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Research Article

We evaluated the effects of acetazolamide on Na+-HCO3- cotransport in basolateral membrane vesicles isolated from the rabbit renal cortex. Na+ uptake stimulated by an imposed inward HCO3- gradient was not significantly reduced by 1.2 mM acetazolamide, indicating that acetazolamide does not directly inhibit Na+-HCO3- cotransport. 4,4'-Diisothiocyanostilbene-2,2'-disulfonate (DIDS)-sensitive Na+-base cotransport was found to be absolutely CO2/HCO3--dependent. We therefore tested whether acetazolamide-sensitive availability of HCO3- at the basolateral membrane could be rate-limiting for Na+-base cotransport under some conditions. In the presence of a CO2/HCO3- buffer system but absence of an initial HCO3- gradient, Na+ influx was stimulated fivefold by an outward NH4+ gradient. This stimulation of Na+ influx by an outward NH4+ gradient was inhibited greater than 75% by 0.6 mM acetazolamide, suggesting that acetazolamide blocked the ability of the NH4+ gradient to generate an inward HCO3- gradient. In the presence of an inward HCO3- gradient, Na+ influx was inhibited greater than 70% by an inward NH4+ gradient. This inhibition of Na+ influx was reduced to only 35% by 0.6 mM acetazolamide, suggesting that acetazolamide blocked the ability of NH4+ to collapse the inward HCO3- gradient. Similarly, Na+ influx in the presence of an inward HCO3- gradient was inhibited greater than 80% by an outward acetate gradient, and this inhibition was reduced to only 50% by acetazolamide. Thus, acetazolamide caused either inhibition […]

Effects of Acetazolamide on Na^+-HCO_3^- Cotransport in Basolateral Membrane Vesicles Isolated from Rabbit Renal Cortex

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Abstract

We evaluated the effects of acetazolamide on Na^+-HCO_3^- cotransport in basolateral membrane vesicles isolated from the rabbit renal cortex. Na^+ uptake stimulated by an imposed inward HCO_3^- gradient was not significantly reduced by 1.2 mM acetazolamide, indicating that acetazolamide does not directly inhibit Na^+-HCO_3^- cotransport. 4,4'-Diisothiocyanostilbene-2,2'-disulfonate (DIDS)-sensitive Na^+-base cotransport was found to be absolutely CO_2/HCO_3^--dependent. We therefore tested whether acetazolamide-sensitive availability of HCO_3^- at the basolateral membrane could be rate-limiting for Na^+-base cotransport under some conditions. In the presence of a CO_2/HCO_3^- buffer system but absence of an initial HCO_3^- gradient, Na^+ influx was stimulated fivefold by an outward NH_4^+ gradient. This stimulation of Na^+ influx by an outward NH_4^+ gradient was inhibited > 75% by 0.6 mM acetazolamide, suggesting that acetazolamide blocked the ability of the NH_4^+ gradient to generate an inward HCO_3^- gradient. In the presence of an inward HCO_3^- gradient, Na^+ influx was inhibited > 70% by an inward NH_4^+ gradient. This inhibition of Na^+ influx was reduced to only 35% by 0.6 mM acetazolamide, suggesting that acetazolamide blocked the ability of NH_4^+ to collapse the inward HCO_3^- gradient. Similarly, Na^+ influx in the presence of an inward HCO_3^- gradient was inhibited > 80% by an outward acetate gradient, and this inhibition was reduced to only 50% by acetazolamide. Thus, acetazolamide caused either inhibition or stimulation of Na^+ uptake depending on the conditions with respect to pH and HCO_3^- gradients. The indirect interaction of acetazolamide with the basolateral membrane Na^+-HCO_3^- cotransport system may be an important mechanism underlying inhibition of proximal tubule acid secretion by this agent.

