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PARTIAL PURIFICATION AND FUNCTIONAL IDENTIFICATION OF A CALMODULIN-ACTIVATED, ADENOSINE 5′-TRIPHOSPHATE-DEPENDENT CALCIUM PUMP FROM SYNAPTIC PLASMA MEMBRANES[1]

DIANE M. PAPAZIAN,* HANNAH RAHAMIMOFF,‡ AND STANLEY M. GOLDIN§[2]

*Departments of *Biological Chemistry and §Pharmacology, Harvard Medical School, Boston, Massachusetts 02115 and ‡Department of Biochemistry, Hadassah Medical School, Hebrew University, Jerusalem, Israel*

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Abstract

Synaptic plasma membranes isolated from rat brain contain a calmodulin-activated Ca^{2+} pump. It has been purified 80- to 160-fold by solubilization with Triton X-100 and affinity chromatography on a calmodulin-Sepharose 4B column. After reconstitution into phospholipid vesicles, the affinity-purified pump efficiently catalyzed ATP dependent Ca^{2+} transport, which was activated 7- to 9-fold by calmodulin.

The major protein component of the affinity-purified preparation had a M_r = 140,000; it was virtually the only band visualized on a Coomassie blue-stained SDS polyacrylamide gel. It has been identified as the Ca^{2+} pump by two functional criteria. First, it was phosphorylated by [γ-^{32}P]ATP in a Ca^{2+}-dependent manner; the phosphorylated protein had the chemical reactivity of an acyl phosphate, characteristic of the phosphorylated intermediates of ion-transporting ATPases. Second, the protein was enriched by transport-specific fractionation, a density gradient procedure which uses the transport properties of the reconstituted Ca^{2+} pump as a physical tool for its purification.

By analogy with calmodulin-activated (Ca^{2+} + Mg^{2+}) ATPases of other cell types, and because of its presence in a synaptic plasma membrane fraction, we hypothesize that the calmodulin-activated Ca^{2+} pump functions *in vivo* to extrude Ca^{2+} from nerve terminals.

Neurotransmitter release from nerve terminals is controlled by the intracellular concentration of Ca^{2+}. When a nerve terminal is depolarized by an invading action potential, voltage-sensitive Ca^{2+} channels open, allowing an influx of Ca^{2+}. In response, neurotransmitter release, believed to occur by exocytosis of synaptic vesicles, is triggered by an unknown mechanism. Release is terminated when the cytoplasmic concentration of Ca^{2+} is reduced to the resting level (reviewed by Kelly et al., 1979).

Several mechanisms have been proposed to act in concert to reduce the intracellular Ca^{2+} concentration. Calcium may be accumulated by mitochondria (Bygrave, 1977). Several groups (Blitz et al., 1977; Blaustein et al., 1978a, b; Rahamimoff and Abramovitz, 1978a, b) have reported the existence of a nonmitochondrial, ATP-dependent Ca^{2+} transport system in synaptosomal lysates. This transport system may sequester Ca^{2+} in organelles within the nerve terminal; synaptic vesicles (Israël et al., 1980; Michaelson et al., 1980), coated vesicles (Blitz et al., 1977), and smooth endoplasmic reticulum (Blaustein et al., 1978a, b; McGraw et al., 1980) have been suggested as possible sites for ATP-dependent Ca^{2+} sequestration. Ca^{2+} may be extruded across the plasma membrane of the nerve terminal by Na^+/Ca^{2+} exchange (Rahamimoff and Spanier, 1979; Gill et al., 1981; Michaelis and Michaelis, 1981) and by an ATP-dependent transport system (Gill et al., 1981).

This report describes the partial purification and functional identification of an ATP-dependent Ca^{2+} pump from synaptic plasma membranes. The pump is activated by calmodulin, a Ca^{2+}-binding protein which regulates several enzymes (Klee et al., 1980), including the erythrocyte Ca^{2+} pump (Jarrett and Kyte, 1979; Niggli et al., 1979) and (Ca^{2+} + Mg^{2+}) ATPases in other cell types (Pershadsingh et al., 1980; Lew and Stossel, 1980; Caroni and Carafoli, 1981; Lichtman et al., 1981). The synaptic plasma membrane pump may function *in vivo* to extrude Ca^{2+} from nerve terminals. A preliminary account of this work has appeared (Papazian et al., 1982).

Material and Methods

Asolectin, a mixture of soybean phospholipids, was obtained from Associated Concentrates (Woodside, NY), stored in a refrigerated desiccator, and used for reconstitutions without any pretreatment. Partially purified phosphatidylcholine (85 to 90% by thin layer chromatography) was prepared from fresh egg yolks by extraction with chloroform-methanol and repeated acetone precipitations from di-

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[2] To whom correspondence should be addressed.

ethylether, similarly to the procedure of Litman (1973). Alternatively, highly purified phosphatidylcholine (99%) was obtained from Avanti Biochemicals (Birmingham, AL). $^{45}CaCl_2$ and [γ-^{32}P]ATP (3000 Ci/nmol) were obtained from New England Nuclear (Boston, MA).

Preparation of synaptic plasma membranes, calmodulin, and calmodulin-Sepharose 4B. Rat brain synaptosomes were prepared essentially by the procedure of Cotman and Matthews (1971), with 0.3 M mannitol, 1 mM EDTA, pH 7.3, employed in the homogenization medium and Ficoll gradient solutions in lieu of 0.32 M sucrose. The 7.5/13% Ficoll interface, previously shown by these investigators to be highly enriched in pinched off nerve terminals, was osmotically lysed (Rahamimoff and Abramovitz, 1978a), and a fraction enriched in synaptic plasma membranes was prepared according to the method of Rahamimoff and Spanier (1979). (These lysis and differential centrifugation procedures are similar to those used by Cotman and Matthews (1971) to prepare crude synaptic plasma membranes.) This fraction was found to possess less than 10% of the α-ketoglutarate dehydrogenase activity of the brain mitochondria-enriched fraction prepared by the method of Haldar (1971).

Toluene sulfonyl fluoride (0.1 mM) was added to all solutions to inhibit proteolysis. The synaptic plasma membranes were stored frozen at −80°C at a protein concentration of 7 to 15 mg/ml in 0.3 M mannitol, 0.1 mM toluene sulfonyl fluoride.

Calmodulin was purified from human erythrocytes by the procedure of Jarrett and Penniston (1978), omitting the last ammonium sulfate precipitation step.

Calmodulin was coupled to Sepharose 4B as follows. Cyanogen bromide-activated Sepharose 4B (Pharmacia, Piscataway, NJ) was swollen in 1 mM HCl and washed once with cold coupling buffer (0.1 M sodium bicarbonate, 0.5 M sodium chloride, pH 8.3). Calmodulin (1 mg dissolved in coupling buffer) was added to 1 ml of swollen, settled CNBr-Sepharose 4B. $CaCl_2$ was added to a final concentration of 50 μM. The reaction volume was brought to 3 ml with coupling buffer. The mixture was agitated gently at 4°C overnight. The resin was allowed to settle, and the reaction supernatant was carefully removed and assayed for remaining calmodulin content by its ability to stimulate the erythrocyte (Ca^{2+} + Mg^{2+}) ATPase (Jarrett and Penniston, 1978). As assayed by depletion of calmodulin from the supernatant, the efficiency of coupling of calmodulin ranged from 83 to 98%. After removal of the supernatant, 2 ml of 1 M ethanolamine, pH 9, were added, and the mixture was gently agitated at room temperature for 2 hr. The calmodulin-Sepharose 4B was alternately washed three times with coupling buffer and 0.1 M sodium acetate, 0.5 M sodium chloride, pH 4, and poured into a small column. Between uses, the column was washed and stored in 50 mM Tris HCl, pH 7.4, containig 0.02% sodium azide and 50 μM $CaCl_2$.

