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SODIUM-DEPENDENT AND CALCIUM-DEPENDENT CALCIUM TRANSPORT BY RAT BRAIN MICROSOMES

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Microsomal vesicles prepared from rat brain contain a Na^+-Ca^{2+} exchange transport system capable of accumulating Ca^{2+} in a time- and temperature-dependent manner. The Ca^{2+} accumulated by these vesicles was released by the Ca^{2+} ionophore A23187 but not by EGTA. The K_m value for Ca^{2+} uptake was 23 μM with a maximal velocity of 21 nmol Ca^{2+}/mg per min. Ca^{2+} uptake was significantly inhibited by La^{3+}, Sr^{2+}, Mn^{2+} and Ba^{2+} and to a lesser extent by Mg^{2+}. $^{45}Ca^{2+}$ accumulated by Na^+-dependent uptake could be released by $^{40}Ca^{2+}$, indicating the presence of a Ca^{2+}-Ca^{2+} exchange activity in the microsomes. Ca^{2+}-Ca^{2+} exchange was stimulated in Li^+- and K^+-containing media as compared to choline$^+$ media. Microsomes also catalyzed ATP-dependent Ca^{2+} uptake (in the absence of Na^+ gradient). The Ca^{2+} sequestered by this mechanism could be released by extravesicular Na^+, indicating that both the ATP-dependent and the Na^+-dependent Ca^{2+} uptake systems are present in the same membrane. The microsomal preparation used did not contain measurable amounts of succinate dehydrogenase activity or oligomycin-azide-dinitrophenol sensitive ATP-dependent Ca^{2+} uptake. Thus, the Ca^{2+} accumulation observed was not due to contaminating mitochondria. The preparation was enriched for 5′-nucleotidase and ($Na^+ + K^+$)-ATPase (plasma membrane markers) as well as antimycin A-resistant NADPH-dependent cytochrome *c* reductase activity (an endoplasmic reticulum marker).

Introduction

Several mechanisms have been proposed for the regulation of cytoplasmic Ca^{2+} concentrations in neuronal cells. Intracellular organelles that are capable of Ca^{2+} uptake such as mitochondria [1], endoplasmic reticulum [2,3] and other vesicular structures [4–9], may be involved in controlling cytoplasmic Ca^{2+} levels by sequestering and releasing Ca^{2+}. Alternately, extrusion of Ca^{2+} by transport systems located in the plasma membrane may be the primary mechanism for controlling cytoplasmic Ca^{2+} concentrations. Whatever the process or combination of processes responsible for Ca^{2+} regulation, the transport systems involved must be capable of maintaining cytoplasmic Ca^{2+} levels at submicromolar concentrations against a large extracellular chemical gradient. These systems must also be capable of rapidly removing Ca^{2+} that enters during depolarization.

At present, much of our knowledge concerning Ca^{2+} extrusion at the plasma membrane comes from experiments with dialyzed giant squid axons. Several laboratories [10–13] have shown that one component of Ca^{2+} efflux in squid axon is dependent on the presence of a Na^+ gradient and occurs by an electrogenic exchange process in which three Na^+ ions are exchanged for one Ca^{2+} ion [11,14]. The apparent K_m value for Ca^{2+} efflux by this system is 8 μM [10,

Abbreviations: EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; SDS, sodium dodecyl sulfate.

15]. In the absence of extracellular Na^+, Ca^{2+} efflux can also be stimulated by external Ca^{2+}. Whether this one-for-one exchange is catalyzed by the same system that exchanges Ca^{2+} for Na^+ is presently not known. A second system that catalyzes net Ca^{2+} efflux in squid axon requires ATP and has a K_m value of 0.18 μM for Ca^{2+} [16,17]. This system is thought to be analogous to the Ca^{2+}-ATPase pump observed in red cell membranes. DiPolo and Beaugé [17] have proposed that at physiological ionized cytoplasmic Ca^{2+} concentrations, (0.02–0.05 μM, Ref. 18) most of the Ca^{2+} efflux is catalyzed by the high affinity ATP-dependent system. In this scheme, the lower affinity Na^+-Ca^{2+} exchange system would only be important when depolarization results in a transient increase in cytoplasmic Ca^{2+} concentrations.

In mammalian brain, Na^+-dependent Ca^{2+} fluxes have been studied in tissue slices [19,20] as well as in synaptosomes [21,22]. Properties of the Na^+-Ca^{2+} exchange activities of these preparations are quite similar to those of the Na^+-dependent system of squid axon. Ca^{2+}-Ca^{2+} exchange has also been observed in synaptosomes [21]. While it is established that Na^+ gradients can drive Ca^{2+} efflux in brain as well as in squid axon it is not presently known whether brain also contains a plasma membrane ATP-utilizing system for Ca^{2+} extrusion analogous to the ATP-dependent system of squid axon. Non-mitochondrial ATP-dependent uptake has been demonstrated in brain subcellular fractions including microscomes [23–27], vesicles from lysed synaptosomes [6], acetylcholine-containing vesicles [4,7,28], coated vesicles [5] and microvesicles [8]. However, whether these activities are exclusively Ca^{2+} sequestering mechanisms or are Ca^{2+} extrusion systems is not present known.

Recently, Reeves and Sutko [29] and others [30–33] have demonstrated the presence of Na^+-Ca^{2+} exhange activity in membrane vesicles (presumably of plasma membrane origin) prepared from heart ventricular tissue. These membrane fragments catalyze the exchange of three Na^+ ions for each Ca^{2+} ion [30] with the uptake being electrogenic [33,34]. The K_m value for Ca^{2+} uptake has been reported as 18 μM [29]. The same preparation carriers out Ca^{2+}-Ca^{2+} exchange [32]. Na^+-Ca^{2+} exchange activity has also been observed in 'synaptic vesicles' prepared from lysed synaptosomes [35].

The following work was undertaken to characterize the Na^+-Ca^{2+} uptake system of brain in the absence of contributions to Ca^{2+} fluxes by mitochondria. Rat brain was fractionated by differential centrifugation and a microsomal fraction was found to be enriched in Na^+-Ca^{2+} exchange activity. The relationship of the Na^+-Ca^{2+} system to ATP-dependent Ca^{2+} uptake was examined and both were found to be present in the same membrane. Our data suggest that these systems are present in the plasma membranes of the central nervous system cells, though a contribution by endoplasmic reticulum cannot yet be entirely excluded. A preliminary account of this work has been previously presented [36].

Experimental Procedures

Preparation of microsomes. Whole brains, removed from adult male rats after decapitation, were immediately homogenized in 10 vol. 0.32 M sucrose (adjusted to pH 7.4 with Tris-HCl) using a motor-driven Teflon-glass homogenizer (ten strokes at 800 rev./min). This crude homogenate was centrifuged at $600 \times g$ for 10 min to yield a cell debris and nuclear pellet. The supernatant was centrifuged at $17\,300 \times g$ for 10 min to obtain a crude synaptosomal and mitochondrial pellet. The microsomes were prepared from this supernatant by centrifugation at $34\,000 \times g$ for 1 h. The resultant pellet consisted of a yellow lower pellet covered by a layer of white-colored material; the upper portion (microsomes) was selectively resuspended in 1–3 ml buffer per brain by gently vortexing. The remaining pellet (mitochondria and mitochondrial fragments) was discarded. Microsomes to be used for ATP-dependent Ca^{2+} transport studies and sucrose gradient experiments were resuspended in 0.32 M sucrose, while microsomes to be used directly for Na^+-Ca^{2+} exchange experiments were resuspended in 160 mM NaCl/20 mM Tris-HCl (pH 7.4), centrifuged at $34\,000 \times g$ for 1 h, and resuspended in the same NaCl buffer (1–2 ml/brain). All the above preparative centrifugation steps were performed at 4°C using a Sorvall SS-34 rotor.

