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Effect of cyclic AMP on hydrogen ion secretion by turtle urinary bladder

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Effect of cyclic AMP on hydrogen ion secretion by the turtle urinary bladder. Parathyroid hormone and its intracellular mediator cyclic AMP play a role in acid excretion by the mammalian kidney. To investigate this effect, we studied cyclic AMP in turtle bladder with the pH-stat method. In carbon dioxide-free and bicarbonate-free solutions, where neither hydrogen ion nor bicarbonate ion gradients existed, serosal cyclic AMP (10 mM) promptly decreased hydrogen ion secretion to 74% of the control value, from 1.22 ± 0.16 to 0.90 ± 0.14 μmoles/hr ($P < 0.01$). The combination of cyclic AMP and theophylline, an inhibitor of phosphodiesterase, decreased hydrogen ion secretion further, to 45% of the control value. The effect of cyclic AMP was additive to that of benzolamide, a known carbonic anhydrase inhibitor. Direct assay of tissue carbonic anhydrase activity demonstrated complete inhibition of the enzyme by benzolamide, but no inhibition by cyclic AMP and theophylline. The decreases in hydrogen ion secretion produced by cyclic AMP and theophylline were not linked to decreased active sodium transport. The results of the present study demonstrate a significant role of intracellular cyclic AMP in the control of hydrogen ion secretion by the turtle bladder. Cyclic AMP decreases active hydrogen ion secretion independently of passive hydrogen ion or bicarbonate ion movement and without coupled decreases in sodium transport. The data also indicate that cyclic AMP does not inhibit carbonic anhydrase in this tissue.

Effet de l'AMP cyclique sur la sécrétion d'ions hydrogène par la vessie de tortue. L'hormone parathyroïdienne et son médiateur cellulaire l'AMP cyclique jouent un rôle dans l'excrétion des acides par le rein de mammifère. Pour étudier cet effet nous avons observé l'action de l'AMP cyclique dans la vessie de tortue au moyen de la méthode du pH-stat. Dans les solutions sans carbon dioxide ni bicarbonate, où il n'existe de gradient ni d'ions hydrogène ni d'ions bicarbonate, l'AMP cyclique (10 mM) du côté séreux diminue rapidement la sécrétion d'ions hydrogène à 74% de la valeur contrôle, de 1,22 ± 0,16 à 0,90 ± 0,14 μmoles/hr ($P < 0,01$). L'association de l'AMP cyclique et de théophylline, un inhibiteur de la phosphodiestérase, diminue encore plus la sécrétion d'ions hydrogène, à 45% de la valeur contrôle. L'effet de l'AMP cyclique est additif avec celui du benzolamide, un inhibiteur de l'anhydrase carbonique. La mesure directe de l'anhydrase carbonique tissulaire montre l'inhibition complète de l'enzyme par benzolamide, mais pas d'inhibition par l'AMP cyclique et theophylline. Les diminutions de la sécrétion d'ions hydrogène produites par l'AMP cyclique et theophylline ne sont pas liées à une diminution du transport actif de sodium. Les résultats de ce travail démontrent un rôle significatif de l'AMP cyclique intracellulaire dans le contrôle de la sécrétion d'ions hydrogène par la vessie de tortue. L'AMP cyclique diminue la sécrétion active d'ions hydrogène de façon indépendante des mouvements passifs d'ions hydrogène ou d'ions bicarbonate et sans diminution couplée du transport de sodium. Enfin, ces observations indiquent que l'AMP cyclique n'inhibe pas l'anhydrase carbonique dans ce tissu.

Considerable clinical and laboratory evidence has indicated that parathyroid hormone (PTH) influences bicarbonate reabsorption by the kidney [1–10]. The hormone stimulates membrane-bound adenylate cyclase, which increases intracellular cyclic adenosine 3′,5′-monophosphate (cyclic AMP) concentrations [11, 12]. Cyclic AMP, the effector substance, mediates the physiologic action of PTH on the renal tubule, and by itself has been demonstrated to reduce bicarbonate reabsorption [13]. The precise mechanism for this is unknown, but several theoretical possibilities can be considered. First, active hydrogen ion secretion by the tubule might be decreased by cyclic AMP. This could occur through inhibition of carbonic anhydrase [14–17], reduction of available substrate (carbon dioxide) for hydrogen ion secretion [18], decreased force of the hydrogen ion pump or decreased active pathway conductance [19]. Second, passive permeability of the renal tubule might be increased by cyclic AMP [20, 21], leading to either increased movement of hydrogen ions from lumen to blood or of bicarbonate ions from blood to lumen. Third, the effects of cyclic AMP on bicarbonate reabsorption might be secondary to changes in transport of other ions; for example decreases in sodium or monovalent phosphate reabsorption [22–26].

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To gain further insight into the basic mechanisms of the action of cyclic AMP, we carried out studies in the isolated turtle urinary bladder. This organ has a well-characterized hydrogen ion transport system [27, 28] which allows direct evaluation of active secretion, passive permeability, and coupled transport. The data demonstrate that cyclic AMP reduced active hydrogen ion secretion significantly by the turtle bladder. This occurred independently of changes in transmembrane electrical, hydrogen ion, or bicarbonate ion gradients. In many instances, simultaneous active sodium transport increased; thus, reduction in hydrogen ion secretion was not linked to a change in sodium transport. The effect of cyclic AMP was additive to the inhibitory effect of benzolamide (a potent inhibitor of carbonic anhydrase) on hydrogen ion secretion, the combination producing nearly total inhibition of active hydrogen ion transport. Direct enzyme assay of bladder tissue failed to show any inhibition of carbonic anhydrase activity by cyclic AMP. The data suggest that the nucleotide inhibits active hydrogen ion secretion either by increasing intracellular pH, or by directly affecting the proton pump.

