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Isolation, purification, and reconstitution of the Na^+ gradient-dependent Ca^{2+} transporter (Na^+–Ca^{2+} exchanger) from brain synaptic plasma membranes

(Ca^{2+} transport/Na^+–Ca^{2+} antiporter/transmitter release/synaptosomes)

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ABSTRACT **A [Na^+]-gradient-dependent Ca^{2+} transporter from brain synaptic plasma membranes has been isolated, purified, and reconstituted into brain phospholipid vesicles. The purification was achieved by sucrose-gradient centrifugation after solubilization of the synaptic membranes in cholate in the presence of a 30-fold excess (by weight) of added brain phospholipids and [Na^+]-gradient-dependent Ca^{2+} loading of the reconstituted vesicles. A 128-fold increase in specific activity of [Na^+]-gradient-dependent Ca^{2+} uptake per mg of protein has been obtained. The purified and reconstituted vesicles took up Ca^{2+} only in response to an outward-oriented [Na^+] gradient. The Ca^{2+} uptake could be inhibited by dissipation of the [Na^+] gradient with nigericin. Successful purification was based on the initial [Na^+]-gradient dependency of the Ca^{2+}-transport process, the magnitude of the [Na^+]-gradient-dependent uptake, and the presence of purified brain phospholipids. Analysis of the sucrose-gradient-purified reconstituted vesicles on $NaDodSO_4$/polyacrylamide gels showed that the activity coincided with enriched appearance of a 70,000-Da protein.**

A Na^+–Ca^{2+} antiporter (exchanger) has been described in most excitable membranes. It was initially found in the heart (1, 2) and, subsequently, in squid axon (3), brain synaptosomes (4), and many other tissues. The Na^+–Ca^{2+} exchanger transports Ca^{2+} ions across the membrane at the expense of the [Na^+] gradient. Most data suggest that 3–5 Na^+ ions are exchanged one way for each Ca^{2+} ion crossing the membrane in the opposite direction (5). Thus, the Na^+–Ca^{2+} exchange process is electrogenic, and under physiological conditions, the direction of the Ca^{2+} flux is modulated by the membrane potential (5, 6) and the relative magnitude of the Na^+ and Ca^{2+} gradient across the cell membrane. In the whole cell or in tissue slices, it is difficult to study the molecular properties of the Na^+–Ca^{2+} antiporter, because both Na^+ and Ca^{2+} ions are involved in a large number of cell processes. Therefore, several years ago, we developed a membrane vesicle preparation derived from synaptic plasma membranes (SPM) in which the ionic gradients could be controlled and in which the [Na^+]-gradient-dependent Ca^{2+} uptake process was not complicated by cellular metabolism (7).

In this work, we report the isolation, purification, and reconstitution of the [Na^+]-gradient-dependent Ca^{2+} transporter from SPM. In other studies, reconstitution of the Na^+-Ca^{2+} exchanger from sarcolemma vesicles (8) or SPM (9) did not lead to purification of the protein(s) of the exchanger. Partial purification of the exchanger has been reported from chicken heart membranes (10). The enriched preparation included 5 major proteins of 20,000, 21,000, 30,000, 32,000, and 36,000 Da; it also included some minor bands. It is not clear which, if any, of these bands corresponds to the exchanger activity itself.

In this work, we have solubilized SPM in detergent (cholate) in the presence of an added excess (30- to 50-fold excess by weight) of purified brain phospholipids, and reconstituted the phospholipid vesicles containing native proteins after removal of the detergent by dialysis. The dialysis was carried out against Na phosphate buffer, which served as the internal medium of the reconstituted vesicles. [Na^+]-gradient-dependent Ca^{2+} uptake served for loading the vesicles containing the relevant carrier. Since the protein-to-phospholipid ratio has been increased from 1:1.4 in the native membrane to ≈1:40 in the reconstituted preparation, we expected that after dialysis and reformation of vesicles the native proteins would be distributed among many liposomes, resulting in none, one, or very few proteins per vesicle. Consequently, we should be able to separate the vesicles containing the [Na^+]-gradient-dependent Ca^{2+} carrier from the vesicles lacking the antiporter on the basis of their differences in density after Ca^{2+} loading, resulting in transport-specific fractionation (11).

