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H^+-ATPase-Mediated Cytoplasmic pH-Responses Associated with Elevation of Cytoplasmic Calcium in Cultured Rabbit Nonpigmented Ciliary Epithelium

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Abstract. Studies were conducted to test whether an increase of cytoplasmic calcium concentration influences H^+-ATPase activity in cultured rabbit nonpigmented ciliary epithelium (NPE). Cytoplasmic calcium concentration or cytoplasmic pH was measured by a fluorescence ratio technique in cells loaded with either Fura-2 or BCECF. Cytoplasmic calcium was increased in three ways; by exposure to BAY K 8644 (1 μM), by exposure to a mixture of epinephrine (1 μM) + acetylcholine (10 μM) or by depolarization with potassium-rich solution. In each case cytoplasmic pH increased significantly. In all three cases 100 nM bafilomycin A_1, a specific H^+-ATPase inhibitor, significantly inhibited the pH increase. These results suggest an increase of cytoplasmic calcium might initiate events that lead to activation of proton export from the cytoplasm by a mechanism involving H^+-ATPase. This notion is supported by the observation that the pH increase was suppressed when either verapamil or nifedipine was used to prevent the cytoplasmic calcium increase in cells exposed to potassium-rich solution. Protein kinase C activation might also be involved in the mechanism of H^+-ATPase stimulation since staurosporine suppressed the pH response to potassium-rich solution. A transient rise of cytoplasmic calcium concentration was observed when cytoplasmic acidification was induced by exposure to high pCO_2. This suggests a rise of cytoplasmic calcium might represent part of a physiological mechanism to stimulate H^+-ATPase-mediated protein export under acid conditions.

Key words: Cytoplasmic pH — H^+-ATPase — Bafilomycin A_1 — BAY K 8644 — Verapamil — Nifedipine

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Introduction

Vacuolar H^+-ATPase is a hetero-oligomeric membrane protein that conducts electrogenic proton transport across cell membranes. It is best known for its ability to acidify cellular compartments such as lysosomes and endosomes (Sundler, 1997; Nelson and Harvey, 1999). However, in many cells including astrocytes, osteoclasts and kidney tubule cells, H^+-ATPase is also found on the plasma membrane and in this location it is thought to actively export protons out of the cell (Pappas & Ransom, 1993; Mattsson et al., 1997; Nelson & Harvey, 1999). In several tissues, H^+-ATPase appears to mediate proton extrusion from the cytoplasm following an acid load (Manger & Koeppen, 1992; Pappas & Ransom, 1993).

In a study of the eye, plasma membrane H^+-ATPase was shown to be immunolocalized in the ciliary epithelium, a bilayer which consists of a nonpigmented ciliary epithelium (NPE) layer and a pigmented ciliary epithelium (PE) layer. H^+-ATPase was found in both cell types but was most abundant at the basolateral surface of NPE layer (Wax et al., 1997). The function of the ciliary epithelium is to secrete aqueous humor and Wax et al. provided evidence suggesting that bafilomycin A_1, a specific inhibitor of H^+-ATPase (Bowman, Siebers & Altendorf, 1988), might suppress fluid production when applied topically to the rabbit eye. The explanation for this action of bafilomycin A_1, is still unclear.

In a recent study of cultured rabbit NPE cells, inhibition of H^+-ATPase by bafilomycin A_1 was reported to cause an increase of intracellular sodium (Hou & Delamere, 2000). If this is the case, modulation of H^+-ATPase activity could change several aspects of NPE function since the sodium gradient provides the driving force for other cotransport and countertransport mechanisms. In other tissues it has been suggested that modulation of H^+-ATPase activity can be triggered by changes

in the concentration of cytoplasmic calcium concentration (Manger, Pappas & Koeppen, 1992). In the present study we present evidence that suggests an increase of cytoplasmic calcium concentration might activate H^+-ATPase in cultured rabbit NPE.

Materials and Methods

Chemicals

All chemicals in this study were purchased from Sigma (St. Louis, MO) except 2′,7′-Bis (carboxyethyl)-5(6)-carboxyfluorescein-acetoxymethyl ester (BCECF-AM), Fura-2, AM and pluronic F-127, which were purchased from Molecular Probes (Eugene, OR).

Cell Culture

The cell line used in this study was kindly provided by Dr. M. Coca-Prados (Yale University, New Haven, CT). The cells, which were derived from SV 40 virus-transformed rabbit nonpigmented ciliary epithelium, have been used previously in studies of H^+-ATPase and cytoplasmic pH regulation (Hou & Delamere, 2000; Wu et al., 1997). Cells were grown on 35 mm petri-dishes in Dulbecco's modified Eagle's medium (GIBCO, Gaithersburg, MD) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin, under a humidified atmosphere of 5% CO_2/95% air at 37°C. The culture medium was changed every 2–3 days. Cells were used prior to confluence.

