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Reconstitution and purification by "transport specificity fractionation" of an ATP-dependent calcium transport component from synaptosome-derived vesicles

(lipid vesicles/central nervous system/neurotransmitter release/presynaptic regulation)

DIANE PAPAZIAN*, HANNAH RAHAMIMOFF†, AND STANLEY M. GOLDIN‡

Department of ‡Pharmacology, 250 Longwood Avenue, and *Department of Biological Chemistry, 25 Shattuck Street, Harvard Medical School, Boston, Massachusetts 02115; and †Department of Biochemistry, Hadassah Medical School, Hebrew University, P.O. Box 1172, Jerusalem, Israel

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ABSTRACT **A synaptosomal ATP-dependent Ca uptake system was reconstituted into artificial vesicles by a cholate dialysis procedure using an 80-fold excess of exogenous phospholipid. Under these conditions, most of these vesicles would be expected to have only one or, at most, a few membrane proteins. The vesicles containing an ATP-dependent Ca transport system were purified from the bulk of the preparation on density gradients by increasing their density by the ATP-dependent intravesicular precipitation of Ca oxalate; a ≈100-fold purification resulted. The purified Ca-transporting vesicles contained two major protein components, of M_r 94,000 and 140,000 according to sodium dodecyl sulfate gel electrophoresis. These components are believed to be responsible for Ca transport in this synaptosome-derived membrane fraction.**

The Ca^{2+} concentration in nerve terminals is a primary factor in the control of neurotransmitter release (1). Action potentials arriving at the nerve terminal elicit an influx of Ca^{2+}; the increased concentration then triggers the exocytosis of neurotransmitter by an as yet undefined mechanism. Removal of Ca from the nerve terminal is then required to terminate the process of neurotransmitter release.

In contrast to our knowledge of regulation of Ca levels in muscle (2, 3), much less is known about control of it in nerve terminals. Ca is probably removed to some extent by mitochondria (4) and by extrusion across the plasma membrane of nerve terminals (5). Recently, several groups (6–8) have presented evidence for the existence of a nonmitochondrial, ATP-dependent Ca transport system derived from lysates of synaptosomes (pinched-off presynaptic nerve terminals). This transport system differs, in its kinetics and inhibition, from the Ca^{2+} ATPase of sarcoplasmic reticulum (9); it may be involved in sequestration of Ca within the nerve terminal. Identification and purification of this Ca transport system is a prerequisite for the use of immunocytochemical techniques to determine its precise location within the nerve terminal; its localization is critical to our understanding of the dynamics of Ca regulation of neurotransmitter release. Evidence for regulation of ATP-dependent Ca transport by an adenylate cyclase-mediated process has been obtained in cardiac muscle (10). Does such regulation of Ca transport occur in nerve terminals? Reconstitution of the synaptosomal Ca transport system into artificial phospholipid vesicles may form the basis of an assay system that can be used to detect such regulatory processes and to define their mode of action.

We report herein the successful reconstitution and purification of this synaptosomal Ca transport component. To purify the Ca transporter, we have used a new technique, "transport specificity fractionation," that has been developed for the purification of membrane transport proteins (11). This approach involves reconstitution of the transport system of interest into artificial vesicles before purification so as to insert only one or, at most, a few membrane proteins into each artificial vesicle. The transport properties of the protein of interest are then used as a physical tool (e.g., transport-specific changes in vesicle density are created) to separate vesicles containing this transport system from the rest of the crude preparation and thus result in its purification.

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METHODS

"Synaptosomal vesicles" were prepared from rat brain as described (7). Reconstitution was akin to the methods used for reconstitution of the purified sarcoplasmic reticulum Ca^{2+}-ATPase (12) and the purified Na^+,K^+-ATPase (13). Acetone-washed (14) soybean phospholipid (Asolectin, Associated Concentrates, Woodside, NY) was suspended to a final concentration of 20 mg/ml in sodium cholate (20 mg/ml)/0.4 M potassium phosphate/5 mM 2-mercaptoethanol/0.2 mM EDTA, pH 7.5. Synaptosomal vesicles were added to this solution as a concentrated suspension (>10 mg/ml protein) to the desired final protein concentration (0.25–1.0 mg/ml). After incubation for 2 min at 22°C, the rechilled mixture was dialyzed at 4°C in a hollow-fiber apparatus (13) against 500 ml of 300 mM K_2 oxalate/5 mM 2-mercaptoethanol, pH 7.8, per ml of mixture over a 12-hr period. The vesicles formed were then dialyzed against "low-oxalate buffer" (5 mM K_2 oxalate/700 mM glycerol/50 mM KCl/50 mM Tris/5 mM 2-mercaptoethanol) for 2 hr.