A positive result of such an experiment depends on the relative impermeability of phospholipid vesicles to passive Ca^{2+} entry in the absence of Na^+–Ca^{2+} antiporter molecules in their membrane and on the ability of the vesicles containing the [Na^+]-gradient-dependent Ca^{2+} carrier to take up large quantities of Ca^{2+} in response to a preformed [Na^+] gradient. A similar strategy was successfully used for the purification of the erythrocyte glucose transporter (11) and the Ca^{2+}-transport ATPase from synaptosomal vesicles (12).

MATERIALS AND METHODS

A preparation enriched in SPM was obtained from Ficoll gradient purified rat or calf synaptosomes after treatment with hypoosmotic (5 mM Tris·HCl, pH 7.4/1 mM EDTA) solution as described (7). Each rat SPM preparation consisted of 30 brains, and each calf SPM preparation consisted of 3 brains. The vesicles were rapidly frozen in liquid N_2 and could be kept in small aliquots for a few months without loss of activity. The [Na^+]-gradient-dependent Ca^{2+}-uptake activity of different native SPM vesicle preparations varied considerably. The average activity was ≈25 nmol of Ca^{2+} transported per mg of protein in 5 min. On occasions, the preparation of SPM vesicles had much higher specific activity, and it reached as high as 60 nmol of Ca^{2+} transported per mg of protein in 5 min. About 0.5 mg of membranes was dissolved per ml of a solution containing 2% Na cholate/0.2 M Na phosphate buffer, pH 7.4/5 mM 2-mercaptoethanol/15 mg of purified brain phospholipids. The solution was clarified by centrifugation at 30,000 × *g* for 20 min at 4°C to pellet any undissolved membranes. The clear supernatant (≈5 ml) was dialyzed overnight against 5000 ml of 0.2 M Na phosphate

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Abbreviation: SPM, synaptic plasma membrane.

buffer (pH 7.4) using either hollow fibers (Bio-Rad) or dialysis tubing (Visking 1-8/32", Medicell International, London). When dialysis was carried out by the latter method, the clear supernatant was diluted 1:4 to decrease the cholate concentration with Na phosphate buffer prior to dialysis overnight. The reconstituted membranes were concentrated by centrifugation at 200,000 × *g* for 180 min at 4°C. Calcium-transport activity was assayed by diluting 3 μl (6 μg of protein) of Na phosphate-loaded vesicles into 100 μl of either 0.2 M KCl/10 mM Tris·HCl, pH 7.4/50 μM $^{45}CaCl_2$ (0.1 μCi; 1 Ci = 37 GBq) or 0.2 M NaCl/10 mM Tris·HCl, pH 7.4/50 μM $^{45}CaCl_2$ (0.1 μCi).

The vesicles were incubated in test solution for various periods of time. The uptake reaction was stopped by pipetting the entire reaction mixture rapidly over a Dowex 50 minicolumn (see ref. 13), to separate intravesicular from extravesicular Ca^{2+} that binds to the column. $^{45}Ca^{2+}$ transport was determined by measuring the radioactivity in an aliquot of vesicles in a 40% Lumax (scintillator) solution.

Purification of the reconstituted [Na^+]-gradient-dependent Ca^{2+} transporter was done by incubating Na phosphate-loaded vesicles for 20 min with $^{45}CaCl_2$. The entire reaction mixture was layered on a linear sucrose gradient (0–0.5 M sucrose) in either 0.2 M KCl or 0.2 M NaCl containing 10 mM Tris·HCl, pH 7.4/5 mM 2-mercaptoethanol. The choice of KCl or NaCl depended on whether the vesicles layered on the gradients were "control" vesicles (Ca^{2+} loading was done in the absence of [Na^+] gradient), or experimental vesicles in which the [Na^+] gradient was present in the initial loading period. The gradients were centrifuged in a Beckman SW 41 rotor at 40,000 rpm for 4.5 hr at 4°C. Fractions of 0.5 ml were collected by piercing the bottom of the tube. From each fraction, 50-μl aliquots were removed for determination of $^{45}Ca^{2+}$ content and phospholipid phosphate (14). For determination of specific $^{45}Ca^{2+}$ content per mg of protein or for analysis of the protein profile on polyacrylamide gels, the following two sets of fractions were pooled: (*i*) The fractions containing the major phospholipid peak in each sucrose gradient; (*ii*) the fractions containing the $^{45}Ca^{2+}$ peak in the [Na^+]-gradient-dependent $^{45}Ca^{2+}$ preloaded vesicles, or the corresponding fractions in the sucrose gradient containing the "control" vesicles.