Purification of the Ca^{2+} pump by affinity chromatography on a calmodulin-Sepharose 4B column. Some aspects of the purification procedure have been adapted from the procedure used by Niggli et al. (1979) to purify the erythrocyte (Ca^{2+} + Mg^{2+}) ATPase. All steps were carried out at 4°C. Synaptic plasma membranes (16 mg of protein) were washed three times with 80 ml of 10 mM Tris-HCl, 1 mM EGTA, 1 mM 2-mercaptoethanol, 0.1 mM toluene sulfonyl fluoride, pH 7.4, by centrifuging at 21,000 × g_{av} for 20 min. The final pellet was resuspended and solubilized in 4 ml of 0.4% Triton X-100 (w/v), 130 mM KCl, 0.5 mM $MgCl_2$, 1 mM EGTA, 2 mM dithiothreitol, 20 mM HEPES, pH 7.4. It was incubated for 10 to 15 min at 4°C with sporadic, vigorous vortexing. Acetone-washed asolectin (Kagawa and Racker, 1971) was added to a concentration of 1 mg/ml. The insoluble material was removed by centrifugation at 50,000 × g for 30 min. The supernatant was concentrated by applying it to 0.8 gm of dry DEAE-Sephadex A25 packed in a 3-ml disposable syringe blocked with filter paper or nylon mesh. After absorption, the sample was equilibrated with the resin for 5 min. Calmodulin present in the sample binds to DEAE-Sephadex at this ionic strength (Jarrett and Penniston, 1978). The calmodulin-depleted, concentrated sample was recovered from the DEAE-Sephadex by placing the syringe in a 13 × 100-mm Pyrex test tube, which was spun at top speed for 3 min in a clinical centrifuge. The concentrate was applied to a Sephadex G75 column (1.0 × 18 cm) and eluted with "G75 buffer": 130 mM KCl, 1 mM $MgCl_2$, 0.05% Triton X-100 (w/v), 0.5 mg/ml of asolectin, 40 μM toluene sulfonyl fluoride, 2 mM dithiothreitol, 20 mM HEPES, pH 7.4 (sonicated to clarity). The void volume peak (determined by monitoring absorbance at 280 nm) was pooled and concentrated with 0.25 gm of dry, coarse Sephadex G25/ml, as described above but without a period for equilibration. $CaCl_2$ was added to a final concentration of 0.1 mM. The sample was applied to the calmodulin-Sepharose column equilibrated with G75 buffer containing 0.1 mM $CaCl_2$. The absorbance of the eluant at 280 nm was monitored, and the column was washed extensively until no more material absorbing at 280 nm eluted. The buffer was then switched to G75 buffer containing 2 mM EGTA in place of 0.1 mM $CaCl_2$, and proteins binding to the column in a Ca^{2+}-dependent manner were eluted. Asolectin (0.5 mg/ml) was added to stabilize the Ca^{2+} pump. The purified preparation was used without further treatment or was concentrated with dry Sephadex G25, and/or treated with Biobeads SM-2, as described below.

Reconstitution procedures. Synaptic plasma membranes and the affinity-purified preparation were reconstituted in asolectin or phosphatidylcholine by procedures adapted from those of Papazian et al. (1979). Samples were solubilized by the detergent sodium cholate, in the presence of an excess of exogenous phospholipid. Subsequent removal of the detergent by hollow fiber dialysis (Goldin, 1977) resulted in the formation of unilamellar vesicles, among which the proteins of the sample have been distributed.

Synaptic plasma membranes were reconstituted in asolectin at a final protein concentration of 0.25 mg/ml with the following minor modifications of the originally published procedure (Papazian et al., 1979). Asolectin was used in the solubilization buffer without any pretreatment. The lipid phosphate content of the asolectin was determined (Goldin, 1977), and, using a molecular weight of 750 gm/mol of lipid phosphate, the concentration of asolectin in the solubilization buffer was adjusted as required. The composition of the initial dialysis buffer was 300 mM oxalate, 10 mM Tris, 5 mM 2-mercaptoethanol, pH 7.8. The composition of "low oxalate buffer," the second dialysis buffer, was 5 mM oxalate, 25 mM KCl, 25 mM ammonium acetate, 50 mM Tris, 5 mM 2-mercaptoethanol, pH 7.5, containing 700 mM glycerol.

Synaptic plasma membranes were reconstituted in phosphatidylcholine at a final protein concentration of 0.25 mg/ml by a similar procedure. The membranes were solubilized in 15 mg/ml of cholate, 20 mg/ml of phosphatidylcholine, 0.4 M potassium phosphate, 5 mM 2-mercaptoethanol, pH 7.5. The mixture was mixed, incubated for 2 min at 24°C, rechilled, and dialyzed overnight at 4°C in a hollow fiber apparatus against 0.5 to 1 liter of 200 mM potassium oxalate, 10 mM Tris, 5 mM 2-mercaptoethanol, pH 7.5. The vesicles were then dialyzed against 500 ml of low oxalate buffer containing 300 mM glycerol.

Before reconstitution of the protein purified by calmodulin affinity chromatography, it was treated overnight with moderate agitation at 4°C with 0.3 gm of Biobeads SM-2/ml to remove Triton X-100 (Holloway, 1973). During the Biobead treatment, 50 μM $CaCl_2$, 2 mM $MgCl_2$, and 0.5 to 1.5 mg/ml of asolectin were also present. The sample was concentrated to 0.5 ml with dry Sephadex G25 (0.25 to 0.3 gm/ml of sample). The concentrated sample (0.5 ml) was added to 1 ml of 30 mg/ml of asolectin, 19.6 mg/ml of cholate, 0.6 M potassium phosphate, 7.5 mM 2-mercaptoethanol, pH 7.5, or, alternatively, to 1 ml of 30 mg/ml of phosphatidylcholine, 22.5 mg/ml of cholate, 0.6 M potassium phosphate, 7.5 mM 2-mercaptoethanol, pH 7.5, mixed, incubated for 2 min at 4°C, and dialyzed as described above.

The BioRad (Richmond, CA) hollow fiber "biofiber" units which were used for reconstitutions are no longer commercially available, but we have successfully substituted Vitafiber Artificial Capillary units (Amicon Corp., Lexington, MA) after preliminary treatment with a buffer containing 10 mg/ml of BSA.

ATP-dependent Ca^{2+} transport and Ca^{2+}-stimulated ATPase assays. ATP-dependent Ca^{2+} transport activity was determined by comparing the amount of vesicle-bound $^{45}Ca^{2+}$ in the presence and absence of ATP. Samples were incubated in low oxalate buffer containing 5.5 mM $MgCl_2$, 0.1 mM $^{45}CaCl_2$ (1.5 × 10^5 cpm/nmol), with or without 2.0 mM MgATP. Free $^{45}Ca^{2+}$ was separated from bound $^{45}Ca^{2+}$ on small Dowex ion exchange columns, as described by Papazian et al. (1979). The buffers used to equilibrate and elute the Dowex columns contained concentrations of glucose sufficient to match the osmolarity of the sample being assayed.

Vesicles reconstituted with synaptic plasma membrane protein contained endogenous calmodulin, which was removed as follows. EGTA was added to a final concentration of 5 mM to the reconstituted vesicles in low oxalate buffer. After 30 min at 4°C the sample was subjected to gel filtration on a 10-ml Sepharose 4B column equilibrated and eluted with low oxalate buffer containing 0.5 mM EGTA. Turbid fractions containing calmodulin-depleted reconstituted vesicles were pooled and dialyzed against EGTA-free low oxalate buffer in a hollow fiber apparatus for 1 hr. To determine the effect of calmodulin on Ca^{2+} transport,

calmodulin (0.2 μM) was added to the vesicles for 5 min at 4°C before proceeding with the transport assay by adding $^{45}CaCl_2$ and $MgCl_2$, with or without Mg ATP and transferring the reaction mixture to a 24°C bath. Phosphatidylcholine vesicles containing the affinity-purified Ca^{2+} pump did not contain a significant amount of endogenous calmodulin; therefore, no treatment was needed before determining the effect of exogenous calmodulin, as described above.