Methods

Adult freshwater turtles (*Pseudemys scripta*) were obtained from Lemburger Co. (Division of Mogul-Ed, Oshkosh, Wisconsin). On the day of the experiment, the animals were decapitated, and the urinary bladders were excised with minimal handling. Excised hemibladders were washed three times in bicarbonate-free turtle Ringer's solution, composed of (in mmoles per liter) sodium 115.0; potassium, 3.5; calcium, 0.9; chloride, 119.7; divalent phosphate, 0.3; dextrose, 2.0, (osmolality, 230 mOsm/kg H_2O), and were mounted between halves of an Ussing-type plastic (Lucite®) chamber, providing an exposed membrane area of 7.3 cm^2. Each side of the mounted bladder was bathed by 15 ml of bicarbonate-free turtle Ringer's solution (pH, 7.4). The two baths were bubbled with air passed through a series of three 3 M potassium hydroxide baths to trap and remove all carbon dioxide, according to the method described by Steinmetz [29]. Mixing was accomplished by air lifts. Transmembrane potential difference (E) was measured through 3 M potassium chloride agar bridges and calomel half-cells, and recorded on a digital multimeter recorder, (Fluke model 8000A, John Fluke Mfg. Co., Inc., Seattle, Washington). The bladders were short-circuited by the method of Ussing and Zerahn [30] with an automatic voltage clamping device; short-circuit current (SCC) was recorded continuously on a recorder (Servo-Graphic, model 2802, Laboratory Data Control, Inc., Riviera Beach, Florida), and transmembrane electrical resistance (R) was estimated intermittently, as described previously [31].

pH-stat method. The pH of the aerated bathing solutions was precisely adjusted to 7.400 ± 0.001. Combination pencil electrodes (Markson Scientific Inc., Del Mar, California) were used to monitor the pH in mucosal and serosal reservoirs; the electrodes were placed well above the plastic chamber containing the short-circuited membrane so that the current from the voltage clamp did not affect the pH reading. Continuous readings were taken with a digital pH meter, (Orion model 801, Orion Research, Cambridge, Massachusetts). Changes in mucosal pH from 7.400 were sensed by an automatic digital controller (Orion model 872). When the mucosal pH fell below 7.400, due to secretion of hydrogen ions by the membrane, the servocontroller activated a pump (Sage Infusion Pump, model 355, Orion Research, Cambridge, Massachusetts), which delivered 0.01 N sodium hydroxide NaOH into the mucosal bath until the pH returned to 7.400. From the time interval and the volume of NaOH delivered, we calculated the hydrogen ion secretion rate (expressed as micromoles of hydrogen per hour per 7.3 cm^2 membrane area).

In preliminary experiments, we determined the accuracy of the pH-stat apparatus. Chambers were assembled with an inert cellophane sheet separating the two halves, which were filled with bicarbonate-free turtle Ringer's solution, mixed and aerated with carbon dioxide-free air. A known amount of 0.01 N hydrochloric acid was added to the mucosal bathing solution, which lowered the pH and instantly activated the servocontroller and pump, leading to the immediate delivery of an equimolar amount of titrant. The response was prompt, complete, and linear over the range of hydrogen ion addition of from 0.1 to 1.0 μmoles, and there was no overshoot; the controller restored the pH exactly to 7.400 ± 0.001.

Measurement of hydrogen ion secretion rate: (A) Control experiments. Using five bladders, bathed on both sides by bicarbonate-free turtle Ringer's solution, we measured hydrogen ion secretion during consecutive periods, each of 30 min's duration. Hydrogen ion secretion and short-circuit current were recorded continuously for 210 min in all experiments, except for periodic determination of electri-

cal potential difference (PD) and tissue resistance (R).

(B) Effect of cyclic AMP on hydrogen ion secretion. After performing three control studies, we added cyclic AMP, 10 mM, to the serosal baths of six bladders. Theophylline, 10 mM, was added to the serosal baths of six additional bladders, after three initial 30-min control periods had been measured. Both cyclic AMP, 10 mM, and theophylline, 10 mM, were added to the serosal baths of another six following three control periods. After these compounds were added, serosal bath pH did not change significantly; thus, there was no change in hydrogen ion gradient across the tissue. In addition, equimolar amounts of dextrose were added to the mucosal bath to balance osmotic increments in the serosal bath and, thus, to prevent any osmotic gradient across the tissue.

(C) Effect of benzolamide. After performing three control studies, we added benzolamide, 3.75 mM, to the serosal baths of five bladders.

(D) Dose-response to cyclic AMP plus theophylline. After performing two 30-min control studies, we added cyclic AMP and theophylline in increasing amounts (0.1 mM, increasing to 10 mM) to both baths of six bladders. Hydrogen ion secretion was measured for 30 min at each dose level.

(E) Additive effects of cyclic AMP and benzolamide. After determining both control and cyclic AMP-inhibited hydrogen ion secretion (see B above), we added benzolamide, 3.75 mM, to the serosal bathing solution of six bladders, and we determined the hydrogen ion secretion an additional 30 to 90 min. Using three other bladders, we added benzolamide, 3.75 mM, to the serosal baths after 60 min of control rates of secretion. When 60 min of hydrogen ion secretion in benzolamide had been measured, cyclic AMP (10 mM) plus theophylline (10 mM) were added to the same serosal solution, and a further 60 min of hydrogen ion secretion was determined.

Measurements of tissue carbonic anhydrase activity. Turtles were perfused with bicarbonate-free turtle Ringer's solution by cardiac puncture until bladder vessels were clear of red cells (25 to 30 min). The urinary bladders were than removed, washed, and divided in pieces. The divided bladders were placed in one of the combinations of Ringer, gas, and test substance (indicated below), selected to parallel the conditions under which hydrogen ion secretion rate had been determined in intact bladders: *(1)* bicarbonate-free turtle Ringer's; *(2)* bicarbonate-free turtle Ringer's plus cyclic (10 mM) plus theophylline (10mM); *(3)* bicarbonate-free turtle Ringer's plus benzolamide (3.75 mM). After 60 min in the solutions, mucosal cells from each piece were removed by scraping with a glass slide, suspended in 1.5 ml of deionized water, and homogenized in a tissue homogenizer (model 45, The Virtis Co., Inc., Yonkers, New York). The homogenate was centrifuged (model HN-S, International Equipment Co.) at > 5000 RPM for 15 min, and the supernatant was filtered through filter paper (no. 1 Whatman) to remove cellular debris. Aliquots of each filtrate were dried and weighed on a Mettler balance (model H/10T, Fisher Scientific Co., Pittsburgh, Pennsylvania) to the nearest 0.001 mg, to give the dry weight per volume of extract.

