EVIDENCE THAT COATED VESICLES ISOLATED FROM BRAIN ARE CALCIUM-SEQUESTERING ORGANELLES RESEMBLING SARCOPLASMIC RETICULUM

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ABSTRACT

Coated vesicles from the brain have been purified to near morphological homogeneity by a modification of the method of Pearse. These vesicles resemble sarcoplasmic reticulum fragments isolated from skeletal muscle. They contain proteins with 100,000- and 55,000-dalton mol wt which co-migrate on polyacrylamide gels, in the presence of sodium dodecyl sulfate, with the two major proteins of the sarcoplasmic reticulum fragment. These vesicles contain adenosine triphosphatase (ATPase) activity which is stimulated by calcium ions in the presence of Triton X-100 (Rohm & Haas Co., Philadelphia, Pa.), displaying maximal activity at 8×10^{-7} M Ca^{++}. They take up calcium ions from the medium, and this uptake is stimulated by ATP and by potassium oxalate, a calcium-trapping agent.

The 100,000-dalton protein of the coated vesicles displays immunological reactivity with an antiserum directed against the 100,000-dalton, calcium-stimulated ATPase of the sarcoplasmic reticulum. As with the sarcoplasmic reticulum fragment, this protein becomes radiolabeled when coated vesicles are briefly incubated with γ-labeled [^{32}P]ATP.

The possible functions of coated vesicles as calcium-sequestering organelles are discussed.

KEY WORDS   brain · coated vesicle · Ca^{++} uptake · Ca^{++} ATPase · sarcoplasmic reticulum

Membrane vesicles which are enclosed by a polyhedral protein lattice (coated vesicles) (14) are involved in several different pathways of intracellular transport, including certain types of endocytosis and exocytosis.

An example of their role in endocytosis is the demonstration that synaptic vesicle components, inserted into the presynaptic plasma membrane during neurotransmitter release, are removed by coated vesicles and recycled into new synaptic vesicles by way of internal cisternae (7, 10).

Coated vesicles also participate in endocytosis during uptake of low density lipoprotein by fibroblasts (1), and in uptake of protein by developing mosquito oocytes (29), by the placental trophoblast (23), and by vascular endothelium (24).

As exocytotic organelles, coated vesicles participate in the secretion of casein by lactating mammary epithelium (6), and in the secretion of serum lipoprotein by hepatocytes (15).

In all these cases, coated vesicles shuttle from one membrane compartment to another, and therefore must be able to undergo repeated membrane fusion and fission.

Because we are interested in studying the mo-

lecular basis of membrane fusion, particularly the role of membrane proteins in this process, we were encouraged by Pearse's successful purification of coated vesicles from brain, adrenal medulla, and lymphoma cells (25, 26). These vesicles have two striking characteristics which distinguish them from other membranous organelles. First, their phospholipid-containing membrane is enclosed within a polyhedral protein coat, and second, their polypeptide composition is extremely simple. In all isolated coated vesicles examined so far, ~70% of the total protein is composed of the 180,000-dalton coat subunit which has been given the name "clathrin" (26). In addition, coated vesicles from the brain of various species, and those from bullock adrenal medulla have polypeptides of 100,000- and 55,000-daltons mol wt, respectively, each comprising ~10% of the total protein (2, 26).

In our preliminary examination of our preparation of coated vesicles from the brain by polyacrylamide electrophoresis, we were struck by the similarity of the peptide composition to that of sarcoplasmic reticulum fragments isolated from skeletal muscle. Here we further document the similarity of the membrane proteins from these two sources, and show that coated vesicles from the brain functionally resemble sarcoplasmic reticulum, in that they sequester calcium ions by an adenosine triphosphate (ATP)-requiring process. The role that these vesicle proteins play in membrane fusion remains an enigma, but we discuss how their ability to sequester calcium might be involved in the control of synaptic transmission.

MATERIALS AND METHODS

Isolation of Coated Vesicles and Sarcoplasmic Reticulum Fragments

Coated vesicles were isolated from the brains of 2-wk-old calves, adult rats, or adult rabbits by a modification of the procedure of Pearse (25). 300 g of brain was homogenized in an equal volume of extraction buffer (MES buffer: 0.1 M MES (2-[N-morpholino]ethane sulfonic acid); 1 mM ethylene glycol bis(β-aminoethyl ether) N,N,N',N'-tetraacetate (EGTA); 0.5 mM $MgCl_2$; 0.02% sodium azide, pH 6.5) in the Waring blender (Waring Products Division, Dynamics Corporation of America, New Hartford, Conn.) employing three 10-s bursts at top speed. The homogenate was centrifuged at 20,000 g (here and elsewhere, the average acceleration is given) for 30 min in the Sorvall RC-2B centrifuge (Ivan Sorval, Inc., Norwalk, Conn.). The supernate was saved, and the pellet was suspended in MES buffer and

homogenized as before. The second homogenate was centrifuged as before, and the resulting supernate was combined with the first. The combined supernates were centrifuged at 85,300 g for 1 h.The resulting pellet was resuspended with the aid of the loose-fitting Dounce homogenizer (Kontes Co., Vineland, N.J.) in MES buffer so as to produce a 20-ml suspension. Equal portions of this suspension were layered on each of six sucrose gradients containing 4.5 ml each of 5, 10, 20, 30, 40, 50, and 60% sucrose prepared in MES buffer. With an SW27 rotor in the Beckman L350 centrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.), the gradients were centrifuged at 50,000 g for 2 h. All the material was removed from the gradients at the end of the centrifugation, exclusive of a red zone at the top and a dense white pad at the bottom. This material was diluted about 3:1 with MES buffer, and pelleted by centrifugation at 100,000 g for 1 h. The pellets were suspended in MES buffer to yield a 12-ml suspension, and layered on a second set of six sucrose gradients containing 5.0 ml each of 20, 30, 40, 50, 55, and 60% sucrose prepared in MES buffer. These gradients were centrifuged at 50,000 g for 16 h.The coated vesicles, which occupied an opalescent band near the 50–55% sucrose interface, were removed with a syringe from the sides of the tubes, diluted ~3:1 with MES buffer, and centrifuged at 100,000 g for 1 h. The pellet was resuspended in a total of 2 ml of MES buffer and layered on a third set of two gradients consisting of 4 ml each of 5, 10, 20, and 30% sucrose prepared in MES buffer. These gradients were centrifuged at 50,000 g for 45 min in the SW27.1 rotor. At the end of the centrifugation, the material of interest formed two opalescent bands found near the 10–20% sucrose interface and near the 20–30% sucrose interface, respectively.The bands were removed and the suspensions of vesicles were diluted ~2:1 with MES buffer, and pelleted by centrifugation at 100,000 g for 1 h, resuspended in 1–2 ml of MES buffer, and centrifuged at low speed for 5 min to remove aggregated material.

