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SOLUBILIZATION AND RECONSTITUTION OF MEMBRANES CONTAINING THE Na^+-Ca^{2+} EXCHANGE CARRIER FROM RAT BRAIN

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The Na^+-Ca^{2+} exchange carrier from brain plasmalemma was solubilized in cholate and reconstituted into asolectin vesicles by the cholate dilution method. Optimal solubilization and reconstitution required the presence of high NaCl ($\geq$1.3 M). The reconstituted vesicles rapidly accumulated $^{45}Ca^{2+}$ in the presence of an outward directed Na^+ gradient. Other monovalent ion gradients (K^+, Li^+ or cholate$^+$) did not drive transport. Further, Mg^{2+}·ATP did not drive Ca^{2+} uptake in the reconstituted vesicles. Uptake was temperature dependent with highest uptake occurring at 37°C. Intravesicular Ca^{2+} accumulated by the Na^+-dependent process could be released by the Ca^{2+} ionophore A23187 or by extravesicular Na^+ but not by external EGTA. Ca^{2+} uptake was inhibited by extravesicular Li^+ or Na^+. The K_i for Na^+ inhibition was 35 mM for both the original membrane vesicles from brain plasmalemma and for the reconstituted vesicles. Ca^{2+} uptake was saturable with respect to extravesicular Ca^{2+} ($K_m(Ca^{2+})=27\ \mu M$).

Introduction

Cellular Ca^{2+} in excitable tissue is thought to be regulated by intracellular organelles that sequester Ca^{2+} and by plasmalemma transport systems that extrude Ca^{2+} [1]. Studies using squid axon [2–4], barnacle muscle [5,6], and heart sarcolemma vesicles [7] demonstrate that two distinct transport mechanisms are present in the plasmalemma of excitable cells which remove Ca^{2+} from the cell: an ATP-dependent Ca^{2+} pump and an Na^+-Ca^{2+} exchange carrier. While both types of transport systems have been shown to mediate Ca^{2+} fluxes in tissue preparations [8], cultured cells [9], subcellular organelles [10,11], and plasmalemma-derived membrane vesicles [7,12–15], the relative role of these two Ca^{2+} transport mechanisms in regulating cytosolic Ca^{2+} in the resting state cell ($[Ca^{2+}]_i < 0.1\ \mu M$) or in the depolarized cell ($[Ca^{2+}]_i > 1\ \mu M$), remains controversial.

Studies with plasmalemma-derived membrane vesicles have been quite useful in characterizing Na^+-Ca^{2+} exchange in the absence of Ca^{2+} fluxes mediated by intracellular organelles such as mitochondria. Vesicles derived from heart sarcolemma and brain plasmalemma have been used to measure Na^+:Ca^{2+} ratios [16], the electrogenicity of Na^+-Ca^{2+} exchange [13,17–19], and the kinetic parameters of transport [12,13,15,17,20]. However, the presence of additional ion channels and pumps in these complex membranes makes it difficult to study the Na^+-dependent carrier in the absence of ion fluxes occurring by other mechanisms. Miyamoto and Racker [21] recently reported solubilization and reconstitution of the unfractionated Na^+-Ca^{2+} exchange carrier from

Abbreviation: EGTA, ethyleneglycol bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′, tetraacetic acid.

heart sarcolemma. The techniques of solubilization and reconstitution developed by these workers should eventually permit the study of the transport properties of the purified Na^+-Ca^{2+} carrier in the absence of all other flux mechanisms. We initiated the following work to define conditions for solubilizing the Na^+-Ca^{2+} carrier from brain and reconstituting it into phospholipid vesicles with the eventual goal of purifying the carrier. The Ca^{2+} transport properties of the reconstituted vesicles were compared to the properties of the original membrane vesicles. As shown below, the Na^+-Ca^{2+} exchange carrier from brain can be solubilized in NaCl and cholate and reconstituted into asolectin vesicles. The properties of Na^+-dependent Ca^{2+} uptake by the reconstituted vesicles are consistent with the characteristics of the exchange carrier in the native membrane.

A preliminary account of this work was presented in abstract form at the Eighth Meeting of the International Society for Neurochemistry.

Experimental procedures

Preparation of membranes

Microsomal membranes containing Na^+-Ca^{2+} exchange activity were prepared from the cerebral cortex of adult male rats. The crude microsomal fraction was further purified by discontinuous sucrose density gradient centrifugation as previously described [12]. When compared to the crude homogenate, the final preparation was enriched in Na^+-Ca^{2+} exchange activity, $(Na^+ + K^+)$-ATPase, 5′-nucleotidase, and antimycin A-resistant NADPH-dependent cytochrome *c* reductase, and was free of mitochondrial contamination [12]. Membranes in 160 mM NaCl, 20 mM Tris-HCl (pH 7.4) could be stored at −70°C for several months without loss of activity.

Preparation of lipid for reconstitution

Aliquots of the stock lipid solution (100 mg/ml in $CHCl_3$) were dried under a stream of N_2 at 25°C followed by two cycles of resuspension in freshly-distilled ether with subsequent drying under N_2. The lipid was resuspended in 1-ml aliquots (40–60 mg/ml) in either 20 mM Tris-HCl (pH 7.4) or in 200 mM sodium oxalate, 20 mM Tris-HCl (pH 7.4). The mixture was clarified by sonication under N_2 at 25°C using a bath type sonicator (Laboratory Supplies Co., Hicksville, NY).

Solubilization and reconstitution

For most of the experiments described in this report, the membranes containing Na^+-Ca^{2+} exchange activity were combined with soybean phospholipid and the mixture was solubilized in cholate. Unless indicated otherwise, the solubilization media contained sonicated soybean phospholipid (20 mg/ml), membranes (2 mg protein/ml), 20 mM Tris-HCl (pH 7.4), 100 mM sodium oxalate, sodium cholate (2.5%) and NaCl (0.5 M). The mixture was incubated at 4°C for 30 min. Insoluble protein and lipid were removed by centrifugation (4°C) for 1 h at $140\,000 \times g$. The resulting supernatant was subsequently diluted 7-fold in either 200 mM sodium oxalate, 20 mM Tris-HCl (for reconstitution experiments using lipid sonicated in the presence of sodium oxalate) or 300 mM NaCl, 20 mM Tris-HCl (pH 7.4) for samples prepared in the absence of oxalate. Vesicles were allowed to form at 4°C overnight. The reconstituted material was removed from the reconstitution media dilution buffer and concentrated by centrifugation at $140000 \times g$ for 2 h at 4°C. The vesicles, resuspended in 300 mM NaCl, 20 mM Tris-HCl, were incubated for 1 h at 4°C to equilibrate intra and extravesicular Na^+, and assayed for $^{45}Ca^{2+}$ uptake the same day.