Introduction

Acetazolamide, a potent inhibitor of carbonic anhydrase, inhibits the process of HCO_3^- reabsorption in the proximal tubule (1–5). This effect has been attributed to inhibition of luminal carbonic anhydrase resulting in an acid disequilibrium pH that then inhibits H^+ secretion across the luminal membrane (6–10). However, recent studies indicate that acetazolamide also inhibits the process of HCO_3^- exit across the basolateral membrane of the proximal tubule cell (11, 12). In the proximal tubule, an electrogenic Na^+-HCO_3^- cotransport (13–22) with a stoichiometry of three bicarbonate per one sodium (16, 23) is the principal pathway for exit of HCO_3^- across the basolateral membrane. The important question then arises of whether acetazolamide inhibition of HCO_3^- exit results from a direct or indirect interaction with the Na^+-HCO_3^- cotransport system. We have studied this question using basolateral membrane vesicles isolated from the rabbit renal cortex, which has been a useful experimental model for characterizing the properties of this transport pathway (21–23). We find that acetazolamide does not inhibit the Na^+-HCO_3^- cotransport system directly but rather inhibits indirectly by retarding the generation of HCO_3^-, a required substrate for operation of this transport mechanism. This interaction of acetazolamide with the Na^+-HCO_3^- cotransport system may be important for explaining the mechanism by which this drug inhibits acid-base transport not only in the proximal tubule but also in other epithelia, such as the cornea and stomach, in which Na^+-HCO_3^- cotransport is a major mechanism for mediating base exit across the plasma membrane (24–26).

Methods

Membrane preparation. Male New Zealand white rabbits were killed by intravenous pentobarbital. Basolateral membrane vesicles were isolated from the renal cortices by differential and Percoll gradient centrifugation, as described previously (21). The membrane vesicles were suspended in a medium consisting of 250 mM sucrose, 10 mM Hepes titrated to pH 7.6 with tetramethylammonium (TMA)[1] hydroxide. The vesicles were then either placed on ice at 0–4°C and used for transport studies within 18 h, or frozen and used within 1 wk of storage at −70°C. No differences were noted in the properties of the Na^+-HCO_3^- cotransport system when fresh and frozen membranes were compared. The purification of the basolateral membrane vesicles relative to the initial homogenate was 10–14-fold based on enrichment in specific activity of Na^+,K^+-ATPase.

Orientation of basolateral membrane vesicles. The specific activity of Na^+,K^+-ATPase was assayed under several different conditions (Table I) in order to estimate the relative amounts of the enzyme in right-side-out vesicles, inside-out vesicles and broken membranes (indistinguishable from unsealed or leaky vesicles) (27). The general procedure for the Na^+,K^+-ATPase assay was modified from Forbush (28). The basolateral membrane vesicles were first preequilibrated in 25 mM KCl, 231 mM sucrose, 9 mM Hepes titrated with TMAOH to pH 7.6 for 24 h at 4°C. Then, 50 μl of membrane suspension (containing 40–60 μg protein) was added to 100 μl of 0.8 mg/ml SDS, 1% BSA, 25 mM imidazole/HCl, pH 7.4, or to 100 μl of the same solution not containing SDS. After 30 min incubation at room temperature

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1. *Abbreviations used in this paper:* DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulfonate; Mes, 2-(*N*-morpholino)ethanesulfonic acid; TMA, tetramethylammonium.

Table I. Orientation of Basolateral Membrane Vesicles

Condition	ATPase activity
	nmol P/min/mg protein
a $Na^+ + K^+$ + SDS	2102.3±122.8
b $Na^+ + K^+$ + ouabain + SDS	99.3±19.1
c $Na^+ + K^+$	601.5±33.9
d $Na^+ + K^+$ + ouabain	475.6±32.5
a–b total Na^+,K^+-ATPase	2002.9±126.3 (100%)
a–c Na^+,K^+-ATPase: RSO vesicles	1500.8±116.1 (75%)
d–b Na^+,K^+-ATPase: ISO vesicles	376.3±14.3 (19%)
c–d Na^+,K^+-ATPase: unsealed vesicles	125.9±12.8 (6%)

The proportions of right-side-out (RSO) vesicles, inside-out (ISO) vesicles, and unsealed vesicles were estimated by assay of Na^+,K^+-ATPase activity in the presence or absence of SDS as explained in the text. Values shown represent the means±SE for three separate experiments.