Suspensions of coated vesicles were stored in MES buffer for several days at 4°C. Before use, they were always centrifuged at 2,000 rpm for 5 min to remove aggregates which continuously formed during storage.

Sarcoplasmic reticulum (SR) fragments were prepared from rabbit back and hindlimb muscle by the method of MacLennan (21).

Preparation of an Antiserum Directed Against the Calcium-Stimulated Adenosine Triphosphatase (Ca-ATPase), and Preparation of Coated Vesicle and SR Antigens

Pelleted SR fragments were dissolved by heating at 100°C in 2% sodium dodecyl sulfate (SDS) containing 10% glycerol. 500 μg of protein in 0.1 ml was applied to

each of a series of 10 × 1.5-cm acrylamide gels containing 7.5% acrylamide, 0.2% N,N'-methylene-bis-acrylamide (BIS), and 0.1% SDS, and subjected to electrophoresis in an apparatus from Hoefer Scientific Instruments, San Francisco, Calif. The tank and gel buffers were 3% Tris-base, 6% glycine, 0.1% SDS, pH 8.6. The sample applied to one of the gels was pretreated with 60 μg of fluorescamine (Fisher Scientific Company, Pittsburgh, Pa.) in 0.02 ml of acetone. At the conclusion of the electrophoresis, the position of the fluorescent SR 100,000-dalton protein was located under UV light, the corresponding regions in the other gels were removed, and the protein contained therein was electrophoretically eluted from the acrylamide slices into the same buffer. The eluant was dialyzed overnight against 0.01 M ammonium bicarbonate, and then lyophilized. The lyophilizate which contained ~500 μg of protein was dissolved in 0.25 ml of saline containing 0.1% SDS, combined with 0.25 ml of Freund's complete adjuvant (Cappel Laboratories, Downingtown, Pa.), and subcutaneously injected into a single rabbit behind the neck. Two more injections at weekly intervals were carried out in the same manner. 1 wk after the last injection, serum was collected and the γ-globulin fraction was recovered by precipitation three times with 33% saturated ammonium sulfate. The final precipitate was taken up in one-half the original volume of saline and dialyzed overnight at 4°C against the same.

The 180,000-, 100,000-, and 55,000-dalton coated vesicle proteins were recovered from polyacrylamide gels in the same manner as the SR protein. The lyophilizates were dissolved in saline containing 0.1% SDS for immunodiffusion studies described in Fig. 8.

Measurement of the Rate of ATP Hydrolysis

The rate of hydrolysis of [U-^{14}C]ATP was measured in a total volume of 0.1 ml of a medium consisting of 0.1 M KCl; 5 mM EGTA; 20 mM imidazole, pH 7.0; 2 mM disodium ATP plus [U-^{14}C]ATP (New England Nuclear, Boston, Mass.; 90,000 cpm/μmol); 0.5 or 2 mM MgCl$_2$ in some experiments; and 20–50 μg of coated vesicle protein. The reactions were started by the addition of coated vesicles, and at appropriate times, were stopped by the rapid addition of 0.9 ml of ice-cold 20 mM EDTA, pH 7.5. The samples were then applied to Dowex-1-Cl (Dow Chemical Co., Midland, Mich.) × 8 columns poured in Pasteur pipettes to a height of 2.2 cm. 1.0 ml of 0.07 N HCl was passed through the columns, followed by an additional 2.0 ml of 0.07 N HCl which removed 86 ± 1% of the [U-^{14}C]adenosine diphosphate (ADP) formed during the course of the reaction, and <1% of the unreacted [U-^{14}C]ATP. An aliquot of the substrate, together with 2.0 ml of 0.07 N HCl and the column eluants themselves, were counted in 4.0 ml of Aquasol II (New England Nuclear). Control incubations were included (identical reaction mixtures without coated vesicles) in order to correct for ~4% [U-

^{14}C]ADP in the commercially prepared substrate, for contamination of the eluted ADP with unreacted ATP, and for nonenzymatic hydrolysis of the substrate. This assay was linear with respect to time for at least 30 min, and linear with respect to the amount of coated vesicle protein added to at least 70 μg.

Measurement of Calcium Uptake

Coated vesicles equivalent to 50–180 μg of protein were incubated for 10 min at room temperature in a total volume of 0.1 ml of the following incubation medium: 0.13 M KCl; 20 mM imidazole, pH 7.0; 2 mM MgCl$_2$, 2 mM disodium ATP (when present); 10 mM potassium oxalate (when present); and 0.2 mM CaCl$_2$ plus ^{45}CaCl$_2$ (9.5 × 10^7 cpm/μmol, New England Nuclear). In some experiments, 0.2 mM EGTA was also present. At the end of this time, the suspensions were treated as follows:

PROCEDURE 1: The incubations were quenched by adding 0.9 ml of 0.13 M KCl, 20 mM imidazole, pH 7.0 (buffer A), and the suspensions were rapidly passed through 13- or 35-mm type HAWP filters (Millipore Corp., Bedford, Mass.), followed by 6 ml of buffer A. The filters were dried at 50°C and counted with 4.0 ml of Aquasol.

PROCEDURE 2: The incubations were quenched with 0.4 ml of 0.375 M sucrose, 20 mM imidazole, pH 7.0, and layered through buffer A onto the tops of 10.5 × 1.0-cm columns of Sephadex G-50 medium previously equilibrated with buffer A. Next, the vesicles and associated calcium ions were eluted in 2.0 ml of buffer A collected after 3.5 ml of buffer A was allowed to pass from the columns. Aliquots of the vesicle-containing fractions were counted in 4.0 ml of Aquasol II. The entire quenching and elution procedure took ~2.5 min.

Urea Treatment of Coated Vesicles

To remove the protein coats from the vesicles, they were treated with 2 M urea as follows: 0.33 ml of 8 M urea was added to 1.0 mg of coated vesicle protein suspended in 1.0 ml of 0.3 M sucrose, 10 mM HEPES (N-2-hydroxyethyl piperazine-N'-ethane sulfonic acid, Sigma Chemical Co., St. Louis, Mo.), pH 7.0. After 5 min at room temperature, the resulting high molecular weight aggregate was pelleted by centrifugation at 55,000 g for 30 min. The pellet was washed by resuspending in the buffered sucrose solution and then centrifuging, as previously explained. The resulting pellet was dissolved in 0.1 ml of buffered sucrose containing 1.0% Triton X-100 (Rohm & Haas Co., Philadelphia, Pa.) to produce a final protein concentration of 3 mg/ml.

