

# Apical Endosomes Isolated from Kidney Collecting Duct Principal Cells Lack Subunits of the Proton Pumping ATPase

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**Abstract.** Endocytic vesicles that are involved in the vasopressin-stimulated recycling of water channels to and from the apical membrane of kidney collecting duct principal cells were isolated from rat renal papilla by differential and Percoll density gradient centrifugation. Fluorescence quenching measurements showed that the isolated vesicles maintained a high,  $\text{HgCl}_2$ -sensitive water permeability, consistent with the presence of vasopressin-sensitive water channels. They did not, however, exhibit ATP-dependent luminal acidification, nor any N-ethylmaleimide-sensitive ATPase activity, properties that are characteristic of most acidic endosomal compartments. Western blotting with specific antibodies showed that the 31- and 70-kD cytoplasmically oriented subunits of the vacuolar proton pump were not detectable in these apical endosomes from the papilla, whereas they were present in endosomes prepared in parallel from the cortex. In contrast, the 56-kD subunit of the proton pump was abundant in papillary endosomes, and was localized at the api-

cal pole of principal cells by immunocytochemistry. Finally, an antibody that recognizes the 16-kD transmembrane subunit of oat tonoplast ATPase cross-reacted with a distinct 16-kD band in cortical endosomes, but no 16-kD band was detectable in endosomes from the papilla. This antibody also recognized a 16-kD band in affinity-purified  $\text{H}^+$  ATPase preparations from bovine kidney medulla. Therefore, early endosomes derived from the apical plasma membrane of collecting duct principal cells fail to acidify because they lack functionally important subunits of a vacuolar-type proton pumping ATPase, including the 16-kD transmembrane domain that serves as the proton-conducting channel, and the 70-kD cytoplasmic subunit that contains the ATPase catalytic site. This specialized, non-acidic early endosomal compartment appears to be involved primarily in the hormonally induced recycling of water channels to and from the apical plasma membrane of vasopressin-sensitive cells in the kidney collecting duct.

**T**HE water permeability of the kidney collecting duct epithelium is regulated by vasopressin-induced recycling of water channels between an intracellular vesicular compartment and the plasma membrane of principal cells. An analogous mechanism exists in other vasopressin-sensitive epithelia such as the toad urinary bladder and the amphibian epidermis (7, 25, 29, 31, 60). We have demonstrated recently that these endosomes are specialized intracellular vesicles which are not acidic, and that they appear to function primarily to recycle membrane components, including water channels, back to the apical membrane during hormonal stimulation. Using FITC-dextran as a marker of endocytosis and acidification, coupled with co-localization of a lysosomal glycoprotein, LGPI20, our data showed that most of the internalized fluorescent probe does not move into lysosomes and that it remains in an apical, non-acidic compartment (36). The passive proton permeability of these endosomes is not sufficiently different from acidifying cortical

endosomes to account for their failure to acidify (36), but alternative explanations remain possible; the endosomes might contain intact, but nonfunctional proton pumps, or the proton pump might be absent from this population of vesicles. It has also been proposed that water and protons may traverse the plasma membrane via the same transmembrane channel, that may resemble or actually be the 16-kD transmembrane subunit of a proton pump (23, 26). To examine more directly the characteristics and composition of these specialized endosomes we have, therefore, used differential and density-gradient centrifugation to prepare a highly enriched population of these vesicles from kidney papilla. Using this preparation, we show that these endosomes, which contain vasopressin-sensitive water channels, are depleted of two of the major cytoplasmic subunits (31 and 70 kD) of the proton pumping ATPase that are required for ATP-dependent proton translocation, as well as the 16-kD transmembrane subunit that acts as a proton translocating channel across the

lipid bilayer. These vesicles, which are derived from the apical membrane by clathrin-mediated endocytosis (12, 56), represent a specialized early endosomal compartment that is distinct from other acidic intracellular vesicles along the endocytotic pathway.

## Materials and Methods

### Preparation of Endocytic Vesicles from Rat Renal Cortex and Papilla

Endocytic vesicles from rat renal cortical homogenates were isolated by differential and Percoll density gradient centrifugation as previously described (47). Endosomes from rat papilla were prepared by a modification of the same method. All steps in the preparation were performed on ice and in refrigerated centrifuges (RC5C, rotor SS34; Sorvall Instruments, Newton, CT) (L8-M ultracentrifuge, rotor T865, and TL-100 ultracentrifuge, rotor TLA 100.2; Beckman Instruments, Palo Alto, CA). Renal cortical brush border membrane vesicles (BBMV)<sup>1</sup> were isolated by the Mg<sup>2+</sup>/EGTA precipitation method (4).

To isolate papillary endosomes, six male Sprague Dawley rats were anaesthetized with sodium pentobarbital (65 mg/100 g body weight, i.p.) and injected via the jugular vein with FITC-dextran (10 kD; Sigma Chemical Co., St. Louis, MO) (50 mg/rat, i.v.). 20 min later, the animals were killed by cervical dislocation; the kidneys were removed and immersed in ice-cold saline. The lower 2/3 of each papilla was excised, cut into small pieces, and homogenized in 14 ml ice-cold buffer (300 mM mannitol, 12 mM Hepes/Tris, pH 7.4) with 30 strokes of a motor-driven Teflon/glass Potter homogenizer (1,200 rpm). The homogenate was diluted with an additional 14 ml of the same buffer, and centrifuged at 2,500 g for 15 min. The pellet was discarded. The supernatant was centrifuged at 20,000 g for 20 min. The resulting supernatant and the upper yellowish layer of the pellet were carefully removed, combined, and centrifuged at 105,000 g for 60 min in an ultracentrifuge (L8-M; Beckman Instruments). The supernatant was decanted. The pellet was resuspended in 1 ml homogenizing buffer using 30–40 strokes of a manual glass/glass Potter homogenizer. A 2-ml polyallomer tube was then loaded with 1.8 g of mixture which contained 1.476 g vesicle suspension, and 0.324 g of concentrated Percoll, to yield 18% wt/wt Percoll. This sample was well mixed and centrifuged at 50,000 g for 50 min in an ultracentrifuge (TL-100; Beckman Instruments) (acceleration setting #9, deceleration #0). The self-formed Percoll density gradient was fractionated from the bottom in fractions of 50  $\mu$ l. The fluorescence and protein profile of each fraction was measured. Fractions incorporating the peak of fluorescence (fractions 2–12) were combined as pool A and fractions 13–24 were combined to make pool B (compare Fig. 1). Routinely, the first 0.1 ml from the gradient with the highest Percoll concentration was discarded; a further 0.5 ml was collected as pool A, and the next 0.7 ml as pool B. These pools were diluted in KCl-buffer (300 mM mannitol, 100 mM KCl, 5 mM MgSO<sub>4</sub>, 5 mM Hepes/Tris, pH 7.4), followed by centrifugation at 50,000 g for 50 min (TL-100 ultracentrifuge; Beckman Instruments). This step also removes the Percoll. The fluffy vesicle pellet was dispersed in a small amount of corresponding buffer, and the resulting suspension of vesicles was used in the assays.

For water transport studies, rats were injected with 6-carboxyfluorescein (6-CF; Sigma Chemical Co.) (20 mM in PBS; 0.75 ml/rat) instead of FITC-dextran, and the papillary endocytic vesicles were isolated subsequently by the same method as described above, except that all buffers were at pH 8.5. Finally, vesicles collected as pools A and B were diluted in and washed with low mannitol buffer (50 mM mannitol, 12 mM Hepes/Tris, pH 8.5).