For some experiments, the microsomal membranes and the soybean phospholipid were solubilized separately. Sonicated phospholipid (60 mg/ml) in 200 mM sodium oxalate, 20 mM Tris-HCl (pH 7.4) was solubilized for 30 min at 4°C in 2.5% sodium cholate. Insoluble material was removed by centrifugation ($140000 \times g$, 1 h at 4°C). The membranes (4 mg/ml) were solubilized in the presence of various concentrations of cholate and NaCl and insoluble material was removed by centrifugation. The soluble lipid and protein were then combined and NaCl and sodium cholate added to give a final mixture of 2.5% sodium cholate, 1.3 M NaCl, 20 mM Tris-HCl (pH 7.4), 20 mg/ml lipid, 2 mg/ml protein, and 100 mM oxalate. This mixture was then diluted with detergent-free buffer, incubated overnight, and concentrated by centrifugation as described above.

Ca^{2+} uptake assays

Reconstituted phospholipid vesicles prepared in the presence of oxalate and microsomes were assayed for Ca^{2+} uptake by the nitrocellulose filtration assay described previously [12]. Uptake was terminated by the addition of 15 mM EDTA followed by rapid filtration. Vesicles prepared in the absence of oxalate were assayed for Ca^{2+} uptake by Sephadex G-50 (medium) gel filtration. The columns were equilibrated and run in 300 mM KCl, 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 0.01% azide. The uptake reaction was performed as described previously [12]. Immediately after the reaction was stopped by EDTA, 0.2 ml was applied to a jacketed column (1 × 9 cm resin bed) maintained at 4°C. Material eluting in the void volume (vesicles containing $^{45}Ca^{2+}$) was collected as a single fraction (drops 35–70). Two additional fractions (10 drops each) were collected after the void volume fraction to insure that the vesicles were completely separated from free $^{45}Ca^{2+}$. The void volume was routinely eluted approx. 0.5–1 min after sample loading.

The nitrocellulose filter assay and the Sephadex gel filtration assay gave similar results when oxalate was used as an intravesicular Ca^{2+} trap (Table I). However, as reported by others [22], in the absence of intravesicular oxalate, a significant amount of Ca^{2+} is lost when vesicles are assayed by nitrocellulose filtration methods.

Other assays

Protein was determined by the method of Lowry et al. [23] as modified by others [24,25]. Bovine serum albumin was used as a standard. Total lipid phosphate was determined by the method of Ames [26].

Materials

Asolectin was purchased from Associated Concentrates (Woodside, NY). Stock solutions of the lipid (100 mg/ml in redistilled $CHCl_3$) were stored at −70°C under N_2 and used within 1–2 months. Ionophore A23187 was obtained from Calbiochem-Behring and $^{45}CaCl_2$ (12 Ci/g) from new England Nuclear. Nitrocellulose filters (0.45 μM) were from Schleicher and Schuell. The $BaCl_2$, $LaCl_3$, $MgCl_2$, $MnCl_2$, $SrCl_2$, and LiCl used in this study were checked for contaminating Ca^{2+} by atomic absorption spectroscopy using a Perkin-Elmer Model 290 spectrophotometer and none of these reagents contained significant levels of Ca^{2+}. Ruthenium red was obtained from Sigma and used without further purification. The Ca^{2+}-selective electrode was from W. Simon as described by Ammann et al. [27].

TABLE I

COMPARISON OF THE NITROCELLULOSE FILTER ASSAY AND THE SEPHADEX G-50 COLUMN ASSAY

Vesicles were loaded with oxalate or NaCl during the reconstitution procedure by diluting the cholate-solubilized protein-lipid mixture with either 200 mM sodium oxalate, 20 mM Tris-HCl (pH 7.4) or 300 mM NaCl, 20 mM Tris-HCl (pH 7.4). After the vesicles were formed, the material was concentrated by centrifugation, resuspended in 300 mM NaCl, 20 mM Tris-HCl (pH 7.4), and incubated for 1 h at 0°C prior to assaying for $^{45}Ca^{2+}$ uptake. Uptake assay media contained 20 mM Tris-HCl (pH 7.4), 2 μM $^{45}Ca^{2+}$, and either 300 mM KCl or 300 mM NaCl. Assays were initiated by diluting the Na^+-loaded vesicles (± internal oxalate) 30-fold into the assay media. Uptake was terminated after 10 min at 23°C as described under Experimental procedures. Values determined in NaCl media were taken as background and subtracted from Ca^{2+} uptake in KCl media. Uptake values are the average ± S.D. of three determinations.

Vesicle content	$^{45}Ca^{2+}$ uptake (nmol/mg)	
	Nitrocellulose filter assay	Sephadex G-50 column assay
200 mM sodium oxalate	2.85 ± 0.45	3.22 ± 0.51
300 mM NaCl	0.68 ± 0.24	3.03 ± 0.44

Results

Solubilization and reconstitution of the Na^+-Ca^{2+} exchange carrier

Previous work from our laboratory [12] and by others [13,14] has demonstrated the presence of Na^+-Ca^{2+} exchange activity in subcellular membrane fractions from brain. The following work was undertaken to define conditions for solubilizing the Na^+-dependent Ca^{2+} carrier from brain and reconstituting it into phospholipid vesicles. The Na^+-Ca^{2+} exchange carrier was routinely solubilized in 2.5% sodium cholate and 0.5 M NaCl in the presence of 20 mg/ml soybean phospholi-

