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Exocytosis regulates urinary acidification in turtle bladder by rapid insertion of H^+ pumps into the luminal membrane

(epithelial transport/proton transport/membrane fusion/membrane recycling)

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ABSTRACT **Urinary acidification by the turtle bladder is due to a H^+-ATPase that is located in the luminal membrane. The rate of H^+ transport is stimulated by an increase in the ambient CO_2. Using the fluorescent dye acridine orange, we showed that the mitochondria-rich cell of this epithelium contains vesicles whose internal pH is acidic. We measured the pH of these vesicles by using endocytosed fluorescein isothiocyanate-labeled dextran and found it to be near 5.0. The pH increased after treatment with protonophores or metabolic inhibitors, suggesting that it was due to a H^+ pump rather than to a Donnan effect. In bladders preloaded with fluorescent dextran, CO_2 stimulated exocytosis and H^+ transport measured simultaneously in the same bladder. The increase in the H^+ current correlated well with the extent of exocytosis, and both were inhibited by pretreatment with colchicine. We conclude that the turtle bladder contains an intracellular reserve of vesicles containing H^+ pumps and CO_2 stimulates rapid fusion of these vesicles with the luminal membrane with consequent insertion of H^+ pumps, thereby stimulating H^+ secretion across the whole epithelium.**

The plasma membrane of epithelial cells is differentiated into two specialized domains (luminal and basolateral) that differ in their permeability characteristics. It is this difference in transport properties of the two surfaces that allows the vectorial translocation of solutes and water across the epithelial layer. Transport across these cells is regulated by changes in the permeability characteristics of the cell membrane and is usually attributed to a change in the kinetic properties of individual transport proteins. An alternative mechanism that could regulate transport is a change in the number of transporting proteins in the membrane. One way by which this could occur is through fusion of vesicles whose membranes contain specific channels or pumps. Exocytotic fusion, known to occur in many cell types, subserves many well-described functions, including export of neurotransmitters and enzymes as well as renewal of membrane constituents. In some epithelia rapid fusion of vesicles has been found in response to physiological stimuli, and there have been suggestions that these vesicles insert transport proteins into the luminal membrane (1–3). However, direct evidence for the presence of functioning transport units in these vesicles does not exist. [The case for fusion of vesicles containing H^+ pumps in the gastric parietal cells remains controversial (3, 4).] Stimulated by the numerous studies which showed that neurosecretory granules and lysosomes contain a proton-translocating ATPase (5, 6) and that these organelles are capable of exocytotic fusion, we studied the role of membrane fusion in the regulation of H^+ transport in a urinary epithelium. This report shows that acid secretion in the turtle urinary bladder in response to CO_2 is regulated by exocytotic insertion of H^+ pumps into the luminal membrane.

Urinary acidification by the turtle bladder is accomplished by a proton-translocating ATPase located in the luminal membrane (7). Several factors affect H^+ transport in this epithelium. An adverse pH or electrical gradient across the membrane reduces the rate of the H^+ pump (8). Increasing ambient CO_2 stimulates H^+ transport by a mechanism that is unrelated to a change in the electrochemical gradient across the luminal membrane (9). This response to CO_2 also occurs in the kidney and may play an important role in the way that the kidney regulates the buffer concentration and pH of the body fluids. We show that the turtle bladder has an intracellular reserve of vesicles containing H^+ pumps and that CO_2 stimulates rapid fusion of these vesicles with the luminal membrane, with consequent insertion of H^+ pumps and stimulation of the rate of H^+ secretion.

MATERIALS AND METHODS

Urinary bladders from the freshwater turtle *Pseudemys scripta elegans* (Mogul, Oshkosh, WI) were bathed by one of two modified Ringer's solutions. The 5% CO_2 Ringer's solution contained 75 mM NaCl, 25 mM $NaHCO_3$, 4.0 mM KCl, 1.0 mM $CaCl_2$, 0.8 mM $MgSO_4$, 6.0 mM NaH_2PO_4, and 10 mM glucose; the pH was 7.4 when the solution was saturated with 5% CO_2 in air. The CO_2-free Ringer's solution contained 100 mM NaCl, 4 mM KCl, 1.0 mM $CaCl_2$, 0.8 mM $MgSO_4$, 6.0 mM NaH_2PO_4, and 10 mM glucose; the solution was titrated to pH 7.4 when saturated with CO_2-free air.