### Enzyme Assays

Protein was measured by the method of Bradford (6). Na/K-ATPase (EC 3.6.1.3) was measured by the coupled spectrophotometric assay as described by Norby (44). The same assay was used to measure oligomycin-sensitive ATPase, i.e., the portion of the total ATPase activity sensitive to 5  $\mu$ g/ml oligomycin. Alkaline phosphatase (EC 3.1.3.1) and leucine arylamidase (EC 3.4.11.2) were measured using conditions described in corresponding com-

mercial kits, i.e., Merckotests 3344 and 3359, respectively. KCN-resistant NADH oxidoreductase (EC 1.6.99.2) was determined by the method of Sotocasa et al. (54). NEM-sensitive ATPase activity was measured by the Pi liberation assay, using experimental conditions described previously (13).

Previously published optimal assay conditions for the H<sup>+</sup> pump in renal cortical endosomes (47, 48, 63) were used to test for the presence of an ATP-driven H<sup>+</sup> pump in papillary endocytic vesicles. An aliquot of isolated vesicles (25–50  $\mu$ g protein) was added to 2.0 ml KCl-buffer, pH 8.5 (endosomes loaded with 6-CF), or pH 7.4 (endosomes loaded with FITC-dextran). The buffers contained 5  $\mu$ M valinomycin. To quench the extravesicular fluorescence signal, an aliquot of polyclonal anti-fluorescein antibody (36) was added to the outside buffer, followed by addition of ATP (final concentration, 1.5 mM). To determine the baseline signal, which was substantial in the samples from the density gradient because of the presence of Percoll (up to 40% of the total), and negligible with vesicles after the Percoll had been removed by washing, the specific fluorescence signal was quenched at the end of each experiment by adding 0.1 ml of 1 M HCl. The total fluorescence signal was always corrected for the contribution from extravesicular and baseline fluorescence. The samples were stirred and the fluorescence was measured continuously at 37°C in an SLM Aminco 8000 fluorometer (Urbana, IL) interfaced to an IBM/PC computer ( $\lambda_{\text{exc}} = 485$  nm;  $\lambda_{\text{em}} > 515$  nm).

### Western Blotting of the Proton Pump Subunits

Western blotting of the proton pump subunits was performed using isolated renal cortical or papillary endocytic vesicles, BBMV, total kidney and papillary homogenates, the pellet and supernatant resulting from centrifugation of the total papillary homogenate at 105,000 g for 60 min, and affinity-purified H<sup>+</sup> ATPase from bovine renal medulla, isolated as previously described (24). Aliquots of these samples were solubilized by boiling for 5 min in sample buffer (1% SDS, 30 mM Tris/HCl, pH 6.8, 5% 2- $\beta$ -mercaptoethanol, 12% vol/vol glycerol). Proteins (3.125–150  $\mu$ g/lane) were separated by Laemmli SDS-PAGE using 10% or 15% gels, and electrophoretically transferred to Immobilon (Millipore Corp., Bedford, MA). The membranes were briefly stained with Coomassie blue to check the efficiency of the transfer, destained, and blocked in blotting buffer (5% non-fat dry milk, 0.15 M NaCl, 1% Triton X-100, 20 mM Tris-HCl, pH 7.4). Incubation at room temperature for 3 h was performed with the following antibodies: a polyclonal antibody (diluted 1:1,000 in blotting buffer) recognizing primarily the 31-, 56-, and 70-kD H<sup>+</sup> pump subunits (10), a monoclonal (undiluted) anti-31-kD subunit antibody (3), a polyclonal antibody (diluted 1:500) against a COOH-terminal peptide from the 56-kD subunit (43), and an antibody (diluted 1:500) against oat tonoplast H<sup>+</sup> ATPase (kindly provided by Dr. H. Sze, Department of Botany, University of Maryland, College Park, MD) that, in addition to other pump subunits, recognizes the 16-kD transmembrane pump subunit (34). The membranes were then washed in several changes of blotting buffer, and incubated for 60 min with 1:1,000 dilution (in blotting buffer) of goat anti-rabbit or anti-mouse IgG conjugated to alkaline phosphatase (Vector Labs., Burlingame, CA). Thereafter, the membranes were extensively washed, and stained for alkaline phosphatase activity using a commercial kit (NBT/BCIB; Kirkegaard & Perry Lab. Inc., Gaithersburg, MD).

### Endosome Water Permeability

The water permeability of endosomes from pool A and pool B was measured by the stopped flow, fluorescence quenching method previously described in detail (35, 59). An endosome suspension was mixed in <1 ms with an equal volume of hyperosmotic buffer to give a 50 mM inwardly directed sucrose gradient. Fluorescence (excitation 470 nm, emission >515 nm) was monitored continuously. For these experiments, 6-CF was infused into rats rather than FITC-dextran, because the fluorescence signal generated by FITC-dextran in the small quantity of material available for each assay was insufficient to produce reliable data. Water permeability coefficients ( $P_f$ , cm/s) were calculated as described previously (59) using average vesicle diameters from the electron microscopic data shown in Fig. 2 to calculate surface/volume ratios.

### Electron Microscopy

EM was performed on ultrathin frozen sections of vesicle preparations that were stained with uranyl acetate according to the method of Tokuyasu (58). Vesicles were fixed in 0.5% glutaraldehyde, washed with PBS, and embedded in 3% agar. The small agar blocks were infiltrated with 2.3 M sucrose, frozen in liquid nitrogen, and 60 nm sections were cut and mounted on car-

1. *Abbreviations used in this paper:* BBMV, brush border membrane vesicles.

bon/Parlodion-coated nickel grids. The grids were then washed with PBS, fixed further with 1% glutaraldehyde, washed with water, and stained with 2% uranyl acetate for 5 min. The grids were then embedded in 2% methyl cellulose, dried, and viewed in an electron microscope (CM10; Philips Electronic Instruments, Mahwah, NJ). Vesicle diameter was estimated with a calibrated eyepiece by measuring the greatest distance between two opposite points at the external surface (round vesicles); in oval vesicles, the diameters along the long and short axes of each vesicle were measured and averaged.

### Immunofluorescence Microscopy

Animals were injected with FITC-dextran, as described above, and following a short rinse of the circulation with HBSS, the kidneys were fixed by intravascular perfusion via the abdominal aorta with paraformaldehyde-lysine-periodate (42). The kidneys from some animals were fixed in PLP without prior FITC-dextran injection. After cryoprotection of tissue pieces in 2.3 M sucrose for at least 1 h, semithin 1- $\mu$ m frozen sections were cut on an ultracycromicrotome. As previously described, this technique allows the detection of internalized fluid phase fluorescent markers, in this case FITC-dextran, in tissue sections (37). Sections were then incubated for 1 h with a mAb against the 31-kD subunit of the proton pump (3). After washing, sections were incubated with a rhodamine-labeled goat anti-mouse IgG (15  $\mu$ g/ml final concentration) for 1 h. Sections were rinsed three times with PBS (5 min each) and mounted in a drop of Tris:HCl (200 mM)/glycerol (50:50), pH 8.0, containing 2% *n*-propyl gallate as an anti-quenching agent (22). For detection of the 56-kD subunit of the proton pump, 1- $\mu$ m cryostat sections were incubated with a 1:100 dilution of the specific antibody, followed by a second step in goat anti-rabbit IgG-FITC (20  $\mu$ g/ml final concentration). Slides were examined using a Nikon FXA fluorescence microscope (DonSanto Corp., Natick, MA) equipped with a 480  $\pm$  10-nm excitation filter and a 520  $\pm$  20-nm barrier filter for fluorescein label, and 546  $\pm$  5-nm excitation filter and a 580-nm barrier filter for rhodamine fluorescence. Photographs were taken on Kodak T-Max 400 film, push processed to 1600 ASA (Eastman Kodak Co., Rochester, NY).

### Results

In a series of previous reports, we have used a crude microsomal vesicle preparation from rat kidney papilla to characterize several functional parameters of principal cell

endosomes (35, 36, 37, 59). Because only endosomes contain internalized fluorescent probes, all other vesicles present in the crude preparation were invisible to the assays. While several functional aspects of endosomes can be measured in this way, such a crude vesicle fraction is clearly of limited use for investigating the molecular composition of these vesicles. In the present report, we describe the preparation of a population of vesicles that is enriched in endosomes from kidney papilla; this now allows us to correlate specific functional parameters with protein composition in these specialized vesicles.