The microsomes used for Ca^{2+} uptake studies (except for Fig. 7) were fractionated by discontinuous sucrose density gradient centrifugation in order to remove residual contamination by mitochondria and mitochondrial fragments. Crude microsomes

in 0.32 M sucrose (3 ml/brain) were layered over 1 M sucrose/10 mM imidazole (pH 7.4) and centrifuged at 25 000 rev./min for 90 min in an SW-27 rotor (Beckman) at 4°C. The material at the 1 M sucrose interface (referred to as 'purified microsomes') was slowly diluted with either 2–3 vol. 160 mM NaCl/20 mM Tris-HCl (pH 7.4) or 0.32 M sucrose (pH 7.4), and pelleted at 100 000 × g for 1 h. The purified microsomes were either resuspended in 0.32 M sucrose for ATP-dependent Ca^{2+} uptake assays or in 160 mM NaCl/20 mM Tris-HCl (pH 7.4) for Na^+-dependent uptake studies.

Uptake assays. Microsomes were prepared for Na^+-Ca^{2+} exchange experiments by overnight incubation at 4°C in 160 mM NaCl/20 mM Tris-HCl (pH 7.4) to allow Na^+ to equilibrate across the microsomal membranes. Unless indicated otherwise, uptake was routinely assayed in media containing 20 mM Tris-HCl (pH 7.4), 160 mM KCl or NaCl, and 1–80 μM $^{45}CaCl_2$ (0.26 mCi/nmol). The reaction mixture and the microsomes were separately preincubated at the final assay temperature and the reaction initiated by dilution of the microsomes 30-fold (3–50 μg protein per assay) into the $^{45}Ca^{2+}$ reaction media. The reaction was terminated by filtration through nitrocellulose filters (Schleicher and Schuell, 0.45 μM) on a 30-place sampling manifold (Millipore); each filter was washed three times with 3 ml aliquots of 160 mM KCl/20 mM Tris-HCl (pH 7.4). The filters were immersed in 1 ml 0.1% SDS and 5 ml Tritosol [37] and radioactivity was determined by scintillation counting.

The K_m value of Na^+-dependent Ca^{2+} uptake was determined by measuring the initial rates (5 s time points) of Ca^{2+} uptake at different concentrations (1–80 μM) of free Ca^{2+}. The ionized Ca^{2+} concentrations in the presence of microsomal membranes and KCl or NaCl media were determined using a Ca^{2+}-selective electrode [38] coupled to a pH meter (Corning 130). The electrode was calibrated in both NaCl and KCl reaction media using solutions of known Ca^{2+} concentration. The amount of microsomal protein used for K_m determinations was accordingly adjusted so that the initial ionized Ca^{2+} concentration in the assay was not reduced by more than 5% by either uptake or binding to the external surface of the membranes or binding to other components of the media. Endogenous Ca^{2+} contributed less than 0.2 μmol/l to the final Ca^{2+} concentration in these assays.

ATP-dependent Ca^{2+} uptake was assayed in 160 mM KCl, 20 mM Tris-HCl (pH 7.4), 2.5 mM $MgCl_2$, 0.01 mM $^{45}Ca^{2+}$, 0.1 mM ouabain, 0.2 mM dinitrophenol, 0.2 mM NaN_3, 0.15 μg/ml oligomycin and either 4 mM Tris · ATP, 4 mM Tris · ADP, or no added nucleotide. The reaction was initiated by the addition of microsomes (1–75 μg/assay) and the reaction was terminated by filtration as described above. ATP-dependent Ca^{2+} uptake studies were always performed using freshly prepared microsomes, since the membranes could not be stored for more than a day without loss of activity. However, the Na^+-Ca^{2+} exchange activity was stable for at least a week at 4°C without significant loss of uptake activity.

Other assays. 5′-nucleotidase [39], $(Na^+ + K^+)$-ATPase [40], succinate dehydrogenase [41], NADPH-dependent antimycin A-resistant cytochrome *c* reductase [42], cytochrome *c* oxidase [43] and inosine diphosphatase [44] were assayed as described by others. Protein was assayed by a modification of the method of Lowry et al. [45] using bovine serum albumin as a standard.

Materials. $^{45}CaCl_2$ (12 Ci/g) was obtained from New England Nuclear. The Ca^{2+} ionophore A23187 was from Calbiochem. Vanadium-free Tris · ATP and disodium ATP were purchased from Sigma. All other chemicals were reagent grade or better. Reagents which inhibited Ca^{2+} uptake including $BaCl_2$, $LaCl_3$, $MgCl_2$, $MnCl_2$ and $SrCl_2$ (Table II) and LiCl (Fig. 1) were checked for contaminating Ca^{2+} by atomic absorption spectroscopy using a Perkin-Elmer model 290 spectrophotometer and none of these reagents were found to contain significant levels of Ca^{2+}. Ruthenium red was obtained from Sigma and used without further purification. The Ca^{2+}-selective electrode was obtained from W. Simons.

Results

Fig. 1 illustrates the time course of Na^+ gradient-dependent Ca^{2+} uptake by purified rat brain microsomes. The Na^+ gradient used to drive Ca^{2+} uptake was established by passively loading the membrane vesicles with NaCl (160 mM) by incubation of the microsomes overnight at 4°C. Uptake was initiated by rapidly diluting the Na^+-loaded microsomes 30-fold into an isoosmotic Na^+-free buffer thus generating a

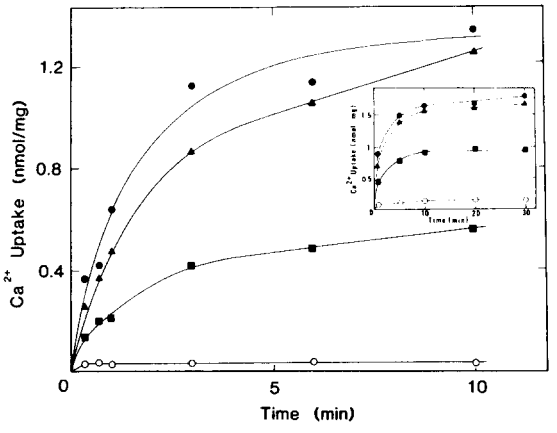

Fig. 1. Na^+-dependent Ca^{2+} uptake by Na^+-loaded microsomes diluted into KCl, LiCl, choline chloride, and NaCl media. Microsomes equilibrated in 160 mM NaCl/20 mM Tris-HCl were diluted 30-fold into 2 μM $^{45}Ca^{2+}$, 20 mM Tris-HCl (pH 7.4) and either 160 mM KCl (•), 160 mM LiCl (■), 160 mM choline chloride (▲) or 160 mM NaCl (○). Uptake was terminated at the indicated times as described under Experimental Procedures. Assays were performed in triplicate at 21°C. The data shown in the main figure and the data shown in the inset were obtained using two different microsomal preparations on different days.

Na^+ gradient. Under these conditions, Ca^{2+} uptake was time dependent with accumulation reaching steady-state levels at approx. 10–20 min (Fig. 1, see inset). When Na^+-loaded microsomes were diluted into Na^+-containing buffer (no Na^+ gradient), little Ca^{2+} uptake occurred (Fig. 1). In addition, replacement of NaCl during the overnight loading incubation by 160 mM LiCl, KCl, choline chloride, or 320 mM sucrose followed by rapid dilution of the microsomes into 160 mM KCl (for LiCl, choline chloride, and sucrose-loaded microsomes), or 160 mM LiCl (for KCl-loaded microsomes) did not result in appreciable Ca^{2+} uptake (data not shown). Thus, a Na^+ gradient was required for Ca^{2+} uptake and other cations could not replace Na^+ and could not drive Ca^{2+} transport. Ca^{2+} uptake was temperature dependent with the initial rate of Ca^{2+} uptake increasing with increasing temperature (Table I). Little uptake occurred at 0°C. In contrast, the Ca^{2+} associated with the microsomes in the absence of a Na^+ gradient remained essentially constant from 0 to 37°C (Table I).

