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Studies on H^+-ATPase in Cultured Rabbit Nonpigmented Ciliary Epithelium

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Abstract: Studies were conducted to examine the influence of the H^+-ATPase inhibitor bafilomycin A_1 on cultured rabbit nonpigmented ciliary epithelial cells (NPE). Cytoplasmic pH and sodium concentrations were measured by digital fluorescence microscopy using BCECF and SBFI respectively. In some experiments, cell sodium content was measured by atomic absorption spectroscopy. Added alone, bafilomycin A_1 (100 nM) failed to change cytoplasmic pH but it caused an increase of cytoplasmic sodium concentration which occurred within 10 min. It is likely that the rise of cytoplasmic sodium concentration was responsible for the stimulation of active sodium-potassium transport which occurred in bafilomycin A_1-treated cells as judged by a 50% increase of ouabain sensitive potassium (^{86}Rb) uptake. In bafilomycin A_1-treated cells, but not in control cells, dimethylamiloride (DMA) inhibited ouabain-sensitive potassium (^{86}Rb) uptake in a dose-dependent manner with an IC_{50} of ~2 μM. DMA (10 μM) also prevented the increase of cytoplasmic sodium caused by bafilomycin A_1. Added alone, DMA (10 μM) failed to change cytoplasmic sodium content but reduced cytoplasmic pH by ~0.4 pH units. In cells that first received 10 μM DMA, the subsequent addition of bafilomycin A_1 (100 nM) caused a further cytoplasmic pH reduction of ~0.3 pH units. Taken together, the results suggest H^+-ATPase might contribute to the regulation of basal cytoplasmic pH in cultured NPE. In the presence of bafilomycin A_1, Na-H exchanger activity appears to be stimulated, so stabilizing cytoplasmic pH but resulting in an increase of cytoplasmic sodium concentration and consequent stimulation of active sodium-potassium transport.

Key words: Cytoplasmic pH — H^+-ATPase — Bafilomycin A_1 — Dimethylamiloride — Cytoplasmic sodium

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Introduction

Vacuolar H^+-ATPases are membrane proteins which conduct electrogenic proton transport. Commonly, H^+-ATPases acidify cytoplasmic compartments such as lysosomes and endosomes (Chou et al., 1997; Sundler, 1997; Bayer et al., 1998; Okhuma et al., 1998). However, in some cells H^+-ATPase localized on plasma membrane conducts the active transport of protons outward from the cytoplasm. Proximal tubule cells and osteoclasts are among the range of cell types in which a functional H^+-ATPase has been described at a plasma membrane location (Reid et al., 1987; Mattson et al., 1997). In several tissues, proton extrusion from the cytoplasm following an acid load appears to be mediated by H^+-ATPase (Manger & Koeppen, 1992; Pappas & Ransom, 1993; Villanger et al., 1995; de Ondarza & Hootman, 1997).

Recently, plasma membrane-localized H^+-ATPase was detected in the ciliary epithelium bilayer of the eye (Wax et al., 1997). Made up of two cell types, nonpigmented ciliary epithelium (NPE) and pigmented ciliary epithelium (PE), the bilayer of cells secretes aqueous humor into the interior of the eye. H^+-ATPase appears to be most abundant at the basolateral surface of the NPE layer (Wax et al., 1997). In studies with cultured rabbit NPE, the specific H^+-ATPase inhibitor bafilomycin A_1 (Bowman et al., 1988) was found to suppress cytoplasmic alkalinization responses associated with cell depolarization or removal of extracellular ammonium chloride (Wu & Delamere, 1997). The sensitivity of these cytoplasmic pH responses to bafilomycin A_1 was consistent with inhibition of H^+-ATPase-mediated outward proton transport. However, bafilomycin A_1 failed to alter the baseline value of cytoplasmic pH in cultured rabbit NPE, suggesting the possibility that H^+-ATPase does not contribute to cytoplasmic pH regulation in resting cells. Based on results of the present study, we propose

this is not the case. Instead, our findings are consistent with a model in which H^+-ATPase actively contributes to cytoplasmic pH regulation by extruding protons under resting conditions but Na-H exchange is activated to stabilize cytoplasmic pH when the cells are exposed to bafilomycin A_1.

