Regulation of Cell pH by Ca^{+2}-mediated Exocytotic Insertion of H^+-ATPases

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Abstract. Exposure to CO_2 acidifies the cytosol of mitochondria-rich cells in turtle bladder epithelium. The result of the decrease in pH in these, the acid-secreting cells of the epithelium, is a transient increase in cell calcium, which causes exocytosis of vesicles containing proton-translocating ATPase. Because mitochondria-rich cells have rapid luminal membrane turnover, we were able to identify single mitochondria-rich cells by their endocytosis of rhodamine-tagged albumin. Using fluorescence emission of 5,6-carboxyfluorescein at two excitation wavelengths, we measured cell pH in these identified mitochondria-rich cells and found that although the cell pH fell, it recovered within 5 min despite continuous exposure to CO_2. This pH recovery also occurred at the same rate in Na^+-free media. However, pH recovery did not occur when luminal pH was 5.5, a condition under which the H^+-pump does not function, suggesting that recovery of cell pH is due to the luminally located H^+ ATPase.

Chelation of extracellular calcium by EGTA prevented the CO_2-induced rise in cell calcium measured with the intracellular fluorescent dyes Quin 2 or Fura 2 and also prevented recovery of cell pH. When the change in cell calcium was buffered by loading the cells with high concentrations of Quin 2, the CO_2-induced decrease in pH did not return back to basal levels. We had found previously that buffering intracellular calcium transients prevented CO_2-stimulated exocytosis. Further, we show here that the increased H^+ current in voltage-clamped turtle bladders, which is directly proportional to the number of H^+-pump-containing vesicles that fuse with the luminal membrane, was significantly reduced in calcium-depleted bladders.

These results suggest that pH regulation in these acid-secreting cells occurs by calcium-dependent exocytosis of vesicles containing proton pumps, whose subsequent turnover restores the cell pH to its initial levels.

ALTHOUGH protons are both generated and consumed during the various chemical reactions that occur in a cell, metabolic balance usually favors net acid production. In addition, most cells have a membrane potential, negative inside, that favors accumulation of protons in the cytoplasm. As many vital biochemical reactions occur at narrowly defined pH optima, cells must extrude these protons. Two mechanisms for regulation of cell pH have been described in a number of tissues (6, 18, 19, 21, 22, 31, 35). Na:H exchange, an electroneutral process driven by the transmembrane Na^+ gradient generated by Na, K ATPase, seems to be the most common mechanism. There is also, although not necessarily in the same cell, a Cl:HCO_3 exchanger that, under some conditions, can move HCO_3^- into cells to neutralize H^+. Both these antiports are driven solely by the transmembrane gradients of the relevant ions rather than by direct coupling of ATP hydrolysis to these transporters. Since proton-translocating ATPases have recently been described in a variety of intracellular organelles, especially endosomes, and since endosomes fuse with and form from the plasma membrane, it is likely these H^+ pumps also participate in cell pH regulation (2, 7–10, 15). We recently discovered that the endosomal H^+-ATPase of urinary epithelia is rapidly inserted into the luminal membrane when the epithelium is stimulated by exposure to CO_2 (5, 11). We show here that exocytotic insertion of proton ATPases regulates cytosolic pH in these cells.

The experiments were performed in the turtle urinary bladder, an epithelium that is composed of two cell types, one of which, the mitochondria-rich cell, is responsible for H^+ transport (11, 26). Transepithelial transport occurs when protons are secreted into the luminal medium by an electrogenic proton-translocating ATPase located in the luminal membrane. The mitochondria-rich cells contain apically located vesicles whose membranes are enriched in this ATPase. CO_2, the major modulator of transepithelial proton transport in this system, causes fusion of these vesicles with the luminal membrane, thereby increasing the rate of transepithelial H^+ transport (5). We recently found that CO_2 produces exocytosis initially by acidifying the cell, which leads to a transient increase in cell calcium. It is this transient increase in cell calcium that is the proximate cause of exocytosis, since buff-

ering intracellular calcium with permeant EGTA derivatives prevents fusion without affecting the cellular acidification (5). These results led us to ask whether this exocytotic event also leads to regulation of cell pH, and the results described below show that this is indeed the case.

Materials and Methods

After freshwater turtles, *Pseudemys scripta elegans* (Lemberger Co., Oshkosh, WI) were pithed, the urinary bladders were excised and placed in CO_2-free turtle Ringer's solution. For some of the experiments, turtles were perfused with CO_2-free Ringer's solution (in millimolars, 110 NaCl, 3.5 KCl, 1.65 Na_2HPO_4, 0.3 NaH_2PO_4, 1 $MgSO_4$, 1 $CaCl_2$, 5 dextrose) for 10 min before removal of the bladder to remove blood from vessels. Bladder epithelial cells were isolated by shaking the bladders in CO_2-free turtle Ringer's solution containing 1 mM EGTA for 20 min. Isolated cells were pelleted at 200 *g* in a table-top centrifuge (E. I. Du Pont de Nemours & Co., Inc., Sorvall Instruments Div., Norwalk, CT) for 5 min. The isolated cells were washed three times with CO_2-free Ringer's solution containing either 1 mM Ca^{+2} or no added calcium (30 μM Ca^{+2} measured with a calcium electrode) and filtered through 30 μm plastic mesh (Spectramesh; Fisher Scientific Co., Pittsburgh, PA).