Na^+-dependent Ca^{2+} uptake was routinely assayed

TABLE I

TEMPERATURE DEPENDENCE OF Na^+-DEPENDENT Ca^{2+} UPTAKE

The Ca^{2+} uptake assays were initiated by diluting gradient-purified microsomes loaded with 160 mM NaCl/20 mM Tris-HCl (pH 7.4) into reaction buffer containing 20 mM Tris-HCl (pH 7.4), 2 μM $^{45}Ca^{2+}$, and either 160 mM KCl or 160 mM NaCl. The microsomes and the media had been preequilibrated at the temperatures indicated below. Uptake was terminated after 10 s by filtration as described in Experimental Procedures. The data presented are the average of four determinations ± S.D.

Temperature (°C)	Ca^{2+} uptake (nmol/mg per min)		
	KCl media	NaCl media	Δ
0	0.34 ± 0.14	0.32 ± 0.10	0.02
6	0.56 ± 0.26	0.24 ± 0.04	0.32
14	0.68 ± 0.06	0.30 ± 0.10	0.38
21	1.14 ± 0.26	0.30 ± 0.14	0.84
29	1.30 ± 0.20	0.38 ± 0.18	0.92
37	1.56 ± 0.20	0.36 ± 0.06	1.20

by diluting Na^+-loaded microsomes into buffer containing 160 mM KCl. However, as illustrated in Fig. 1, K^+ is not required for Na^+-dependent Ca^{2+} uptake; when Na^+-loaded microsomes were diluted into 160 mM LiCl buffer or 160 mM choline chloride buffer (containing no K^+), time-dependent Ca^{2+} uptake was observed. While the time course of Ca^{2+} uptake in KCl media was nearly identical to Ca^{2+} uptake in choline chloride media, uptake in Li^+-containing buffer was reduced in both the initial rate of accumulation and in the amount of Ca^{2+} associated with the microsomes at equilibrium. The LiCl used in these experiments did not contain significant amounts of contaminating $^{40}Ca^{2+}$ as determined by atomic absorption spectroscopy; thus, the inhibition of Ca^{2+} uptake in Li^+ media was not due to the dilution of the specific activity of the added $^{45}Ca^{2+}$ by $^{40}Ca^{2+}$ present in the commercial LiCl preparation.

Although accumulation of Ca^{2+} was linear for only short time periods, initial rates of uptake could be approximated by using very short assay times (5–10 s). Based on 5-s time points, Ca^{2+} uptake was saturable with respect to Ca^{2+} concentration. A typical experiment is shown in Fig. 2. When the data were analyzed by linear transformation plots (S/V versus S,

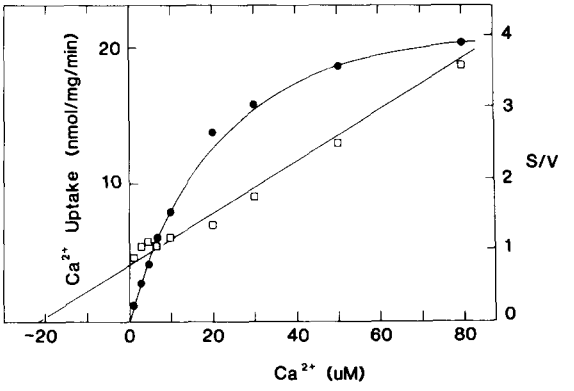

Fig. 2. Ca^{2+} concentration dependence of Na^+-dependent Ca^{2+} uptake. Assays were initiated by diluting Na^+-loaded microsomes 30-fold into 20 mM Tris-HCl (pH 7.4), $^{45}Ca^{2+}$ (1–80 μM) and either 160 mM KCl or 160 mM NaCl. Ca^{2+} uptake was terminated after 5 s at 21°C. The free Ca^{2+} concentration in the presence of microsomes was determined as described under Experimental Procedures. The data points presented (•) are the difference between Ca^{2+} uptake in KCl media minus Ca^{2+} uptake in NaCl media and each point is the average of three determinations. Kinetic parameters (K_m and maximal velocity) were determined from the S/V versus S linear transformation (□) of the Ca^{2+} uptake data. S is the substrate concentration (μM) and V the velocity of Ca^{2+} uptake (nmol Ca^{2+} taken up/mg protein per min).

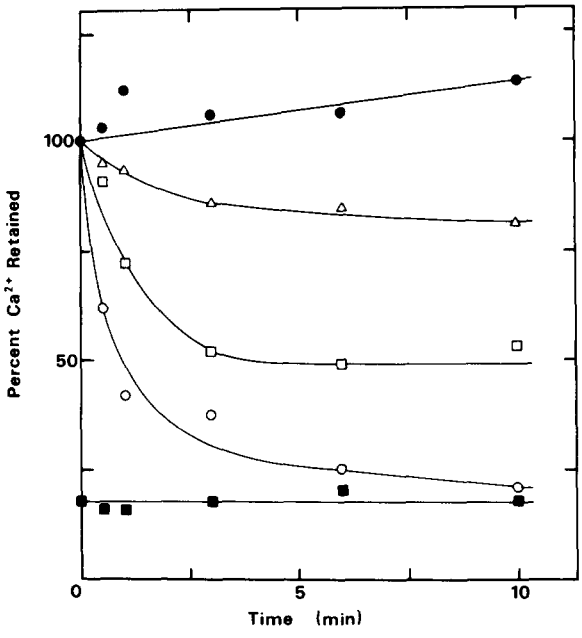

Fig. 3. Na^+-stimulated release of Ca^{2+} accumulated by microsomes in response to a Na^+ gradient. Na^+-loaded microsomes (equilibrated in the presence of 160 mM NaCl) were diluted 30-fold into buffer containing 160 mM KCl and 2 μM $^{45}Ca^{2+}$ and incubated for 10 min. These $^{45}Ca^{2+}$-loaded microsomes were then diluted 5-fold ($t = 0$) into assay media containing 2 μM $^{45}Ca^{2+}$ and various concentrations of NaCl. The microsomes were assayed over the next 10 min at the times indicated as described under Experimental Procedures. The osmolarity of the different reaction mixtures was maintained by varying the KCl concentration in the media. The final NaCl concentrations were 5.3 mM (•), 16 mM (△), 32 mM (□) and 96 mM (○). Na^+-loaded microsomes were also initially diluted 1 : 30 into media containing 2 μM $^{45}Ca^{2+}$ and 160 mM NaCl (no Na^+ gradient). After 10 min, the reaction mixture was diluted 5-fold into 160 mM NaCl, 20 mM Tris-HCl and 2 μM $^{45}Ca^{2+}$ and assayed over a 10-min period (▪). Assays were performed in triplicate at 21°C.

Ref 46) a $K_{m,Ca^{2+}}$ of 23 μM (S.D. = 3.3, $n = 3$) and a maximal velocity of 21 nmol Ca^{2+} uptake/mg per min was determined (see Experimental Procedures for details).

The Na^+-Ca^{2+} exchange system is capable of catalyzing both Ca^{2+} accumulation and Ca^{2+} release from microsomes. The Ca^{2+} taken up in response to an outwardly directed Na^+ gradient could be released by extravesicular Na^+. Addition of Na^+ to reduce the Na^+ gradient from an original internal to external ratio of 30 (160 mM inside, 5.3 mM outside) to ratios of 10 or 1.7, resulted in the release of 41% and 91%, respectively, of the Ca^{2+} originally sequestered by the microsomes (Fig. 3). These experiments demonstrate that the amount of Ca^{2+} accumulated at equilibrium is dependent on the Na^+ gradient present and that the Na^+-Ca^{2+} carrier can catalyze Ca^{2+} fluxes in both directions.

