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Structure of the Novel Membrane-coating Material in Proton-secreting Epithelial Cells and Identification as an H^+ATPase

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Abstract. Specialized proton-secreting cells known collectively as mitochondria-rich cells are found in a variety of transporting epithelia, including the kidney collecting duct (intercalated cells) and toad and turtle urinary bladders. These cells contain a population of characteristic tubulovesicles that are believed to be involved in the shuttling of proton pumps (H^+ATPase) to and from the plasma membrane. These transporting vesicles have a dense, studlike material coating the cytoplasmic face of their limiting membranes and similar studs are also found beneath parts of the plasma membrane. We have recently shown that this membrane coat does not contain clathrin. The present study was performed to determine the structure of this coat in rapidly frozen and freeze-dried tissue, and to determine whether the coat contains a major membrane protein transported by these vesicles, a proton pumping H^+ATPase. The structure of the coat was examined in proton-secreting, mitochondria-rich cells from toad urinary bladder epithelium by rapidly freezing portions of apical membrane and associated cytoplasm that were sheared away from the remainder of the cell using polylysine-coated coverslips. Regions of the underside of these apical membranes as large as 0.2 μm^2 were decorated by studlike projections that were arranged into regular hexagonal arrays. Individual studs had a diameter of 9.5 nm and appeared to be composed of multiple subunits arranged around a central depression, possibly representing a channel. The studs had a density of ~16,800 per μm^2 of membrane. Similar arrays of studs were also found on vesicles trapped in the residual band of cytoplasm that remained attached to the underside of the plasma membrane, but none were seen in adjacent granular cells. To determine whether these arrays of studs contained H^+ATPase molecules, we examined a preparation of affinity-purified bovine medullary H^+ATPase, using the same technique, after incorporation of the protein eluted from a monoclonal antibody affinity column into phospholipid liposomes. The affinity-purified protein was shown to be capable of ATP-dependent acidification. In such preparations, large paracrystalline arrays of studs identical in appearance to those seen in situ were found. The dimensions of the studs as well as the number per square micrometer of membrane were identical to those of toad bladder mitochondria-rich cells: 9.5 nm in diameter, 16,770 per μm^2 of membrane. Finally, we used affinity-purified polyclonal antibodies against the 70- and 56-kD subunits of the protein for immunocytochemical localization in sections of tissue; the stud-coated membrane regions in kidney intercalated cells were heavily labeled with protein A–gold. We conclude that the coat material associated with plasma membrane segments and with a population of transport vesicles in these proton-secreting epithelial cells contains protein subunits that are closely related to, or are an actual part of the cytoplasmic domain of the H^+ATPase molecule.

To maintain the functional polarity of epithelial cells, membrane proteins such as transporting enzymes, ion channels, and hormone receptors must be selectively inserted into and removed from specific membrane domains at the cell surface. This requires the concerted interaction of exocytotic and endocytotic events involving different classes of carrier vesicles that transport defined cargoes to precise destinations in the cell. Of these vesicles, those with clathrin coats have been the most extensively studied, and their role in the endocytosis of membrane proteins as well as in the selective intracellular transport of macromolecules is well established (6, 36). However, it has become clear that not all vesicle coats are composed of clathrin, indicating that other components may be involved in the regulation of intracellular traffic (39). It has recently been shown that non-clathrin-coated vesicles are involved in protein transport through the Golgi apparatus (31). In kidney collecting duct intercalated cells (and related cells in other transporting epithelia), we

have found a specialized population of coated vesicles lacking clathrin (3) that are involved in the shuttling of membrane components, including an H^+ATPase, to and from the plasma membrane in response to different physiological conditions (13, 26, 27, 44, 46). It is not known whether these novel membrane-coating materials are formed of the cytoplasmic domains of molecules transported by vesicles, or whether the coat is involved in vesicle function, by analogy with clathrin.

In this report, we have examined the membrane-coating material found in mitochondria-rich proton-secreting cells from the toad urinary bladder (cells that are structurally and functionally analogous to kidney intercalated cells) by rapid-freeze, freeze-drying technology, and have compared its structure with that of a protein complex incorporated into phospholipid liposomes, which was affinity-purified using a monoclonal antibody raised against bovine medullary H^+ATPase. In both cases we found striking hexagonal arrays of studs 9.5 nm in diameter that were morphologically identical. Using affinity-purified polyclonal antibodies against 70- and 56-kD subunits of the purified protein, we have, in addition, demonstrated that these subunits are localized in the studlike coating material underlying regions of the kidney intercalated cell membrane. These results provide strong evidence that the coat associated with plasma membranes and a population of transport vesicles in specialized proton-secreting epithelial cells contains the cytoplasmic domains of protein subunits that are associated with the H^+ATPase molecule.

Materials and Methods

Animals

The toads used in this study were adult female *Bufo marinus*, obtained from National Reagent, Bridgeport, CT. They were kept on damp wood shavings at room temperature until death. Male Sprague-Dawley rats had access to food and water ad libitum before death.

Thin Sections

Partially stretched bladders, excised from doubly pithed toads, were fixed by immersion in 4% glutaraldehyde containing 0.1% tannic acid, in 0.1 M sodium phosphate buffer, pH 7.4. After rinsing in buffer alone, bladders were postfixed for 1 h in 2% OsO_4 in distilled water, dehydrated in ethanol, and embedded in LX-112 embedding medium (Ladd Research Industries, Inc., Burlington, VT). Sections were stained with uranyl acetate and lead citrate before examination.

After a short rinse of the circulation with Hank's balanced salt solution, rats were perfused with 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4, via the left ventricle, for 10 min. After this time, pieces of kidney were fixed for 1 h more by immersion in the tannic acid/saponin/glutaraldehyde solution described by Maupin and Pollard (28). Further processing for electron microscopy was as described above.

Rapid Freezing and Freeze-drying of Apical Membrane Fragments from Toad Bladder Epithelium

Glass coverslips 5 mm in diameter were coated with 1% hydrobromide polylysine (M_r 70,000), and air-dried. The mucosal surfaces of toad bladders were lightly brushed with filter paper to remove the mucous layer, and were adhered to these coverslips at room temperature for 30 s, after which the coverslip was removed, washed briefly for 30 s in 60 mM Pipes, 25 mM Hepes, 10 mM EGTA, and 2 mM $MgCl_2$, pH 6.9 (40), and then fixed in 10 mM sodium phosphate buffer containing 1% glutaraldehyde and 0.2% tannic acid, pH 7.0, for 10 min. Some specimens were incubated for 10 min with 2 mg/ml myosin subfragment 1 in the above buffer, containing 10% glycerol, before fixation as described by Hartwig and Shevlin (19). After fixation, salt was removed from the specimens by washing in distilled water. Coverslips with adherent apical membrane fragments and associated cytoskeletal elements were prepared for the electron microscope by rapid freezing and freeze-drying. Coverslips were placed in 15% methanol, mounted on the stage of the plunger arm of the rapid freezing device (Med-Vac, Inc., St. Louis, MO), blotted to near dryness with filter paper, and then frozen on a liquid helium-cooled copper block as described by Heuser and Kirschner (20). Frozen specimens were freeze-dried in a freeze-fracture apparatus (model 400; Balzers Union, Hudson, NH) at a stage temperature of −80°C for 45 min and then rotary coated with platinum at a 25° angle and with carbon at 60°. To separate the replicas from glass, the coverslip was dissolved in 25% hydrofluoric acid, transferred to bleach for 1 h, washed in distilled water, and picked up on carbon/formvar-coated copper grids. The replicas were examined and photographed at 100 kV in a JEOL 1200-EX electron microscope.