Ca-transporting reconstituted vesicles were prepared as above (final concentration, 0.25 mg of protein per ml) for transport specificity fractionation of the Ca transport system. Linear gradients were formed (in tubes for the Beckman VTI 50 rotor) from 18 ml of 5 mM K_2 oxalate/700 mM sucrose/50 mM KCl/50 mM Tris/5 mM 2-mercaptoethanol, pH 7.5 (heavy phase); and 16 ml of low-oxalate buffer (light phase). The gradient was layered over a 2-ml 50% sucrose cushion. Vesicles were incubated for 20 min at 23°C after $MgCl_2$ and $CaCl_2$ were added to concentrations of 5.5 mM and 0.1 mM, respectively, from concentrated stock solutions; vesicles were then rechilled, layered on top of the gradient (4 ml of vesicles per tube), and overlayered with about 1 ml of gradient light phase. The gradient was centrifuged for 1.5 hr at 50,000 rpm at 2°C; 1-ml fractions were collected by piercing the bottom of the tube. The main artificial vesicle peak consisted of four to six easily identifiable turbid fractions; the top three-fourths of these peak fractions were pooled and reincubated for 20 min at 23°C with 0.1 mM $CaCl_2$/5.5 mM $MgCl_2$/2 mM MgATP (MgATP was omitted from control preparations); the rechilled vesicle peak

was then diluted with an equal volume of low-oxalate buffer, overlayered onto a second identical gradient, and centrifuged as before. Fractions (1 ml) of the second gradient were collected and assayed for ATPase activity, ATP-dependent Ca transport activity, or ^{45}Ca incorporation during the second preincubation.

[γ-^{32}P]ATPase assays were performed as described (13). ^{45}Ca transport assays were performed by separation of intravesicular from extravesicular ^{45}Ca on ion exchange resin (15): 2-ml disposable columns of Dowex 50W-X2-200 (Tris form) were equilibrated with 700 mM glycerol/10 mM Tris, pH 7.5, and treated with 1 ml of bovine serum albumin (10 mg/ml) in the same buffer. Aliquots (50 μl) of the material to be assayed were incubated at 23°C in the presence of 5.5 mM $MgCl_2$/0.1 mM $^{45}CaCl_2$ (110,000 cpm/nmol) with or without 2 mM MgATP; 45 μl of material was then eluted from the column with 2 ml of glycerol/Tris at a flow rate of about 1.5 ml/min; 15 ml of Aquasol was added for scintillation counting. Preincubation of control portions of the reconstituted transport system for 20 min at 23°C with Ca and ATP as above resulted in irreversible ≈4-fold inhibition of the transport activity. This was shown by removal of external Ca and ATP from the preincubated vesicles on Sephadex G-50 M columns equilibrated with low-oxalate buffer, followed by reincubation of the void fractions containing the vesicles with the same concentrations of Mg, Ca (this time radiolabeled with ^{45}Ca), and ATP; the resulting Ca uptake rates were compared with those of control preparations. This inhibition is presumably due to chelation of incorporated unlabeled Ca, by intravesicular oxalate, decreasing the availability of oxalate for subsequent chelation of ^{45}Ca; for estimation of the recovery and purification of the transport activity, this inhibition was taken into account.

Electrophoresis in sodium dodecyl sulfate slab gels (1.6 mm thick; 10% acrylamide in the separating slab) was performed by the method of Laemmli (16). Material in regions of the gradient to be electrophoretically examined was first pelleted by centrifugation for 4 hr at 4°C and 40,000 rpm in a Beckman 50.2 Ti rotor. Pellets were resuspended in 35 μl of 1 mM EDTA saturated with phenylmethylsulfonyl fluoride, to inhibit proteolysis (17); lipid phosphate was determined (11) on aliquots of the pellets. Sample buffer (final sodium dodecyl sulfate concentration, 2%) was added from concentrate, and samples were incubated for 30 min at room temperature. Application of more than 0.6 mg of phospholipid per gel lane was avoided to prevent overloading. All crude vesicle fractions and molecular weight markers were treated with EDTA/phenylmethylsulfonyl fluoride prior to sodium dodecyl sulfate treatment.