In order to distinguish between Ca^{2+} transported into closed membrane vesicles and Ca^{2+} bound to external membrane sites on microsomes, the effects of the Ca^{2+} ionophore A23187 and EGTA on Na^+-dependent Ca^{2+} uptake were examined. A23187 released 86% of the Ca^{2+} accumulated by microsomes in the presence of a Na^+ gradient, leading to the conclusion that most of the Ca^{2+} associated with this preparation is sequestered in membrane vesicles (Fig. 4). Since this ionophore equilibrates the intravesicular Ca^{2+} with Ca^{2+} in the external media, release of Ca^{2+} from the microsomes also demonstrates that under the conditions of Fig. 4, the Na^+-Ca^{2+} exchange sys-

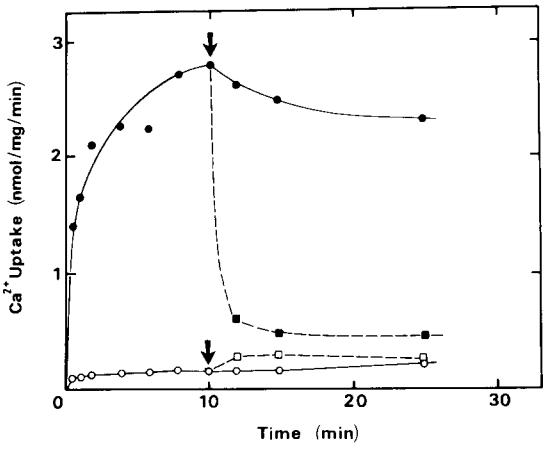

Fig. 4. Release of $^{45}Ca^{2+}$ by the ionophore A23187. Microsomes equilibrated in 160 mM NaCl were rapidly diluted (10-fold) into media containing 20 mM Tris-HCl (pH 7.4), 2 μM $^{45}Ca^{2+}$ and either 160 mM KCl (closed symbols) or 160 mM NaCl (open symbols). After 10 min of $^{45}Ca^{2+}$ accumulation, either A23187 in ethanol (■, □) or an equivalent volume of ethanol (●, ○) was added (arrows). The final A23187 concentration was 50 μM. Assays were performed in triplicate at 21°C as described under Experimental Procedures.

tem is able to concentrate Ca^{2+} above the level of the media. In contrast, EGTA (1 mM) which would be expected to remove externally bound Ca^{2+}, released only 5–22% of the Ca^{2+} associated with the microsomes after a 5-min incubation (Fig. 5A). Similar results were obtained with 10 mM EGTA (data not shown).

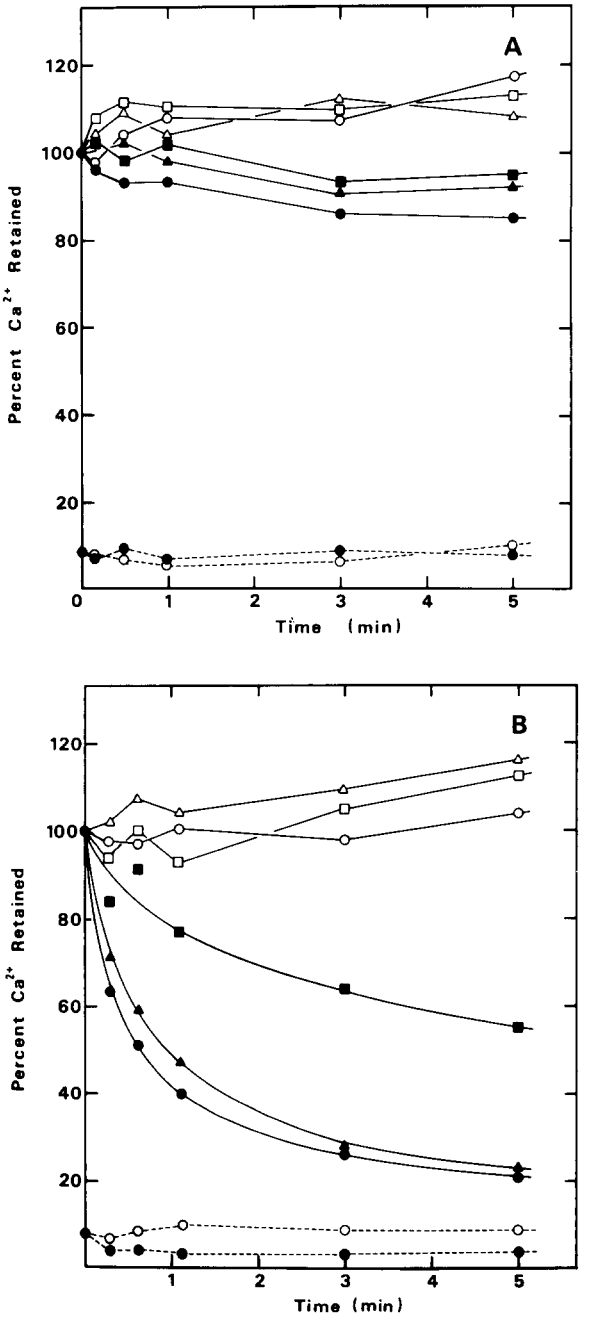

Fig. 5. (Panel A) Release of $^{45}Ca^{2+}$ from microsomes by EGTA. Na^+-loaded microsomes (equilibrated in 160 mM NaCl/20 mM Tris-HCl, pH 7.4) were rapidly diluted 30-fold into 2 μM $^{45}Ca^{2+}$, 20 mM Tris-HCl (pH 7.4) and either 160 mM choline chloride (solid lines) or 160 mM NaCl (dashed lines). After 15 min of $^{45}Ca^{2+}$ accumulation, the samples loaded in choline chloride media were added to an equal volume of 2 μM $^{45}Ca^{2+}$, 20 mM Tris-HCl (pH 7.4) and either 160 mM KCl (△, ▲), 160 mM LiCl (○———○, ●———●) or 160 mM choline chloride (□, ■). EGTA (1 mM final concentration) was added to some samples (closed symbols), and an equal volume of reaction media to the remaining samples (open symbols) and the microsomes were assayed as described under Experimental Procedures over the next 5 min. Samples, which were allowed to accumulate $^{45}Ca^{2+}$ for 15 min in NaCl media, were added to an equal volume of 20 mM Tris-HCl, 2 μM $^{45}Ca^{2+}$ and 160 mM NaCl. EGTA (1 mM final concentration, ○- - - - -○) or an equal volume of reaction media (●- - - - -●) was added at time 0 and the microsomes were assayed over the next 5 min. Assays were performed in triplicate at 21°C. (Panel B) Release of $^{45}Ca^{2+}$ from microsomes by extravesicular $^{40}Ca^{2+}$. Microsomes were loaded with $^{45}Ca^{2+}$ and assayed as described for panel A except that $^{40}Ca^{2+}$ (0.5 mM final concentration, closed symbols) rather than EGTA was added to the same samples.

Inhibition of Na^+-Ca^{2+} exchange

Mitochondria prepared from brain contain a very active Ruthenium red-sensitive, energy-dependent Ca^{2+} uptake system as well as a Na^+-Ca^{2+} exchange system [1]. In order to determine that the Na^+-Ca^{2+} exchange system studied here was not of mitochondrial origin, the effects of inhibitors known to affect Ca^{2+} transport by mitochondria were studied (Table II). The mitochondrial poisons dinitrophenol, oligomycin and azide had no effect on Na^+-dependent Ca^{2+} uptake. $LaCl_3$, which inhibits both the Ruthenium red-sensitive Ca^{2+} uptake system and the Na^+-stimulated release of Ca^{2+} from mitochondria at submicromolar concentrations did not inhibit Na^+-Ca^{2+} exchange in microsomes until 100 μM La^{3+} was present. Curiously, Reeves and Sutko [29] found that La^{3+} inhibited Na^+-Ca^{2+} exchange in heart sarcolemma vesicles with half-maximal inhibition occurring at 10 μM. As seen in Table II, no inhibition occurred in brain at 10 μM, and the difference in sensitivity to this ion may indicate a difference in the Na^+-Ca^{2+} systems of brain and heart. Ruthenium red, a compound which inhibits Ca^{2+} uptake but not Na^+-Ca^{2+} exchange in brain mitochondria, did not significantly affect Ca^{2+} uptake even at 400 μg/ml. The same preparation of Ruthenium red completely inhibited energy-dependent Ca^{2+} uptake by rat brain mitochondria [47]. Other inhibitors of nonmitochondrial transport were also examined. Taurine, which inhibits Ca^{2+} uptake by rat cortical synaptosomes [48] and by subcellular fractions from chick retina [49] did not inhibit Na^+-dependent Ca^{2+} uptake in either Tris-buffered media or bicarbonate-buffered media. Ouabain, an inhibitor of the $(Na^+ + K^+)$-ATPase had no effect on Ca^{2+} uptake. Several divalent cations were found to be potent inhibitors of Ca^{2+} uptake. At a $^{45}Ca^{2+}$ concentration of 50 μM, 1 mM $MnCl_2$, $SrCl_2$ or $BaCl_2$ inhibited Ca^{2+} uptake by 79, 75 and 57%, respectively (Table II).