Fluorescence Microscopy. Small pieces of bladder (≈1 cm in diameter) supported luminal side up on nylon mesh were stretched tightly over small polyethylene cylinders and held in place by polyethylene rings that fitted over the cylinders. The mounted bladders were bathed in Ringer's solution until used. The cylinders were attached to a plastic holder on a microscope slide, and a glass cover slide was placed on the luminal surface. Microscopy was done using a Zeiss epifluorescence microscope equipped with a mercury lamp and a ×40 objective. The dye 3,3′-dipentyloxacarbocyanine [Di-O-C_5 (3)] or acridine orange was dissolved in the appropriate Ringer's solution and the bladders were stained by flushing the luminal side immediately before observation. In some experiments the serosal side was continuously perfused during the microscopy by using a constant infusion pump. For microscopy with fluorescent dextran the bladders were initially mounted in Ussing chambers and bathed by 5% CO_2 Ringer's solution with fluorescent dextran (M_r 70,000) at 100 mg/ml added to the luminal solution. After 2 hr the bladders were washed and small pieces of each bladder

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Abbreviations: Di-O-C_5(3), 3,3′-dipentyloxacarbocyanine; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

were mounted over the cylinders and examined as above. (Endocytosis of dextran by these cells occurred both in the presence and in the absence of CO_2. This probably represents membrane recycling in these cells—see *Discussion* for details. CO_2 appears to increase the surface area of these cells, and this facilitates photography and increases the fluorescent signal in the microfluorometer.)

To measure the pH of the endocytosed dextran we used a microfluorometer attached to the microscope (microscope spectrum analyzer; Farrand Optical, Valhalla, NY). Cells containing fluorescent dextran were centered in the microscope field and the diaphragm for the light source was closed down so that only single cells were illuminated. The sample was excited alternately with 490-nm and 458-nm light by using interference filters placed in front of the exciting light beam. The fluorescence emission intensity was measured at 520 nm with the microfluorometer; the slit was 0.22 μm. A calibration curve of the ratio of fluorescence intensities at the two exciting wavelengths versus the pH was constructed by using droplets of buffer at pH values varying between 4 and 7 placed on a microscope slide.

Exocytosis of Fluorescent Dextran. The purpose of these experiments was to show that an impermeant molecule, 70,000 M_r dextran coupled to fluorescein isothiocyanate (fluorescent dextran), was released from intracellular vesicles into the luminal solution when the bladder was stimulated with CO_2. The bladders were mounted in Ussing chambers having an exposed surface area of 8 cm^2. The volume of the luminal side was 3 ml and its lateral wall contained a magnet-driven disc that allowed vigorous stirring. The bladders were perfused on both sides by continuously circulating freshly bubbled buffer through the chamber with a peristaltic pump (Buchler Instruments, Ft. Lee, NJ). The transepithelial potential difference was set to zero with an automatic voltage-clamp and the short-circuit current was recorded continuously.