### Preparation of a Vesicle Fraction Enriched in Principal Cell Endosomes

Using a combination of differential and density gradient separation, endosomal vesicles containing internalized fluorescent markers were enriched in a vesicle preparation from kidney papilla, and formed a sharp peak in an 18% wt/wt Percoll gradient. As shown in Fig. 1, the fractionation of the gradient in portions of 0.05 ml demonstrates that proteins distributed in two peaks whereas intravesicular fluorescence, indicating localization of papillary endosomes, colocalized with a smaller protein peak at the bottom of the gradient. Fractions 2-12, which represent the major fluorescent peak, were pooled for further study and are referred to as pool A. Fractions 13-24 are referred to as pool B. Because of the small amount of material obtained in each preparation, it proved difficult to perform reliable functional or biochemical assays on smaller-sized pools. While the characteristics of pools A and B were qualitatively similar, quantitative data showed that pool A represents a vesicle population that is the most enriched in the functional parameters of interest. Table I shows various enzyme activities in papillary homogenates and isolated vesicles as well as the enrichment factors for these enzymes in vesicles collected as pools A and B. As judged from the enrichment factors for Na/K-ATPase,

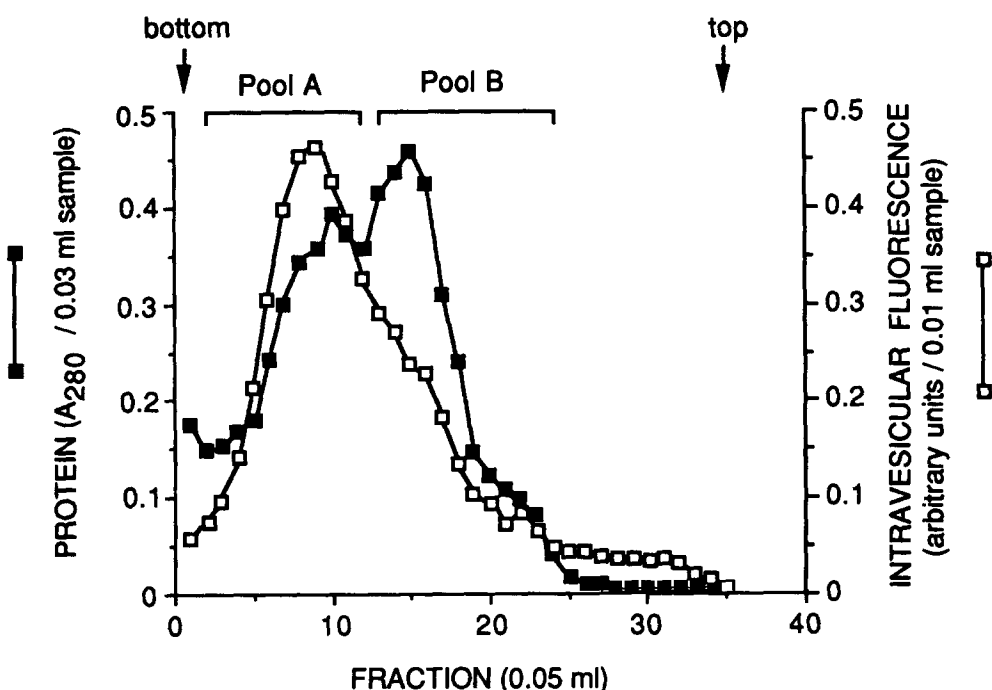


Figure 1. Separation of papillary endosomes from a crude vesicle preparation on 18% Percoll gradient. 50- $\mu$ l samples were taken from the entire gradient and their intravesicular fluorescence intensity and protein content were plotted. A clear peak of fluorescence intensity, indicating the location of endosomes, was distinct from the major protein peak on the gradient.

**Table I. Enzyme Characteristics of Vesicles in Pool A and Pool B**

Enzyme	Enzyme activity		
	Homogenate	Pool A (EF)*	Pool B (EF)*
		<i>(nmol/min × mg protein)</i>	
Na/K-ATPase	114 ± 13	18 ± 11 (0.2 ± 0.1)	10 ± 9 (0.1 ± 0.06)
Alkaline phosphatase	6.6 ± 1.9	31 ± 0.4 (4.7 ± 0.1)	18.5 ± 3.1 (2.8 ± 0.5)
Leucine arylamidase	20 ± 2	207 ± 5 (10.3 ± 0.3)	65 ± 9 (3.3 ± 0.5)
NEM-sensitive ATPase†	431 ± 112	Not detectable	Not detectable
KCN-resistant NADH oxidoreductase	109 ± 31	9 ± 7 (0.1 ± 0.06)	13 ± 11 (0.12 ± 0.08)
Oligomycin-sensitive ATPase	113 ± 11	101 ± 22 (0.9 ± 0.2)	118 ± 35 (1.1 ± 0.3)

Data are means ± SD of three to four vesicle preparations.

\* EF, Enrichment factor.

† nmol P<sub>i</sub>/min·mg protein.

NEM-sensitive ATPase, oligomycin-sensitive ATPase, and KCN-resistant NADH oxidoreductase, both pools were devoid of significant contamination by basolateral plasma membranes, various membranes that possess a vacuolar type H<sup>+</sup> ATPase, mitochondrial membranes, and ER, respectively. Furthermore, Western blotting of proteins with a lysosomal membrane marker, LGP120 (39), showed an insignificant contamination by these membranes in vesicles from both pools (data not shown). However, the apical membrane markers, alkaline phosphatase, and leucine arylamidase, were enriched in both pools and were two- to threefold greater in pool A than in pool B. Finally, Western blots with antibodies against the Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> anion-exchanger AE1 and the brain/erythroid facilitated glucose transporter Glut 2, both basolateral membrane proteins that are concentrated in collecting duct intercalated cells in the initial portion of the rat papilla (1, 16, 49, 57), showed that these proteins were absent from papillary endosomal preparations (data not shown).

Ultrathin frozen sections of the two vesicle pools A and B confirmed that the preparations were relatively free of contaminating organelles, but showed that the vesicles in pool A were only slightly smaller and more homogeneous than those in pool B (Fig. 2).

### Endosome Water Permeability

Measurements of osmotic water permeability were carried out to evaluate whether functional water channels were present in vesicle fractions A and B. Fig. 3 shows the time course of fluorescence quenching in response to an osmotic gradient which caused endosome shrinkage. As described previously, the labeled endosomes contained a subpopulation of vesicles with fast water transport (containing water channels), as shown by the fluorescence decrease over 200 ms, and a subpopulation with slower water transport, with a fluorescence decrease over several seconds (36, 59). In three sets of measurements on fractionated endosomes, the endosomes with fast water transport accounted for 39 ± 4% (SEM) of the total signal in fraction A, and only 17 ± 3% in fraction B. Average P<sub>f</sub> (osmotic permeability coefficient) for the faster and slower components of water transport were 0.028 cm/s and 0.001 cm/s, respectively.