Measurement of Cytoplasmic Calcium Using Digital Fluorescence Microscopy

The fluorescent calcium-sensitive dye Fura-2 (Molecular Probes, Eugene, OR) was used to measure the cytoplasmic calcium concentration in cells superfused at a rate of 1 ml/min with Krebs solution containing (in mM) 119 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 25 $NaHCO_3$, 2.5 $CaCl_2$, 1 $MgCl_2$, and 5.5 glucose, at pH 7.4, equilibrated with 5% CO_2/95% air. Water insoluble materials were dissolved in a minimum volume of dimethyl sulfoxide (DMSO), 20% pluronic F-127 in DMSO or ethanol (< 0.1% final concentration). An equal amount of DMSO or ethanol was added to control solutions. Potassium-rich Krebs solution was prepared by increasing the concentration of KCl to 80 mM at the expense of an equimolar amount of NaCl. To load the dye, the cells were incubated in Krebs solution containing 10 μM Fura-2 AM for 1 hour under humidified 5% CO_2/95% air at 37°C. Then the cells were washed three times with Krebs solution and the petri dish was placed on the stage of a fluorescence microscope (Zeiss, Thornwood, NY) equipped with a digital imaging system (Attofluor Instruments, Rockville, MD). A water jacket system was used to warm the microscope stage to 37°C and a flow-through automatic temperature controller (Warner Instrument, Hamden, CT) was used to maintain the temperature of the incoming superfusate at 37°C. Fura-2 fluorescence intensity was measured using an emission wavelength of 520 nm and alternating dual excitation wavelengths of 334 nm and 380 nm. At the end of each experiment, the relationship between cytoplasmic calcium and the ratio of fluorescence intensity at 334 nm to that at 380 nm was calibrated by first adding 1 μM ionomycin to the superfusate to permit calcium equilibration with the external solution in order to obtain the maximum ratio. Then 30 mM EGTA was added to the superfusate in the continued presence of ionomycin to obtain minimum ratio.

Measurement of Cytoplasmic pH Using Digital Fluorescence Microscopy

Cytoplasmic pH was measured using the pH-sensitive dye BCECF with alternating excitation wavelengths of 460 nm and 488 nm. The methodology was similar to that described above for cytoplasmic calcium measurements. At the end of each experiment, the relationship between cytoplasmic pH and the ratio of fluorescence intensity at 488 nm to that at 460 nm was calibrated by using a potassium-rich buffer containing 10 μM nigericin. In combination with potassium-rich buffer, nigericin mediates K^+/H^+ exchange and so equilibrates the extracellular pH and intracellular pH. The potassium-rich buffer contained (in mM) 110 KCl, 20 NaCl, and 20 of a buffer selected to control pH. 2-(N-morpholino) ethanesulfonic acid (MES; $pK_a = 6.1$) was used to set pH in the range 6.0–6.5; piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES; $pK_a = 6.8$) for a pH of 7.0; N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES; $pK_a = 7.5$) for a pH of 7.4; N-tris[hydroxymethyl]-3-aminopropanesulfonic acid (TAPS; $pK_a = 8.4$) for a pH of 8.4.

Data Analysis

Student's *t*-test was used for the statistical analysis. Values of $P < 0.05$ were considered as significantly different.

Results

To examine whether elevation of cytoplasmic calcium might be linked to an increase of cytoplasmic pH, the calcium channel agonist BAY K 8644 was tested for its ability to alter cytoplasmic pH. When cells preloaded with Fura-2 were exposed to 1 μM BAY K 8644, a marked increase of cytoplasmic calcium concentration was observed (Fig. 1*A*). The cytoplasmic calcium concentration measured 5 min after BAY K 8644 addition was 92.55 ± 2.60 nM, which was significantly ($P < 0.01$) higher than the control cytoplasmic calcium concentration of 55.87 ± 2.15 nM (mean ± SEM; $n = 6$). In parallel experiments, cells preloaded with BCECF were exposed to 1 μM BAY K 8644. Under these conditions an increase of cytoplasmic pH was observed (Fig. 1*B*). Cytoplasmic pH measured 5 min after BAY K 8644 addition had risen to 7.28 ± 0.08, which was significantly ($P < 0.05$) higher than the resting cytoplasmic pH value of 6.99 ± 0.08 (mean ± SEM; $n = 6$). To examine the contribution of H^+-ATPase to the alkalinization response, some cells were superfused first with bafilomycin A_1 (100 nM) for 5 min before being exposed to 1 μM BAY K 8644 in the continued presence of 100 nM bafilomycin A_1. In the presence of bafilomycin A_1, 1 μM BAY K 8644 failed to increase cytoplasmic pH (Fig. 1*B*).

In some experiments, cytoplasmic calcium concentration was elevated by a receptor-mediated mechanism as described by Ciluffo and coworkers (1998) who demonstrated that exposing NPE to a mixture of epinephrine and acetylcholine causes a large rise of cytoplasmic calcium. Exposing the cells to 1 μM epinephrine + 10 μM

