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Constitutive and Transport-Related Endocytotic Pathways in Turtle Bladder Epithelium

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Summary. Proton secretion in the urinary bladder of the freshwater turtle is mediated by a proton pump located in the apical membrane of a population of cells characteristically rich in carbonic anhydrase. Earlier studies have demonstrated that these cells exhibit apical-membrane endocytotic and exocytotic processes which are thought to be involved in the regulation of the rate of proton transport via alterations in the number of pumps within the apical membrane. In this study, we sought to characterize these processes using two different methods. Analysis of transepithelial impedance yielded estimates of membrane capacitance which could be related to membrane area, thereby allowing one to monitor net changes in apical-membrane area resulting from changes in the net rates of endo- and exocytosis. Uptake of the fluid-phase marker FITC-dextran provided a measure of net extracellular volume uptake which was related to net rates of endocytosis. Our major conclusions are summarized as follows. The bladder cells exhibit a high baseline rate of endocytosis which appears to be a constitutive process similar to pinocytosis. This process is completely inhibited when ambient temperature is reduced to 15°C. In addition, serosal application of 0.5 mM acetazolamide causes a transient increase in the rate of endocytosis, concomitant with a decrease in the rate of transport. Reduction of ambient temperature to 15°C reduces the rate of acetazolamide-induced endocytosis, but does not abolish it. Addition of 1 mM serosal azide not only prevents the acetazolamide-induced increase in endocytosis, but also prevents the decrease in transport caused by acetazolamide. Azide has no effect on the baseline rate of endocytosis, nor does it prevent inhibition of carbonic anhydrase by acetazolamide. The specificity of azide, coupled with the different temperature sensitivities, demonstrate that the constitutive and transport-dependent endocytotic pathways are distinct processes. The observation that azide prevents both the acetazolamide-induced increase in endocytosis and the decrease in transport strongly supports the notion that endocytosis of proton-pump-containing membrane is requisite for the inhibition of transport by acetazolamide. Finally, the results also demonstrate that acetazolamide does not inhibit proton secretion simply by inhibiting carbonic anhydrase.

Key Words acetazolamide · azide · carbonic anhydrase · endocytosis · exocytosis · FITC-dextran · impedance analysis · pinocytosis · proton transport · turtle bladder

Introduction

The urinary bladder of the freshwater turtle is a useful model for studying cellular mechanisms involved in the regulation of acid secretion by the collecting duct of the kidney. In turtle bladder epithelium, proton transport is an active process carried out by a pump located in the apical membrane [5, 8]. Proton transport is mediated by a population of cells which amount to 10 to 20% of the total cell number, and which are characteristically rich in carbonic anhydrase.

Gluck, Cannon and Al-Awqati [7] suggested that one means of regulating the rate of proton secretion is by altering the number of proton pumps present in the apical membrane. They showed that stimulation of proton secretion by CO_2 is dependent upon the exocytotic fusion of proton-pump-containing cytoplasmic vesicles with the apical membrane. We subsequently showed [1, 6] that the inhibition of transport by the carbonic-anhydrase inhibitor acetazolamide is accompanied by endocytosis of intracellular vesicles derived from apical membrane. This observation also supports the notion that the regulation of proton secretion involves alterations in the number of apical-membrane pumps.

In addition to the transport-associated changes in rates of endo- and exocytosis, we have recently observed a constitutive membrane shuttling process located in the carbonic-anhydrase-rich cells [6]. This process is independent of the rate of proton secretion and involves the continuous endocytosis and exocytosis of cytoplasmic vesicles with the apical membrane. Under baseline conditions, the membrane surface area remains constant, hence the rates of endocytosis and exocytosis must be identical. Inhibition of transport by acetazolamide is associated with a concomitant decrease in apical membrane surface area, resulting from transient alterations in the rates of endocytosis and exocytosis.

The studies presented in this paper were done to investigate further the endo- and exocytotic processes observed in turtle urinary bladder. Measurements of uptake of an extracellular marker were used to determine rates of endocytosis, and transepithelial impedance-analysis techniques were used

to measure changes in exposed membrane areas. Specifically, we wished to determine whether the increased endocytosis observed after inhibition of carbonic anhydrase with acetazolamide was required for inhibition of proton secretion. If this were found to be the case, then we also hoped to determine whether the net endocytosis of apical membrane involved a transient uncoupling of the constitutive membrane shuttling process, or if it was mediated by a separate regulated pathway.

Materials and Methods

Dissection, Solutions and Chambers

Freshwater turtles, *Pseudymys scripta elegans*, were double pithed and their urinary bladders removed with a minimal amount of handling. The mucosa and serosa were both bathed in a modified phosphate Ringer's solution having the following composition (in mM): 110 NaCl, 3.5 KCl, 1.0 $MgCl_2$, 1.0 $CaCl_2$, 1.5 Na_2HPO_4, and 1.0 NaH_2PO_4. The serosal solution also contained 2% bovine serum albumin and 5.0 mM D-glucose. The pH of all solutions was adjusted to 7.0 and the solutions were bubbled with room air which had been passed through KOH traps to remove CO_2. In some experiments either acetazolamide (Sigma, St. Louis, Mo.) or sodium azide (NaN_3) was added to the serosal solution in doses described in the text.

For all impedance experiments and endocytosis experiments done at reduced temperature, hemibladders were mounted in modified Ussing chambers specifically designed to eliminate mounting damage [11]. These chambers had a nominal exposed surface area of 2 cm^2 and mucosal and serosal volumes of 15 ml. A small amount of silicon oil (Antifoam A, Dow Corning, Midland, Mich.) was sprayed atop the serosal bath to control the foam produced as a result of bubbling the albumin-containing solution. The chambers also possessed surrounding water jackets permitting control of temperature to better than ±0.5°C.