ATPase activity was determined in the presence of Ca^{2+}, Mg^{2+}, and EGTA and compared to that in the presence of Mg^{2+} and EGTA (the net difference was Ca^{2+}-stimulated ATPase activity). To measure ATPase activity in a "Ca^{2+}-free" medium, we found it necessary to include EGTA, so the ATPase assays were performed in low oxalate buffer containing 5.5 mM $MgCl_2$, 2.0 mM Mg ATP, 0.1 mM EGTA, 0.1 mM strophanthidin, with or without 0.2 mM $CaCl_2$, in a final volume of 0.5 ml. ATPase activities obtained with these conditions were directly comparable to transport activities determined in parallel as described above: control experiments indicated that ATP-dependent Ca^{2+} transport activity was the same when assayed either in the presence of 0.1 mM EGTA, 0.1 mM strophanthidin, and 0.2 mM $CaCl_2$, or using the standard assay conditions. Except for ATP, all components, including calmodulin (0.4 μM) where indicated, were pre-incubated on ice for 15 min before starting the reaction by adding ATP and transferring the reaction mixture to a 24°C bath. The ATPase reaction was quenched, and inorganic phosphate was determined as described by Jarrett and Penniston (1978). Reconstituted vesicles and affinity-purified material were assayed using [γ-^{32}P]ATP (5000 cpm/nmol), in which case the reaction volume was reduced to 0.1 ml, and $^{32}P_i$ was determined as described by Goldin (1977).

Before determining the effect of calmodulin on the Ca^{2+}-stimulated ATPase activity of synaptic plasma membranes, the membranes were depleted of endogenous calmodulin by washing by centrifugation at 21,000 × g_{av} for 20 min (4°C) three times in 0.3 M mannitol, 10 mM Tris, 1 mM EGTA, 1 mM 2-mercaptoethanol, 0.1 mM toluene sulfonyl fluoride, pH 7.4, and once in the same solution lacking EGTA.

Phosphorylation of the affinity-purified Ca^{2+}-stimulated ATPase. Biobead-treated, affinity-purified protein in G75 buffer (10 to 15 μg of protein in a final volume of 250 μl) was incubated for 15 sec at 4°C in the presence of 12 μM $MgCl_2$, 1.6 mM EGTA (final concentration), 2 μM [γ-^{32}P]ATP (5 to 10 Ci/mmol) with or without 2.3 mM $CaCl_2$. Bovine serum albumin (1 mg) was added from a concentrated solution, and the phosphorylation reaction was immediately quenched by the addition of 5 ml of ice-cold 5% trichloroacetic acid, 2.5 mM ATP, 5 mM phosphate. The precipitated protein was pelleted by centrifuging at top speed in a clinical centrifuge for 10 min at 4°C. The pellet was washed twice more with the trichloroacetic acid solution. Solubilization of the pellet in SDS at pH 2.4 and electrophoresis at pH 2.4 and 15°C were performed according to the procedures of Avruch and Fairbanks (1972) and Drickamer (1975). Approximately 1 μg of affinity-purified protein was applied to each gel. The gels were sliced into 2-mm slices, which were soaked for 24 hr in 1 ml of 25 mM Tris base, 0.5% SDS, before adding 8 ml of Hydrofluor (National Diagnostics, Somerville, NJ) for scintillation counting. To determine the reactivity of the incorporated phosphate with hydroxylamine, the purified material was phosphorylated in the presence of Ca^{2+}, pelleted once, and then resuspended in 1 ml of 0.8 mM hydroxylamine, 0.1 mM sodium acetate, pH 5.4, and incubated for 10 min at 30°C. After the incubation, the solution was chilled, 5 ml of the trichloroacetic acid solution were added, and the procedure was continued as usual. In control experiments, 0.8 M NaCl replaced hydroxylamine; under these conditions 50% of the phosphate incorporated in the presence of Ca^{2+} survived the incubation. Other experiments showed that the hydroxylamine step did not affect the efficiency of protein pelleting.

Transport-specific fractionation of the affinity-purified Ca^{2+} pump. The affinity-purified preparation was reconstituted in asolectin, as described above. Transport-specific fractionation was performed as previously described (Papazian et al., 1979), except that a Beckman VTi 65 rotor was used, and gradient and sample volumes were scaled down accordingly by a factor of about 7. The gradients were centrifuged at 50,000 rpm for 60 min at 4°C.

Other procedures. Electrophoresis in SDS slab gels (6.5% acrylamide separating gel, 4% acrylamide stacking gel, 1.6 mm thick) was performed by the method of Laemmli (1970). Before electrophoresis, affinity-purified protein, aggregated by Triton X-100 removal using Bio-beads SM-2, was pelleted by centrifugation at 200,000 × g for 3 hr at 4°C. Pellets were resuspended, and other samples were prepared as previously described (Papazian et al., 1979). Silver staining of SDS gels was performed as described by Oakley et al. (1980).

Protein determination was by the method of Lowry (1951) as modified by Goldin (1977) and Bensadoun and Weinstein (1976). Alternatively, protein was determined by the amido black method (Schaffner and Weissmann, 1973). Protein determinations were corrected for interference by asolectin or phosphatidylcholine present in the samples. Lipid phosphate was determined as previously described (Goldin, 1977).

Results

Demonstration of calmodulin-activated Ca^{2+} transport and ATPase activities in synaptic plasma membranes. Synaptic plasma membranes were prepared from osmotically lysed synaptosomes. The membranes catalyzed Ca^{2+}-stimulated ATP hydrolysis, which was about 30% of the total ATPase activity (Table I). The mean specific Ca^{2+}-stimulated ATPase activity of nine different synaptic plasma membrane preparations at 24°C was 30.8 (SEM 20.7) nmol/mg/min. After the synaptic plasma membranes were washed repeatedly with a buffer containing EGTA to remove endogenous calmodulin, the Ca^{2+}-stimulated ATPase activity was activated about 2-fold by added calmodulin (0.4 μM). Similar results have been reported previously by others (Sobue et al., 1979; Sorensen and Mahler, 1981).

Ca^{2+}-stimulated ATPase activities in membrane fractions from other sources have been implicated in ATP-dependent Ca^{2+} transport (MacLennan and Holland, 1975; Niggli et al., 1981b). Synaptic plasma membranes, incubated with MgATP and $^{45}CaCl_2$, accumulated Ca^{2+} slowly, with a typical rate of about 0.1 nmol/mg/min at 24°C. Transport was extremely inefficient: from 0.001 to 0.003 Ca^{2+} ion was taken up/ATP molecule hydrolyzed. Since isolated membrane fractions may be improperly oriented or too leaky to take up and retain ions, solubilization and reconstitution of the putative ion pump were necessary to demonstrate clearly the transport activity. The reconstitution procedure used here, which has been described in detail elsewhere (Papazian et al., 1979), distributes the membrane proteins among tightly sealed vesicles composed of exogenous phospholipids.

Reconstitution of the synaptic plasma membranes in soybean phospholipids (asolectin) dramatically enhanced the rate and efficiency of ATP-dependent Ca^{2+} transport. After reconstitution, the ATP-dependent Ca^{2+} transport activity of 12 different reconstituted synaptic plasma membrane preparations at 24°C was 18.7 (SEM 4.9) nmol/mg/min. The initial rates of ATP-dependent transport, (Ca^{2+} + Mg^{2+}) ATPase, and Ca^{2+}-stimulated ATPase activities at 24°C of a representative reconstituted preparation are given in Tables I and II. Comparison of the rates indicates that the ratio of Ca^{2+} transport to Ca^{2+}-stimulated ATP hydrolysis for the preparation was 1.5. Coupling ratios of 1 or 2 have been proposed for other ATP-dependent Ca^{2+} pumps (MacLennan and Holland, 1975; Niggli et al., 1981b).

Negatively charged phospholipids, including some present in asolectin, have been shown to substitute for calmodulin in activating the erythrocyte Ca^{2+} pump (Niggli et al., 1981a, b). Similarly, the synaptic plasma membrane Ca^{2+}-stimulated ATPase activity was not further activated by calmodulin in the presence of asolectin (data not shown). To determine whether calmodulin activates the synaptic plasma membrane Ca^{2+} transport activity, it was necessary to reconstitute the activity in phosphatidylcholine, a neutral phospholipid. The resulting vesicles transported Ca^{2+} as actively as asolectin vesicles (Table II). After removal of calmodulin from the phosphatidylcholine vesicles by Sepharose 4B gel filtration in the presence of EGTA, the specific activity of transport was reduced. Readdition of calmodulin (0.2 μM) activated transport about 2.5-fold, with virtually 100% recovery of the original specific activity.