Materials and Methods

Cell Culture

Studies were conducted using a cell line derived from SV40 virus-transformed rabbit nonpigmented ciliary epithelium which was generously provided by Dr. Miguel Coca-Prados (Department of Ophthalmology, Yale University). This cell line has been used previously in studies of Na,K-ATPase and other ion transport mechanisms (Delamere, Coca-Prados & Aggarwal, 1993; Dong et al., 1994; Wu, Pierce & Delamere, 1998). Cells were grown at 37°C in Dulbecco's modified Eagle's medium (Sigma Chemical, St. Louis, MO) supplemented with 10% fetal bovine serum (Sigma), under a humidified atmosphere of 5% CO_2/95% air. Penicillin (100 U/ml) and streptomycin (100 ng/ml) were added to the growth medium as antibacterial agents. It should be noted that under these conditions the cells are probably not polarized and are unlikely to have distinct apical and basolateral surfaces. At least 12 hr before each experiment, the growth medium was replaced with penicillin and streptomycin-free Dulbecco's modified Eagle's medium. Confluent monolayers of cells grown in 24-well plates were used for ^{86}Rb uptake experiments and studies to measure cell sodium content by atomic absorption spectrophotometry. For measurement of intracellular sodium concentration and cytoplasmic pH using fluorescent dyes, cell monolayers were grown on mini petri dishes (Greire Labortechnik, Germany).

Potassium (^{86}Rb) Uptake

Ouabain-sensitive potassium (^{86}Rb) uptake was used as an index of Na, K-ATPase-mediated sodium-potassium transport. Experiments were conducted using monolayers of cultured rabbit NPE cells bathed in Krebs solution (119 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$ 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, and 5.5 mM glucose, at pH 7.4, equilibrated with 5% CO_2/95% air). Cells were equilibrated in Krebs solution for at least 30 min before the start of each experiment. Cell monolayers were treated with test agents for a specified time, then half the cells received 1 mM ouabain for 10 min, before ^{86}Rb (1 μCi/ml) was added for a further 10 min. At the end of the ^{86}Rb uptake period, the cell monolayers were lysed with 500 μl 0.5 N NaOH and 200 μl aliquots of the lysate were used to quantify ^{86}Rb using a scintillation counter. In addition, 100 μl aliquots of the lysate was used to measure protein content. Na, K-ATPase activity is expressed as nmol potassium (^{86}Rb) uptake/mg protein/10 min.

Measurement of Cell Sodium Content by Atomic Absorption Spectrophotometry

Following specified pretreatment with test agents, cells monolayers were washed with ice-cold isotonic magnesium chloride solution (100 mM $MgCl_2$, adjusted to pH 7.4 with Tris base). The cells were then lysed by adding 200 μl 30% nitric acid to each well. Then 1.8 ml deionized water was added and the sodium content of the diluted cell lysates was measured using an atomic absorption spectrophotometer (Perkin-Elmer, Norwalk, CT) at a wavelength of 566.5 nm.

Measurement of Cytoplasmic Sodium Concentration Using SBFI

The fluorescent dye SBFI (Molecular Probes, Eugene, OR) was used to measure cytoplasmic sodium concentration. To load the cells, SBFI-AM was dissolved in 20% pluronic F-127 in dimethyl sulfoxide (Molecular Probes, Eugene, OR) and added to the Dulbecco's modified Eagle's medium cell culture medium for 3 to 4 h at a final concentration of 10 μM SBFI-AM and <0.1% pluronic F-127 and <0.5% dimethyl sulfoxide. After the dye-loading period, the cells were washed with SBFI-free Krebs solution. SBFI fluorescence was measured with a microscope (Zeiss, Carl Zeiss, Thornwood, NY) equipped with a digital fluorescence imaging system (Attofluor; Attofluor Instruments, Rockville, MD). The cells were continuously superfused with Krebs solution on a warmed microscope stage that maintained temperature close to 37°C. The emission wavelength was 520 nm. Alternating excitation wavelengths were 340 nm and 380 nm. The ratio of fluorescence intensity at 340 nm and 380 nm was continuously recorded. To calibrate the signal at the end of each experiment, the cells were permeablized by exposure to Krebs solution containing a mixture of 10 μM nigericin, 5 μM monensin, and 5 μM gramicidin and a range of sodium concentrations. The fluorescence ratio signals were thus measured in the permeablized cells equilibrated to different external sodium concentrations.