For endocytosis experiments performed at room temperature, a different set of chambers were used. These chambers had an exposed surface area of 6.6 cm^2, and mucosal and serosal volumes of 6 ml. We verified that the choice of chambers did not significantly affect the area-adjusted rates of endocytosis.

Measurement of the Rate of Proton Secretion

Proton transport was measured using the short-circuit current technique [23]. The turtle bladder possesses an active sodium transport process which results in a mucosa-to-serosa short-circuit current (I_{sc}). In all experiments, this transport process was inhibited by the mucosal addition of 0.1 mM amiloride (provided as a gift from Merck, Sharp and Dohme, Rahway, N.J.). Complete inhibition of sodium transport causes a reversal of the polarity of I_{sc}, and this resulting current (sometimes referred to as the reversed short-circuit current, or RSCC) has been shown to be equivalent to the rate of proton secretion [23]. Amiloride results in near complete inhibition of sodium transport. Rates of proton transport will be referred to as positive values of I_{sc}, despite the fact that this current is directed from serosa to mucosa.

Endocytosis experiments were performed under short-circuit conditions using an automatic voltage clamp circuit. Impedance experiments were performed under open-circuit conditions, and I_{sc} was measured intermittently by passing brief (ca. 1 sec) depolarizing currents which abolished the transepithelial potential. We reported earlier [6] that rates of endocytosis did not differ significantly when measured under open- versus short-circuit conditions.

Measurement and Analysis of Transepithelial Impedance

Transepithelial impedance was measured using the method of Clausen and Fernandez [2]. A detailed description of the technique can be found in Clausen, Reinach and Marcus [3], and the details and validation of its use in turtle bladder can be found in Clausen and Dixon [1]. Briefly, the impedance was determined by measuring the transepithelial voltage resulting from a small wide-band constant applied current. The voltage was measured differentially using a high-impedance amplifier connected to a pair of Ag-AgCl electrodes mounted close to the preparation. A second set of Ag-AgCl electrodes was mounted at opposite ends of the chamber and was used to pass transepithelial current. Constant current was generated using a calibrated 1 MΩ resistor in series with the current electrodes. Data were acquired by a computer which calculated the impedance at 100 discrete frequencies logarithmically spaced between 2.2 Hz and 8.6 kHz. The phase angle and log impedance magnitude were computed at each frequency, resulting in a total of 200 data points.

Estimates of the electrical parameters of the epithelial membranes were determined by fitting the measured data (phase angle and magnitude points) by the impedance calculated from a morphologically based equivalent-circuit model (for complete details, *see* ref. 1). The electrical properties of the apical membrane were represented by a parallel resistor-capacitor circuit. An analogous circuit was used to represent the electrical properties of the basolateral membrane. The basolateral membrane was treated as a distributed circuit which explicitly considers the path resistance of the lateral spaces adjacent to the lateral region of the basolateral membrane. Finally, a small series resistance was included to represent the resistance between the voltage electrodes and the epithelial surfaces.

A nonlinear least-squares minimization algorithm was used to determine the values of the circuit elements which minimized the error between the model and the data. Standard statistical tests were subsequently performed to assess the quality of the fits, as well as the accuracy of the circuit-element values. The fitting procedure resulted in estimates of the ionic conductances of the apical and basolateral membranes (G_a and G_b, respectively), and the corresponding membrane capacitances (C_a and C_b, respectively). The membrane capacitances were used as indirect measurements of the exposed membrane areas, since the specific capacitance of biological membranes is remarkably constant at approximately 1 $\mu F/cm^2$ [4]. Measurements of capacitance also allow one to estimate the specific conductances of the membranes by normalizing G_a and G_b to unit area (i.e., $G_{a\text{-norm}} = G_a/C_a$ and $G_{b\text{-norm}} = Gb/C_b$). Finally, the procedure also resulted in estimates of the path resistance (R_p) of the lateral spaces, which provided an indirect measure of lateral space geometry (R_p is directly proportional to lateral-space length, and inversely proportional to the cross-sectional area).

Measurement of the Rate of Endocytosis

Cellular uptake of FITC-dextran, used as a fluid-phase marker, was measured in order to determine the rate of endocytosis; a detailed description of the technique can be found in Ref. 6.

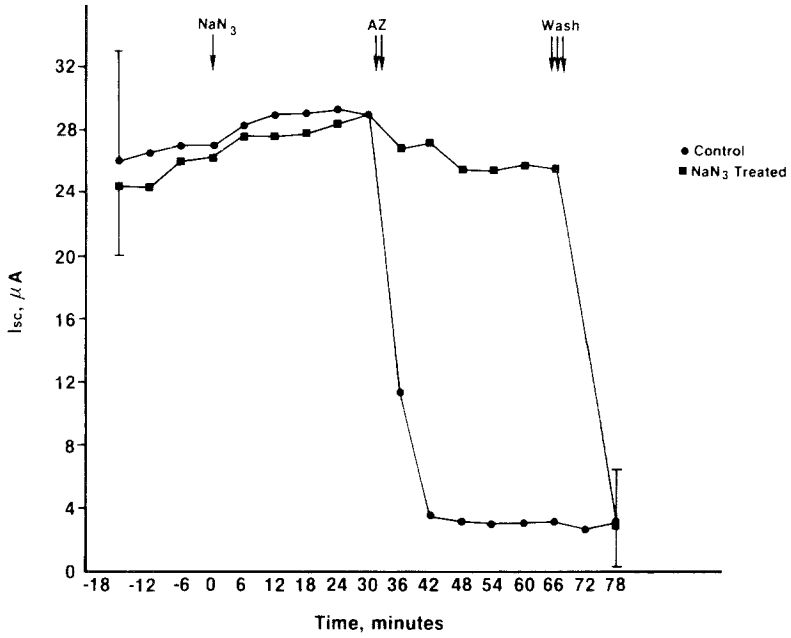

Fig. 1. Mean rate of proton transport measured as I_{sc} determined from four paired hemibladders (6.6 cm^2 chamber area); the bars on the first and last points represent standard errors. At time zero (single arrow) the serosal solution of one group was changed to one which contained 1 mM azide (squares). The other group served as a control and was continued in baseline serosal Ringer's solution (circles). Thirty minutes after the addition of azide (double arrows), 0.5 mM acetazolamide was added to serosal solutions of both groups. Forty minutes after the addition of acetazolamide (triple arrows) the mucosal and serosal solutions of both groups were washed with normal Ringer's solution that contained no acetazolamide or azide (the mucosal solution contained amiloride)