Protein of the purified vesicle fractions was determined by the amido Schwarz procedure (18). When less sensitivity was required, the Lowry method was used.

RESULTS AND DISCUSSION

The cholate/hollow-fiber dialysis treatment of the synaptosomal vesicle fraction reproducibly resulted in a vesicle preparation (termed "reconstituted") that exhibited a marked ATP-dependent stimulation of Ca uptake (Fig. 1). Oxalate (a Ca-sequestering agent) was trapped in the vesicles to enhance the observed uptake. Adenosine 5'[β,γ-imido]triphosphate, a nonhydrolyzable analog of ATP that supports passive but not active ion transport in some systems (13, 19), did not significantly stimulate Ca uptake. There was marked enhancement of ATP-dependent Ca uptake relative to that of untreated "synaptosomal vesicles" [a mitochondria-depleted membrane fraction obtained from lysates of synaptosomes (7)] measured under the same conditions (Table 1); this enhancement, shown

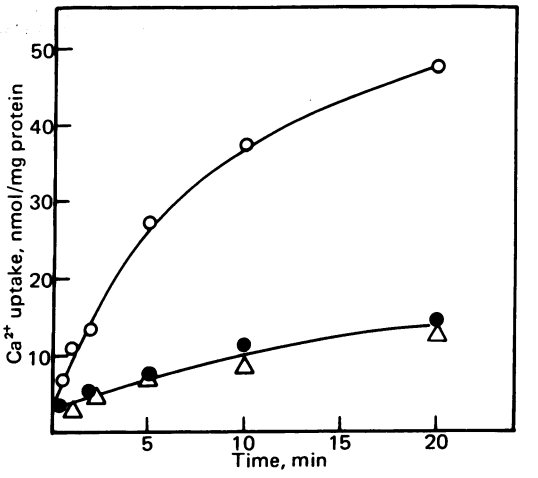

FIG. 1. Stimulation by ATP (○) of Ca uptake into artificial vesicles formed by exogenous lipid/cholate dialysis treatment ("reconstitution") of the synaptosomal vesicle fraction. △, Ca uptake in the absence of ATP; ●, Ca uptake in the presence of 2 mM Mg adenosine [β,γ-imido]triphosphate prepared as in ref. 14.

at an exogenous lipid/endogenous protein ratio of 76:1, was comparable to the level obtained when the cholate dialysis operation was performed at severalfold lower lipid/protein ratios (as in Fig. 1).

ATP hydrolysis was measured in parallel under the same incubation conditions used in the transport assay, to determine the ratio of Ca transport to ATP hydrolysis. Table 1 summarizes these data; untreated synaptosomal vesicles have a low Ca/ATP ratio when incubated in oxalate-free medium. Assay of the untreated synaptosomal vesicles in the same oxalate-containing medium used in measurements on the reconstituted preparation demonstrated a 30-fold increase in the Ca/ATP ratio; this was due both to increased resolvable ATP-dependent Ca transport and to decreased ATPase activity in the oxalate incubation medium. The reconstituted transport system exhibited a 15-fold higher Ca/ATP ratio than did the oxalate-incubated synaptosomal vesicles; the ATPase activity was about half that of the synaptosomal vesicles, perhaps because about half of the "reconstituted" ATP-hydrolyzing sites face the interior rather than the exterior of the artificial vesicle (13) and hence are inaccessible to external ATP.

Demonstration That the Ca Transport System Has Been Reconstituted into an Artificial Vesicle. The observed enhancement of ATP-dependent Ca uptake that resulted from the reconstitution treatment conceivably could be due to some kind of modification of the synaptosomal vesicles rather than to the actual insertion of the Ca transport system into an artificial lipid vesicle. To eliminate this ambiguity, the following observations were made. The lipid/cholate-treated preparation was eluted on a Bio-Gel A-150 m gel filtration column. The ATP-dependent Ca transport activity closely paralleled the elution profile of the artificial vesicles on this column (Fig. 2). As determined by the elution position on the column (11), the vesicles were of ≈550 Å mean diameter. Native synaptosomal vesicles and associated transport activity eluted in the void volume of the column. The lipid/cholate-treated preparation was subjected to isopycnic isoosmotic density gradient centrifugation (Fig. 3). Again, the position of ATP-dependent Ca uptake on the gradient precisely paralleled the gradient profile of the artificial vesicles ($\rho \approx 1.035$ g/cm^3; 50–75% recovery of transport activity). The Ca transport activity of the untreated synaptosomal vesicles sedimented to a position just above the 50% sucrose cushion on this gradient, as shown.