Aliquots of each extract were assayed for carbonic anhydrase activity by the method of Maren, Ash, and Bailey [33, 34]. Five hundred microliters of indicator (12.5 mg of phenol red dissolved in 1000 ml of 2.6 mM sodium bicarbonate), 200 μl of buffer (300 ml of 1 M dibasic sodium carbonate added to 206 ml of 1 M sodium bicarbonate and made up to 1000 ml), and 100 μl of sample were placed in a reaction tube. One hundred percent carbon dioxide was bubbled through the solution at a constant rate regulated by a precise flowmeter (model no. 10A4139M, Fisher and Porter Co., Warminster, Pennsylvania). Reagents, samples, and the reaction chamber were cooled in a constant temperature water bath to 1° C. Indicator and sample were added to the reaction vessel first. The buffer was rapidly added, and timing was begun with a stopwatch. The time from addition of buffer until a change in color of indicator from red to yellow was recorded for each sample. After each run, the apparatus was rinsed six times with deionized water. With this method, the control uncatalyzed reaction time, using distilled water, was 104 ± 3 seconds, and solutions of known carbonic anhydrase content gave faster reaction times, proportional to the amount of enzyme added. The results of tissue assays are reported as arbitrary units of carbonic anhydrase activity per milligram of dry weight, using a standard purified carbonic anhydrase preparation.

Results are expressed as means ± SEM. Comparisons were made with the paired Student's *t* test. Theophylline and 3'5'-cyclic adenosine monophosphoric acid as the sodium were obtained from Sigma Chemical Co., St. Louis, Missouri. Purified carbonic anhydrase was obtained from ICN Pharmaceuticals, Cleveland, Ohio. Benzolamide was supplied by Dr. Thomas Maren.

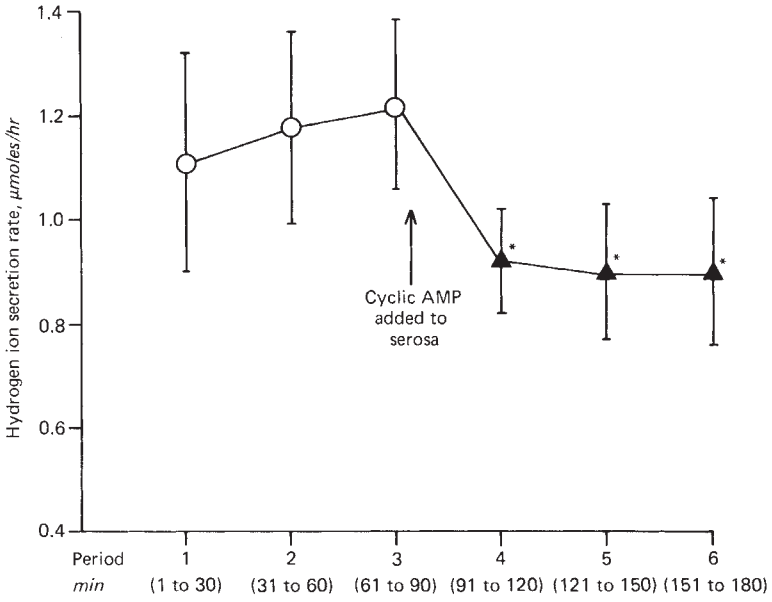

Fig. 1. *Effect of serosal cyclic AMP on active hydrogen ion secretion in six turtle urinary bladders bathed in carbon dioxide-free amphibian Ringer's solution.* Results are means ± SEM. *Asterisk* denotes $P < 0.01$.

Results

Control experiments. In five tissues, bathed with carbon dioxide-free turtle Ringer's solution, a stable rate of hydrogen ion secretion was maintained over a period of 210 min. The rates of hydrogen ion secretion measured in these control experiments (1.0 to 1.2 μM/hr) are comparable to those previously reported by Steinmetz [29]. Short-circuit current remained constant or declined slightly and transmembrane electrical resistance (R) remained stable throughout the period of observation in all control studies.

Effect of cyclic AMP on active hydrogen ion secretion. Cyclic AMP (10 mM) was added to the serosal baths of six bladders, after three initial control periods had been measured. The bathing solutions were bicarbonate-free turtle Ringer's solution on both sides of the bladder. The results are presented in Fig. 1. Control hydrogen ion secretion rate, which had been stable at 1.1 to 1.2 μmoles/hr during the 90 min of periods 1 to 3, fell promptly in all experiments to 0.92 ± 0.10 μmoles/hr after 30 min (period 4), and 0.9 ± 0.14 μmoles/hr in periods 5 and 6 ($P < 0.01$). The decrease in hydrogen ion secretion began within a few minutes of addition of cyclic AMP and persisted over the 90 min of observation. The percent change from period 3, the last control period before addition of cyclic AMP, to period 5 is shown in Fig. 2. Hydrogen ion secretion rate was reduced to 74 ± 4% of the control value. For comparison, data from the control experiments are represented as the percent change from period 3 to period 5. In the five control studies, the secretion rate in period 5 rose slightly to 109 ± 11% (NS) of the rate in period 3.

Effect of theophylline on active hydrogen ion secretion. Addition of theophylline (10 mM) to the serosal bath, without addition of exogenous cyclic AMP (bicarbonate-free turtle Ringer's solution on both sides of bladder), produced a prompt and sustained fall in hydrogen ion secretion rate. The percent change from period 3, the last control period, to period 5 is shown in Fig. 2. Theophylline, which prevents breakdown of endogenous intracellular cyclic AMP by inhibition of phosphodiesterase [32], produced an even greater fall in hydrogen ion secretion than did exogenous cyclic AMP alone. The rate of hydrogen ion secretion after addition of theophylline was 55 ± 4% in period 5, as compared with the control rate in period 3 ($P < 0.01$).