The lower curve in Fig. 3 shows the effect of 0.3 mM HgCl<sub>2</sub> on endosome water permeability. This concentration of HgCl<sub>2</sub> was shown to inhibit water permeability by 85% in vasopressin-induced endosomes from toad urinary blad-

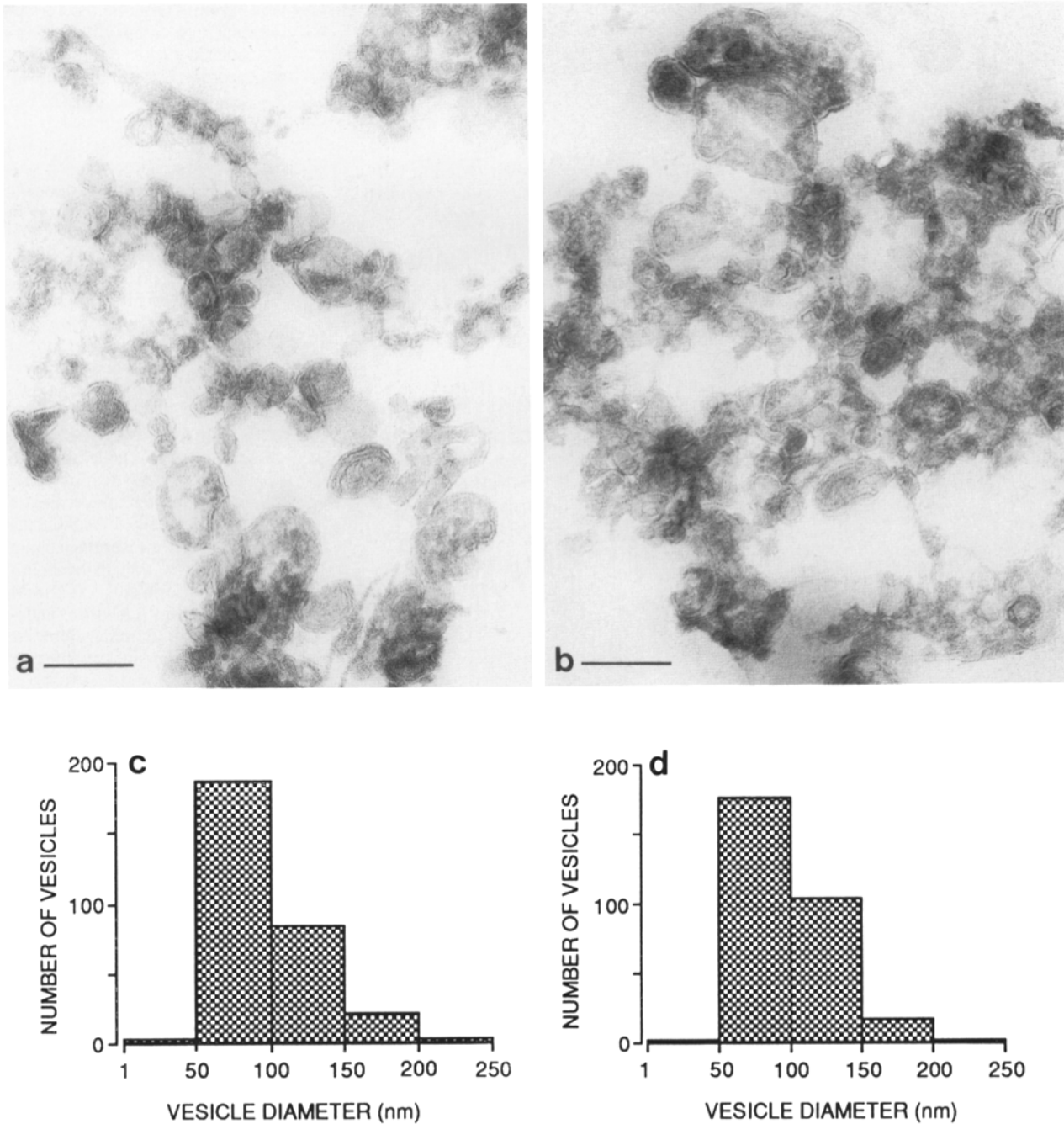
der (51). In three sets of measurements, P<sub>f</sub> was decreased by 60–75% in the presence of HgCl<sub>2</sub>, supporting the interpretation that the fast component of water transport indicates the presence of functional water channels.

### Endosome Acidification

As for the bulk papillary vesicle population we have previously described (36, 59), the vesicle populations in pools A and B showed no detectable ATP-dependent proton pumping activity as opposed to a large ATP-driven accumulation in renal cortical vesicles (Fig. 4). In accord with this, no NEM-sensitive ATPase activity could be detected in either pool A or in pool B, while such activity was readily detectable in the crude papillary homogenate from which the endosomes were isolated (Table I).

### Proton Pump Subunits in Endosomes

Westerns blots of cortical endosomes with a polyclonal anti-proton pump antibody raised against the holoenzyme revealed three major bands at 31, 56, and 70 kD (Fig. 5). From previous work, it is known that these three subunits are part of the cytoplasmic domain of the proton pumping ATPase, including the ATPase catalytic site (18, 55). In contrast, the 31- and 70-kD subunits were virtually undetectable in endosomes isolated from the papilla when the same amount of vesicle protein was added to the gel; the absence of the 31-kD subunit was confirmed using a specific mAb raised against a defined peptide sequence from the cloned 31-kD subunit (3). Surprisingly, however, a strong band corresponding to the position of the 56-kD subunit in cortical endosomes was still present in papillary endosomes. Confirmation of the presence of the 56-kD subunit was achieved using a specific antipeptide antibody that was raised against a COOH-terminal domain of an isoform of the 56-kD subunit of the bovine vacuolar proton pump. This isoform is not found in the proximal tubule. Characterization of this antibody is described in detail in a separate publication (43). This subunit was detectable in both cortical and medullary endosomes (pool A), but was much less abundant in brush border membranes from the cortex (Fig. 6A). Surprisingly, the isoform-specific anti-56-kD antibody gave only a weak staining in pool B of the papillary vesicle preparation, whereas the non-specific polyclonal antibody raised against holo-ATPase gave a strong staining of a 56-kD band (Fig. 5). This may indicate that multiple forms of the 56-kD subunit are also present in the papilla, and that these forms are local-



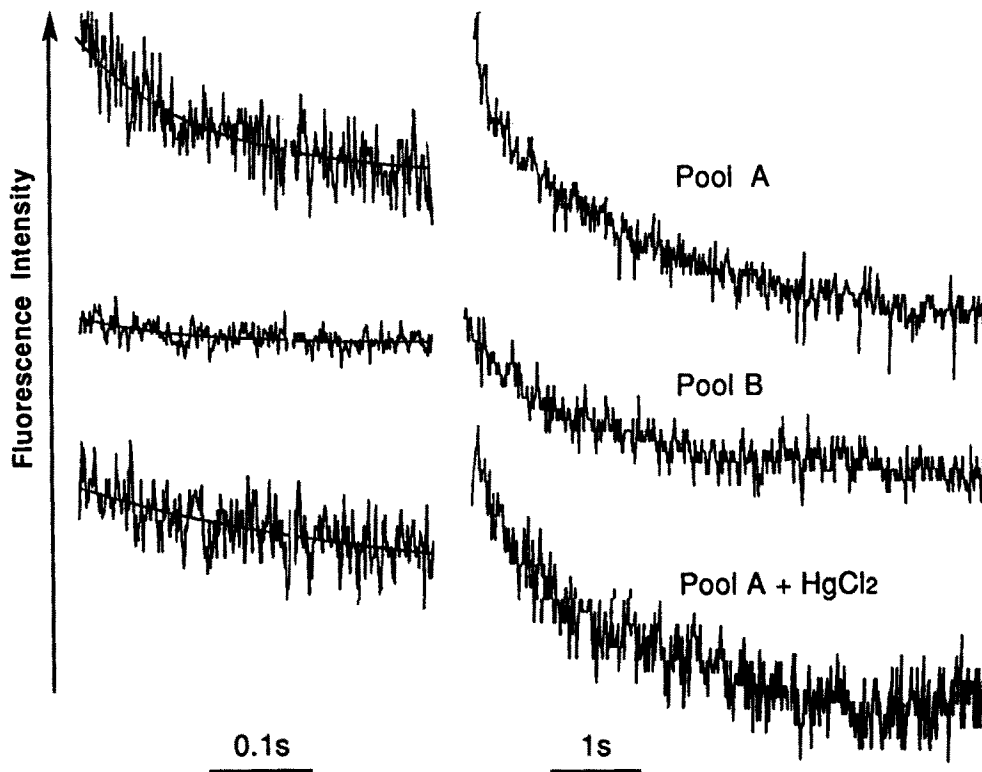
**Figure 2.** Electron micrographs of ultrathin (60 nm) frozen sections of vesicles in pool A (a) and pool B (b) negatively stained with uranyl acetate. The vesicles in both preparations are sharply delineated by a membrane bilayer. Histograms of vesicle diameters in pool A (c) and pool B (d), calculated from analysis of electron micrographs, indicate that in both preparations the vesicles exhibit a similar size distribution;  $97 \pm 1.4$  nm ( $n = 300$ ) and  $101 \pm 3.4$  nm ( $n = 300$ ), respectively. Bar,  $0.25 \mu\text{m}$ .

ized on different populations of vesicles that can be separated by Percoll gradient centrifugation.