Freeze-etching of Reconstituted H^+ATPase-containing Liposomes

Phospholipid vesicles into which an H^+ATPase protein complex affinity purified from bovine kidney medulla had been incorporated (see details below) were placed on the surface of polylysine-coated coverslips (5 μl per coverslip) and allowed to settle for 1 or 2 min. The coverslips were then washed sequentially in inside buffer containing 60 mM Pipes, 25 mM Hepes, 10 mM EGTA, and 2 mM $MgCl_2$, pH 6.9, distilled water, and 15% methanol, and then rapidly frozen and processed as described above.

Morphological Measurements

To determine the packing density of the studs and the diameter of individual studs, hexagonally packed studs in flat areas of membrane were selected. The area of membrane was determined using a PDP 11/73 (19) and all studs within the area were counted. The number of studs per square micrometer was calculated by dividing the total membrane area by the number of studs. A total of six areas containing 784 and 1,023 studs were analyzed from mitochondria-rich cell membranes and reconstituted liposomes, respectively. Stud diameter was measured with a micrometer eyepiece.

Purification of Bovine Medullary H^+ATPase

H^+ATPase was affinity purified from a bovine kidney medulla extract (10) on a Sepharose column to which a monoclonal antibody against a bovine medullary H^+ATPase (15) had been covalently coupled (see below). The eluate from the column was a multisubunit protein whose subunit composition was virtually identical to that obtained using a separation procedure based on conventional and HPLC chromatography (11). Details of the production of the monoclonal antibody, and the affinity purification procedure and characterization of the enzyme are described elsewhere (11, 12, 53).

Incorporation of H^+ATPase into Liposomes

Approximately 250 μg of protein was eluted from the affinity column with 50 mM glycine, 5 mM sodium azide, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1 propanesulfonate (CHAPS), 0.1% nonyl glucoside, pH 2.5 in a volume of 3 ml, and neutralized immediately with 1 M Tris. The solubilized protein was mixed with 250 μg of soybean phospholipid (23), solubilized in 1% CHAPS, and dialyzed overnight against PBS. The liposomes were stored at 4°C in PBS. Before rapid freezing, liposomes were fixed for 10 min by the addition of sufficient 25% glutaraldehyde to an aliquot of liposomes to give a final concentration of 2%. Some liposomes were rapidly frozen without prior fixation. The structure of fixed and nonfixed material was identical.

Reconstitution of Proton Transport

H6.1, a monoclonal antibody specific for the kidney vacuolar H^+ATPase (53) was purified from mouse ascites fluid and coupled covalently at 3 mg/ml to protein A–Sepharose beads as previously described (41). 50 μl of H6.1 protein A–Sepharose beads was incubated with 1 ml of bovine kidney medulla microsomes (15) solubilized in 10 mM Tris-Cl, 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.6% CHAPS, 1.5% nonyl glucoside, and 10% glycerol, pH 7.0. The beads were washed twice with 1 ml of 10 mM Tris-Cl, 5 mM sodium azide, 1% CHAPS, pH 7.0. 50 μl of the solubilized phospholipid was added to the beads, and then 1 ml of 10 mM Tris-Cl, 10 mM 2-(*N*-morpholino)ethane sulfonic acid (MES), 150 mM KCl, pH 7.0, was added to allow liposome formation by dilution. A final concentration of 2

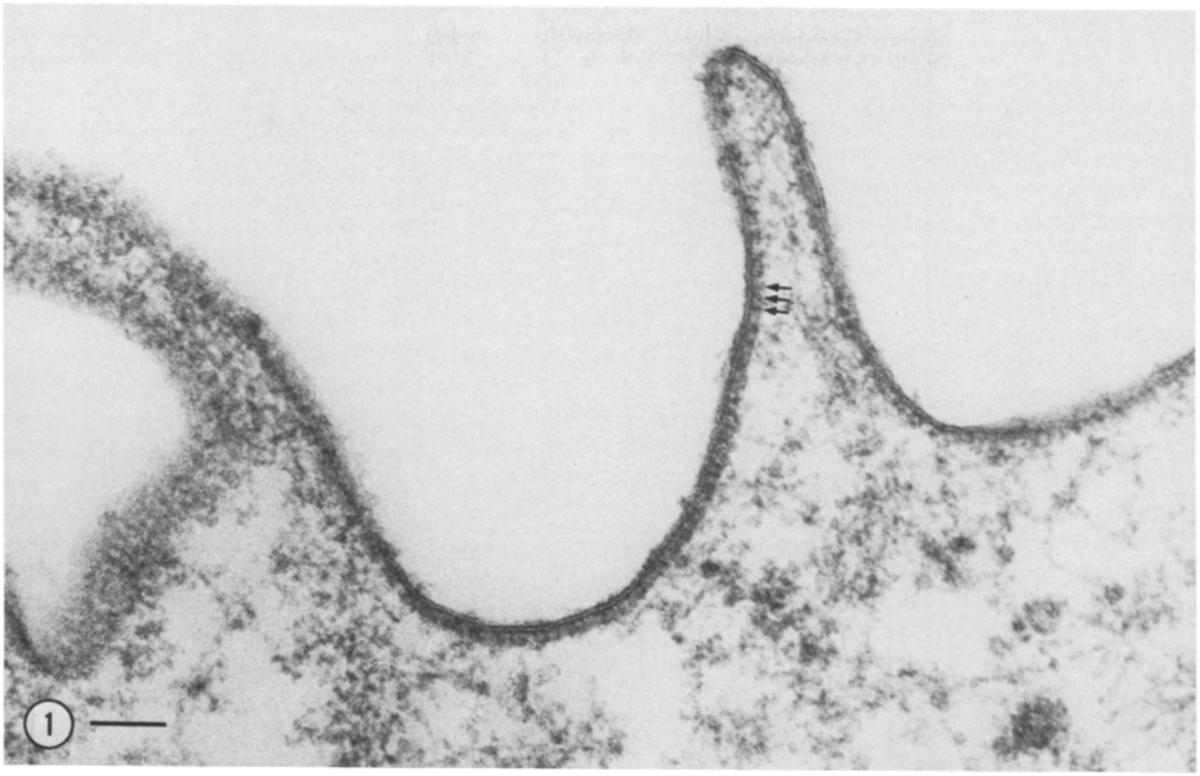


Figure 1. Thin section of the apical region of an intercalated cell from rat kidney medulla (inner stripe), fixed using the glutaraldehyde/tannic acid/saponin procedure (28). The cytoplasmic side of the apical plasma membrane is decorated by a coat of electron-dense studs (*arrows*). Identical studs are found to be associated with regions of the plasma membrane and cytoplasmic vesicles in toad bladder mitochondria-rich, proton-secreting cells (47). Bar, 0.1 μm.

mM $MgCl_2$ was added, and the entire sample was transferred to a cuvette. To measure directional proton transport, 10 μM acridine orange and 1 μM valinomycin were added, and the sample was placed in a dual wavelength spectrophotometer monitoring the 492–540-nm absorbance difference. Proton transport was initiated by addition of 1 mM ATP.