Effect of theophilline plus cyclic AMP on active hydrogen ion secretion. In six bladders, both theophylline (10 mM) and cyclic AMP (10 mM) were added to the bicarbonate-free turtle Ringer's serosal medium after three control periods. There was a slightly but not significantly additive effect of this combination on hydrogen ion secretion when compared to the studies with theophylline alone. Hydrogen ion secretion rate in period 5 (after addition)

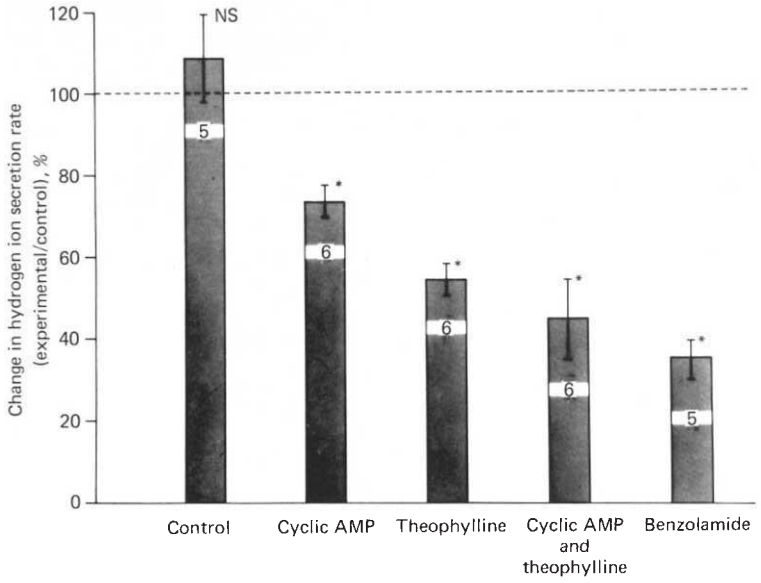

Fig. 2. *Effect of serosal cyclic* AMP, *theophylline, and benzolamide on hydrogen ion secretion in turtle urinary bladders bathed in carbon dioxide-free amphibian Ringer's solution.* Rates after treatment are expressed as a percent of control rates. Results are means ± SEM. *Numbers in bars* indicate numbers of bladders. *Asterisk* denotes $P < 0.01$.

was 45 ± 10% of the control rate in period 3 ($P < 0.01$). The results are shown in Fig. 2.

Effect of benzolamide on active hydrogen ion secretion. To compare the magnitude of the effects of cyclic AMP and theophylline to that of a known potent inhibitor of hydrogen ion secretion, we added 3.75 mM benzolamide to the serosal bath of five bladders mounted in bicarbonate-free turtle Ringer's solution. The results are shown in Fig. 2. After benzolamide was added, the hydrogen ion secretion rate in period 5 was 35 ± 5% ($P < 0.01$) of the control value in period 3, a maximal response similar to that found previously by Schwartz, Rosen, and Steinmetz for acetazolamide [36]. This degree of inhibition was comparable to that observed with cyclic AMP plus theophylline.

Dose response to cyclic AMP plus theophylline. The effect of increasing doses of cyclic AMP plus theophylline is shown in Fig. 3. Addition of small amounts (0.1 mM) of these compounds resulted in inhibition of hydrogen ion secretion, which became progressively greater at increasing doses. Maximum effect was observed at 5 mM, and further increment had no additional inhibitory effect on hydrogen ion secretion.

Additive effect of benzolamide plus cyclic AMP and theophylline on active hydrogen ion secretion. To investigate whether or not benzolamide and cyclic AMP share a similar mechanism of action, that is, inhibition of carbonic anhydrase, we added these compounds sequentially to the serosal baths of tissues bathed by bicarbonate-free turtle Ringer's solution. The results are presented in Table 1. In part A of Table 1, cyclic AMP alone inhibited hydrogen ion secretion in six bladders from 1.17 ± 0.18 to 0.91 ± 0.12 μM/hr ($P < 0.05$). The subsequent administration of serosal benzolamide (3.75 mM) reduced hydrogen ion secretion rate further from 0.91 ± 0.12 to 0.41 ± 0.14 μM/hr ($P < 0.01$). No significant effects on short-circuit currents or transmembrane electrical resistance (R) were noted. The additive effect of the two compounds was not dependent on the order in which they were introduced. In part B of Table 1 are shown three experiments in which benzolamide was added first. Hydrogen ion secretion declined to 0.26 ± 0.03 μM/hr ($P < 0.01$) from the control value. The subsequent addition of cyclic AMP (10 mM) and theophylline (10 mM) in the serosal bath completely abolished hydrogen ion secretion. There was a small but significant decline in short-circuit current after treatment of membranes with benzolamide in these studies, associated with the decrease in hydrogen ion secretion. Addition of cyclic AMP with or without theophylline, however, did not reduce short-circuit current significantly. From these ob-

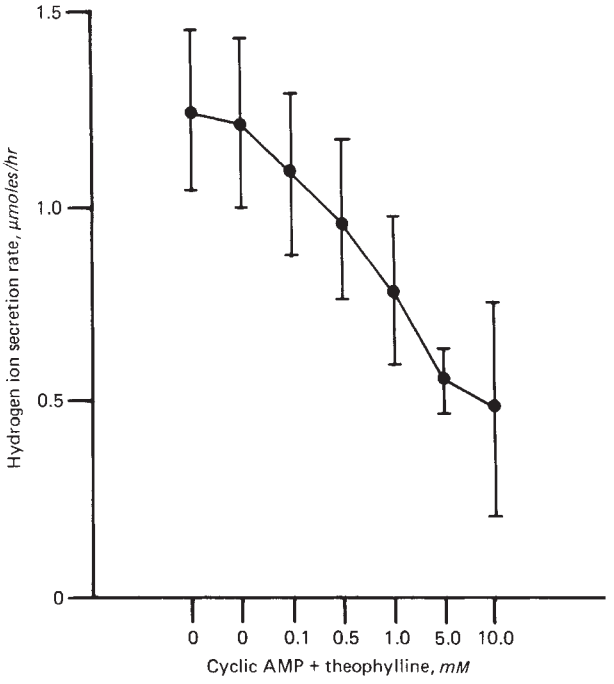

Fig. 3. *Dose response hydrogen ion secretion rate in six turtle urinary bladders, treated with increasing doses of cyclic AMP plus theophylline.* Results are means ± SEM.