Intermediate fractions, in which the lower shoulder of the major phospholipid peak overlapped considerably with the upper shoulder of the $^{45}Ca^{2+}$ peak, were pooled separately, although some overlap probably occurred. The pooled fractions were dialyzed overnight against two changes of ≈2000 ml each of double-distilled water, and they were lyophilized prior to analysis of their protein profiles on $NaDodSO_4$/polyacrylamide slab gels (15). For determination of the specific activity of the purified [Na^+]-gradient-dependent Ca^{2+} transporter, the pooled fractions were diluted with 0.2 M NaCl/10 mM Tris·HCl, pH 7.4, and concentrated by centrifugation for 3 hr at 200,000 × *g*. The small pellet thus obtained has been redissolved by addition of a solution containing purified brain phospholipids (30 mg/ml) in 2% cholate (1/4th of its volume) and dialyzed for 12 hr against 2000 ml of 0.2 M Na phosphate (pH 7.4). At the end of the second dialysis, the phospholipid vesicles were again concentrated by centrifugation at 200,000 × *g* for 3 hr and the specific activity of [Na^+]-gradient-dependent Ca^{2+} transport was determined. Brain phospholipids were prepared by a modification of the Bligh and Dyer extraction of lipids: 20-g pieces of calf brain were extracted with a mixture of chloroform (20 ml)/methanol (40 ml) (16). Lipids were purified further by chromatography on a K-oxalate-washed column of silicic acid to separate neutral lipids from phospholipids. Total chloroform/methanol (20:80) extractable phospholipids were used for reconstitution. The composition of the phospholipid mixture was determined by thin-layer chromatography. It contained 31.4% phosphatidylcholine/35.8% phosphatidylethanolamine/17.7% phosphatidylserine/15.1% sphingomyelin. Protein was determined either by the method of Lowry *et al.* (17) or by the amido black method (18), depending on the amount of protein expected. $^{45}CaCl_2$ was purchased from New England Nuclear; nigericin was a gift from Ely Lilly; all biochemicals were purchased from Sigma, Israel, and the chemicals were analytical grade reagents. Lumax was purchased from Lumac B.V. (The Netherlands).

RESULTS

The time course of the uptake of $^{45}Ca^{2+}$ into reconstituted vesicles is shown in Fig. 1. In the experiment presented here, the Ca^{2+} taken up by the vesicles reached 50 nmol per mg of protein in 5 min. In the absence of a [Na^+] gradient, when the Na phosphate-loaded reconstituted vesicles were diluted into a medium in which the 0.2 M KCl was replaced by 0.2 M NaCl, <5 nmol of Ca^{2+} was taken up per mg of protein in 5 min. The importance of the [Na^+] gradient in Ca^{2+} uptake is shown by adding nigericin (an ionophore exchanging $Na^+/H^+/K^+$) to the uptake medium of the Na phosphate-loaded vesicles diluted into KCl. This led to a considerable decrease in Ca^{2+} uptake by the vesicles; only 10 nmol of Ca^{2+} was taken up per mg of protein in 5 min. The [Na^+]-gradient dependency of the Ca^{2+} transport by SPM vesicles has been repeated with >30 different reconstituted preparations. The average [Na^+]-gradient-dependent Ca^{2+}-uptake activity of reconstituted SPM vesicles was 37 nmol per mg of protein per 5 min. In the absence of a [Na^+] gradient, 3.06 nmol per mg of protein per 5 min was, on average, associated with the vesicles.

Table 1 shows the results obtained in four experiments in which highly purified brain phospholipids were used. In addition, the initial [Na^+]-gradient-dependent Ca^{2+}-uptake activity of the native SPM vesicle preparation used was 50 nmol per mg of protein. It can be seen that the Ca^{2+} uptake in the presence of an outward-directed [Na^+] gradient is about 10-fold greater than in its absence. This increment in the amount of Ca^{2+} associated with the vesicles in response to a [Na^+] gradient has a special importance in the subsequent purification of the carrier based on transport-specific fractionation.