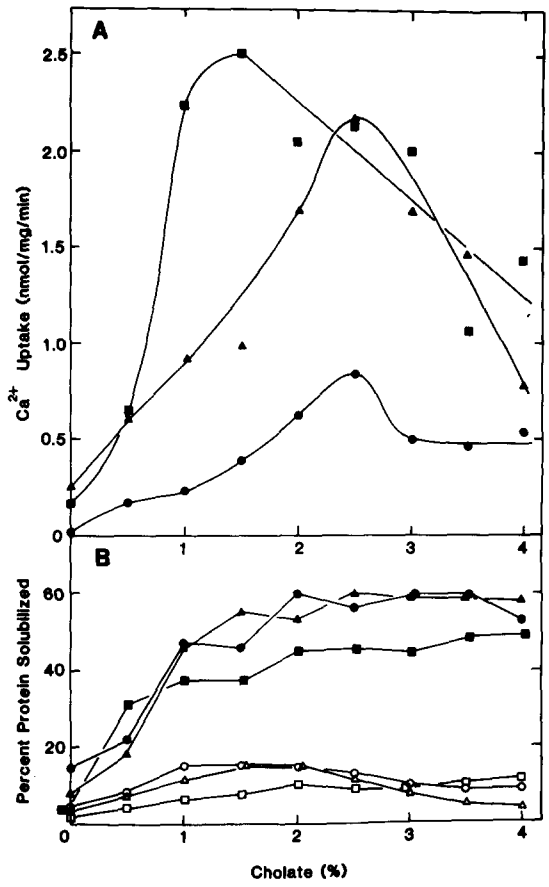

Fig. 1. The effect of cholate concentration on solubilization-reconstitution of Na^+-Ca^{2+} exchange activity. Membranes (2 mg/ml) solubilized in the presence of added lipid (20 mg/ml) were incubated in 20 mM Tris-HCl, 100 mM disodium oxalate, various amounts of sodium cholate, and either 0.3 M NaCl (●, ○) (0.5 M Na^+ from disodium oxalate + NaCl) or 1.1 M NaCl (▲, △) (1.3 M Na^+ from disodium oxalate + NaCl). The resulting soluble protein-lipid mixture was reconstituted as described under Experimental procedures. Membranes (4 mg/ml) solubilized in the absence of added lipid (■, □) were incubated in 1.3 M NaCl, 20 mM Tris-HCl (pH 7.4), and various amounts of sodium cholate. The solubilized protein was then added to cholate-solubilized asolectin and reconstituted as described under Experimental procedures. The reconstituted vesicles were assayed (30-s time points) as described in Table I. The values presented in A are Ca^{2+} uptake in KCl media minus uptake in NaCl media and are the average of four determinations. The percent total protein recovered (B) was determined in the solubilization mixture after insoluble material was removed by centrifugation (closed symbols) and in the reconstituted vesicles after removal from the solubilization-reconstitution media by centrifugation (open symbols).

pid and 100 mM oxalate essentially as described by Miyamoto and Racker [21] for sarcolemma vesicles. The solubilized protein-phospholipid mixture was diluted with detergent-free media thereby lowering the cholate concentration and inducing the formation of vesicles. Fig. 1 illustrates the dependence of the solubilization-reconstitution process on the concentration of cholate used to solubilize the lipid-protein mixture. The highest specific activity of Na^+-dependent Ca^{2+} uptake was recovered from material solubilized in 2.5% cholate.

High NaCl was necessary for optimal solubilization and reconstitution. The amount of NaCl present during solubilization did not appreciably alter the amount of protein solubilized or the amount of protein recovered in the vesicles. However, the Ca^{2+} uptake specific activity of the vesicles formed from material solubilized in high NaCl (1.3 M) was approx. 1.7-fold and 2.6-fold greater than material solubilized in 0.5 M NaCl (Fig. 1) or no added NaCl, respectively (data not shown). KCl could not replace NaCl in stimulating solubilization and reconstitution.

The conditions described above for solubilizing the carrier in the presence of added lipid could also be adapted to solubilizing the carrier in the absence of exogenous lipid. In these experiments (Fig. 1), membranes were solubilized in various cholate concentrations without added asolectin. Solubilized protein was then combined with cholate-solubilized phospholipid and the final detergent concentration adjusted to 2.5%. Optimal Ca^{2+} uptake activity was obtained with a cholate concentration of 1.5% and NaCl concentrations of 0.5 M or greater. Increasing the Na^+ concentration from 0.5 M to 1.5 M did not result in higher specific uptake activities. However, the amount of protein solubilized and recovered in the reconstituted vesicles was increased (data not shown); at 0.5 M and 1.5 M NaCl, the portion of the starting protein recovered in the reconstituted vesicles was 6% and 20%, respectively.

Additional factors which have been reported to affect the efficiency of reconstitution by the cholate dilution method were investigated. Racker et al. [28] reported that efficient reconstitution of cytochrome oxidase required incubation of the protein-lipid-cholate solubilization mixture for 20 h

prior to formation of vesicles by dilution in detergent-free buffer. For reconstitution of the Na^+-Ca^{2+} exchange carrier, lengthy pre-incubation in detergent did not enhance the recovery of Na^+-dependent Ca^{2+} uptake; incubation of the cholate-protein-lipid mixture for periods from 1.5 h up to 24 h did not affect the specific activity of Ca^{2+} uptake or the amount of protein recovered in the vesicles (data not shown). Another variable which has been reported to affect reconstitution [21] is the volume of detergent-free buffer added to the solubilization mixture to induce formation of the vesicles. Miyamoto and Racker [21] reported that at dilution factors greater than 7-fold, the Ca^{2+} uptake specific activity was reduced and that the inhibition of Ca^{2+} uptake by extravesicular Na^+ was reduced. We observed that formation of vesicles required at least a 3-fold dilution. Optimal activity and recovery were achieved at dilutions of 5-fold or greater. However, no reduction in activity or Na^+ sensitivity was observed at increased dilution up to 15-fold.

Under optimal conditions for solubilizing the Ca^{2+} carrier in the absence of added lipid (1.3 M NaCl, 2.5% cholate), 67% (S.D. = 16%, $n = 3$) of the total protein was solubilized and 15% (S.D. = 3%, $n = 3$) of the total protein was recovered in the vesicles. The specific activity of Na^+-dependent Ca^{2+} uptake at 2 μM $^{45}Ca^{2+}$ was 1.86 nmol/mg per min (S.D. = 0.59, $n = 3$) for the reconstituted vesicles. This was comparable to the specific activity of Na^+-Ca^{2+} exchange by the original membrane vesicles which was 1.78 nmol/mg per min (S.D. = 0.24, $n = 3$) under the same conditions (see Experimental procedures for details). The maximal rate of Ca^{2+} uptake by the reconstituted vesicles at 100–200 μM $^{45}Ca^{2+}$ was approx. 20 nmol/mg per min.