Preparation of Polyclonal Antibodies to H^+ATPase

Immunoaffinity-purified H^+ATPase was suspended at 1 mg/ml in PBS, mixed 1:1 with keyhole limpet hemocyanin (KLH), 1 mg/ml in PBS, and 0.5 mg of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide was added. The mixture was left at room temperature overnight, and was then dialyzed exhaustively against PBS at 4°C. 1 mg (1 ml) of the coupled protein was mixed with 1 ml Freund's complete adjuvant, and New Zealand White rabbits were immunized with 2 ml of suspension administered in 20 separate intradermal injections. After 1 m, animals were boosted with 2 ml of the KLH-coupled antigen in incomplete adjuvant, and were given booster immunizations every 3 wk thereafter. Serum was screened by immunoblotting (51); the antisera used in this study were obtained after 6–9 mo of immunization. Specific antibodies against the 70- and 56-kD subunits were prepared from the serum by using strips cut from nitrocellulose filters containing specific subunit bands separated by SDS-PAGE as immunological dipsticks, as previously described (2). The specificity of the antibodies prepared in this way was demonstrated by Western blotting against the affinity-purified, SDS-denatured enzyme and bovine kidney microsomes. In Fig. 11, the specificity of the anti-70-kD subunit polyclonal antibody (used for immunolabeling in Figs. 9 and 10), is demonstrated.

Immunocytochemistry

Rat kidneys were perfusion-fixed via the left ventricle with a paraformaldehyde/lysine/periodate (PLP) fixative (29). After 10 min perfusion, small pieces of kidney were fixed overnight by immersion at 4°C in the same fixative.

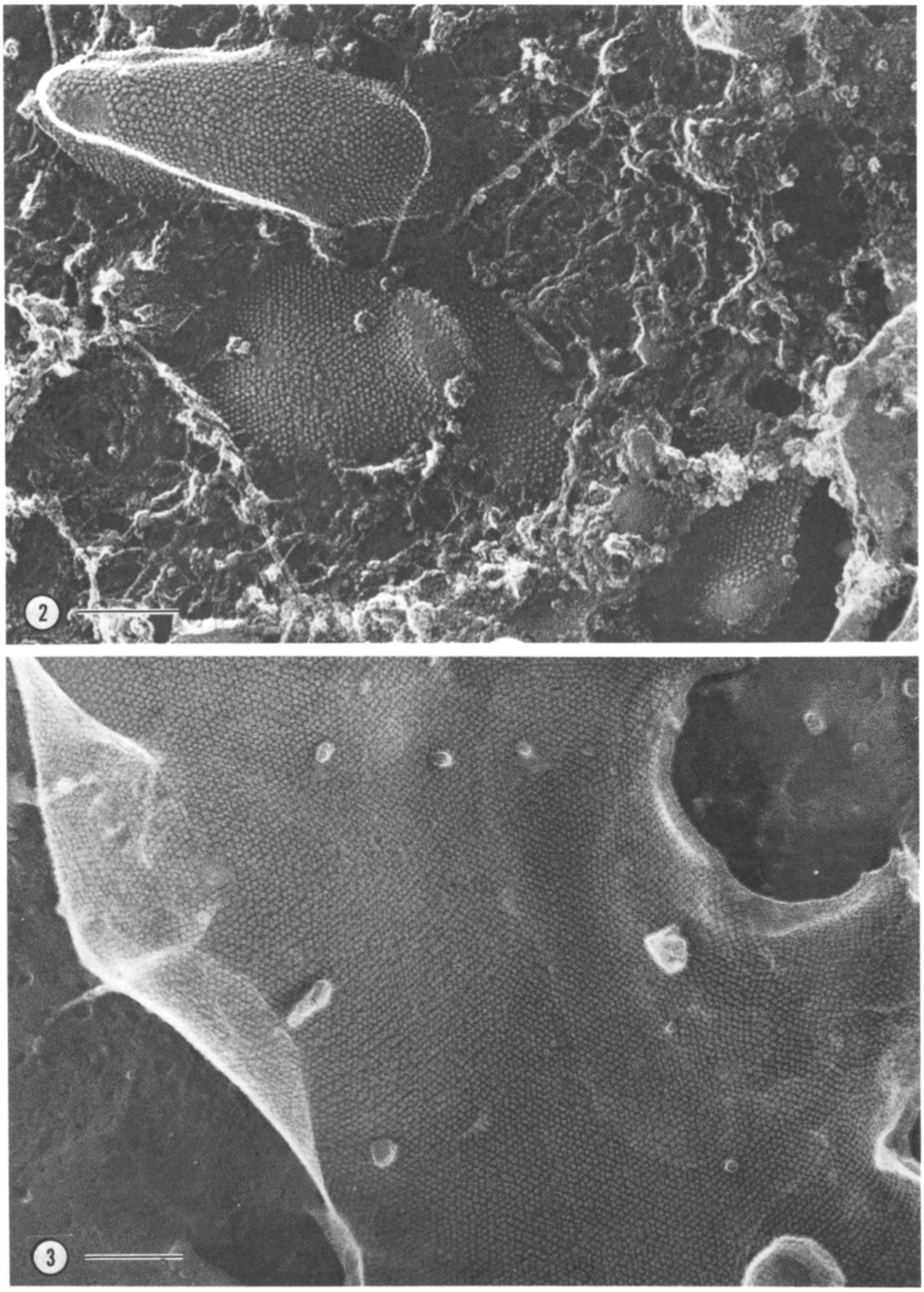
Electron Microscopy. Samples were then dehydrated in dimethylformamide and embedded in Lowicryl K4M by the rapid procedure described previously (1). Thin sections were picked up on nickel grids and used for immunocytochemistry. Sections were incubated for 10 min on a drop of PBS (0.9% NaCl in 10 mM sodium phosphate buffer, pH 7.4), followed by 10 min in PBS containing 1% BSA. The sections were transferred to 10-μl drops of undiluted affinity-purified antibody, specific for either the 70- or the 56-kD subunit of the H^+ATPase. After a 2-h incubation at room temperature, in a moist chamber, sections were rinsed three times for 5 min by flotation on drops of PBS, and transferred to 20-μl drops containing protein A bound to 8-nm gold particles (45). The concentrated stock solution of protein A–gold was diluted 1:70 in PBS containing 1% BSA. After 1 h at room temperature, sections were washed twice for 5 min with PBS, and 5 min with distilled water. The sections were stained for 7 min with 2% aqueous uranyl acetate and 1 min with lead citrate before examination in a JEOL 1200-EX electron microscope.

Light Microscopy. PLP-fixed kidneys were dehydrated in ethanol without prior osmification, and then embedded in LX-112 resin. Semithin (1 μm) sections were treated for 2 min with a mixture of 2g KOH, 5 ml propylene oxide, and 10 ml methanol to remove resin, and after rinsing in PBS/methanol (50:50) and PBS alone, were incubated for 2 h at room temperature with 20 μl of the specific anti-70-kD subunit polyclonal antibody. After three 5-min rinses in PBS, a biotinylated goat anti-rabbit IgG (Vector Laboratories Inc., Burlingame, CA) was applied to the sections for 1 h. Antigenic sites were then revealed by the ABC procedure (Vector Laboratories). After sites of peroxidase activity were revealed, sections were dehydrated in ethanol and mounted in Permount (Fisher Scientific Co., Fair Lawn, NJ). Photographs were taken on Kodak Tri-X Pan film using Plan Neofluar objectives on a Zeiss Ultraphot III microscope.

Results

Thin Sections of the Membrane Studs

As previously described, the mitochondria-rich cells of toad urinary bladder and kidney intercalated cells contain a popu-



Figures 2 and 3. (Fig. 2) Replica of a rapidly frozen, freeze-dried toad bladder apical plasma membrane that was torn from the remainder of the cell with a polylysine-coated coverslip. Coverslips were briefly washed in an intracellular buffer (40), fixed, rapidly frozen against

lation of tubulovesicles in their apical cytoplasm that have an unusual appearance in thin sections (3, 16, 26, 27, 47, 48). The cytoplasmic leaflet of the vesicle membrane is lined with arrays of dense, club-shaped projections, or studs and portions of the apical plasma membrane of these cells have similar arrays of studs (Fig. 1). The membrane studs in toad bladder mitochondria-rich cells (not shown) are identical to the novel membrane-coating material we have previously described (3) in proton-secreting, intercalated cells of mammalian kidney. In thin sections, the studs are 10–12 nm in diameter and project ~12 nm from the plasma membrane.