Phosphorylation of the 100,000-dalton Protein

The phosphorylation of coated vesicle protein was carried out by allowing [γ-^{32}P]ATP to react with either intact coated vesicles or with coated vesicles which had been treated with 2 M urea and 1.0% Triton X-100 as

described above. The phosphorylation reactions were carried out in 0.13 M KCl; 20 mM imidazole, pH 7.0; 1 mM EGTA; 0.5 mM $CaCl_2$ (free calcium ion concentration, 1 μM); 2 μM disodium ATP; [γ-^{32}P]ATP (New England Nuclear), 3.08×10^8 cpm; pH 7.0. The reactions, carried out in a total volume of 0.1 ml, were initiated by the addition of either 0.013 ml of a suspension of intact coated vesicles in buffered sucrose (100 μg of protein), or 0.034 ml of a solution of urea-treated coated vesicles in buffered sucrose plus 1.0% Triton X-100 (100 μg of protein). A duplicate of each of the two reaction mixtures was run, except that the radiolabeled ATP was omitted. After 10 s at room temperature, 0.4 ml of 6.25% trichloroacetic acid was added to each, and the suspensions were incubated in ice for 5 min. They were then centrifuged at 59,000 g for 30 min. The supernates were removed as thoroughly as possible, and the pellets were dissolved in 0.08 ml of 2% SDS, 0.1 M sodium phosphate, pH 5.6, with the aid of the Vortex mixer (Scientific Industries, Inc., Bohemia, N.Y.). Next, 0.02 ml of 55% glycerol, 0.001% bromphenol blue was added to each protein solution. Finally, 1.0 N NaOH was added in 1-μl portions until the color of the solutions changed from yellow to purple (pH 4–5). The solutions were then applied to 2 × 40-mm polyacrylamide gels containing 7.5% acrylamide, and 0.2% BIS, with 0.1% SDS, 0.1 M sodium phosphate, pH 5.6, as both gel and tank buffers. The electrophoresis was carried out at 50 V for 3 h after which time the gels containing the radioactive samples were removed and frozen. The gels containing the unlabeled samples were stained in 45% methanol, 10% acetic acid, 0.25% Coomassie Blue R (Merck Chemical Div., Merck Co., Inc., Rahway, N.J.) at room temperature for 2 h, and then destained overnight at 50°C in 5% methanol, and 7.5% acetic acid. The frozen gels were sliced into 0.8-mm sections, and the slices were separately counted in 4.0 ml of Aquasol II after each had been incubated overnight in 0.2 ml of 30% hydrogen peroxide at 50°C.

Electron Microscopy

Suspensions of coated vesicles and urea-treated coated vesicles were fixed in 0.1 M sodium phosphate, pH 7.2, 2.5% glutaraldehyde, and 2% paraformaldehyde in ice for 30 min, and then pelleted by centrifugation at 56,000 g for 20 min. The pellets were treated with 1.0% osmium tetroxide in 0.1 M sodium phosphate, pH 7.2, in ice for 5–10 min, dehydrated in ethanol solutions, and embedded in Araldite-dodecenyl-succinic anhydride (DDSA) (1:1). Silver sections were cut with a diamond knife and an LKB ultramicrotome (LKB Instruments, Inc., Rockville, Md.), collected on 200- or 400-mesh copper grids, stained with 1.0% phosphotungstic acid for 5 min, with 10% methanolic uranyl acetate for 10 min, and with Reynold's lead citrate (28) for 10 min. The stained sections were viewed with a Philips 300 electron microscope.

Polyacrylamide Gel Electrophoresis

This was essentially carried out as described by Weber and Osborn (32) under the exact conditions described in the figure legends. Special conditions were employed for the phosphorylation experiment as previously described.

Determination of Protein Concentration

The method of Lowry et al. (20) was employed, but was scaled down in volume by a factor of 2, and with samples dissolved in 2% SDS.

RESULTS

Isolated Coated Vesicles

The 5–30% sucrose step gradient employed as the final step in the isolation procedure separates the preparation into a densely banding fraction near the 10–20% interface, and a more diffusely banding fraction of greater bouyant density near the 20–30% interface. Electron micrographs (Fig. 1) of the upper fraction (fraction 1) reveal a nearly morphologically homogeneous preparation, partly containing vesicles enclosed in polygonal coats with a total diameter of about 600 Å. In addition, some empty coats appear to be present. Compared to the upper fraction, the lower fraction (fraction 2) contains, in addition to coated vesicles, larger, frequently dumbbell-shaped membrane profiles without coats (Fig. 2). The two fractions, although appearing morphologically dissimilar, have nearly identical polypeptide compositions (Fig. 4a, b). This result suggests that the two fractions represent different modes of organization of the same intracellular organelle.

Some of the experiments reported below were carried out by using preparations of coated vesicles which had not been separated into two fractions. These will be referred to below as unfractionated coated vesicles. Other experiments separately employed either fraction 1, the wholly coated fraction, or fraction 2, which contained a mixture of coated and smooth vesicles.