Properties of Ca^{2+} uptake by reconstituted vesicles

The Ca^{2+} uptake properties of the Na^+-Ca^{2+} exchange carrier in the reconstituted vesicles were examined by comparing Ca^{2+} uptake in the presence of an outward-directed Na^+ gradient to uptake in the absence of an Na^+ gradient. The Na^+ gradients were generated by diluting Na^+-loaded vesicles into iso-osmotic Na^+-free buffers. Vesicles were formed in the presence of oxalate as a Ca^{2+} trap. Fig. 2A shows the time dependence of Ca^{2+}

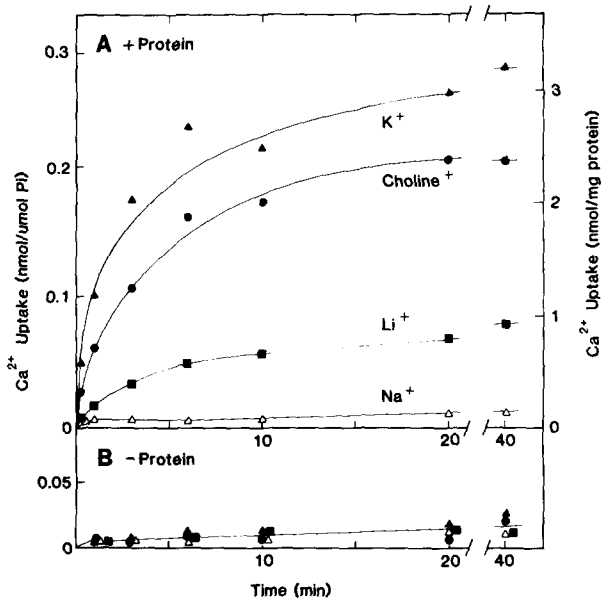

Fig. 2. Time-dependence of Na^+-dependent Ca^{2+} accumulation by vesicles reconstituted in the presence (A) or absence (B) of solubilized protein. Vesicles, loaded with sodium oxalate and suspendedin 300 mM NaCl, were prepared as described under Experimental procedures. Ca^{2+} uptake was assayed at 23°C by diluting (30-fold) the vesicles into media containing 2 μM $^{45}Ca^{2+}$, 20 mM Tris-HCl (pH 7.4), and either 300 mM KCl (▲), LiCl (■), choline chloride (●), or NaCl (△). Ca^{2+} accumulation was determined at the indicated times by the nitrocellulose filter assay. Values are the average of four determinations and are expressed as nmol Ca^{2+} taken up per mg protein (A) and nmol Ca^{2+} taken up per μmol total phospholipid phosphate (A and B).

uptake. The rate of Na^+-dependent Ca^{2+} uptake was extremely rapid for 15–30 s and leveled off to steady-state levels of Ca^{2+} accumulation after 10–20 min. The rate of uptake was greatest when vesicles were diluted into KCl media. K^+ was not, however, required for uptake since Na^+-dependent uptake was also observed in choline chloride media in the absence of K^+. Previous work from our laboratory [12] indicates that in the original plasmalemma vesicles, Na^+-dependent Ca^{2+} uptake is inhibited when external Li^+ is present. The properties of the reconstituted carrier were identical to the behavior of the exchange system in the original membrane in that Ca^{2+} uptake was greatly reduced in the presence of Li^+ (Fig. 2A). The Na^+-dependent uptake observed was not a property of phospholipid vesicles. Vesicles formed in the absence of added protein did not exhibit Na^+-

TABLE II

TEMPERATURE DEPENDENCE OF Ca^{2+} UPTAKE BY RECONSTITUTED PHOSPHOLIPID VESICLES

Vesicles were prepared in the presence of sodium oxalate. $^{45}Ca^{2+}$ uptake was assayed over 1 min by the nitrocellulose filter assay as described in Table I. Na^+-loaded vesicles were diluted 30-fold into either KCl- or NaCl-containing media and the difference between uptake in these buffers is given in the last column. The values given are the average±S.D. of four determinations.

Temp. (°C)	Ca^{2+} uptake (nmol/mg/min)		
	KCl media	NaCl media	Δ
0	0.03±0.017	0.018±0.016	0.012±0.023
6	0.11±0.011	0.012±0.015	0.098±0.019
14	0.26±0.025	0.019±0.007	0.24 ±0.026
21	0.65±0.049	0.035±0.018	0.62 ±0.052
29	0.95±0.045	0.036±0.007	0.91 ±0.046
37	1.01±0.045	0.040±0.013	0.97 ±0.047
45	0.68±0.024	0.060±0.017	0.62 ±0.029

dependent Ca^{2+} uptake (Fig. 2B). Other outwardly directed gradients of Li^+ or K^+ could not drive Ca^{2+} uptake; when vesicles loaded with Li^+ or K^+ were diluted into buffer containing $^{45}Ca^{2+}$ and NaCl, KCl or LiCl, no gradient-dependent Ca^{2+} uptake occurred (data not shown). Gradient-driven uptake was observed only when Na^+-loaded vesicles were diluted into Na^+-free buffers (Fig. 2A). In the absence of an Na^+ gradient, 1 mM Mg^{2+}·ATP did not stimulate Ca^{2+} uptake, indicating the absence of an ATP-dependent Ca^{2+} pump in these vesicles.

Table II illustrates the temperature-dependence of Na^+-Ca^{2+} exchange. Ca^{2+} uptake increased with increasing temperature reaching a maximum at 37°C. At 45°C, uptake was decreased. The temperature dependence of Ca^{2+} uptake is consistent with Ca^{2+} uptake being a carrier-mediated process. The Na^+-Ca^{2+} carrier was inactivated at high temperatures. When cholate solubilized protein was incubated at 60° for 15 min or at 100°C for 2 min and then added to lipid and reconstituted, 82% and 83%, respectively, of the Na^+-dependent Ca^{2+} uptake activity (assayed at 25°C) was destroyed.

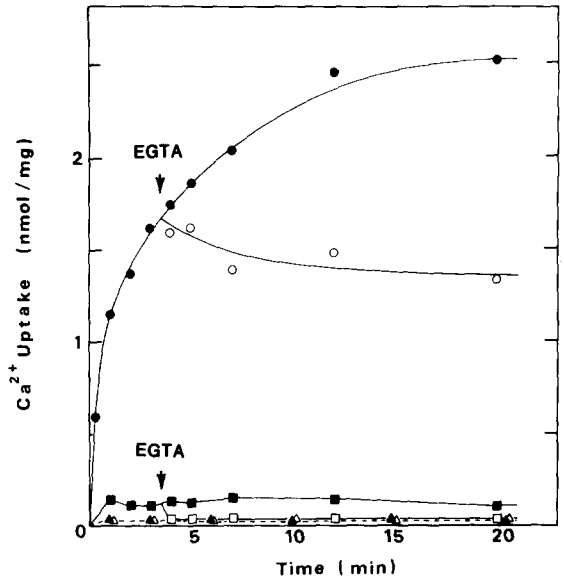

Fig. 3. The effect of EGTA on Na^+-dependent Ca^{2+} uptake by reconstituted vesicles. Vesicles loaded with sodium oxalate and suspended in 300 mM NaCl, 20 mM Tris-HCl were prepared as described under Experimental procedures. Ca^{2+} uptake was assayed at 23°C by diluting (30-fold) the vesicles into media containing 2 μM $^{45}Ca^{2+}$, 20 mM Tris-HCl (pH 7.4), and either 300 mM KCl (●, ○), 300 mM NaCl (■, □), 300 mM KCl, 5 mM EGTA (△), or 300 mM NaCl, 5 mM EGTA (▲). At the arrows EGTA (5 mM final) was added to some samples (○, □). Uptake assays were performed by the nitrocellulose filter assay essentially as described in Table I and under Experimental procedures except that uptake was not terminated by EDTA addition but rather simply by rapid filtration. Values are the average of three determinations.