In addition, the papilla contained a broad band between the 56- and 70-kD subunits that was detected with the polyclonal anti-proton pump antibody; its identity is unknown and it varied in intensity among different preparations (Fig. 5). In contrast to isolated endosomes from the papilla, the 31-, 56-, and 70-kD subunits of the proton pump were detectable in the papilla homogenate, as well as in total mem-

branes isolated from the papillary homogenate, and in the supernatant from the membrane pellet, although the 31-kD subunit was extremely weakly labeled (Fig. 7).

Serial dilutions of cortical endosomes showed that the polyclonal antiserum was able to detect subunits of the proton pump in these vesicles when as little as  $3.25 \mu\text{g}$  of total vesicle protein was run on the gel (Fig. 7, lane f), although the ability of this antibody to detect the 31-kD subunit was somewhat less than for the other two subunits; in particular



**Figure 3.** Osmotic water transport in 6-carboxyfluorescein-labeled endosomes from rat papilla. Endosomes were labeled with 6-CF *in vivo*, isolated, and fractionated as described in Materials and Methods. Endosomes were subjected to a 50 mM inwardly directed gradient of sucrose in a stopped-flow apparatus. The time course of decreasing fluorescence corresponds to osmotic water efflux, endosome shrinkage, and fluorescence self-quenching of entrapped 6-CF. Data are shown for measurements performed at two time scales and represent averages of four to eight individual experiments. Single exponential fits for the data obtained on the shorter time scale are shown; averaged results for a series of measurements are given in the text. In the inhibition experiments, 0.3 mM  $\text{HgCl}_2$  was added to endosomes for 5 min before the stopped flow measurement.

the 56-kD subunit yielded a strong band even at the lowest protein concentration applied to the gel, and the 70-kD subunit was also clearly visible at this low concentration (Fig. 7, lane *f*). For the papilla, 100  $\mu\text{g}$  of papillary protein was applied to the gel (Fig. 7, *papilla*); the 31-kD band was undetectable, and the 70-kD band was only weakly stained in the papillary vesicle pellet. The vesicle pellet used for this gel was the 105,000 *g* pellet, before Percoll gradient separation of purer endosomes. As mentioned above, the 70-kD band was virtually undetectable in the purer endosomal preparation (Fig. 5). The detection technique used would, therefore, have revealed proton pump subunits in the papillary preparation, even if this preparation was markedly (up to 30-fold) less rich in proton pump-containing endosomes than the cortical preparation.

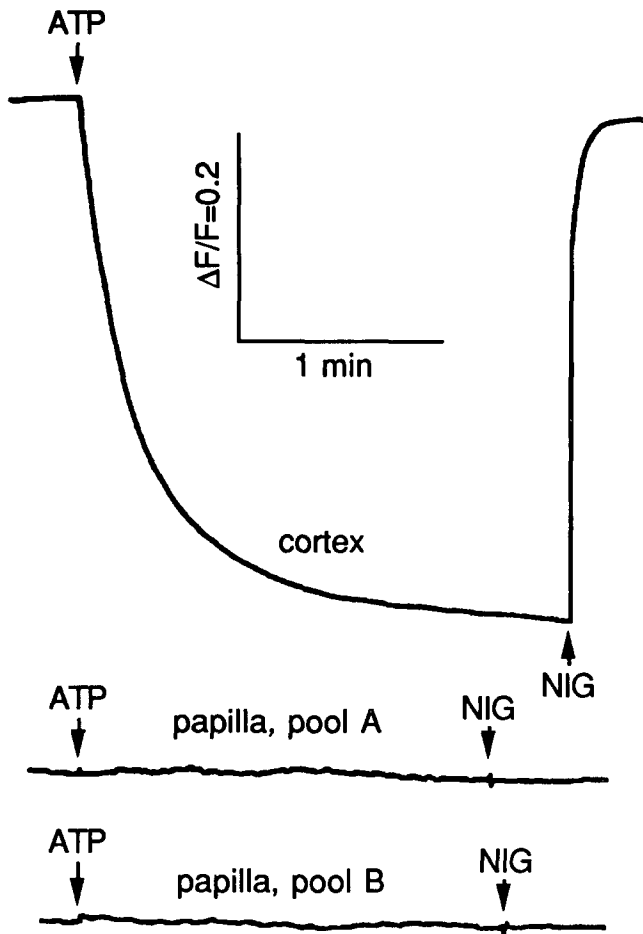
Finally, to determine whether the transmembrane 16-kD subunit of the proton pump was present in endosomal preparations, we used a previously characterized antibody raised against oat tonoplast  $\text{H}^+$  ATPase, which recognizes several pump subunits, including the 16-kD subunit (34). The 16-kD subunit is the DCCD binding subunit of the proton pump, and is a proteolipid that forms the transmembrane proton pore within the lipid bilayer (18, 55). As shown in Fig. 6 *B*, a distinct 16-kD band was present in both endosomes and BBMV derived from rat kidney cortex; this band was absent from papillary endosomes (pool A and pool B) even when 150  $\mu\text{g}$  of protein was added per lane. These results demonstrate that this antibody cross-reacts with a protein of similar molecular weight to the 16-kD subunit of the oat tonoplast vacuolar proton pump, and that this subunit is detectable only in endosomes from the kidney cortex. To determine

whether this antibody cross-reacted with the 16-kD subunit of the proton pump, affinity-purified  $\text{H}^+$  ATPase was separated by SDS-PAGE and blots were performed with this antibody. As seen in Fig. 6 *C*, in two separate preparations of purified  $\text{H}^+$  ATPase, a 16-kD band was detected that ran in parallel with the 16-kD band from renal BBMV. This result provides direct evidence that the antibody obtained from Dr. Heven Sze (Department of Botany, University of Maryland) recognizes a 16-kD band present in immunopurified  $\text{H}^+$  ATPase, most probably the 16-kD transmembrane subunit.

#### **Immunolocalization of Proton Pumps in Kidney Papilla**

Our previous results have shown that proton pumps are abundant in collecting duct intercalated cells, but that principal cells show a considerably weaker staining (10). Fig. 8 shows a cryostat section of a collecting duct from rat papilla, in which endosomes were labeled by *in vivo* infusion with FITC-dextran. Double staining with a mAb against the 31-kD subunit of the proton pumping ATPase shows colocalization of FITC-dextran and the proton pump in intercalated cell endosomes, whereas endosomes in adjacent principal cells are unlabeled. However, while this immunocytochemical result is confirmatory of the Western blotting data, a low level of proton pump 31-kD subunit on principal cell endosomes would probably not be detected by immunocytochemistry, because the anti-proton pump antibody also fails to label lysosomes in sectioned tissues (11), probably due to the low number of pumps on lysosomal membranes.