The bladder was initially perfused with 5% CO_2 Ringer's solution with fluorescent dextran added to the luminal solution at 100 mg/ml and ouabain added to the serosal solution at 1 mM. After 45–60 min the short-circuit current reversed in polarity and reached a steady level. The short-circuit current under these conditions has been shown to be due solely to H^+ transport. After the initial steady H^+ current was attained, the perfusing solutions were changed to CO_2-free Ringer's solution containing fluorescent dextran at 100 mg/ml in the luminal side and 1 mM ouabain in the serosal side. During the next 45 min the H^+ current fell, as expected, and reached a new steady level. At this time the luminal solution was changed to CO_2-free Ringer's solution *without* fluorescent dextran. Fresh CO_2-free Ringer's solution was pumped continuously through the luminal side and the effluent was passed through a flow cell in a fluorometer (ratio fluorometer; Farrand Optical). The fluorescence of the effluent (470-nm excitation, 520-nm emission) was continuously recorded. After 20–30 min the fluorescence of the effluent fell to a very low level with either a steady or a slightly decreasing value. At this time the perfusion solution was changed to 5% CO_2 Ringer's solution, which produced a rise in the H^+ current and release of fluorescent dextran into the luminal solution detected as a small broad peak in the fluorescence tracing. Because of the vigorous stirring the effluent always contained small air bubbles, which appeared on the fluorescence tracing as small spikes at irregular intervals. The recording was continued until the H^+ current reached a new steady level. Measured amounts of Ringer's solution containing fluorescent dextran were then injected into the luminal chamber to obtain a calibration curve for each experiment. Plotting the area of these calibrating injections against the volume injected gave the standard curve that was used to estimate the exocytosed volume. The areas of peaks were measured by cutting out and weighing tracings of them.

Fluorescent dextran, acridine orange, ouabain, and colchicine were purchased from Sigma Chemicals. Di-O-C_5(3) was a generous gift from Alan Waggoner (Amherst College, Amherst, MA).

RESULTS

To demonstrate insertion of H^+ pumps in the luminal membrane required first showing the presence of intracellular compartments containing H^+ pumps. For this we used the fluorescent pH probe acridine orange. This dye is thought to accumulate in lysosomes because of their low pH (5, 10), and it has also been used to mark the appearance of acid secretion in the lumen of isolated gastric glands (11). Acridine orange is a weak base with a tendency to form dimers and higher-order multimers as its concentration increases, a characteristic shared by other planar dyes. The fluorescent emission maximum has a red-shift with increasing concentration, as shown in Fig. 1, so that the fluorescent color of the dye changes from green at low concentrations to red-orange at high concentrations. When living turtle bladders are stained with 100 μM acridine orange, some cells are seen to contain reddish-orange vacuoles in the cytoplasm (Fig. 2*a*). The accumulation of acridine orange in these vacuoles is probably due to a low pH. To rule out the possibility of binding of the dye, we showed that adding 10 mM NH_4Cl to the acridine orange solution caused a loss of the orange color in these vacuoles (Fig. 2*b*), an effect probably due to diffusion of NH_3 into the vacuoles with a consequent rise in pH. Because the low pH in the vacuole could result either from the action of a H^+ pump or from a Donnan equilibrium, we examined the effect on the orange color of adding the proton ionophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). Fig. 2 *c* and *d* shows that CCCP also caused a loss of orange color, an effect that supports the existence of a H^+ pump in these vesicles, because increasing the proton permeability could only increase the rate of development of a pH difference caused by a Donnan equilibrium. Johnson *et al.* (12) used similar experiments to show that the accumulation of 9-aminoacridine in mast cell granules was the result of an acid internal pH arising from a proton pump.

Because the orange vacuoles appeared in only some of the cells, our next experiments were designed to define which cell type contained these vacuoles. The turtle bladder, like the renal

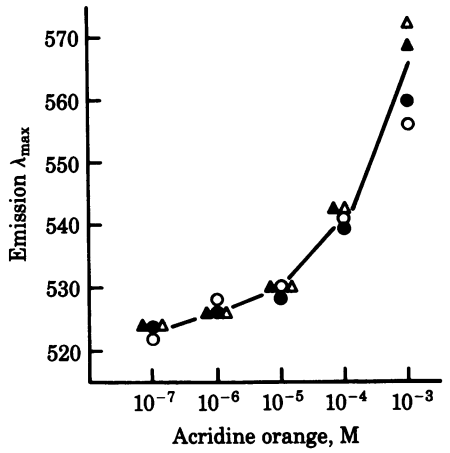

FIG. 1. Concentration and pH dependence of the emission maxima of acridine orange. The spectra were uncorrected. pH values: ○, 7.0; ●, 6.0; △, 5.15; ▲, 4.1.