Fig. 3 shows the effects of EGTA on Ca^{2+} uptake by the reconstituted vesicles. While Na^+-dependent Ca^{2+} uptake was abruptly halted by the addition of EGTA, the majority of the Ca^{2+} already associated with the vesicles was not released. These results indicate that Ca^{2+} taken up by the Na^+-dependent mechanism is sequestered within the vesicles and is not bound to extravesicular sites. EGTA also reduced the amounts of Ca^{2+} associated with the vesicles in the absence of an Na^+-gradient. Addition of EGTA prior to the start of Ca^{2+} uptake resulted in complete inhibition of Ca^{2+} uptake.

Release of accumulated Ca^{2+} from vesicles

In the experiments described above, the vesicles were prepared in the presence of oxalate as a Ca^{2+}

TABLE III

THE EFFECT OF A23187 ON $^{45}Ca^{2+}$ ACCUMULATED BY RECONSTITUTED VESICLES

Vesicles were prepared in the presence of 300 mM NaCl (no oxalate) and assayed by the sephadex G-50 column assay. The Na^+-loaded vesicles were diluted 30-fold into either KCl or NaCl as described in Table I. Vesicles were allowed to accumulate $^{45}Ca^{2+}$ for 15 min. At this time, ethanol or A23187 (1 mg/ml in ethanol) was added to some samples to give a final ethanol content of 1%. $^{45}Ca^{2+}$ associated with the vesicles was determined after 10 min. Uptake values are the average±S.D. of three determinations. Assays were performed at 23°C.

Addition	Ca^{2+} uptake (nmol/mg)		
	KCl media	NaCl media	Δ
None	4.10±0.56	1.10±0.16	3.0 ±0.58
Ethanol	3.91±0.51	1.13±0.11	2.78±0.52
A23187 in ethanol	0.48±0.01	0.17±0.01	0.31±0.01

trap and accumulated Ca^{2+} was assayed by nitrocellulose filtration. Attempts to release accumulated Ca^{2+} by extravesicular Na^+ or by the Ca^{2+} ionophore A23187 from oxalate-loaded vesicles were unsuccessful. Presumably, the intravesicular Ca^{2+} was essentially completely complexed with the oxalate, leaving little free Ca^{2+} available for exchange. In order to demonstrate Ca^{2+} release, vesicles were prepared in the absence of oxalate. Since these preparations could not be assayed by nitrocellulose filtration (see Experimental procedures, Table I), a Sephadex G-50 gel filtration assay was used. Comparable rates of $^{45}Ca^{2+}$ uptake by oxalate-loaded vesicles were obtained by both methods (Table I).

Ca^{2+} accumulated by oxalate-free vesicles could be released by the addition of the Ca^{2+} ionophore A23187 (Table III). The ionophore released 88% of the Ca^{2+} accumulated in the presence of an Na^+ gradient and 85% of the Ca^{2+} accumulated in the absence of a Na^+ gradient. The release of Ca^{2+} by A23187 indicates that the Ca^{2+} associated with the reconstituted vesicles is sequestered and not merely bound to the external surface of the membranes.

Work with Na^+-dependent Ca^{2+} uptake by membrane vesicles derived from heart [7,15] and brain plasmalemma [12,13] has indicated that the

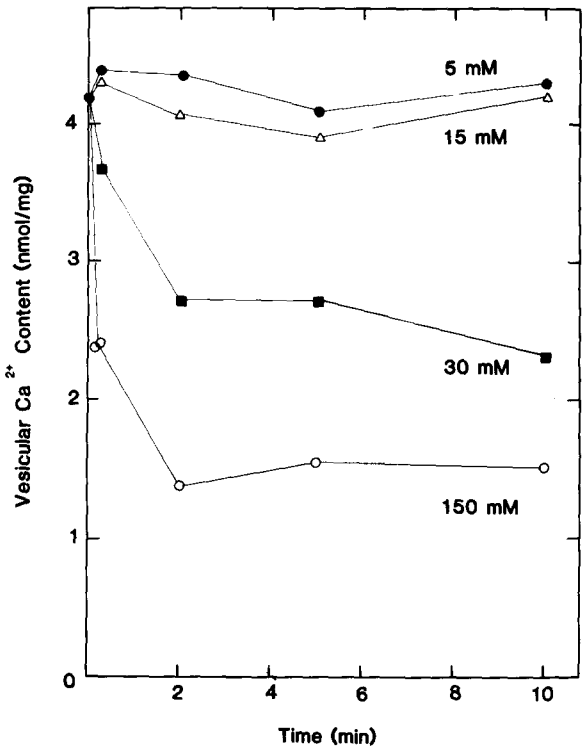

Fig. 4. Release of $^{45}Ca^{2+}$ from reconstituted vesicles by extravesicular Na^+. Vesicles were reconstituted in the absence of oxalate and suspended in 300 mM NaCl as described under Experimental procedures. The vesicles were loaded with $^{45}Ca^{2+}$ at 25°C by diluting the Na^+-loaded vesicles into media containing 2 μM $^{45}Ca^{2+}$, 20 mM Tris-HCl (pH 7.4) and 300 mM KCl. After 15 min of Ca^{2+} accumulation, an equal volume of reaction media was added ($t=0$) containing 2 μM $^{45}Ca^{2+}$, 20 mM Tris-HCl (pH 7.4), and various concentrations of NaCl and KCl ([KCl]+[NaCl]=300 mM). Uptake was assayed at the indicated times by the Sephadex-G 50 column assay. The final NaCl concentrations were 5 mM (●), 15 mM (△), 30 mM (■) and 150 mM (○). $^{45}Ca^{2+}$ uptake by Na^+-loaded microsomes diluted into 2 μM $^{45}Ca^{2+}$, 20 mM Tris-HCl (pH 7.4) and 300 mM NaCl was assayed over the same period and subtracted as background from the values obtained in KCl media. Data points are the average of three determinations.