(20–22°C), 600 μl 0.3% BSA, 25 mM imidazole/HCl, pH 7.4 was added, and aliquots were taken for ATPase assay as described by Forbush (28). These assays were performed for 1 min at 37°C in a medium with final composition 120 mM NaCl, 25 mM KCl, 4 mM $MgCl_2$, 4 mM Na_2ATP, 1 mM EDTA, 60 mM Tris/HCl, pH 7.4. In addition, to avoid any contribution from ouabain-insensitive Na^+-ATPase activity, 2 mM furosemide was added to the assay medium (27). Subsequently, we found that neither ouabain-insensitive Na^+-ATPase activity nor furosemide-sensitive ATPase activity could be detected in rabbit renal basolateral membrane vesicles. The values for ATPase activities given in Table I represent means±SE for three separate experiments on different membrane preparations.

Treatment of the vesicles with SDS provides access of substrates and inhibitors to all ATPase present in the membrane preparation. Therefore, the total Na^+,K^+-ATPase is the difference between the activity measured in the presence of SDS (*a*) less that measured in the presence of SDS with ouabain (*b*). The latter (*b*) represents the ouabain-insensitive ATPase activity (i.e., Mg-ATPase). Na^+,K^+-ATPase in right-side-out vesicles requires SDS to provide access of ATP to its site on the internal face of the membrane. Thus, the proportion of right-side-out vesicles is the increment in apparent Na^+,K^+-ATPase activity resulting from the addition of SDS (a–c), which was equal to 75% of the total Na^+,K^+-ATPase activity. Na^+,K^+-ATPase in inside-out vesicles requires SDS to provide access of ouabain to its inhibitory site. Accordingly, the proportion of inside-out vesicles is the decrement in ATPase resulting from the addition of SDS in the presence of ouabain (*d–b*), which was equal to 19% of the total Na^+,K^+-ATPase activity. Na^+,K^+-ATPase in broken membranes does not require SDS to provide access of substrates and ouabain to their respective sites. Hence, the proportion of broken or leaky vesicles is the ouabain-sensitive ATPase measured in the absence of SDS (*c–d*), which was equal to 6% of the total Na^+,K^+-ATPase activity.

^{22}Na transport measurements. In general, the timed uptake of ^{22}Na by aliquots of membrane vesicle suspension (160–220 μg protein/sample) were assayed in triplicate at room temperature by the rapid filtration technique previously described (21). The ice-cold medium used to dilute and wash the vesicles at the termination of the period of uptake consisted of 170 mM K gluconate, 10 mM Hepes titrated to pH 7.5 with TMA hydroxide. Each Millipore filter (0.65 μm, DAWP) was placed in 3 ml Opti-Fluor (Packard Instrument Co., Inc., Downers Grove, IL) and radioactivity assayed by scintillation spectroscopy. The final composition of the experimental media and other details of the protocols are given in the figure legends. The membrane suspension and all experimental media were continuously gassed with 5% CO_2/95% N_2, or 10% CO_2/90% N_2. All experiments were performed using vesicles treated with valinomycin (0.5 mg/ml) and preequilibrated in media of appropriate composition to ensure that $[K^+]_i = [K^+]_o$ during uptake measurements.

Materials. We purchased ^{22}Na from Amersham Corp. (Arlington Heights, IL); Percoll from Pharmacia Fine Chemicals (Piscataway, NJ); and valinomycin, 4,4′-diisothiocyanostilbene-2,2′-disulfonate (DIDS), amiloride and acetazolamide from Sigma Chemical Co. (St. Louis, MO). Valinomycin was dissolved in 95% ethanol and added to the membrane vesicles in a 1:100 dilution.

Results

In the first experiment (Fig. 1), we tested for a direct effect of acetazolamide on Na^+ influx stimulated by an inward HCO_3^- gradient. The inward HCO_3^- gradient was imposed by setting an inside-acid pH gradient in the presence of a CO_2/HCO_3^- buffer system. Different concentrations of acetazolamide were added to the extravesicular medium at the initiation of the 5-s uptake measurement and were also preincubated with the membrane vesicles for 120 min at room temperature to provide access to the intravesicular space. As illustrated, no significant inhibition resulted from acetazolamide concentrations ≤ 0.6 mM, and maximal inhibition at 1.2 mM drug was only 17%. These results suggest that acetazolamide has little effect as a direct inhibitor of the Na^+-HCO_3^- cotransport system in renal basolateral membrane vesicles.