Measurement of Cytoplasmic pH Using BCECF

The fluorescent pH sensitive dye, 2′,7′-biscarboxyethyl-5(6)-carboxyfluorescein (BCECF) was used to measure cytoplasmic pH. Using BCECF-AM at a final concentration of 1.5 μM, the cells were loaded with dye in the same manner as that described above for SBFI experiments. After loading, the cells were washed with BCECF-AM free Krebs solution. BCECF fluorescence was measured as described above, using an emission wavelength of 520 nm. Alternating dual excitation wavelengths were 460 nm and 488 nm. The ratio of fluorescence intensity of 488 nm and 460 nm was continuously recorded. To calibrate the signal at the end of each experiment, the cells were superfused with a potassium-rich buffer containing 10 μM nigericin (Sigma Chemical, St. Louis, MO). Nigericin effects K^+-H^+ exchange and in combination with potassium-rich external solution serves to equilibrate intracellular and extracellular pH. The relationship between the ratio (I_{488}/I_{460}) and intracellular pH was established using specific potassium-rich buffers: 2-(N-morpholino) ethanesulfonic acid buffer (MES, pK_a = 6.1) was used to set pH range from 6.0–6.5; piperazine—N,N′—bis (2-ethanesulfonic acid) buffer (PIPES, pK_a = 6.8) was to set pH at 7.0; N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid buffer (HEPES, pK_a = 7.5) was used to set pH at 7.4; N-tris (hydroxymethyl) methyl-3-aminopropanesulfonic acid (TAPS, pK_a = 8.4) was used to set pH at 8.0.

Results

In an earlier study of cultured rabbit NPE, the selective H^+-ATPase inhibitor bafilomycin A_1 was found to suppress cytoplasmic pH responses to depolarization and to an ammonium chloride prepulse (Wu & Delamere, 1997). However, bafilomycin A_1 does not cause a sig-

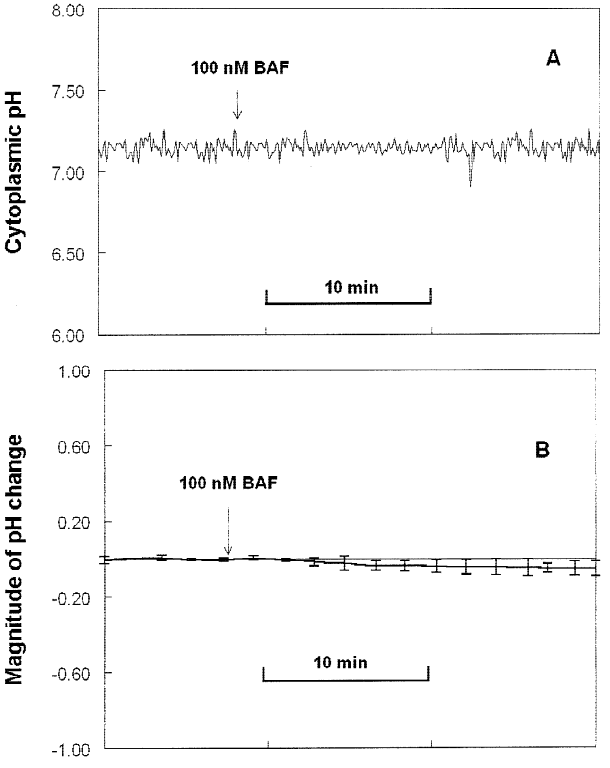

Fig. 1. The influence of bafilomycin A_1 on cytoplasmic pH. Cytoplasmic pH was measured by a BCECF fluorescence technique. BCECF-loaded cells were first superfused with control Krebs solution, then the superfusate was switched to contain 100 nM bafilomycin A_1 (arrow). Panel (*A*) shows the result of a typical experiment. Panel (*B*) shows the mean ± SE (vertical bar) of results from 10 different experiments.