Briefly, the tissue was incubated for 15 min with mucosal solution containing 50 mg/ml of FITC-dextran (70,000 molecular weight, Sigma). The mucosal solution was then removed and all endo- and exocytosis processes were halted by flushing the mucosal surface with ice-cold saline. Following brief mucosal washes with ice-cold saline and acid solutions, procedures which remove extracellular FITC-dextran and adherent mucus, the epithelial cells were lysed in a buffered 0.1% SDS solution, and the bladder surface was scraped lightly with a metal spatula. The FITC-dextran content of the lysate was measured using a fluorescence spectrophotometer at an excitation wavelength of 495 nm and an emission wavelength of 520 nm. The fluorescence signal of the apical lysate was converted to a volume of endocytosed fluid by constructing standard curves measuring the fluorescence of known volumes of FITC-dextran incubation media in lysing solution. Finally, the combined protein content of the hemibladder and lysate was determined, and the rate of endocytosis was expressed as the volume of uptake per unit time per weight of tissue protein.

Earlier, we reported control experiments designed to test the efficacy of the FITC-dextran method for measuring endocytosis in the turtle bladder [6]. Notably, 50 mg/ml mucosal FITC-dextran does not affect the rate of proton transport, FITC-dextran is selectively endocytosed by the carbonic-anhydrase-rich cells, and intracellular FITC-dextran is retained following the washing procedures which removes extracellular FITC-dextran. We have also verified by fluorescence microscopy that cellular uptake of FITC-dextran is inhibited at 0°C, an expected finding if FITC-dextran uptake is dependent upon endocytosis.

Measurement of Carbonic-Anhydrase Activity

Excised bladders were rinsed extensively in Ringer's solution to remove red blood cells. Bladder cells were then isolated using a collagenase-treatment procedure described by Schwartz et al. [20]. The isolated cells were washed twice by suspending them in 20 mM imidazole buffer (pH 7.5) for 5 min, with subsequent centrifugation for 2 min at 1000 rpm in a clinical centrifuge. Care was taken after each wash to exclude any red blood cell pellet. The cells were then resuspended in imidazole buffer and homogenized using a Potter Elvehjem glass dounce.

Carbonic anhydrase activity was measured at 0°C in aliquots of the homogenate using the method described by Kernohan [9]. The method was modified to use a pH electrode (Radiometer, Copenhagen, Denmark) as opposed to using *p*-nitrophenol as a pH indicator. The protein content of the homogenate was determined by a protein assay (Bio-Rad Laboratories, Richmond, Calif.), using bovine serum albumin as a standard. The carbonic-anhydrase activity is reported as units/mg cell protein, using the definition of unit activity described by Maren [12].

Results

Effect of Azide on Inhibition of Proton Transport by Acetazolamide

If the increased rate of endocytosis and the membrane remodeling processes that are noted following acetazolamide treatment are causally related to the decline in proton transport, then a maneuver that alters the endocytotic response should also alter the transport response. Azide (N_3^-) is an agent that has been shown to inhibit endocytotic processes in several different cells [17, 22]. We tested whether this agent affected the ability of acetazolamide to inhibit proton transport in the turtle bladder.

Fig. 1 shows measurements of proton transport rates in four paired hemibladders. The serosal addition of 1 mM azide had no significant effect on the baseline rate of proton transport (filled boxes). In the azide pretreated hemibladders, a 40-min incuba-

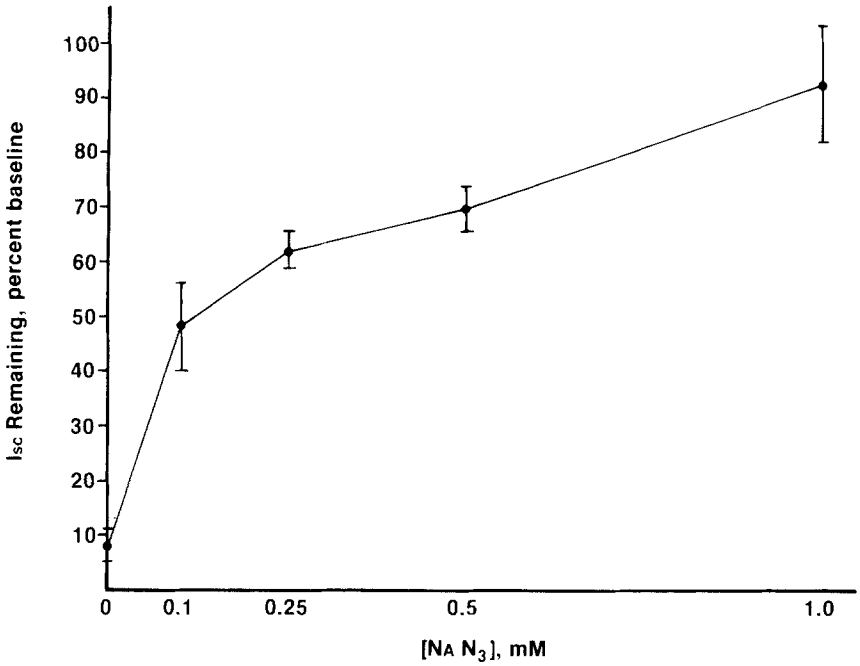

Fig. 2. Mean values (±SEM) of proton transport normalized as a percentage of the baseline rate of transport which remained following serosal treatment with 0.5 mM acetazolamide for 45 min. Hemibladders were bathed with either zero (n = 7), 0.1 (n = 4), 0.25 (n = 4), 0.5 (n = 7), or 1.0 mM (n = 4) azide in the serosal solution