We next considered the possibility that the inhibitory effect of acetazolamide on base exit from the proximal tubule cell as observed in the intact tubule might result from its ability to inhibit the carbonic anhydrase-mediated generation of HCO_3^- at the basolateral membrane of the cell. This would inhibit the process of base exit via the Na^+-HCO_3^- cotransporter if base transport via this system were highly HCO_3^--dependent. We therefore evaluated the HCO_3^--dependence of this transport system in basolateral membrane vesicles.

Shown in Fig. 2, imposing an inside-acid pH gradient in the absence of CO_2/HCO_3^- (*center group of three bars*) modestly stimulated Na^+ influx compared to that measured in the absence of a pH gradient (*right*). This suggested that Na^+-OH^-

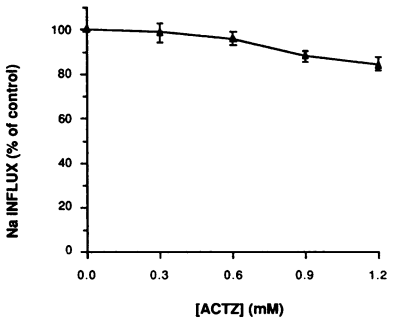

Figure 1. Effect of acetazolamide on HCO_3^- gradient-stimulated Na^+ influx. Basolateral membrane vesicles were preequilibrated for 120 min at 20°C in a medium consisting of 52 mM TMA-gluconate, 71 mM potassium-gluconate, 52 mM Mes, 42 mM Hepes, 21 mM TMA-OH, pH 6.0, gassed with 95% N_2, 5% CO_2. Uptake of 1 mM ^{22}Na was then assayed after 2 s incubation of the vesicles in a medium of final composition 52 mM TMA-gluconate, 43 mM potassium-gluconate, 28 mM $KHCO_3$, 42 mM mannitol, 5 mM Mes, 42 mM Hepes, 26 mM TMA-OH, pH 7.5, gassed with 95% N_2, 5% CO_2. The indicated concentrations of acetazolamide were added both to the preequilibration media and the incubation media. Values shown represent means±SE for three separate experiments performed in triplicate on different membrane preparations, and are expressed as percent of the control value measured in the absence of acetazolamide.