Protein Composition of Coated Vesicles

The protein compositions of coated vesicles (Fig. 3a, b, c) isolated from calf, rabbit, and rat brain are quite similar, and closely resemble the protein composition of coated vesicles isolated from pig brain (25), and from the brain and adrenal medulla of bullock (26), as reported earlier by Pearse. The major protein estimated to comprise ~70% of the total protein in these vesicles has a
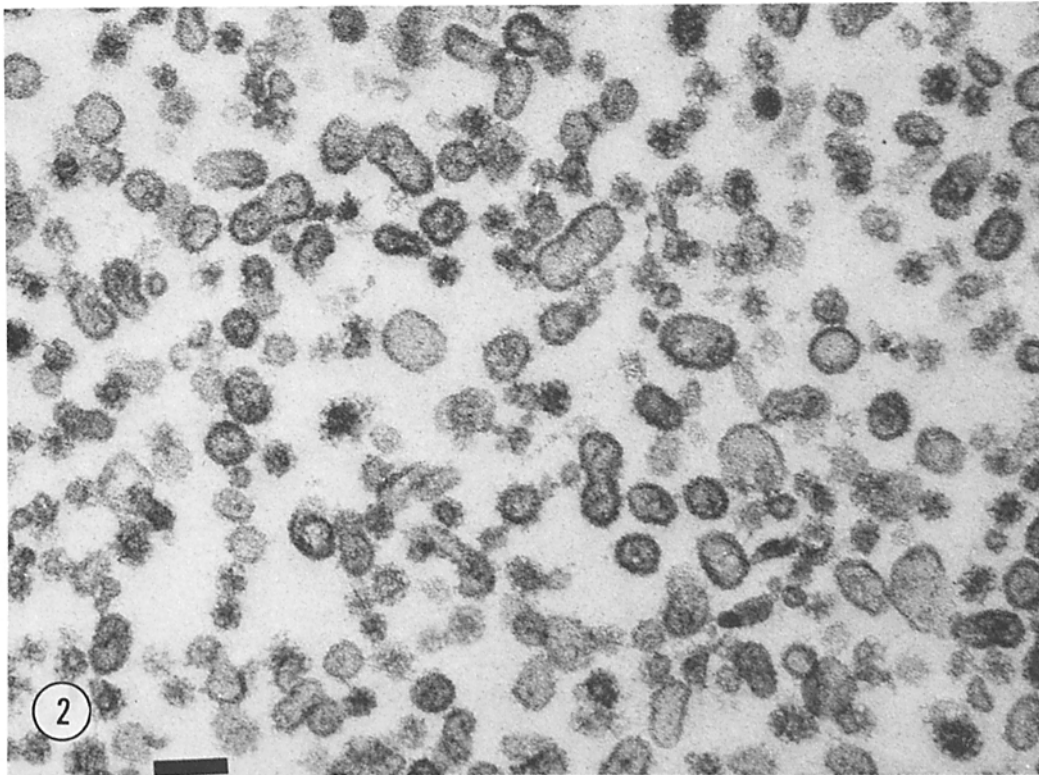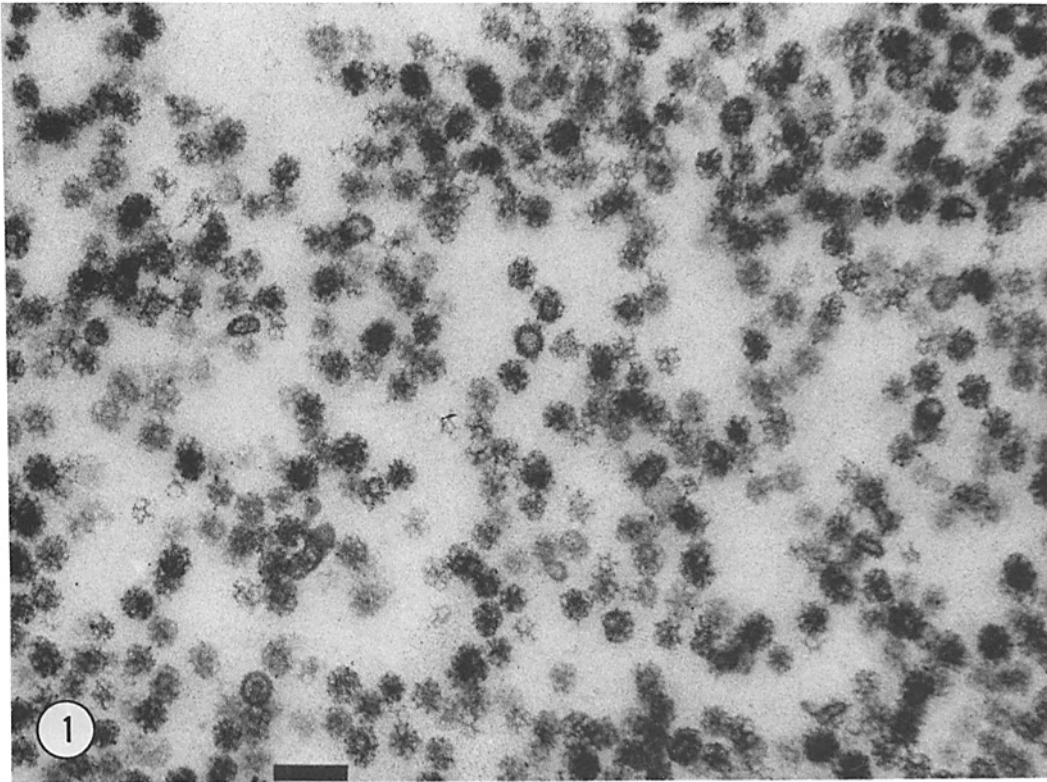
FIGURE 1 Electron micrograph of coated vesicles from rabbit brain. Fraction 1, × 100,000. Bar, 0.1 μm.

FIGURE 2 Electron micrograph of coated vesicles from rabbit brain. Fraction 2, × 100,000. Bar, 0.1 μm.
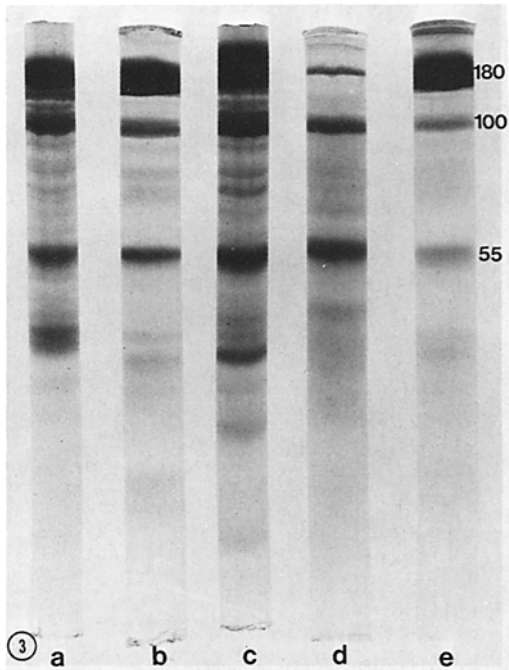
FIGURE 3 Polyacrylamide gels of coated vesicles from brain and urea-treated coated vesicles. Coated vesicles isolated from (a) calf brain, (b) rat brain, and (c) rabbit brain. The unfractionated coated vesicles from rabbit were suspended in 0.1 M MES; 1 mM EGTA; 0.5 mM MgCl$_2$; 0.02% sodium azide, pH 6.5, and were treated with 2 M urea followed by low-speed centrifugation to form a pellet (d) and a supernate (e). Each sample contained 40 μg of protein. The gels contained 7.5% acrylamide, 0.2% BIS, and 0.1% SDS. The gel and tank buffer was 0.1 M sodium phosphate, pH 7.2, and 0.1% SDS. The gels were stained with 0.25% Coomassie Blue R in 45% methanol, 10% acetic acid, and destained at 50°C. The gel samples were dissolved in 2% SDS, 5% mercaptoethanol, and heated at 100°C for 1~2 min.

subunit molecular weight of ~180,000 daltons. It is most likely clathrin, the coat subunit (25, 26). Two smaller proteins with ~100,000- and 55,000-dalton mol wt are also present, and each is estimated to represent ~10% of the total protein.