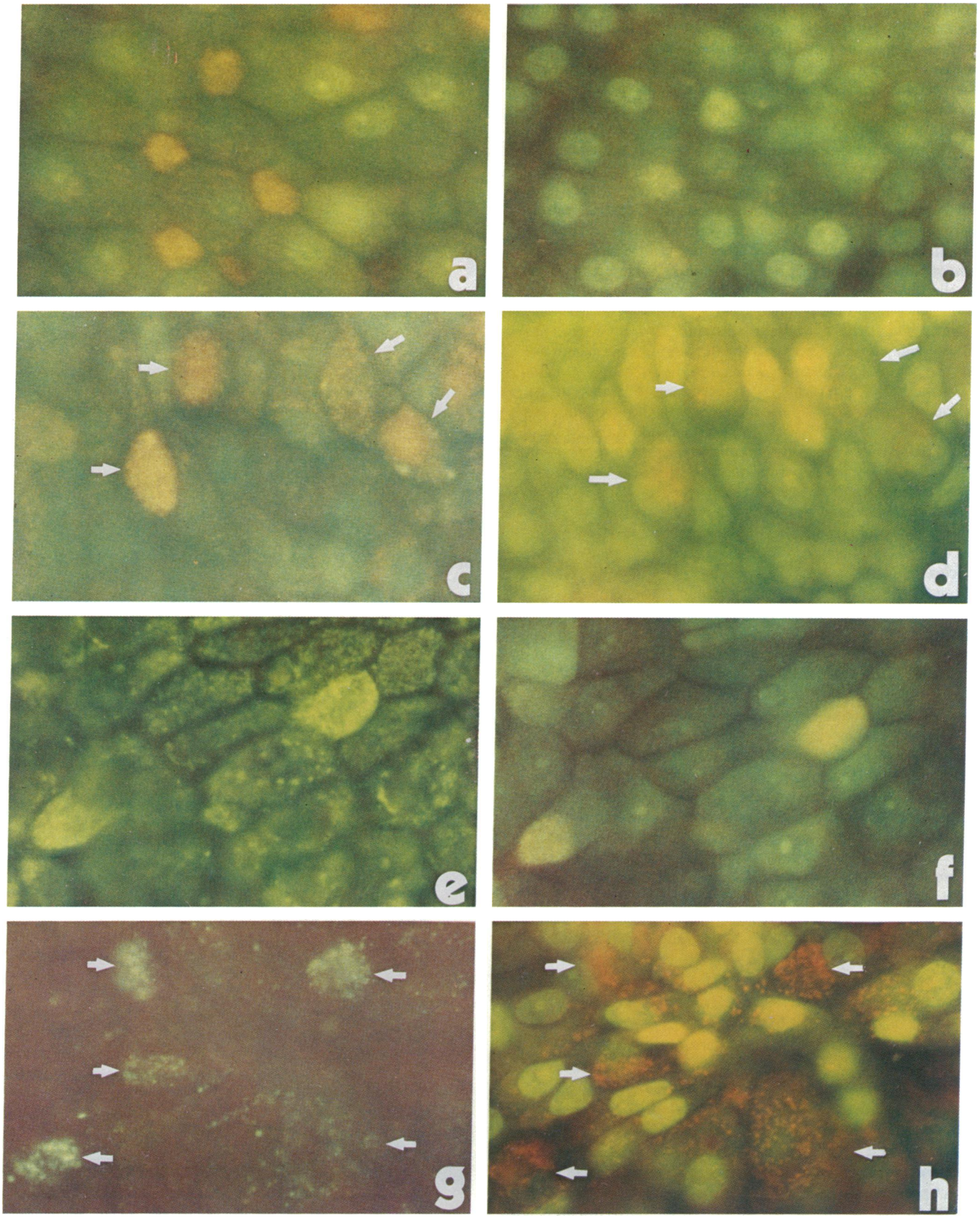

FIG. 2. Fluorescence micrographs of living turtle bladders. Each pair of results represents the same field. (×400.) (*a*) Bladder stained with 100 μM acridine orange; (*b*) same bladder as in *a* after perfusion with buffer containing 10 mM NH_4Cl. (*c*) Bladder stained with 100 μM acridine orange; (*d*) same bladder as in *c* after perfusion with buffer containing 100 μM CCCP; arrows indicate the same cells in each photograph. (*e*) Bladder stained with 1 μM Di-O-C_5(3), a mitochondrial stain, showing mitochondria-rich cells; (*f*) same bladder after additional staining with acridine orange. (*g*) Bladder loaded with fluorescent dextran; (*h*) same field stained with 100 μM acridine orange; arrows point to the same cells in *g* and *h*. See text for details.