tion with 0.5 mM serosal acetazolamide (still in the presence of azide) failed to cause a significant decline in transport (which decreased only 7 ± 11%). In the paired control bladders, which were not treated with azide, acetazolamide resulted in a 92 ± 10% decline in proton transport. These results demonstrate that 1 mM serosal azide protects the bladder from the inhibitory action of acetazolamide.

The protective action of azide was found to be reversible, and this is also shown in Fig. 1. Following 40 min incubation with acetazolamide, the mucosal and serosal solutions of the azide-treated hemibladders were washed and replaced with solutions devoid of both acetazolamide and azide. This led to a rapid decline in the rate of proton transport to a level not significantly different from that of the control hemibladders. In the control hemibladders, washing and replacing the solutions with acetazolamide-free media failed to restore transport, as has previously been reported [19]. We presume that the fall in transport following the wash in the azide-treated bladders was a manifestation of prior inhibition of carbonic anhydrase by acetazolamide, which was taken up by the cells during the incubation with both acetazolamide and azide. The rapidity and extent of inhibition following the wash in acetazolamide-free media argues that the mechanism of azide protection does not involve prevention of cellular uptake of acetazolamide.

Figure 2 shows a dose-response curve of the protection offered by azide against the inhibition of proton transport by 0.5 mM acetazolamide. A serosal concentrations of azide as low as 100 μM leads to significant protection, and a dose of 1 mM leads to near complete protection. Azide results in a 50% prevention of acetazolamide-induced transport inhibition at a concentration of 209 μM.

Effect of Azide and Acetazolamide on Carbonic-Anhydrase Activity

The results presented thus far suggest that azide affects a cellular step in the inhibition of transport by acetazolamide. One possibility is that azide directly affects carbonic-anhydrase activity, or that azide prevents carbonic-anhydrase inhibition by acetazolamide. In order to investigate these possibilities, we measured the activity of carbonic anhydrase in homogenates of epithelial cells isolated from turtle bladders.

In measuring carbonic-anhydrase activity in an epithelial-cell homogenate, one must be concerned about possible contamination from red blood cells (RBC's) which contain large amounts of carbonic anhydrase. RBC's present in the homogenate will not only affect the measured activity, but can also affect the apparent sensitivity to pharmacological agents, since RBC's contain carbonic anhydrase I and bladder cells contain carbonic anhydrase II [24], a different isoenzyme. In order to estimate the carbonic-anhydrase activity attributed to contaminating RBC's, we first measured the activity and hemoglobin content in RBC's from two turtles, and found an average activity of 50 units/mg hemoglobin. We then used the hemoglobin content of blad-

der-cell homogenates to estimate the degree of contamination from carbonic anhydrase derived from RBC's. In homogenates derived from four bladders, we found the carbonic-anhydrase activity attributable to contaminating RBC's to be only 2.6 ± 1.3% of the total activity.

Carbonic-anhydrase activity was measured in aliquots of homogenates from four separate bladders. The mean carbonic-anhydrase activity in control aliquots was 18.9 ± 3.0 units/mg protein. Addition of 1 mM azide 2 min prior to assay caused the activity to decline to 6.0 ± 0.7 units/mg protein (P = 0.004 compared to control by paired analysis). Assuming azide enters the cell by passive diffusion, the intracellular concentration is likely to be far less than the 1 mM serosal concentration used to protect transport from acetazolamide inhibition. If the basolateral membrane potential is ca. −70 mV [14], then one can predict that the intracellular azide concentration at equilibrium will be approximately ten times lower than the serosal concentration, or 0.1 mM. Incubation of aliquots of the homogenates with 0.1 mM azide also produced a significant decline in the carbonic-anhydrase activity to a level of 11.5 ± 2.2 units/mg protein (P = 0.02). Hence, azide alone acts as an inhibitor of carbonic-anhydrase activity, an observation that is seen in other tissues [10].

Inhibition of carbonic anhydrase by acetazolamide under control conditions, and in the presence of 0.1 and 1.0 mM azide, is shown in Fig. 3. As expected, the carbonic-anhydrase activity is quite sensitive to acetazolamide, exhibiting complete inhibition at a concentration of 100 nM. In addition, the combination of azide and acetazolamide led to increased inhibition of carbonic-anhydrase activity compared to the azide-free case. Hence, azide does not prevent the inhibition of carbonic anhydrase by acetazolamide. Therefore, the protection offered by azide against acetazolamide-induced inhibition of transport can not be explained by a direct interaction with carbonic anhydrase.