Table 1. Coupling of Ca uptake to ATPase activity

Preparation	ATPase activity, nmol/min/mg	ATP-dependent Ca^{2+} transport, nmol/min/mg	Ratio, Ca uptake/ ATP hydrolysis, mol/mol	Lipid/protein mass ratio
Synaptosomal vesicles	145	0.264	0.00189	0.66
Synaptosomal vesicles + 5 mM oxalate	20.3	2.5	0.06	0.66
Reconstituted transport system + 5 mM oxalate	8.56	8.1	0.95	76

These results, taken together, demonstrate that the synaptosome-derived Ca transport system has indeed been translocated into the unilamellar artificial vesicles of relatively homogeneous dimensions (13) that result from the cholate dialysis method. A method was then devised for separating specifically those vesicles containing the Ca transport system from the rest of the preparation.

Transport Specificity Fractionation of the Ca Transport System. The strategy for purification of the Ca-transporting vesicle fraction takes advantage of the observation that—as is the case with isolated skeletal sarcoplasmic reticulum (20)—oxalate greatly augments ATP-dependent Ca uptake by this synaptosomal transport system. This is presumably due to the formation of a calcium oxalate complex within the vesicle. The formation of this complex can be expected to significantly increase the density of those vesicles exhibiting active uptake of Ca and could enable their separation from the rest of the vesicle population on isopycnic density gradients.

To achieve this purification, a two-stage gradient separation procedure was devised (details in *Methods* section). Reconstituted vesicles with oxalate trapped inside were incubated first with Mg and Ca but without ATP. These vesicles were applied to a linear density gradient. This first gradient produced a well-defined major band of artificial lipid vesicles (as in Fig. 3) above a diffuse region of membrane fragments and other debris. The purpose of the first gradient was to remove this dense material which would interfere with the subsequent purification. The artificial vesicle peak was pooled, incubated in the presence of Ca, Mg, and ATP, and resedimented on a second, similar gradient. Fig. 4 shows the transport activity and lipid phosphate profiles on the second gradient. Inclusion of ATP in the incubation before the second gradient moved the peak of ATP-dependent Ca transport activity to a position of higher density; the density of the bulk of the artificial vesicles was unaffected. When the vesicles from the first gradient were incubated with ^{45}Ca (in the absence of ATP) before they were subjected to the second gradient step, a peak of ^{45}Ca was resolved coinciding with the position of the main vesicle peak (Fig. 5, arrow); when MgATP was included in this ^{45}Ca preincubation, a shoulder of ^{45}Ca appeared below the main vesicle peak. Preincubation of the vesicles with ATP before the second gradient centrifugation moved about half the ATPase activity from a position that coincided with the main vesicle peak to a new peak in the denser region of the gradient (Fig. 6).

The positions of the ATP-dependent shift in the peak of Ca transport and ATPase activities—and the ATP-dependent shoulder of ^{45}Ca incorporation—all coincide. Taken together,