collecting tubule, has two major types of cells. One of these types stains intensely for carbonic anhydrase (13) and decreases its surface area as the rate of H^+ secretion of the whole bladder decreases (14), and it is thus felt to be the cell responsible for H^+ secretion. This cell also contains more mitochondria than the other type. To identify these mitochondria-rich cells we stained the bladders with the permeant fluorescent cation Di-O-C_5(3), which has been used previously to stain mitochondria in rat fibroblasts (15, 16). Bladders stained with this dye (Fig. 2*e*) showed a granular-filamentous pattern of staining typical of mitochondria that disappeared when the mitochondrial potential was collapsed with 1 μM valinomycin (not shown). (The filamentous nature of the staining does not photograph well in these cells because the plane of focus cuts through many of the mitochondria.) Fig. 2*e* shows that the fluorescent intensity of the mitochondrial stain is much greater in some cells* and Fig. 2*f*, which shows the same field subsequently stained with acridine orange, demonstrates that the mitochondria-rich cells are the ones containing the orange vacuoles.

To examine the effect of CO_2 on increasing H^+ transport, we perfused the serosal side of the bladders with CO_2-free Ringer's solution followed by 5% CO_2 Ringer's solution during observation under the microscope, hoping to observe loss of the orange vacuoles from the cytoplasm. Instead, with CO_2 we observed discrete bursts of green fluorescence, which appeared as scintillations, over the cells containing orange vacuoles. These bursts probably represented individual exocytotic events that were visible because acridine orange was present in these vesicles in high concentrations and was subsequently released during fusion with the luminal membrane. Unfortunately this phenomenon could not be photographed because of the low light intensity and bleaching of acridine orange on prolonged exposure to light. A large number of orange vacuoles remained in the cells even after prolonged exposure to CO_2.

As an alternative method of demonstrating exocytosis we used an impermeant large molecular weight dextran labeled with fluorescein isothiocyanate (fluorescent dextran) as a marker for fluid-phase endocytosis and exocytosis. When bladders are bathed with fluorescent dextran on the luminal side for 2 hr, they take up the dextran into endocytic vesicles. Fig. 2 *g* and *h* shows that under these conditions fluorescent dextran is endocytosed only by the cells containing orange vacuoles. To show that these endocytic vesicles contain proton pumps, we measured the intravesicular pH by using the pH sensitivity of the excitation spectrum of fluorescein isothiocyanate. Fig. 3 *Inset* shows a calibration curve generated by measuring the ratio of fluorescence intensity at two exciting wavelengths. This method was used previously to measure the average pH of lysosomes in living macrophages (17). Using this method, we found the mean (± SD) vesicle pH measured in 12 cells to be 4.99 ± 0.19. As shown in Fig. 3, the pH is quite stable in each cell over a prolonged period of observation. When these cells were exposed to 10 mM NH_4Cl a rapid rise in the vesicle pH occurred. The same effect was seen when CCCP was added, and when cellular metabolism was inhibited by cyanide and iodoacetate (Fig. 4). These results show that fluorescent dextran is endocytosed into vesicles whose internal pH is lowered by metabolism-dependent proton pumps rather than by a Donnan effect produced by fixed negative charges.