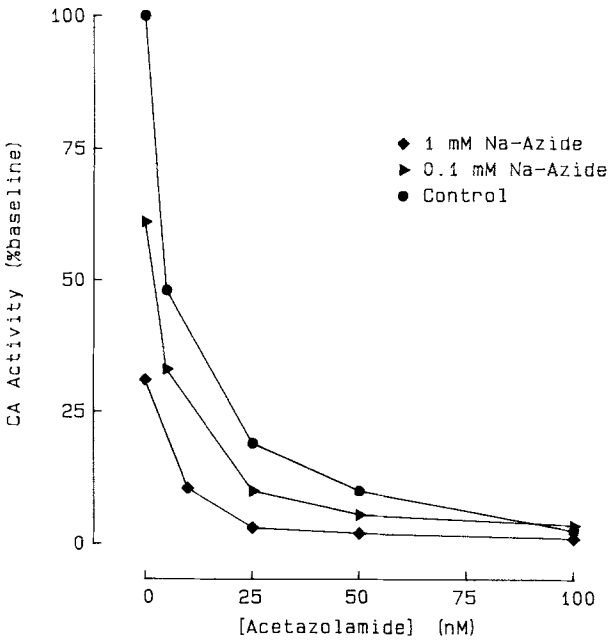

Fig. 3. Carbonic-anhydrase (CA) activity in homogenates of turtle bladder shown as a percentage of activity found in the baseline state. The inhibitory effect of acetazolamide on the activity of carbonic anhydrase was tested in samples which were preincubated for 2 min with either zero (circle), 0.1 (square), or 1 mM (triangle) azide. Each point represents the mean value obtained from four experiments

Effect of Azide on Membrane Electrical Characteristics

In our earlier study [6], we showed that treatment of hemibladders with 0.5 mM acetazolamide is associated with a decrease in the apical-membrane surface area resulting from endocytosis of apical membrane. This was determined from analyses of transepithelial impedance which showed a decrease in C_a (the apical-membrane capacitance) following application of acetazolamide. If azide exerts its protective action by inhibiting endocytosis of pump-containing membrane, then this should be evident in impedance studies which measure membrane electrical characteristics.

Membrane electrical parameters determined from analyses of transepithelial impedance in five hemibladders are shown in Table 1. Incubation with 1 mM serosal azide for 30 min produced no significant changes in either the apical or basolateral membrane parameters. We did note, however, a small but significant increase in R_p (the lateral space resistance) which may be indicative of changes in lateral-space geometry, cell volume, or ionic composition of the fluid bathing the lateral spaces.

In the presence of azide, serosal addition of 0.5 mM acetazolamide was associated with a small decline in proton transport which was not statistically significant (P = 0.09 by paired analysis). In addition, acetazolamide resulted in a small decline in C_a which also was not statistically significant (P = 0.07). We previously reported that in the absence of azide, acetazolamide produces a significant decrease in C_a amounting to 0.47 μF/cm^2 [6].[1] In the

[1] In the presence of azide, the *relative* decrease in C_a following acetazolamide is 5 ± 1% which is significant (P = 0.01), but this decrease is much less than the 16 ± 3% decrease (P = 0.005, unpaired analysis) following acetazolamide in the absence of azide [6]. Viewing the data in this manner, it is clear that 1 mM azide markedly blunts the effects of acetazolamide, but does not completely prevent either the inhibition of transport, or the change in C_a.

Table 1. Effect of azide on proton transport and membrane electrical parameters[a]

	I_{sc} (μA/cm^2)	G_a (mS/cm^2)	C_a (μF/cm^2)	$G_{a\text{-norm}}$ (μS/μF)	G_b (mS/cm^2)	C_b (μF/cm^2)	$G_{b\text{-norm}}$ (μS/μF)	R_p (Ωcm^2)
Control	6.8 ± 1.5	0.19 ± 0.01	3.0 ± 0.7	78 ± 17	5.2 ± 1.1	8.3 ± 2.5	829 ± 325	260 ± 68
Azide	7.5 ± 1.8	0.22 ± 0.02	2.8 ± 0.6	89 ± 12	5.8 ± 1.0	9.1 ± 2.7	869 ± 332	270 ± 68
Δ	0.8 ± 0.5	0.03 ± 0.02	−0.2 ± 0.1	12 ± 8	0.6 ± 0.4	0.8 ± 0.5	50 ± 26	10 ± 2
P	NS	NS	NS	NS	NS	NS	NS	0.004
Azide + AZ	5.9 ± 1.6	0.23 ± 0.03	2.6 ± 0.6	97 ± 13	5.7 ± 0.9	8.7 ± 2.8	855 ± 281	271 ± 76
Δ	−1.6 ± 1.0	0.01 ± 0.02	−0.1 ± 0.1	8 ± 10	−0.2 ± 0.2	−0.4 ± 0.2	−14 ± 57	1 ± 12
P	NS	NS	NS	NS	NS	NS	NS	NS
Azide removed	1.1 ± 0.7	0.15 ± 0.02	2.3 ± 0.5	71 ± 7	5.0 ± 0.7	7.8 ± 2.5	804 ± 221	260 ± 77
Δ	−4.8 ± 1.0	−0.08 ± 0.03	−0.3 ± 0.1	−26 ± 10	−0.7 ± 0.2	−0.9 ± 0.3	−51 ± 66	−11 ± 11
P	0.004	0.02	0.03	0.03	0.02	0.02	NS	NS

[a] Mean values (±SEM) from measurements in five hemibladders. The first two rows show values under control conditions, and following the serosal addition of 1 mM azide, respectively. The third row shows the mean difference between these states, and the fourth row shows the probabilities comparing the control and azide-treated states. The fifth row shows the results of incubating the azide-pretreated hemibladders with 0.5 mM serosal acetazolamide (AZ). The sixth row shows the mean difference between the azide-treated and the azide-plus-acetazolamide-treated states, and the seventh row shows the probabilities comparing the two conditions. The eighth row shows the effect of removing azide from the hemibladders while continuing to bathe them in 0.5 mM serosal acetazolamide. The ninth row shows the mean difference between the azide-plus-acetazolamide and the azide-free acetazolamide states, and the last row shows the probabilities comparing these two conditions. All probabilities are based on paired t-tests. NS denotes probabilities that are not significant ($P > 0.05$).

presence of azide, acetazolamide also produced no change in G_a (the apical-membrane conductance), which again is different from our earlier result in which a decrease was observed.

