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Vacuolar H^+-ATPase in ocular ciliary epithelium

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ABSTRACT **The mechanisms controlling the production of aqueous humor and the regulation of intraocular pressure are poorly understood. Here, we provide evidence that a vacuolar H^+-ATPase (V-ATPase) in the ocular ciliary epithelium is a key component of this process. In intracellular pH (pH_i) measurements of isolated ciliary epithelium performed with 2′,7-biscarboxyethyl-5(6)-carboxyfluorescein (BCECF), the selective V-ATPase inhibitor bafilomycin A_1 slowed the recovery of pH_i in response to acute intracellular acidification, demonstrating the presence of V-ATPase in the plasma membrane. In isolated rabbit ciliary body preparations examined under voltage-clamped conditions, bafilomycin A_1 produced a concentration-dependent decrease in short-circuit current, and topical application of bafilomycin A_1 reduced intraocular pressure in rabbits, indicating an essential role of the V-ATPase in ciliary epithelial ion transport. Immunocytochemistry utilizing antibodies specific for the B1 isoform of the V-ATPase 56-kDa subunit revealed localization of V-ATPase in both the plasma membrane and cytoplasm of the native ciliary epithelium in both rabbit and rat eye. The regional and subcellular distribution of V-ATPase in specific regions of the ciliary process was altered profoundly by isoproterenol and phorbol esters, suggesting that change in the intracellular distribution of the enzyme is a mechanism by which drugs, hormones, and neurotransmitters modify aqueous humor production.**

In glaucoma, a disease characterized by elevated intraocular pressure, a primary therapeutic strategy is to decrease the secretion of aqueous humor by the ciliary epithelium. Aqueous humor production requires active ion transport. The ciliary epithelium is a double layer with two cell types: the outer nonpigmented epithelial (NPE) layer and the inner pigmented epithelial (PE) layer, both of which exhibit properties of transporting epithelia (1–8). The two cell layers have juxtaposed apical membranes (Fig. 1), and the NPE and PE are coupled through an extensive network of gap junctions; consequently, the bilayer is thought to function electrogenically as a syncytium (5, 9–11). In current models, solute entry into the dual epithelium is postulated to occur at the basolateral surface of the PE cells through several sodium-dependent cotransporters, including Na^+–H^+ exchange, Na^+-dependent $NaHCO_3^-$ exchange, electroneutral Na^+/Cl^- cotransport, and others (refs. 12–17, for review, see ref. 18). The NPE is thought to provide the main ion-motive force for sodium-dependent cotransporters, as physiologic and immunocytochemical evidence indicates that Na^+/K^+-ATPase resides in the basolateral membrane of the NPE (19–23). Electroneutrality is thought to be maintained by anion channels in the NPE basolateral membrane, and recently it has been demonstrated that the β-adrenergic antagonist timolol, which reduces aqueous humor formation, inhibits cAMP-dependent, 4,4′-diisothiocyanotostilbene-2,2′-disulfonic acid (DIDS)-sensitive chloride efflux (24).

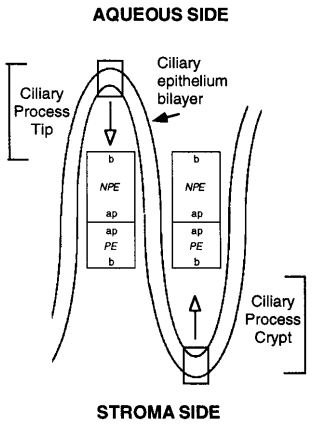

FIG. 1. Schematic representation of the ocular ciliary epithelium. The dual layer epithelium consists of a columnar nonpigmented epithelium (NPE) cell layer and a cuboidal pigmented epithelium (PE) cell layer. The two layers of the epithelium have their apical (ap) surfaces in apposition, whereas the basal (b) pole of NPE cells lies adjacent to aqueous humor and the basal pole of the PE cells lies adjacent to the stroma of the ciliary processes. The apex-to-apex orientation of the epithelial bilayer remains consistent throughout the ciliary epithelium, whose tissue architecture contains "tips" and "crypts" characterisitc of invaginated secretory epithelium.