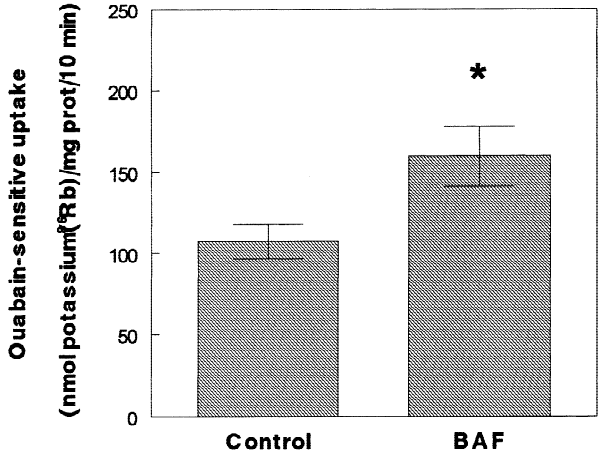

Fig. 2. The influence of bafilomycin A_1 on ouabain-sensitive potassium (^{86}Rb) uptake by cultured rabbit nonpigmented ciliary epithelium. Cell monolayers were incubated in Krebs solution containing 100 nM bafilomycin A_1 for 20 min then ^{86}Rb was added for a further 10 min. Half the cells also received 1 m ouabain. The data represent the difference between potassium (^{86}Rb) uptake measured in the presence or absence of ouabain. The data are the mean ± SE of results from 24 cell monolayers. *Indicates a significant difference ($P < 0.05$) from the value measured in control (no bafilomycin A_1) cells.

nificant change in the cytoplasmic pH baseline (Fig. 1). Lack of sensitivity of baseline cytoplasmic pH to bafilomycin A_1 could signify that under resting conditions, vacuolar H^+-ATPase does not contribute to cytoplasmic pH regulation. However, when the cultured NPE cells were exposed to bafilomycin A_1 an unexpected increase was observed in the rate of ouabain sensitive-potassium (^{86}Rb) uptake. Cells were exposed to 100 nM bafilomycin A_1 for 20 min and then rate of potassium (^{86}Rb) uptake was measured over a subsequent 10 min time period in the presence or absence of 1mM ouabain. The ouabain-sensitive potassium (^{86}Rb) uptake rate was increased by approximately 50% (Fig. 2), indicating that in the presence of bafilomycin A_1, the rate of active sodium-potassium transport is stimulated. The total rate of potassium (^{86}Rb) uptake (measured in the absence of ouabain) increased from 588 ± 8 to 645 ± 7 nmol potassium/mg protein/10 min in the presence of 100 nM bafilomycin A_1 (significant; $P < 0.01$) whereas the ouabain-insensitive component of potassium (^{86}Rb) was unchanged (486 ± 6 and 481 ± 7 nmol potassium/mg protein/10 min in the presence or absence of bafilomycin A_1, respectively; data as mean ± SE, $n = 24$ cell monolayers).

Since the rate of active sodium-potassium transport is determined in large part by the cytoplasmic sodium concentration, some cells were loaded with the sodium-sensitive fluorescent dye SBFI prior to being exposed to bafilomycin A_1. In the presence of bafilomycin A_1, a prompt increase in cytoplasmic sodium concentration was observed (Fig. 3). To examine the mechanism responsible for the increase of cytoplasmic sodium concentration in bafilomycin A_1-treated cells, some cells were exposed to the Na-H exchange inhibitor dimethylamiloride. Using atomic absorption spectrophotometry to measure sodium, the cell sodium content was found to be unchanged in monolayers exposed to 10 μM dimethylamiloride for 20 min but the value was significantly increased in cells that received bafilomycin A_1. Importantly, cell sodium content was not significantly increased in cells that received bafilomycin A_1 in the presence of dimethylamiloride (Fig. 4).

Sodium measurements suggest that bafilomycin A_1 causes an increase in cytoplasmic sodium concentration via a dimethylamiloride-sensitive mechanism. If this is correct, the increase in the ouabain-sensitive potassium (^{86}Rb) uptake rate that occurs in bafilomycin A_1-treated cells should be suppressed by dimethylamiloride. This was the case. In cells exposed to 100 nM bafilomycin A_1, dimethylamiloride produced a dose-dependent inhibition in the rate of ouabain-sensitive potassium (^{86}Rb) uptake rate (Fig. 5). In contrast, dimethylamiloride did not cause a detectable change in the rate of ouabain sensitive potassium (^{86}Rb) uptake in control cells that did not receive bafilomycin A_1.