Ca^{2+}-Ca^{2+} exchange

The presence of a Ca^{2+}-dependent Ca^{2+} efflux system (presumably Ca^{2+}-Ca^{2+} exchange) has been observed in squid axons [11] and rat brain synaptosomes [21]. Recently, Ca^{2+}-Ca^{2+} exchange has also been demonstrated in sarcolemma vesicles prepared from heart [32]. The existence of Ca^{2+}-Ca^{2+} exchange activity in brain microsomes was investigated using

TABLE II

INHIBITION OF Na^+-DEPENDENT Ca^{2+} UPTAKE

Ca^{2+} uptake was initiated by diluting microsomes loaded with 160 mM NaCl in 20 mM Tris-HCl (pH 7.4) into reaction media containing 50 μM $^{45}Ca^{2+}$, 20 mM Tris-HCl (pH 7.4), inhibitors when indicated, and either 160 mM NaCl or 160 mM KCl. Ca^{2+} uptake was terminated after 5 s at 21°C by filtration as described under Experimental Procedures. Na^+-dependent Ca^{2+} uptake was taken as Ca^{2+} accumulated in KCl media minus Ca^{2+} accumulated in NaCl media. The data are expressed as the percent of Ca^{2+} uptake relative to the uninhibited control and each determination was the average of four assays ± S.D. Inhibitor concentrations are given in mM units except for Ruthenium red which is presented in μg/ml units in parenthesis below the Ca^{2+} uptake values. In addition to the inhibitors listed in this table, the following inhibitors were tested and did not significantly affect Ca^{2+} uptake: dinitrophenol, 0.2 mM; azide, 0.2 mM; oligomycin, 0.15 μg/ml; ouabain, 0.1 mM; and taurine, 25 mM. Inhibition of Na^+-dependent Ca^{2+} uptake by taurine was also examined in microsomes loaded with 160 mM NaCl/20 mM bicarbonate buffer (pH 7.4) and diluted into 160 mM KCl/20 mM bicarbonate buffer. No inhibition of Ca^{2+} uptake was observed.

Addition	Inhibitor concn. (mM)	$^{45}Ca^{2+}$ uptake (% control)			
		0.01	0.1	1	10
None		100 ± 10	100 ± 10	100 ± 10	100 ± 10
$LaCl_3$		104 ± 4	33 ± 11	4 ± 2	1 ± 1
$MnCl_2$		123 ± 11	90 ± 22	28 ± 3	6 ± 3
$SrCl_2$		63 ± 22	41 ± 9	25 ± 10	1 ± 2
$BaCl_2$		65 ± 11	76 ± 8	43 ± 17	17 ± 7
$MgCl_2$		91 ± 15	110 ± 19	93 ± 12	32 ± 10
Ruthenium red		102 ± 3 (0.8 μg/ml)	110 ± 20 (8 μg/ml)	128 ± 12 (80 μg/ml)	83 ± 4 (400 μg/ml)

vesicles loaded with $^{45}Ca^{2+}$ by Na^+-Ca^{2+} exchange. Na^+-loaded microsomes were allowed to accumulate $^{45}Ca^{2+}$ until equilibrium was reached (15 min). As shown in Fig. 5B, when excess $^{40}Ca^{2+}$ was added, the $^{45}Ca^{2+}$ sequestered in the vesicles was rapidly released. Under these conditions, the loss of $^{45}Ca^{2+}$ from the microsomes would be the summation of the following processes: (1) Ca^{2+}-Ca^{2+} exchange where extravesicular $^{40}Ca^{2+}$ would replace the $^{45}Ca^{2+}$ present in the vesicles; (2) displacement of externally bound $^{45}Ca^{2+}$ by excess added $^{40}Ca^{2+}$; (3) $^{45}Ca^{2+}$ efflux coupled to Na^+ uptake via the Na^+-Ca^{2+} exchange system; and (4) non-carrier-mediated $^{45}Ca^{2+}$ efflux due to $^{45}Ca^{2+}$ leaking across the membrane. The contribution of processes 2–4 to total $^{45}Ca^{2+}$ efflux in Fig. 5B was estimated as the loss of $^{45}Ca^{2+}$ in the presence of EGTA (Fig. 5A); under these conditions, the loss of $^{45}Ca^{2+}$ by Ca^{2+}-Ca^{2+} exchange would not occur since only very low concentrations of external Ca^{2+} would be present (process 1). $^{45}Ca^{2+}$ release and efflux by processes 2–4, however, still would occur since in the absence of significant amounts of extravesicular Ca^{2+} (+EGTA), the Ca^{2+} bound to nonspecific low-affinity sites on the external surface of the membrane (process 2) would most likely be released. In addition, EGTA would not alter Na^+-dependent Ca^{2+} efflux (process 3) or Ca^{2+} leaking out of the vesicles nonspecifically (process 4), since these efflux events do not require the presence of extravesicular Ca^{2+}. Thus, by comparing Fig. 5A to 5B, it is evident that Ca^{2+}-Ca^{2+} exchange is the principal mechanism of $^{45}Ca^{2+}$ release in these experiments. Whether the Ca^{2+}-Ca^{2+} exchange activity is catalyzed by the same carrier as Na^+-Ca^{2+} exchange cannot be determined by these experiments. However, the observation that external $^{40}Ca^{2+}$ can release $^{45}Ca^{2+}$ that had been accumulated by Na^+-dependent uptake indicates that both activities are present in the same vesicles. Ca^{2+} efflux studies with synaptosomes [21] have raised the possibility that Li^+ stimulates Ca^{2+}-Ca^{2+} exchange; when LiCl was used in place of KCl in Ca^{2+}-Ca^{2+} exchange experiments, no difference in $^{45}Ca^{2+}$ efflux was observed. However, $^{45}Ca^{2+}$ efflux into choline-containing media was significantly slower compared to KCl or LiCl media.

Subcellular localization of the Na^+-Ca^{2+} exchange activity

The demonstration of Na^+-dependent Ca^{2+} fluxes in synaptosomes [21,22] and squid axon [10–14] suggests that the Na^+-Ca^{2+} carrier is a plasma membrane protein. Whether the same Na^+-Ca^{2+} exchange system is also present in nonmitochondrial intracellular membrane systems possibly functioning in Ca^{2+} sequestering, is not presently known. In order to determine the subcellular origin of the membranes containing Na^+-Ca^{2+} exchange transport, subcellular fractions of rat brain were examined with respect to Na^+-Ca^{2+} exchange activity and the activities of several membrane marker enzymes. While Na^+-Ca^{2+} exchange activity was found in low levels in both the nuclear-cell debris pellet as well as the crude mitochondrial pellet, only the crude microsomes were significantly enriched in this activity (Table III). The microsomes were also enriched in antimycin A-resistant NADPH-dependent cytochrome *c* reductase, an endoplasmic reticulum enzyme. No significant enrichment, however was seen with 5′-nucleotidase or ($Na^+ + K^+$)-ATPase which are enzymes thought to be associated with plasma membranes. Inosine diphosphatase was also not significantly enriched in the microsomal fraction. This enzyme, though clearly associated with the endoplasmic reticulum of liver [50], appears, from histochemical studies, to be associated with the golgi apparatus and plasma membrane of brain [51]. Although brain mitochondria are known to contain a Na^+-Ca^{2+} exchange system [1], the low level of Na^+-dependent Ca^{2+} uptake seen in the crude mitochondrial fraction (Table III) indicates that under the conditions used in our study for measuring Ca^{2+} uptake, the mitochondrial Na^+-Ca^{2+} system has little or no activity. (Compare the rates in Table III to those reported by others [47,52] for Na^+-dependent Ca^{2+} transport.) Mitochondrial functions including Ca^{2+} uptake and release are best measured in freshly prepared material and the mitochondrial Na^+-Ca^{2+} system may not be stable to the overnight incubations used to load membrane organelles with Na^+. Alternately, the Na^+ loading and Ca^{2+} uptake methods used in our study may not be optimal for measuring Na^+-Ca^{2+} exchange (see Refs. 47 and 52 for examples of conditions for measuring Na^+-dependent Ca^{2+} release in mitochondria).

