

# Carbon Dioxide Causes Exocytosis of Vesicles Containing H<sup>+</sup> Pumps in Isolated Perfused Proximal and Collecting Tubules

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## Abstract

In the turtle bladder it has recently been shown that CO<sub>2</sub> stimulates H<sup>+</sup> secretion, at least in part, by causing fusion of vesicles enriched in H<sup>+</sup> pumps with the luminal plasma membrane. To test for the presence of this mechanism in the kidney we perfused collecting ducts and proximal straight tubules on the stage of an inverted epifluorescence microscope with fluorescein isothiocyanate dextran (70,000 mol wt) in CO<sub>2</sub>-free medium. After washout we noted punctate fluorescence in endocytic vesicles in some collecting ducts and in all proximal straight tubule cells. More cells took up fluorescent dextran in outer medullary than in cortical collecting ducts. Using the pH dependence of the excitation spectrum of fluorescein we found the pH of the vesicles to be acid (~pH 6). Addition of proton ionophores increased vesicular pH by 0.6±0.1 U, suggesting that the acidity of the vesicles was caused by H<sup>+</sup> pumps. CO<sub>2</sub> added to the medium (25 mmHg, pH 7.6 at 37°C) reduced fluorescence intensity by 24±5% in cortical collecting ducts, 27±5% in medullary collecting ducts, and 25±5% in proximal straight tubules. Since this effect was prevented by the prior addition of colchicine to the bath, we believe that CO<sub>2</sub> caused a decrease in cytoplasmic fluorescence by stimulating exocytotic fusion of the vesicles and thereby secretion of fluorescent dextran. This exocytotic fusion also occurred when tubules that were loaded with fluorescent dextran at a pCO<sub>2</sub> of 37 mmHg were exposed isohydrally to a pCO<sub>2</sub> of 114 mmHg; the mean decrease was 53±4%.

We conclude that some cells in the collecting ducts and all cells in the proximal straight tubule incorporate fluorescent dextran into the apical cytoplasmic vesicles and acidify them with H<sup>+</sup> pumps. CO<sub>2</sub> causes fusion of these vesicles with the luminal membrane, but whether CO<sub>2</sub> stimulates H<sup>+</sup> secretion by increasing the number of functioning H<sup>+</sup> pumps remains to be determined.

## Introduction

Many cells secrete and ingest macromolecules by the process of exocytosis and endocytosis (1, 2). There is increasing evidence that these processes are used to recycle the plasma membrane. Further, recent studies have identified rapid fusion

Dr. Schwartz is an Established Investigator of the American Heart Association; funds were contributed in part by the New York chapter. Dr. Al-Awqati was a Career Scientist of the Irma T. Hirschl Trust.

Received for publication 30 January 1984 and in revised form 10 December 1984.

J. Clin. Invest.

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0021-9738/85/05/1638/07 \$1.00

Volume 75, May 1985, 1638-1644

as a mechanism for regulation of the number of surface membrane proteins. In epithelia, rapid changes in the number of water channels (3, 4), H<sup>+</sup> pumps (5-7), and possibly sodium channels (8), occurs in response to a wide variety of stimuli. Among these epithelia, studies in the turtle urinary bladder have shown that CO<sub>2</sub> stimulates H<sup>+</sup> secretion, in part by exocytotic insertion of H<sup>+</sup> pumps into the luminal membrane (5). These studies showed that the acid secreting cells of the turtle bladder contained an intracellular reserve of vesicles whose contents were acidified by H<sup>+</sup> pumps. Addition of CO<sub>2</sub> caused fusion of some of these vesicles with the apical plasma membrane leading to an increase in the number of these functional H<sup>+</sup> pumps exposed to the luminal fluid. This insertion of H<sup>+</sup> pumps was correlated with enhanced H<sup>+</sup> secretion across the bladder (5), and ultrastructurally, with increased luminal membrane area and decreased number of cytoplasmic vesicles in carbonic anhydrase-containing cells (6). Recently, similar ultrastructural changes were seen in the intercalated cells of the rat collecting tubule (9).

The purpose of this study was to determine whether CO<sub>2</sub> stimulates exocytotic insertion of H<sup>+</sup> pumps in the renal tubule. Since the turtle bladder is analogous to the collecting tubule, it was also important to study a different H<sup>+</sup> transporting segment, the proximal straight tubule, to test the generality of this process. Our results show that CO<sub>2</sub> causes fusion of vesicles enriched in H<sup>+</sup> pumps in three known acidifying segments of the nephron, raising the possibility that CO<sub>2</sub> is a general stimulus for exocytosis.