Dye Loading

To label cells with fluorescent dyes, 4–5 × 10^6 cells were incubated in 1 mM Ca^{+2} CO_2-free Ringer's solution containing 40 μM acetoxymethyl Quin 2 (Amersham Corp., Arlington Heights, IL) and/or 12 μM 5,6 dicarboxyfluorescein diacetate (Molecular Probes, Junction City, OR) for 60 min. Whole bladders were incubated on the luminal side only with 10 μM 5,6 dicarboxyfluorescein diacetate for 10 min or with 10 μM acetoxymethyl Fura 2 (Molecular Probes) for 20 min. These compounds are ester derivatives of the H^+-(5,6 carboxyfluorescein) or Ca^{+2}-binding (Quin 2 and Fura 2) forms of these fluorescent dyes. The esters are uncharged and diffuse into cells, where they are cleaved to their active forms by cytoplasmic esterases (12). Stock solutions of the esters were made in dimethylsulfoxide; final dimethylsulfoxide concentration in the incubation media never exceeded 1%. After loading, the cells were washed three times with 1 mM Ca^{+2} CO_2-free buffer, resuspended to final concentration of 1 × 10^6, and filtered again to remove clumped cells.

The acid-secreting cells of the turtle urinary bladder account for ~15% of the epithelial cell population and are enriched in carbonic anhydrase II (28), which is also a potent esterase that cleaves these dyes (14). Therefore, they concentrate these dyes preferentially compared with other cells in the epithelium. These cells also have very high turnover of the luminal plasma membrane and therefore exhibit rapid endocytosis of impermeant fluorophores which are sequestered in acid vesicles (5, 11). To identify mitochondria-rich cells, the luminal surfaces of bladders mounted on rings were exposed for 20 min to 1–2 mg/ml rhodamine isothiocyanate (Sigma Chemical Co., St. Louis, MO) covalently bound to albumin (34). Only cells with rhodamine-albumin–labeled vesicles had acid vesicles when stained with acridine orange, a fluorescent weak base that accumulates in acid compartments and displays a spectral shift at high concentration, confirming that these cells were the mitochondria-rich cells. After labeling with rhodamine-albumin, the bladder rings were labeled with the esters of 5,6-carboxyfluorescein or Fura 2. As shown in Fig. 1, only mitochondria-rich cells, identified by the uptake of rhodamine-albumin, were significantly labeled with 5,6-carboxyfluorescein or Fura 2. The differential labeling by 5,6 carboxyfluorescein or acetoxymethyl Fura 2, but not that of rhodamine-albumin, was abolished by 1 mM acetazolamide, a potent inhibitor of carbonic anhydrase but not of endocytosis.

To confirm that mitochondria-rich cells were labeled preferentially with these dyes, bladder epithelial cells were labeled with 5,6 dicarboxyfluorescein diacetate in the presence and absence of 1 mM acetazolamide and analyzed with a cytofluorograph (Ortho Diagnostic Systems, Raritan, NJ) equipped with an argon laser. Fig. 2 shows that acetazolamide shifts the peak representing the population of highly labeled cells to a position of lower relative fluorescence. Since only mitochondria-rich cells contain carbonic anhydrase, the highly fluorescent cells whose labeling was inhibited by acetazolamide are the mitochondria-rich cells. Cells incubated with the ester of Quin 2 in the presence of 1 mM acetazolamide contained 1.3 nmol/10^6 cells intracellular Quin 2. Cells incubated with the ester of Quin 2 alone contained 2.6 nmol/10^6 cells Quin 2. As only the mitochondria-rich cells contain carbonic anhydrase, these results show that at least half of the total dye content of the suspended cells was in the mitochondria-rich cells. Since they represent only 10–20% of the total cell population, the dye concentration in the cells was at least 5–10 times higher than in the other cells. This is likely to be a gross underestimate since the effect of acetazolamide is unlikely to be complete even at this concentration and the turnover of carbonic anhydrase II is probably the highest of any enzyme. The results are underestimated further by inclusion of extracellular dye in the acetazolamide-insensitive fraction.