Table 1. Effect of combination of cyclic AMP and benzolamide on hydrogen ion secretion by turtle bladder

	Hydrogen ion secretion *μmoles/hr*
A (N = 6)	
Control	1.17 ± 0.18
Cyclic AMP (10 mM)	0.91 ± 0.12[a]
Benzolamide (3.75 mM)	0.41 ± 0.14[b]
B (N = 3)	
Control	0.57 ± 0.05[c]
Benzolamide (3.75 mM)	0.26 ± 0.03[d]
Cyclic AMP (10 mM) plus theophylline (10 mM)	0.0

[a] $P < 0.05$, when compared with the control value.
[b] $P < 0.01$, when compared with the cyclic AMP (10 mM) value.
[c] $P < 0.02$, when compared with the control value.
[d] $P < 0.02$, when compared with the benzolamide (3.75 mM) value.

servations, the effect of cyclic AMP appeared to be additive to that of benzolamide on hydrogen ion secretion.

Effect of cyclic AMP plus theophylline on short-circuit current (SCC) and transmembrane resistance (R). As noted above, SCC and R remained quite stable throughout control experiments, with SCC declining slowly with time as is usual for untreated epithelial tissues. In those bladders treated with cyclic AMP plus theophylline, two patterns of response of SCC and R were noted. A representative example of each response is presented in Fig. 4. In three of the six bladders, SCC declined slowly during the experiment without being clearly affected by cyclic AMP plus theophylline (Fig. 4, panel A). In these same membranes, simultaneously measured hydrogen ion secretion rates invariably decreased after addition of cyclic AMP and theophylline; R rose slightly as SCC decreased. In the three other bladders treated with cyclic AMP and theophylline, SCC rose after addition of the test substances with concomitant inhibition of hydrogen ion secretion (Fig. 4, panel B). The increase in SCC was prompt and often persisted for 60 to 90 min. Associated with the rise in SCC, there was a fall in R in these tissues. The rise of SCC and the coincident fall in R is the response often described for cyclic AMP plus theophylline in other epithelia [32]. The data on SCC from these experiments suggests that active sodium transport (total SCC minus hydrogen ion secretion) was preserved well in bladders treated with cyclic AMP plus theophylline, and that inhibition of hydrogen ion secretion was not associated with decreased active sodium transport.

Effect of cyclic AMP and theophylline on tissue carbonic anhydrase activity. Tissue carbonic anhydrase activity was assayed in homogenates of bladder mucosal cells that had been treated identically to the bladders used in the titration studies described above. The results are presented in Table 2. One portion of each of seven bladders was incubated in bicarbonate-free turtle Ringer's solution, and the mucosal cells were scraped and homogenized. The supernatants contained significant carbonic anhydrase activity. Another portion from each of the seven bladders was incubated in bicarbonate-free turtle Ringer's solution containing 3.75 mM benzolamide, and then they were scraped and homogenized. The supernatants from benzolamide-treated tissues contained minimal enzyme activity in only one instance, and were otherwise indistinguishable from the distilled-water controls. In contrast, a third portion of seven bladders exposed to cyclic AMP (10 mM) plus theophylline (10 mM), then scraped and homogenized, did not show any enzyme inhibition. There was preservation of enzyme activity identical to the activity of carbonic anhydrase noted in the control homogenates. The data thus suggest that cyclic AMP and theophylline do not inhibit carbonic anhydrase activity in this tissue.