Fig. 2*A* shows a typical sucrose-gradient profile obtained when Na phosphate-containing reconstituted vesicles were loaded with $^{45}Ca^{2+}$ and centrifuged on a linear 0–0.5 M sucrose gradient containing 0.2 M KCl/5 mM 2-mercapto-

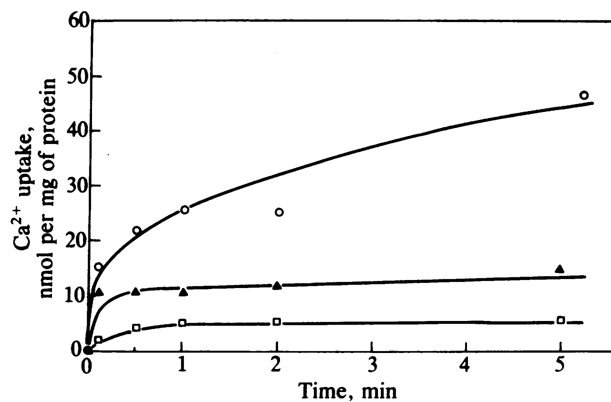

FIG. 1. [Na^+]-gradient-dependent Ca^{2+} uptake in reconstituted SPM vesicles. Three microliters of Na phosphate-loaded reconstituted vesicles were diluted into 100 μl of 0.2 M KCl/10 mM Tris·HCl, pH 7.4/50 μM $^{45}CaCl_2$ (○), or into the same medium except that 30 μM nigericin was also added (▲), or into a medium containing 0.2 M NaCl/10 mM Tris·HCl, pH 7.4/50 μM $^{45}CaCl_2$ (□). The reaction was terminated at time points shown, and $^{45}Ca^{2+}$ uptake was measured.

Table 1. [Na^+]-gradient-dependent Ca^{2+} uptake in reconstituted SPM vesicles

	Ca^{2+} uptake, nmol per mg of protein per 5 min	*n*
$[NaP_i]^{in}$ $[KCl]^{out}$	53.7 ± 4.1 (SD)	4
$[NaP_i]^{in}$ $[NaCl]^{out}$	5.8 ± 2.3 (SD)	4
$[NaP_i]^{in}$ $[KCl]^{out}$/ 30 μM nigericin	12.1 ± 3.9 (SD)	4

Reconstituted SPM vesicles (≈6 μg of protein and ≈180 μg of highly purified brain phospholipids) were preloaded with 0.2 M Na phosphate and diluted into 100 μl of 0.2 M KCl (or NaCl)/10 mM Tris·HCl, pH 7.4/50 μM $^{45}CaCl_2$. *n*, number of experiments. NaP_i, Na phosphate buffer.

ethanol/10 mM Tris·HCl, pH 7.4.

Fig. 2*B* shows the control sucrose gradient when identical amounts of phospholipid vesicles were exposed to $^{45}CaCl_2$ in the absence of a [Na^+] gradient and centrifuged on identical linear sucrose gradients, except that the 0.2 M KCl was replaced with 0.2 M NaCl. Most of the $^{45}Ca^{2+}$ added to the reaction mixture is not associated with the vesicles and remains on top of the gradients (fractions 21–24) (not shown).

Fractions corresponding to those marked by bars in Fig. 2*A*, (6–11 and 14–16) and in Fig. 2*B*, (7–13 and 16–18) and the intermediate fractions (no bars) were pooled, and the phospholipid vesicles were collected by centrifugation. The ratio of $^{45}Ca^{2+}$ content to protein and phospholipid in the pooled fractions is compared in Table 2. It can be seen that, while 47% of the $^{45}Ca^{2+}$ associated with the vesicles was in the denser region of the sucrose gradient than the majority of the phospholipid vesicles, only 0.9% of the total protein and 1.3% of the total phospholipid was to be found there. The increase in specific $^{45}Ca^{2+}$ content (based on protein) was 105-fold (average of five experiments) and 101-fold (based on phospholipid) compared to the initial [Na^+]-gradient-dependent Ca^{2+} loading of the unfractionated vesicle population before the sucrose gradient.

To directly determine the specific [Na^+]-gradient-dependent Ca^{2+}-transport activity, the fractions below the phospholipid peak containing most of the $^{45}Ca^{2+}$ associated with the vesicles (corresponding to fractions 6–11 in Fig. 2*A*) were diluted with buffered 0.2 M NaCl, and the phospholipid vesicles were collected by centrifugation. The pellets thus obtained were redissolved in detergent and fortified by added purified brain phospholipids, and a second reconstitution was carried out by dialysis against 0.2 M Na phosphate. This was done to empty the vesicles of previous Ca^{2+} content remaining from the initial purification step. The [Na^+]-gradient-dependent Ca^{2+}-transport activity of these purified and