Although carbonic anhydrase inhibitors are among the most potent inhibitors of aqueous humor formation, the mechanism of their effect is obscure (4, 25). Since carbonic anhydrase inhibitors are potent antagonists of bicarbonate reabsorption in proton-transporting epithelia, we suspected that a proton pump might have an essential function in the formation of aqueous humor. Here we report the results of *in vitro* studies on intracellular pH, as well as immunocytochemical, electrophysiological, and *in vivo* experiments that support a role for active membrane-bound H^+-ATPase in the ciliary epithelium as an important ion-motive force in aqueous humor production.

MATERIALS AND METHODS

Measurement of Intracellular pH (pH_i). Measurement of pH_i was performed on the ciliary epithelial bilayer (CEB)

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Abbreviations: NPE, nonpigmented epithelium (or epithelial); PE, pigmented epithelium (or epithelial); pH_i, intracellular pH; CEB, ciliary epithelial bilayer; BCECF-AM, 2′,7-biscarboxyethyl-5(6)-carboxyfluorescein acetoxymethyl ester; V-ATPase, vacuolar H^+-ATPase.

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isolated from New Zealand White rabbits as described (26). Hepes-buffered Ringer's solution consisted of (in mM): 110 NaCl, 3.5 KCl, 1.4 $CaCl_2$, 1.0 $MgSO_4$, 1.5 $H_2PO_4^-$, 10 glucose, 0.01 EDTA, 38 Hepes hemisodium salt, 14 sodium gluconate; pH = 7.48, 292 mosmol·kg^{-1}. In the bicarbonate Ringer's solution, 28 mM hemisodium Hepes was replaced by 28 mM $NaHCO_3$ and a 5% CO_2 atmosphere, and the sodium gluconate was omitted. In the gluconate/bicarbonate Ringer's solution all Cl^- was replaced by gluconate. The isolated CEB was incubated in Hepes-buffered Ringer's solution containing 25 mM precursor acetoxymethyl ester of 2′,7-biscarboxyethyl-5(6)-carboxyfluorescein (BCECF-AM; Molecular Probes) at room temperature for 60 min. After washing, dye-loaded CEB was placed epithelial-side down over a coverslip using a tissue adhesive, Cell-Tak (Collaborative Biomedical Products, Bedford, MA). The coverslip was placed in a thermocontrolled chamber (Biophysica Technologies, Sparks, MD). Fluorescence spectroscopy measurements were made in a four-channel microfluorometer with excitation at 440 nm and 495 nm and emission at 530 nm at 37°C with a perfusion rate of 2–4 ml/min. A field of about 20 cells in the pars plana–pars plicata boundary was selected for the measurement. At the end of the measurement, calibration was achieved utilizing nigericin and KCl as described (27).

Immunocytochemistry. Sections (1 μm) from tissue embedded in Epon were treated with saturated NaOH and absolute ethanol to remove resin. Sections were incubated in PBS containing 1% bovine serum albumin (BSA) to reduce nonspecific background staining and then with a rabbit polyclonal antibody to the B1 subunit of vacuolar H^+-ATPase (V-ATPase). Fluorescein isothiocyanate-labeled goat anti-rabbit immunoglobin was used as the secondary antibody. The sections were mounted in 50% glycerol/PBS and photographed with an epifluorescent microscope. Preimmune sera failed to reveal any significant staining (not shown).

For drug treatment, rabbit iris/ciliary bodies (I/CB) were freshly dissected, sectioned, and maintained in modified oxygenated Krebs buffer for 30 min, in which buffer was changed twice prior to drug treatment. The tissue was then treated for 20 min with drug as indicated in the various panels. I/CB sections were embedded in Epon, and 1-μm sections were stained with a polyclonal antibody to the B1 subunit of V-ATPase.