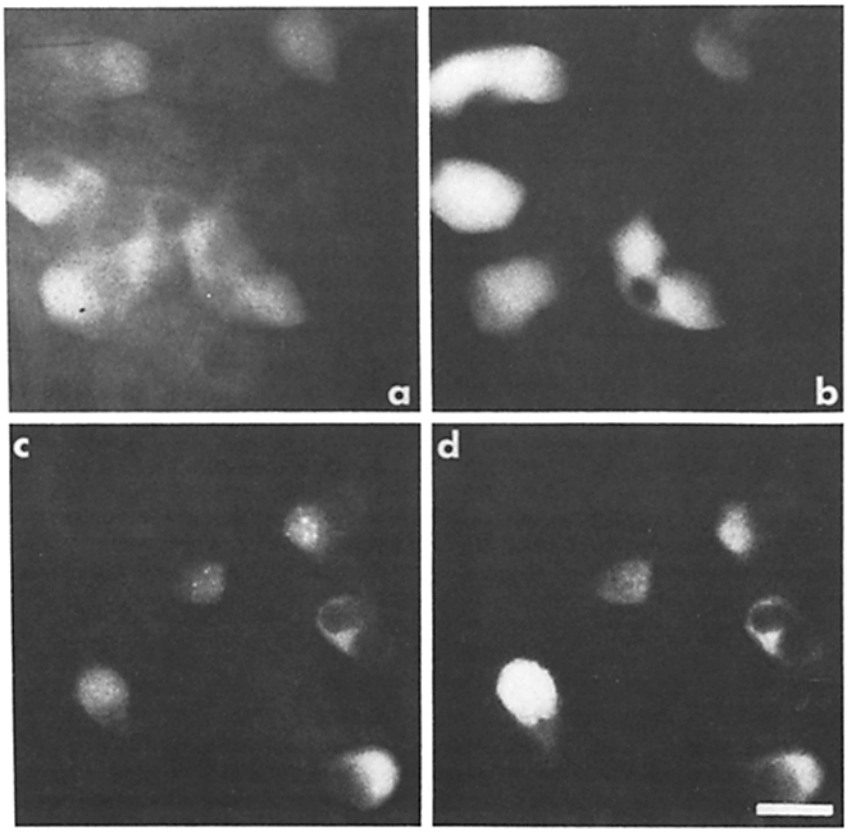

Figure 1. Photographs of whole turtle bladder preparations. (*a* and *b*) Bladder labeled with rhodamine-albumin and 5,6 carboxyfluorescein (*a*) photographed using rhodamine filter. (*b*) Same field photographed using fluorescein filter. (*c* and *d*) Bladder labeled with rhodamine-albumin and Fura 2. (*c*) Photographed using rhodamine filter. (*d*) Same field photographed using Fura 2 filter. Bar, 6 μm.

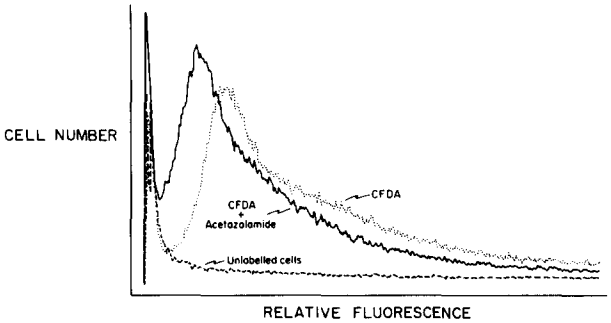

Figure 2. Histogram of relative fluorescence of suspended bladder epithelial cells labeled with 5,6 carboxyfluorescein diacetate in the presence and absence of 1 mM acetazolamide. After labeling, all samples were suspended in CO_2-free Ringer's solution containing 1 mM EGTA with and without 1 mM acetazolamide to prevent clumping. Each tracing represents a total count of 100,000 cells with relative fluorescence measured by flow cytofluorometry.

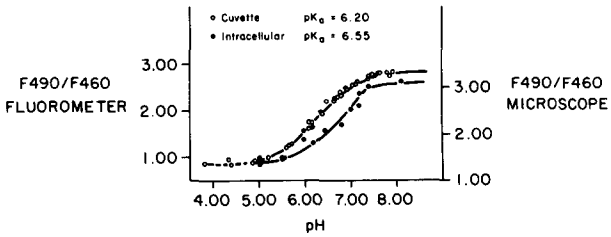

Figure 3. Calibration curves relating fluorescence of 5,6 carboxyfluorescein to pH. F490/F460 represents fluorescence emission intensity of 5,6 carboxyfluorescein measured at 530 nm using excitation at 490 nm divided by emission intensity at 530 nm using excitation at 460 nm. F490/F460 calculated from measurements made on the microscope differed from those made in the fluorometer. There was no difference between pK_a calculated using 5,6 carboxyfluorescein alone or cells labeled with 5,6 carboxyfluorescein when calibration was done in the fluorometer (data points labeled "cuvette"). In these experiments, in the presence of nigericin and valinomycin, as much as 50% of 5,6 carboxyfluorescein was extracellular, so that no reliable estimate of the pK_a of intracellular dye could be made.

Measurement of Cell pH and Cell Calcium in Suspended Cells

Cell pH was measured by excitation ratio fluorometry as previously described (5, 30). All measurement of fluorescence in suspended cells were made in a spectrofluorometer (Farrand Optical Co., Valhalla, NY) with 2.5-nm slits for excitation and emission. To obtain an estimate for cell pH, F490/F460 (see Fig. 3, legend) was compared with a calibration curve obtained with the free dye in cuvettes and also in isolated dye-loaded cells and whole epithelia incubated for 5 min in 90 mM KCl, 2 mM $KHPO_4$, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 20 μM valinomycin, 30 μM nigericin, and 20 mM acetate, 2-*N*-morpholino ethane sulfonic acid, morpholinopropane sulfonic acid, or Hepes at the desired pH. F490/F460 was calculated from measurements made on suspended cells as well as in single cells mounted on an epifluorescence microscope, as described below. The apparent intracellular pK_a of 5,6-carboxyfluorescein was 6.55, 0.35 pH units higher than that measured extracellularly.

To measure dye leakage, 5,6 carboxyfluorescein-labeled cells were stirred for up to 2 h with a magnetic stir bar and the concentration of extracellular dye was measured after pelleting the cells. Assuming that 50% of the dye was in 15% of the cells, the intracellular dye concentration was 0.55 mM in the mitochondria-rich cells. The rate coefficient of dye leakage was 0.0016 min^{-1} and was not affected by CO_2. After centrifugation, 10% of the dye was extracellular, presumably due to cell damage during centrifugation. This background of extracellular dye obtained by centrifugation at time 0 was subtracted from timed samples to calculate dye leakage.

To measure intracellular calcium, suspended cells labeled with Quin 2 were exposed to 340 nm light and fluorescence emission at 480 nm was measured. The cells were lysed at the end of the experiment with 2 μM digitonin (final concentration) in ethanol, and Quin 2 fluorescence with intracellular Ca^{+2} concentration was calibrated as previously described (32).