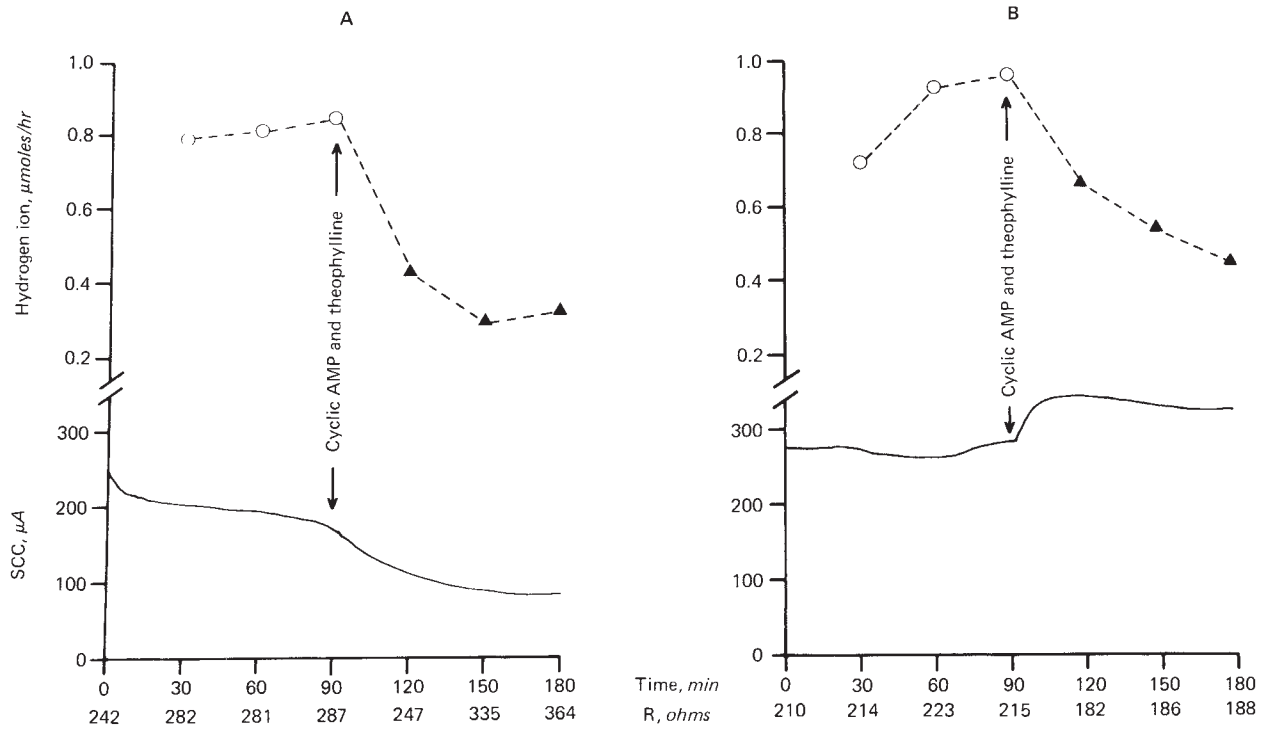

Fig. 4. *Different effects of serosal cyclic AMP and theophylline on short-circuit current (SCC) and transmembrane electrical resistance (R) in representative turtle urinary bladders.* Despite similar inhibition of hydrogen ion secretion, SCC decreased slightly in three experiments (*panel A*) and increased in three experiments (*panel B*).

Discussion

The results of the present study demonstrate that cyclic AMP, the intracellular messenger substance for many hormones, including PTH, inhibits active hydrogen ion secretion by the turtle urinary bladder. Enhancement of this effect by the concomitant administration of theophylline, an inhibitor of the intracellular enzyme which accelerates breakdown of cytosol cyclic AMP (phosphodiesterase), supports the idea that intracellular concentrations of cyclic AMP are critical in mediating the effect on hydrogen ion secretion. Although it would have been desirable to test the effect of PTH directly, preliminary studies indicated that bovine PTH is not capable of stimulating turtle bladder adenylate cyclase or influencing hydrogen ion secretion in intact turtle urinary bladders.[1] Since this species of water turtle is known to have parathyroid glands [37], the lack of response to bovine PTH probably reflects a species difference, similar to that which has been described by Dousa for amphibian kidney and urinary bladder [38]. Nonetheless, the results with cyclic AMP provide a possible explanation for the in vivo effects of PTH and cyclic AMP on renal acid excretion [1–10, 13].

Several possible mechanisms to explain the effect of cyclic AMP on hydrogen ion secretion can be considered. A decrease in hydrogen ion secretion has been observed to occur when there is a reduction in transepithelial electrical potential difference [29]. However, since the cyclic AMP-mediated re-

Table 2. Assay of carbonic anhydrase activity in scraped mucosal cells of turtle bladders[a]

	Carbonic anhydrase activity *U/mg dry wt*[b]
Control (NaR)	13.4 ± 1.8
Cyclic AMP + theophylline (NaR)	13.1 ± 1.1[c]
Benzolamide (NaR)	1.5 ± 0.0[d]

[a] Bladders were quartered, and each quarter was bathed in turtle Ringer's solution (NaR) with addition of the compounds before the mucosal cells were scraped for analysis.

[b] See text.

[c] NS, when compared to the control value.

[d] $P < 0.001$, when compared to either the cyclic AMP + theophylline value or the control value.

[1] Studies performed for us by Dr. Detleff Schlondorff, Department of Medicine, Albert Einstein College of Medicine, demonstrated that bovine PTH failed to stimulate membrane-bound adenylate cyclase in turtle urinary bladder.

duction in hydrogen ion secretion occurred in short-circuited tissues, transepithelial electrical potential changes can be excluded as a mechanism. An increase in intracellular potential, not measured in the present studies, could have altered the potential across the apical cell membrane and, thus, influenced secretion. The present data do not exclude this possibility.

An increase in passive pathway permeability leading to either an increase in mucosal to serosal hydrogen ion backleak, or an increase in serosal to mucosal bicarbonate transport might have reduced net hydrogen ion secretion into the mucosal bath. Such a mechanism has been suggested by Lorentz to explain the effect of PTH or cyclic AMP on proximal renal tubular transport [20, 21]. The present studies in which cyclic AMP inhibited hydrogen ion secretion were carried out, however, in the absence of either hydrogen ion gradients or bicarbonate gradients across the whole bladder wall. The results, thus, cannot be easily accounted for by an increase in passive hydrogen ion or bicarbonate permeability across the whole tissue. The present data, however, do not exclude alterations in either mucosal or serosal cell membranes. Thus, it is possible that there was a change in backflux of hydrogen ions from the unstirred layer adjacent to the mucosal membrane into the cells or alternatively of bicarbonate or hydroxide ions from the cell interior into the mucosal solution. Measurements of intracellular pH and estimates of gradients for hydrogen ions across the cell membrane might shed light on these possibilities.