As mentioned above, brain mitochondria are

TABLE III

DISTRIBUTION OF ENZYMATIC ACTIVITIES AFTER FRACTIONATION OF BRAIN BY DIFFERENTIAL CENTRIFUGATION

Rat brains were fractionated as described under Experimental Procedures. The values presented are the average of assays performed on three separate preparations. The S.D. is given in parenthesis. Na^+-dependent Ca^{2+} uptake was determined at 21°C by diluting NaCl-loaded microsomes 30-fold into 20 mM Tris-HCl (pH 7.4), 2 μM $^{45}Ca^{2+}$, and either 160 mM KCl or 160 mM NaCl. After 1 min incubation, uptake was terminated by filtration as described under Experimental Procedures. The difference between Ca^{2+} uptake in KCl media and Ca^{2+} uptake in NaCl media was taken as Na^+-dependent Ca^{2+} uptake. All other assays were performed as described under Experimental Procedures. ($Na^+ + K^+$)-ATPase activity was taken as the fraction of ATP hydrolysis which was inhibited by 0.1 mM ouabain. 5'-Nucleotidase and ($Na^+ + K^+$)-ATPase activities are expressed as μmol P_i formed/mg protein per h. Succinate dehydrogenase is presented as μmol bis-formazan formed/mg protein per h. Antimycin A-resistant NADPH cytochrome *c* reductase is given as nmol cytochrome *c* reduced/mg protein per h.

Subcellular fraction	Succinate dehydrogenase (μmol/mg per h)	5'-Nucleotidase (μmol/mg per h)	($Na^+ + K^+$)-ATPase (μmol/mg per h)	Inosine diphosphatase (μmol/mg per h)	Antimycin A-resistant NADPH cytochrome *c* reductase (nmol/mg per h)	Na^+-Ca^{2+} exchange uptake (nmol/mg per h)
Crude homogenate	0.28	1.17	19.6	1.26	43	0.37
	(0.06)	(0.30)	(4.9)	(0.06)	(28)	(0.19)
Nuclear and cell debris pellet	0.18	1.26	15.6	1.22	24	0.29
	(0.02)	(0.18)	(4.5)	(0.29)	(5)	(0.21)
Mitochondrial and synaptosomal pellet	0.67	1.2	18.8	0.56	83	0.40
	(0.09)	(0.27)	(6.3)	(0.10)	(26)	(0.28)
Crude microsomes (top pellet)	0.071	1.50	16.1	1.34	202	1.53
	(0.02)	(0.27)	(3.5)	(0.31)	(77)	(0.75)
Crude microsomes (bottom pellet)	0.41	1.75	12.1	0.99	110	0.43
	(0.18)	(0.30)	(5.8)	(0.04)	(18)	(0.15)

capable of Ca^{2+} uptake and Na^+-dependent Ca^{2+} release. However, the Na^+-Ca^{2+} exchange observed in the crude microsomal preparation does not appear to be due to mitochondrial contamination, since the distribution of Na^+-Ca^{2+} exchange in the subcellular fractions shown in Table III does not parallel the distribution of the mitochondrial marker succinate dehydrogenase. In addition, further fractionation of the crude microsomes by isopycnic discontinuous sucrose density gradient centrifugation (Fig. 6) demonstrates that the Na^+-Ca^{2+} exchange activity has a lower density than the membranes containing succinate dehydrogenase and cytochrome *c* oxidase (mitochondrial enzymes). Curiously, the 5′-nucleotidase activity peak was not coincident with the Na^+-Ca^{2+} exchange peak in the experiment shown in Fig. 6, as well as in other experiments not shown.

The microsomes used in the above Ca^{2+} uptake studies were prepared by a discontinuous sucrose density gradient centrifugation procedure. As shown in Table IV, the residual succinate dehydrogenase activity present in the crude microsomes is removed by this fractionation step, indicating the removal of contaminating mitochondrial fragments. The presence of mitochondrial contamination was also assessed by measuring ATP-dependent Ca^{2+} uptake in the microsomal preparations. When ATP-dependent Ca^{2+} uptake was measured in crude microsomes, 70% of the activity was inhibited by a combination of the mitochondrial poisons azide, dinitrophenol and oligomycin, indicating that this portion of the ATP-dependent Ca^{2+} uptake was mediated by mitochondrial fragments. In contrast, ATP-dependent Ca^{2+} uptake in gradient-purified microsomes was resistant

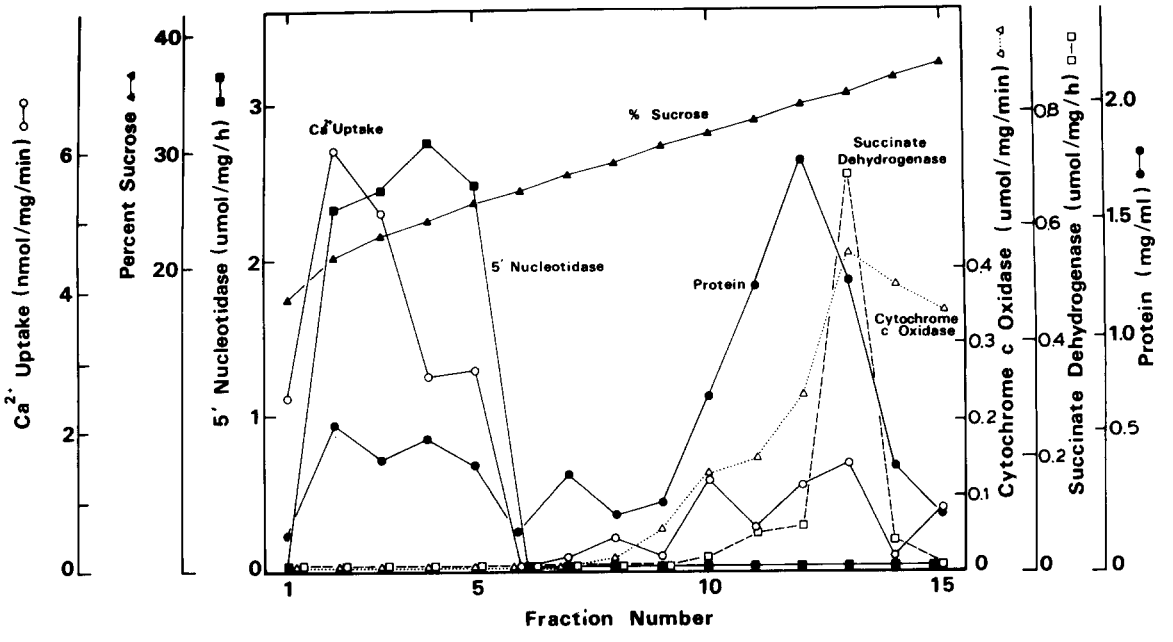

Fig. 6. Isopycnic sucrose density gradient fractionation of microsomal membranes. Crude microsomes (top pellet, 1 mg) in 0.32 M sucrose were layered on top of a linear sucrose gradient formed by mixing 0.6 and 1.2 M sucrose solutions. The sample were centrifuged for 2.5 h at 25 000 rev./min using an SW 27 rotor (Beckman) and 17-ml buckets. The gradient was fractionated as described by Olenick and Lorenz [53] and the percent sucrose (▲) of each fraction was determined by refractive index using a Bausch and Lomb refractometer. The fractions were prepared for enzyme assays and Ca^{2+} uptake determinations by slowly diluting each fraction to 0.32 M sucrose, pelleting the membranes by centrifugation at 100 000 × *g* for 1 h, followed by resuspension in 160 mM NaCl and 20 mM Tris-HCl (pH 7.4). Samples were incubated overnight at 4°C to equilibrate the vesicles with NaCl. Ca^{2+} uptake was determined by diluting the Na^+-loaded vesicles 10-fold into 2 μM $^{45}Ca^{2+}$, 20 mM Tris-HCl (pH 7.4) and either 160 mM KCl or 160 mM NaCl. Na^+-dependent Ca^{2+} uptake (○) was taken as the Ca^{2+} accumulated in KCl media minus Ca^{2+} accumulated in NaCl media. 5′-Nucleotidase (■), cytochrome *c* oxidase (△), protein (●) and succinate dehydrogenase (□) were determined as described under Experimental Procedures. Enzyme activity is expressed as described in Table III.