Also shown in Table 1 is the effect of removing azide from the serosa while continuing to bathe the hemibladders in 0.5 mM serosal acetazolamide. This maneuver resulted in a significant decline over 45 min in the rate of proton transport, which decreased by 4.8 ± 1.0 μA/cm^2 ($P = 0.004$). The decline in proton transport was accompanied with significant decline in both C_a ($P = 0.03$) and G_a ($P = 0.02$), and in $G_{a\text{-norm}}$ (an estimate of the specific conductance of the apical membrane, $P = 0.03$). The acetazolamide-induced declines in G_a C_a, and $G_{a\text{-norm}}$ are statistically indistinguishable ($P > 0.2$, 0.5, and 0.5, respectively, by unpaired analysis) from those reported in our earlier study [6] in the absence of azide pretreatment.

Following the removal of azide, small but significant declines in C_b and G_b (the basolateral membrane capacitance and conductance, respectively) were also noted, but no change in $G_{b\text{-norm}}$ was observed. The physiological significance of these apparent changes in the basolateral parameters are not known.

Effect of Azide on Rates of Endocytosis

The impedance data presented demonstrates that azide, in essence, prevents the acetazolamide-induced decline in C_a and G_a, and is thus compatible with the notion that azide prevents the endocytotic removal of proton-pump-containing apical membrane. In order to quantify the effect of azide on endocytosis, we measured the cellular uptake of FITC-dextran. In the absence of azide, we have previously shown that 15 to 30 min following application of 0.5 mM serosal acetazolamide. the rate of FITC-uptake nearly doubles [6].

Each of seven bladders was divided into three parts; one part was used as a control tissue, and the other two were experimental tissues. In the control tissue, we measured a baseline rate of endocytosis of 0.54 ± 0.12 nl/min/mg protein, a value that is not different from the baseline endocytosis rate reported earlier [6]. In the experimental tissue that was treated with 1 mM serosal azide for 30 min, we measured a rate of endocytosis of 0.51 ± 0.11 nl/min/mg protein, a value which was not different from the control rate. This shows that azide does not affect the constitutive endocytotic process described previously.

The second experimental tissue was similarly treated with azide for 30 min, and then 0.5 mM serosal acetazolamide was added. In the 15- to 30-min time period following addition of acetazolamide, the measured rate of endocytosis was 0.62 ± 0.14 nl/min/mg protein. This rate was not significantly different from either the control, or the azide-treated experimental tissue (mean difference 0.11 ± 0.15 nl/min/mg protein, $P > 0.2$ by paired analysis). These results, which are consistent with the impedance data presented above, show that azide prevents the

Table 2. Effect of reduced temperature on proton transport and electrical parameters[a]

	I_{sc} (μA/cm^2)	G_a (mS/cm^2)	C_a (μF/cm^2)	$G_{a\text{-norm}}$ (μS/μF)	G_b (mS/cm^2)	C_b (μF/cm^2)	$G_{b\text{-norm}}$ (μS/μF)	R_p (Ωcm^2)
Control	11.1 ± 4.8	0.39 ± 0.07	5.3 ± 1.3	79 ± 11	6.6 ± 0.7	12.9 ± 2.5	603 ± 152	207 ± 80
15°C	3.6 ± 1.8	0.22 ± 0.03	5.1 ± 1.2	48 ± 7	5.1 ± 1.5	12.1 ± 2.5	463 ± 131	266 ± 98
$\bar{\Delta}$	−7.5 ± 3.0	−0.17 ± 0.06	−0.2 ± 0.2	−32 ± 11	−1.5 ± 1.3	−0.8 ± 0.7	−140 ± 162	59 ± 20
P	0.03	0.02	NS	0.02	NS	NS	NS	0.02
15°C + AZ	0.7 ± 1.1	0.18 ± 0.03	4.8 ± 1.2	42 ± 8	4.3 ± 0.9	11.3 ± 2.3	450 ± 126	272 ± 86
$\bar{\Delta}$	−2.8 ± 1.1	−0.04 ± 0.02	−0.3 ± 0.1	−5 ± 3	−0.8 ± 0.7	0.7 ± 0.9	−13 ± 24	6 ± 17
P	0.03	0.04	0.02	0.07	NS	NS	NS	NS

[a] Mean values (±SEM) from measurements in five hemibladders. The first two rows show values under control conditions (21°C) and after reducing the temperature to 15°C, respectively. The third row shows the mean difference between the two conditions, and the fourth row shows probabilities comparing the control and low-temperature states. The fifth row shows mean values obtained after the serosal addition of 0.5 mM acetazolamide (AZ) to the hemibladders maintained at 15°C. The sixth row shows mean differences between the hemibladders at 15°C and in the presence of acetazolamide at 15°C, and the last row shows probabilities comparing these two states. All probabilities are based on paired t-tests. NS denotes probabilities that are not significant ($P > 0.05$).

acetazolamide-induced increase in the rate of endocytosis and are therefore consistent with the notion that the protective effect of azide is mediated by inhibition of endocytosis.

Effect of Reduced Temperature on Endocytosis

It has been shown that endo- and exocytotic processes in other systems show a high temperature sensitivity. Notably, reducing temperature to 18°C inhibits phagocytosis in mammalian macrophages [21]. We therefore decided to investigate the temperature sensitivity of the endocytotic processes observed in turtle urinary bladder.