Structure of the Membrane Studs in Toad Bladder Seen by Rapid Freezing and Freeze-drying

Apical surfaces of toad bladders were adhered to poly-L-lysine–coated coverslips, torn off, and subjected to rapid freezing with a helium-cooled copper block, followed by freeze-drying (20). Rotary-shadowed replicas of the cytoplasmic side of the apical surface were examined in the electron microscope. Apical membranes from mitochondria-rich cells were readily identified by their small size compared with adjacent granular cells, and by their distinctive highly convoluted structure. In these cells, a striking membrane feature was present. Large regions of the underside of the apical plasma membrane were decorated by studlike projections, often arranged into regular, hexagonal arrays (Fig. 2). As shown in stereo in Fig. 4, individual studs appeared to have a central depression, which possibly corresponds to a central channel, and many studs appeared to be formed of smaller subunits arranged around this central depression. Stereo viewing also reveals that the studs have a symmetrical shape (i.e., they are as tall as they are wide), and that they can be interconnected near their bases by thin strands of material (Fig. 4). The diameter of the studs was 9.5 nm, and their density was 16,800/μm^2 of membrane area. In addition to plasma membrane–associated studs, similar structures were found on vesicles trapped in the residual band of apical cytoplasm that remained attached to the underside of the plasma membrane after the ripping procedure (Fig. 6). In some cases, these vesicles appeared to have been fixed during the process of fusion with noncoated membrane segments, possibly the plasma membrane.

This membrane-coating material corresponds to the dense studs seen in thin sections of these cells, and has a structure that, as previously demonstrated by conventional electron microscopy (3) is quite different from that of clathrin. A clathrin-coated vesicle from the same cell type is shown at the same magnification, for comparison, in the inset of Fig. 6.

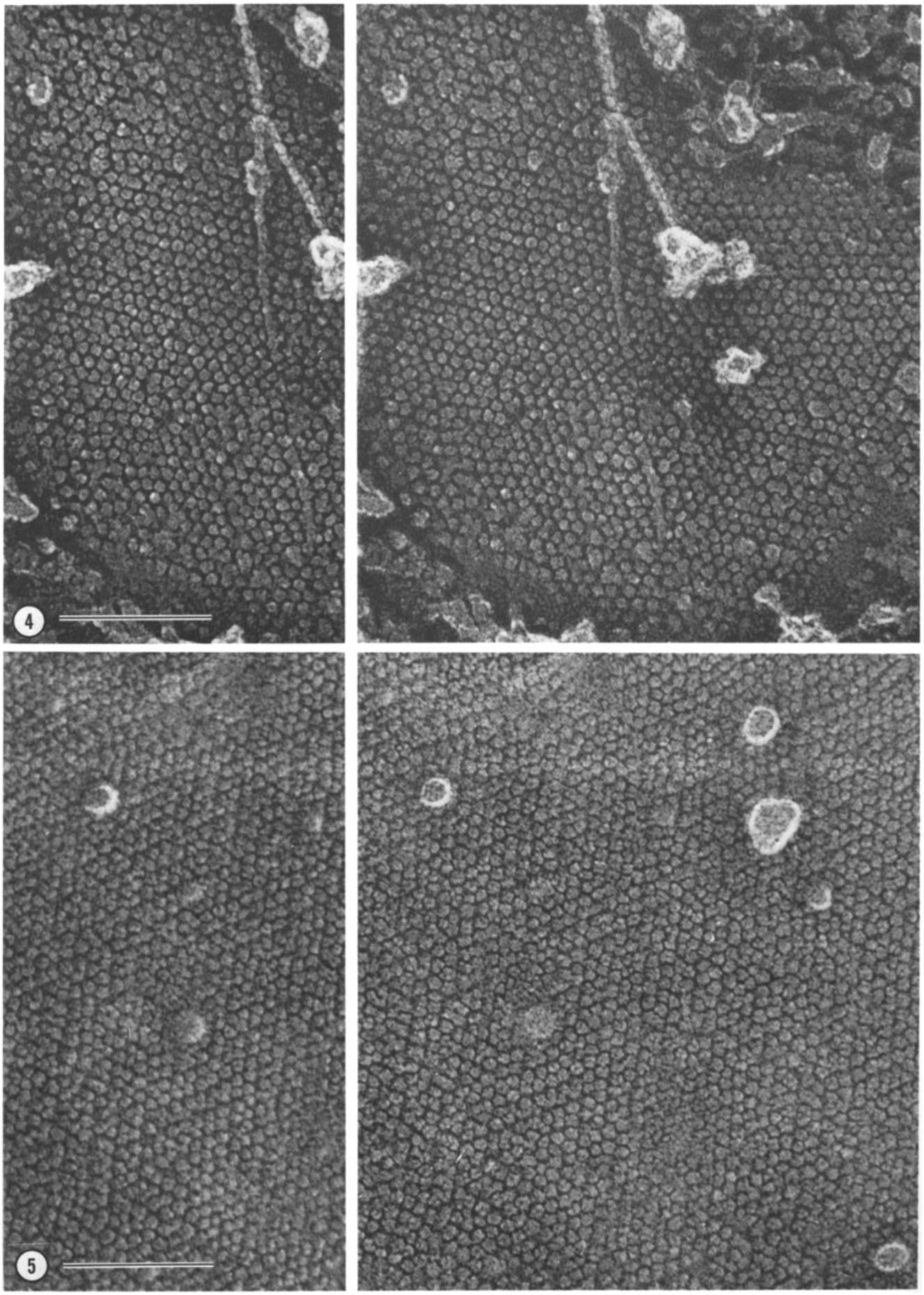
Structure of H^+ATPase in Liposomes

Because the studded tubulovesicles of specialized proton-secreting cells are believed to transport proton pumps to and from the apical plasma membrane, we explored the possibility that the studs represent the cytoplasmic domains of H^+ATPase molecules. We examined directly, by rapid freezing, the structure of a protein complex, incorporated into liposomes, that had been affinity purified on a column prepared from monoclonal antibodies raised against partially purified bovine medullary H^+ATPase. This multisubunit protein was capable of ATP-dependent acidification and potential generation when reconstituted into liposomes while still bound to the immunoaffinity beads (Fig. 8; reference 12). The purified protein was reconstituted into phospholipid liposomes at a protein/lipid ratio of 1:1. Both unfixed liposomes, and liposomes fixed in 2% glutaraldehyde, were rapidly frozen, freeze-dried, and rotary shadowed. The resulting replicas showed studlike projections on the liposomes (Figs. 3, 5, and 7). In many cases, large sheets of hexagonally packed studs were found (Fig. 3) in addition to vesicles coated with the studs (Fig. 7). These arrays of studs were identical to those found in situ in toad bladder mitochondria-rich cell membranes. Studs had a diameter of 9.5 nm, and were present in the paracrystalline arrays at a density of 16,800 per μm^2, exactly the same values as seen for the in situ bladder preparation.