TABLE IV

FRACTIONATION OF MICROSOMES BY DISCONTINUOUS SUCROSE DENSITY GRADIENT CENTRIFUGATION

Crude microsomes and gradient-purified microsomes were prepared as described under Experimental Procedures. Na^+-dependent Ca^{2+} uptake was assayed by diluting NaCl-loaded microsomes 30-fold into 2 μM $^{45}Ca^{2+}$, 20 mM Tris-HCl (pH 7.4), and either 160 mM KCl or NaCl; Na^+-dependent uptake was taken as $^{45}Ca^{2+}$ accumulated in KCl media minus $^{45}Ca^{2+}$ accumulated in NaCl media. ATP-dependent Ca^{2+} uptake was assayed as described under Experimental Procedures except that when indicated, the inhibitors were omitted. Other assays were performed as described under Experimental Procedures. Units of enzyme activity are expressed as described in Table III.

Fraction	Na^+-dependent Ca^{2+} uptake (nmol/mg per min)	ATP-dependent Ca^{2+} uptake (nmol/mg per min)		Succinate dehydrogenase (μmol/mg per h)	Antimycin A-resistant NADPH-dependent cytochrome *c* reductase (nmol/mg per h)	$(Na^+ + K^+)$-ATPase (μmol/mg per h)	5′-Nucleotidase (μmol/mg per h)
		–	+				
Crude microsomes (top pellet)	1.3	1.72	0.53	0.071	185	13.9	1.5
Gradient-purified microsomes	3.7	0.86	0.87	0 [a]	406	37.5	2.6

[a] Less than 0.01 μmol/mg per h.

to these inhibitors, leading to the conclusion that mitochondrial fragments have been completely removed from this preparation. When compared to crude microsomes (Table IV), the purified preparation was enriched in Na^+-Ca^{2+} exchange activity (2.8-fold), ($Na^+ + K^+$)-ATPase activity (2.7-fold), 5′-nucleotidase activity (1.7-fold), antimycin A-resistant NADPH cytochrome *c* reductase (2.2-fold) and azide-dinitrophenol-oligomycin resistant ATP-dependent Ca^{2+} uptake (1.6-fold).

ATP-dependent Ca^{2+} uptake

The above Na^+-dependent Ca^{2+} uptake experiments were performed in the absence of ATP with the Na^+ gradient being the driving force of uptake. Microsomes from rat brains have also been reported to have nonmitochondrial ATP-dependent Ca^{2+} uptake [23–26]. Fig. 7 illustrates the time course of ATP-dependent Ca^{2+} uptake compared to Ca^{2+} accumulation when ADP was present. (Values similar to the ADP control were obtained in the absence of added nucleotide). In this experiment, no Na^+ gradient was initially present. After Ca^{2+} uptake had reached equilibrium, NaCl was added (arrow, Fig. 7),

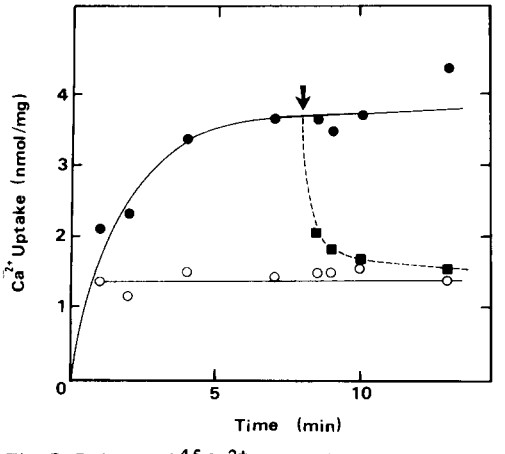

Fig. 7. Release of $^{45}Ca^{2+}$ accumulated by an ATP-dependent transport system by extravesicular Na^+. Determination of ATP-dependent $^{45}Ca^{2+}$ uptake was performed as described under Experimental Procedures either in the presence of Tris · ATP (closed symbols) or Tris · ADP (open symbols) and in the absence of Na^+ or a Na^+ gradient. After 8 min of $^{45}Ca^{2+}$ uptake, either NaCl (▪) or KCl (•) was added (arrow) to give a final concentration of 50 mM salt without altering the osmolarity of the reaction mixture. Each data point is the average of three determinations at 21°C.

resulting in an inwardly directed Na^+ gradient. The Ca^{2+} accumulated by the ATP-dependent mechanism was rapidly released presumably via the Na^+-Ca^{2+} exchange system. This experiment demonstrates that the ATP-dependent uptake system occupies the same membrane vesicles as the Na^+-Ca^{2+} exchange system.

Discussion

The experiments described in this report demonstrate the presence of a Na^+-Ca^{2+} exchange transport system in microsomes prepared from rat brain. In these membrane fragments, Ca^{2+} uptake can be driven by an outwardly directed Na^+ gradient but not by gradients of other cations. Operating in the reverse direction, this carrier also mediates the efflux of Ca^{2+} when external Na^+ is present. A Ca^{2+}-Ca^{2+} exchange activity is also present in the same microsomal vesicles.

Na^+-dependent Ca^{2+} transport by microsomes is not the result of mitochondrial contamination for the following reasons. The gradient-purified microsomal membranes used in our studies have no detectable succinate dehydrogenase activity or dinitrophenol-azide-oligomycin inhibitable ATP-dependent Ca^{2+} uptake which would indicate the presence of mitochondrial fragments in this preparation (Table IV). In addition, the specificity of the mitochondrial Na^+-Ca^{2+} exchange system differs from that of the microsomal system. Na^+-dependent Ca^{2+} release from heart or brain mitochondria is inhibited by extremely low concentrations of La^{3+} (Na^+-Ca^{2+} exchange is inhibited 90% at 3 μM La^{3+} in heart mitochondria, Ref. 52), whereas the microsomal system is inhibited only at higher La^{3+} concentrations (Table II). The mitochondrial and microsomal systems also differ in terms of the specificity of the Na^+ interaction site(s); as pointed out by Reeves and Sutko [29], Li^+ can replace Na^+ in heart mitochondria [51] but not in the plasma membrane Na^+-Ca^{2+} system of heart [29] or the microsomal system of brain (this study). As in heart, experiments with brain mitochondria also indicate that Li^+ can replace Na^+ in Ca^{2+} efflux experiments (Clark, A.F. and Roman, I.J., unpublished data).

