0 60 μmol Ca^{2+}/g protein)					
Ca^{2+}-free Li^+ (+EGTA)	0	1 26±0 30	14	—	
Li^++5 K^++Ca^{2+}	0	4 60±0 28	51	—	
Li^++5 K^++Ca^{2+}	2 4	3 10±0 24	34	−33	< 0 05
Li^++5 K^++Ca^{2+}	5 0	1 92±0 32	21	−59	< 0 01
Experiment 2† ($^{45}Ca^{2+}$ load = 8 43±0 69 μmol Ca^{2+}/g protein)					
Choline+5 K^++Ca^{2+}	0	1 77±0 98	21	—	
Li^++5 K^++Ca^{2+}	0	4 92±0 17	58	—	
Li^++5 K^++Ca^{2+}	2 4	3 31±0 61	39	−33	< 0 05
Li^++5 K^++Ca^{2+}	5 0	2 25±0 47	27	−54	< 0 01
Experiment 3 ($^{45}Ca^{2+}$ load = 12 54±0 82 μmol Ca^{2+}/g protein)					
Ca^{2+}-free choline+5 K^+ (+EGTA)	0	0 18±0 18	1	—	
$\frac{1}{2}$ Na^+−$\frac{1}{2}$ choline+5 K^++Ca^{2+}	0	8 02±0 36	64	—	
$\frac{1}{2}$ Na^+−$\frac{1}{2}$ choline+5 K^++Ca^{2+}	2 4	7 42±0 50	59	− 7	< 0 05
$\frac{1}{2}$ Na^+−$\frac{1}{2}$ choline+5 K^++Ca^{2+}	5 0	5 18±0 56	41	−36	< 0 05
Experiment 4 ($^{45}Ca^{2+}$ load = 9 40±0 20 μmol Ca^{2+}/g protein)					
Ca^{2+}-free $\frac{1}{2}$ Na^+−$\frac{1}{2}$ choline+5 K^+ (+EGTA)	0	7 82±0 22	83	—	
$\frac{1}{2}$ Na^+−$\frac{1}{2}$ choline+5 K^+ (0 Ca^{2+})	0	6 38±0 20	68	—	
$\frac{1}{2}$ Na^+−$\frac{1}{2}$ choline+5 K^+ (0 Ca^{2+})	2 4	5 66±0 32	60	−11	< 0 05
$\frac{1}{2}$ Na^+−$\frac{1}{2}$ choline+5 K^+ (0 Ca^{2+})	5 0	4 38±0 40	47	−31	< 0 05

* Each value is the mean of three determinations ± S E

** Percent inhibition of $^{45}Ca^{2+}$ efflux due to the addition of $MnCl_2$ to the efflux solution

*** Confidence limit for the inhibition of efflux by Mn^{2+}, calculated by student's t-test

† Incubation temperature for this experiment was 37 °C

DISCUSSION

The data described above indicate that the efflux of Ca^{2+} from synaptosomes depends upon both monovalent and divalent cations in the bathing medium In the absence of external Ca^{2+}, Ca^{2+} efflux is almost exclusively external Na^+ dependent in the absence of Na^+ and presence of Ca^{2+}, Ca^{2+} efflux is stimulated by Li^+ Both the Ca^{2+}-dependent Li^+-stimulated Ca^{2+} efflux and the Na^+-dependent Ca^{2+} efflux are inhibited by Mn^{2+} These properties are strikingly similar to the properties of the Ca^{2+} extrusion mechanism in invertebrate nerve and muscle fibers [8, 19 20, 25], this suggests that a very similar, if not identical, mechanism is involved in Ca^{2+} extrusion in these evolutionally divergent preparations