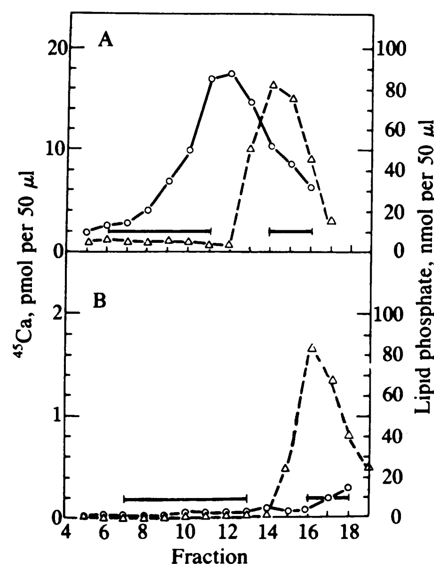

FIG. 2. Sucrose-gradient profile of reconstituted SPM vesicles after loading with $^{45}Ca^{2+}$. Na phosphate-loaded reconstituted vesicles were exposed to $^{45}Ca^{2+}$ in the presence of buffered 0.2 M KCl (*A*) or buffered 0.2 M NaCl (*B*) for 20 min and then centrifuged. Fractions (0.5 ml) were collected from the bottom. $^{45}Ca^{2+}$ (○) and phospholipid phosphate (△) were determined in each fraction.

reconstituted phospholipid vesicles is shown in Table 3. To determine whether a decrease in activity occurred after this rather lengthy procedure (≈60 hr), unfractionated reconstituted $^{45}Ca^{2+}$-loaded SPM vesicles were also redissolved and subjected to a second reconstitution in parallel to the purified vesicles. In two such experiments, an average loss of 40% of the overall activity of the vesicles was measured. Assuming a parallel loss in activity of the purified preparation, a 128-fold increase in specific [Na^+]-gradient-dependent Ca^{2+}-transport activity was obtained.

Analysis of the protein profile of the purified [Na^+]-gradient-dependent Ca^{2+}-transport activity was carried out on pooled fractions after dialysis to remove sucrose, lyophilization, and $NaDodSO_4$/polyacrylamide gel electrophoresis (bars in Fig. 2). Between two and six gradients were pooled for this purpose, depending on the total amount of phospholipid, the ^{45}Ca content available, and the overlap between the $^{45}Ca^{2+}$-containing peak and the phospholipid peak.

Fig. 3 shows a typical gel pattern obtained from purified and reconstituted calf SPM stained with Coomassie blue. In lane 3, pooled fractions 6–11 from Fig. 2*A* are shown; lane 2 shows pooled fractions 7–13 from Fig. 2*B*; identical amounts

Table 2. $^{45}Ca^{2+}$, phospholipid, and protein distribution after sucrose-gradient centrifugation of [Na^+]-gradient-dependent $^{45}Ca^{2+}$-loaded reconstituted SPM vesicles

Sucrose-gradient fraction	$^{45}Ca^{2+}$, nmol	Phospholipid, μg	Protein, μg	Specific $^{45}Ca^{2+}$ content, nmol/mg: Protein	Specific $^{45}Ca^{2+}$ content, nmol/mg: Phospholipid
1	10.8	125	3.2	3465	84.4
2	7.9	3110	94	84	4.3
3	4.1	5906	268	15.8	0.69

Reconstituted SPM vesicles were loaded with $^{45}Ca^{2+}$ in a [Na^+]-gradient-dependent manner and centrifuged on a sucrose gradient. The following gradient fractions were pooled: 1, major $^{45}Ca^{2+}$-containing peak; 2, intermediate fractions in which the upper shoulder of the $^{45}Ca^{2+}$ peak overlaps with the lower shoulder of the phospholipid peak; 3, major phospholipid-containing fractions. Control gradients (vesicles exposed to Ca^{2+} in the absence of [Na^+] gradient) yielded the following: 1, 1 nmol of $^{45}Ca^{2+}$ per 88 μg of phospholipid; 2, 1.3 nmol of $^{45}Ca^{2+}$ per 3640 μg of phospholipid; 3, 0.8 nmol of $^{45}Ca^{2+}$ per 6804 μg of phospholipid. Data represent mean of five experiments. Average initial [Na^+]-gradient-dependent Ca^{2+} uptake in these experiments was 33 nmol per mg of protein in 5 min and 0.83 nmol of $^{45}Ca^{2+}$ per mg of phospholipid.