Blaustein and Ector [21] have demonstrated the presence of Na^+-dependent Ca^{2+} transport, as well as Ca^{2+}-dependent Ca^{2+} fluxes in rat brain synapto-

somes. The properties of this synaptosomal system are similar, if not identical, to those of the Na^+-Ca^{2+} exchange system characterized in our study. As in brain microsomes (Fig. 5B), Ca^{2+}-Ca^{2+}-exchange in synaptosomes [21] is stimulated in Li^+ media compared to experiments in choline media. Based on these data and assuming the brain Na^+-Ca^{2+} system exchanges three Na^+ ions per Ca^{2+} ion, Blaustein and Ector [21] have proposed that the substrate requirements of the brain exchange system can be fulfilled either by three Na^+ ions per Ca^{2+} ion when the system acts in the Na^+-Ca^{2+} exchange mode or by one Ca^{2+} ion and one Li^+ ion exchanged for a Ca^{2+} ion when the carrier acts in the Ca^{2+}-Ca^{2+} exchange mode. However, as shown in Fig. 5, K^+ is also effective in stimulating Ca^{2+}-Ca^{2+} exchange. Thus, it is possible that either K^+ or Li^+ is cotransported with Ca^{2+} during Ca^{2+}-Ca^{2+} exchange with K^+ and Ca^{2+} occupying the same sites as 3 Na^+ ions would during Na^+-Ca^{2+} exchange. However, since a K^+ gradient cannot replace a Na^+ gradient in driving Ca^{2+} uptake, it seems unlikely that stimulation of Ca^{2+}-Ca^{2+} exchange by Li^+ or K^+ occurs at the Na^+ binding sites. If indeed Ca^{2+}-Ca^{2+} exchange does include transport of a monovalent cation such as Li^+ or K^+, it should be possible to demonstrate that Ca^{2+}-Ca^{2+} exchange is electrogenic. Curiously, while Li^+ stimulates Ca^{2+}-Ca^{2+} exchange in microsomes (Fig. 5B), it partially inhibits Na^+-dependent Ca^{2+} uptake in this preparation (Fig. 1). The cause of this inhibition is currently being investigated.

Rahamimoff and Spanier [35] have also investigated Na^+-dependent Ca^{2+} uptake in subcellular preparations from rat brain. These workers demonstrated the presence of Na^+-dependent Ca^{2+} uptake in vesicles obtained by the hypoosmotic lysis of synaptosomes. This preparation differs from the brain microsomal system in that it does not appear to catalyze Ca^{2+}-Ca^{2+} exchange. Recently Gill et al. [54] have reported that characterization of Na^+-Ca^{2+} exchange in vesicles obtained from guinea-pig brain synaptomes by hypotonic lysis. Consistent with the data shown in Fig. 7, these workers found that Ca^{2+} accumulated by ATP-dependent transport could be released by Na^+. Whether the same vesicles were also capable of Ca^{2+}-Ca^{2+} exchange was not reported.

Studies with heart sarcolemma vesicles have shown that these membranes contain a Na^+-dependent Ca^{2+} transport system [29–33], as well as Ca^{2+}-dependent Ca^{2+} transport activity [32]. The K_m value for Ca^{2+} uptake by these vesicles (18 μM, Ref. 29) is similar to the 23 μM value (Fig. 2) determined in our study with brain microsomes. However, the heart and brain systems may not be identical. La^{3+} has been reported to inhibit Na^+-dependent Ca^{2+} uptake in heart sarcolemma vesicles with half-maximal inhibition occurring at 10 μM La^{3+} in the presence of 40 μM Ca^{2+}. In contrast, no inhibition of Ca^{2+} uptake in brain was observed at 10 μM La^{3+} in the presence of 50 μM Ca^{2+} (Table II).

Since Na^+-Ca^{2+} exchange activity has been demonstrated in Ca^{2+} efflux studies of both squid axon [10–13] and synaptosomes [21,22], it is probable that this Ca^{2+} transport system is located in the plasma membrane and thus would be involved in the extrusion of Ca^{2+} from the cell. In agreement with these studies, the gradient-purified microsomes which are enriched for Na^+-Ca^{2+} exchange also are significantly enriched in the plasma membrane marker 5′-nucleotidase (Tables III and IV). However, both crude and purified microsomes are also enriched for the endoplasmic reticulum marker antimycin A-resistant NADPH-dependent cytochrome *c* reductase, raising the possibility that some or all of the Na^+-Ca^{2+} exchange activity of microsomes may be of endoplasmic reticulum origin. The unambiguous determination of the cellular localization of the Na^+-Ca^{2+} carrier by conventional subcellular fractionation techniques will be difficult. The presence of Na^+-Ca^{2+} exchange activity in a given membrane fraction is dependent not only on the presence of the carrier but also on whether the original membrane organelle resealed into closed vesicular structures after the initial homogenation. Whether vesicles reseal into right-side-out or inside-out structures is probably not important in Na^+-Ca^+ transport determinations, since the Na^+-Ca^{2+} exchange mechanism appears to function in both directions. In contrast, other membrane markers such as 5′-nucleotidase or antimycin A-resistant NADPH-dependent cytochrome *c* reductase do not require the presence of closed structures but only that the active sites of the enzymes be accessible to the added substrate and not sequestered inside a closed vesicle. Whereas the latter two marker enzymes probably require substrate access to only one side of the membrane, measurement of the $(Na^+ + K^+)$-

ATPase requires access of the substrates Mg^{2+}-ATP and Na^+ to one side of the membrane and K^+ and ouabain to the other side. Thus, under the assay conditions used, only activity in unsealed fragments would be measured. Recent work on Ca^{2+} transport in synaptosomes [3,55–57] has suggested that non-mitochondrial intracellular organelles (possibly endoplasmic reticulum) contain ATP-dependent uptake activity and may be important in sequestering and regulating intracellular Ca^{2+}. Thus, it is conceivable that endoplasmic reticulum contain Na^+-dependent as well as ATP-dependent Ca^{2+} transport mechanisms. The influx of Na^+ during depolarization could release Ca^{2+} from the endoplasmic reticulum into the cytoplasm where it would function in stimulating Ca^{2+}-dependent events such as neurotransmitter release. Identification of the intracellular origins of the Na^+-dependent and the ATP-dependent Ca^{2+} transport system awaits further fractionation of endoplasmic reticulum and plasmalemma membrane fragments. A more unambiguous approach would be to localize the Na^+-Ca^{2+} transport protein by immunochemical techniques using specific antibody prepared against the purified carrier.

The relative importance of the Na^+-Ca^{2+} system in Ca^{2+} extrusion compared to other Ca^{2+} pumps located in the plasma membrane remains an unanswered question. Ca^{2+} extrusion and/or Ca^{2+} sequestering processes are thought to maintain free cytoplasmic Ca^{2+} concentrations at submicromolar levels [58]. However, the K_m value of Ca^{2+} uptake by the Na^+-Ca^{2+} carrier observed in our study was 23 μM and thus would be expected to effect very low Ca^{2+} fluxes at submicromolar Ca^{2+} concentrations. However, Caroni et al. [59], using heart sarcolemma vesicles, have suggested that the K_m value for Ca^{2+} uptake by the Na^+-Ca^{2+} carrier may be significantly lower than previously reported values of 18 μM [29]. Under certain conditions where charge buildup by the electrogenic exchange of 3 Na^+ ions per Ca^{2+} ion is prevented by including K^+ and valimomycin in the reaction media, a K_m value for Ca^{2+} of 1.5 μM was obtained [59]. Thus, the $K_{m,Ca^{2+}}$ of this system may be regulated by the membrane potential. In contrast to the Na^+-Ca^{2+} exchange system, the ATP-dependent Ca^{2+} pumps of brain and heart sarcolemma have Ca^{2+} K_m values in the order of 0.2–0.6 μM [31,55]. These higher affinity systems thus would be expected to be active, even at extremely low (submicromolar) Ca^{2+} concentrations. The lower affinity Na^+-Ca^{2+} system does appear to have a higher total capacity for Ca^{2+} transport, compared to ATP-dependent Ca^{2+} uptake. Thus, the Na^+-dependent system may be more important in clearing large amounts of Ca^{2+} from the cytoplasm during transient increases in the Ca^{2+} concentration occurring during depolarization events.

The microsomal system decribed above is a good source of membrane vesicles for studying the properties of the Na^+-Ca^{2+} exchange system of brain in the absence of contaminating mitochondria. The specific activity of Na^+-dependent Ca^{2+} uptake in our preparation is comparable to [54] or higher [35] than other subcellular membrane preparations from brain. The similarities of the properties of the Na^+-Ca^{2+} system of microsomes and synaptosomes make this a good system for studying the role of Ca^{2+} transport systems in regulating intracellular Ca^{2+} levels during the depolarization-repolarization cycle occurring at nerve endings.

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