Immunocytochemical Localization of H^+ATPase

The relationship between the 70- and 55-kD components of the purified H^+ATPase and the membrane-coating studs was investigated using subunit-specific IgGs (see Fig. 11) and protein A–gold immunocytochemistry on thin sections of kidney. As shown in Fig. 9, dense gold particle labeling was found at the level of the apical plasma membrane of medullary (inner stripe) intercalated cells; in sections perpendicular to the membrane, gold label was found directly over the band of membrane-coating material. The cytoplasmic side of the stud-coated intracellular vesicles was also labeled with gold particles (not shown). We have previously reported that the individual studs in K4M-embedded sections are difficult to discern. Instead, the coat appears as a continuous dense band on the cytoplasmic side of the membrane (3). As shown in Fig. 9, the labeling of mitochondria is no greater than background, indicating that our antibodies do not cross-react with the mitochondrial F_0 F_1 ATPase. By light microscopy (Fig. 10), a mosaic pattern of epithelial cell staining was seen, reflecting the intense reaction of our antibody with intercalated cells. The adjacent principal cells, in which exten-

a helium-cooled copper block, and freeze-dried at −80°C. Dried specimens were rotary shadowed with platinum at 25° and carbon at 60°. Large areas of the underside of the plasma membrane of this mitochondria-rich cell are coated with numerous studlike projections. These projections are packed into a hexagonal array. Much of the apical membrane is hidden beneath residual cytoplasmic and cytoskeletal material that remains attached to the apical membrane during the cleaving procedure. Bar, 0.1 μm. (Fig. 3) Micrograph of a large sheet of material found in the liposomes that were reconstituted with protein eluted from an H^+ATPase monoclonal IgG-Sepharose affinity column. When examined by rapid freezing and freeze-drying, many such sheets of studlike material were found in these preparations, in addition to spherical vesicles bearing studs, as shown in Fig. 7. The studs have exactly the same diameter (9.5 nm) as those found in toad bladder mitochondria-rich cells (Fig. 2) and are packed at the same density (16,800 per μm^2). These studs were not found in liposomes lacking purified H^+ATPase protein. Bar, 0.1 μm.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sive stud-coated membrane domains have not been detected, are only weakly stained. In this region of the collecting duct (the inner stripe of the outer medulla), all intercalated cells are labeled at their apical pole. Identical results were obtained using antibodies against the 70- or the 56-kD subunits of the affinity-purified protein. Staining was absent when antibodies were preincubated with the purified protein before incubation with the sections, and there was no staining when the specific antisera were replaced with preimmune rabbit serum in the first incubation step.

The similar morphology of the protein-reconstituted liposomes and the membrane-coating material seen in situ, together with the immunocytochemical labeling of the studs with polyclonal, subunit-specific antibodies, provides strong evidence that this material contains the cytoplasmic domains of H^+ATPase molecules that are present in specialized membranes of proton-secreting epithelial cells.

Discussion

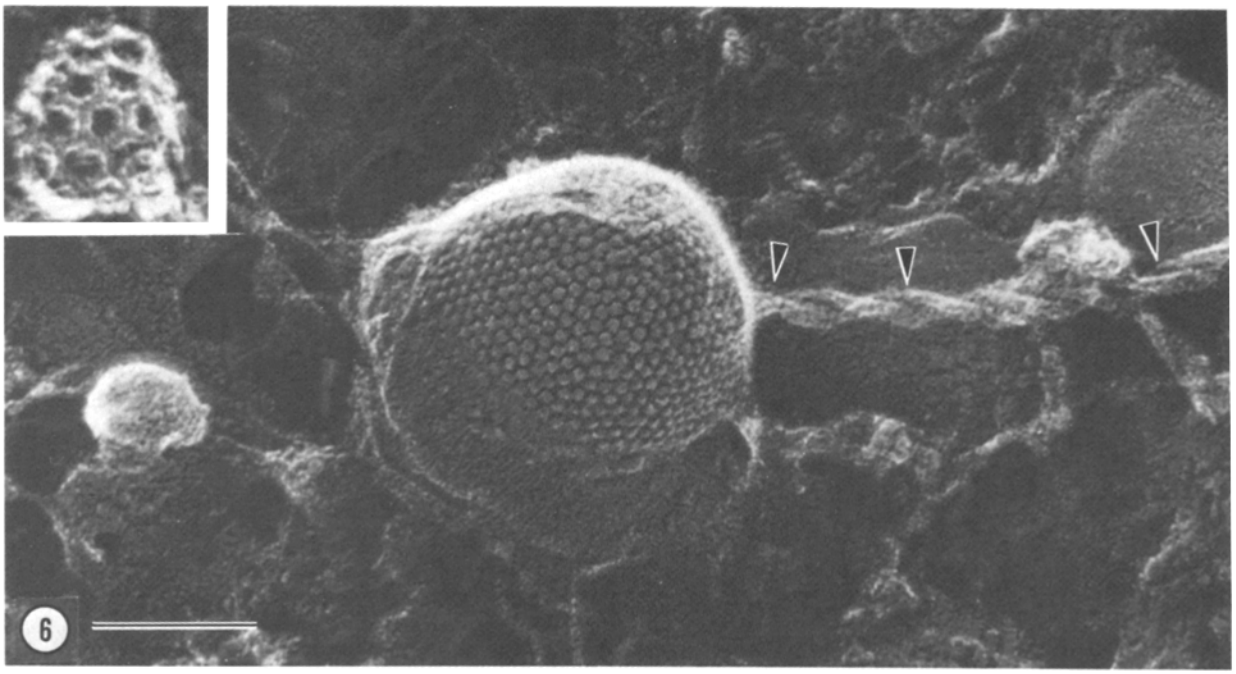
Several studies have defined morphological changes in a family of specialized, carbonic anhydrase–containing (4, 25, 38), mitochondria-rich cells that occur when they are stimulated to secrete H^+, including an increase in the number of apical microvilli coupled with a reduction in the number of cytoplasmic tubulovesicles (13, 18, 24, 26, 27, 44, 46). Gluck et al. (13) demonstrated that in the turtle bladder apical cytoplasmic vesicles present in mitochondria-rich cells, presumed to be tubulovesicles, maintain an internal acidic pH, suggesting the presence of proton pumps on their limiting membrane. When H^+ secretion was stimulated, fusion of these vesicles with the apical plasma membrane was seen, and similar fusion events have more recently been described for intercalated cells in isolated, perfused collecting ducts during stimulation of H^+ secretion (44). These observations suggest that fusion (exocytosis) of tubulovesicles with the plasma membrane during H^+ secretion results in the incorporation of specific membrane segments containing proton pumps into the apical membrane. Tubulovesicles have, in addition, been shown to be capable of the bulk endocytotic uptake of lectins (37), horseradish peroxidase (5), and other macromolecules tagged with fluorescent tracers (13, 43, 44), and hence also appear to remove proton pumps from the cell surface. Therefore, these specialized vesicles may be involved in an exocytotic–endocytotic cycle during which the H^+ pumps and other membrane components are moved to and from the plasma membrane. One notable feature of tubulovesicles is the decoration of the cytoplasmic face of their limiting membrane with an array of 10–12-nm electron-dense projections (3, 16, 26, 27, 47, 48). The apical plasma membranes of cells stimulated to secrete H^+ also have extensive regions that are decorated by these studs, presumably inserted into the membrane by tubulovesicle exocytosis events. These projections are a unique type of membrane-coating material that is antigenically distinct from clathrin (3). Furthermore, the polygonal lattice of clathrin has not been found in thin sections of tubulovesicles.