Immunoelectron Microscopy. *Rabbit*. Antibody 865 (provided by N.N.) against the B subunit of bovine brain H^+-ATPase diluted 1:50 and followed by staphylococcal protein A conjugated to 15-nm colloidal gold particles as described (28). Briefly, 5-μm thin sections were labeled at room temperature by the flotation of grids on droplets. Grids were treated with 0.1 M NH_4Cl for 1 hr, rinsed with buffers, partially dried, and exposed to primary antibody 865 overnight at 4°C in a moist chamber. The grids were treated with buffers for 1 hr, partially dried, and treated with secondary antibody consisting of goat anti-rabbit IgG conjugated to 15-nm diameter colloidal gold particles for 1 hr, rinsed with buffers and water, and counterstained with uranyl acetate and lead citrate. Sections were examined and photographed with a Zeiss 10A transmission electron microscope. *Rat*. Antibodies against B1 subunit isoform (29) were used followed by protein A conjugated to 8-nm colloidal gold particles as described (30). Briefly, 1-μm sections picked up on nickel grids were processed with no prior etching procedure. Sections were incubated for 10 min on a drop of PBS, and 10 min on a drop of PBS containing 1% BSA to reduce background staining. The sections were incubated for 2 hr at room temperature with primary antibody against the B1 subunit isoform of H^+-ATPase in a moist chamber. Sections were rinsed and incubated in 1:70 dilution of protein A-gold solution in PBS/1% BSA for 1 hr, washed in PBS and distilled water, stained for 7 min in 2% uranyl acetate and for 1 min with lead citrate, and examined and photographed on a JEOL 1200-EX electron microscope.

RESULTS

To determine if the ciliary epithelium contains an active membrane-bound H^+-ATPase, we examined the effect of bafilomycin A_1, a selective V-ATPase inhibitor (31), on restoration of pH_i in isolated ciliary epithelium. Bafilomycin A_1 was purchased from K. Altendorf (University of Osnabruck, Osnabruck, Austria). As shown in Fig. 2, changing the medium from bicarbonate-free Hepes solution to CO_2/HCO_3^- buffer produced a rapid fall in pH_i that recovered upon change to a chloride-free solution (tracing A) at a rate of 0.003 ± 0.0008 pH unit/sec, a response that has been attributed to Cl^-/HCO_3^- exchange (14). Treatment of the epithelium with 1 mM amiloride had no effect on pH_i recovery (0.003 ± 0.0007 pH unit/sec), suggesting that it does not involve Na^+–H^+ exchange (tracing B). Pretreatment of the isolated rabbit CEB with bafilomycin A_1 (tracing C), however, significantly reduced the rate of pH_i recovery to 0.001 ± 0.0002 pH unit/sec ($P < 0.05$), indicating that at least part of the pH_i recovery is dependent on proton extrusion by a V-ATPase. These findings