Na^+-Ca^{2+} exchange mechanism can transport Ca^{2+} in either direction; Ca^{2+} accumulated by Na^+-dependent uptake can be released by increasing extravesicular Na^+. The reconstituted vesicles showed similar properties (Fig. 4). Oxalate-free vesicles loaded with 300 mM Na^+ were diluted into media containing 300 mM KCl. Final KCl and NaCl concentrations were 290 mM and 10 mM, respectively. When the steady state was

reached after approx. 15 min of Ca^{2+} accumulation, extravesicular Na^+ was added, resulting in the reduction of the outward-directed Na^+ gradient. As seen in Fig. 4, addition of extravesicular Na^+ resulted in the rapid release of Ca^{2+} and the establishment of new steady-state levels of intravesicular Ca^{2+}. The amount of Ca^{2+} retained by the vesicles was related to the ratio of intra to extravesicular Na^+; at 10 mM external Na^+, little of the accumulated Ca^{2+} was released, while at 150 mM external Na^+, approx. 63% of the Ca^{2+} was released.

Inhibition of Ca^{2+} uptake by extravesicular Na^+

Extravesicular Na^+ inhibits Na^+-dependent Ca^{2+} uptake by membrane vesicles from heart sarcolemma [21] and from brain plasmalemma (Fig. 5A). Presumably Na^+ and Ca^{2+} compete for the same ion binding site(s) on the exposed surface of the membrane. The effect of external Na^+ on Ca^{2+} uptake by reconstituted vesicles was examined by measuring Na^+-dependent Ca^{2+} uptake at constant intravesicular Na^+ (300 mM) and differing amounts of extravesicular Na^+ (5–300 mM). External KCl was varied such that the total extravesicular NaCl plus KCl concentration was constant at 300 mM salt. Inhibition was measured at both 2 μM and 50 μM external Ca^{2+}. As observed in the original membrane vesicles, external Na^+ inhibited Ca^{2+} uptake by the reconstituted vesicles (Fig. 5B). When plotted by the linear transformation method of Dixon [29] the data from the reconstituted vesicles was linear (Fig. 5B), while the values from the membrane vesicles yielded curved plots (Fig. 5A). The K_i for NaCl from the reconstituted vesicles, taken as the negative of the x-axis value below the intersection of the 2 μM and 50 μM lines, was approx. 35 mM. For the membrane vesicle data, the intersection of the 2 μM and 50 μM plots was estimated using low NaCl values (Fig. 5A) indicating again a K_i for NaCl of approx. 35 mM.

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

The specificity of Na^+-dependent Ca^{2+} uptake by the reconstituted vesicles was examined (Table IV). Ruthenium red is a potent inhibitor of mitochondrial Ca^{2+} uptake [30] and a somewhat less potent inhibitor of plasma membrane Na^+-

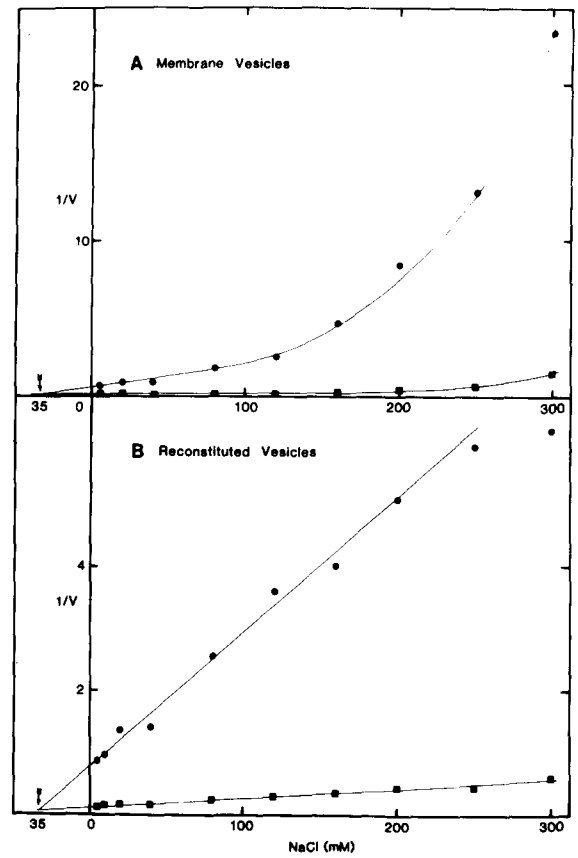

Fig. 5. Inhibition of Na^+-dependent Ca^{2+} uptake by extravesicular Na^+. Ca^{2+} uptake by Na^+-loaded membrane vesicles (A) and sodium oxalate-loaded reconstituted vesicles (B) was assayed in the presence of 20 mM Tris-HCl (pH 7.4), 2 μM (●) or 50 μM (■) $^{45}Ca^{2+}$, and various amounts of NaCl and KCl. For all assays the total salt concentration (KCl+NaCl) was maintained at 300 mM. Uptake was assayed at 23°C for 15 s (A) or 30 s (B) by the nitrocellulose filter method described under Experimental procedures. Each point presented is the average of three determinations. The data are plotted by the method of Dixon [29] with $1/V$ indicating the inverse of the Ca^{2+} uptake velocity. Ca^{2+} uptake is expressed as nmol Ca^{2+} taken up per mg protein per min.

Ca^{2+} exchange [12,13]. Inhibition by this compound was evident only at high concentrations (80 μg/ml). The same preparation of Ruthenium red completely inhibited energy-dependent Ca^{2+} uptake in rat brain mitochondria at 1 μg/ml [31]. Sodium vanadate, which inhibits $(Na^+ + K^+)$-ATPase [32] and Ca^{2+}-ATPase [3,4,6] had little effect at 1 mM. Of the di- and trivalent cations examined, $SrCl_2$ was the most potent inhibitor,

TABLE IV

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

Vesicles were prepared in the presence of sodium oxalate and assayed by the nitrocellulose filter assay. Assays were performed as described in Table I except that 50 μM $^{45}Ca^{2+}$ was used and uptake was terminated after 30 s. The above values are the averages of four determinations. All inhibitor concentrations are given in mM except for Ruthenium red which is presented as μg/ml. In addition to the above inhibitors, taurine was examined for its ability to inhibit uptake; no inhibition occurred at 5 mM or 25 mM. Assays were performed at 23°C. n.d., uptake could not be determined at these values since the presence of the di- or tri-valent cation caused aggregation of the vesicles and high background levels of Ca^{2+} binding to the filters.