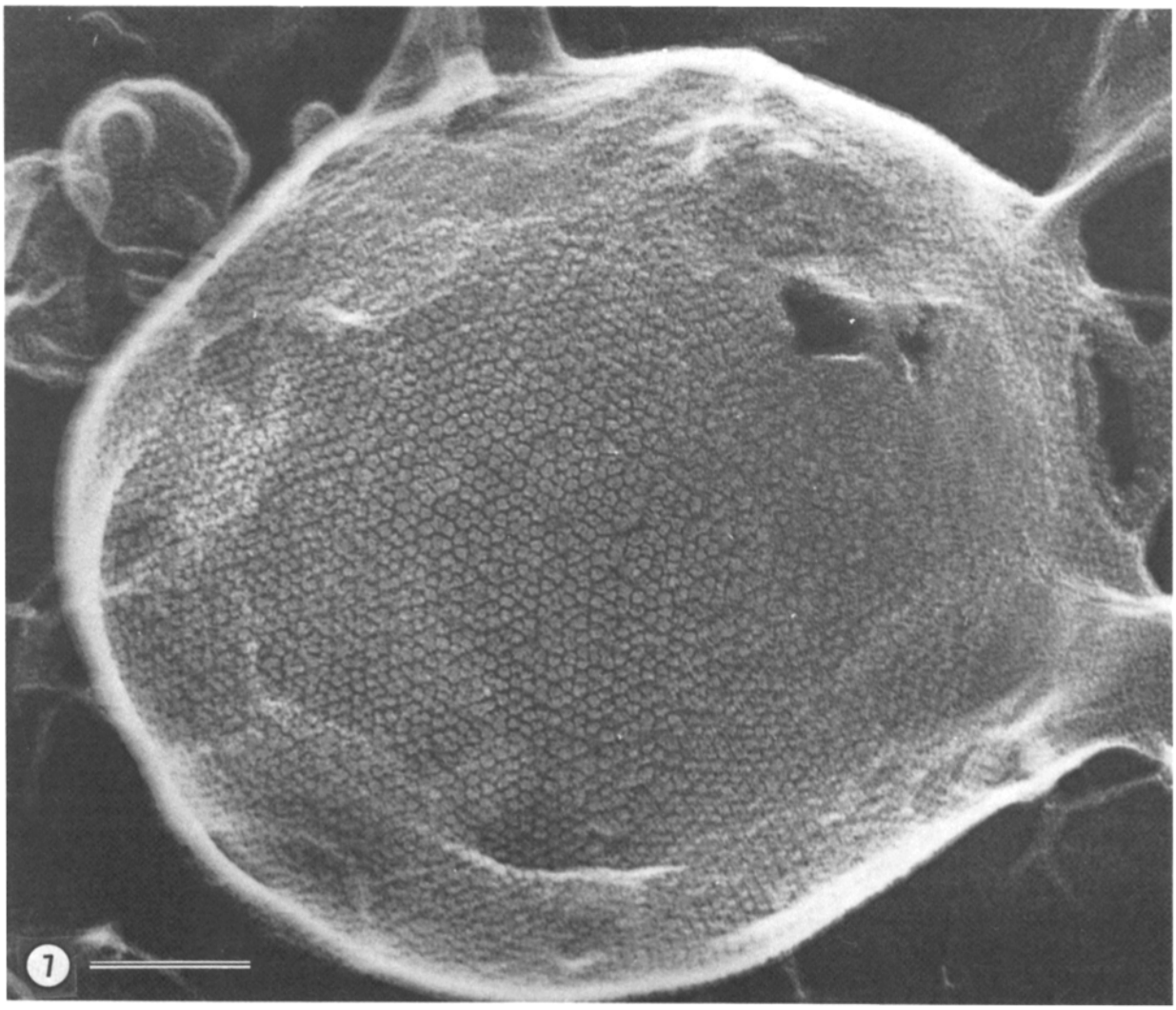
In this report, we first examined the structure of this novel material coating tubulovesicles in mitochondria-rich cells from rapidly frozen and freeze-dried toad bladders. We used toad bladder, instead of kidney tissue, because large sheets of apical membrane from the luminal surface of the bladder, together with associated cytoplasmic elements, can be sheared from the surface using polylysine-coated coverslips, and replicas of the cytoplasmic side of apical membranes can be obtained. Our main finding is that the unique coat material found on plasma membranes and vesicles is composed of protrusions or studs 9–10 nm in diameter that are, in turn, clustered into large hexagonally packed arrays. Since several lines of evidence suggest that the mitochondria-rich cell of anuran bladders is a proton-secreting cell with a proton-translocating ATPase in the luminal membrane (13, 38, 46), we went on to examine a protein fraction eluted from a monoclonal affinity column with the same rapid freezing technique. The antibody was prepared using partially purified bovine medullary H^+ATPase (11, 15) as an antigen. Remarkably, liposomes containing the purified bovine enzyme complex contained hexagonal paracrystalline arrays of 9–10-nm studs that were identical in appearance to those observed on the membranes of mitochondria-rich cells.

These results provide strong evidence that the coating material found on mitochondria-rich cell plasma and tubulovesicle membranes is formed of a protein(s) that is closely related to, or is an actual part of, the cytoplasmic domain of the H^+ATPase molecule. This conclusion is supported by our immunocytochemical data using polyclonal antibodies specific for either the 70- or 56-kD subunits of the affinity-purified protein. Both antibodies gave extensive labeling of intercalated cell plasma and some intracellular vesicle membranes that was concentrated directly over the studded membrane-coating material. Adjacent principal cell membranes were only poorly labeled, indicating the specificity of the antibodies for the stud-coated membrane domains.

The studlike morphology of the bladder and kidney membrane-coating material is strikingly similar to the mitochondrial proton pump. The stalked sphere structure is characteristic of the entire class of H^+ATPases, of which the mitochondrial pump is one, known as the F_0 F_1 ATPases (22). In addition to their distinctive morphology, these enzymes have a complex structure (10 different polypeptides on average), and do not form a phosphorylated intermediate in their reaction cycle. The bovine medullary H^+ATPase also has a complex structure with over 10 different polypeptides, but is not immunologically cross-reactive with the known F_0 F_1 ATPases. These multiple polypeptides are consistently found following two different methods of enzyme purifica-

Figures 4 and 5. (Fig. 4) Stereoelectron micrographs showing the structure of the membrane-coating studs in the apical plasma membrane of a toad bladder mitochondria-rich cell. From stereo viewing, the studs appear to be as tall as they are wide; they appear to be composed of multiple subunits surrounding a central channel. Individual studs often appear to be interconnected at their bases by strands of thin, filamentous material. Bar, 0.1 μm. (Fig. 5) Stereoelectron micrographs of a sheet of studs from the liposome preparation into which protein eluted from the H^+ATPase affinity column was incorporated. The structure and packing of the affinity-purified protein in liposomes is identical to the membrane-coating studs from toad bladder mitochondria-rich cells shown in Fig. 2. Bar, 0.1 μm.





Figures 6 and 7. (Fig. 6) Small, stud-coated vesicle from the apical region of a toad bladder mitochondria-rich cell examined by rapid freezing and freeze-drying. The hexagonally packed array of studs is identical to that found on the apical plasma membranes of these cells.