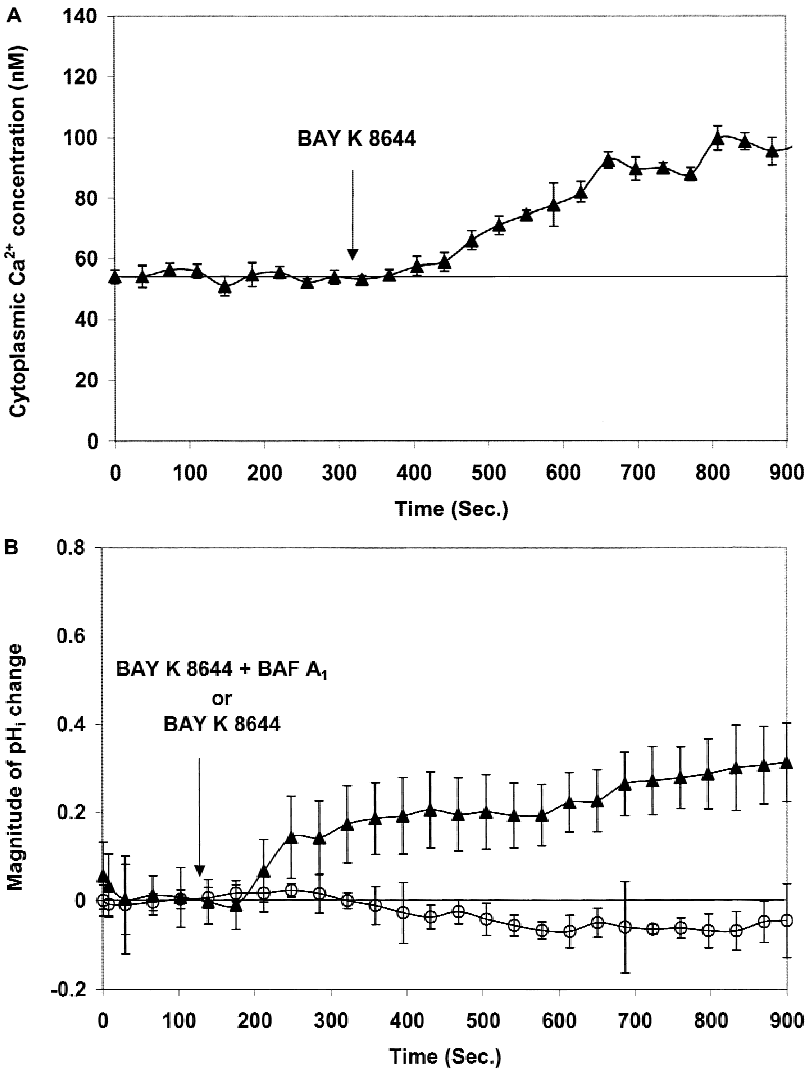

Fig. 1. The influence of BAY K 8644 on cytoplasmic calcium concentration and cytoplasmic pH. Cells were first superfused with control Krebs solution (▲) for 5 min to obtain a stable baseline, then 1 μM BAY K 8644 was added to the superfusate (*arrow*). Cytoplasmic calcium concentration (*A*) and cytoplasmic pH change (*B*) were monitored continuously. (*B*) also shows the cytoplasmic pH change measured in cells that were first superfused with 100 nM bafilomycin A_1 prior to the addition of BAY K 8644 (○). The data are the mean ± SEM of results from 6 independent experiments.

acetylcholine also caused a significant rise of cytoplasmic pH (Table 1). Importantly, bafilomycin A_1 inhibited the pH increase.

Experiments were conducted to test whether changes of cytoplasmic calcium concentration are associated with the cytoplasmic alkalinization response to an increase of external potassium concentration. Exposure to potassium-rich solution caused a marked increase of cytoplasmic pH that was suppressed by the specific H^+-ATPase inhibitor bafilomycin A_1 (Fig. 2*A*). This result is consistent with the earlier proposal that H^+-ATPase is activated to shift protons outward when NPE cells are depolarized (Wu et al., 1997). When cells preloaded with Fura-2 were similarly exposed to potassium-rich solution, a rapid increase of cytoplasmic calcium was observed (Fig. 2*B*). Following the addition of potassium-rich solution, the concentration of cytoplasmic calcium rose to attain a value of 119.38 ± 4.51 nM which is significantly higher ($P < 0.01$) than the baseline value of 63.78 ± 3.49 nM (mean ± SEM; $n = 6$). Importantly, the increase of cytoplasmic calcium was not suppressed when cells were exposed to potassium-rich solution in the presence of 100 nM bafilomycin A_1 (Fig. 2*C*). In the presence of bafilomycin A_1 alone, the concentration of cytoplasmic calcium was 56.87 ± 4.59 nM which was not significantly different from the baseline value of 55.91 ± 3.94 nM measured prior to bafilomycin A_1 addition. When the external potassium concentration was raised to 80 mM in the continued presence of bafilomycin A_1, the concentration of cytoplasmic calcium rose to attain a value of 118.69 ± 3.63 nM which was no different from the concentration observed in cells exposed to 80 mM external potassium in the absence of bafilomycin A_1.

One possible explanation for the above results is that an increase of cytoplasmic calcium in some way brings about the activation of H^+-ATPase in cells exposed to potassium-rich solution. To further explore this possibility, L-type calcium channel antagonists verapamil and

Table 1. The influence of epinephrine and acetylcholine on cytoplasmic pH and calcium concentration

	Krebs		Krebs + BAF	
	Control	Epinephrine + acetylcholine	Control	Epinephrine + acetylcholine
pH	7.12 ± 0.08	7.42 ± 0.06[a,b]	7.09 ± 0.03	7.21 ± 0.03[a]
Ca^{2+} (nM)	63.82 ± 6.61	216.16 ± 15.38[c]	ND	ND

Cells were first exposed to control Krebs solution to establish a stable pH or calcium baseline and then exposed to Krebs solution containing 1 μM epinephrine + 10 μM acetylcholine. pH was also measured in a group of cells that was first exposed to Krebs solution containing 100 nM bafilomycin A_1 (BAF) before exposure to epinephrine + acetylcholine in the continued presence of 100 nM bafilomycin A_1. The data were obtained 100 sec before (control) or after epinephrine + acetylcholine addition. The pH and calcium data are the mean ± SEM of results from 4 and 6 independent experiments respectively. ND, not determined.

[a] indicates a significant difference ($P < 0.05$) between cytoplasmic pH measured before and after exposure to epinephrine + acetylcholine.

[b] indicates a significant difference ($P < 0.05$) between cytoplasmic pH measured in bafilomycin A_1-treated and -untreated cells in the presence of epinephrine + acetylcholine.