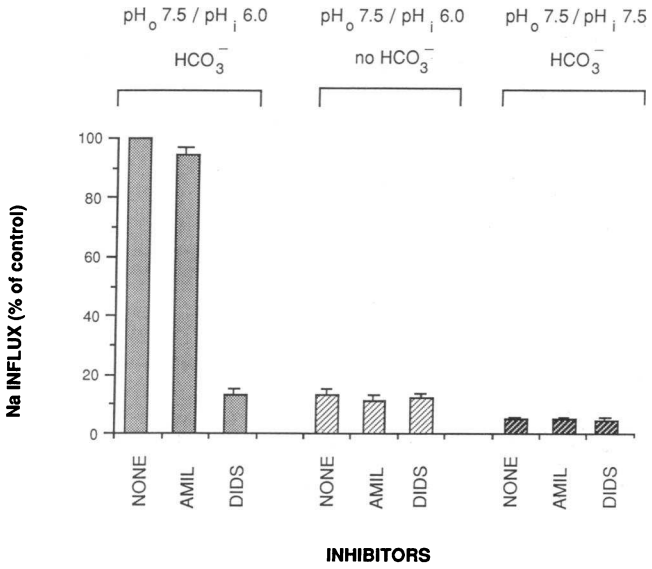

Figure 2. HCO_3^--dependence of DIDS-sensitive Na^+ influx. pH_0 7.5/pH_i 6.0: Basolateral membrane vesicles were preequilibrated for 120 min in a medium consisting of 52 mM TMA-gluconate, 71 mM potassium-gluconate, 52 mM Mes, 42 mM Hepes, 21 mM TMA-OH, pH 6.0, and gassed either with 100% N_2 (no HCO_3^-) or with 90% N_2, 10% CO_2 (HCO_3^-). Uptake of 2 mM ^{22}Na was then assayed after 5 s incubation of the vesicles in a medium of final composition 52 mM TMA-gluconate, 14 mM potassium-gluconate, 31 mM mannitol, 10 mM Mes, 42 mM Hepes, 31 mM TMA-OH, pH 7.5, that either contained an additional 57 mM potassium-gluconate and was gassed with 100% N_2 (no HCO_3^-), or contained 57 mM $KHCO_3$ and was gassed with 90% N_2, 10% CO_2 (HCO_3^-). pH_0 7.5/pH_i 7.5: Basolateral membrane vesicles were preequilibrated in a medium consisting of 52 mM TMA-gluconate, 57 mM $KHCO_3$, 52 mM mannitol, 42 mM Hepes, 21 mM TMA-OH, pH 7.5, gassed with 90% N_2, 10% CO_2. The 5-s uptake of ^{22}Na was then assayed in the same medium. As indicated, 1 mM amiloride hydrochloride or 1 mM disodium DIDS were added to the incubation media. A Na^+ concentration of 2 mM was achieved in the incubation media by appropriate addition of sodium gluconate. Values shown represent means±SE for three separate experiments performed in triplicate on different membrane preparations, and are expressed as percent of the control uptake measured under conditions of pH_0 7.5/pH_i 6.0, HCO_3^-. ^{22}Na uptake values under conditions of pH_0 7.5/pH_i 7.5 were not different whether performed in the presence or absence of CO_2/HCO_3^- (not shown).

cotransport might take place in these membrane vesicles. However, 1 mM DIDS, a potent inhibitor of the Na^+-HCO_3^- cotransport system (21), did not inhibit the component of Na^+ uptake stimulated by an inside-acid pH gradient in the absence of CO_2/HCO_3^-, suggesting that this component did not represent Na^+-OH^- cotransport via the Na^+-HCO_3^- cotransport system. In contrast, imposing the same inside-acid pH gradient in the presence of a CO_2/HCO_3^- buffer system greatly stimulated Na^+ uptake (*left*), and the HCO_3^--stimulated component of Na^+ influx was completely abolished by DIDS. Thus, DIDS-sensitive base-coupled Na^+ transport in basolateral membrane vesicles is absolutely HCO_3^--dependent. Finally, it may be noted in Fig. 2 that amiloride did not significantly inhibit Na^+ influx under any condition, confirming the absence of amiloride-sensitive Na^+-H^+ exchange in these membrane vesicles.

In view of the high degree of HCO_3^--dependence of the Na^+-HCO_3^- cotransport system, one would predict that acetazolamide-sensitive availability of HCO_3^- at the basolateral membrane could be rate-limiting for base transport under some conditions. To test this hypothesis, we measured the effect of acetazolamide on Na^+ influx under conditions of the experiment schematically illustrated in Fig. 3, in which the NH_4^+ pre-pulse method (29) was adapted to membrane vesicles. An outward NH_4^+ gradient was imposed in the presence of CO_2/HCO_3^- but in the absence of an initial pH or HCO_3^- gradient. The outward NH_4^+ gradient would acidify the intravesicular space by liberating H^+ as NH_3 diffuses down its concentration gradient. This in turn would consume internal OH^-, which would reduce internal HCO_3^-. The resulting inward HCO_3^- gradient would then stimulate Na^+ influx. If the rate of consumption of internal HCO_3^- is carbonic anhydrase-dependent, as shown in Fig. 3, then inhibition of carbonic anhydrase with acetazolamide should inhibit the generation of an inward HCO_3^- gradient and thereby inhibit the stimulation of Na^+ influx otherwise resulting from imposition of the outward NH_4^+ gradient.

The results of the experiment schematically illustrated in Fig. 3 are shown in Fig. 4. As predicted in the preceding paragraph, imposing an outward NH_4^+ gradient stimulated Na^+ influx greater than fivefold, reflecting the generation of an inward HCO_3^- gradient. This stimulation of Na^+ influx by the outward NH_4^+ gradient was minimally inhibited when 0.6 mM acetazolamide was added to the extravesicular medium only, but was inhibited > 75% when 0.6 mM acetazolamide was, in addition, preloaded into the vesicles by preincubation for 120 min. These findings indicate that acetazolamide can indeed interact with the Na^+-HCO_3^- cotransport system, in this case causing inhibition of influx by blocking the carbonic anhydrase-mediated generation of an inward HCO_3^- gradient.