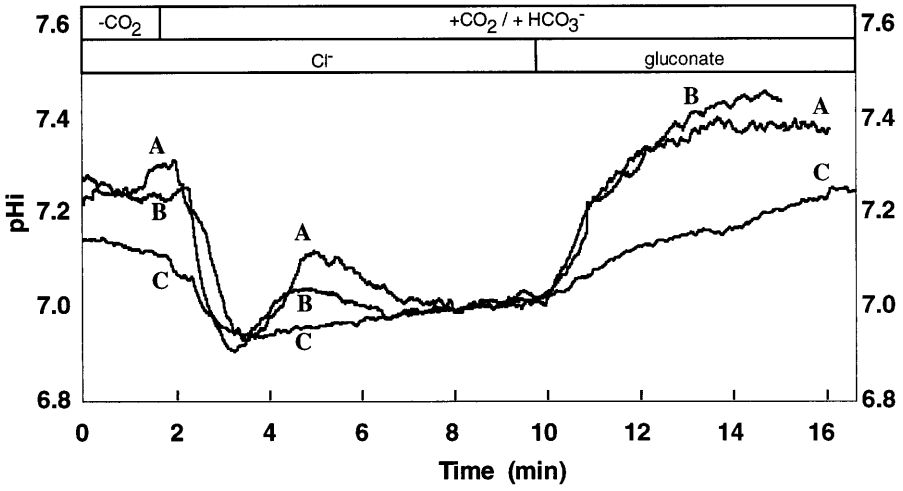

FIG. 2. pH_i measurement in isolated rabbit CEB. Dye-loaded CEB was initially perfused with Hepes buffer in the absence of CO_2. Perfusion buffer was changed from Hepes to bicarbonate containing CO_2, then to Cl^--free (gluconate) buffer. Preincubation of CEB with 1 mM amiloride or 2 μM bafilomycin A_1 was performed for 20 min at room temperature. A, B, and C are representative traces from control, amiloride-treated, and bafilomycin A_1-treated CEB, respectively.

demonstrate that a V-ATPase is present and active on the epithelial plasma membrane.

We next examined whether the plasma membrane V-ATPase has an essential function in aqueous humor formation by testing the effect of bafilomycin A_1 on intraocular pressure (IOP), a measurement which reflects a steady-state relationship between aqueous production and aqueous outflow. Topical application of bafilomycin A_1 to rabbit corneas lowered IOP in a dose-dependent manner (Fig. 3). To determine whether the ocular hypotensive effect of bafilomycin was due to reduced aqueous inflow or to enhanced outflow facility, we performed tonography on rabbits 1 hr after treatment with either 0.2% or 0.4% bafilomycin ($n = 3$). Outflow facility (μl/min per mmHg) was 0.25 ± 0.01 after 50 μl of topical vehicle (0.5% dimethyl sulfoxide); 0.25 ± 0.01 for 0.2% bafilomycin; and 0.27 ± 0.01 for 0.4% bafilomycin ($P > 0.5$). The results indicate bafilomycin does not affect outflow, indicating that the ciliary epithelial V-ATPase provides a primary ion-motive force for aqueous humor formation.

As a second method for determining if the ciliary epithelial plasma membrane V-ATPase is essential for aqueous humor formation, we examined the effect of bafilomycin on ion transport in isolated rabbit iris/ciliary body preparations mounted in an Ussing–Zerahn chamber. As shown in Table 1, addition of bafilomycin A_1 to the "aqueous" (internal) side produced a concentration-dependent inhibition of both the open-circuit transepithelial potential difference (PD) and the short-circuit current, a direct index of active transport (32), demonstrating that V-ATPase in the NPE cells generates the ion-motive force for transport in the intact ciliary epithelium. Addition of bafilomycin to the "blood" (external) side increased the PD, consistent with the presence of V-ATPase in the PE cells, as confirmed below.

To determine directly whether a V-ATPase is present in the basolateral membranes of the NPE or PE cells of the rabbit ciliary epithelium, we performed immunocytochemistry on intact ciliary tissue from freshly dissected eyes (Fig. 4*A*) using antibodies specific for the B1 ("kidney") and B2 ("brain") isoforms of the V-ATPase B (56-kDa) subunit (29). Staining for the V-ATPase B1 subunit isoform was observed in the plasma membrane region of both the NPE and PE layers in rabbit ciliary epithelia (Fig. 4*A*); no plasma membrane staining was observed for the B2 isoform (not shown). Although immunostaining was observed primarily in the basolateral surfaces of the NPE and PE, there was a marked immunostaining difference in the ciliary process tip as compared with the ciliary process crypt. In the ciliary process tip, the B1 subunit staining was localized mainly in the basolateral domain in both the NPE and PE layers. In the crypt, however, staining in the basolateral domain was minimal or absent from the NPE layer. In addition, immunostaining was observed in the apical domain of certain NPE cells of the ciliary tips, whereas minimal apical staining was observed in either cell layer of the ciliary crypts.