Measurement of Cell pH and Cell Calcium in Single Identified Cells

To measure cell pH, bladders were mounted on rings 0.5 cm in diameter with the basolateral side facing a chamber, continuously perfused during experiments, containing ~0.3 ml of media. Bladders labeled with rhodamine-albumin and 5,6-carboxyfluorescein were mounted luminal side down on an inverted epifluorescence microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with a mercury lamp and 63× fluorite objective (Carl Zeiss, Inc.). Mitochondria-rich cells (cells containing rhodamine-albumin because of endocytosis) were identified using a 510–560-nm band pass excitor filter in a cassette containing a 580-nm dichroic and a 590-nm barrier filter (rhodamine filter, Ditric Optics Inc., Hudson, MA). After identification of mitochondria-rich cell, the filter set was changed to a 510-nm reflector with a 520 barrier and 440–490 excitor filter (fluorescein filter, Ditric Optics Inc.) and F490/F460 was measured by using a microfluorometer (Farrand Optical Co.) as previously described (5). After initial measurement of F490/F460, bladders were perfused on the basolateral side with CO_2-free or 5% CO_2 buffers at 12 ml/min and serial measurements of F490/F460 were performed. F490/F460 was compared with the calibration curve in Fig. 3 to obtain cell pH.

To measure cell calcium, bladders mounted on rings were labeled sequentially with rhodamine-albumin and Fura 2 and were mounted on the epifluorescence microscope. Mitochondria-rich cells were identified as above and then were excited alternately with 340 nm and 380 nm light using band pass filters (Ditric Optics Inc.), measuring emission at 490 nm. The filter set for these measurements was a 420-nm reflector with a 365-nm excitor and 470-nm barrier (Fura 2 filter). The ratio of emission at 490 nm of these excitation wavelengths in cells labeled with Fura 2 is proportional to cell calcium (12). Serial measurements during basolateral perfusion by media with or without 5% CO_2 were performed.

Measurement of Transepithelial H^+ Transport

When the turtle bladder is short-circuited by an automatic voltage clamp and Na^+ absorption is abolished by 1 mM ouabain, a reversed short circuit current develops. This current has been shown to be an H^+ current and is greatly increased by perfusing the bladder with buffer containing CO_2 (25). The change in H^+ current resulting from exposure to CO_2 is directly proportional to the volume of H^+ pump-containing vesicles that fuse with the luminal membrane (5, 11). Paired hemibladders were incubated in CO_2-free turtle Ringer's solution with 1 mM ouabain and 5 mM dextrose on the serosal side until the H^+ current reached a steady state (1, 3, 5, 11). The bladders were then washed three times with either a Ca-EGTA CO_2-free Ringer's buffer calculated to contain 1 μM free Ca^{+2} or 1 mM Ca^{+2} CO_2-free Ringer's solution. This low calcium wash lasted 20 min. Measurements of open circuit potential difference were made every 5 min during the washes and the final incubation to calculate transepithelial resistance. Finally, the bladders were stimulated isohydrically with 5% CO_2 buffered with 25 mM $NaHCO_3$ in either 0.5 mM EGTA Ringer's solution or 1 mM Ca^{+2} Ringer's solution. Pairs were excluded from analysis either if the H^+ current in the control hemibladder did not increase by 10 μA or if the resistance in the calcium-depleted hemibladder fell by >100 Ω.

Results

Regulation of Cell pH in Response to an Acid Load

In the mitochondria-rich cell of the turtle urinary bladder, CO_2 acidifies the cytosol by rapid diffusion into the cell followed by hydration to carbonic acid, a reaction catalyzed by carbonic anhydrase which is present in high concentration in these cells. Acidification causes fusion of vesicles containing membrane-associated H^+-ATPases with the luminal membrane. This fusion event increases the electrogenic transport of H^+ from serosa to lumen. Transepithelial transport of H^+ remains high during continued exposure of the bladder to CO_2.

An obvious question arising from these observations is whether continued increased H^+ transport requires the maintenance of cytosolic acidification. The fusion event, which