Fig. 5 shows that dextran is released from these vesicles when the bladder is stimulated with CO_2. The tracing shows the effect of CO_2 in a single bladder that was initially loaded with fluorescent dextran. After washout of the dextran the luminal surface was perfused with CO_2-free buffer and the perfusate was passed continuously through the flow cell in a fluorometer. The addition of CO_2 resulted in appearance of dextran in the perfusate simultaneously with an increase in the rate of H^+ transport. (Note that the signal produced by the released dextran is the area between the tracing and the broken line in the figure and not the frequent spikes, which are artifacts produced by small air bubbles.) The release of dextran is transient, probably because it represents the unloading of vesicles, whereas the increase in H^+ transport persists, probably because it represents insertion of pumps in the luminal membrane rather than simply the unloading of the acid from these vesicles. (The amount of acid contained in these vesicles is small, amounting to no more than 1 pmol in 100 nl. The increase in the rate of H^+ transport is 30 μA, which is a rate of ≈20 nmol/min. The

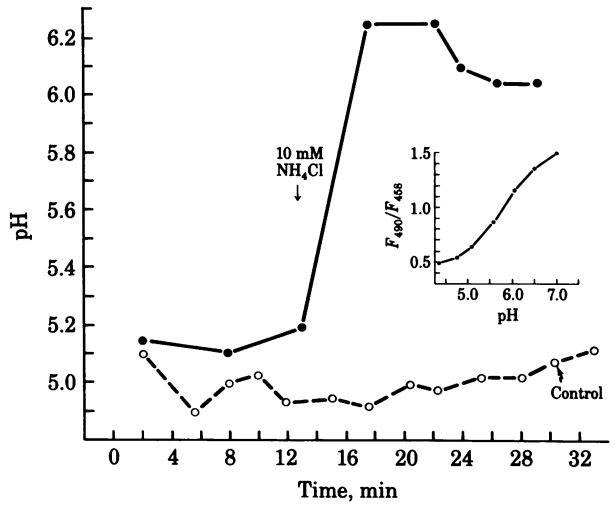

FIG. 3. Intravesicular pH in individual mitochondria-rich cells of turtle bladder measured with a microfluorometer. Emission intensity was measured at 520 nm, using excitation at 490 and 458 nm. A calibration (*Inset*) was performed on a microscope slide before each experiment. One bladder was washed on the mucosal surface with Ringer's solution containing 10 mM NH_4Cl at arrow (●).

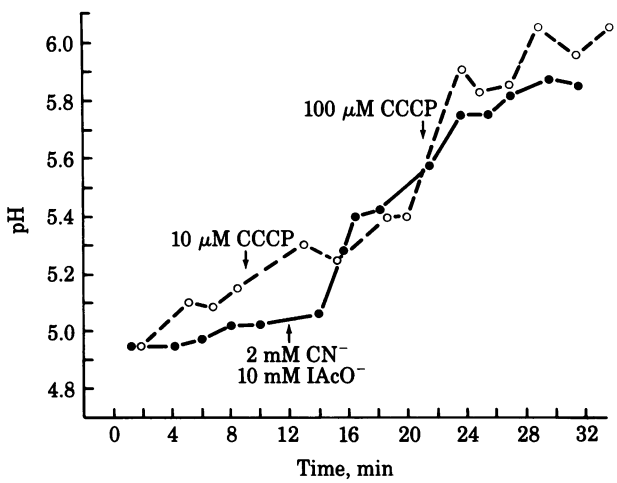

FIG. 4. Effect of CCCP and metabolic inhibitors (iodoacetate and cyanide) on intravesicular pH in individual mitochondria-rich cells in the turtle bladder. Reagents were added at the time indicated by arrows. Measurements were as in Fig. 3.

* Of course, it is not possible by this method to tell whether the intensity is due to an increased number of mitochondria or whether the mitochondrial membrane potential is higher in these cells. However, the surface morphology of these cells is sufficiently characteristic to allow us to be confident that they are the mitochondria-rich cells.