## Methods

The left kidney was removed from New Zealand white rabbits weighing 1.8-2.5 kg and sliced sagittally. From the medullary rays in the cortex we obtained cortical collecting ducts and proximal straight tubules. Medullary collecting ducts were isolated from rays in the outer stripe of the outer medulla. Each tubule was perfused and held with a concentric glass pipette (10) on the stage of an inverted epifluorescence microscope (Carl Zeiss, Inc., Thornwood, NY). Transepithelial voltage was measured between calomel cells using the perfusion pipette as the luminal electrode and the bath as the ground (10, 11). Each tubule was perfused and bathed initially with a solution that was free of exogenous CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> and contained NaCl, 145 mM; K<sub>2</sub>HPO<sub>4</sub>, 2.5 mM; CaCl<sub>2</sub>, 2.0 mM; MgSO<sub>4</sub>, 1.2 mM; Na lactate, 4.0 mM; Na<sub>3</sub> citrate, 1.0 mM; L-alanine, 6.0 mM; glucose, 5.5 mM; pH 7.4. Collecting ducts were perfused by gravity feed at 5 cm of water, compared with 10-15 cm for the proximal tubules.

Fluorescent excitation was accomplished using a 50-W high pressure mercury arc lamp and a 450-490 nm interference filter. The emitted fluorescent light of >510 nm was reflected from a dichroic mirror through a 520-nm long-pass filter and into the eyepieces or into the camera port. When photometric analysis was performed, we attached a microscope spectrum analyzer assembly (Farrand Optical Co., Valhalla, NY) to the camera port. Emitted light passed through a 0.55-mm target hole and into the monochromator; maximum emission was

obtained at 520 nm. The monochromatic light passed through a 20- $\mu$ m exit slit and into a photomultiplier tube, connected in series to a photometer (Farrand Optical Co.). We alternately placed 460 nm and 490 nm bandpass interference filters (Ditric Optics, Inc., Hudson, MA) in front of the fluorescent beam and recorded the emission on the photometer at 520 nm in microamperes. The cells were not exposed to fluorescent light except during the few seconds required for the measurements, and then only with the additional bandpass filters placed in the light path. These latter filters reduced the transmitted light by  $\sim$ 55%. By adhering to this technique, we were able to minimize or prevent bleaching of the fluorophore.

To test for endocytosis we added fluorescein isothiocyanate coupled to a 70,000 mol wt dextran (Sigma Chemical Co., St. Louis, MO) to the perfusate in a concentration of 10 mg/ml. Uptake of this fluorescent dextran, like the uptake of other macromolecules, occurs by endocytosis (1, 2, 12, 13). After 20–60 min, the fluorescent dextran was removed and the tubule was perfused with an acidic (pH 5) fluorescent dextran-free solution for 10 min. This maneuver served to wash away fluorescent material that was bound to the luminal surface. Then the segment was perfused with the initial solution (pH 7.4 and free of fluorophore) and examined under light and fluorescence microscopy ( $\times$  400).

To demonstrate that  $H^+$  pumps were present in the endocytic vesicles containing fluorescent dextran, we made use of the pH sensitivity of fluorescein, which shows a pH dependence of the excitation spectrum (Fig. 1 A). We calibrated aliquots of the perfusing solution in the specimen chamber over the pH range 4.5 to 7.5 by measuring the ratios of fluorescent light emitted at 520 nm after excitation at 490 and 460 nm (Fig. 1 B). The concentration of fluorescent dextran used in the calibration solutions was chosen to produce the same fluorescence intensity as observed in the perfused tubules. We estimated the apparent intravesicular pH in the same way by alternately exciting a patch of cytoplasm containing endocytosed fluorescent dextran at 490 and 460 nm and measuring photometrically the light emitted at 520 nm (5). In general, we used the 0.03  $\mu$ amp range to analyze the proximal tubular cells and the 0.01  $\mu$ amp range for the collecting tubular cells. We studied multiple areas (usually 3–9 cells) and obtained a mean value for each tubular segment.

To show that  $CO_2$  causes fusion of the vesicles with the luminal

membrane (exocytosis), we then exposed both luminal and basolateral surfaces of the tubule segment to 5%  $CO_2$  using a solution that contained NaCl, 120 mM;  $NaHCO_3$ , 25 mM;  $K_2HPO_4$ , 2.5 mM;  $MgSO_4$ , 1.2 mM;  $CaCl_2$ , 2.0 mM; Na lactate, 4.0 mM;  $Na_3$  citrate, 1.0 mM; L-alanine, 6.0 mM; and glucose, 5.5 mM; pH 7.4 after gassing with 95%  $O_2/5\%$   $CO_2$  at room temperature. At 37°C, the pH of this solution was 7.62, the total  $CO_2$  was 26 mM, and the calculated  $pCO_2$  was 25 mmHg. We performed additional experiments where the cortical collecting tubules were loaded with fluorescent dextran at an ambient  $pCO_2$  of 37 mmHg at pH 7.1. Then we increased the  $pCO_2$  of perfusate and bath isohydrically to 114 mmHg. We tested for fusion in these two types of experiments by measuring the fluorescent intensity at 520 nm while exciting at 460 nm those cells that internalized the dextran. A reduction in fluorescence would imply loss of fluorescent dextran, presumably by exocytosis.