Acrylamide gels of coated vesicles from rabbit brain closely resemble gels of SR fragments isolated from rabbit skeletal muscle (Fig. 4a, b, c). Although, as expected, SR fragments contain no clathrin, they do contain proteins with ~100,000- and 55,000-dalton mol wt which co-migrate with the corresponding coated vesicle proteins. When SDS-solubilized coated vesicles and SR fragments are together subjected to electrophoresis (Fig. 4d, e), the resulting pattern is the same. Additional

bands do not appear, suggesting that the coated vesicle proteins and SR proteins have identical or nearly identical molecular weights.

The SR 100,000-dalton protein has been identified as a Ca-ATPase which is believed to catalyze the accumulation of calcium ions by SR fragments and by the intact SR (21). The 55,000-dalton SR protein is believed to be a calcium-binding protein (22).

In the remaining experiments, coated vesicles from calf brain were used.

Distribution of Proteins in Coated Vesicles
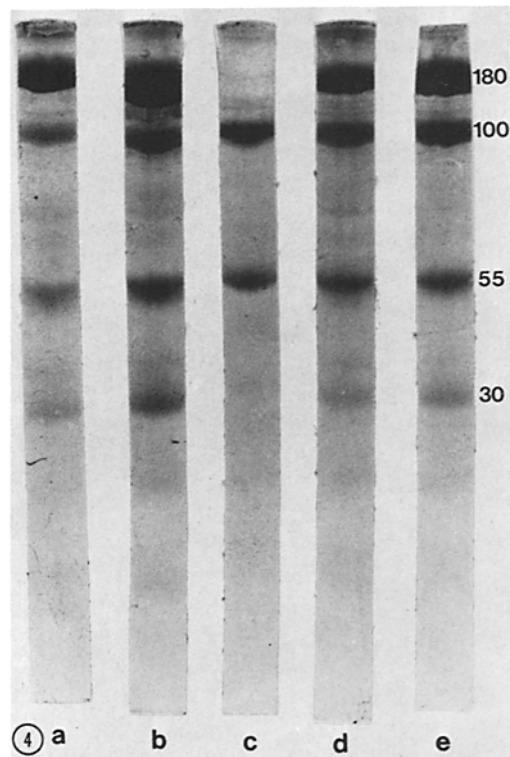
When intact, unfractionated coated vesicles sus-

FIGURE 4 SDS containing polyacrylamide gels of coated vesicle fractions from rabbit brain and SR fragments from rabbit skeletal muscle. (a) coated vesicle fraction 2, (b) coated vesicle fraction 1, (c) SR fragments, (d) mixture of coated vesicle fraction 2 plus SR fragments, and (e) mixture of coated vesicle fraction 1 plus SR fragments. Each sample contained 40 μg of protein, (d) and (e) contained 20 μg each of coated vesicle protein and SR protein. Electrophoresis was carried out as in Fig. 3, except the gel and tank buffer was 3% Tris, 6% glycine, and 0.1% SDS, pH 8.6.

pended in either buffered sucrose or in MES buffer were treated with 2 M urea, the suspension momentarily cleared, followed by the rapid appearance of a high molecular weight aggregate which could be pelleted by low-speed centrifugation. In electron micrographs (Fig. 5), this aggregate appears as uncoated membrane vesicles.

The protein composition of both the aggregate and the supernate, which remained after the aggregate was pelleted by centrifugation, is shown in Fig. 3 d, e. The bulk of the coat protein remains in the supernate, and a large portion of the lower molecular weight proteins remains associated with the uncoated vesicles which make up the aggregate. It has not been possible to resuspend this aggregate by a variety of methods, and therefore, this aggregate appears to consist of largely uncoated vesicles tightly bound to one another by unknown means.

These results suggest that the coat protein is extrinsically associated with the lipid bilayer which makes up the vesicle membrane. They further suggest that the lower molecular weight proteins are intrinsic membrane-associated proteins.

ATPase Activity Associated with Coated Vesicles

All preparations of unfractionated coated vesicles examined so far have contained ATPase activity. This activity displayed a sharp magnesium ion dependence with maximal activity at 0.5–0.6 mM $MgCl_2$ when assayed in the presence of 2 mM ATP (Fig. 6). Higher magnesium ion concentrations were markedly inhibitory.

The ATPase activity of intact unfractionated coated vesicles did not increase in the presence of added calcium ions (Fig. 7). However, when the coat protein was removed with urea, and.the aggregate was dissolved in 1.0% Triton X-100, calcium ion activation of the ATPase could be seen (Fig. 7). When the uncoated vesicles were assayed at 0.5 mM $MgCl_2$, maximal activity occurred at
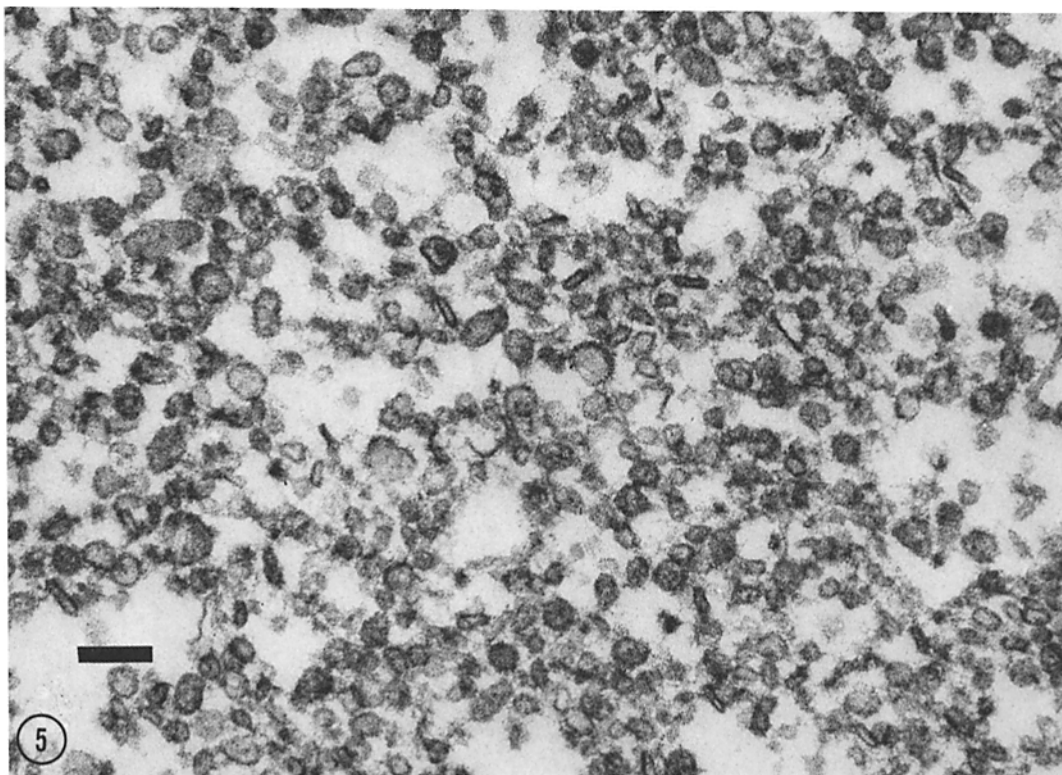