TABLE I

The Ca^{2+}-stimulated ATPase activity of synaptic plasma membranes

The ATPase activity of synaptic plasma membranes or reconstituted vesicles was assayed in low oxalate buffer in the presence of 5.5 mM $MgCl_2$ and 0.1 mM EGTA (Mg^{2+} ATPase), or 5.5 mM $MgCl_2$, 0.1 mM EGTA, and 0.2 mM $CaCl_2$ ((Ca^{2+} + Mg^{2+}) ATPase). Ca^{2+}-stimulated ATPase was the difference between the two. Reaction mixtures contained 50 to 150 μg of membrane protein and were incubated for 20 min at 24°C. The values shown are the averages of two or three determinations. Replicates varied by less than 7%.

	Mg^{2+} ATPase	(Ca^{2+} + Mg^{2+}) ATPase	Ca^{2+}-stimulated ATPase
		nmol/mg/min	
Synaptic plasma membranes	53.9	77.1	23.2[a,b]
EGTA-washed synaptic plasma membranes	67.5[c]	83.0[c]	15.5
EGTA-washed synaptic plasma membranes + calmodulin	62.0	97.5	35.5
Synaptic plasma membranes reconstituted in asolectin	23.1	34.2	11.1

[a] Using these assay conditions, the Ca^{2+}-stimulated component of ATP hydrolysis was about 30% of the total ATPase activity. This, as percentage of total ATPase, was reduced if the conditions of Jarrett and Penniston (1978) for assaying the erythrocyte (Ca^{2+} + Mg^{2+}) ATPase were used (at 24°C) instead, due to an increase in the Mg^{2+} ATPase activity. (The net Ca^{2+}-stimulated ATPase activity was unchanged.)

[b] The Ca^{2+}-stimulated ATPase activity of untreated synpatic plasma membranes was not activated by exogenous calmodulin.

[c] The increase in specific activity seen after washing with EGTA may have been due to the selective loss of unrelated proteins.

TABLE II

The ATP-dependent Ca^{2+} transport activity of synaptic plasma membranes after reconstitution

Synaptic plasma membranes were reconstituted in asolectin or phosphatidylcholine. ATP-dependent Ca^{2+} transport activity at 24°C was determined by comparing vesicle-bound $^{45}Ca^{2+}$ in the presence and absence of ATP. Transport assay replicates varied by less than 5%.

	No ATP	+ ATP	ATP-dependent CA^{2+} Transport Activity
		nmol/mg/min	
Asolectin vesicles	4.1	20.8	16.7
Phosphatidylcholine vesicles	1.5	18.0	16.5
Calmodulin-depleted phosphatidylcholine vesicles	1.8	8.3	6.5
Calmodulin-depleted phosphatidylcholine vesicles, plus exogenous calmodulin	1.7	17.8	16.1

Affinity purification of the Ca^{2+} pump on a calmodulin-Sepharose 4B column. We have demonstrated that synaptic plasma membranes contained Ca^{2+}-stimulated ATPase and ATP-dependent Ca^{2+} transport activities, both of which were activated by calmodulin. These observations indicated the presence of an ATP-dependent Ca^{2+} pump which might be purified by its interaction with calmodulin. Calmodulin affinity chromatography has been used previously to purify several enzymes (Adelstein et al., 1978; Westcott et al., 1979; Sharma et al., 1980), including the erythrocyte Ca^{2+} pump (Niggli et al., 1979).

The synaptic plasma membranes were solubilized with Triton X-100; 80 to 95% of the Ca^{2+}-stimulated ATPase activity, but only about 30% of the membrane protein, was solubilized. The concentrated, solubilized sample was subjected to gel filtration on a Sephadex G75 column to reduce rapidly the bulk concentration of Triton and to remove EGTA. The Ca^{2+}-stimulated ATPase activity eluted at the void volume of the column (data not shown). The peak of activity was pooled, concentrated, and, after addition of $CaCl_2$, applied to a calmodulin-Sepharose 4B column (Fig. 1). The absorbance of the protein in the eluant at 280 nm was monitored. After the absorbance returned to base line, indicating that unbound protein had been thoroughly washed through, protein bound to the column in a Ca^{2+}-dependent manner was eluted with a buffer containing EGTA.

Protein which passed through the column in the presence of Ca^{2+} and protein which eluted with EGTA were separately pooled, as indicated by the *bars* in Figure 1. The Ca^{2+}-stimulated ATPase and Mg^{2+} ATPase activities in the two peaks were compared. Figure 2*b* shows that the peak eluted with EGTA primarily catalyzed Ca^{2+}-stimulated ATP hydrolysis. In contrast, the peak which passed through in the presence of Ca^{2+} mainly catalyzed Mg^{2+} ATPase (Fig. 2*a*); this peak contained 90% of the Mg^{2+} ATPase recovered from the column. Since the bulk of the Mg^{2+} ATPase activity did not co-purify with the Ca^{2+}-stimulated ATPase, Ca^{2+}-stimulated ATP hydrolysis appears to be catalyzed by an enzyme separate from the one catalyzing most of the Mg^{2+} ATPase activity in synaptic plasma membranes.

In a representative preparation, the peak of protein eluted with EGTA catalyzed Ca^{2+}-stimulated ATP hydrolysis with initial rates of 300 nmol/mg/min at 24°C (Fig. 2*b*) and 870 nmol/mg/min at 37°C. The mean Ca^{2+}-stimulated ATPase activity of four different affinity-purified preparations was 316 (SEM 108) nmol/mg/min at 24°C. The affinity-purified peak was treated with Biobeads SM-2 to remove Triton X-100 and was reconstituted in asolectin. As shown in Figure 3, the reconstituted vesicles efficiently pumped Ca^{2+}; the initial rates of ATP-dependent Ca^{2+} transport and Ca^{2+}-stimulated ATPase activities at 24°C of this preparation were 381 and 374 nmol/mg/min, respectively. These results indicate a coupling ratio of about 1. Comparison of the results obtained before and after reconstitution indicates that the affinity-purified Ca^{2+} pump can be reconstituted without loss of Ca^{2+}-stimulated ATPase activity.

To determine whether the affinity-purified Ca^{2+} pump was activated by calmodulin, the peak eluted with EGTA was reconstituted in phosphatidylcholine. The resulting vesicles had a 60-fold excess of phosphatidylcholine over asolectin, which had been added during the purification to stabilize the enzyme. Addition of calmodulin activated ATP-dependent Ca^{2+} transport for three different preparations by 8.2 ± 0.7-fold. The calmodulin concentration needed for maximal activation (0.4 μM) was unchanged by reconstitution. When maximally acti-

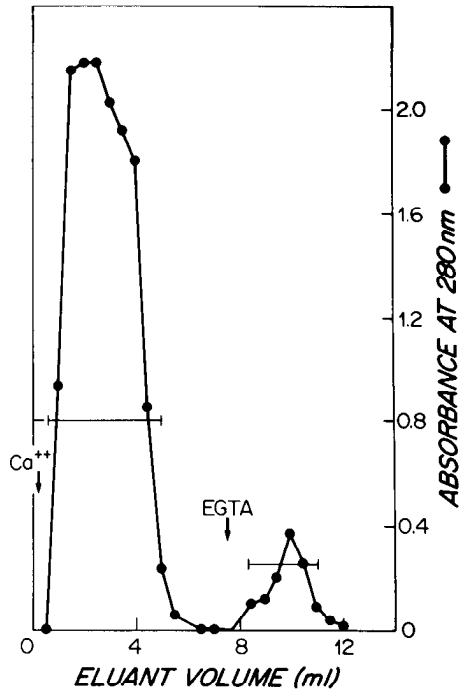

Figure 1. Purification of the Ca^{2+} pump from synaptic plasma membranes by affinity chromatography on a calmodulin-Sepharose 4B column. A concentrated sample of calmodulin-depleted, Triton-solubilized synaptic plasma membrane protein was applied to a Sephadex G75 column. The eluant was monitored for protein content and Ca^{2+}-stimulated ATPase activity. The void volume peak containing the Ca^{2+}-stimulated ATPase activity was pooled and concentrated. After addition of $CaCl_2$, the concentrated sample was applied to the calmodulin-Sepharose 4B column, which was eluted with a Ca^{2+}-containing buffer (*first arrow*) and then an EGTA-containing buffer (*second arrow*). Absorbance of the eluant at 280 nm was monitored. The fractions containing protein eluted with Ca^{2+} or with EGTA were separately pooled, as indicated by the *horizontal bars*, and were used in subsequent experiments.

vated, the rate of transport was equal (± 10%) to that of the corresponding affinity-purified pump reconstituted in asolectin. The time course of ATP-dependent Ca^{2+} uptake by the calmodulin-activated, reconstituted preparations (not shown) was roughly linear for the first minute.