Table 1. Effect of bafilomycin A_1 on short circuit current (SCC) and potential difference (PD) in the isolated rabbit/ciliary body

	Bafilomycin added to aqueous side		Bafilomycin added to blood side	
Bafilomycin	SCC, μA	PD, mV	SCC, μA	PD, mV
None	−8.1 ± 0.8	0.55	−8.5 ± 0.6	0.45
1 nM	−7.7 ± 1.3	0.55	−8.5 ± 0.8	0.45
10 nM	−7.8 ± 0	0.55	−8.5 ± 0.6	0.45
100 nM	−7.7 ± 0.8	0.55	−8.5 ± 0.6	0.45
1 μM	−6.0 ± 0.9*	0.40	−10.4 ± 0.6	0.45
10 μM	−4.2 ± 0.9**	0.40	−12.4 ± 0.9*	0.40

Bafilomycin was added to the bath in increasing concentrations every 30 min and transepithelial measurements (26) were obtained. *, $P < 0.05$; **, $P < 0.01$.

To confirm the presence of V-ATPase in the plasma membrane, we performed immunoelectron microscopy on native ciliary epithelium from rabbit and rat eyes. Freshly dissected eyes were fixed with periodate/lysine/paraformaldehyde, embedded in Lowacryl K4M, sectioned by ultramicrotome, and mounted on carbon/Formvar-coated nickel grids; sections were labeled with immunogold using primary antibodies followed by incubation with protein A conjugated to 15-nm colloidal gold particles. Utilizing antibodies to the B subunit of V-ATPase (provided by N.N.) and to the B1 subunit isoform (29), we identified vacuolar H^+-ATPase on the basolateral membrane of the rabbit and rat nonpigmented epithelium (Fig. 5).

The observed differences in V-ATPase distribution between the ciliary process tips and crypts suggested that these regions of the epithelium might have functional differences important for the control of aqueous humor secretion. We therefore performed experiments to determine whether the subcellular distribution of V-ATPase is altered by drugs known to affect aqueous humor production (33). The isolated fields shown in Fig. 4 are representative of the of the characteristic changes in V-ATPase distribution induced by protein kinase A or protein kinase C activating in the tip or crypt of the rabbit ciliary processes. In freshly dissected ciliary processes treated with 100 μM isoproterenol, an agent that activates adenylyl cyclase, V-ATPase staining in the apical pole of the NPE layer in both the ciliary process tips appears modestly enhanced, and staining the basolateral pole in the ciliary body crypts was greatly enhanced (Fig. 4*B*); these effects were blocked by the coincubation of the tissue with 1 μM propranolol during isoproterenol treatment. In ciliary processes treated with 1 μM phorbol ester (phorbol 12,13-dibutyrate), an agent that activates protein kinase C, V-ATPase staining in the basolateral domain of the NPE was also enhanced in the ciliary process crypt and tip region. No change in the regional or subcellular distribution of V-ATPase staining was observed in tissue treated with the inactive 4α-phorbol didecanoate.

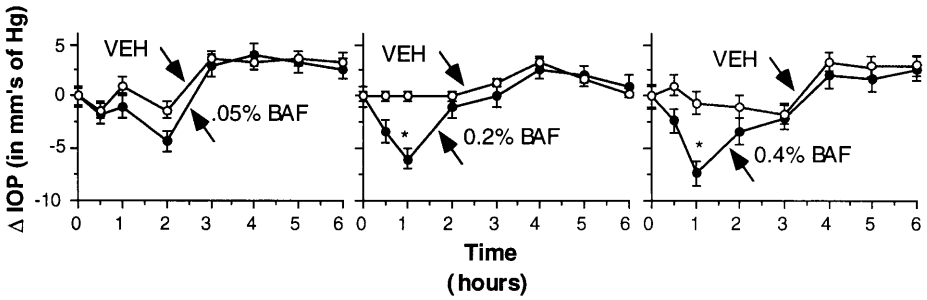

FIG. 3. Intraocular pressure (IOP) in rabbits after topical bafilomycin A_1. A 50-μl sample of bafilomycin (BAF) in 0.5% dimethyl sulfoxide, or dimethyl sulfoxide vehicle (VEH), was applied topically to (paired) rabbit eyes ($n = 3$), and intraocular pressures were recorded for 6 hr and reported as differences from baseline values (approximately 24 mmHg). *, $P < 0.05$.