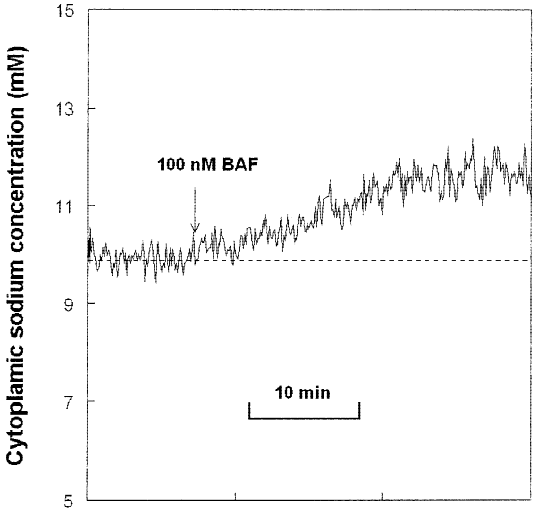

Fig. 3. The increase of cytoplasmic sodium concentration in cells exposed to bafilomycin A_1. Cytoplasmic sodium concentration was measured by an SBFI fluorescence technique. SBFI-loaded cells were first superfused with control Krebs solution, then the superfusate was switched to contain 100 nM bafilomycin A_1 (arrow). The data represent the average of results from 3 separate experiments, each comprising pooled measurements from >20 cells.

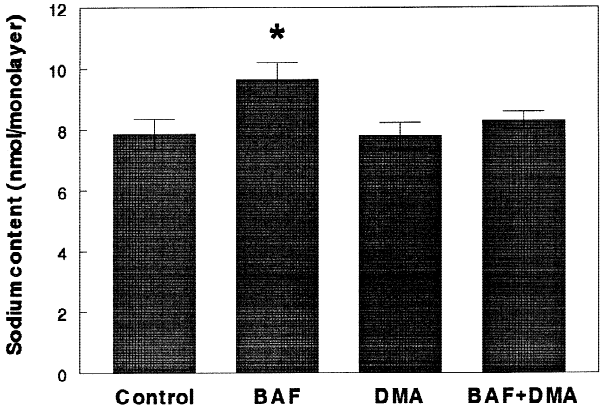

Fig. 4. The influence of bafilomycin A_1 and dimethylamiloride on cell sodium content. Cell sodium content determined by atomic absorption spectrophotometry in cell monolayers exposed to 100 nM bafilomycin A_1 (BAF) for 20 min. A second group of cells was exposed to bafilomycin A_1 in the presence of 10 μM dimethylamiloride (BAF + DMA). A third group of cells was exposed to dimethylamiloride alone (DMA) while control cells received neither bafilomycin A_1 nor DMA. The data are the mean ± SE of results from 11 cell monolayers. *Indicates a significant difference from control ($P < 0.05$).

Although bafilomycin A_1 does not change the baseline value of cytoplasmic pH, the above findings are consistent with the possible activation of a dimethylamiloride-sensitive Na-H exchange mechanism which stabilizes cytoplasmic pH in the bafilomycin A_1-treated cell. If this is the case, bafilomycin A_1 should reduce cytoplasmic pH in cells where dimethylamiloride pre-

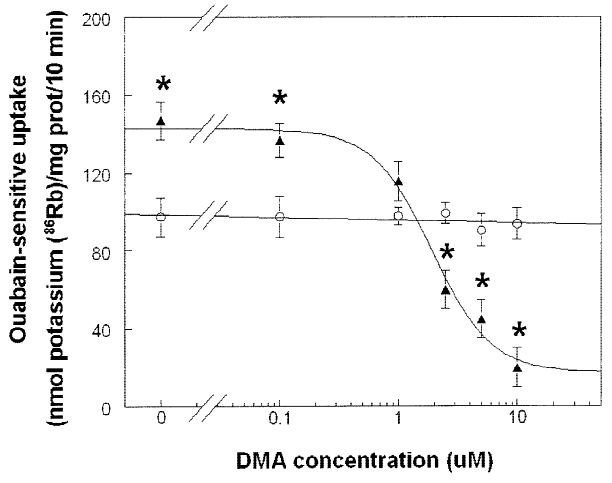

Fig. 5. The influence of dimethylamiloride (DMA) on ouabain-sensitive potassium (^{86}Rb) uptake. Cell monolayers were incubated 20 min in Krebs solution containing DMA (0.1–10 μM) either in the presence (▲) or absence (○) of 100 nM bafilomycin A_1 and then ^{86}Rb was added for a further 10 min. Half the cells also received 1 mM ouabain. The data represent the difference between potassium (^{86}Rb) uptake measured in the presence or absence of ouabain. The data are the mean ± SE of results from 24 cell monolayers. *Indicates a significant difference ($P < 0.05$) between the value measured in the presence or absence of bafilomycin A_1 at each DMA concentration.

treatment prevents Na-H exchanger activation. Such a response was observed. Although dimethylamiloride alone produced a significant decrease of cytoplasmic pH, the subsequent addition of bafilomycin elicited a significant further reduction of cytoplasmic pH (Fig. 6; Table).