FIGURE 5   Electron micrograph of urea-treated coated vesicles. The coated vesicle suspension in MES buffer was treated with 2 M urea, the resulting aggregate was pelleted at 55,000 g for 30 min, and the pellet was washed once with extraction buffer, resuspended, and processed for electron microscopy as described in the text. × 100,000. Bar, 0.1 μm.
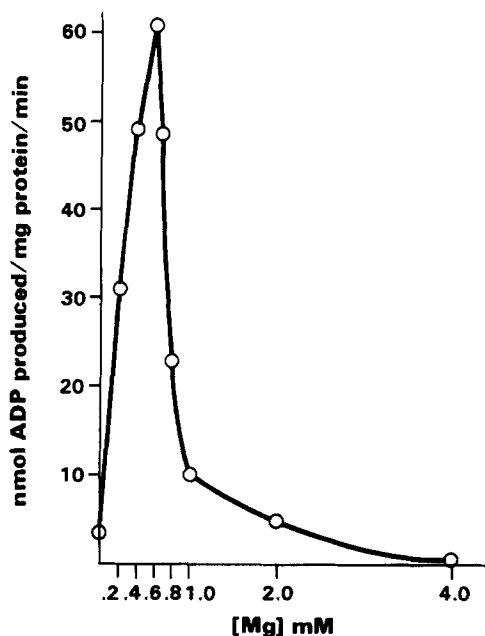
FIGURE 6 Magnesium dependence of the coated vesicle ATPase from calf brain (fraction 1 plus 2). The assay was carried out in 0.1 ml of 0.1 M KCl; 5 mM EGTA; 20 mM imidazole, pH 7.0; 2 mM disodium ATP plus [U-^{14}C]ATP (90,000 cpm/μmol); and 20 μg of coated vesicle protein for 20 min at room temperature. The amount of [U-^{14}C]ADP produced was determined as described in the text.

~0.8 μM calcium ion, and higher calcium ion concentrations were markedly inhibitory. The Ca-ATPase of SR is also inhibited at higher than optimal calcium ion concentrations (12).

When the ATPase activity of fractions 1 and 2 were separately examined (Table I), it was found that both fractions exhibited similar ATPase activity in the presence of Triton X-100 at 0.8 μM free calcium ion. In this case, the assay was carried out in the presence of higher magnesium ion concentration (2 mM) which was equimolar with the ATP concentration. Under these conditions, the apparent enhancement of the ATPase activity by calcium ions was magnified, as the activity in the absence of calcium was much reduced in the case of fraction 2 and undetectable with fraction 1. It should be noted that the calcium sensitivity of the SR ATPase is uniformly assayed with equimolar Mg^{++} and ATP, a condition which is presumed to be physiological.

Because inhibition by calcium ion is also a feature of the plasma membrane sodium-potassium-stimulated ATPase (11), we tested for the pres-

ence of this enzyme in our preparations. Evidence against its presence was the finding that the unfractionated coated vesicle ATPase was maximally active in 0.1 M KCl, and was not stimulated by replacing either a portion or all of the potassium ions with sodium ions. Furthermore, the activity was not inhibited by 0.1 mM ouabain. These determinations were carried out in the presence of 1.0% Triton X-100 to disrupt the vesicle mem-
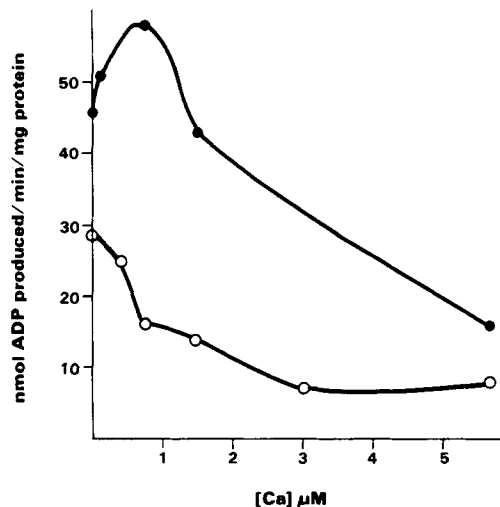

FIGURE 7 Calcium ion dependence of the ATPase of intact and uncoated, Triton-solubilized coated vesicles. The reactions were carried out as described in Fig. 6, except that 0.5 mM MgCl$_2$ was present. O, intact coated vesicles. ●, coated vesicles in extraction buffer treated with 2 M urea and pelleted by centrifugation. The pellet was dissolved in 0.3 M sucrose, 10 mM HEPES, pH 7.0, 0.1% Triton X-100 before assay.

TABLE I

ATPase Activity of Coated Vesicles

Additions	Fraction 1	Fraction 2
	nmol ATP split/mg protein/min	
None	ND*	21.5
Calcium	76.1	78.8

The reaction mixture contained 0.1 M KCl; 2 mM MgCl$_2$, 2 mM disodium ATP +[U-^{14}C]ATP (90,000 cpm/μmol); 5 mM EGTA; 2.2 mM CaCl$_2$ when present (free [Ca] equals 0.8 μM), 20 mM imidazole, pH 7.0, in a total volume of 0.1 ml. The reactions were initiated with coated vesicles, fraction 1 or 2, pretreated with 1.0% Triton X-100 equivalent to 20 μg of protein. After 20 min at room temperature, the reactions were stopped and the [U-^{14}C]ADP produced was determined as described in the text.

* Not detectable

brane, because ouabain and ATP act on opposite faces of the membrane-associated sodium–potassium-stimulated ATPase (11).

These results suggest that coated vesicles contain an ATPase which is similar to the one associated with the membrane of the SR. To obtain further evidence for a biochemical similarity between these enzymes, an antiserum directed against the 100,000-dalton calcium ATPase of SR was prepared. The 100,000-dalton coated vesicle protein, present in both fractions, formed a precipitate with this antiserum, as revealed by Ouchterlony double diffusion (Fig. 8). When the two proteins were placed in adjacent wells, a line of identity was formed. Neither the 180,000-dalton coat protein nor the 55,000-dalton protein formed a precipitate with the antiserum.