In contrast to the failure to immunolocalize 31-kD

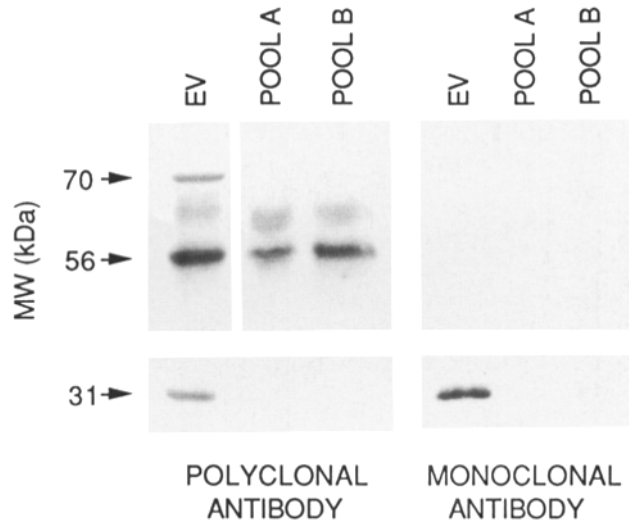


**Figure 4.** ATP-dependent acidification in FITC-dextran loaded vesicles from kidney cortex, papillary pool A, and papillary pool B. Vesicles loaded with FITC-dextran in vivo were equilibrated with KCl buffer, pH 7.4, and diluted in the same buffer. 50  $\mu$ g protein from each vesicle preparation was used in the assay. Before addition of ATP, extravesicular fluorescence was quenched with anti-FITC antibodies. Where indicated, nigericin (*NIG*) was added to dissipate the  $\Delta$ pH. ATP-driven  $H^+$  uptake is vigorous in cortical endosomes, but is absent from both pools of papillary vesicles.

subunits in principal cells, the monospecific anti 56-kD antibody gave a marked apical labeling in principal cells (Fig. 9), in addition to the expected heavy staining of intercalated cells (not present in this section of papillary collecting duct). The intensity of apical staining varied from cell to cell along the same tubule segment, and appeared to be present either in the apical plasma membrane, or in closely apposed sub-apical vesicles. Some vesicles in other regions of the cytoplasm were also labeled with this antibody. This result is consistent with Western blotting data in Fig. 6 A showing the retention of the 56-kD subunit of the proton pump in endosomes (pool A) from principal cells.

## Discussion

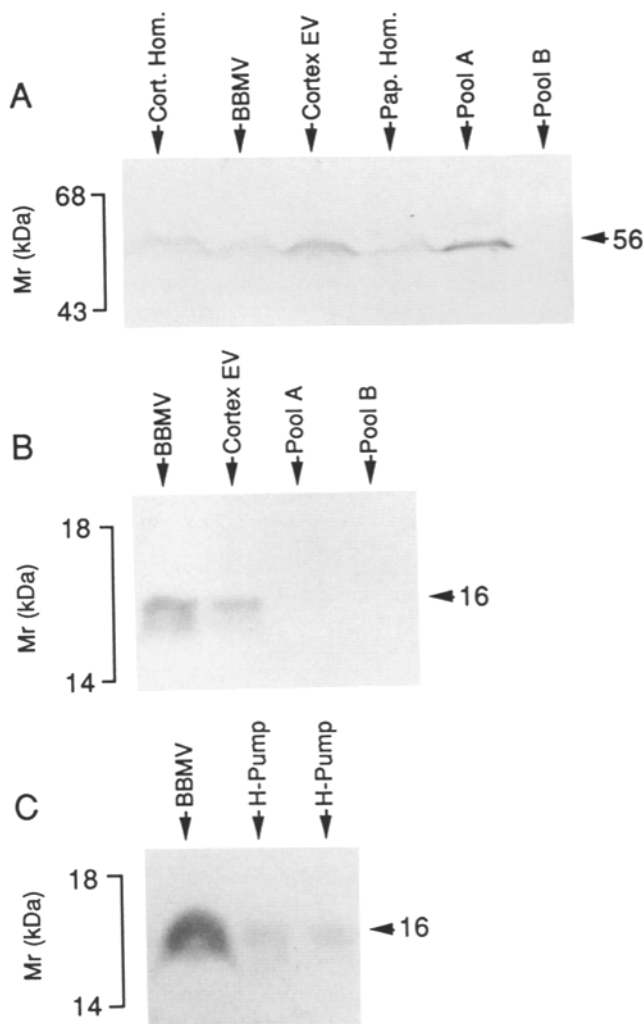
Our previous data showed that papillary endosomes that are involved in the internalization of vasopressin-induced water channels are specialized, non-acidic intracellular compartments (36). Potential reasons for this failure to develop an



**Figure 5.** Western blot of endosomes from kidney cortex (*EV*), and from pools A and B from the papilla, using a polyclonal antibody that recognizes several pump subunits, including the 31-, 56-, and 70-kD subunits, and a mAb that is specific for the 31-kD pump subunit. The 31- and 70-kD subunits are not detectable in papillary vesicles. In some endosome preparations, a diffuse band intermediate between the 70- and 56-kD subunits was detected. The identity of this band is unknown. The 56-kD protein pump subunit is present in endosomes from both cortex and papilla.

acidic intraluminal pH include the presence of a high passive proton permeability in the endosomal membrane, the presence of a nonfunctional proton pumping ATPase in the endosome membrane, or the absence of the entire proton pump, or of one or more pump subunits from the endosome membrane. Our present results show that two major cytoplasmic subunits of the proton pump are absent from papillary endosomes, and that a 16-kD protein identified by antibodies to oat tonoplast  $H^+$  ATPase is also absent from these vesicles; this protein is probably the 16-kD transmembrane proton conducting channel of the vacuolar ATPase (18, 24, 34, 55). These findings account for the failure of papillary apical endosomes to acidify in an ATP-dependent manner.

The identification of early endosomes devoid of proton pump subunits suggests that the vesicles that initially pinch off from the apical plasma membrane of collecting duct principal cells also do not acidify. Because, in the case of water channel endocytosis, this is a clathrin-mediated process (8, 12, 56), our results imply that endocytotic clathrin-coated vesicles lack the capability to acidify. However, when total cellular clathrin-coated vesicles are isolated from various sources, including the kidney, proton pump subunits can be detected in these vesicles, and ATP-dependent acidification can be measured in the total vesicle population (19, 55, 62). The most likely explanation for these results is that the coated vesicles that are specifically involved in (apical) endocytosis are not acidic vesicles, but that other clathrin coated vesicles within the cell, that subserve other transport functions, do contain membrane-associated proton pumps, and are capable of generating an internal acidic pH. In support of this, staining of fibroblasts with the morphological pH marker, DAMP, has demonstrated intracellular heterogeneity of coated vesicle labeling (2). In addition, it has been



**Figure 6.** (A) Western blot of endosomal and BBMVs and kidney homogenates with antibodies that recognize an isoform of the 56-kD subunit of the proton pump that is not present in proximal tubules, but is present in endosomes from other cell types. BBMVs stain weakly, confirming the relative absence of this isoform from proximal tubules. Endosomes from the cortex show a stronger staining, indicating the presence of vesicles from a variety of cell types in the cortex (this isoform is not detected on proximal tubule endosomes by immunocytochemistry). Each lane contained 150  $\mu$ g of protein. (B) Western blot of BBMVs, cortical endosomes (EV), and endosomes from pool A and pool B of the papilla with an antibody that recognizes the 16-kD subunit of oat tonoplast ATPase. Whereas a strong signal is detected in BBMVs and cortical endosomes, signal is absent from both pool A and pool B of the papilla. Each lane contained 150  $\mu$ g of protein. (C) Western blot of BBMVs (150  $\mu$ g protein) and affinity-purified H<sup>+</sup> ATPase protein from bovine renal medulla (two separate preparations, 20  $\mu$ g protein per lane) using the anti-oat tonoplast ATPase antibody that recognizes the 16-kD H<sup>+</sup> ATPase subunit. A strong 16-kD band is present in BBMVs, and a distinct 16-kD band is also present in both preparations of purified H<sup>+</sup> ATPase (H pump). This result indicates that the antibody cross-reacts with the mammalian 16-kD proton pump subunit.