Discussion

In rabbit alveolar macrophages, H^+-ATPase has been identified as the principal mechanism responsible for regulation of basal cytoplasmic pH (Bidani, Brown & Heming, 1994). However in hepatocytes and pancreatic acinar cells, it has been suggested that H^+-ATPase does not contribute significantly to regulation of baseline cytoplasmic pH even though the H^+-ATPase inhibitor bafilomycin A_1 does modify cell responses (Wadsworth & van Rossum, 1994; Gonzalez et al., 1998). Results from an earlier study suggested that a functional H^+-ATPase is capable of extruding protons to alkalinize the cytoplasm of cultured rabbit NPE (Wu & Delamere, 1997). However, lack of a detectable effect of bafilomycin A_1 on baseline cytoplasmic pH raised doubts as to whether H^+-ATPase contributes to cytoplasmic pH regulation in the resting cell. In the present study, we present evidence for an increase of cytoplasmic sodium concentration in cells exposed to bafilomycin A_1. One possible interpretation of the sodium increase is that it stems from activation of Na-H exchanger when H^+-ATPase is inhibited

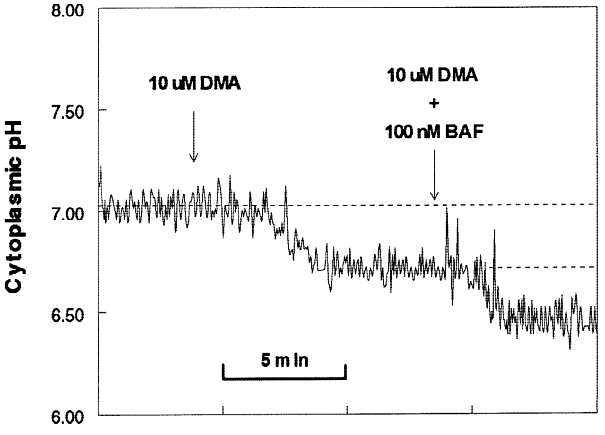

Fig. 6. The influence of dimythelamiloride and bafilomycin A_1 on cytoplasmic pH. The result of a typical experiment in which data was pooled from >20 cells. Cytoplasmic pH was measured by a BCECF fluorescence technique. BCECF-loaded cells were first superfused with control Krebs solution, then the superfusate was switched to contain 10 μM DMA (left hand arrow). After an interval of ~15 min, 100 nM bafilomycin A_1 was introduced in the continued presence of DMA (right hand arrow). The Table shows the mean of results from 7 independent experiments.

Table. The influence of bafilomycin A_1 and dimethylamiloride on cytoplasmic pH

	Cytoplasmic pH
Control	7.12 ± 0.09
Dimethylamiloride	6.72 ± 0.04*
Dimethylamiloride + bafilomycin A_1	6.46 ± 0.05**

BCECF-loaded cells were first superfused with control Krebs solution to establish the baseline pH (Control) then the superfusate was switched to contain 10 μM DMA for a periof of ~15 min. Then 100 nM bafilomycin A_1 was introduced in the continued presence of DMA. The data are the mean ± SE of results from 7 independent experiments. * Indicates a significant difference from control ($P < 0.05$). ** Indicates a significant difference from control ($P < 0.01$) and a significant difference from dimethylamiloride alone ($P < 0.05$).

by bafilomycin A_1. In keeping with this model, the increase in cell sodium content was not observed in cells exposed to bafilomycin A_1 in presence of dimethylamiloride.