[c] indicates a significant difference ($P < 0.01$) between cytoplasmic calcium measured before and after exposure to epinephrine + acetylcholine, $P < 0.01$.

nifedipine were used to inhibit the increase of cytoplasmic calcium. Cells preloaded with Fura-2 were first superfused with Krebs solution containing either verapamil or nifedipine to establish a stable cytoplasmic calcium baseline and then exposed to potassium-rich solution in the continued presence of either verapamil or nifedipine. In the presence of either verapamil (5 μM) or nifedipine (100 μM) the cytoplasmic calcium increase was abolished (Figs. 3*A*, 4*A*).

In parallel studies, cells preloaded with BCECF were exposed to potassium-rich solution in the presence of either verapamil (5 μM) or nifedipine (100 μM). Both verapamil and nifedipine were found to suppress the cytoplasmic pH increase caused by depolarization (Figs. 3*B;* 4*B;* Table 1). The value of cytoplasmic pH measured 5 min following the exposure of either verapamil- or nifedipine-treated cells to 80 mM external potassium was almost identical to the value recorded following exposure of bafilomycin A_1-treated cells to 80 mM external potassium (Table 2).

In other cell types, activation of protein kinase C has been associated with H^+-ATPase stimulation. To test whether this is the case in NPE, cells were exposed to potassium-rich solution in the presence of staurosporine (100 nM), a protein kinase C inhibitor. Staurosporine inhibited the pH response (Fig. 5; Table 2) but did not prevent the increase of cytoplasmic calcium caused by potassium-rich solution (Fig. 5 *inset*). Colchicine, a microtubule disruptor, also inhibited the pH response. In the presence of 10 μM colchicine, the pH response to potassium-rich solution was abolished (Fig. 6). Added alone, colchicine did not change cytoplasmic pH.

The results suggest NPE cells might be primed to activate H^+-ATPase-mediated proton export in response to a rise of cytoplasmic calcium concentration. To test whether an episode of cytoplasmic acidification causes a calcium rise that could activate the mechanism, cells were exposed to an increase of CO_2 from 5% to 100%. This acidifies the cell when additional CO_2 enters and generates more protons through a carbonic anhydrase-catalyzed reaction. Under these conditions, cell pH fell from 7.17 ± 0.07 to 6.81 ± 0.05 (mean ± SEM; $n = 4$). This maneuver caused a transient increase of cytoplasmic calcium (Fig. 7).

Discussion

We present evidence for a parallel increase of cytoplasmic calcium and cytoplasmic pH in NPE cells exposed to BAY K 8644, in cells exposed to potassium-rich solution and in cells exposed to a mixture of epinephrine and acetylcholine. The ability of bafilomycin A_1 to suppress the pH increase suggests the alkalinization responses are mediated at least in part by H^+-ATPase. Based on these findings we suggest that an increase of cytoplasmic calcium concentration initiates events that lead to an increase in the activity of H^+-ATPase. A link between cytoplasmic calcium and H^+-ATPase activity has been proposed in other tissues but in some cells, a cytoplasmic calcium increase activates H^+-ATPase (Manger et al., 1992) while in other cells a calcium rise causes H^+-ATPase inhibition (Sabatini, 1997).

The observation that potassium-rich solution causes a significant increase of cytoplasmic calcium in NPE is consistent with the depolarization response in other cell types (Cartin, Lounsbury & Nelson, 2000; Sena et al., 1995; Zhang et al., 1995). Importantly, when the L-type calcium channel antagonist verapamil was used to inhibit the cytoplasmic calcium increase in NPE cells depolarized by potassium-rich solution, the cytoplasmic pH increase was also suppressed. Nifedipine produced a similar inhibition of the cytoplasmic pH increase. This adds support for the notion that an increase of cytoplasmic calcium could be a trigger for activation of H^+-ATPase-mediated proton extrusion. In cells exposed to potas-