Although acetazolamide has been most frequently characterized as an inhibitor of acid-base transport mechanisms, one would predict that under certain conditions its effects on HCO_3^- generation and consumption might actually lead to a stimulation of transport activity. An experiment to test this prediction is schematically illustrated in Fig. 5. An inward NH_4^+ gradient was imposed in the presence of an initial inside-acid pH gradient and inward HCO_3^- gradient. The inward NH_4^+ gradient would alkalinize the intravesicular space by consuming internal H^+ as NH_3 diffuses inward down its concentration gradient. This in turn would generate internal OH^-, which, in the presence of a CO_2/HCO_3^- buffer system, would generate internal HCO_3^-. The resulting collapse of the inward HCO_3^- gradient would then inhibit Na^+ influx. If the rate of generation of internal HCO_3^- is carbonic anhydrase-dependent, as shown in Fig. 5, then inhibition of carbonic anhydrase with

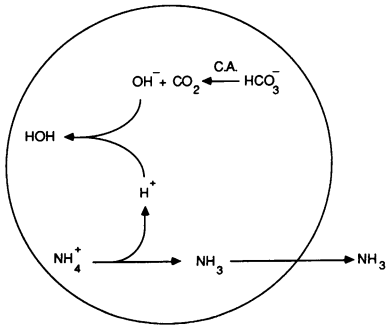

Figure 3. Use of an outward NH_4^+ gradient to generate an inward HCO_3^- gradient.

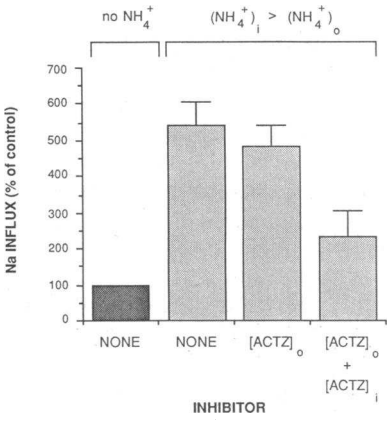

Figure 4. Effect of acetazolamide on Na^+ uptake in the presence of an outward NH_4^+ gradient. Basolateral membrane vesicles were preequilibrated for 120 min in a medium consisting of 52 mM TMA-gluconate, 86 mM potassium-gluconate, 28 mM choline-bicarbonate, 10 mM Hepes, 5 mM TMA-OH, pH 7.5, that contained in addition either 60 mM TMA-gluconate (no NH_4^+) or 60 mM NH_4^+- gluconate [$(NH_4^+)_i > (NH_4^+)_o$]. The uptake of 1 mM ^{22}Na was then assayed 1 s after incubation in a medium of final composition 106 mM TMA-gluconate, 86 mM potassium-gluconate, 28 mM choline-bicarbonate, 10 mM Hepes, 5 mM TMA-OH, pH 7.5, that contained in addition either 6 mM TMA-gluconate (no NH_4^+) or 6 mM NH_4^+-gluconate [$(NH_4^+)_i > (NH_4^+)_o$]. All media were gassed with 95% N_2, 5% CO_2. Acetazolamide (0.6 mM) was added either to the incubation medium only ($[ACTZ]_o$) or to both the preequilibration medium and the incubation medium ($[ACTZ]_o + [ACTZ]_i$). Values shown represent means±SE for three separate experiments performed in triplicate on different membrane preparations, and are expressed as percentage of the control measured in the absence of NH_4^+. For the condition $(NH_4^+)_i > (NH_4^+)_o$, $P < 0.01$ for $[ACTZ]_o + [ACTZ]_i$ vs. None or $[ACTZ]_o$ by use of the Scheffe F test.