We made an effort to examine the same cells photometrically during sequential 10–15-min periods, first in 100%  $O_2$  and then in 95%  $O_2/5\%$   $CO_2$ , and expressed the mean values after  $CO_2$  per tubule as a percentage of the control in the absence of  $CO_2$ . To examine more carefully the time course of change in fluorescence intensity in some experiments, we focused the photometer on a single cell and took readings at 460 nm excitation before and during exposure to  $CO_2$ . Although this wavelength is not the isosbestic point of fluorescein, it shows a smaller change in intensity with a change of pH making it useful for the measurement of total dye content (Fig. 1 A). To test for spontaneous declines in the fluorescence signal we performed time control experiments in which the fluorescence intensity was measured at the same ambient  $pCO_2$  for periods of time comparable in length to those of the  $CO_2$ -induced exocytosis experiments.

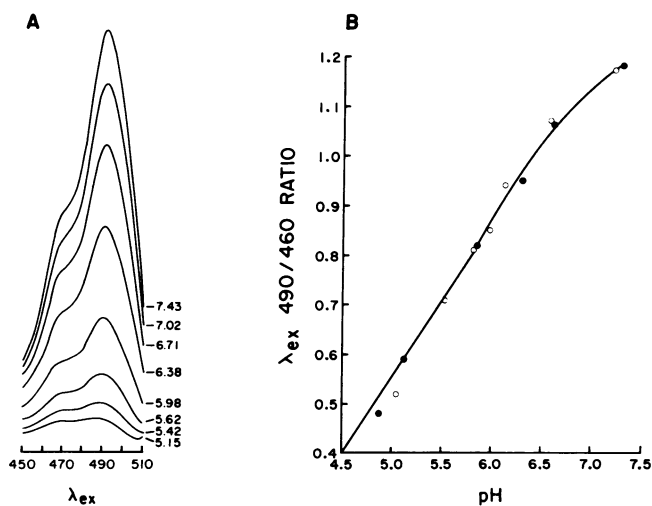
Proximal tubular uptake of fluorescent dextran was also studied in the absence of  $Na^+$  in the medium. Tetramethyl ammonium chloride replaced NaCl; Na lactate and  $Na_3$  citrate were replaced by Ca lactate, and  $Mg_3$  citrate. The chemical concentration of  $Na^+$  measured by flame photometry (using a 5 mM standard) was zero. When  $CO_2$  was added to stimulate fusion, 25 mM  $NaHCO_3$  was added concomitantly to maintain the pH. Thus, 25 mM  $Na^+$  was present in the  $CO_2$ -containing solution. Amiloride,  $5 \times 10^{-5}$  M, was added to both solutions to minimize any luminal  $Na^+-H^+$  exchange resulting from traces of  $Na^+$  in the  $CO_2$ -free solution, and to reduce  $Na^+-H^+$  exchange in the  $CO_2$ -containing solutions.

Data are expressed as mean  $\pm$  SE. Statistical significance was asserted if  $P < 0.05$ , with the paired or unpaired  $t$  tests, as appropriate.

## Results

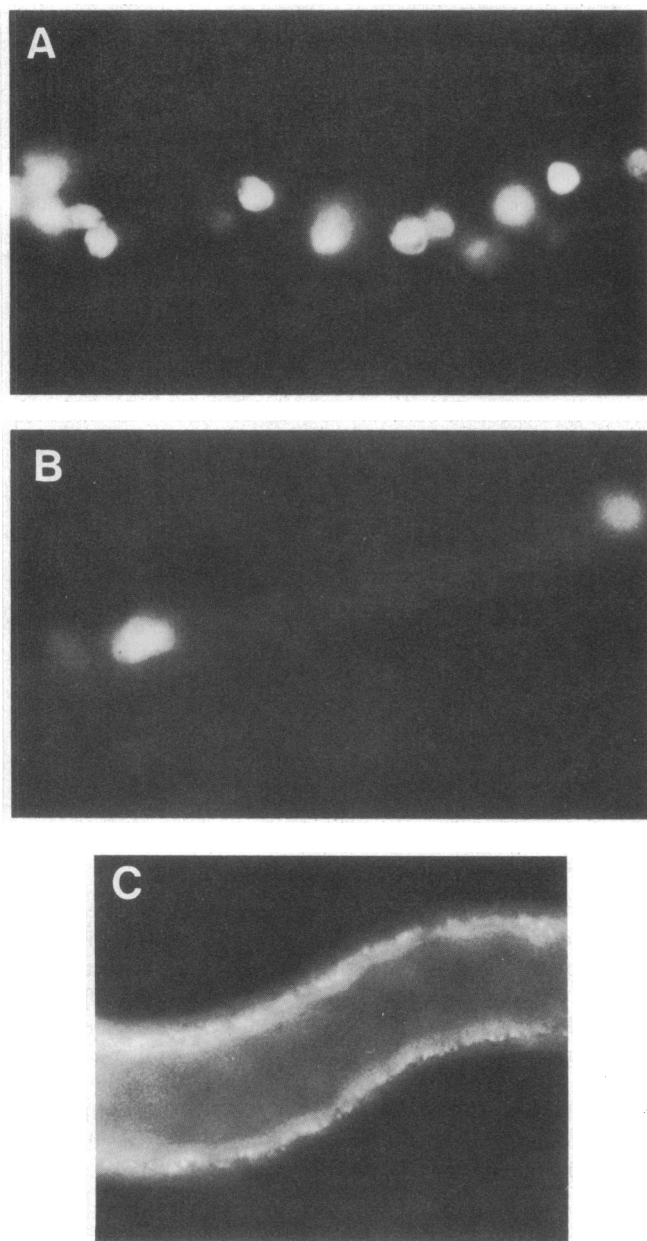
Collecting ducts were isolated from the cortex and outer stripe of the outer medulla; the location of origin was identified by inspection and confirmed by the transepithelial potential difference. In ducts from the cortex the potential difference averaged  $-26 \pm 5$  mV, while those from the outer stripe had a mean value of  $-5 \pm 3$  mV. These values are similar to those reported as a function of position within the kidney by Stokes (14) and for the cortical segment by one of us (10)<sup>1</sup>. The transepithelial voltage of the proximal tubule averaged  $-2 \pm 1$  mV in the absence of  $CO_2$  and  $HCO_3^-$ .