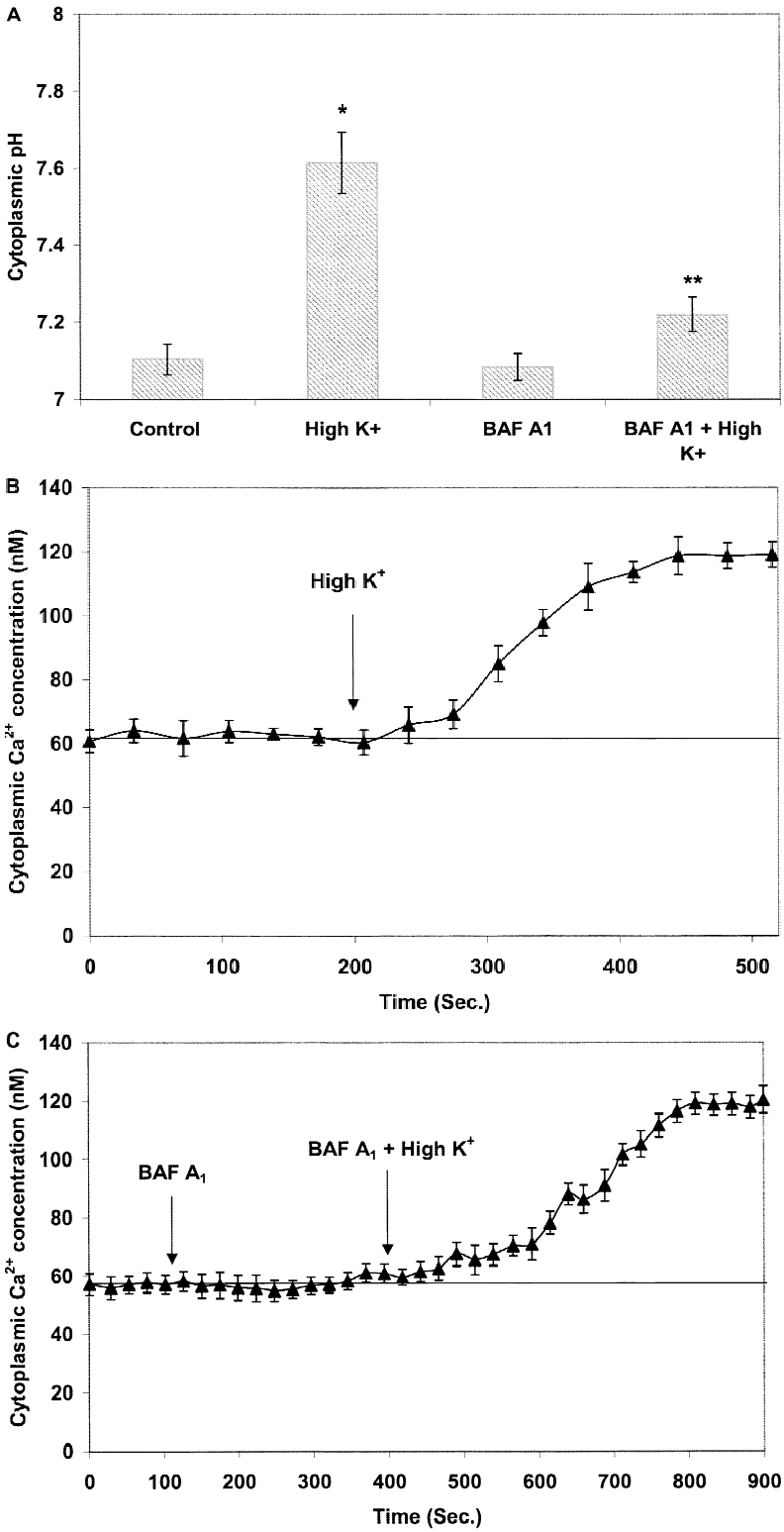

Fig. 2. The influence of potassium-rich solution on cytoplasmic pH and Ca^{2+} concentration. (*A*) Cytoplasmic pH. Cytoplasmic pH was measured in cells that were first superfused with Krebs solution for 5 min to obtain a stable baseline (*Control*) before the potassium concentration in the superfusate was increased to 80 mM (*High* K^+). A different group of cells was first superfused with Krebs solution containing 100 nM bafilomycin A_1 (*BAF A*1) and then the superfusate was switched to contain 80 mM potassium in the continued presence of 100 nM bafilomycin A_1 (*BAF A*1 + *High* K^+). The data are the mean ± SEM of results from 6 independent experiments. * indicates a significant difference from the baseline pH ($P < 0.05$). ** indicates a significant difference ($P < 0.01$) between the pH measured in the presence of 80 mM potassium + bafilomycin A_1 and the pH measured in the presence of 80 mM potassium alone. (*B*) Cytoplasmic calcium concentration. Cells were first superfused with control Krebs solution to obtain a stable baseline (indicated in this and other figures by the straight horizontal line), then the potassium concentration in the superfusate was increased to 80 mM (*arrow*). Cytoplasmic calcium concentration was monitored continuously. (*C*) Cytoplasmic calcium concentration. Some cells were first exposed to Krebs solution containing 100 nM bafilomycin A_1 (*first arrow*) and then the superfusate was switched to contain 80 mM potassium plus 100 nM bafilomycin A_1 (*second arrow*). The data are the mean ± SEM of results from 6 independent experiments.

sium-rich solution, bafilomycin A_1 suppresses the increase of cytoplasmic pH but not the increase of cytoplasmic calcium concentration. This makes it unlikely that the rise of cytoplasmic calcium is triggered by the increase of cytoplasmic pH in depolarized cells. Instead, the results are consistent with rise of cytoplasmic calcium initiating events that lead to H^+-ATPase stimulation. Possibly a rise of cytoplasmic calcium might rep-