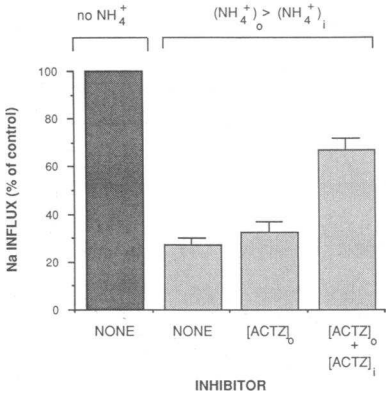

Figure 6. Effect of acetazolamide on Na^+ uptake in the presence of an inward NH_4^+ gradient. Basolateral membrane vesicles were preequilibrated for 120 min in a medium consisting of 131 mM TMA-gluconate, 73 potassium-gluconate, 45 mM mannitol, 6 mM Mes, 5 mM Hepes, 2.5 mM TMA-OH, pH 6.0. Uptake of 1 mM ^{22}Na was then assayed after 2 s incubation in a medium of final composition 73 mM potassium-gluconate, 28 mM choline-bicarbonate, 52 mM mannitol, 0.6 mM Mes, 5 mM Hepes, 3.1 mM TMA-OH, pH 7.5, that contained in addition either 103 mM TMA-gluconate (no NH_4^+), or 43 mM TMA-gluconate and 60 mM NH_4^+-gluconate [$(NH_4^+)_o > (NH_4^+)_i$]. All media were gassed with 95% N_2, 5% CO_2. Acetazolamide (0.6 mM) was added either to the incubation medium only ($[ACTZ]_o$) or to both the preequilibration medium and the incubation medium ($[ACTZ]_o + [ACTZ]_i$). Values shown represent means±SE for three separate experiments performed in triplicate on different membrane preparations, and are expressed as percentage of the control measured in the absence of NH_4^+. For the condition $(NH_4^+)_o > (NH_4^+)_i$, $P < 0.005$ for $[ACTZ]_o + [ACTZ]_i$ vs. None or $[ACTZ]_o$ by use of the Scheffe F test.

acetazolamide should inhibit the collapse of the inward HCO_3^- gradient and thereby release the inhibition of Na^+ influx otherwise resulting from imposition of the inward NH_4^+ gradient.

The results of the experiment schematically illustrated in Fig. 5 are shown in Fig. 6. As predicted in the preceding paragraph, imposing an inward NH_4^+ gradient inhibited Na^+ influx > 70%, reflecting the collapse of the inward HCO_3^- gradient. This inhibition of Na^+ influx by the inward NH_4^+ gradient was virtually unchanged when 0.6 mM acetazolamide was added to the extravesicular medium only, but was reduced to only 35% when 0.6 mM acetazolamide was, in addition, preloaded into the vesicles by preincubation for 120 min. Thus, in this experiment acetazolamide actually caused stimulation of influx via the Na^+-HCO_3^- cotransport system, as it blocked the carbonic anhydrase-mediated collapse of the initially imposed inward HCO_3^- gradient. These findings underscore the indirect nature of the interaction of acetazolamide with the Na^+-HCO_3^- cotransport system, as the drug can cause either inhibition or stimulation depending on the conditions with respect to pH and HCO_3^- gradients.

An important assumption underlying this approach is that the imposed NH_4^+ gradients have no direct effect on Na^+ transport other than via alterations in the transmembrane pH and HCO_3^- gradient. We tested this assumption in two ways. In the first approach, we evaluated the effects of NH_4^+ gradients on Na^+ transport in the presence of higher buffer capacity (Table II). In Fig. 4, an outward NH_4^+ gradient of 60 mM:6 mM was found to stimulate Na^+ uptake fivefold into vesicles preequilibrated with 10 mM Hepes and 28 mM HCO_3^- at pH 7.5. As shown in Table II, imposing the same outward NH_4^+ gradient failed to significantly stimulate Na^+ uptake into vesicles pre-

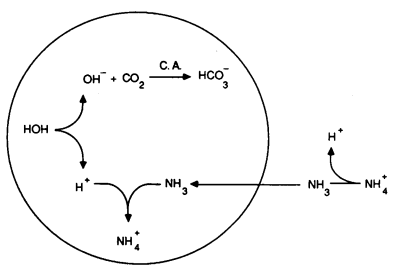

Figure 5. Use of an inward NH_4^+ gradient to collapse an inward HCO_3^- gradient.