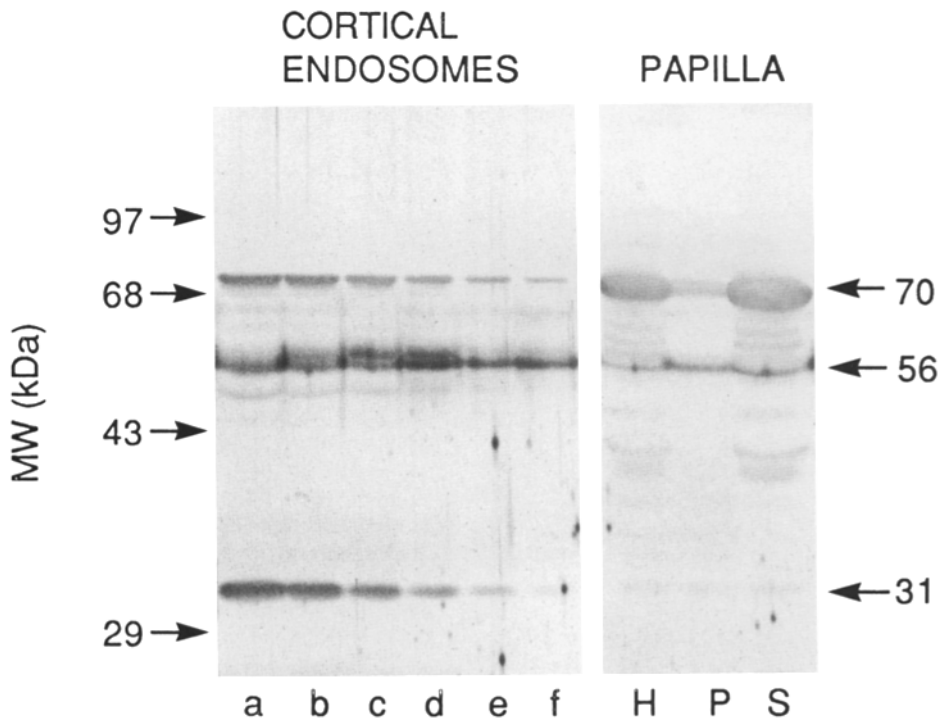
reported that clathrin-coated vesicles involved in endocytosis in other cell types are non-acidifying vesicles (Fuchs, R., A. Ellinger, M. Pavelka, M. Peterlik, and I. Mellman. 1987. *J. Cell Biol.* 105:91a).

An unexpected finding was that the 56-kD subunit of the vacuolar proton pumping ATPase is still detectable in papillary endosomes, despite the absence of the 16-, 31-, and 70-kD subunits. The 31-, 56-, and 70-kD subunits are all located on the cytoplasmic face of the membrane, where they form a morphologically detectable stud-like structure that projects several nanometers from the cytoplasmic leaflet of the membrane. In specialized epithelial cells, such as kidney intercalated cells, that display an extremely high density of proton pumps on vesicles and on their plasma membrane (10, 41, 50), these studs are grouped together in a paracrystalline hexagonal array that has been visualized by rapid-freezing and deep-etch microscopy of these membranes (9). Similar stud-like structures have been more recently found on other acidifying organelles (Heuser, J. E., H. Padh, and T. L. Steck. 1991. *J. Cell Biol.* 111:315a). Models of the vacuolar proton pump, by analogy with the F<sub>0</sub>F<sub>1</sub> mitochondrial proton pump, have predicted that the 56- and 70-kD subunits are attached to the plasma membrane via the 31-kD subunit, which links these subunits to the smaller molecular weight 16-kD transmembrane proton conducting channel (18). If this model is correct, it is difficult to understand how the 56-kD subunit could remain membrane-attached to papillary endosomes, in the absence of the 31-kD subunit. Our results suggest, therefore, that the 56-kD subunit is capable of attaching to the endosomal membrane without the need to interact with the 31- or 70-kD subunits.

Although the 56- and 70-kD pump subunits can be readily detected in the total papillary homogenate and supernatants during the vesicle isolation procedure, this probably reflects the presence of free, soluble components of the V<sub>1</sub> portion of the ATPase in the cytosol of all cells in the papilla, rather than separation of the V<sub>0</sub> and the V<sub>1</sub> components of membrane-associated pumps induced by the vesicle preparation technique. Cortical endosomes isolated in parallel contain all functional pump subunits, and medullary proton pumping vesicles contain V<sub>1</sub> subunits that remain in place during vesicle isolation, but which can be removed by stripping vesicles at pH 11.3 (10). Furthermore, immunocytochemical data clearly demonstrate that the 56-kD subunit is localized on principal cell membranes and vesicles in the intact, fixed tissue, while the absence of other pump subunits from these membranes was confirmed by our present and previous immunocytochemical studies using affinity-purified antibodies (11). In addition, recent work has also shown the presence of a 56-kD subunit on non-acidifying, water channel-containing vesicles from toad urinary bladder using the anti-holo-ATPase polyclonal antibody (27). Although one of the antibodies used in our studies was raised against a peptide that appears to be specific for the COOH-terminal domain of one 56-kD isoform of the proton pump (the "kidney" isoform), it remains possible that the 56-kD subunit in principal cells may be performing a function that is unrelated to the proton pump. This subunit may, therefore, be a promiscuous subunit that can become membrane associated via an interaction with other membrane proteins or multisubunit transporters.

Our observation that the 16-kD subunit is also not detectable in papillary endosomes is important for two reasons. First, this subunit was believed to be required by the 56-kD subunit for membrane association and pump assembly. Second, others have suggested that the vasopressin-induced water channel may have analogies to this proton conducting