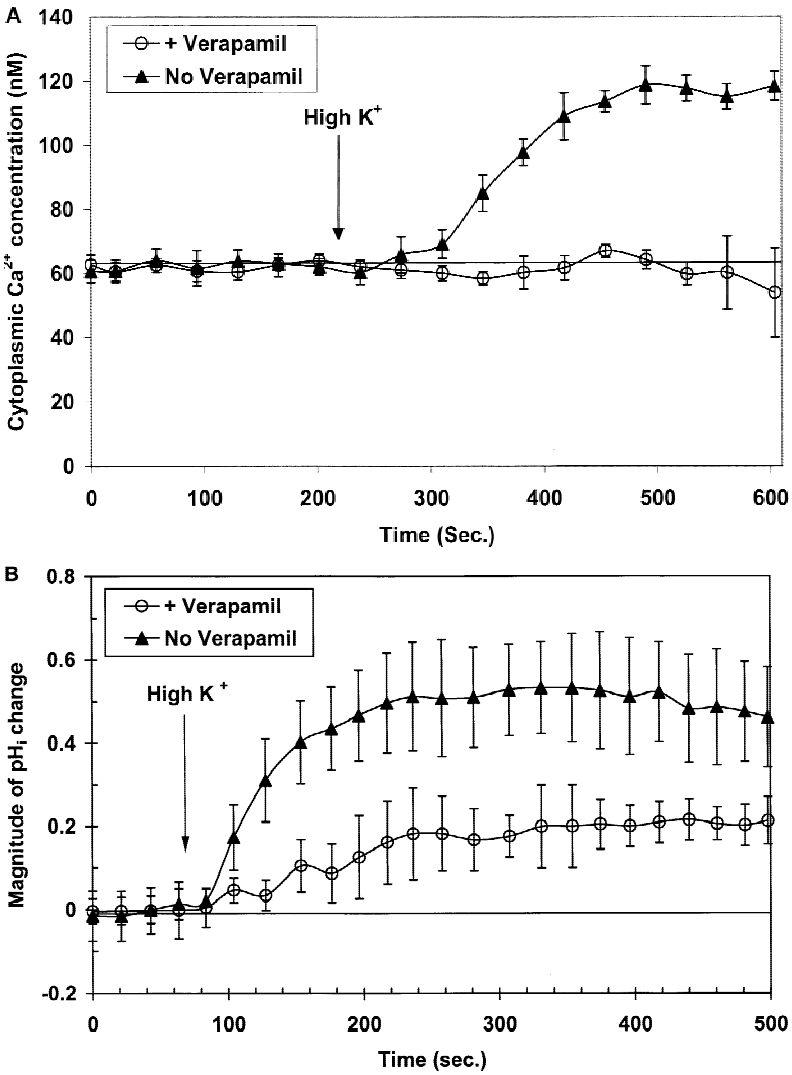

Fig. 3. The influence of verapamil on the cell response to potassium-rich solution. Cells were first exposed to control Krebs solution (▲) or Krebs solution containing 5 μM verapamil (○). After a stable baseline was established, the potassium concentration in the superfusate was increased to 80 mM (*arrow*). Cytoplasmic calcium concentration (*A*) and cytoplasmic pH (*B*) were monitored continuously. The baseline cytoplasmic pH was 7.09 ± 0.03 and 7.08 ± 0.04 in the presence and absence of verapamil, respectively. Each data set represents the mean ± SEM of results from 6 independent experiments.

resent part of a physiological mechanism to stimulate H^+-ATPase-mediated proton extrusion under acid conditions since cytoplasmic acidification itself has been suggested to cause an increase of cytoplasmic calcium (Swallow, Grinstein & Rotstein, 1990). In the present study, acidification of NPE cells following an increase of pCO_2 was indeed found to cause a transient cytoplasmic calcium increase.

Several agonists including acetylcholine, epinephrine, carbachol, the adenosine A3 receptor agonist IB-MECA and endothelin cause an increase of cytoplasmic calcium in the NPE (Ohuchi et al., 1992; Ciluffo et al., 1998; Tao et al., 1998; Hirata, Nathanson & Sears, 1999; Mitchell et al., 1999). It is possible that a receptor-mediated increase of cytoplasmic calcium could be associated with an increase of cytoplasmic pH similar to that observed in the present study when NPE cells were exposed to a mixture of epinephrine and acetylcholine. Interestingly, in vascular and coronary smooth muscle cells as well as trabecular meshwork cells, endothelin receptor stimulation also causes an increase of cytoplasmic pH as well as a transient increase of cytoplasmic calcium concentration (Kohmoto, Matsumoto & Serizawa, 1994; Rubanyi & Polokoff, 1994). However, not all cells respond to endothelin in the same way. In bovine corneal epithelium, endothelin caused an apparent inhibition of proton export (Wu et al., 1997).

Elevation of cytoplasmic calcium causes a wide variety of cell responses including activation of protein kinase C-dependent events. In outer medullary collecting duct, stimulation of bafilomycin A_1-sensitive proton secretion by metabolic acidosis is inhibited by not only the cytoplasmic calcium chelator BAPTA but also by the protein kinase C inhibitor staurosporine (Tsuruoka & Schwartz, 1998). In bovine corneal epithelium, protein kinase C was also reported to be involved in the mechanism of H^+-ATPase activation (Wu et al., 1998). The same holds true for NPE cells since staurosporine was