1. Lombard et al. found that cortical collecting tubules taken from normal rabbits did not significantly absorb bicarbonate (15). On the other hand, medullary collecting tubules taken from the inner stripe absorbed bicarbonate at a rate of  $11.3 \pm 1.4$  pmol/min  $\cdot$  mm and had a transepithelial voltage of  $+9.4 \pm 1.1$  mV (15). In five preliminary studies on collecting tubules taken from the outer stripe of the outer medulla, we found the mean bicarbonate absorption rate to be  $4.2 \pm 1.1$  pmol/min  $\cdot$  mm, with an average voltage of  $+1.4 \pm 0.7$  mV. The values for bicarbonate absorption ( $H^+$  secretion) and for transepithelial voltage are intermediate between those for cortical and those for outer medullary (from inner stripe) collecting ducts.



**Figure 1.** Calibration curve for fluorescein isothiocyanate (FITC) dextran. A, excitation spectrum of FITC-dextran as a function of pH as analysed in a spectrofluorometer at an emission wavelength of 520 nm. B, the ratio of fluorescent light emitted at 520 nm after excitation at 490 and 460 nm is plotted as a function of medium pH in the microscope chamber using the photometer (Farrand). The concentration of FITC-dextran was chosen to approximate the average fluorescent intensity of stained tubules.

Fluorescent dextran was internalized into some, but not all, collecting duct cells. The outer medullary collecting ducts showed from 10 to more than 100 fluorescent cells per millimeter (Fig. 2 *A*), while the cortical collecting ducts showed ~0–5 fluorescent cells per millimeter (Fig. 2 *B*). The fluorescent pattern was punctate, suggesting uptake into endocytic vesicles (Fig. 3 *A*). In contrast, all of the cells of the proximal straight tubule endocytosed fluorescent dextran (Fig. 2 *C*). The punctate appearance of the fluorescence was evident underneath the



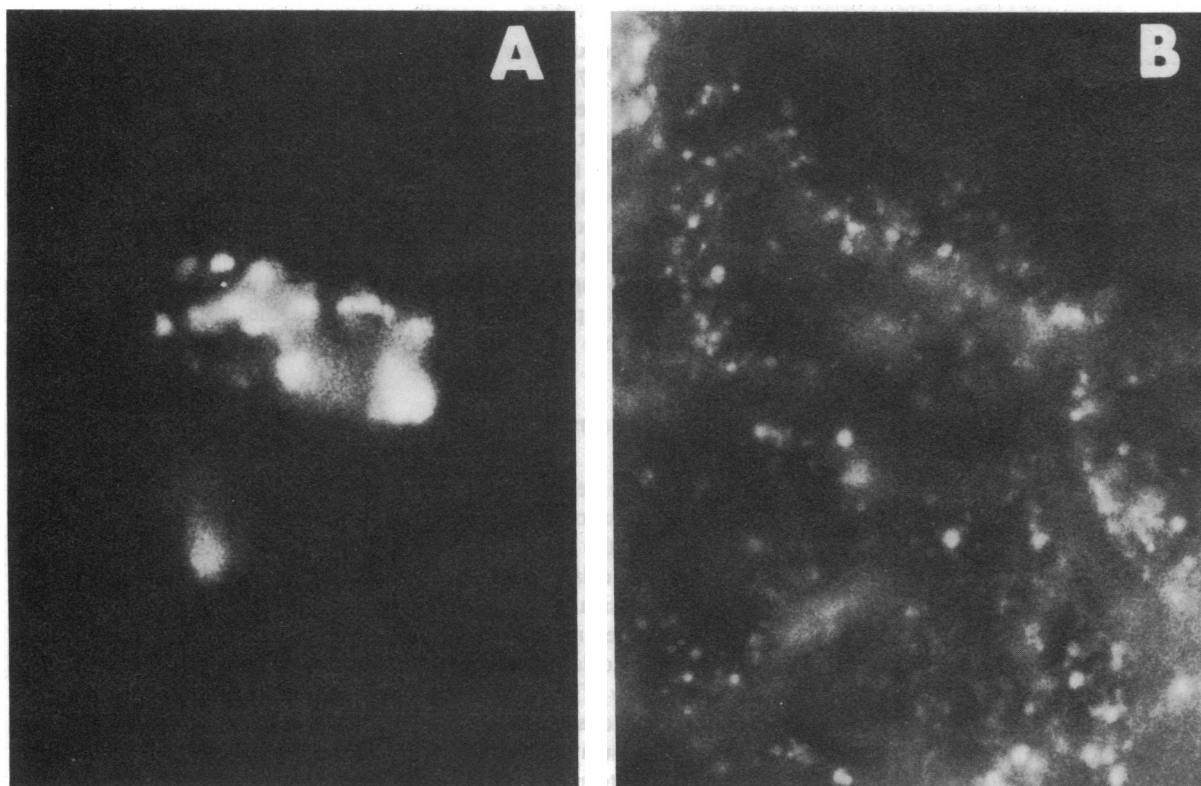
**Figure 2.** Fluorescent micrograph of isolated nephron segments after 20 min of perfusion with 10 mg/ml of fluorescent dextran. The fluorescent material was removed from the perfusate 10 min before photography at  $\times 200$ . The fluorescent pattern shows punctate and perinuclear distribution. *A*, medullary collecting duct that shows uptake in many cells. *B*, cortical collecting duct that shows uptake in few cells. *C*, proximal straight tubule that shows a punctate distribution beneath the brush border of all cells; no fluorescence is evident in the basolateral area.