Table 3. [Na^+]-gradient-dependent Ca^{2+} transport activity of the sucrose-gradient purified reconstituted phospholipid vesicles after solubilization and second reconstitution

Phospholipid vesicles	External medium	$^{45}Ca^{2+}$ uptake, nmol per mg of protein per 5 min
Sucrose gradient	KCl	3786
purified vesicles*	NaCl	1390
Unfractionated	KCl	32
control vesicles†	NaCl	13.28

The internal medium of the vesicles was 0.2 M Na phosphate (pH 7.4), and they were diluted into 0.2 M KCl/10 mM Tris·HCl, pH 7.4/50 μM $^{45}CaCl_2$, or 0.2 M NaCl/10 mM Tris·HCl, pH 7.4/50 μM $^{45}CaCl_2$.

*Pooled fractions (corresponding to fractions 6–11 in Fig. 2*A*) were redissolved in detergent and reconstituted.

†Reconstituted unfractionated control vesicles were redissolved in detergent and reconstituted exactly as the gradient purified vesicles. Initial Ca^{2+} transport activity was 53.5 nmol per mg of protein per 5 min in the presence of [Na^+] gradient. The decrease in activity after the entire procedure was 40%.

of phospholipid were compared on the $NaDodSO_4$/polyacrylamide gel profiles shown in lanes 2 and 3. Lane 1 contains 40 μg of the native calf SPM. In lane 3, where the pooled fractions of the vesicles loaded with $^{45}Ca^{2+}$ in the presence of a [Na^+] gradient were analyzed, one protein band is highly enriched. In the corresponding pooled fractions of the vesicles exposed to $^{45}CaCl_2$ in the absence of a [Na^+] gradient, this band does not appear (lane 2). The major phospholipid peaks could not be analyzed by $NaDodSO_4$/polyacrylamide gels directly because of the high phospholipid content.

To decrease the phospholipid content of the major phospholipid peak (fractions 14–16 in Fig. 2*A* and 16–18 in Fig. 2*B*), all the combined sucrose-gradient fractions were extracted with a mixture of chloroform/methanol (1:2), and the delipidated protein precipitates were subjected to analysis on $NaDodSO_4$/polyacrylamide gels. Under these conditions, no enriched appearance of any protein band was visible on either of the gels. In addition, although after delipidation the major phospholipid peak could be analyzed by polyacrylamide gel electrophoresis, several protein bands that were clearly noticeable in the native synaptic plasma membrane vesicle preparation were absent in the Coomassie blue-stained protein profiles obtained.

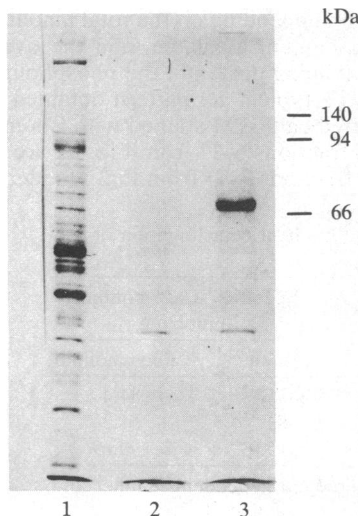

FIG. 3. $NaDodSO_4$/polyacrylamide gel electrophoresis pattern of purified [Na^+]-gradient-dependent Ca^{2+} transporter stained with Coomassie blue. Lanes: 1, 40 μg of native calf SPM; 2, combined fractions 7–13 from sucrose-gradient profile shown in Fig. 2*B*; 3, combined fractions 6–11 from sucrose-gradient profile shown in Fig. 2*A*.