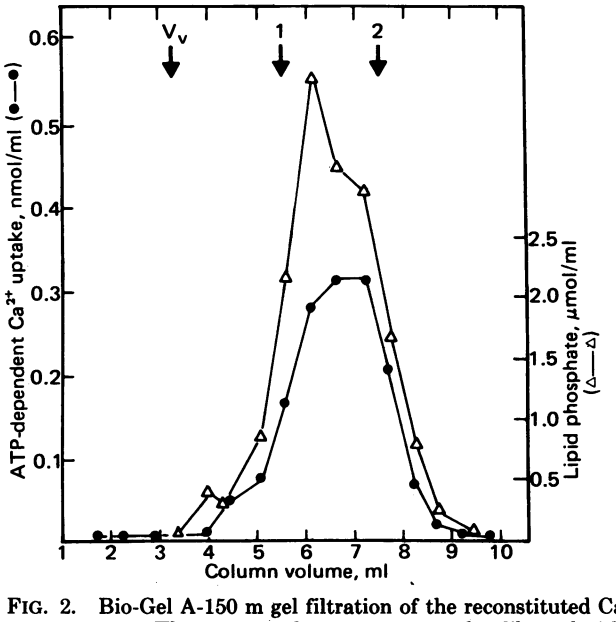

FIG. 2. Bio-Gel A-150 m gel filtration of the reconstituted Ca transport system. The agarose column was prepared, calibrated with uniform polystyrene latex spheres, and operated as described (12). The column fractions were assayed for lipid phosphate (△) and ATP-dependent Ca uptake (●). Recovery of phospholipid from the column was 94%; recovery of transport activity was 110%. Arrows denote void volume (V_v) and peak positions of 850 Å (arrow 1) and 380 Å (arrow 2) diameter uniform latex spheres on the column.

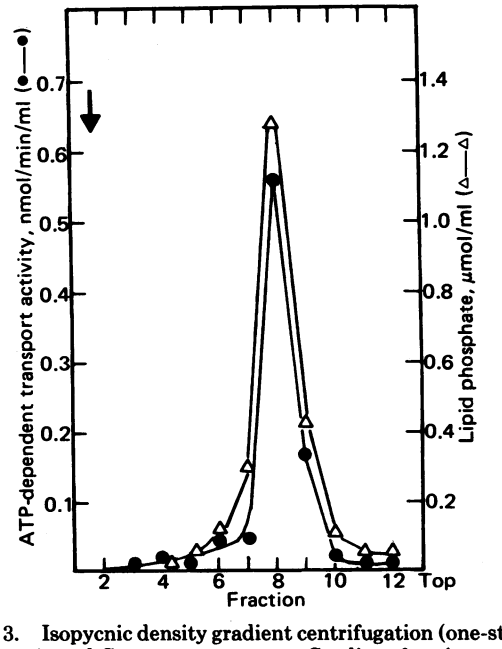

FIG. 3. Isopycnic density gradient centrifugation (one-stage) of the reconstituted Ca transport system. Gradient fractions were assayed for lipid phosphate (△) and ATP-dependent Ca uptake (●), 1 min at 23°C. The position of the single peak of ATP-dependent Ca transport activity associated with the untreated synaptosomal vesicle fraction on a parallel, identical gradient is shown by the vertical arrow. Recovery of transport activity was 52%.

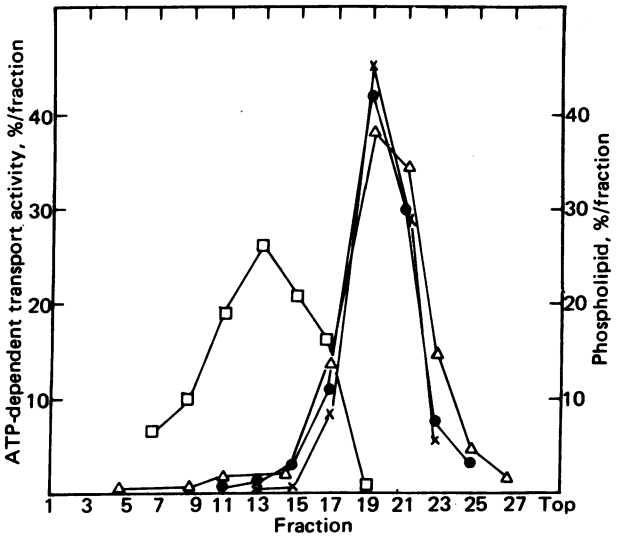

FIG. 4. Results of the two-stage gradient purification of the reconstituted Ca transport system. Before being placed on the second gradient, the vesicle peak obtained from the first gradient was preincubated with Mg, Ca, and MgATP; the positions of phospholipid (●) and ATP-dependent Ca uptake (□), assayed for 5 min at 23°C, were determined on the second gradient. A control preparation exposed to Mg and Ca but not ATP before the second gradient step exhibited coinciding phospholipid (x) and ATP-dependent Ca uptake (△) profiles.

these results clearly demonstrate that the density of a small fraction of the artificial vesicles (less than 1% by lipid phosphate determination) is increased owing to ATP-dependent, oxalate-enhanced incorporation of Ca; this permits that vesicle fraction to be separated from the bulk of the reconstituted preparation on density gradients.