brush border (Fig. 3 *B*). The basolateral cytoplasm did not show fluorescence even after prolonged loading with the fluorescent dextran. Although the punctate pattern of fluorescent staining of the perfused tubules was visible, the photographs shown in Fig. 2 do not convey the presence of discrete vesicles. This was probably caused by artifacts resulting from movement, difficult positioning, and light scattering. To obtain optimal photographs of endocytic vesicles, we had to remove the loaded tubule from the perfusion apparatus and place it between two coverslips (Fig. 3).

To test whether the dextran was internalized into vesicles that were acid, we placed a small patch of cytoplasm in the aperture of the photometer and measured light intensity emitted at 520 nm while alternately exciting at 460 and 490 nm. The ratio of the light intensity is a reasonable function of pH as shown in Fig. 1 *B*. The apparent pH of the vesicles was  $5.74 \pm 0.07$  in the cortical collecting ducts,  $6.00 \pm 0.15$  in the medullary collecting ducts, and  $5.95 \pm 0.14$  in the proximal straight tubules (Fig. 4). Addition of 10 mM  $\text{NH}_4\text{Cl}$ , which provided a sufficient amount of the permeant weak base  $\text{NH}_3$ , caused an alkalization of the intravesicular pH (Fig. 4, *left*). Similar results were obtained using the neutral  $\text{K}^+\text{-H}^+$  ionophore, nigericin (Fig. 4, *middle*). These results suggest that an increase in pH can be measured using this method. The absolute value of the acid pH must await corroboration by an independent method but is in reasonable agreement with that recently measured in endosomes of mouse 3T3 fibroblasts (16).

To distinguish whether this low pH results from acidification by  $\text{H}^+$  pumps or from a Donnan potential caused by fixed negative charges, we added the electrogenic protonophore, carbonylcyanide *m*-chlorophenylhydrazone in concentrations of 50  $\mu\text{M}$  to the bathing solution. The pH increased rapidly (Fig. 5), indicating that the endocytic vesicles in these segments were acidified by  $\text{H}^+$  pumps rather than by a Donnan potential. The mean increase in the intravesicular pH of proximal straight tubules was  $0.75 \pm 0.09$  pH units,  $n = 7$ ,  $P < 0.001$ , and that for the cortical collecting tubules was  $0.74 \pm 0.03$  pH units,  $n = 6$ ,  $P < 0.005$ . On the other hand, no significant change was noted in proximal tubules exposed only to the diluent, the difference being  $+0.05 \pm 0.06$  pH units,  $n = 4$ . Note that increasing the  $\text{H}^+$  conductance would be expected, if anything, to accelerate the development of a low pH due to a Donnan potential.

The addition of  $\text{CO}_2$  (25 mmHg) did not significantly change the apparent intravesicular pH (Fig. 4, *right*). Thus, any decrease in fluorescence intensity caused by  $\text{CO}_2$  would imply loss of fluorescent dextran, presumably by exocytosis, rather than by a quenching of the fluorescent signal by  $\text{CO}_2$ -induced acidification of the vesicular contents. A decrease in fluorescent signal was observed in all segments (Fig. 6). The mean decrease in fluorescence during the 10–15-min exposure to  $\text{CO}_2$  was  $24 \pm 6\%$  in the cortical collecting ducts,  $27 \pm 5\%$  in the medullary segments, and  $25 \pm 5\%$  in the proximal straight tubules (Fig. 6). A similar decrease of  $33 \pm 6\%$  was seen in 12 cells in which the fluorescence intensity over each cell was measured for 5 min after exposure to  $\text{CO}_2$ . The effect was rapid, the major part of the decline occurring within 1 min, and it was associated with the appearance of fluorescent material in the lumen. The decrease in fluorescence was also associated with a decrease in the electronegativity of the transepithelial voltage in the cortical collecting ducts from  $-26$  to  $-17$  mV (mean difference,  $+8 \pm 2$ ,  $n = 6$ ,  $P < 0.01$ ).