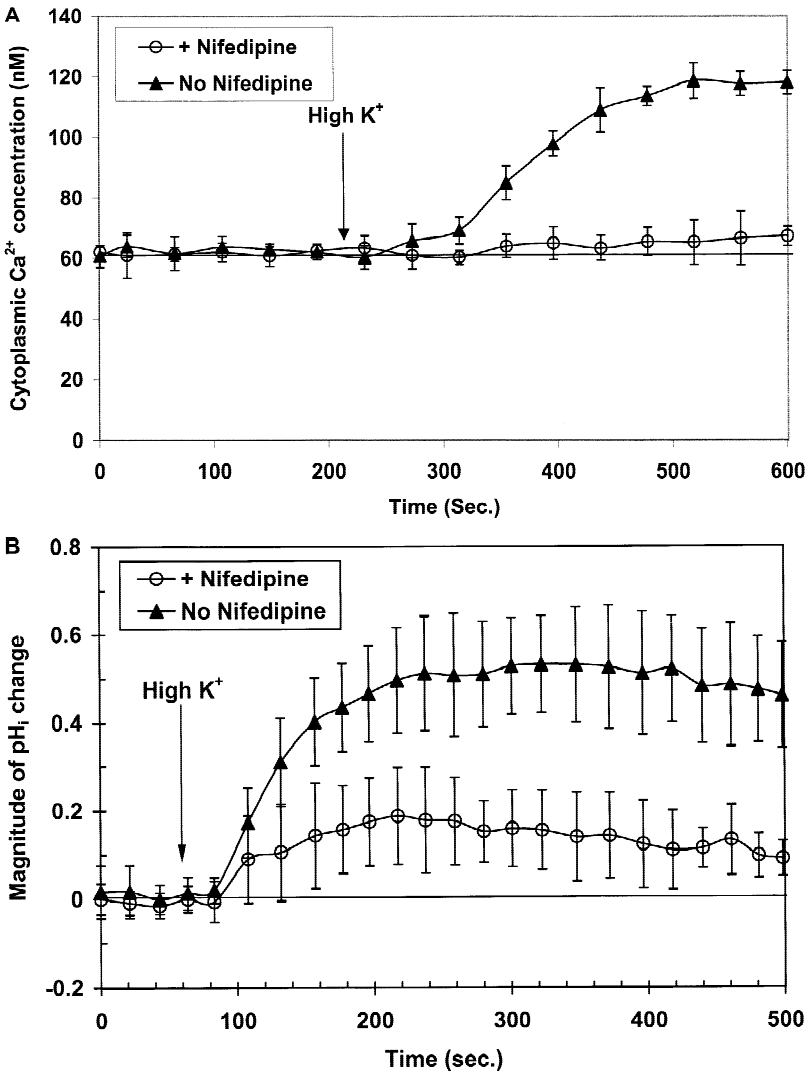

Fig. 4. The influence of nifedipine on the cell response to potassium-rich solution. Cells were first exposed to control Krebs solution (▲) or Krebs solution containing 100 μM nifedipine (○). After a stable baseline was established, the potassium concentration in the superfusate was increased to 80 mM (*arrow*). Cytoplasmic calcium concentration (*A*) and cytoplasmic pH (*B*) were monitored continuously. The baseline cytoplasmic pH was 7.08 ± 0.04 and 7.10 ± 0.03 in the presence and absence of nifedipine respectively. Each data set represents the mean ± SEM of results from 6 independent experiments.

found to prevent the bafilomycin A_1-sensitive pH rise caused by depolarization with potassium-rich solution. Together with a requirement for protein kinase C activation, there could be a number of intervening steps between elevation of cytoplasmic calcium and stimulation of H^+-ATPase. In some cells, the mechanism of H^+-ATPase activation involves calcium-induced exocytotic insertion of H^+-ATPase sites into the plasma membrane (Swallow et al., 1990; Tsuruoka & Schwartz, 1998). The ability of the microtubule disrupter colchicine to abolish the pH response to high potassium suggests this might also be the case in NPE.

L-type calcium channels could contribute to the observed calcium entry to NPE cells depolarized by potassium-rich solution. However, the concentrations of verapamil and nifedipine required to prevent the increase of cytoplasmic calcium concentration were higher than those reported necessary for inhibition of L-type calcium channels in aorta (Ko, Huang & Teng, 1997). This sug-

Table 2. The influence of potassium-rich Krebs solution on cytoplasmic pH

	Cytoplasmic pH	
	5.9 mM external $[K^+]$	80 mM external $[K^+]$
Control	7.10 ± 0.04	7.62 ± 0.08
Bafilomycin A_1	7.08 ± 0.04	7.22 ± 0.05*
Verapamil	7.09 ± 0.03	7.26 ± 0.04*
Nifedipine	7.08 ± 0.04	7.24 ± 0.08*
Staurosporine	6.98 ± 0.04	7.13 ± 0.08*

Mean cytoplasmic values measured before and 5 min after increasing the concentration of external potassium from 5.9 to 80 mM. Some cells were also exposed to either bafilomycin A_1 (100 nM), verapamil (5 μM), nifedipine (100 μM) or staurosporine (100 nM) throughout the experiments. The data are the mean ± SEM of results from 6 independent experiments. A significant difference ($P < 0.01$) between the cytoplasmic pH value measured at 80 mM external potassium and the value measured at 80 mM external potassium in the presence of either bafilomycin A_1, verapamil, nifedipine or staurosporine is indicated by *.

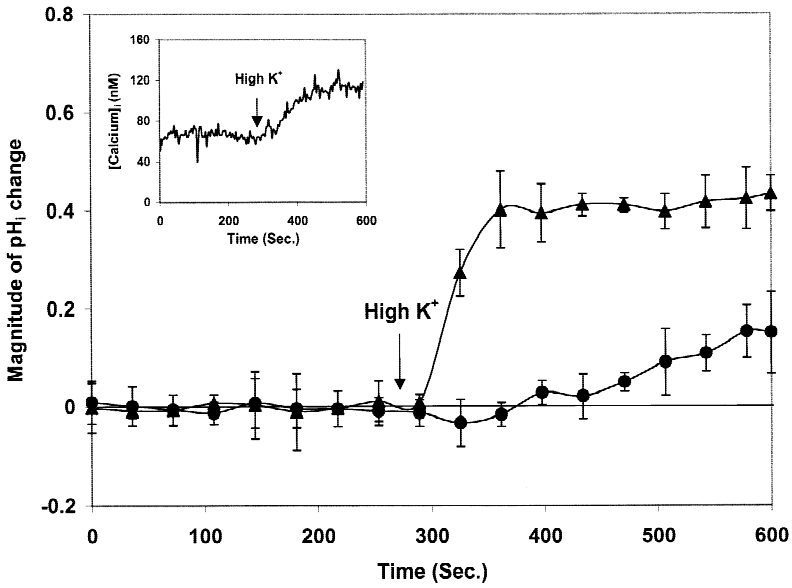

Fig. 5. The influence of staurosporine on the cell response to potassium-rich solution. Cells were first exposed to control Krebs solution (▲) or to Krebs solution containing 100 nM staurosporine (●). After a stable baseline was established, the potassium concentration in the superfusate was increased to 80 mM (*arrow*). Cytoplasmic pH or cytoplasmic calcium concentration (*inset*) were monitored continuously. The baseline cytoplasmic pH was 6.98 ± 0.04 and 7.07 ± 0.06 in the presence and absence of staurosporine respectively. The pH data represent the mean ± SEM of results from 4 independent experiments. A typical calcium response in the presence of staurosporine is shown in the inset panel.