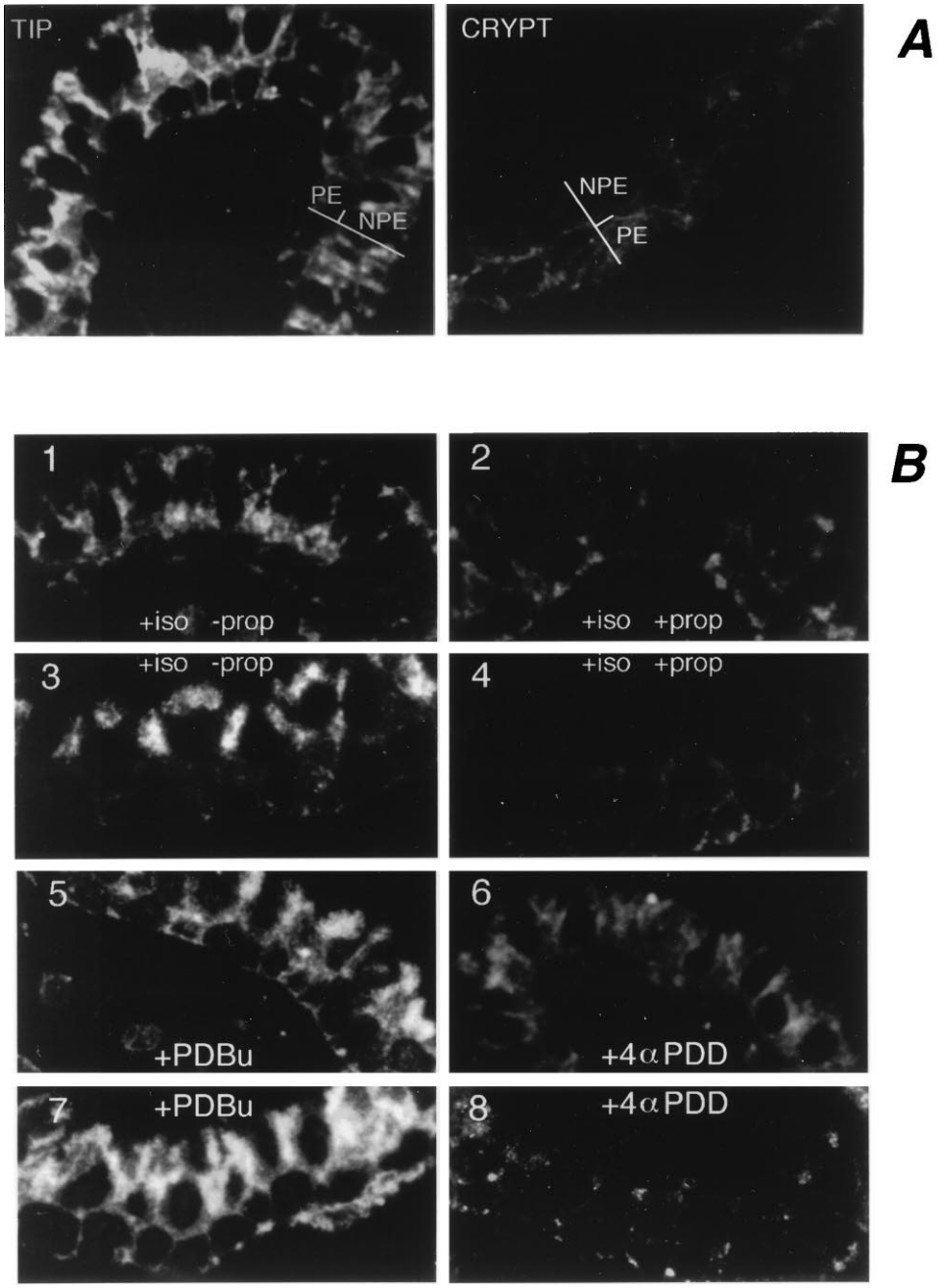

FIG. 4. (*A*) Immunocytochemistry of the rabbit ciliary process. (×2,500.) (*B*) Immunocytochemistry of rabbit ciliary epithelium tips or crypts treated with isoproterenol (iso) at 100 μM, propranolol (prop) at 1 μM, active phorbol ester phorbol 12,13-dibutyrate (PDBu) at 1 μM, or the inactive phorbol ester 4α-phorbol didecanoate (4αPDD) at 1 μM as indicated in the following panels: *1*, tip +iso −prop; *2*, tip +iso −prop; *3*, crypt +iso −prop; *4*, crypt +iso +prop; *5*, tip +PDBu; *6*, tip +4αPDD; *7*, tip +PDBu; and *8*, crypt +4αPDD. (×2,500.)

DISCUSSION

Our results support the hypothesis that the plasma membrane V-ATPase provides a driving force for aqueous humor formation. Although V-ATPases are found in intracellular compartments of all eukaryotic cells, their presence at high levels in the plasma membrane is rare and found only cells specialized for proton secretion, such as the intercalated cell in kidney and the osteoclast in bone (34). The identification of the B1 isoform of the V-ATPase in the ciliary epithelium is significant, as expression and plasma membrane localization of this isoform have been described previously only in kidney collecting duct (29) and epididymis (35). In the collecting duct of the kidney the specialized proton pump of the intercalated cell may be polarized to either the apical or basolateral pole in subsets of cells, imparting a capacity for either net proton or net bicarbonate secretion and is ultimately thought to be important for bicarbonate resorption from urine (34). We hypothesize that in the eye, membrane-bound H^+-ATPase may be similarly important in bicarbonate transport in the ciliary epithelium.

Carbonic anhydrase inhibitors are potent suppressors of H^+ transport by V-ATPases in renal and other epithelia (36). It has been argued by different investigators that carbonic anhydrase inhibitors act either directly on bicarbonate transport mechanisms (37) or indirectly by providing buffering capacity for hydroxide ions, thereby affecting pH_i (25, 38, 39). An implication of the model proposed here is that the potent suppressive effect of carbonic anhydrase inhibitors on aqueous production may be due to inhibition of H^+ transport by the ciliary epithelium V-ATPase.