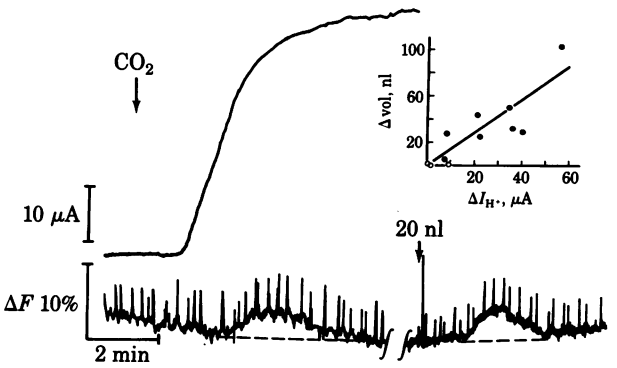

FIG. 5. Simultaneous measurement of H^+ current and exocytosis of fluorescent dextran in a turtle bladder. Upper trace is the short-circuit current, lower trace is the fluorescence signal in arbitrary units of % of full scale. Difference in time between arrow indicating perfusion with CO_2 and increase in H^+ current shows delay time for buffer to flow into chamber. Spikes in lower trace are artifacts due to bubbles. Exocytosis signal is the area between tracing and broken line. The second peak in the tracing is a calibration signal produced by injecting the equivalent of 20 nl of the loading solution. Difference between arrow and appearance of the signal gives the delay time for passage through the dead space of the apparatus. (*Inset*) Correlation between increase in H^+ current and volume extruded in seven control bladders (●) and three bladders pretreated with 0.1 mM colchicine (○).

unloading of acid into the luminal medium also would not increase the transepithelial H^+ current.)

By injecting known volumes of the fluorescent dextran solution used to load the bladder, we were able to estimate the volume of vesicles fusing with the luminal membrane after CO_2 stimulation. Fig. 5 *Inset* shows the results of seven experiments, demonstrating a reasonable correlation between the increase in H^+ transport and the apparent vesicle volume released in response to CO_2. (The variable response of the H^+ current in these experiments arose from spontaneous differences among individual bladders.) In three additional experiments the bladders were treated with 0.1 mM colchicine after loading with fluorescent dextran. Addition of CO_2 did not result in an increase in the H^+ current [as shown previously (18)], nor was there any detectable release of fluorescent dextran.

DISCUSSION

The results show that the mitochondria-rich cells of the turtle bladder contain vesicles whose internal pH is acidic due to metabolism-dependent H^+ pumps. On stimulation by CO_2, these vesicles fuse with the luminal membrane, thereby inserting the pumps and increasing transepithelial H^+ transport. In a preliminary report Stetson and Steinmetz used ultrastructure morphometry to show that CO_2 reduced the volume fraction of vesicles in the mitochondria-rich cells (19). Recently we presented direct evidence for the presence of H^+ pumps in these vesicles when we obtained a plasma membrane fraction from turtle bladder that contained an electrogenic proton-translocating ATPase that is inhibited by dicyclohexyl carbodiimide but not oligomycin. This ATPase-containing fraction increased its density when the bladders were allowed to endocytose luminal buffer made dense with metrizamide before cell fractionation (20). Endocytosis probably removes H^+ pumps from the luminal membrane and may also participate in the regulation of H^+ transport. The demonstration that fluorescent dextran is endocytosed into vesicles that later fuse with the luminal membrane suggests that membrane recycling may occur at the luminal membrane similarly to the process that occurs in macrophages and other cells. Whether the endocytic vesicles and vesicles fusing with the luminal membrane represent lysosomes or other specialized membranes containing H^+ pumps needs to be determined.

The initial cellular signal by which CO_2 induces exocytosis is unknown at present. An initial lowering of cell pH or a rise in cytosolic calcium (21) is possible, but no direct measurement of either is available in these cells. Both a reduced pH (22) and increased calcium (23) have demonstrable effects on microtubule assembly *in vitro*.

Rapid insertion of transport proteins into cell membranes by fusion of vesicles is an attractive mechanism for regulation of transport across membranes. The recent demonstration that insulin stimulates fusion of vesicles containing glucose carriers in fat cells (24) suggests that this mechanism is more widespread than initially suspected. While insertion of H^+ pumps into urinary membranes has obvious implications for urinary acidification, the insertion of the H^+-ATPase may also play a role in the fusion of neurosecretory vacuoles of the synapse. One possible function would be to alkalinize the synaptic cytosol, which in turn might reduce the extent of exocytosis by acting as a negative feedback signal.

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