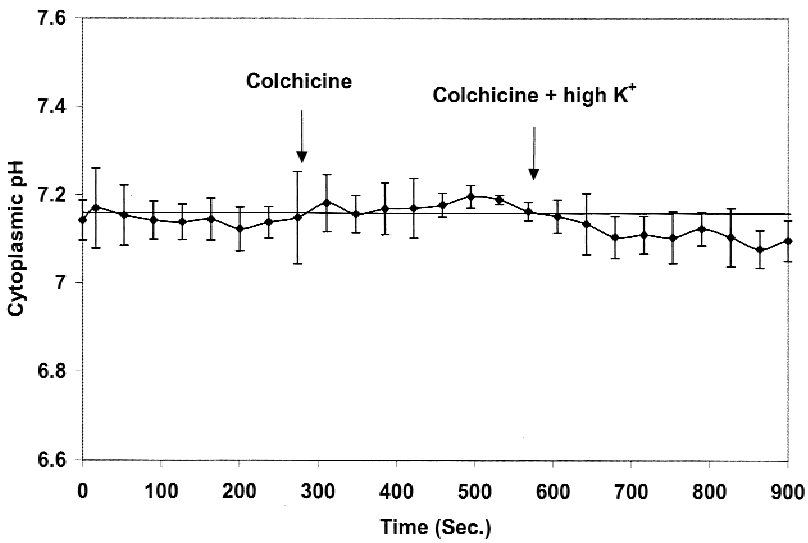

Fig. 6. The influence of colchicine on the cell response to potassium-rich solution. Cells were first exposed to control Krebs solution. After a stable baseline was established, 10 μM colchicine was added (1st *arrow*). After 5 min the potassium concentration in the superfusate was increased to 80 mM in the continued presence of colchicine (2nd *arrow*). Cytoplasmic pH was monitored continuously. The data represent the mean ± SEM of results from 6 independent experiments.

gests other calcium entry pathways could also be involved. It is known that blockade of high-threshold T-type calcium channels in rat neostriatal neurons requires concentrations of verapamil as high as 50–150 μM (Hoehn, Watson & MacVicar, 1993). In depolarized chromaffin cells, N-, P-, and Q-type calcium channels were found to be responsible for 70 percent of calcium entry with only 10 percent mediated by L-type calcium channels; in these cells, 3 μM verapamil was required to inhibit calcium entry (Villarroya et al., 1997).

In a previous study, H^+-ATPase was found to contribute to cytoplasmic pH regulation in resting NPE cells and inhibition by bafilomycin A_1 was observed to cause an increase of cytoplasmic sodium concentration that occurred as Na-H exchange became activated to stabilize cytoplasmic pH (Hou & Delamere, 2000). It is possible that stimulation of H^+-ATPase could have an opposite effect on the cytoplasmic sodium concentration. On this basis, prolonged H^+-ATPase activation or inhibition in vivo could potentially lead to changes in the magnitude of the sodium gradient driving force that energizes solute transport across the ciliary epithelium bilayer. Changes in cytoplasmic pH of the NPE could also have a direct impact on ion channels and transporters. For example, Skou (1982) has presented evidence suggesting a significant decrease of Na, K-ATPase activity might be caused by an alkaline shift in pH. In living rabbits, bafilomycin A_1 was found to cause a decrease of intraocular pressure (IOP) that could have been the result of reduced aqueous humor secretion since the resistance to fluid outflow

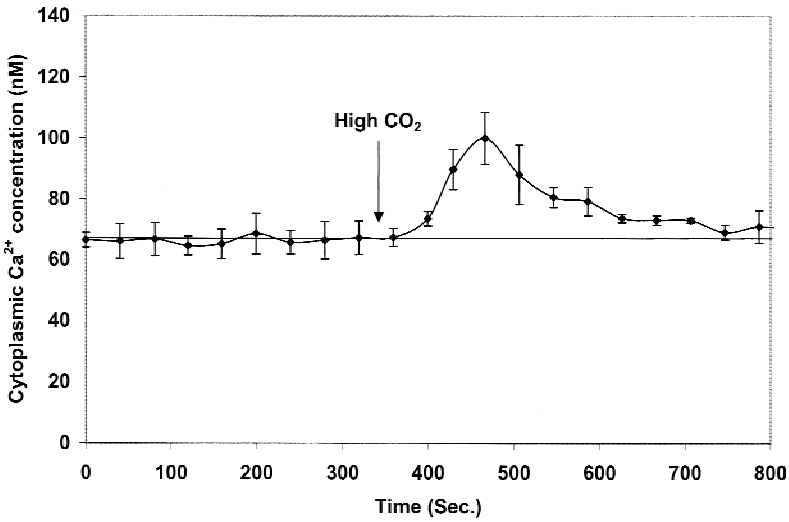

Fig. 7. The influence of high CO_2 on cytoplasmic calcium concentration. Cells were first exposed to control Krebs solution equilibrated with 5% CO_2/95% air (pH 7.4). After a stable baseline was established, the superfusate was switched (*arrow*) to Krebs solution equilibrated with 100% CO_2 (pH 7.4), a maneuver that reduced cytoplasmic pH from 7.17 ± 0.07 to 6.81 ± 0.05 (mean ± SEM; $n = 4$). Cytoplasmic calcium concentration was monitored continuously. The data represent the mean ± SEM of results from 6 independent experiments.

from the eye was unaltered (Wax et al., 1997). It is noteworthy that verapamil and nifedipine have also been reported to lower IOP (Payne et al., 1990; Segarra et al., 1993).

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