Based on what is known about the kinetics of Na,K-ATPase, an increase in the concentration of cytoplasmic sodium explains the observed increase in the rate of ouabain-sensitive active sodium-potassium transport in cells exposed to bafilomycin A_1. Consistent with a model in which the increase of cytoplasmic sodium results from Na-H exchanger activation following H^+-ATPase, the rate of ouabain-sensitive potassium (^{86}Rb) uptake in bafilomycin A_1-treated cells could be inhibited by dimethylamiloride in a dose-dependent manner. The IC_{50} for dimethylamiloride was approximately 2 μM which is consistent with Na-H exchanger inhibition; at this concentration, dimethylamiloride is not effective as an inhibitor of epithelial sodium channels (Wang et al., 1993). Importantly, dimethylamiloride failed to change the rate of ouabain-sensitive potassium (^{86}Rb) uptake in cultured NPE cells which did not receive bafilomycin A_1. This is consistent with the observation that dimethylamiloride alone did not increase cell sodium content.

It seems likely that the Na-H exchanger is active in resting NPE cells because dimethylamiloride exposure produces cytoplasmic acidification. However, if this is the case, it is difficult to explain why blockade of the Na-H exchange sodium entry pathway by dimethylamiloride fails to decrease either the cytoplasmic sodium content or the rate of active sodium-potassium transport. Perhaps, under resting conditions, the magnitude of the Na-H exchange sodium entry pathway is small compared to other sodium entry pathways such as the Na/K/2Cl cotransporter which is active in cultured NPE (Dong et al., 1994; Dong & Delamere, 1994). Importantly, in cells exposed to dimethylamiloride, the application of bafilomycin A_1 produces an additional reduction of cytoplasmic pH. This confirms H^+-ATPase inhibition is capable of causing a change of cytoplasmic pH under conditions where activation of the Na-H exchanger is unable to compensate. Together, dimethylamiloride and bafilomycin A_1 cause a large reduction of cytoplasmic pH. It has been suggested that changes of cytoplasmic pH could alter Na,K-ATPase (Huang & Askari, 1984; Breitweiser, Altamirano & Russel, 1987) and it may be the case that cytoplasmic acidification contributes to the large degree of ouabain-sensitive ^{86}Rb uptake inhibition observed in cells exposed simultaneously to dimethylamiloride and bafilomycin A_1 compared to the uptake in cells exposed to dimethylamiloride alone even though cytoplasmic sodium content is similar under both conditions.

Results from the present study suggest that in cultured NPE, activation of Na-H exchange might compensate for cytoplasmic pH changes which otherwise would occur when H^+-ATPase is inhibited. A cooperative interaction between H^+-ATPase and Na-H exchange has also be proposed in cardiomyocytes where it was suggested that H^+-ATPase-mediated proton efflux may serve to spare cytoplasmic sodium overload due to sodium entry via Na-H exchange (Karwatowska-Prokopczuk et al., 1998). However, in frog skin, the situation is different because apical sodium entry is reduced following H^+-ATPase inhibition with bafilomycin A_1; the inward passage of sodium ions through frog skin apical sodium channels apparently depends on the electrochemical driving force established by H^+-ATPase (Ehrenfeld & Klein, 1997).

It remains to be determined whether H^+-ATPase

plays a specific functional role in the mechanism of solute transport and aqueous humor fluid secretion by NPE cells. In studies with living rabbits, Wax et al. (1997) discovered that bafilomycin A_1 applied topically to the eye produced a reduction of intraocular pressure which appeared to stem from a decrease in the rate of aqueous humor secretion. These authors detected a bafilomycin A_1-sensitive component of short circuit current across the isolated iris-ciliary body and speculated that H^+-ATPase might provide a driving force for fluid movement into the eye across the ciliary epithelium. However, this is unlikely since the magnitude of the measured short-circuit current was insufficient to drive the known rate of fluid entry into the rabbit eye and, moreover, the polarity of the observed short circuit current was such that it would drive fluid in the opposite direction. It is more likely that H^+-ATPase function at the basolateral surface of the NPE might impact the function of other ion transport mechanisms that support fluid movement. In the present study, we find that H^+-ATPase inhibition can lead to an increase of the cytoplasmic sodium concentration in cultured rabbit NPE. If such a response were to occur in vivo, solute transport across the ciliary epithelium might be affected since many solute transporters are driven by the intracellular-extracellular gradient of sodium. However, the present findings do need to be interpreted cautiously because the study was conducted in a cell line that is unlike native NPE in many respects. H^+-ATPase responses and the functional role of H^+-ATPase might be different in native NPE.

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