To determine recovery of biological activity, the initial Triton-solubilized synaptic plasma membrane fraction was treated with Biobeads and reconstituted by the same procedure employed for the affinity-purified material. By these criteria, about 60% of both the ATP-dependent Ca^{2+} transport activity and the Ca^{2+}-stimulated ATPase activity, but only about 1.5% of the protein recovered from the calmodulin-Sepharose column, was present in the peak eluted with EGTA. Thus, we estimate that the Ca^{2+} pump has been purified about 40-fold by the affinity chromatography step. The partially selective solubilization of the enzyme, and its concentration on DEAE-Sephadex, as described under "Materials and Methods," may have contributed an additional 2- to 4-fold to the overall enrichment, which we estimate to be 80- to 160-fold.[3]

[3] Comparison of the specific Ca^{2+}-stimulated ATPase activity of the affinity-purified preparation with that of the synaptic plasma membranes gives a lower estimate of the purification achieved. However, this value is an underestimate because of the instability of the enzyme. Before solubilization, about 50% of the Ca^{2+}-stimulated ATPase activity of synaptic plasma membranes was lost in 5 days at 4°C. After removal of endogenous calmodulin, solubilization in Triton, and addition of asolectin, the rate of decay was dramatically accelerated; 50% of the solubilized activity was lost in several hours at 4°C.

To examine the protein composition of the affinity-purified preparation, a sample of the peak eluted with EGTA was subjected to electrophoresis on an SDS-polyacrylamide gel (Fig. 4). As visualized by staining with Coomassie blue, the major component was a broad protein band with a mean $M_r = 140{,}000$ (Fig. 4*a*). When a more sensitive silver staining technique was used, minor bands became visible (Fig. 4*b*). Densitometry of the silver-stained gel showed that about 55% of the protein in the peak eluted with EGTA corresponded to the major component. None of the minor bands was more than 10% of the total protein.

Functional identification of the Ca^{2+} pump in the affinity-purified preparation. Because silver staining has shown that the affinity-purified preparation had a heterogeneous protein composition, we have used functional criteria to identify the Ca^{2+} pump. The catalytic cycles of ion pumping ATPases, such as the sarcoplasmic reticulum Ca^{2+} ATPase and the (Na^+ + K^+) ATPase proceed via unstable, covalent, acylphosphate intermediates, which can be captured at low pH and temperature (Hokin et al., 1965; Makinose, 1969; Pang and Briggs, 1973). To identify which of the affinity-purified proteins had the properties expected of a Ca^{2+}-stimulated ATPase, the purified, Biobead-treated preparation was incubated with [γ-^{32}P] ATP in the presence of Ca^{2+} or EGTA and was subjected to electrophoresis at pH 2.4 and 15°C. Comparison of parts *a* and *b* of Figure 5 indicates a Ca^{2+}-dependent incorporation of phosphate at $M_r = 140{,}000$; the amount of phosphate incorporated per microgram of purified protein was comparable to that reported previously for the affinity-purified erythrocyte Ca^{2+} pump (Niggli et al., 1979). When the affinity-purified preparation (without carrier BSA) was subjected to electrophoresis at pH 2.4, the Coomassie blue-stained major protein band, located at the *arrow* in Figure 5*a*, had the same mobility as the phosphorylated component. The phosphorylated protein was dephosphorylated by hydroxylamine (Fig. 5*c*). This hydroxylamine sensitivity is a chemical property of acylphosphates (Hokin et al., 1965) but not of phosphate esters (Tada et al., 1975). A protein with the same mobility was phosphorylated in a Ca^{2+}-dependent, hydroxylamine-sensitive manner in synaptic plasma membranes (data not shown).

The phosphate-containing material which migrated near the tracking dye in Figure 5 may have been a mixture of [γ-^{32}P] ATP and $^{32}P_i$ (Avruch and Fairbanks, 1972). This material was present in a control gel which contained carrier BSA but none of the affinity-purified preparation (Fig. 5*d*) and was, therefore, unrelated to the Ca^{2+}-stimulated ATPase.

To determine which components of the affinity-purified preparation were responsible for ATP-dependent Ca^{2+} transport, the preparation was reconstituted in asolectin and was subjected to transport-specific fractionation. This method has been described in detail elsewhere (Papazian et al., 1979). It can be summarized as follows. The reconstitution procedure randomly distributes individual, solubilized proteins among small, unilamellar vesicles. Due to the large excess of lipid over protein used in the procedure, vesicles containing the Ca^{2+} pump would be expected to contain few, if any, extraneous proteins. The vesicles contain a high concentration of oxalate; ATP-dependent precipitation of Ca^{2+} oxalate, a dense complex of low solubility, occurs only inside those vesicles containing the Ca^{2+} pump. Thus, Ca^{2+}-transporting vesicles are made more dense than other vesicles and can be separated on a density gradient.

The results of transport-specific fractionation are shown in Figure 6. The reconstituted vesicles were incubated with Ca^{2+} and Mg^{2+}, with or without ATP, and were subjected to equilibrium density gradient centrifugation. The fractions of the gradients were assayed for ATP-dependent Ca^{2+} transport activity. The position of the phospholipid vesicles was determined by analysis of the total phosphate content of the fractions. After

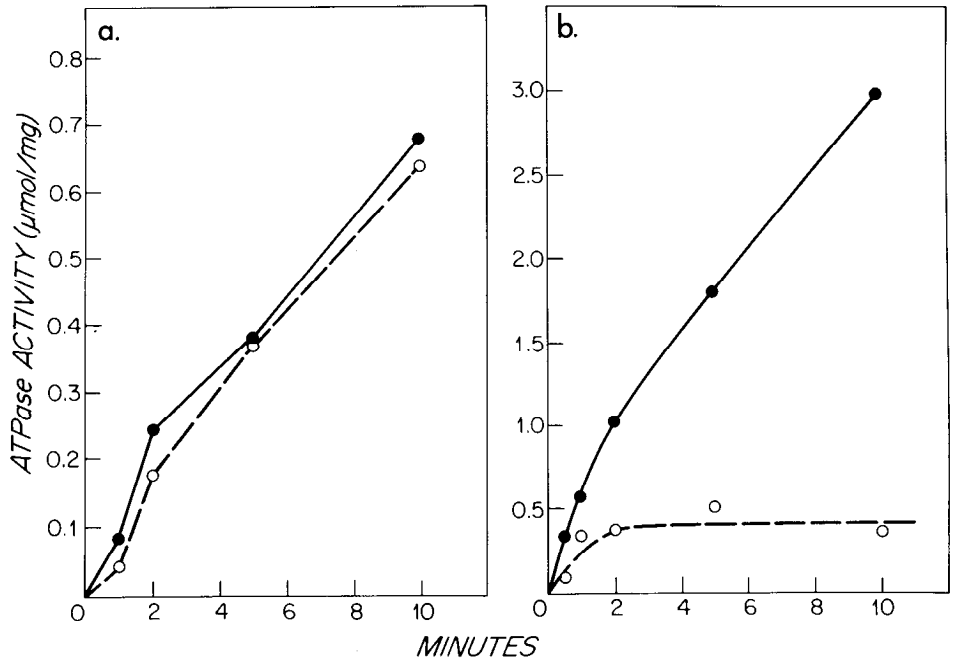

Figure 2. Separation of the Ca^{2+}-stimulated ATPase activity from most of the Mg^{2+} ATPase activity of synaptic plasma membranes by affinity chromatography. The pooled fractions eluted from the calmodulin-Sepharose 4B column by (*a*) Ca^{2+} (20 μg of protein) or (*b*) EGTA (2 μg of protein) were assayed at 24°C for ATPase activity in the presence (●) or absence (○) of Ca^{2+}. The assay conditions of Jarrett and Penniston (1978) were used, except that the final concentrations of EGTA and $CaCl_2$ were 0.8 mM and 1.25 mM, respectively, in a volume of 0.1 ml. The ATPase reaction was monitored by the release of $^{32}P_i$ from [γ-^{32}P]ATP (Goldin, 1977).