If this view is correct, it would mean that some of the detailed kinetic and stoichiometric Ca^{2+} flux data obtained in squid axons may also be applicable to the mammalian central nervous system For example, several types of data from squid axons [19, 20] indicate that the Ca^{2+} efflux may involve an exchange of three Na^+-for-one Ca^{2+} Some of the synaptosome Ca^{2+} efflux data (see Fig 1A and ref 17) are much more difficult to evaluate than are comparable squid data (see Fig 3 of ref 19) and appropriate reliable Na^+ influx [20] and voltage dependence [19, 26] data are not obtainable in synaptosomes Consequently, estimates of Na^+-Ca^{2+} exchange stoichiometry in this preparation cannot be made with any degree of confidence However in view of the obvious similarities between Ca^{2+} efflux from synaptosomes and squid axons it seems reasonable to at least consider the possibility that a three-for-one exchange mechanism may also occur in synaptosomes This idea is supported primarily by the evidence for Ca^{2+}-Ca^{2+} exchange reported above (Table II and Fig 2) Perhaps the most straightforward model which can account for this Li^+-stimulated, external Ca^{2+}-dependent Ca^{2+} efflux is a transport mechanism which must be loaded with both Ca^{2+} and Li^+ at the external face of the membrane in order to effect a complete cycle of the Ca^{2+} transport system In squid axons there is evidence [19, 20] that Na^+ may also be effective at the site which accommodates Li^+, although this may not be the case in synaptosomes, where (see Table II) the addition of Ca^{2+} to external media containing Na^+ appears to inhibit Ca^{2+} efflux

Previous studies on internal Na^+-dependent Ca^{2+} influx (presumably, the Na^+-Ca^{2+} exchange mechanism working in reverse) in synaptosomes [17] may shed some light on the external site which accommodates Ca^{2+} In the latter experiments, external Na^+ was found to compete with Ca^{2+}, the Ca^{2+} influx was inversely related to the square of the external Na^+ concentration, which may imply that two Na^+ compete with one Ca^{2+}

On the assumption that a single carrier mechanism can move Ca^{2+} either in or out, across the synaptosome plasma membrane, the afore-mentioned observations, taken together, suggest that the external sites must be occupied by ions with a net charge of 3 + (for example, three Na^+, or one Ca^{2+} and one Li^+) in order to permit the transport mechanism to cycle If this is the case, and if the mechanism is electrogenic (that is, it effects the net entry of one positive charge in each cycle, as may occur in squid axons [19, 20, 26]), then the energy liberated by the influx of three Na^+, moving down their electrochemical gradient, may, in principle, be coupled to Ca^{2+} extrusion, so as to drive Ca^{2+} up its electrochemical gradient At equilibrium the relationship between the ionized Na^+ and Ca^{2+} concentration gradients would then be given by [9]

$$\frac{[Ca^{2+}]_o}{[Ca^{2+}]_i} = \frac{[Na^+]_o^3}{[Na^+]_i^3} \exp\left(\frac{-V_{memb}F}{RT}\right) \quad (1)$$

With a membrane potential, V_{memb}, of −60 mV, and an Na^+ concentration ratio, $[Na^+]_o/[Na^+]_i$ of about 10, the proposed mechanism could use energy from the Na^+ gradient to maintain $[Ca^{2+}]_i$ in the physiological range of $1\ 10^{-6}$–$1\ 10^{-7}$ M [7, 8] in intact neurons On thermodynamic grounds, a coupled mechanism of this type would not necessarily need a direct input of metabolic energy (clearly, metabolic energy is required at least to maintain the Na^+ gradient, via the sodium pump) However, available data [17] are insufficient to rule out a direct metabolic energy input to the Ca^{2+} extrusion mechanism In squid axons, where intracellular ATP and inorganic ion concentrations can be independently controlled [27, 28], DiPolo [29] has found that although it is not absolutely required, ATP does facilitate Na^+-dependent Ca^{2+} efflux This effect might be due to an ATPase, analogous to the $(Na^+ + K^+)$-dependent ATPase which is involved in Na^+ extrusion from most animal cells [30] One other possibility is that an energy-rich molecule may catalyze the transport process without actually being degraded, as occurs with ATP during Na^+-Na^+ exchange mediated by the sodium pump [31] Unfortunately, these alternatives cannot be rigorously explored in synaptosomes or most other mammalian preparations Nevertheless, the evidence that similar Ca^{2+} extrusion mechanisms may operate in synaptosomes and in squid axons suggests that synaptosomes may be a convenient source of plasma membranes from which to isolate and characterize Ca^{2+} "carriers" with the properties noted above

ACKNOWLEDGEMENTS

We thank Dr N C Kendrick for helpful discussion and for critical comments concerning the manuscript This research was supported by grant NS-08442 from the National Institutes of Health

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