To obtain still further evidence that the 100,000-dalton protein is an ATPase, coated vesicles (fractions 1 plus 2) were allowed to briefly react with ATP labeled in the terminal phosphate with isotopic phosphorus. When this experiment was carried out by others on SR fragments, radiolabeled phosphate was found covalently bound to the 100,000-dalton ATPase (22). A similar result was obtained with unfractionated coated vesicles treated with urea and with Triton X-100 (Fig. 9). Several attempts to phosphorylate the 100,000-dalton protein of intact coated vesicles have failed. The radiolabel was consistently found associated with higher molecular weight material which remained at or near the top of the gel.

Calcium Uptake by Coated Vesicles

SR fragments accumulate calcium ions in vitro in the presence of ATP, and the amount of calcium ion which can be taken up is enhanced when sodium or potassium oxalate, calcium-trapping agents, are included in the medium (31). We have found that intact coated vesicles display similar behavior.

The determinations reported in Table IIa were obtained by two independent methods. In the first, calcium associated with unfractionated coated vesicles was separated from free calcium by millipore filtration. Because the coated vesicles are ~600 Å in diameter, and the filter pores average ~4,500 Å, the separation may depend upon binding of the vesicles to the filter. Because it was not certain that this binding was quantitative, an alternate procedure was also employed. Here the vesicles and associated calcium were separated from free calcium by gel filtration on
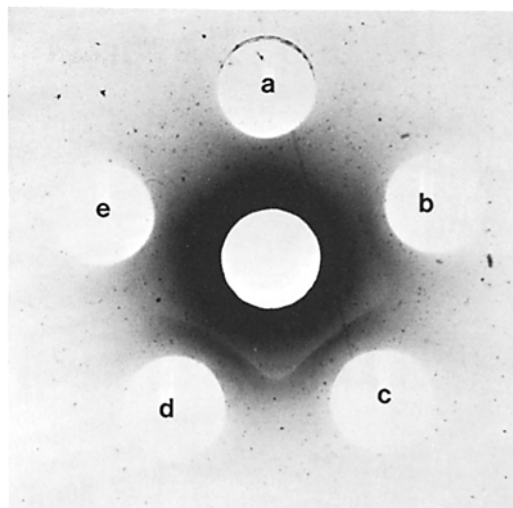

FIGURE 8 Double diffusion in agar, immunoprecipitate formation between anti-SR calcium ATPase and coated vesicle 100,000 mol wt protein. The central well contained rabbit anti-SR Ca-ATPase in saline. The outer wells contained: (a) coated vesicle 180,000 mol wt protein (45 μg); (b) 100,000 mol wt coated vesicle protein of rabbit brain (2.5 μg); (c) rabbit SR Ca-ATPase (5 μg); (d) 100,000 mol wt coated vesicle protein of rabbit brain (5 μg); (e) 55,000 mol wt coated vesicle protein of rabbit brain (50 μg). The outer well solutions were diluted to the indicated protein concentrations with saline containing 0.1% SDS. After 48 h at room temperature, the plate was stained with 1% thiazine red and destained with 1% acetic acid. The preparation of the antiserum and the antigens is described in the text.

Sephadex G-50. The results of the two determinations were in reasonable agreement. The amount of calcium associated with the vesicles increased 10- to 20-fold when ATP was present, and increased by an additional 5- to 10-fold when potassium oxalate was present as well. The results are consistent with an ATP-dependent calcium accumulation by the coated vesicles. They further suggest that active calcium accumulation, rather than calcium binding, was being measured.

The calcium uptake capacity of coated vesicle fractions 1 and 2, in the presence and absence of ATP, was also separately determined (Table IIb). Both fractions displayed similar calcium uptake in the presence of ATP. The results also illustrate the enhancement of calcium uptake when the concentration of free calcium in the medium was reduced from 2×10^{-4} M (Table IIa) to 2×10^{-5} M (Table IIb) by the inclusion of EGTA in the assay solution.
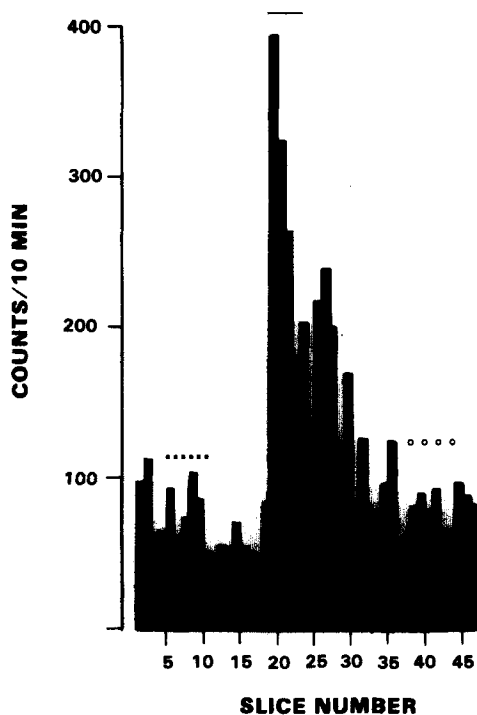
FIGURE 9 Phosphorylation of the coated vesicle 100,000-dalton protein. Urea-treated, Triton-solubilized coated vesicles were allowed to react with [γ-^{32}P]ATP, dissolved in SDS, and subjected to electrophoresis on polyacrylamide gels as described in the text. Radioactivity associated with 0.8-mm slices of the gel is displayed. The bar indicates the position of migration of the 100,000-dalton coated vesicle protein during gel electrophoresis , position of 180,000-dalton protein. Bar, position of 100,000-dalton protein. O, position of 55,000-dalton protein.

DISCUSSION

Coated Vesicles and SR Fragments Compared

Our evidence strongly suggests that the membrane-associated proteins of the coated vesicle isolated from brain, and the SR fragment isolated from skeletal muscle are closely related. The polypeptide composition of the two organelles, except for the presence of clathrin in coated vesicles, are indistinguishable when examined by polyacrylamide gel electrophoresis in the presence of SDS. The 100,000-dalton proteins associated with both organelles display immunological cross-reactivity as revealed by Ouchterlony double-diffusion analysis. With both organelles, this protein can be phosphorylated by [γ-^{32}P]ATP. In the case of SR, this protein is known to be the Ca-ATPase (22).