Carbonic anhydrase inhibitors are known to inhibit hydrogen ion secretion in the turtle bladder [36], and because it has been suggested that cyclic AMP inhibits carbonic anhydrase in the rat renal cortex [17], we examined the effect of cyclic AMP on this enzyme in the turtle bladder. Two approaches were used, both comparing cyclic AMP with benzolamide, a known inhibitor of carbonic anhydrase. First, we found an additive effect of cyclic AMP and benzolamide in reducing hydrogen ion secretion in the intact bladder, regardless of the sequence of exposure of the tissues to the two substances (Table 1). Of particular interest is the *total* inhibition of hydrogen ion secretion noted when cyclic AMP plus theophylline was added to benzolamide-treated tissues (Table 1, part B), because maximal carbonic anhydrase inhibition should have reduced hydrogen ion secretion by no more than 80% [29]. Second, we demonstrated by tissue analysis that there was no inhibition of carbonic anhydrase in bladders exposed to cyclic AMP plus theophylline, in contrast to bladders exposed to benzolamide, where the enzyme was totally inhibited (Table 2). We added cyclic AMP plus theophylline to the intact tissue, in physiologic doses identical to those which had inhibited hydrogen ion secretion. Although it is not possible to determine the final intracellular concentration of cyclic AMP in these experiments, based on the known effect of theophylline on amphibian epithelial cell cyclic AMP concentrations, we estimate that cyclic AMP was present in 10 to 20 pmoles/mg dry weight (Dr. D. Schlondorff, personal communication). Our findings that cyclic AMP does not inhibit endogenous tissue carbonic anhydrase activity, although contrary to the data of Beck et al in rat kidney homogenates [17], are entirely consistent with the studies of Puschett and Goldberg [39] and Beck and Goldberg [24] on erythrocyte carbonic anhydrase, with the observations of Garg on renal cortical carbonic anhydrase of rat [40, 41], and with the studies of Schlondorff on dog renal cortical carbonic anhydrase (personal communication). On the basis of the present studies, as well as most of the published data, we suggest that the effect of cyclic AMP on hydrogen ion secretion is not mediated by inhibition of carbonic anhydrase.

In the absence of exogenous carbon dioxide, hydrogen ion secretion by the turtle bladder is limited by carbon dioxide derived from metabolism, primarily that formed as the result of energy utilized by sodium transport, but also from other metabolic processes [19, 42, 43]. In the present studies, the reduction in hydrogen ion secretion by cyclic AMP was not associated with a significant change in sodium transport (estimated as short-circuit current minus hydrogen ion secretion), suggesting that the main source of endogenous carbon dioxide was not impaired by cyclic AMP.

These considerations leave two additional possibilities to explain our findings. First, cyclic AMP might have raised intracellular pH by release of buffers from the mitochondria, as postulated by Rasmussen, Goodman, and Tenenhouse [44] and Rasmussen [45]. This would have made less hydrogen ions available for active transport. Second, cyclic AMP might have directly affected the hydrogen ion pump, for example by changing the protonmotive force or active pathway conductance [46]. Our data do not permit a choice between these possibilities.

The present findings in the turtle bladder are in conflict with the recent report by Frazier [47], who found that serosal bovine PTH or cyclic AMP *in-*

creased hydrogen ion secretion by the urinary bladder of the Colombian toad. The reasons for the discrepant results are not clear, but may be due to important species differences, or to sodium transport-mediated generation of more carbon dioxide. It is also possible that PTH, or cyclic AMP, or both stimulated other metabolic sources of carbon dioxide. High and Hersey [48] have shown that theophylline mobilizes exogenous fatty acids in frog gastric mucosa, which are then oxidized and produce carbon dioxide for participation in secretion of acid by that tissue. Whatever the mechanism, the paradoxical finding of enhanced hydrogen ion secretion by Frazier is opposite to the observed effects of PTH and cyclic AMP on hydrogen ion secretion in the intact mammalian kidney and those of cyclic AMP in the present study in the turtle urinary bladder.

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References

1. Ellsworth R, Nicholson WM: Further observations upon the changes in the electrolytes of the urine following the injection of parathyroid extract. *J Clin Invest* 14:823–827, 1935
2. Nordin BEC: The effect of intravenous parathyroid extract on urinary pH, bicarbonate and electrolyte excretion. *Clin Sci* 19:311–319, 1960
3. Hellman DE, Au WYW, Bartter FC: Evidence for a direct effect of parathyroid hormone on urinary acidification. *Am J Physiol* 209:643–650, 1965
4. Muldowney FP, Donohoe JF, Freaney R, Kampff C, Swan M: Parathormone-induced renal bicarbonate wastage in intestinal malabsorption and in chronic renal failure. *Israel J Med Sci* 3:221–231, 1970
5. Muldowney FP, Carroll DV, Donohoe JF, Freaney RF: Correction of renal bicarbonate wastage by parathyroidectomy: Implications in acid-base homeostasis. *Q J Med* 40:487–498, 1971
6. Crumb CR, Martinez-Maldonado M, Eknoyan G, Suki WN: Effects of volume expansion, purified parathyroid extract, and calcium on renal bicarbonate absorption in the dog. *J Clin Invest* 54:1287–1294, 1974
7. Diaz-Buxo JA, Ott CE, Cuche JL, Marchand GR, Wilson DM, Knox FG: Effects of extracellular fluid volume contraction and expansion on the bicarbonaturia of parathyroid hormone. *Kidney Int* 8:105–109, 1975
8. Bank N, Aynedjian HS: A micropuncture study of the effect of parathyroid hormone on renal bicarbonate reabsorption. *J Clin Invest* 58:336–344, 1976
9. Puschett JB, Zurbach P: Acute effects of parathyroid hormone on proximal bicarbonate transport in the dog. *Kidney Int* 9:501–510, 1976
10. Arruda JAL, Nascimento L, Westenfelder C, Kurtzman NA: Effect of parathyroid hormone on urinary acidification. *Am J Physiol* 232:F429–F433, 1977
11. Chase LR, Aurbach GD: Renal adenyl cyclase: Anatomically separate sites for parathyroid hormone and vasopressin. *Science* 159:545–547, 1968
12. Liddle GW, Hardman JG: Cyclic adenosine monophosphate as a mediator of hormone action. *N Engl J Med* 285:560–566, 1971
13. Karlinsky ML, Sager DS, Kurtzman NA, Pillay VKG: Effect of parathormone and cyclic adenosine monophosphate on renal bicarbonate reabsorption. *Am J Physiol* 227:1226–1231, 1974
14. Clapp JR, Watson JF, Berliner RW: Effect of carbonic anhydrase inhibition on proximal tubular bicarbonate reabsorption. *Am J Physiol* 205:693–696, 1963
15. Bernstein BA, Clapp JR: Micropuncture study of bicarbonate reabsorption by the dog nephron. *Am J Physiol* 214:251–257, 1968
16. Kunau RT Jr: The influence of the carbonic anhydrase inhibitor, benzolamide (CL-11,366), on the reabsorption of chloride, sodium and bicarbonate in the proximal tubules of the rat. *J Clin Invest* 51:294–306, 1972
17. Beck NS, Kwang K, Wolak M, Davis BB: Inhibition of carbonic anhydrase by parathyroid hormone and cyclic AMP in rat renal cortex in vitro. *J Clin Invest* 55:149–156, 1975
18. Schwartz JH, Steinmetz PR: CO_2 requirements for H^+ secretion by the isolated turtle bladder. *Am J Physiol* 220:2051–2057, 1971
19. Beauwens R, Al-Awqati Q: Active H^+ transport in the turtle urinary bladder: Coupling of transport to glucose oxidation. *J Gen Physiol* 68:421–439, 1976
20. Lorentz WB Jr: The effect of cyclic-AMP and dibutyryl cyclic-AMP on the permeability characteristics of the renal tubule. *J Clin Invest* 53:1250–1257, 1974
21. Lorentz WB Jr: Effect of parathyroid hormone on renal tubular permeability. *Am J. Physiol* 231:1401–1407, 1976
22. Agus ZS, Puschett JB, Senesky D, Goldberg M: Mode of action of parathyroid hormone and cyclic adenosine 3′,5′-monophosphate on renal tubular phosphate reabsorption in the dog. *J Clin Invest* 46:95–102, 1971
23. Agus ZS, Gardner LB, Beck LH, Goldberg M: Effects of parathyroid hormone on renal tubular reabsorption of calcium, sodium, and phosphate. *Am J Physiol* 224:1143–1148, 1973
24. Beck LH, Goldberg M: Effects of acetazolamide and parathyroidectomy on renal transport of sodium, calcium and phosphate. *Am J Physiol* 224:1136–1142, 1973
25. Hamburger RJ, Lawson NL, Dennis VW: Effects of cyclic adenosine nucleotides on fluid absorption by different segments of proximal tubule. *Am J Physiol* 227:396–401, 1974
26. Baumann K, Chan YL, Bode F, Papavassilion F: Effect of parathyroid hormone and cyclic adenosine 3′5′-monophosphate on isotonic fluid reabsorption: Polarity of proximal tubular cells. *Kidney Int* 11:77–85, 1977
27. Steinmetz PR: Cellular mechanisms of urinary acidification. *Physiol Rev* 54:890–956, 1974