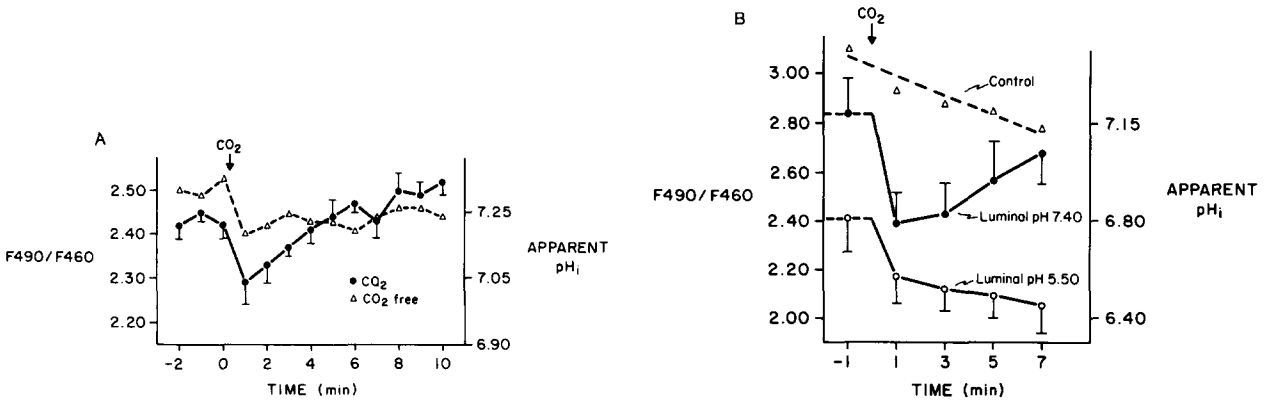

Figure 4. (*A*) Cell pH (pH_i) measured with 5,6 carboxyfluorescein in suspended cells exposed immediately after $t = 0$ to 5% CO_2 buffered with 25 mM HCO_3^- in 1 mM Ca^{+2} turtle Ringer's solution (in millimolars; 85 NaCl, 25 $NaHCO_3$, 3.5 KCl, 1.65 Na_2HPO_4, 0.3 NaH_2PO_4, 1 $MgSO_4$, 1 $CaCL_2$). F490/F460 defined as in Fig. 3. Points represent mean of five experiments ± SE. All solutions were pH 7.35. The measured ratio was compared with the calibration curve of dye in cuvette from Fig. 3 to obtain cell pH. To adjust for the shift in K_a of intracellular dye, 0.35 was added to this pH to obtain apparent cell pH. (*B*) Cell pH measured with 5,6 carboxyfluorescein in mitochondria-rich cells identified by endocytosis of rhodamine-albumin. Luminal solutions were CO_2-free Ringer's solution at pH 7.35 for center and upper tracings and CO_2-free Ringer's solution with 20 mM 2-*N*-morpholino ethane sulfonic acid, pH 5.50, for bottom tracing. Bladders were perfused with 5% CO_2 buffered with 25 mM HCO_3 at $t = 0$ in both sets of experiments. For each point, $n = 5$ and bars represent SE.

inserts more H^+ ATPases into the luminal membrane, is transient. If the increase in H^+ transport were due solely to an increased number of H^+ pumps, then continued cytosolic acidification would not be required to maintain H^+ secretion. However, if increased turnover induced by the low cell pH were required for the increase in H^+ transport, then cellular acidification should continue throughout the period of exposure to CO_2. When carboxyfluorescein-labeled epithelial cells were acidified by CO_2, their cell pH promptly returned to initial levels (Fig. 4*A*). Since most of the fluorescent signal in this heterogeneous cell suspension is from mitochondria-rich cells, the observed changes in fluorescence were interpreted as changes in cell pH of mitochondria-rich cells. To ensure that the effects observed indeed reflected changes in the intracellular pH of mitochondria-rich cells, we repeated these experiments in single, identified mitochondria-rich cells (Fig. 4*B*, center tracing), and again found recovery of cell pH. This suggests that despite continuous exposure to CO_2, the cell pH is rapidly regulated back to the original level. These results also show that most of the signal observed in suspensions of unseparated cells originates in the mitochondria-rich cells and suggest that continued acidification and therefore increased turnover is not necessary to increase H^+ pumping.

In many other systems, cells regulate intracellular pH by Na:H exchange (6, 21, 23, 31, 35). However, when mitochondria-rich cells were incubated in Na^+-free buffers, pH recovery after exposure to CO_2 occurred at the same rate as that seen in the presence of Na^+, suggesting that Na:H exchange was not involved in cell pH recovery (Fig. 5).[1] Addition of CO_2-free isosmotic Na^+-Ringer's solution to cells suspended in Na^+-free buffers did not affect cell pH, further documenting the absence of a Na:H exchanger. Since these cells are enriched in proton-translocating ATPases, we tested the hypothesis that these H^+ pumps are responsible for regulation of cell pH. The H^+-ATPase in these cells is located in the luminal plasma membrane, which is impermeant to protons (3, 28). The rate of turnover of the pump is affected by the proton electrochemical gradient across that membrane; an adverse gradient of 3 pH units or 180 mV is sufficient to stop proton transport (1, 3). We mounted epithelia on the stage of a fluorescence microscope and reduced the luminal pH to 5.5. The basolateral side was continuously perfused with buffer at pH 7.35. When the cell pH of individual identified mitochondria-rich cells was measured, we found that addition of CO_2 acidified the cytoplasm but cell pH did not recover (Fig. 4*B*, bottom trace). These data show that mitochondria-rich cells regulate cell pH by the action of H^+ ATPases located in the luminal membrane.[2]

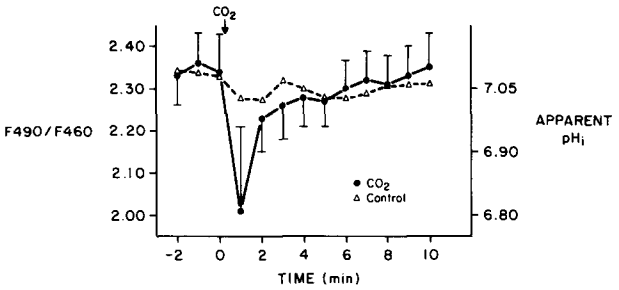

Figure 5. Suspended cells labeled with 5,6 carboxyfluorescein stimulated with 5% CO_2 buffered with 25 mM HCO_3 in sodium-free solutions. F490/F460 defined as in Fig. 3. Composition of buffers (in millimolars) CO_2-free: 110 choline Cl, 1.65 K_2HPO_4, 0.3 KH_2PO_4, 1 $CaCl_2$, 1 $MgSO_4$, bubbled with CO_2-free air (paired control, $n = 6$). CO_2 buffer: 85 choline Cl, 25 choline HCO_3, 0.55 K_2HPO_4, 2.40 KH_2PO_4, 1 $CaCl_2$, 1 $MgSO_4$, bubbled with 5% CO_2 ($n = 6$).

Calcium-mediated Regulation of Cell pH

The observation that mitochondria-rich cell pH recovered after acidification by CO_2 due to activity of the H^+ pump

1. Of note is the fact that resting cell calcium was higher (133 ± 26 nM, $n = 4$) in cells suspended in Na-free buffers and returned to baseline values of 92 ± 21 nM ($n = 3$) within 10 s upon the addition of isosmotic Na-containing buffers. These data suggest that these cells possess an Na:Ca exchanger.