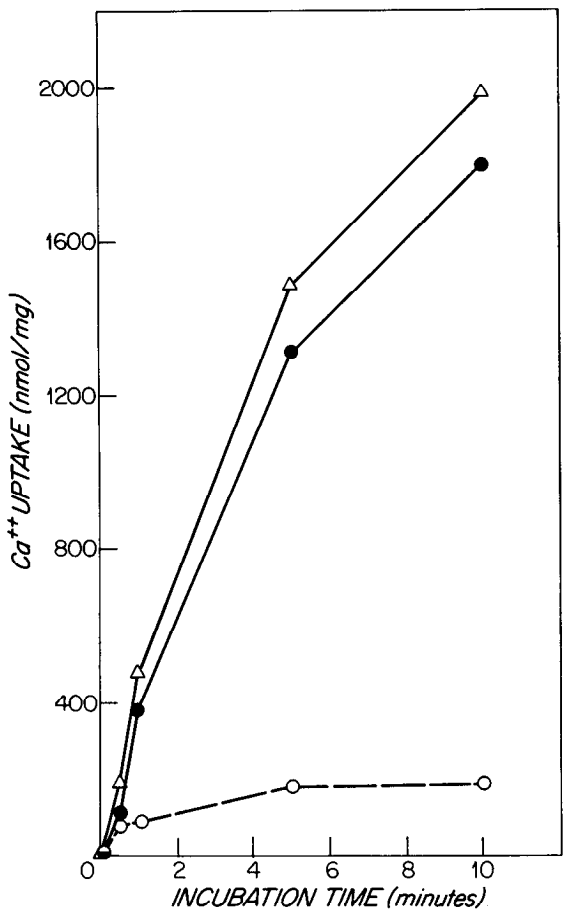

Figure 3. Time course of Ca^{2+} uptake at 24°C by the affinity-purified Ca^{2+} pump reconstituted into asolectin vesicles. Shown are Ca^{2+} uptake measured in the presence (Δ–Δ) and absence (○–○) of ATP and the net ATP-dependent Ca^{2+} uptake (●–●). The mean initial rates of ATP-dependent Ca^{2+} uptake, measured after 1 min of incubation, for four different reconstituted preparations was 268 (SEM 76) nmol/mg/min. Reconstitution and transport assays were performed as described under "Materials and Methods."

incubation with ATP, the transport activity was found below the bulk of the phospholipid vesicles on the gradient (Fig. 6*a*). Displacement of the activity to a region of higher density was dependent on ATP; if the vesicles were incubated without ATP, the positions of the transport activity and the phospholipid vesicles were identical (Fig. 6*b*).

The *bars* in Figure 6 indicate regions of the gradients which were pooled, concentrated by centrifugation, and subjected to electrophoresis on an SDS-polyacrylamide gel. A silver-stained gel is shown in Figure 7. The protein of M_r = 140,000 is substantially enriched in an ATP-dependent manner by transport-specific fractionation. Comparison of *lanes 1* and *2* indicates that other protein bands of variable identity and intensity were present in the pooled regions of both gradients. *Lane 3* shows that most of these bands were contributed by asolectin, which is present in the vesicles in such excess over the affinity-purified protein that minor protein contaminants in the asolectin become prominent bands on the gel. Based on the results of several experiments, the lower molecular weight bands present in *lane 1* appeared in extremely variable amounts from experiment to experiment; the reasons for this variability are as yet not understood. The M_r = 140,000 component was the only protein specifically and reproducibly enriched by transport-specific fractionation.

Discussion

Identity and purity of the Ca^{2+} pump. A protein component with M_r = 140,000 was substantially purified by calmodulin affinity chromatography. It was implicated in catalyzing Ca^{2+}-stimulated ATP hydrolysis by phosphorylation experiments and in ATP-dependent Ca^{2+} transport by reconstitution and transport-specific fractionation. These results identify the pro-

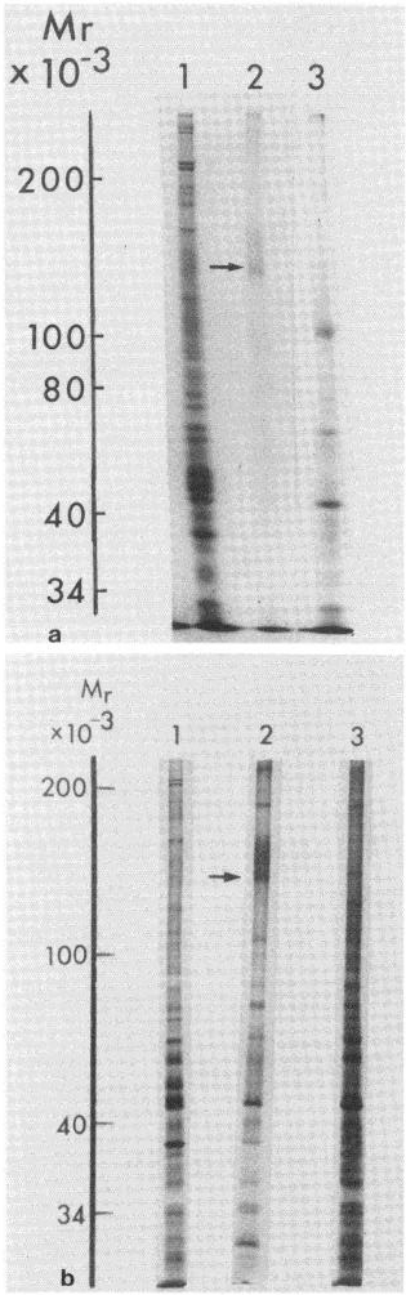

Figure 4. Electrophoresis of the affinity-purified preparation on SDS-polyacrylamide gels. Polyacrylamide gel (*a*) stained with Coomassie blue or (*b*) silver stained by the method of Oakley et al. (1980). Both gels consist of: *lane 1*, the synaptic plasma membrane fraction; *lane 2*, the affinity-purified preparation (eluted from the calmodulin column by EGTA); *lane 3*, protein passing through the calmodulin column in the presence of Ca^{2+} (i.e., unbound protein). *Lanes* contain approximately 15 μg and 3 μg of protein in *gels a* and *b*, respectively. The *arrows* in *lanes 2* denote the most intensely stained region of the broad band purified by affinity chromatography.

tein as a calmodulin-activated Ca^{2+} pump of synaptic plasma membranes.

On SDS-polyacrylamide gels, the component with a mean M_r = 140,000 consisted of a broad, diffusely stained band and a sharper, more intensely stained band with a slightly higher mobility. We have not determined precisely whether all or only part of the stained region corresponds to the active Ca^{2+} pump. Phosphate was incorporated in a Ca^{2+}-dependent manner into a wide band with the same mobility as the stainable component.