28. MALNIC G, STEINMETZ PR: Transport processes in urinary acidification. *Kidney Int* 9:172–188, 1976
29. STEINMETZ PR: Characteristics of hydrogen ion transport in urinary bladder of water turtle. *J Clin Invest* 46:1531–1540, 1967
30. USSING HH, ZERAHN K: Active transport of sodium as the source of electric current in the short-circuited isolated frog skin. *Acta Physiol Scand* 23:110–127, 1951
31. LIEF PD, MUTZ BF, BANK N: Effect of stretch on passive transport in toad urinary bladder. *Am J Physiol* 230:1722–1729, 1976
32. ORLOFF J, HANDLER JS: The similarity of effects of vasopressin, adenosine 3′5′-phosphate (cyclic-AMP) and theophylline on the toad bladder. *J Clin Invest* 41:702–709, 1962
33. MAREN TH, ASH VI, BAILEY EM JR: Carbonic anhydrase inhibition. *Bull Johns Hopkins Hosp* 95:244–255, 1954
34. MAREN TH: A simplified micromethod for the determination of carbonic anhydrase and its inhibitors. *J Pharm Exp Therap* 130:26–29, 1960
35. LESLIE BR, SCHWARTZ JH, STEINMETZ PR: Coupling between Cl^- absorption and HCO_3^- secretion in turtle urinary bladder. *Am J Physiol* 225:610–617, 1973
36. SCHWARTZ JH, ROSEN S, STEINMETZ PR: Carbonic anhydrase function and the epithelial organization of H^+ secretion in turtle urinary bladder. *J Clin Invest* 51:2653–2662, 1972
37. CLARK NB: Experimental and histological studies of the parathyroid glands of fresh-water turtles. *Gen Comp Endocrinol* 5:297–312, 1965
38. DOUSA TP: Effects of hormones on cyclic AMP formation in kidneys of nonmammalian vertebrates. *Am J Physiol* 226:1193–1197, 1974
39. PUSCHETT JB, GOLDBERG M: The relationship between the renal handling of phosphate and bicarbonate in man. *J Lab Clin Med* 73:956–969, 1969
40. GARG LC: Effect of parathyroid hormone and adenosine 3′5′-monophosphate on renal carbonic anhydrase, *Biochem Pharmacol* 24:437–439, 1975
41. GARG LC: Failure of parathyroid hormone and cyclic AMP to inhibit renal carbonic anhydrase. *Pfluegers Arch* 367:103–104, 1976
42. STEINMETZ PR: Acid-base relations in epithelium of turtle bladder: Site of active step in acidification and role of metabolic CO_2. *J Clin Invest* 48:1258–1265, 1969
43. SCHWARTZ JH, FINN JT, VAUGHAN G, STEINMETZ PR: Distribution of metabolic CO_2 and the transported ion species in acidification by turtle bladder. *Am J Physiol* 226:283–289, 1974
44. RASMUSSEN H, GOODMAN DBP, TENENHOUSE A: The role of cyclic AMP and calcium in cell activation. *CRC Crit Rev Biochem* 1:95–148, 1972
45. RASMUSSEN H: Secondary hyperparathyroidism. *Mt Sinai J Med* 40:462–473, 1973
46. AL-AWQATI Q: H^+ transport in urinary epithelia. *Am J Physiol* 235(2):F77–F88, 1978
47. FRAZIER LW: Effects of parathyroid hormone on H^+ and NH_4^+ excretion in toad urinary bladder. *J Memb Biol* 30:187–196, 1976
48. HIGH WL, HERSEY SJ: Mechanism of theophylline stimulation of acid secretion by frog gastric mucosa. *Am J Physiol* 226:1408–1412, 1974