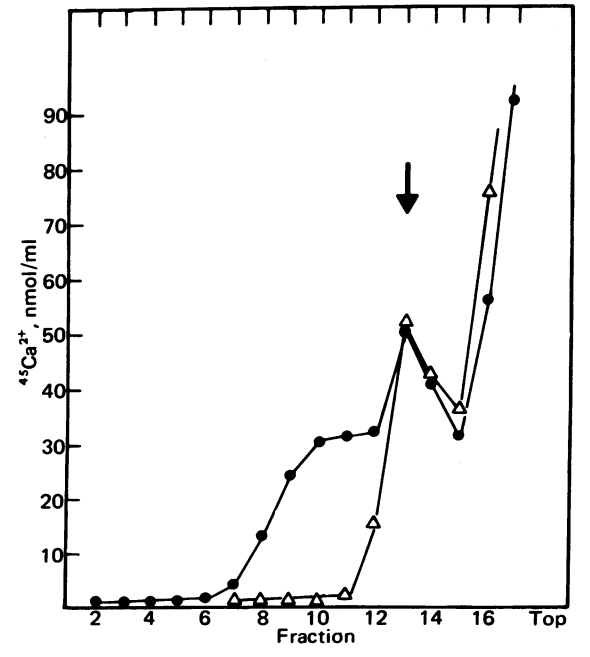

FIG. 5. Distribution of ^{45}Ca within vesicles on the second gradient. Before being placed on the second gradient, vesicles were preincubated with $MgCl_2$ and 0.1 mM ^{45}Ca (110,000 cpm/nmol) in the presence (●) or absence (△) of 2 mM MgATP. ^{45}Ca content of gradient fractions was then determined. Procedure was otherwise identical to that described in *Methods* and the legend to Fig. 4. Arrow, position of the main artificial vesicle peak in both gradients.

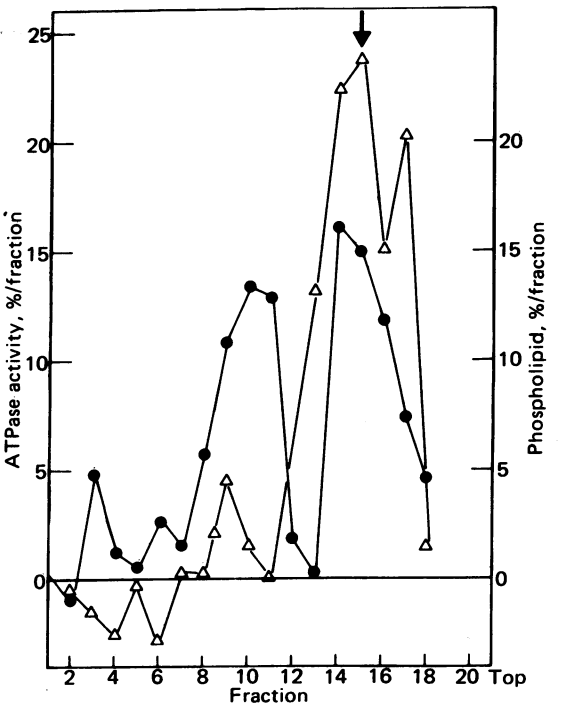

FIG. 6. ATPase activity distribution on the second gradient. Before being placed on the gradient, the vesicles were preincubated in the presence (●) or absence (△) of MgATP. Recovery of ATPase activity was 32–39%. Incubation conditions for the ATPase assay were 20 min at 23°C. Arrow, position of the main artificial vesicle peak on both gradients.

Degree of Purification of Transport System. The lower two-thirds of the gradient peak of the purified Ca-transporting vesicle fraction typically had 2.4 μg of protein (of 1.0 mg of starting material) with a specific ATPase activity (at 23°C) of 0.5 μmol/mg per min. This activity is about the same as that of efficiently reconstituted, purified sarcoplasmic reticulum Ca^{2+}-ATPase (21). Thus, in terms of enrichment of specific activity over that of the initial reconstituted preparation (Table 1), a 60-fold purification was achieved. This degree of purification is a minimal estimate because it assumes that all the ATPase activity in the original preparation is potentially capable of Ca transport. Considered in terms of purification of actual ATP-dependent Ca-transport activity, a 140-fold purification has resulted. In terms of ATPase activity and transport activity, the yield of purified vesicles was 14 and 33%, respectively. ATP-dependent incorporation of vesicle phospholipid into this region of the gradient was only 0.6% of the total phospholipid.