2. Resting cell pH at a luminal pH at 5.5 is less than that at a luminal pH of 7.4. Since the turtle bladder luminal membrane is impermeant to protons, these data suggest that proton pumps already in the luminal membrane are inhibited, so that OH^- equivalents are no longer generated.

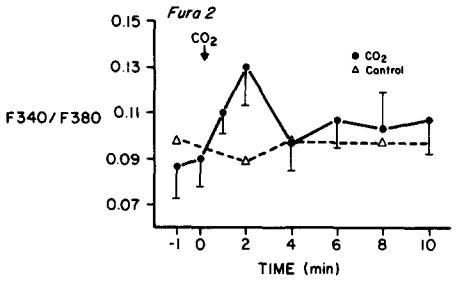

Figure 6. Measurement of intracellular calcium in identified mitochondria-rich cells identified by endocytosis of rhodamine-albumin using Fura 2. Bladders were perfused beginning at $t = 0$ on the basolateral side with either 5% CO_2 Ringer's solution or CO_2-free Ringer's solution. F340/F380 represents fluorescence emission intensity measured at 490 nm using excitation at 340 nm divided by emission intensity at 490 nm using excitation at 380 nm. For each point, $n = 3$ and bars represent SE. There is a lag between initiation of perfusion and actual exposure of the individual cell to CO_2.

Table I. Cells Suspended in CO_2-free Ringer's Solution Labeled with Both Quin 2 and 5,6 Carboxyfluorescein

	Change in cell $[H^+]$	Change in cell $[Ca^{+2}]$
	nM	*nM*
1 mM acetazolamide ($n = 3$)	-15 ± 21	-62 ± 6
10 mM NH_4Cl ($n = 4$)	-28 ± 9	-34 ± 9
10 mM butyrate ($n = 8$)	$+61 \pm 10$	$+62 \pm 15$

Cellular calcium and pH were measured at the beginning of the experiment, acetazolamide, NH_4Cl, or butyrate were added isohydrically and isosmotically. Cell calcium and pH were remeasured at the end of 5 min. The pH in the acetazolamide experiments rose by ≥0.5 in two experiments and fell by 0.05 in one. Initial cell calcium was 108 ± 7 nM and initial H^+ was 63 ± 3 nM in these experiments. Results represent means ± SE.

prompted us to investigate further whether pH recovered because of increased number of H^+ pumps or because of increased turnover of H^+ pumps. The fact that H^+ transport continues after cell pH has returned to the initial level suggests that pH recovery is due to increased numbers of pumps, inserted by calcium-mediated exocytosis. To confirm our previously published experiments, we repeated the measurements of cell calcium using Quin 2 and found that addition of CO_2 to suspended cells indeed increased cell calcium transiently. We also performed experiments on single identified mitochondria-rich cells using the new "second generation" calcium-sensitive dye, Fura 2 (12). We found that addition of CO_2 increased the F340/F380 ratio, which indicates that CO_2 indeed increases cell calcium in mitochondria-rich cells (Fig. 6). We also measured cell calcium and cell pH simultaneously in the same cells and found that cell acidification by the permeant weak acid butyrate also increased cell calcium. Alkalinization of the cell by adding either NH_4Cl, which releases the permeant weak base NH_3 intracellularly, or acetazolamide, an inhibitor of carbonic anhydrase, reduced cell calcium (Table I).[3] These results demonstrate that cell pH is an important regulator of cell calcium.

We used another experimental approach to confirm the role of calcium-mediated exocytosis in cell pH regulation. Since the increase in cell calcium was transient, we were able to prevent exocytosis by increasing the calcium buffering power of the cytoplasm (5). Quin 2 acts as a Ca^{+2} indicator by reversibly binding Ca^{+2} and therefore can also act as an intracellular calcium buffer. We found that recovery of cell pH in mitochondria-rich cells labeled with Quin 2 is proportional to the intracellular concentration of Quin 2 (Fig. 7). At high concentrations, cell pH recovery is prevented. In a previous study we found that when the epithelium was loaded with the Quin 2 analogue, MAPTAM, in an effort to blunt the transient increase in cell calcium, the increase in the H^+ current induced by CO_2 was abolished (5). These results suggested that exocytotic insertion of the proton ATPase was the major, or sole, cause of the increase in the proton current. Using another experimental approach, we reasoned that removal of calcium from the bathing medium should also block the CO_2-mediated increase. Unfortunately, the results with this maneuver were not straightforward since calcium is necessary for the integrity of the bladder. Indeed, the epithelial cells were isolated for our experiments by shaking bladders with EGTA. Complete removal of calcium resulted in an increase in the conductance of the bladder (likely due to dissociation of the cells), preventing adequate electrophysiological studies. To minimize this problem we exposed the bladders to low calcium media and measured the effect of CO_2 on transepithelial H^+ transport only in bladders whose conductance did not increase. Obviously, these bladders were not severely calcium-depleted, otherwise their cells would have dissociated. Hence, we did not expect a complete abolition of exocytosis but rather a diminution in the number of H^+ pumps reaching the luminal membrane. The increase in H^+ current resulting from exposure of the bladder to CO_2 was significantly lower (by 18 ± 7 μA) in the calcium-depleted hemibladders when compared with paired controls (Table II). Whether this partial inhibition is due to partial calcium depletion or whether the increase in cell calcium is due to movement of calcium from intracellular stores remains to be determined. However, the fact that increasing the calcium-