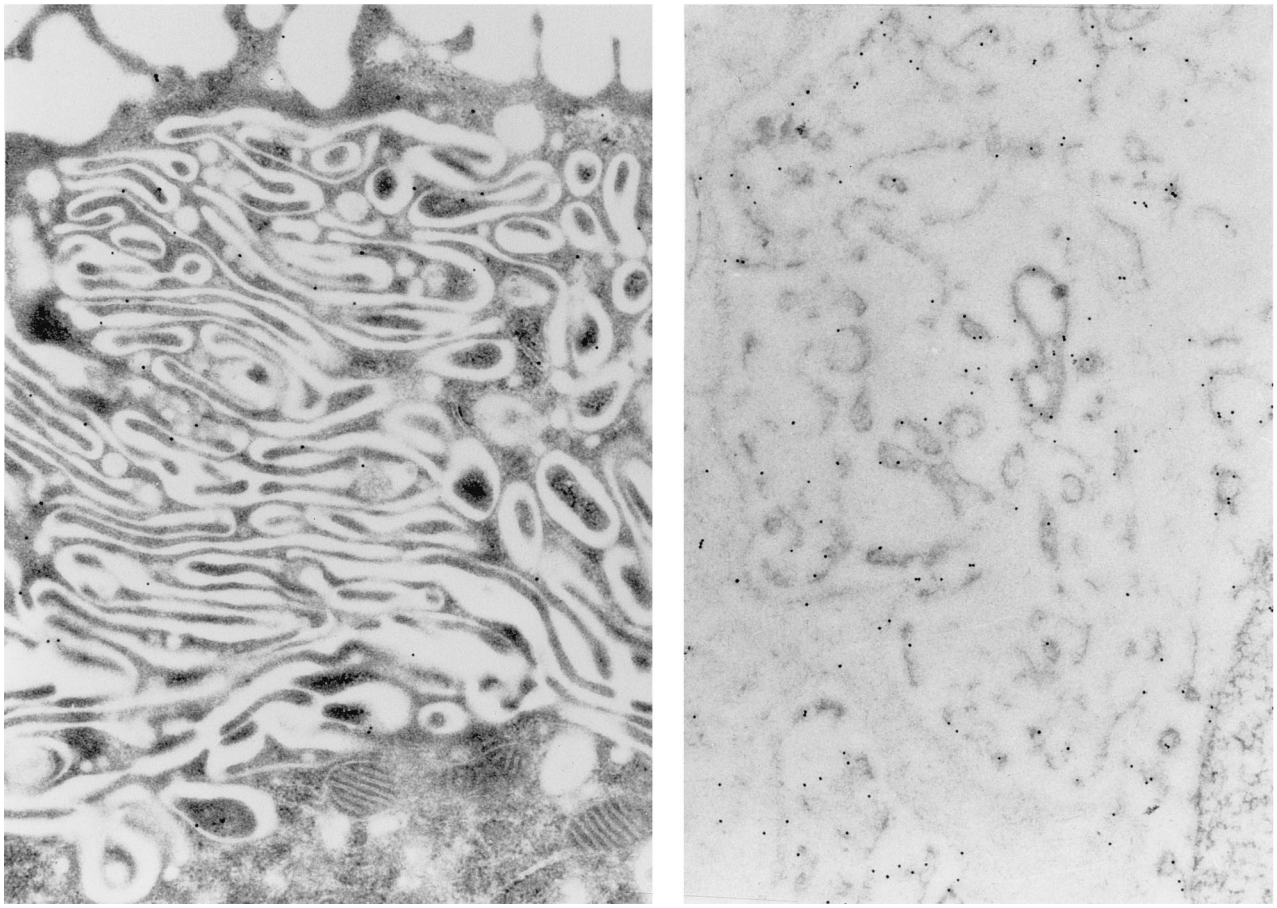

FIG. 5. Immunoelectron microscopy of NPE cell from rabbit (*Left*) (×16,000) and rat (*Right*) (×19,200) ciliary processes using antibodies against the B subunit of V-ATPase. Label is prominent on basolateral plasma membrane.

One surprising result of these studies is that bafilomycin A_1 had little sustained effect on pH_i. Transporters other than the V-ATPase may be involved in the restoration of cell pH in the ciliary epithelium (18), but they are not necessarily involved in the steady-state transport activity needed for formation of aqueous humor. A similar example is found in the proton-secreting α intercalating cell of the kidney collecting duct, in which inhibition of the lumenal vacuolar H^+-ATPase completely inhibits bicarbonate reabsorption, but pH_i is maintained by a basolateral Na^+/H^+ antiporter (40, 41). A second surprising finding is that the V-ATPase is abundant on the basolateral membrane of the NPE cells, as aqueous humor in mammalian species has a pH of 7.2, similar to plasma. This suggests that V-ATPase of the ciliary epithelium may function in a manner resembling the goblet cell of the *Manduca* foregut (42), in which a V-ATPase in the lumenal membrane provides an essential electrical driving force for alkali secretion by a K^+/H^+ antiporter.

Last, our findings suggest that changes in subcellular distribution of V-ATPase such as by vesicle-mediated translocation or regulated assembly and disassembly by drugs which activate protein kinases A and C may be a mechanism central to the production and regulation of aqueous humor. To our knowledge, changes in V-ATPase distribution in response to agents that activate protein kinase A and C have not been reported previously for any tissue. The finding that the β-adrenergic antagonist drug propranolol inhibited the observed change in V-ATPase staining in the ciliary epithelium suggests the possibility that the V-ATPase may be a key pharmacologic target of several clinically used glaucoma medications, and it may lead to novel treatments for glaucoma.

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