Biochemically, the two organelles are similar in that they both display calcium uptake which is enhanced by ATP and further enhanced by the calcium-trapping agent, potassium oxalate. SR fragments will accumulate 150-300 nmol Ca^{++}/mg protein in the presence of ATP (4, 16). Our values of 22.2 and 21.3 nmol Ca^{++}/mg protein for coated vesicle fractions 1 and 2 are 10% of the reported values for SR fragments. Our values were obtained when the free calcium concentration in the medium was 2×10^{-5} M. At a 10-fold higher free calcium concentration, the calcium uptake was reduced, most likely because the Ca-ATPase of the vesicles was strongly inhibited at elevated calcium concentration.

When assayed in the presence of equimolar Mg^{++} and ATP, the ATPase activity associated with Triton-treated coated vesicle fractions 1 or 2 was activated by calcium ions. In the case of fraction 2, the activity increased about four times

TABLE II

Calcium Uptake by Coated Vesicles of the Brain

Additions	Procedure 1	Procedure 2
	nmol Ca/mg protein	
(a)		
None	0.20	0.45
ATP	5.60	4.0
ATP + K$^+$ oxalate	53.0	21.5
	Fraction 1	Fraction 2
(b)		
None	3.5	ND*
ATP	22.2	21.3

(a) Unseparated coated vesicles equivalent to 50 μg were incubated in 0.13 M KCl; 20 mM imidazole, pH 7.0; 2 mM MgCl$_2$; 2 mM ATP when present; 10 mM potassium oxalate when present, and 0.2 mM CaCl$_2$ + ^{45}CaCl$_2$ (9.5 $\times$ 10^7 cpm/μmol) in a total volume of 0.1 ml for 10 min at room temperature. The mixtures of the vesicles and associated calcium ion were separated from free calcium ion by ultrafiltration through membranes (procedure 1) or by gel filtration (procedure 2) as described in the text.

(b) Coated vesicles, fraction 1 or 2, equivalent to 180 μg of protein were incubated as described above, except that 0.2 mM EGTA was included in the reaction mixture. The free calcium ion concentration was 2×10^{-5} M. Procedure 2 was used. Fraction 1, coated fraction recovered from the 10-20% sucrose interface of the third sucrose gradient employed in the purification procedure. Fraction 2, the fraction containing uncoated and coated vesicles recovered from the 20-30% sucrose interface of the above gradient.

* Not detectable

when 0.8 μM calcium ion was added. In the case of fraction 1, the activity in the absence of added calcium was undetectable under these conditions. At a lower magnesium concentration (0.5 mM), the apparent calcium activation was much lower because the activity in the absence of calcium was considerably higher. It is difficult to compare the calcium sensitivity of the SR ATPase with these values, because widely differing assay conditions have been employed, but values of 5- to 10-fold activation by calcium ion have been reported (9).

In some respects, the coated vesicle ATPase differs from the SR ATPase. First, the specific activity of the coated vesicle ATPase is considerably lower. We found values near 70 nmol ATP split/mg protein per min at optimal calcium concentration (0.8 μM). A value of 250 nmol ATP split/min per mg protein has been reported for the SR ATPase near this calcium concentration (5). At higher calcium ion concentration, where the coated vesicle ATPase is inhibited, SR displays a higher activity. However, it is likely that the specific activity of the coated vesicle ATPase compares more favorably with the specific activity of the SR ATPase than these data would imply. This is because, in SR, the Ca-ATPase comprises $\sim$ 70% of the total protein. In contrast, the 100,000-dalton coated vesicle protein comprises only $\sim$ 10% of the total protein.

Perhaps the most significant difference between the two enzymes is the total insensitivity of the coated vesicle ATPase to calcium ions when assayed in the absence of Triton X-100. We do not yet understand why Triton treatment is necessary to unmask the calcium sensitivity. Significantly, however, Inesi et al. (13) have recently reported the isolation of an SR fraction which has maximal ATPase activity in the absence of Ca^{++}, whereas upon addition of Triton X-100, the ATPase activity in the absence of Ca^{++} is very low, returning to its original value upon addition of Ca^{++}.

The Calcium-Sequestering Membrane is a Constituent of Coated Vesicles, not a Contaminant

The finding that the coated vesicle fraction isolated from the second sucrose gradient contains both coated vesicles and smooth vesicles suggested the possibility that the calcium-sequestering membrane is a contaminant, possibly resulting from the co-isolation of coated vesicles, and a neuronal or glial microsomal membrane fraction. We believe

that this possibility is unlikely for the following reasons. (a) Fractions 1 and 2 isolated from the third sucrose gradient contain vesicles having the identical polypeptide composition, even though fraction 1 almost entirely consists of coated vesicles and empty coats, and fraction 2 contains, in addition, a considerable number of uncoated vesicles. (b) Both fractions 1 and 2 display similar Ca^{++} ATPase and Ca^{++} uptake activities. (c) Coated vesicles isolated from pancreas, adrenal medulla, and parotid glands, all calcium-dependent secretory tissues, contain membranes having the identical peptide compositions as brain coated vesicles.[1] These vesicles also have ATPase activity.

A possible explanation for the frequent larger, dumbbell-shaped smooth vesicles seen in fraction 2 is that they have arisen during the isolation procedure by fusion of coated vesicles and subsequent loss of coat material. This process occurs in vivo in several cell types (3, 10, 18). Consistent with this explanation, we have also observed that coated vesicles are unstable. Upon prolonged storage, larger uncoated vesicles are seen in greater numbers. Because all the presumed functions of coated vesicles depend on their ability to undergo fusion with or fission from other cell membranes, this instability in isolated preparations is not unexpected.

Possible Functions of the Coated Vesicle in Calcium-Associated Processes

Perhaps the most obvious possible functions of coated vesicles would be the sequestering of calcium ions, thereby helping to reduce the calcium level after neurotransmitter release triggered by calcium influx, thus preventing uncontrolled exocytosis. Consistent with this possibility, evidence has recently been obtained that isolated presynaptic nerve terminals contain an ATP-dependent nonmitochondrial calcium storage system which is stimulated by oxalate (17).

It is of interest here that coated vesicles increase in number in frog motor nerve terminals after prolonged stimulation (10). This phenomenon has been interpreted as a mechanism by which synaptic vesicle membrane is recaptured from the presynaptic membrane and recycled into synaptic vesicles (10). In light of our results, this increase in the coated vesicle number could also serve as a response to the elevated cytoplasmic calcium concentration which may result from prolonged nerve stimulation (19).

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[1] Fine, R., and A. Blitz. Unpublished results.

Finally, calcium uptake by coated vesicles may be involved in fusion with other cellular membranes. There is considerable evidence that calcium promotes fusion of various types of membranes in vitro (8, 27) and in vivo (30). Whether or not transmembrane movement of calcium is involved in this type of fusion has not yet been established in most cases, although this may occur during the fusion of human erythrocyte ghosts (33).

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