**Figure 3.** Fluorescent micrographs of cortical collecting tubule loaded with rhodamine albumin (*A*) and proximal straight tubule (*B*) loaded with FITC-dextran. To obtain these photographs we perfused the tubule with the fluorophore for 10 min followed by perfusion with nonfluorescent medium for 3 min. The segment was then removed

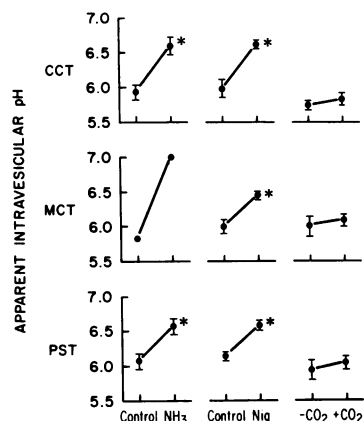
from the perfusion pipette, placed on a coverslide, and photographed under oil,  $\times 500$ . Note in *B* the punctate appearance of fluorescence beneath the brush border and absence of fluorescence at the basolateral border.

and in the medullary collecting ducts from  $-5$  to  $-3$  mV (mean difference,  $+2 \pm 0.5$ ,  $n = 8$ ,  $P < 0.01$ ). The change of voltage is consistent with an increase in electrogenic  $H^+$  secretion by these segments, although this flux was not measured. The loss of fluorescence in response to  $CO_2$  was most likely due to the secretion of fluorescent dextran by a process of exocytosis. Since the contents of these vesicles were acidified by  $H^+$  pumps, it follows that  $CO_2$  caused an exocytotic insertion of  $H^+$  pumps into the luminal membrane.

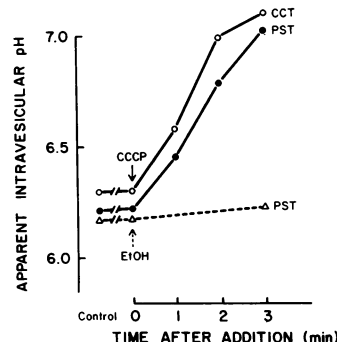
To rule out spontaneous declines in the fluorescence intensity we focused the photometer on six cells; each was observed for  $7 \pm 1$  min (range, 4–11) in the absence of  $CO_2$ . There was no reduction in fluorescence intensity, as the final

experimental readings averaged  $99.5 \pm 5.6\%$  of the initial values; nor was there any significant change in transepithelial voltage (mean difference,  $-0.9 \pm 0.6$  mV). These experiments provide an appropriate time control for the  $CO_2$  studies and also rule out bleaching of the fluorophore as an explanation for the observed decrease in the fluorescence intensity. Since we did not study single cellular areas for more than 11 min, we cannot make any conclusions regarding the base-line recycling of endocytic vesicles from the cytoplasm to the luminal membrane over longer periods of time; however, this method would be useful in the study of this important process.

To provide further evidence for the effect of  $CO_2$  on fusion, we pretreated the tubules with colchicine. We added 0.5 mM

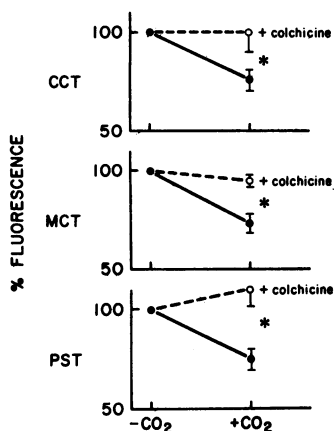


**Figure 4.** Apparent intravesicular pH. Effect of  $NH_3$ , nigericin, and  $CO_2$  on the pH of vesicles in cells of the cortical collecting duct (CCT), medullary collecting duct (MCT), and proximal straight tubule (PST). Significant paired changes ( $P < 0.05$ ) are indicated by \*. The number of tubules studied varied between six and nine except for MCT,  $NH_3$  where it was two.



**Figure 5.** Effect of carbonyl cyanide m-chloromethylhydrazone (CCCP) on the apparent intravesicular pH of isolated perfused cortical collecting tubules (CCT) and proximal straight tubules (PST) loaded with FITC-dextran. CCCP, dissolved in ethanol, was added to the bath to a final concentration of  $50 \mu M$ . Ethanol was added to the control tubules to reach a similar concentration of 0.5% (vol/vol). The displayed results are the averages obtained from four tubules. The standard error of these measurements is  $\sim 0.1$  pH units.

from the perfusion pipette, placed on a coverslide, and photographed under oil,  $\times 500$ . Note in *B* the punctate appearance of fluorescence beneath the brush border and absence of fluorescence at the basolateral border.