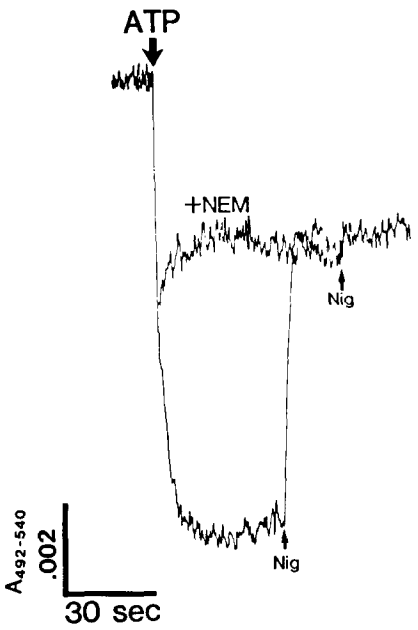


Figure 8. Demonstration of acidification by immunoprecipitated H^+ATPase. The protein was reconstituted into liposomes as described in Materials and Methods. 1 mM ATP was added at the time indicated to initiate acidification. The negative deflection in the bottom trace indicates uptake of acridine orange into the reconstituted liposomes and reflects liposome acidification (10). The proton gradient was collapsed by the addition of the electroneutral ionophore nigericin (1 μM). The addition of 1 mM *N*-ethyl-maleimide, an inhibitor of the urinary epithelial H^+ATPase (10) and vacuolar H^+ATPase (30) inhibited ATP-dependent acidification (*top trace*).

tion; the affinity-purified protein had a subunit composition that was virtually identical to that of H^+ATPase activity purified by a conventional chromatographic technique (11). These data suggest that the isolated polypeptides are integral components of the proton pump, but we cannot rule out the possibility that some of them may be peptides that co-purify with the enzyme. However, we are now in a position to correlate H^+ATPase function with its structure and to determine which of the observed subunits are associated with the membrane-coating studs by selective elimination of defined polypeptides. If the studs do indeed represent an integral subunit(s) of the proton pump, its morphological appearance indicates that the kidney H^+ATPase may represent a new subclass of F_0 F_1 ATPases, but additional work will be required to determine this.

Proton pumps are associated with other intracellular compartments, such as lysosomes (42), endosomes (8, 30), Golgi (9), and clathrin-coated vesicles (7, 49). The array of studs that we describe here has not been found on the cytoplasmic surface of these other intracellular compartments. Therefore, the characteristic paracrystalline array could result from an unusually high density of proton pumps on specialized membranes in proton-secreting cells, making the presence of studs easy to detect by microscopic techniques. The small size of individual studs would make them difficult to identify definitively at low density. Alternatively, the structure of the proton pump could be different in the other cellular compartments in which studs have not yet been detected. The nonclathrin coat associated with transporting vesicles in the Golgi region (17, 31, 34) is morphologically different to the membrane-coating material described here, and its molecular nature has not yet been determined. The origin of the stud-coated vesicles in the proton-secreting cell is also obscure at present. Clathrin-coated vesicles have been seen directly fusing with tubulovesicles, and it was proposed that the clathrin-coated vesicles may carry proton pumps from the Golgi to the specialized tubulovesicles, which, in turn shuttle to and from the plasma membrane (26, 27).

An additional characteristic feature of mitochondria-rich cell tubulovesicle membranes, as well as the plasma membrane, is the presence of a population of distinctive rod-shaped intramembranous particles (IMPs)[1] that are found by conventional freeze-fracture in all cells of the mitochondria-rich cell family (21, 32, 33, 52). These IMPs appear to be located in the same membrane domains that are decorated by the studs, although at present no technique is available to allow the stimultaneous demonstration of studs and IMPs. However, the idea that studs and rod-shaped IMPs are present in the same membrane domains is supported by recent work in turtle bladder in which a subpopulation of mitochondria-rich cells is shown to have studs and rod-shaped IMPs not on the apical, but on the basolateral plasma membrane (47). These cells are believed to be bicarbonate-secreting cells. Although the rod-shaped IMPs may, therefore, be related to the studs, perhaps representing the transmembrane domain of H^+ATPase molecules, we have not yet found extensive areas of reconstituted liposome membranes that contain large numbers of these particles, corresponding to the paracrystalline array of studs. It is possible that our reconstitution conditions did not preserve the characteristic morphology of these particles (which appear to be formed, in situ, by the tight juxtaposition of two or three globular subunits), or that the fracturing properties of the membranes bearing the paracrystalline array is such that they escape detection in conventional freeze-fracture. Finally, it is also possible that the rod-shaped IMP may be a distinct protein that associates with or is targeted to the same membrane compartments as proton pumps. A previous freeze-fracture study on H^+ATPase reconstituted into liposomes also did not reveal rod-shaped IMPs (50). A direct relationship between the membrane-

1. *Abbreviation used in this paper*: IMP, intramembranous particles.

In many cases, actin filaments, identified here by the binding of myosin subfragment 1 (*arrowheads*), are associated with these vesicles. A clathrin-coated vesicle from a mitochondria-rich cell is shown, for comparison, in the inset. Bar, 0.1 μm. (Fig. 7) Rapidly frozen, freeze-dried liposome into which protein eluted from the H^+ATPase monoclonal antibody affinity column was incorporated. The studs formed by the purified protein are identical to the studs found on a population of vesicles in toad bladder mitochondria-rich cells (Fig. 6). In the reconstituted liposome preparation, studs were found on spherical vesicles, such as illustrated here, and on large membrane sheets, as shown in Fig. 5. In both cases, the morphology and packing of the studs was the same. Bar, 0.1 μm.