Transport rate and FITC-dextran uptake were measured in 10 paired hemibladders. The control bladder was maintained at room temperature (20 to 21°C), and an experimental bladder was cooled to 15°C. Proton transport decreased significantly from 11.5 ± 1.5 to 5.9 ± 0.7 μA/cm^2. The baseline rate of endocytosis exhibited a marked temperature sensitivity. The rate measured in the control hemibladders was 0.66 ± 0.12 nl/min/mg protein, whereas in the cooled bladder, endocytosis was nearly abolished and exhibited a rate of 0.04 ± 0.01 nl/min/mg protein.

In order to determine whether reduced temperature affects the transport-related endocytosis, we examined the effect of inhibiting transport with acetazolamide in 10 paired hemibladders all maintained at 15°C. The control hemibladders exhibited a rate of endocytosis of 0.03 ± 0.01 nl/min/mg protein. In the experimental hemibladders, 0.5 mM acetazolanide resulted in a decline in proton transport from 6.6 ± 0.7 to 1.9 ± 0.7 μA/cm^2. The rate of endocytosis measured 15 to 30 min following the addition of acetazolamide, 0.13 ± 0.04 nl/min/mg protein, was significantly higher than that in the control hemibladders (mean difference 0.10 ± 0.04 nl/min/mg protein, $P = 0.02$). These results demonstrate that although the constitutive endocytotic pathway is nearly abolished at 15°C, activity in the transport-related pathway can still be observed.[2]

Effect of Reduced Temperature on Membrane Electrical Characteristics

As shown above, reducing the ambient temperature inhibits the constitutive endocytotic pathway to a much greater degree than the transport-related pathway. In order to investigate this further, we measured the membrane electrical characteristics present under these conditions. Results from these experiments, determined by analyzing transepithelial impedance, are shown in Table 2.[3]

Reducing the temperature to 15°C results in a marked reduction in proton transport, and is accompanied by a reduction in G_a and an increase in

[2] The increase in the rate of endocytosis following acetazolamide in the cooled bladders is less than that noted following acetazolamide at room temperature (*see* Table 2 in ref. 6). Not surprisingly, the transport-related pathway also exhibits temperature sensitivity, but not to the degree exhibited by the constitutive pathway (*see* Discussion).

[3] Some of the control values shown in Table 2 differ significantly from values reported in Table 1 and in our earlier study [6]. In these experiments, hemibladders were mounted more loosely, hence these values reflect the presence of a greater amount of tissue per unit chamber area. It is notable that the area-normalized parameters ($G_{a\text{-norm}}$ and $G_{b\text{-norm}}$) are statistically identical ($P > 0.5$ by unpaired analysis) to the values found in Table 1 and those reported earlier [6].

R_p. Since C_a remains unchanged, the reduction in G_a signifies a decrease in $G_{a\text{-norm}}$ (i.e., the specific conductance of the apical membrane). The lack of change in C_a under these conditions implies that the decline in the rate of proton transport is not due to a decline in the number of apical-membrane pumps. Moreover, since reducing temperature results in a near-complete inhibition of the baseline rate of endocytosis (*see above*), the lack of change of C_a also implies that the baseline rate of exocytosis is similarly inhibited, again demonstrating that the constitutive shuttling process involves a tight coupling of endo- and exocytotic processes. The increase in R_p could simply reflect an increase in the resistivity of the fluid bathing the lateral spaces, or could possibly reflect a small degree of cell swelling which would be expected to change the geometry of the lateral spaces.

Also shown in Table 2 are the results of adding 0.5 mM acetazolamide to the serosal bath in hemibladders maintained at 15°C. Acetazolamide produced a further reduction in the rate of proton transport, which was associated with a significant decline in G_a and C_a. The acetazolamide-induced reduction in C_a (hence, apical membrane area), coupled with the increased rate of endocytosis (*see above*), demonstrate that the transport-dependent endocytotic process is functional at reduced temperature, providing further evidence that this process is distinct from the baseline constitutive shuttling process.

Discussion

Endocytosis and exocytosis have long been recognized as a means of transporting lipid-insoluble substances across cell membranes. Recently, there has been a growing appreciation that these mechanisms can regulate transport processes by altering the number of transporting proteins present in the cell membrane. The purpose of this study was to investigate further the relationships between proton transport, endocytosis, and exocytosis, in turtle bladder epithelium.

Azide Protection

We showed that 1 mM serosal azide prevents the inhibition of proton transport by 0.5 mM acetazolamide. Moreover, this protection appears to be mediated by preventing the endocytotic removal of proton-pump-containing apical membrane, a conclusion that strongly supports the notion that proton transport is regulated by altering the apical-membrane pump number as opposed to altering the activity of the individual pumps. We arrived at this conclusion from the observations that azide prevents transport dependent uptake of extracellular marker and, in addition, prevents the decrease in apical-membrane area reflected by a decrease in C_a.

Although azide clearly inhibits transport-related endocytosis, the agent has no effect on the baseline constitutive rate of endocytosis. This finding supports the notion that the cells possess two different endocytotic pathways. Moreover, it suggests that under baseline conditions, when the rate of proton transport is constant, the transport-related vesicles have a low rate of shuttling as compared to the rate of shuttling found in the constitutive pathway.