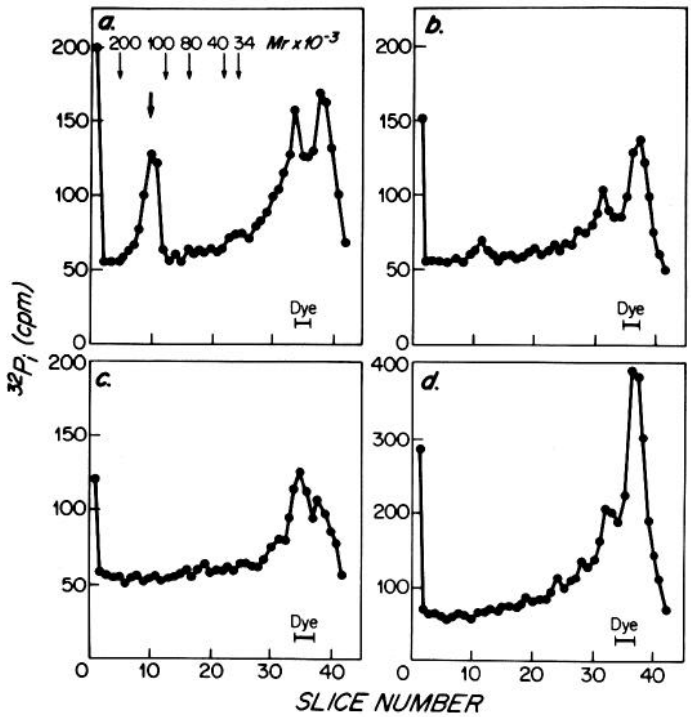

Figure 5. Phosphorylation of the purified Ca^{2+}-stimulated ATPase by [γ-^{32}P]ATP. The affinity-purified preparation was incubated with [γ-^{32}P]ATP and 1.6 mM EGTA under various conditions, precipitated with tricholoroacetic acid in the presence of carrier BSA, and subjected to electrophoresis at pH 2.4 and 15°C. Approximately 1 μg of affinity-purified protein was applied to each gel. Phosphate incorporation is shown after: (*a*) incubation with 2.3 mM $CaCl_2$ (The *heavy arrow* denotes the position of the major protein component of the affinity-purified preparation, subjected to electrophoresis at pH 2.4 and 15°C, in the absence of carrier BSA, and stained with Coomassie blue. The component had a mobility of 140,000 in a standard gel system at pH 7.5. Under acidic conditions, the component had an apparent mobility of 125,000.); (*b*) incubation without added Ca^{2+}; (*c*) incubation with 2.3 mM $CaCl_2$, followed by treatment with hydroxylamine; (*d*) control incubation of buffer with [γ^{32}P]ATP, 2.3 mM $CaCl_2$, and 1.6 mM EGTA in the absence of affinity-purified protein. The variable number of counts present in the first slice of each of the gels represents material that did not enter the gels.

Transport-specific fractionation resulted in the ATP-dependent enrichment of a band with M_r = 140,000; its breadth was difficult to determine because it was relatively weakly stained on an SDS gel. We attempted to increase its staining intensity by applying more protein. However, this resulted in overloading the gel with phospholipid (Papazian et al., 1979), since the reconstituted vesicles used for transport-specific fractionation had a 2000-fold excess of asolectin over affinity-purified protein. This composition also resulted in the appearance on the gel of minor protein contaminants of asolectin in amounts comparable to that of the Ca^{2+} pump. The concentration of these contaminants varied among different gradients; the cause of the variation has not yet been determined.

Other membrane proteins, such as the voltage-sensitive Na^+ channel of eel electroplax, and bands 3 and 4.5, the erythrocyte anion and sugar transporters, respectively, electrophorese as broad bands on SDS gels (Agnew et al., 1983; Drickamer, 1978; Goldin and Rhoden, 1978). The breadth of band 3 is due to a variation in the extent of glycosylation among individual molecules of one protein component (Tsuji et al., 1980; Markowitz and Marchesi, 1981). The voltage-sensitive Na^+ channel also exhibits stable microheterogeneity on SDS gels. Treatment of the Na^+ channel with glycosidases before electrophoresis con-

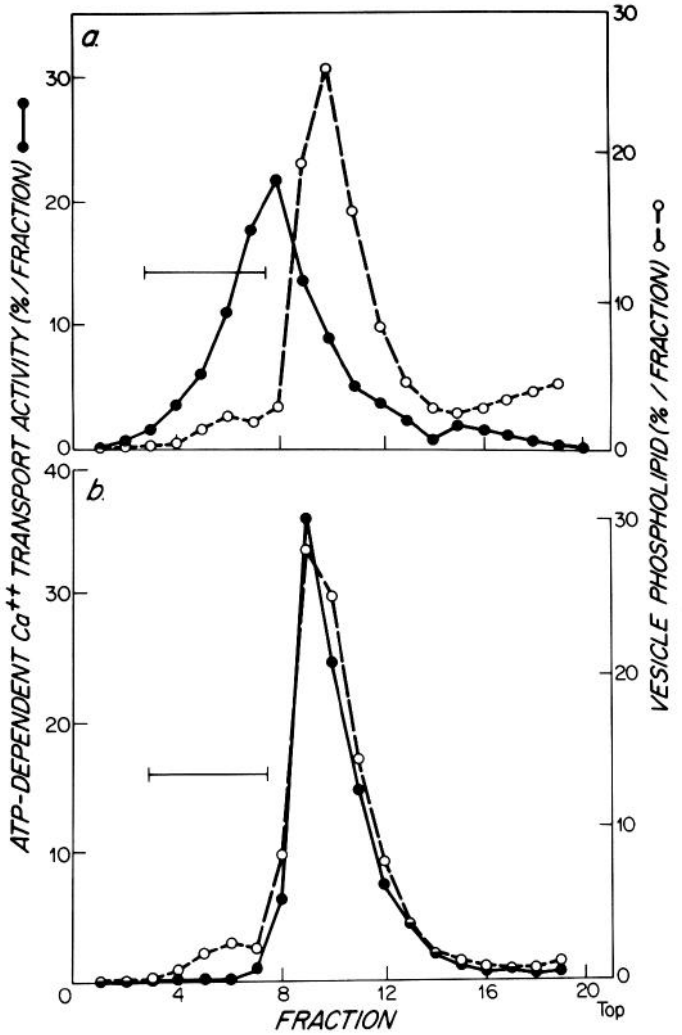

Figure 6. Transport-specific fractionation of the affinity-purified, ATP-dependent Ca^{2+} pump. The Biobead-treated purified preparation was reconstituted in asolectin, incubated with $CaCl_2$ and $MgCl_2$ with (*a*) or without (*b*) ATP, and subjected to equilibrium density gradient centrifugation (Papazian et al., 1979). Fractions of the gradients were assayed for ATP-dependent Ca^{2+} transport activity (●) and phospholipid content (○). *Horizontal bars* indicate the regions of the gradients pooled for SDS-polyacrylamide gel electrophoresis.

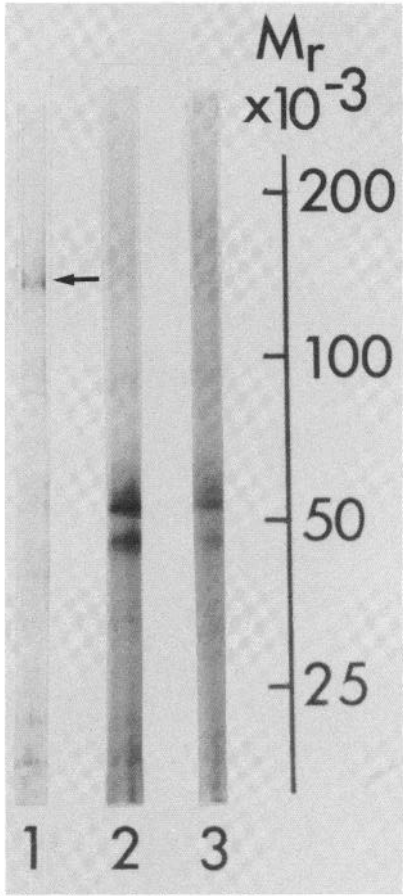

Figure 7. SDS-polyacrylamide gel electrophoresis of the affinity-purified, reconstituted Ca^{2+} pump subjected to transport-specific fractionation. Each *lane* is the protein profile of a region of a density gradient corresponding to the *horizontal bars* depicted in Figure 6. *Lane 1* is the gradient region of Figure 6*a*, resulting from centrifugation of vesicles incubated with Ca^{2+}, Mg^{2+}, and ATP. The *arrow* corresponds to the *arrows* of Figure 4*a* and *b*. *Lane 2* is the gradient region of Figure 6*b*, resulting from centrifugation of vesicles incubated with Ca^{2+} and Mg^{2+} but without ATP. *Lane 3* is a corresponding gradient region obtained after the centrifugation of vesicles composed of asolectin but containing no affinity-purified protein; these vesicles were formed by hollow fiber dialysis of cholate-solubilized asolectin in the absence of added protein. The gel has been silver stained by the method of Oakley et al. (1980).

verts the broad band to a narrower one of higher mobility (Agnew et al., 1983). We have not yet determined whether the breadth of the affinity-purified component with a mean M_r = 140,000 is due to a heterogeneous sugar content.