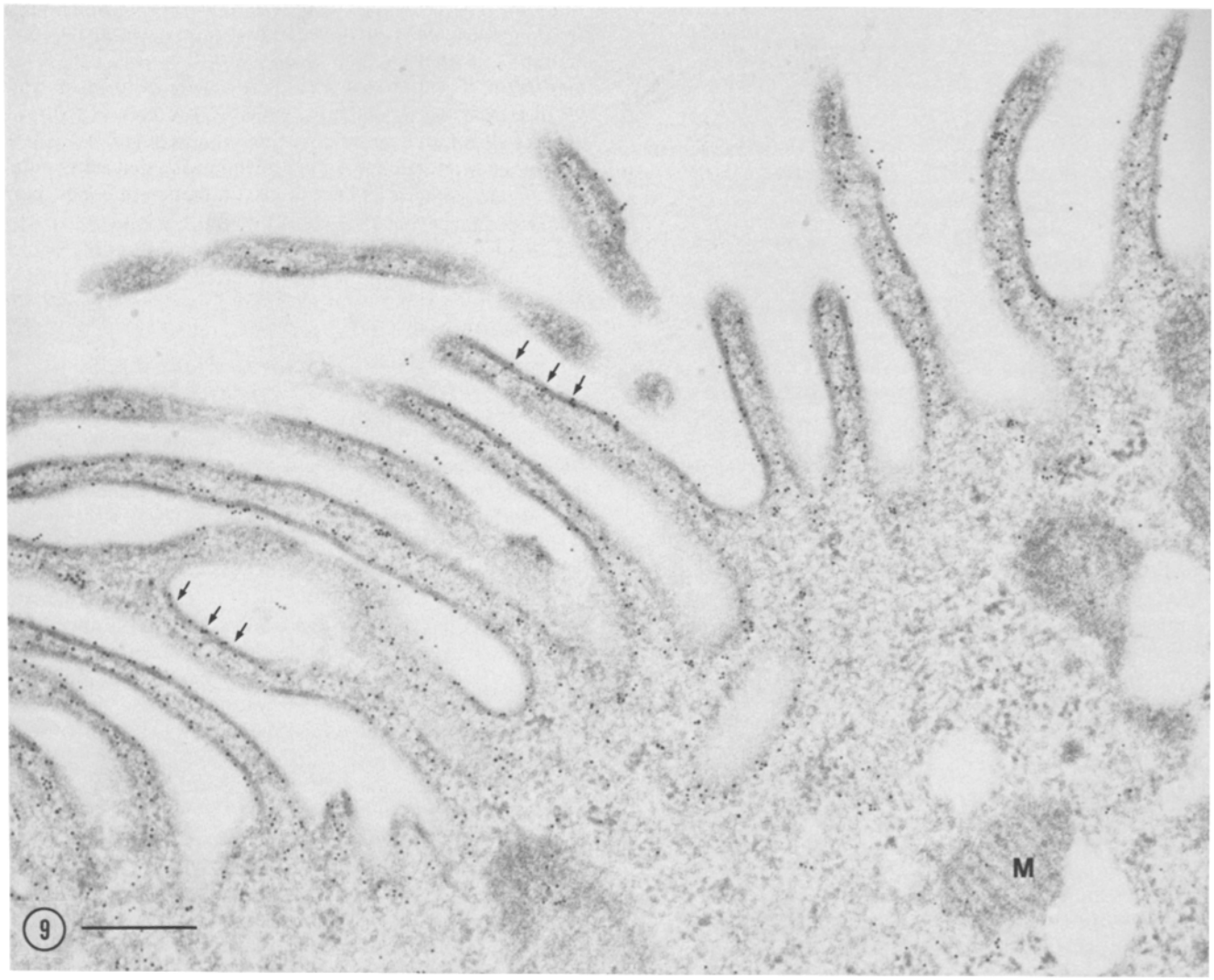


Figure 9. Thin section of the apical region of an intercalated cell from the inner stripe of the outer medulla of kidney collecting duct. The tissue was fixed in the PLP fixative, embedded in Lowicryl K4M, and sections were incubated with an affinity-purified, polyclonal IgG antibody against the 70-kD subunit of the purified H^+ATPase. A second incubation with protein A–gold (8-nm diameter gold particles) was performed to reveal antigenic sites. The gold particles are concentrated over the apical plasma membrane of this cell, and in areas where the membrane is sectioned perpendicularly (*arrows*), the gold particles are preferentially localized over a dense band on the cytoplasmic face of the plasma membrane, corresponding to the membrane-coating studs (cf. Fig. 1). The mitochondria (*M*) show only background levels of labeling. Bar, 0.25 μm.

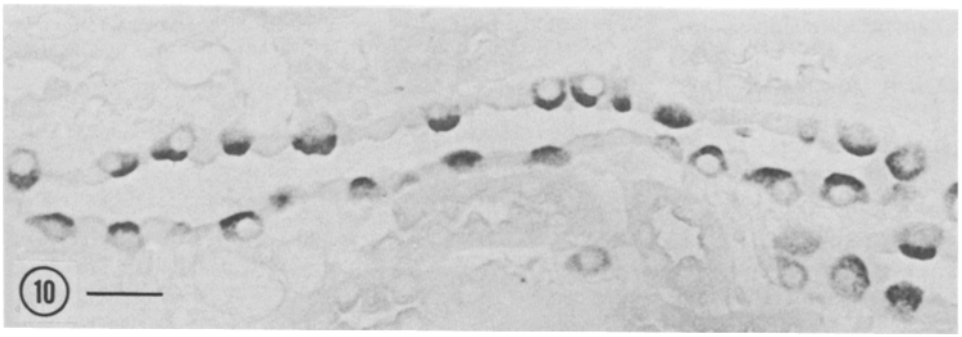


Figure 10. Semithin section of Epon-embedded rat kidney, fixed in the PLP fixative, showing the inner stripe of the outer medulla. After removal of the resin (see Materials and Methods), the section was incubated for 2 h with a polyclonal antibody against the 70-kD subunit of an affinity-purified H^+ATPase (see Fig. 11), followed by visualization of antigenic sites with the ABC-peroxidase procedure. The intercalated cells, shown by electron microscopy in Fig. 9, are heavily labeled and the label is concentrated at their apical pole in this region of the kidney, reflecting labeling of both the apical plasma membrane and the population of stud-coated vesicles in the apical cytoplasm of these cells; the adjacent principal cells of the collecting duct epithelium are only weakly stained. Bar, 20 μm.

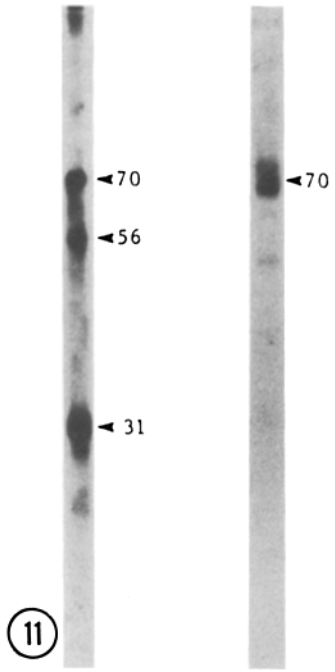


Figure 11. 5 μg of affinity-purified H⁺ATPase (*right lane*) or 50 μg of bovine kidney microsomes (*left lane*) were separated on a discontinuous 10% SDS-polyacrylamide gel, transferred to nitrocellulose (51), and cut into strips. The strips were incubated in PBS with 5% BSA and 0.2% Triton X-100 (Tr-BSA buffer) for 1 h, and then for 2 h in unpurified polyclonal antiserum (1:25; *left*) or affinity-purified anti-70K (1:100; *right*), diluted in Tr-BSA to the concentrations indicated. Strips were then washed three times with PBS containing 0.2% Triton X-100, then once with PBS containing 1% Triton, 2 M urea, and 0.1% glycine, and finally six times in PBS. The strips were incubated with Tr-BSA for 5 min, and then with ^{125}I-labeled protein A (500,000 cpm/ml) in Tr-BSA for 1 h. The strips were then washed as above except for omission of the urea wash, and were dried and visualized by overnight autoradiography. Whereas the unpurified polyclonal antiserum recognized three major bands from microsomal membranes, the affinity-purification procedure yielded antibodies (in this case against the 70-kD band) that were subunit specific.

coating studs and rod-shaped IMPs, therefore, remains to be demonstrated.

Since our previous data showed that clathrin is undetectable on H⁺ATPase-transporting tubulovesicles (3), these vesicles must recycle to and from the plasma membrane in a clathrin-independent fashion. Payne and Schekman (35) have shown that mutant yeast cells from which the clathrin gene was deleted are still viable, and able to secrete the enzyme invertase. Clathrin is, therefore, not necessary for the intracellular processing and transport of all proteins; indeed, non–clathrin-coated vesicles have also been shown to participate in vesicular traffic in the Golgi (31). In the case of the H⁺ATPase-transporting vesicles, what structural aspects of the protein and what intracellular components regulate their insertion into specific membrane domains? The large cytoplasmic portion of molecules forming a coat around these vesicles seems to be a reasonable candidate for initiating specific recognition processes between the vesicles and precise target membrane domains. If the proton pumps in different intracellular compartments are structurally identical, then other proteins may interact with the pump to provide the needed targeting information. Alternatively, proton pumps in different compartments may have structural differences that provide a basis for selective compartmentalization. The signaling process that directs the stud-coated, H⁺ATPase-carrying vesicles to specific membrane domains is particularly intriguing in view of recent data suggesting that some intercalated cells can change their functional polarity under different physiological conditions, inserting proton pumps into either the apical or the basolateral plasma membrane (43). Using studs as a morphological marker of the H⁺ATPase-containing membrane domains and using polyclonal anti-H⁺ATPase antibodies against different subunits of the proton pump, we are now in a position to directly monitor this process, and our preliminary results indicate that subpopulations of intercalated cells with proton pumps in either their apical or basolateral plasma membranes coexist in the cortical collecting duct (14).

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