Laemmli sodium dodecyl sulfate gel electrophoresis of the purified Ca-transporting vesicle fraction (Fig. 7) reproducibly showed that two components of the original synaptosomal vesicle fraction, of apparent M_r 94,000 and 140,000, had been purified. Two-step gradient procedures were performed without preincubation with ATP; material from the region of the control second gradient that corresponded to the position of the purified vesicle fraction was subjected to gel electrophoresis. No significant amount of the 94,000 or 140,000 M_r peptides was present, but there was some lower molecular weight material. This demonstrates that the small amount of this lower molecular weight protein in the purified Ca-transporting vesicle fraction is most likely a contaminant not associated with the transport function, and that ATP is specifically required to cause the two polypeptides (M_r 140,000 and 94,000) to appear in the denser region of the gradient; this is strong

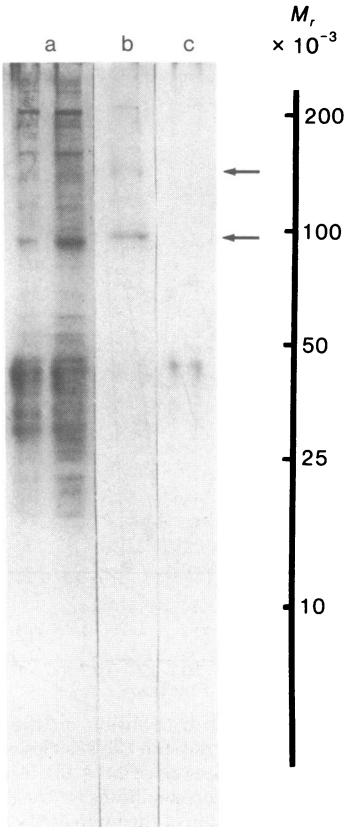

FIG. 7. Coomassie blue-stained sodium dodecyl sulfate slab gels of the crude synaptosomal vesicle fraction (lane a: right, 12 μg of protein; left, 6 μg) and of purified Ca-transporting vesicle fraction (lane b)—material from the region of the second gradient step containing the lower two-thirds of the peak of purified Ca transport activity (fractions 8–14 of curve □ in Fig. 4). One-half of the material in these fractions (≈1.2 μg of protein; 0.45 mg of phospholipid) was applied to the gel. Lane c was material obtained from fractions 8–14 of the control second gradient (△ in Fig. 4); the sample was layered on the second gradient after preincubation in the absence of ATP. All of the material in these fractions (containing 0.15 mg of phospholipid) was applied to the gel. Arrows denote positions of 140,000 and 94,000 M_r components in lane b. A third component in lane b at M_r 200,000 did not appear consistently and may be a dimer of the 94,000 M_r component.

evidence that the two polypeptides are components of the Ca transport system(s). The gels of the crude synaptosomal vesicles reveal a major protein band (several percent of the total protein) of mobility similar to that of the purified 94,000 M_r polypeptide; considering the estimated ≈100-fold purification, it appears that the purified 94,000 M_r component may be only a fraction of the protein of this electrophoretic mobility. It has not escaped our attention that 140,000 is approximately the apparent M_r of the Ca-dependent ATPase in human erythrocytes as determined by the phosphorylation studies of Drickamer (22) and that 94,000 is approximately the M_r of the sarcoplasmic reticulum Ca^{2+}-ATPase (9). Further investigation is required to elucidate the relationship of the two peptides to one another: are they both part of the same Ca transport system, or have we copurified two distinct Ca transport proteins from separate regions of the nerve terminal?

The successful application of the transport specificity fractionation technique to the purification of a class of ion transport proteins has been reported here. It is hoped that this approach may be of general utility for the purification of other ion transport proteins—to our knowledge, no comparable degree of purification of any ion transport protein from the central nervous system has yet been demonstrated. Because preservation of transport activity is *required* for the viability of this approach, the ambiguities regarding the relationship of ligand binding to transport function that have been encountered [e.g., in studies of the purified acetylcholine receptor (discussed in ref. 23)] are avoided.

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