The mechanism of action of azide protection is unclear. The effect of azide is readily reversible, whereas inhibition of transport by acetazolamide (at the dose used) is not. Exploiting these facts, we demonstrated that the protective action of azide does not involve prevention of cellular uptake of acetazolamide. Moreover, our results show that azide protection does not involve an effect on carbonic anhydrase. In the presence of azide, acetazolamide is still a potent inhibitor of carbonic-anhydrase activity. In fact, azide itself was found to be an inhibitor of carbonic anhydrase, albeit at higher concentrations than acetazolamide. Azide is a well known inhibitor of oxidative metabolism, but our results suggest that the mechanism of protection is not a manifestation of alterations in the rate of metabolism or ATP production. The finding that azide does not affect the baseline rate of proton transport would be unexpected if cellular ATP levels had decreased significantly. One could argue that the endocytotic process has a relatively high K_m for ATP and that even small changes in the ambient ATP concentration could decrease the endocytotic response. This, however, is unlikely since even under complete anaerobic conditions, acetazolamide has been shown to inhibit proton transport [25]. Moreover agents which inhibit ATP production (e.g., iodoacetate and cyanide) fail to offer protection against the inhibitory effects of acetazolamide on proton transport (*unpublished observation*). Our results are most consistent with the notion that azide prevents the transport-associated endocytosis of apical membrane. This could occur either by preventing the intracellular signal for endocytosis, or by preventing recognition or transduction of the signal, or by inhibiting the endocytotic machinery directly. Azide is known to inhibit endocytotic processes in several different cell types [17, 22], and in at least one of these, azide's action was found to be independent of alterations in intracellular levels of ATP.

We should note that azide is also known to be a potent stimulator of guanylate-cyclase activity in a variety of cells [13]. The enzymatic processes involved in the regulation of endocytosis and exocytosis in the turtle bladder are currently unknown, but given the dramatic effect of azide in this tissue, it was tempting to speculate that cGMP might be involved in the regulation of these processes. However, recent studies involving the use of 8-bromo-cGMP (a permeable cGMP analog), or other substances known to stimulate guanylate cyclase (e.g., nitroprusside and nitrite), failed to alter acetazolamide-induced inhibition of proton transport (*unpublished observation*). Hence, the specific pathways involved in azide protection remain unknown.

Role of Carbonic Anhydrase in Proton Transport

The rate of proton transport does not change after azide and acetazolamide treatment, a situation where carbonic anhydrase is believed to be completely inhibited. If carbonic-anhydrase activity is truly inhibited, then the rate of proton transport must not be dependent on carbonic-anhydrase-catalyzed production of protons. It should be noted, however, that the bladders were not transporting protons at a maximal rate, such as the case when transport is stimulated with CO_2. It is possible that at maximal rates of proton transport, carbonic-anhydrase activity is required to sustain transport. Nevertheless, it is clear that, under the conditions used in this study, inhibition of proton transport by acetazolamide does not result solely from the inhibition of carbonic anhydrase.

Effects of Reduced Temperature on Endocytotic Pathways

Reducing the ambient temperature to 15°C nearly abolished the baseline uptake of FITC-dextran, thereby indicating that the constitutive endocytotic pathway is highly temperature sensitive. We noted, however, that acetazolamide-induced inhibition of transport resulted in a significant increase in FITC-dextran uptake even at 15°C, although this rate of uptake was less than that measured at room temperature. To compare the effect of temperature on the two processes, we computed their apparent Q_{10} values [18].

At room temperature (ca. 21°C), the transport-dependent rate of FITC-dextran uptake is 0.46 nl/min/mg protein (see Table 2 in ref. 6). At 15°C, the rate decreases to 0.10 nl/min/mg protein (*see above*). From these values, we compute a Q_{10} of 13. This value indicates that the transport-dependent endocytotic process is highly temperature sensitive, as compared to simple diffusive processes which exhibit Q_{10} values near 1.3 [16]. However, the temperature sensitivity of this process is far less than that computed for the baseline constitutive endocytotic process which exhibits a Q_{10} of 102. The apparent difference in the temperature sensitivity of the two processes provides additional evidence that they are mediated by different biochemical pathways, and hence are distinct processes.[4] Since the function of the baseline membrane shuttling (which resembles pinocytosis) is unknown in turtle bladder, we hesitate to even speculate on the significance of its apparent extreme temperature sensitivity.

Effect of Reduced Temperature on Pump Activity and Electrical Parameters

Since reduction of the ambient temperature results in a dramatic reduction in the rate of proton transport, but is not accompanied by a significant reduction in apical membrane area, this reduction of transport must reflect the temperature dependence of the activity of individual pumps. We therefore estimate the Q_{10} of the pump to be 7.

Although temperature-related changes in membrane conductances are expected, it is difficult to predict the direction or magnitude of a change in conductance since it is not only dependent on membrane ionic permeability (which decreases with temperature reduction), but it is also dependent on the transmembrane ionic gradients (which may increase or decrease with temperature reduction). The decrease in $G_{a\text{-norm}}$ is therefore not unexpected. On the other hand, the decrease in R_p is completely expected and can be explained solely by the temperature-dependent change in the resistivity of the solution bathing the lateral spaces. The change in R_p exhibits a Q_{10} of 1.5 which is close to that expected for an electrolyte solution (ca. 1.3, *see* ref. 16).

Conclusions

In summary, we conclude from these studies that the turtle bladder possesses two distinct endocy-

[4] One must be cautious in interpreting these Q_{10} values as being statistically different, especially considering that the low-temperature uptake rates were low which invariably results in large Q_{10} values. The standard error of the transport-dependent rate of FITC-dextran uptake is unavailable, and hence one cannot perform a propagation-of-error analysis and subsequent *t*-test.

totic pathways. A constitutive process resembling pinocytosis exhibits a high turnover rate, insensitivity to azide, and an apparent extreme temperature sensitivity. The physiological function of this pathway remains to be determined. The second pathway is involved in the regulation of proton transport, it exhibits a low rate of shuttling, and is selectively inhibited by azide. Earlier studies by us and others [6,15] showed a correlation between endocytotic processes and proton transport rate, which led to the notion that the rate of transport might be regulated by altering the number of proton pumps in the apical membrane. The results presented herein demonstrate that stimulation of this process is requisite for the inhibition of transport using acetazolamide. The difference in the kinetic properties and pharmacologic sensitivities of the two pathways will foster the design of experiments to elucidate further the details of each pathway.

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