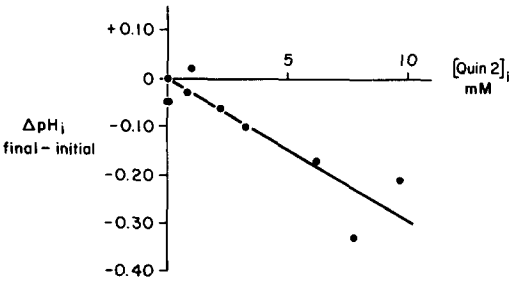

Figure 7. Change in pH in suspended cells at end of 5-min incubations with 5% CO_2. Cell pH was measured at beginning and end of incubation. Changes in pH were calculated by subtracting initial from final pH. Intracellular concentration of Quin 2 was calculated by lysing Quin 2–loaded cells in 1 mM Ca^{+2} and comparing the fluorescence of dye to a standard curve relating fluorescence to known concentrations of Quin 2 in 1 mM Ca^{+2}. This figure was divided by cell number and average cell volume of mitochondria-rich cells measured in a channel analyzer (Coulter Electronics, Inc., Hialeah, FL).

3. Changes in measured cell calcium are not due to an effect of pH on the fluorescence of Quin 2. Fluorescence of Quin 2 is quenched only at very low pH (<5), levels which were not reached in these experiments. Also, decreasing pH quenches the fluorescence of Quin 2, which should result in an apparent decrease in cell calcium. As the changes in cell pH and cell calcium occurred in opposite directions, a decrease in pH, if it had any effect, would tend to underestimate the increase in cell calcium.

Table II. Paired Hemibladders Stimulated with 5% CO_2 in 1 mM Ca^{+2} or 0.5 mM EGTA

	1 mM Ca^{+2}		Ca^{+2} Depleted
Resistance (Ω/8 cm^2)			
Pre CO_2	574 ± 74	(NS)	586 ± 84
Post CO_2	510 ± 45	(NS)	611 ± 88
H^+ Current (μA/8 cm^2)			
Pre CO_2	19.9 ± 4.3	(NS)	17.0 ± 2.4
Post CO_2	66.7 ± 16.3	($P < 0.05$)	46.3 ± 8.1

Results are expressed as mean ± SE of six experiments.

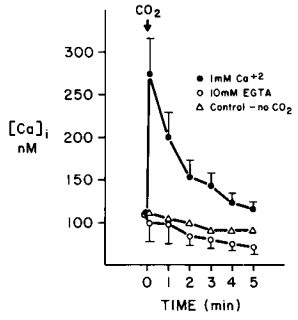

Figure 8. Measurement of cell calcium (Ca_i) in suspended cells labeled with Quin 2. Ringer's solution containing 0 or 5% CO_2 was added to suspended cells at $t = 0$. CO_2 buffers were either 1 mM Ca^{+2} or 10 mM EGTA. Control ($n = 13$), CO_2 ($n = 13$), EGTA ($n = 6$).

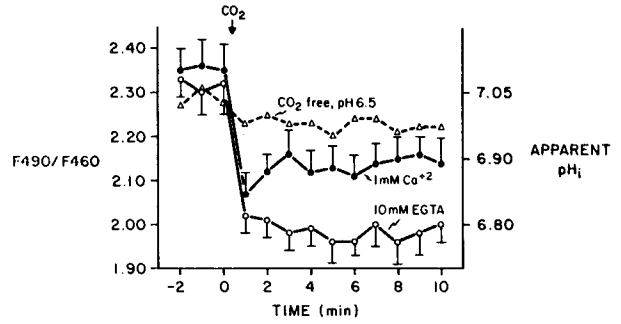

Figure 9. Cell pH (pH_i) measured with 5,6 carboxyfluorescein in suspended cells. After $t = 0$, 5% CO_2 buffered with 2.5 mM HCO_3^- to pH 6.50 with 10 mM EGTA or 1 mM Ca^{+2} was added to cells suspended in CO_2-free Ringer's solution. 1 mM Ca^{+2} ($n = 5$), EGTA ($n = 4$). F490/F460 and apparent pH_i as in Figs. 3 and 4.

buffering power of the cytoplasm prevented the CO_2-mediated increase in H^+ current implies that the present experiments caused only partial depletion of epithelial calcium.

We found that the CO_2-stimulated increase in cell calcium was prevented by adding 10 mM EGTA simultaneously with CO_2 to suspended cells (Fig. 8). Fig. 9 shows that cells exposed to CO_2 in the presence of 10 mM EGTA in low HCO_3^- solutions did not recover cell pH but remained acid. These results demonstrate that cell pH regulation in response to an acid load is calcium dependent. We believe calcium dependence is due to inhibition of exocytosis, rather than an effect of EGTA on the activity of proton pumps already resident in the membrane. Note that high concentrations of extracellular EGTA did not significantly reduce cell calcium below basal levels during the short period of observation so that reduced activity of H^+-ATPase due to extremely low cell calcium is unlikely to account for the inhibition of cell pH recovery in these experiments. Although these results also suggest that the increase in cell calcium comes from outside the cell as EGTA was added simultaneously with CO_2, more experiments need to be done to directly test the role of intracellular stores, which may have been rapidly depleted by EGTA. These data in the aggregate show that pH recovery depends on increases in cell calcium and exocytotic insertion of H^+ pumps into the luminal membrane.