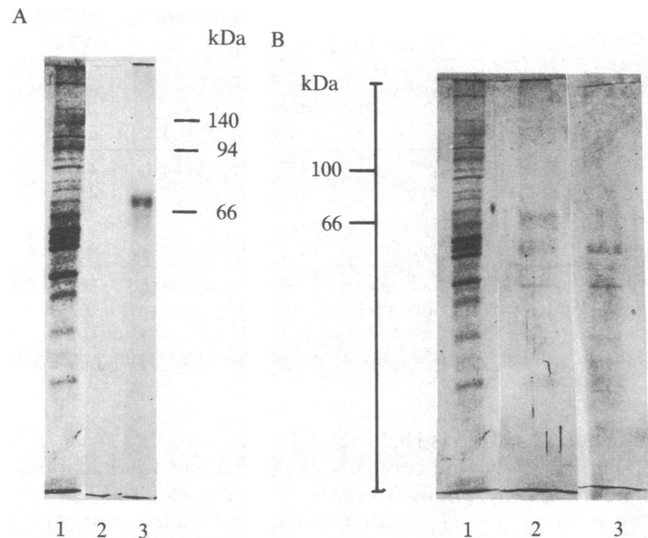

FIG. 4. $NaDodSO_4$/polyacrylamide gel electrophoresis pattern of purified reconstituted rat SPM vesicles stained with Coomassie blue. (*A*) Lane 1, native rat SPM (≈30 μg); lane 2, pooled sucrose-gradient fractions of the reconstituted rat SPM vesicles below the phospholipid peak (corresponding to fractions 7–13 in Fig. 2*B*). Vesicles were previously loaded with $^{45}Ca^{2+}$ in the absence of [Na^+] gradient. Lane 3, pooled sucrose-gradient fractions of reconstituted rat SPM vesicles previously loaded with $^{45}Ca^{2+}$ in the presence of [Na^+] gradient (corresponding to fractions 7–11 in Fig. 2*A*). (*B*) Lane 1, native rat SPM (≈30 μg); lane 2, pooled sucrose-gradient fractions of reconstituted rat SPM vesicles previously loaded with Ca^{2+} in a [Na^+]-gradient-dependent manner. Fractions containing the $^{45}Ca^{2+}$ peak (below the major phospholipid peak) are shown. Lane 3, same vesicles as shown in lane 2 except that the Ca^{2+} loading before the sucrose gradient was done in the presence of [Na^+] gradient and nigericin. Fractions of the sucrose gradient corresponding to those in lane 2 were pooled.

Fig. 4 *A* and *B* shows $NaDodSO_4$/polyacrylamide gels similar to the one shown in Fig. 3 except that the initial reconstitution was done with rat SPM. The reconstituted rat SPM vesicles were loaded with Ca^{2+} in the presence (Fig. 4A, lane 3) or in the absence (lane 2) of [Na^{2+}] gradient. The sucrose-gradient fractions with the major $^{45}Ca^{2+}$-containing peak, or their corresponding fractions in the control sucrose gradient, were pooled. Fig. 4*B* shows a similar experiment in which reconstituted rat SPM vesicles were exposed to $^{45}Ca^{2+}$ in the presence of [Na^+] gradient only (lane 2) or in the presence of [Na^+] gradient and nigericin (lane 3). It should be noted that when there is no [Na^+] gradient or when the [Na^+] gradient is dissipated and, consequently, the driving force for Ca^{2+} is decreased or absent, the enriched appearance of the 70,000-dalton protein band is also abolished.

DISCUSSION

Transport-specific fractionation is a powerful tool for purification of transport-associated integral membrane proteins (11, 12). In this work, we have taken advantage of the specific transport properties of a carrier that rendered the phospholipid vesicles into which it was reconstituted denser than other phospholipid vesicles in the preparation. This was done by specific [Na^+]-gradient-dependent Ca^{2+} loading. The density difference obtained between vesicles containing the [Na^+]-gradient-dependent Ca^{2+} carrier and other vesicles was sufficient to displace the [Na^+]-gradient-dependent Ca^{2+} transporter to a region of higher sucrose density on the

isopycnic sucrose gradient. Early experiments (not shown here), showed that successful purification of [Na^+]-gradient-dependent Ca^{2+} carrier was directly related to its initial activity. If Ca^{2+} uptake of reconstituted vesicles in the presence of a [Na^+] gradient were greater than 10 times the uptake in the absence of the [Na^+] gradient, we could expect good separation of the vesicles on the isopycnic sucrose gradient and consequent enrichment of the 70,000-dalton band. However, when the [Na^+]-gradient-dependent activity of the reconstituted vesicles was <10 times the activity observed in the absence of a [Na^+] gradient, no significant displacement of vesicles occurred. In addition, the total amount of activity was also important to obtain a successful separation; if the [Na^+]-gradient-dependent Ca^{2+}-uptake activity after reconstitution was <30 nmol of Ca^{2+} per mg of protein per 5 min, the separation obtained on the isopycnic sucrose gradient between the $^{45}Ca^{2+}$-containing peak and the phospholipid peak was rather poor. Another important finding of our early experiments was that high [Na^+]-gradient-dependent Ca^{2+} uptake activity and subsequent purification of the Na^+–Ca^{2+} antiporter could be obtained only by using purified brain phospholipids for reconstitution. Soy bean phospholipids (asolectin), purified egg yolk phosphatidylcholine, or a mixture of purified egg yolk phosphatidylcholine/phosphatidylethanolamine (1:1), did not make suitable preparations. In these cases, the [Na^+]-gradient-dependent Ca^{2+}-uptake activity after reconstitution was lower, and the [Na^+]-gradient-independent radioactive Ca^{2+} content was higher than when brain lipids were used.