Addition	$^{45}Ca^{2+}$ uptake (% control)			
Inhibitor concn.	0.01 mM	0.1 mM	1 mM	10 mM
None			100±11	
$SrCl_2$	96±10	62± 5	28±15	n.d.
$LaCl_3$	109±15	104±19	n.d.	n.d.
$MgCl_2$	102±50	107±20	109± 3	72±16
$BaCl_2$	93± 9	82± 2	76±16	44± 6
$MnCl_2$	111± 9	104± 9	108±16	70± 5
Ruthenium red	106±35 (0.8 μg/ml)	129±25 (8 μg/ml)	17±10 (80 μg/ml)	n.d.
$NaVO_4$	-	-	82 (19)	-

reducing uptake to 28% of the control at 1 mM. $BaCl_2$, $MnCl_2$ and $MgCl_2$ inhibited uptake slightly at 10 mM levels.

Ca^{2+} concentration dependence of Ca^{2+} uptake

Fig. 6 illustrated the Ca^{2+} concentration dependence of Na^+-dependent Ca^{2+} uptake. For the experiment shown in Fig. 6, the $K_m(Ca^{2+})$ of activation was 23 μM. The average $K_m(Ca^{2+})$ from 3 experiments was 27 μM (S.D. = 12, $n = 3$). This value is comparable with the $K_m(Ca^{2+})$ value of 23 μM (S.D. = 3.3, $n = 3$) determined in the original membranes vesicles [12]. This experiment illustrates that Na^+-dependent Ca^{2+} uptake is saturable with respect to extravesicular Ca^{2+}.

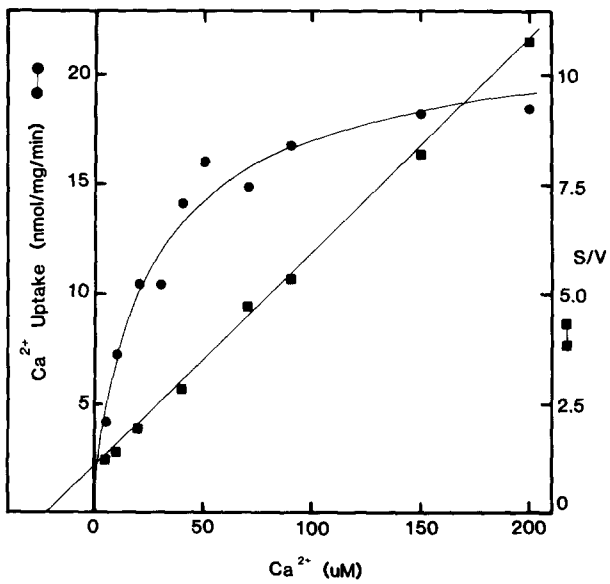

Fig. 6. Ca^{2+} concentration dependence of Na^+-dependent Ca^{2+} uptake. Assays were initiated by diluting sodium oxalate loaded vesicles 30-fold into 20 mM Tris-HCl (pH 7.4), $^{45}Ca^{2+}$ (5–200 μM), and either 300 mM KCl or 300 mM NaCl. Ca^{2+} uptake was terminated after 30 s at 23°C. The free Ca^{2+} concentrations in the presence of reconstituted vesicles and NaCl or KCl media were determined using a Ca^{2+}-selective electrode [27]. The electrode was calibrated in both NaCl and KCl media using solutions of known Ca^{2+} concentrations. The quantity of vesicles used to determine K_m values 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. The data presented represent Ca^{2+} uptake in KCl media minus Ca^{2+} uptake in NaCl media. Values are the average of four determinations (●). The $K_{1/2}$ was determined from the S/V versus S linear transformation plot of the Ca^{2+} uptake data (■). S is the substrate Ca^{2+} concentration and V the velocity of Ca^{2+} uptake (nmol Ca^{2+} taken up per mg protein per min).

Discussion

The above work demonstrates that the Na^+-Ca^{2+} exchange carrier from mammalian brain can be solubilized in an active state and reconstituted into phospholipid vesicles. Optimal solubilization requires cholate concentrations of 2.5% for membranes solubilized in the presence of lipid and 1.5% for membranes solubilized in the absence of exogenous lipid. Higher levels of cholate do not increase the amount of protein released from the membranes and result in inactivation of the carrier (Fig. 1). High NaCl also facilitates cholate solubilization of the carrier in the most active form. The specific activity of Ca^{2+} uptake by the reconstituted vesicles is comparable to that of Na^+-dependent Ca^{2+} uptake by the original membranes. However, while approximately 60% of the total protein is solubilized, only 10–20% of the total Na^+-dependent Ca^{2+} uptake is recovered in the reconstituted material. Since the specific activities of the starting material and the reconstituted vesicles are similar, the low recovery of activity is probably due to inefficient reconstitution rather than denaturation of the carrier during solubilization.

The Na^+-dependent Ca^{2+} uptake observed in the reconstituted vesicles is a carrier-mediated process as shown by the following observations: (1) Na^+-dependent Ca^{2+} uptake is time-dependent (Fig. 2); (2) no Na^+-dependent uptake is observed when vesicles are prepared in the absence of added membrane protein (Fig. 2); (3) Na^+-dependent Ca^{2+} uptake increases 81-fold when the temperature is raised from 0°C to 37°C while Na^+-independent Ca^{2+} leakage across the vesicular membrane increases by a factor of 2.2 (Table II); and (4) Na^+-dependent Ca^{2+} uptake is saturable with respect to Ca^{2+} concentration (Fig. 6) while Na^+-independent Ca^{2+} leakage across the membrane is not (data not shown). Uptake experiments with EGTA (Fig. 3) and A23187 (Table III) demonstrate that the $^{45}Ca^{2+}$ associated with the vesicles in response to a Na^+-gradient is transported across the membrane and is present in the intravesicular space. The carrier solubilized in this study appears to be an intrinsic membrane protein since little activity is released in the absence of added detergent (Fig. 1). Further, high salt alone, which releases some extrinsic proteins, does not solubilize Na^+-Ca^{2+} exchange activity.

The properties of Na^+-dependent Ca^{2+} uptake by the vesicles are consistent with the properties of the Na^+-Ca^{2+} carrier in the native membrane. Particularly striking is the inhibition of Ca^{2+} uptake by the presence of extravesicular Li^+ in both the reconstituted vesicles (Fig. 2) and the native membranes [12,13]. As in other systems where Na^+-Ca^{2+} exchange has been examined, only Na^+ gradients can drive Ca^{2+} uptake while other monovalent cation gradients of Li^+, K^+, or choline$^+$ do not support Ca^{2+} influx. In addition, the carrier in the reconstituted vesicles can mediate Na^+-dependent Ca^{2+} uptake (Fig. 2), as well as Na^+-dependent efflux (Fig. 4). Similar properties have been observed in plasmalemma vesicles [12,13,15], indicating that the carrier can move Ca^{2+} in both directions. The pattern of divalent cation inhibition of Ca^{2+} uptake is quite similar in the membrane vesicles and the reconstituted preparation. With the original membranes, the order of effectiveness of inhibition is $Sr > Ba > Mn > Mg$ while with the reconstituted vesicles, the order is $Sr > Ba > Mg \simeq Mn$. The most significant difference observed in the inhibition data is that ruthenium red at 80 μg/ml inhibits 83% of the Ca^{2+} uptake in reconstituted vesicles (Table IV), but only 17% of the Ca^{2+} uptake in the original membranes.