**Figure 6.** Effect of CO<sub>2</sub> on the fluorescent signal in cells of the cortical collecting duct (CCT), medullary collecting duct (MCT), and proximal straight tubule (PST). The number of tubules studied ranged from eight to ten in the controls (solid lines) and four to five in the colchicine experiments (dashed lines). Significant unpaired changes are indicated by \*. The photometer outputs that represent 100% of the scale were for CCT, 4.4±1 namp; MCT, 12.2±3 namp; and PST, 16.3±5 namp. In

colchicine-treated tubules the intensity of fluorescence was generally lower than in control tubules, but it was not statistically different; for CCT, 3.4±1 namp, MCT, 7.0±1.7 namp, and PST, 6.4±1.4 namp.

colchicine to the bathing solution 10 min after the fluorescent dextran loading procedure was begun. This agent is known to inhibit exocytosis in response to CO<sub>2</sub> in the turtle urinary bladder (5, 6). Colchicine is also known to inhibit other exocytotic events but not endocytosis (17). We found that the initial fluorescent signal was of similar magnitude but that the decrease in fluorescence in response to CO<sub>2</sub> was prevented by preincubation of the segment in colchicine (dashed lines in Fig. 6). CO<sub>2</sub> also failed to change the transepithelial potential difference in colchicine-treated tubules (two cortical and four medullary collecting ducts: control, -7 mV vs. colchicine -5; mean difference, +3±2, *P* = 0.2). The lack of effect of CO<sub>2</sub> in colchicine-treated tubules indicates that the decrease in fluorescence in response to CO<sub>2</sub> is not a CO<sub>2</sub>-induced artifact; rather, it is due to exocytotic fusion of vesicles with consequent discharge of their contents.

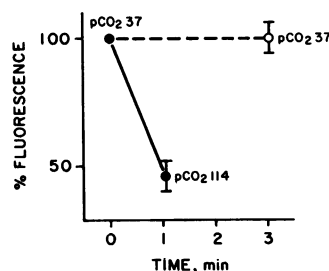
We also studied tubules at higher pCO<sub>2</sub>s than in the above experiments. We loaded cortical collecting tubules with fluorescent dextran at a pCO<sub>2</sub> of 37 mmHg and increased the pCO<sub>2</sub> isohydrically to 114 mmHg (Fig. 7). Within 1 min of the elevation of the pCO<sub>2</sub>, the fluorescence intensity decreased to an average of 47±4% of the initial level. Parallel studies in which the fluorescent cells were observed for as long as 5 min (average, 3±1) did not show any decrease in the signal; the average was 100±4% of the initial value. Recent ultrastructural studies by Madsen and Tisher (9) showed that acute respiratory acidosis using similar changes in pCO<sub>2</sub> caused fusion of vesicles with the apical plasma membrane of the intercalated cell of the rat renal medullary collecting tubule.

Finally, since the proximal tubule is believed to acidify the luminal fluid primarily by Na<sup>+</sup>-H<sup>+</sup> exchange (11, 18, 19), we tested whether the Na<sup>+</sup>-H<sup>+</sup> exchangers were responsible for acidifying the endocytic vesicles. To ensure that the endocytic vesicles did not contain Na<sup>+</sup>, we perfused and bathed four proximal straight tubules with Na<sup>+</sup> free solutions and added 50 μM amiloride to the perfusate to inhibit any residual Na<sup>+</sup>-H<sup>+</sup> exchange. The apparent intravesicular pH was 5.86±0.11, not different from that observed in the presence of Na<sup>+</sup>, and the fluorescent pattern was similar, albeit somewhat less intense in the absence of Na<sup>+</sup>. In response to CO<sub>2</sub>, the fluorescent signal decreased to 81.3±1.4% (*P* < 0.001) of the initial value,

approximately the same value as observed in the presence of Na<sup>+</sup> (74.7±5.2%) and significantly less than that observed in the presence of Na<sup>+</sup> and colchicine (*P* < 0.02). These results suggest that the endocytic vesicles budding off the brush border of the proximal tubule are acidified at least in part by proton pumps, rather than exclusively by Na<sup>+</sup>-H<sup>+</sup> exchangers, and respond to CO<sub>2</sub> by fusing with the luminal membrane. Previous studies by Kinne-Saffran et al. (20) and one of us (11) have also suggested that the proximal tubular brush border contains a H<sup>+</sup> pump. What is not clear is the quantitative role that such a pump might play in net H<sup>+</sup> secretion in this segment.

## Discussion

The results presented above demonstrate that CO<sub>2</sub> causes exocytotic fusion of vesicles with the luminal plasma membrane of the renal tubule. Since the proton pumps acidified the vesicular contents, they must have been inserted into the plasma membrane in response to CO<sub>2</sub>. These pumps, like other membrane proteins, are probably synthesized in the endoplasmic reticulum before their transport to the surface by way of the golgi apparatus. Such proteins are carried there by vesicles that move on cytoskeletal tracks and fuse with the plasma membrane (1, 2, 17). Retrieval of the membrane proteins occurs by endocytosis; some are then taken to the lysosomes to be degraded, whereas others are recycled back to the surface (1, 2). Our results show that the movement of proton pumps to the plasma membrane is regulated by CO<sub>2</sub>. Recent studies have also identified regulated pathways for the movement of other proteins. These proteins are stored in an intracellular reserve of vesicles and rapidly fuse with the plasma membrane in response to the stimulus. Vasopressin, for instance, causes the appearance of intramembrane particles (widely suspected to be markers of the water channels if not actually the aqueous pores themselves) in the apical membrane of toad bladder (4). Insulin stimulates the fusion of light vesicles enriched in glucose carriers with the plasma membrane of fat cells (21, 22). Similarly, it is likely that histamine stimulates exocytotic insertion of the H<sup>+</sup>-K<sup>+</sup>-ATPase into the luminal membrane of the oxyntic cell of the stomach (7). Recent studies in the turtle urinary bladder have shown that CO<sub>2</sub> causes the fusion of vesicles enriched in the H<sup>+</sup>-ATPase with the luminal membrane of the mitochondria-rich cell (5). Further, removal of CO<sub>2</sub> stimulates endocytic removal of these