Direct evidence that the vesicles that were displaced toward the denser region of the isopycnic sucrose gradient after [Na^+]-gradient-dependent Ca^{2+} loading are the vesicles that contain the [Na^+]-gradient-dependent Ca^{2+} transporter is obtained by recovery of an increased specific-uptake activity. This was done by resolubilizing the sucrose-gradient-purified transporter and measuring its activity after a second reconstitution step. The 128-fold increase in specific activity was obtained by displacing ≈0.8% of the protein and 1.3% of the phospholipid of Ca^{2+}-loaded reconstituted phospholipid vesicles (in a [Na^+]-gradient-dependent manner) to a denser region on an isopycnic sucrose gradient than the rest of the vesicles.

It is interesting that, starting with either calf or rat SPM, we purified a 70,000-Da protein that appears to be associated with [Na^+]-gradient-dependent Ca^{2+}-transport activity. Such a similarity in apparent molecular size and transport properties between two Ca^{2+}-transporting proteins purified from rat and calf brain synaptosomal vesicles has also been observed with the purified and reconstituted Ca^{2+}-transport ATPase (19). We do not know whether this enriched protein band is the Na^+–Ca^{2+} exchanger or if its appearance coincides with the denser region (below the major phospholipid peak) of the sucrose gradient of the reconstituted SPM vesicles loaded with $^{45}Ca^{2+}$ in a [Na^+]-gradient-dependent manner. But since only 0.8% of the total protein is found in the region where most of the [Na^+]-gradient-dependent Ca^{2+}-transport activity is found, this procedure is a powerful tool in the purification of the Na^+–Ca^{2+} exchanger, and it could prove to be valuable in preparing antibodies against Ca^{2+}-transporting protein from excitable membranes.

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1. Lüttgau, H. C. & Niedergerke, R. (1958) *J. Physiol. (London)* **143,** 486.
2. Reuter, H. & Seitz, N. (1968) *J. Physiol. (London)* **195,** 451–470.
3. Baker, P. F., Blaustein, M. P., Hodgkin, A. L. & Steinhardt, R. A. (1969) *J. Physiol. (London)* **200,** 431–458.
4. Blaustein, M. P. & Ector, A. C. (1976) *Biochim. Biophys. Acta* **419,** 295–308.
5. Mullins, L. J. (1979) Am. J. Physiol. **5,** c103–c110.
6. Mullins, L. J. (1981) *Ion Transport in Heart* (Raven, New York).
7. Rahamimoff, H. & Spanier, R. (1979) *FEBS Lett.* **104,** 111–114.
8. Miyamoto, H. & Racker, E. (1980) *J. Biol. Chem.* **255,** 2656–2658.
9. Schellenberg, G. C. & Swanson, P. D. (1982) *Biochim. Biophys. Acta* **690,** 133–144.
10. Wakabayashi, S. & Goshima, K. (1982) *Biochim. Biophys. Acta* **693,** 125–133.
11. Goldin, S. M. & Rhoden, V. (1978) *J. Biol. Chem.* **253,** 2575–2583.
12. Papazian, D., Rahamimoff, H. & Goldin, S. M. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 3708–3712.
13. Gasko, O. D., Knowles, A. F., Shertzer, H. G., Suolinna, E. H. & Racker, E. (1976) *Anal. Biochem.* **72,** 57–65.
14. Ames, B. (1966) *Methods Enzymol.* **8,** 115–118.
15. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
16. Sheltawy, A. & Dawson, R. M. C. (1969) *Chromatographic and Electrophoretic Techniques*, ed. Smith, I., Vol. 1, pp. 450–493.
17. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
18. Schaffner, W. & Weissmann, C. (1973) *Anal. Biochem.* **56,** 502–514.
19. Chan, S. Y., Hess, E. J., Rahamimoff, H. & Goldin, S. M. (1984) *J. Neurosci.* **4,** 1468–1478.