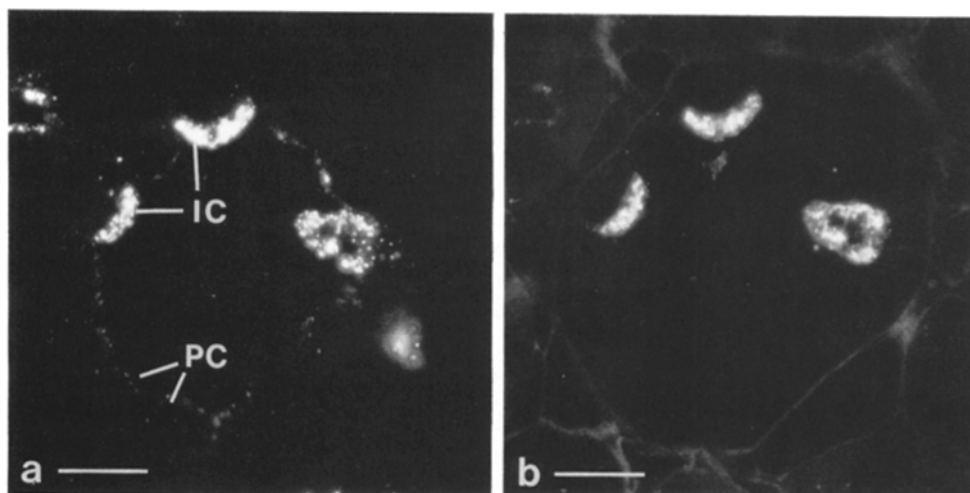




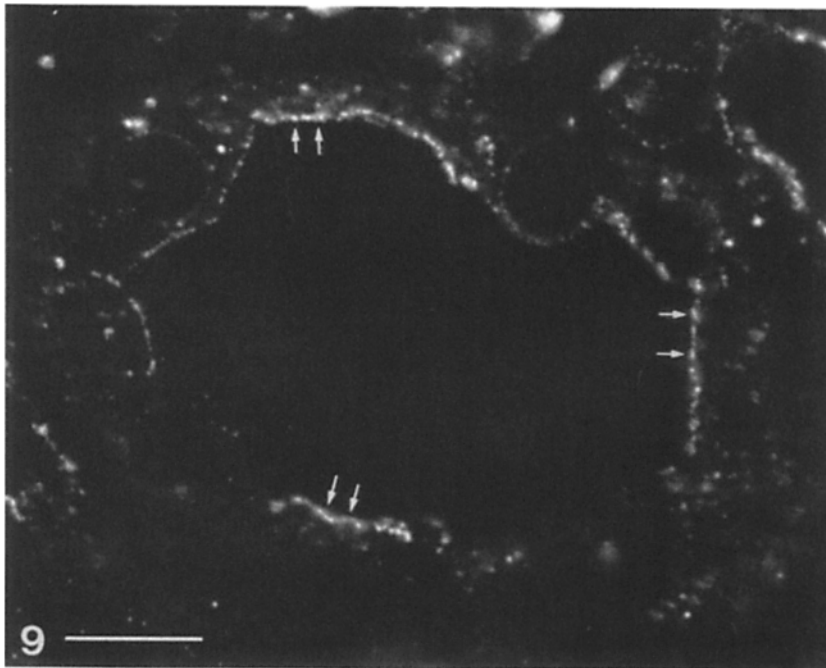
**Figure 7.** Western blots of  $H^+$  pump subunits in renal cortical endosomes, papillary homogenate (*H*), total membranes present in the 105,000 *g* pellet of the papilla (*P*), and the 105,000 *g* supernatant (*S*). Blotting was performed with polyclonal antibodies against 31-, 56-, and 70-kD  $H^+$  pump subunits using decreasing amounts of cortical endosomal proteins (*a*, 100  $\mu$ g; *b*, 50  $\mu$ g; *c*, 25  $\mu$ g; *d*, 12.5  $\mu$ g; *e*, 6.225  $\mu$ g; and *f*, 3.125  $\mu$ g), and 100  $\mu$ g protein per lane in fractions from papilla. Mol wt markers are indicated on the left, whereas the position of  $H^+$  pump subunits is labeled on the right.

channel, and have demonstrated a high proton flux through toad bladder membranes containing vasopressin-induced water channels (28). Gluck and Al-Awqati (23) first showed that the transepithelial passive proton permeability of the toad bladder increases in parallel with vasopressin-induced increase in osmotic water permeability. More recently, it has been shown that some inhibitors of proton pumping ATPases (DCCD, NEM, diethylstilbesterol, and oligomycin) also inhibit vasopressin-induced water flow in toad skin and toad urinary bladder (30). These data are consistent with a com-

mon pathway for water and protons across these membranes, and that this pathway may be via an  $F_0$ -like transmembrane proton pore. While papillary endosomes may indeed contain such a transmembrane pore, this channel does not cross-react with antibodies against the 16-kD transmembrane subunit of a proton pumping ATPase. In contrast, this subunit was readily detectable in cortical endosomes, derived mainly from the proximal tubule, as well as in renal brush border membrane vesicles and in affinity-purified  $H^+$  ATPase protein, showing that the antibody does recognize a



**Figure 8.** Cryostat section of collecting duct from the inner stripe of a rat, injected for 15 min with FITC dextran and double-labeled to reveal proton pumps by immunocytochemistry. In *A*, endosomes in principal cells (*PC*) and intercalated cells (*IC*) are labeled with FITC dextran. The intercalated cells show a much greater uptake of the probe than principal cells. Fig. 8 *B* shows the same section, incubated with a monoclonal antibody against the 31-kD subunit of the proton pumping ATPase. Whereas endosomes in intercalated cells are heavily labeled, principal cell endosomes are unlabeled, supporting the functional data showing a lack of ATP-dependent acidification by these vesicles. Bar, 10  $\mu$ m.



*Figure 9.* Semithin frozen section of rat papillary collecting duct, incubated with antibody against the 56-kD subunit of the proton pumping ATPase. The principal cells show a concentration of immunoreactive sites on or close to the apical plasma membrane, that is consistent with the Western blotting data (Fig. 5) showing the presence of the 56-kD subunit in endosomal vesicles from these cells. Vesicles scattered in other regions of the cytoplasm are also stained. Bar, 10  $\mu\text{m}$ .

mammalian 16-kD subunit in membranes that contain a functional proton pumping ATPase. Other reports have suggested that the transmembrane water channel may be, or is related to a facilitated glucose transporter (17) or a band 3-like anion exchange protein (53). However, we were unable to detect either of these proteins in papillary endosomes using specific antibodies that recognize glucose transporters (57) and band 3 (1) in kidney epithelial cells (data not shown). In addition, immunocytochemical studies have shown these proteins to be localized on basolateral, but not apical, plasma membrane in kidney epithelial cells (1, 16, 49, 57).

Recently, a 28,000-mol wt integral membrane protein found in erythrocyte and proximal tubule membranes, and which is related to the 26-kD major intrinsic protein of the lens, has been isolated and sequenced (15, 45, 52). This protein, known as CHIP 28, is a member of an ancient family of transmembrane channel forming proteins and probably functions as the proximal tubule and erythrocyte water channel (45, and A. N. van Hoek and A. S. Verkman, manuscript submitted for publication). At least in the cortex, therefore, proximal tubule water channels are distinct from the 16-kD subunit of the  $\text{H}^+$  ATPase. However, the nature of the vasopressin-sensitive water channel in collecting ducts has not yet been established.

Whereas endosomes derived from the apical plasma membrane of principal cells are specialized structures that appear not to be primarily involved in the transport of internalized fluid-phase molecules to lysosomes, basolateral endocytosis probably does result in the rapid delivery of internalized molecules to lysosomes. Fluorescent vasopressin analogs that bind to basolateral receptors on principal cells are internalized and the fluorescent marker can be detected in large, perinuclear, lysosome-like structures after a chase period at 37°C (33, 40). A similar difference between apical and basolateral pathways of endocytosis has been reported for filter-

grown, MDCK cells, in which a calculated 90% of apical endosomes are either recycled back to the apical plasma membrane, or else undergo transcytosis to the base of the cell without entering a later endosomal compartment (5). However, the fate of apically endocytosed material is likely to be cell-specific, because endocytosis from the luminal pole of proximal tubule epithelial cells in the kidney clearly results in delivery of markers to an acidic compartment (36, 46, 63), and eventually to lysosomal degradation (14). If basolateral endosomes from papillary tubule cells do, indeed, contain a functional proton pump, then they do not appear to co-purify with our FITC-dextran-loaded apical vesicles. Alternatively, the extent of basolateral endocytosis in these cells may be considerably less than at the apical pole, so that the contribution of basolateral endosomal membranes to the total vesicle preparation is too small to be detectable by Western blotting.

Finally, endosomes that are involved in internalizing water channels in the toad urinary bladder also fail to acidify their lumen. This has been demonstrated by ATP-dependent fluorescence quenching assays (29, 61), as well as by morphological studies using the DAMP-labeling procedure (Franki, N., G. Ding, and R. M. Hays. 1988. *Am. Soc. Nephrol.* 39a). The specialized nature of apical endosomes in vasopressin-sensitive cells presumably reflects the superficial vesicle shuttling mechanism that characterizes their physiological response to hormonal stimulation. However, other cell types also use a similar process to recycle functionally important cell surface molecules between cytoplasmic vesicles and the plasma membrane in response to a variety of stimuli. For example, the insulin-sensitive glucose transporter Glut-4 has a similar recycling mechanism, as does the  $\text{H}^+/\text{K}^+$  ATPase in gastric parietal cells. In the mammalian urinary bladder, sodium channels are inserted into the apical membrane of surface epithelial cells as a result of stretch-induced exocytosis (38). Whether these and

other vesicles involved in specialized recycling processes also lack the capacity to acidify remains to be determined.

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