**Figure 7.** Effect of increasing the ambient pCO<sub>2</sub> from 37 mmHg to 114 mmHg on the fluorescence intensity of endocytosed fluorescent dextran in cortical collecting tubular cells. The pCO<sub>2</sub> was increased isohydrically (pH 7.0-7.1) in five isolated perfused tubules. The fluorescence intensity was measured after 1.2±0.1 min of exposure to the high pCO<sub>2</sub>. Time control experiments are also shown, but here the fluorescence intensity was measured for an average of 3.1±0.3 min to maximize the potential for spontaneous declines in the signal. The initial photometric readings of the cells were 6.4±1.1 namp.

Time control experiments are also shown, but here the fluorescence intensity was measured for an average of 3.1±0.3 min to maximize the potential for spontaneous declines in the signal. The initial photometric readings of the cells were 6.4±1.1 namp.

pumps from the apical membrane (23). This parallel regulation of endocytosis and exocytosis by a single agent makes CO<sub>2</sub> a particularly attractive tool to study the process of regulated membrane recycling. The present demonstration that CO<sub>2</sub> causes fusion of vesicles with the brush border of the proximal tubule is surprising and suggests that the effect of CO<sub>2</sub> is perhaps a general stimulus for the fusion of vesicles with the plasma membrane. Recent studies on the mechanism of action of antidiuretic hormone on water permeability (another fusion mediated event [3, 4]) indeed showed that CO<sub>2</sub> increased the water permeability response to vasopressin in the collecting tubule (24). It would be important to verify this notion in nonpolar cells as well as in other epithelia.

One surprising finding was the paucity of endocytosing cells in the cortical collecting tubule. It is well known that as many as 30% of cells in this segment are intercalated (i.e., carbonic anhydrase-rich or mitochondria-rich cells) (25). We have recently studied this issue and have preliminary evidence to suggest that the endocytosing intercalated cell is an acid secreting cell while the nonendocytosing intercalated cell secretes HCO<sub>3</sub><sup>-</sup> (26).

The signal by which CO<sub>2</sub> causes exocytosis is unknown at present. Preliminary evidence in the turtle bladder suggests that it acts as a trigger for a cascade of events; initially the cell is acidified, which in turn causes an increase in cell calcium, which is the proximate cause of fusion. It is well known that changes in cytosolic calcium can cause fusion. Further, recent studies in nerve, muscle, and Necturus tubule show that addition of CO<sub>2</sub> changes cytosolic calcium activity (27–29). However, the direction of change has been variable. Hence, one will have to measure cytosolic calcium activity in the renal tubular cell simultaneously with the fusion event before any conclusions about the role of cell calcium in H<sup>+</sup> transport can be made.

The observation that the pattern of fluorescence is punctate does not imply that all of the fluorescein isothiocyanate dextran is located in endocytic vesicles. Studies in a number of different cells have now established that the contents of endocytic vesicles are transported to a variety of organelles such as golgi and lysosomes, as well as recycled back to the cell surface. Even the endosomes themselves seem to be composed of a number of subcompartments, each with characteristic time constants of equilibration with externally infused label (2). There is now very good evidence that all of these compartments are acidified by a proton-translocating ATPase (30–35). Recently, it has been shown that the endocytic vesicles budding off the apical plasma membranes of proximal tubules are coated with clathrin (36). Such vesicles in other tissues are acidified by H<sup>+</sup> translocating ATPases (32–34). Hence, it is likely that the proton pumps identified in the endocytic vesicles of the proximal tubule in the present study are also proton translocating ATPases.

The kidney responds to elevation of ambient pCO<sub>2</sub> with an increase in acid excretion (18, 37). The extent to which fusion events mediate this will have to await the development of methods that allow the quantitation of its role. Simultaneous measurements of the rate of transport and the fusion event will be needed before and after the use of agents that can completely block exocytotic insertion of H<sup>+</sup> pumps. That fusion can be demonstrated ultrastructurally in the collecting tubule during acute respiratory acidosis in whole animals suggests that this process may contribute to urinary acidification

in the collecting tubule (9), if not in the proximal tubule as well.

## Acknowledgments

We are grateful to J. Fischer, D. Spray, and R. White for their help in adapting the fluorescence microscope for use in these experiments. We thank F. Andrade and M. Coleman for excellent secretarial assistance and Beth Zvilowitz for preparing the solutions.

Dr. Schwartz was supported by U. S. Public Health Service grant HD13232. Dr. Al-Awqati was supported by U. S. Public Health Service grant AM 20999, and by a grant in aid from the New York Heart Association.

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