The Ca^{2+} pump has been enriched 80- to 160-fold by the purification procedure. The major component was virtually the only one visible on an SDS gel stained with Coomassie blue. However, silver staining has shown that the affinity-purified preparation was not homogeneous; the minor components of the preparation may be other calmodulin-binding proteins from brain (Kakiuchi et al., 1981). Since the homogeneity of a purified preparation can be overestimated when gels are stained with Coomassie blue, we believe that silver staining is, in general, a more rigorous test of the homogeneity of purified proteins.

Hakim et al. (1982) have reported the affinity purification of a Ca^{2+} ATPase from synaptic plasma membranes. After electrophoresis of their preparation on an SDS-polyacrylamide gel, a broad protein band with M_r = 138,000 was visualized by staining with Coomassie blue, a result consistent with our own observations (Papazian et al., 1982). Although its ability to catalyze Ca^{2+} transport was not studied, they found that it cross-reacted with antibodies to the erythrocyte (Ca^{2+} + Mg^{2+}) ATPase. It is difficult to assess the homogeneity of their preparation because a more sensitive staining procedure was not used.

Properties of the affinity-purified, reconstituted Ca^{2+} pump. We have focused much of our attention on the ATP-dependent Ca^{2+} transport activity of the purified Ca^{2+} pump. Transport was efficiently associated with Ca^{2+}-stimulated ATP hydrolysis. Our results are consistent with a coupling ratio (Ca^{2+}/ATP) of 1, but we cannot rule out higher values, since some Triton, which is difficult to remove from proteins entirely, may remain in the reconstituted vesicles, making them slightly leaky. The Ca^{2+}-stimulated ATPase activity of the purified Ca^{2+} pump was separable from most of the Mg^{2+} ATPase of synaptic plasma membranes. However, we cannot conclude that the Ca^{2+} pump is incapable of any Mg^{2+} ATPase activity, since a small amount remained in the purified preparation.

After reconstitution of the purified pump in phosphatidylcholine, transport was activated 7- to 9-fold by calmodulin *in*

vitro at a total Ca^{2+} concentration of 100 μM. The Ca^{2+}-stimulated ATPase activity and the reconstituted Ca^{2+} transport activity of the unpurified pump from synaptic plasma membranes were activated to a lesser degree. This difference may indicate that some endogenous calmodulin remained in the unpurified material, despite our efforts to remove it.

The possible role of the Ca^{2+} pump in nerve terminals. Having established the existence and molecular identity of the calmodulin-activated Ca^{2+} pump, the next important question is its localization. We hypothesize that the pump is situated in the plasma membrane of nerve terminals. Evidence for a Ca^{2+} pump localized to synaptic plasma membranes in mammalian brain has been reported by Gill and co-workers (1981). Ca^{2+} taken up by isolated brain membrane vesicles in an ATP-dependent manner can be released by an outwardly directed Na^+ gradient, indicating that a Ca^{2+} pump is present in the same population of vesicles as a Na^+/Ca^{2+} exchange activity (Gill et al., 1981). Analogous experiments have shown that these same vesicles also contain the voltage-sensitive Na^+ channel and the (Na^+/K^+) ATPase, components of the plasma membrane (Gill, 1982). The synaptic plasma membrane fraction employed for our purification also contained Na^+/Ca^{++} exchange activity as well as Na^+-dependent γ-aminobutyric acid uptake activity (Erdreich et al., 1983). Isolated rat brain fractions enriched in mitochondria contained insignificant amounts of both of these activities when assayed under the same conditions (H. Rahamimoff, unpublished observations).

The calmodulin-activated Ca^{2+} pump with M_r = 140,000 described herein is strikingly similar to that previously purified from erythrocyte ghosts (Niggli et al., 1979; Graf et al., 1982). Since erythrocytes have no internal organelles, that pump has been assigned unambiguously to the plasma membrane. Similar calmodulin-activated Ca^{2+} pumps have also been described in plasma membrane fractions derived from a wide variety of other cell types including cardiac cells (Caroni and Carafoli, 1981), lymphocytes (Lichtman et al., 1981), adipocytes (Pershadsingh et al., 1980), and macrophages (Lew and Stossel, 1980). Thus, the synaptic plasma membrane Ca^{2+} pump may function to extrude Ca^{2+} from the cell, in conjunction with other mechanisms, to maintain a low intracellular concentration of Ca^{2+}. However, subcellular immunocytochemical localization of this Ca^{2+} pump is necessary to determine conclusively its distribution.

Extrusion of Ca^{2+} also occurs by Na^+/Ca^{2+} exchange, driven by the Na^+ gradient (Rahamimoff and Spanier, 1979; Gill et al., 1981; Michaelis and Michaelis, 1981; Gill, 1982). Until the kinetic parameters of the Na^+/Ca^{2+} antiporter and the ATP-dependent Ca^{2+} pump are studied more completely, the relative importance of the two processes under various physiological conditions will not be known. Our results with synaptic plasma membranes indicate that the transport capability of the Ca^{2+} pump can be drastically underestimated in unreconstituted membrane fractions.

By analogy with the erythrocyte Ca^{2+} pump, which the neuronal Ca^{2+} pump closely resembles (Niggli et al., 1979, 1981a, b), the extent of activation by calmodulin *in vivo* probably depends on the free concentration of Ca^{2+} and the levels of negatively charged phospholipids (Niggli et al., 1981a, b). At free Ca^{2+} concentrations between 10^{-7} and 10^{-6} M, calmodulin activation of the erythrocyte Ca^{2+} pump becomes significant *in vitro* (Niggli et al., 1981a, b). It is noteworthy that the deformability of the erythrocyte, critical for its passage through capillaries, is decreased at intracellular Ca^{2+} concentrations greater than 10^{-6} M (Kirkpatrick et al., 1975); activation by calmodulin of Ca^{2+} extrusion *in vivo* at 10^{-7} to 10^{-6} M Ca^{2+} may play a role in maintaining erythrocyte deformability. In neurons, the resting internal concentration of Ca^{2+} has been estimated to be 10^{-7} M; an increase of 10- to 100-fold or more triggers neurotransmitter release (Kelly et al., 1979). Thus, calmodulin activation of Ca^{2+} extrusion from nerve terminals may occur in a range of Ca^{2+} concentrations critical to regulating neurotransmitter release.

Relationship of this pump to the Ca^{2+} pump associated with synaptosomal vesicles. We have previously used transport-specific fractionation to purify ATP-dependent Ca^{2+} transport components from synaptosomal vesicles (Papazian et al., 1979). This is a membrane fraction derived from synaptosomal lysates by high speed centrifugation after removal of the synaptic plasma membrane fraction. The vesicular fraction includes, but is not exclusively composed of, neurotransmitter-containing "synaptic vesicles" (Rahamimoff and Abramovitz, 1978a). Synaptosomal vesicles possess a nonmitochondrial, ATP-dependent Ca^{2+} uptake activity (Rahamimoff and Abramovitz, 1978a, b). Transport-specific fractionation purifies three proteins from synaptosomal vesicles: a major component with M_r = 94,000, a minor one of 140,000, and a third of 230,000, which may be a dimer of the first (Papazian et al., 1979).

Since synaptosomal vesicles and synaptic plasma membranes are derived from the same synaptosomal lysate and are separated only by differential centrifugation, the membrane fractions are probably cross-contaminated with one another. Thus, it is likely that the protein with M_r = 140,000, purified as a minor component from synaptosomal vesicles, is the same calmodulin-activated Ca^{2+} pump which we have now purified and characterized from synaptic plasma membranes, a richer source of the protein.

Preliminary evidence indicates that the Ca^{2+} uptake activity of synaptosomal vesicles is not directly activated by calmodulin (Papazian et al., 1981). Our working hypothesis is that the M_r = 94,000 and 230,000 proteins may comprise a calmodulin-insensitive Ca^{2+} pump located in vesicles specialized for Ca^{2+} sequestration, whereas the M_r = 140,000 protein is a calmodulin-activated pump extruding Ca^{2+} through the plasma membrane, as discussed above. Proteins from bovine brain, comparable to those with M_r = 94,000 and 230,000 from rat synaptosomal vesicles, have been found to be immunologically related and may comprise a Ca^{2+} transport system independent of the protein with M_r = 140,000 (Goldin et al., 1983).

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