The kinetic constants of Na^+-dependent Ca^{2+} uptake by the reconstituted vesicles are similar to the properties of the carrier in the original membrane. The $K_m(Ca^{2+})$ values of Na^+-dependent Ca^{2+} uptake are 27 μM (Fig. 6) and 23 μM [12] for the reconstituted vesicles and the original membrane vesicles, respectively. Others have reported values of 7–10 μM for vesicles from lysed synaptosomes [13], 18 μM for sarcolemma vesicles [15] and 8 μM for squid axon (in the absence of ATP, Ref. 2). However, Caroni et al. [17] suggested that the $K_m(Ca^{2+})$ in heart plasmalemma vesicles is affected by the electrical potential across the membrane and may be significantly lower than the 18 μM value previously reported [15]. These workers found that when Ca^{2+} transport-induced charge buildup across the membrane (assuming an electrogenic exchange ratio of 3 Na^+/Ca^{2+}) is prevented by including valinomycin and K^+ in the

assay, the initial rate of Ca^{2+} uptake is stimulated and the $K_m(Ca^{2+})$ was reduced to 1.5 μM [17]. In agreement with these results, we have found in brain microsomes that valinomycin does stimulate Na^+-Ca^{2+} exchange by approx. 30% when uptake is measured over short time periods (5 s). Thus in brain as in heart, Na^+-Ca^{2+} exchange is electrogenic. However, we did not observe any significant change in the $K_m(Ca^{2+})$ values in the presence or absence of valinomycin (Schellenberg, G.D., unpublished data). This Ca^{2+} transport difference between heart and brain vesicles may reflect an intrinsic difference in the properties of the Na^+-Ca^{2+} carrier from these two tissues.

The K_i for Na^+ inhibition of Ca^{2+} uptake for the reconstituted vesicles and the original membrane vesicles are also in agreement. As shown in Fig. 5, identical K_i values of 35 mM are obtained for both types of vesicles. However, in agreement with the results of Miyamoto and Racker [21], the Dixon plots of the data from native membranes are curvilinear, while the data from the reconstituted vesicles yield straight lines. This difference in inhibition data may indicate that a factor(s) which causes cooperative Na^+ binding in the original membranes is lost during the solubilization-reconstitution process. The missing subunit or factor which is not required for Ca^{2+} transport would regulate the Na^+-interaction properties of the holo Na^+-Ca^{2+} carrier.

Intracellular ATP, though not essential for Na^+-Ca^{2+} exchange, stimulates Na^+-dependent Ca^{2+} efflux in dialyzed squid axons [2] and in barnacle muscle [5,6]. Reinlib et al. [20] have also demonstrated ATP stimulation of Na^+-Ca^{2+} exchange in plasmalemma membrane vesicles prepared from dog heart. In squid axon, this ATP effect is seen at low internal Ca^{2+} concentrations, but not at saturating Ca^{2+} levels; ATP lowers the $K_m(Ca^{2+})$ of the system from 8 μM in the absence of ATP to 0.73 μM in the presence of ATP [2]. Recently Nelson and Blaustein [6] demonstrated that ATP-stimulated Na^+-dependent Ca^{2+} efflux from barnacle muscle is inhibited by orthovanadate. These workers suggested that a phosphorylation step is involved in the ATP stimulation of Na^+-Ca^{2+} exchange. In the present study, the effect of ATP on Na^+-dependent Ca^{2+} uptake by the reconstituted vesicles was examined. ATP at 1, 10, 100 or 1000 μM did not stimulate Na^+-dependent Ca^{2+} uptake by the reconstituted vesicles at external Ca^{2+} concentrations of 0.5 or 2 μM (data not shown). Mg^{2+} at concentrations equal to the ATP concentration was included in these assays. The failure of ATP to stimulate Na^+-Ca^{2+} exchange in the reconstituted vesicles suggests several explanations. The orientation of the reconstituted Na^+-Ca^{2+} system in the vesicles may be primarily right-side out. Thus an ATP-interaction site on the cytoplasmic side of the Na^+-Ca^{2+} carrier would not be accessible to extravesicular ATP. Alternately, ATP stimulation may involve a regulatory protein or subunit that is not solubilized and/or reconstituted along with the carrier protein.

The microsomal membranes from brain utilized in this study contain both an ATP-dependent Ca^{2+} pump and an Na^+-dependent Ca^{2+} carrier. Both transport systems are located in the same membrane vesicle population [12]. However, in the reconstituted system when no Na^+ gradient is present, ATP does not drive Ca^{2+} uptake. Thus the ATP dependent system is not solubilized and reconstituted under the same conditions which result in incorporation of the active Na^+-Ca^{2+} carrier into the vesicles. These data support the conclusion that the ATP-dependent system and the Na^+-dependent system are distinct mechanisms for transporting Ca^{2+}.

The solubilization conditions developed for the Na^+-Ca^{2+} exchange activity from brain should facilitate attempts to fractionate and purify the Na^+-Ca^{2+} exchange carrier. In particular, preparations of the carrier solubilized in the absence of added lipid will be useful for subsequent fractionation procedures. The specific activity of the solubilized and reconstituted material is comparable to the activity of the transport system in the original membrane indicating that the carrier has not been denatured during solubilization. In addition, the maximal velocity of Ca^{2+} uptake achieved in this study (20 nmol/mg/min) is comparable to the uptake activity achieved in reconstituted preparations from heart [21]. Studies with the purified carrier reconstituted in vesicles should be extremely useful in determining the properties of Na^+-Ca^{2+} transport in the absence of other ion channels and pumps.

Note

Recently, the potassium-sparing diuretic amiloride has been identified as an inhibitor of Na^+-Ca^{2+} exchange [33,34]. The drug at 1 mM inhibits Na^+-dependent Ca^{2+} uptake by rat brain microsomes and by membrane vesicles from lysed synaptosomes by approx. 56% [34]. Similarly, 1 mM amiloride inhibits Na^+-dependent Ca^{2+} uptake by reconstituted vesicles by 54% (Schellenberg, G.D., unpublished data). These findings support the conclusion that the Na^+-Ca^{2+} exchange carrier has been successfully reconstituted by the methods described here.

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