Role of Cl:HCO_3 Exchanger

The mitochondria-rich cell is a polar epithelial cell in which proton ATPases are present on the luminal domain of the plasma membrane. The other domain contains a Cl:HCO_3 exchanger which disposes of the base generated by the secretion of H^+ (28). To directly demonstrate the presence of this process we prevented Cl:HCO_3 exchange by using CO_2-free, Cl^--free media, and found that the cell pH was 7.51 ± 0.04 (SE, $n = 17$), significantly higher than the resting cell pH of 7.20 ± 0.01 ($n = 21$) measured in cells suspended in CO_2-free Ringer's solution containing Cl^-. Because of the presence of this anion exchanger, isohydric addition of CO_2 buffered with HCO_3^- should initially reduce pH_i by rapid diffusion of CO_2 across the cell membrane. However, even in the absence of exocytosis and H^+ transport, cell pH might recover since the extracellular solution now contains a much higher concentration of HCO_3^-, which could enter the cell by reversal of the usual action of the Cl:HCO_3 exchanger. Hence the recovery of cell pH noted could be a composite of the exocytotic insertion of proton pumps as well as the entry of HCO_3^- into the cell. Recovery of cell pH occurred when CO_2 was added to the media at constant HCO_3^- concentration (Figs. 3–5), implying that exocytotic insertion is the primary mechanism by which cell pH recovers from an acid load. That this reversal of transport by the exchanger can indeed occur was tested in experiments where the exocytotic insertion was prevented by adding CO_2 buffered with 25 mM HCO_3^- with 10 mM EGTA. The cell pH recovered but the time course was quite slow, occurring in 8–9 min (data not shown) rather than the 2–3 min as observed in Fig. 4*A*. Hence, the results suggest that Cl:HCO_3 exchange can also participate in the process of regulation of the pH of these cells provided the transmembrane ion gradients are favorable for HCO_3^- entry into the cell.

Discussion

Our results demonstrate a mechanism for regulation of intracellular pH that has not been described previously. Exocytotic insertion of H^+-ATPase is a kind of feedback loop in which the product of the reaction, luminal proton ATPase, tends to restore cell pH to its original level. Therefore, one might expect that other acid-secreting cells would display cell pH regulation by exocytotic insertion of proton ATPases. Osteoclasts in bone and oxyntic cells in gastric glands secrete H^+ and contain apical cytoplasmic vesicles like mitochondria-rich cells of turtle urinary bladder; either of these cell types might display cell pH regulation by exocytosis of H^+ pump. Whole gastric glands have Na:H exchange (9), but these glands contain two cell types, only one of which, the oxyntic cell, secretes acid; the cell type(s) containing Na:H exchange is therefore obscure.

The effect of exocytotic insertion of proton pumps is likely to be more general than the special case of acid-secreting cells, as many secretory cells contain vesicles whose contents are

made acid by the action of membrane-associated H^+ ATPases (8, 16, 29, 33). In the resting state, these proton pumps have no effect on cytoplasmic pH because they are "turned off" by the transmembrane proton electrochemical gradient generated by the ATPase. However, when secretion occurs, these pumps will be inserted into the plasma membrane and will no longer face an insurmountable electrochemical gradient. The expected result of exocytosis of acid vesicles is proton extrusion and cell alkalinization until the H^+-ATPases are inactivated by endocytic retrieval from the luminal membrane or by other mechanisms like enzymatic modification or degradation. Note that repetitive stimulation, by causing repeated waves of secretion, would insert more H^+ pumps into the plasma membrane and cause a greater and more prolonged increase in cell pH. In support of this thesis, recent experiments have shown that alkalinization of pancreatic islets in *ob/ob* mice whose islets are ≥90% B cells occurred 5 min after stimulation by glucose (19).

Alkalinization of the cytosol as a result of exocytotic insertion of proton pumps is likely to be functionally significant. Endocytosis in turtle urinary bladder is stimulated by removal of CO_2 which is expected to increase intracellular pH (22). Alkalinization therefore tends to restore initial conditions by removing membrane-associated H^+-ATPase from the apex of the cell. One of the major problems faced by secretory cells is the regulation of membrane surface area, which increases after secretion. Cytosolic alkalinization may be a general signal for retrieval of plasma membrane and its associated proteins. In addition, many important enzymes and structural proteins, for example, phosphofructokinase, calmodulin, and tubulin, have very narrowly defined pH optima, so that a change in cell pH could act to shift cellular metabolism or structure to a new steady state (4, 24, 37). Prolonged increases in cell pH accompany many biological events involving changes in phenotype that also involve DNA and protein synthesis, for example, fertilization of echinoderm eggs, spore germination, refeeding of starved *Saccharomyces*, shift from anaerobic to aerobic metabolism, growth stimulation of fibroblasts by epidermal growth factor and insulin, and mitogenesis of lymphocytes by concanavalin A (4, 13, 20). Whether the changes in cell pH upon activation of growth are necessary for that event or whether the cell pH increase is a by-product of cell activation is not yet clear, although the observations that protein synthesis stimulated by A23187 in fertilized eggs (36), DNA synthesis in cultured fibroblasts (17), and protein synthesis in cell-free systems (37) are all pH dependent suggest that cell pH changes have functional significance. Note that cell pH returns to "normal" with time after alkalinization in the events described above, implying that synthesis of new proteins or modification of existing proteins must have taken place so that the new phenotype can be maintained without a continued high pH. Prolonged alkalinization, such as that which would occur in a frequently stimulated secretory cell with acid vesicles and proton pumps, might therefore trigger a cascade of events leading to a new phenotype. An example of this kind of phenotypic modulation might be the reversal of polarity in H^+ and HCO_3^--secreting epithelia noted recently by Schwartz et al. (27).

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