Table II. Effects of NH_4^+ Gradients on Na^+ Uptake in the Presence of Increased Buffering Capacity

Gradient	Na^+ uptake
	% control
$(NH_4^+)_i > (NH_4^+)_o$	115.9±8.6
$(NH_4^+)_o > (NH_4^+)_i$	97.3±5.7

The experiments shown in Figs. 4 and 6 were repeated using media of higher buffer capacity. "$(NH_4^+)_i > (NH_4^+)_o$": Uptake of ^{22}Na was assayed as described for Fig. 4 except that the concentrations of Hepes, TMA-OH, and choline-bicarbonate were 52, 26, and 57 mM, respectively. Media were gassed with 90% N_2, 10% CO_2. "$(NH_4^+)_o > (NH_4^+)_i$": Uptake of ^{22}Na was assayed as described for Fig. 6 except that the concentrations of TMA-gluconate, Mes, Hepes, and TMA-OH in the preequilibration medium were 160 mM, 64 mM, 52 mM, and 26 mM, respectively; and the concentrations of mannitol, Mes, Hepes, TMA-OH, and choline-bicarbonate in the incubation media were 52, 6, 52, 32, and 57 mM, respectively. Media were gassed with 90% N_2, 10% CO_2. Each datum represents the mean±SE for three experiments performed in triplicate on different membrane preparations, and is expressed as percentage of the control Na^+ uptake measured in the absence of NH_4^+ under the same conditions.

equilibrated with 52 mM Hepes and 57 mM HCO_3^- at pH 7.5. Similarly, in Fig. 6, an inward NH_4^+ gradient of 60:0 mM was found to inhibit Na^+ uptake > 70% into vesicles preequilibrated with 5 mM Hepes and 6.5 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes) at pH 6.0. As shown in Table II, imposing the same inward NH_4^+ gradient failed to significantly inhibit Na^+ uptake into vesicles preequilibrated with 52 mM Hepes and 64 mM Mes at pH 6.0. Thus, the effects of NH_4^+ gradients on Na^+ transport were virtually abolished when the intravesicular buffering capacity was increased, strongly arguing against a direct interaction of NH_4^+ with the Na^+-HCO_3^- cotransport system.

Another approach was to use an acetate gradient rather than an NH_4^+ gradient to alter the transmembrane pH gradient. The protocol of an experiment to evaluate the effect of an outward acetate gradient on Na^+ uptake in the presence of an inward HCO_3^- gradient is schematically illustrated in Fig. 7. The outward acetate gradient would alkalinize the intravesicular space by consuming internal H^+ as undissociated acetic acid diffuses outward down its concentration gradient. This in turn would generate internal OH^-, which, in the presence of a CO_2/HCO_3^- buffer system, would generate internal HCO_3^-. The resulting collapse of the inward HCO_3^- gradient would then inhibit Na^+ influx. If the rate of generation of internal HCO_3^- is carbonic anhydrase-dependent, as shown in Fig. 7, then inhibition of carbonic anhydrase with acetazolamide should inhibit the collapse of the inward HCO_3^- gradient and thereby release the inhibition of Na^+ influx otherwise resulting from imposition of the outward acetate gradient.

The results of the experiment schematically illustrated in Fig. 7 are shown in Fig. 8. As predicted in the preceding paragraph, imposing an outward acetate gradient inhibited Na^+ influx > 80%, reflecting the collapse of the inward HCO_3^- gradient. This inhibition of Na^+ influx by the outward acetate gradient was virtually unchanged when 0.6 mM acetazolamide was added to the extravesicular medium only, but was reduced to only 50% when 0.6 mM acetazolamide was, in addition, preloaded into the vesicles by preincubation for 120 min. Thus, as previously shown in Fig. 6, acetazolamide again caused significant stimulation of influx via the Na^+-HCO_3^- cotransport system, as it blocked the carbonic anhydrase-mediated collapse of the initially imposed inward HCO_3^- gradient.

Discussion

Many studies have demonstrated that the carbonic anhydrase inhibitor acetazolamide reduces the rate of H^+ secretion and HCO_3^- reabsorption in the proximal tubule by at least 80% (1–5). Historically, major attention has focused on the role of luminal membrane carbonic anhydrase in catalyzing the breakdown of intratubular carbonic acid formed by the titration of filtered HCO_3^- with secreted H^+ (6–10). Thus, selective inhibition of externally facing, luminal membrane carbonic anhydrase by use of a dextran-bound, nonpenetrating carbonic anhydrase inhibitor reduces proximal HCO_3^- reabsorption by almost 80% and produces an acid disequilibrium pH, reflecting the accumulation of intratubular carbonic acid (30).

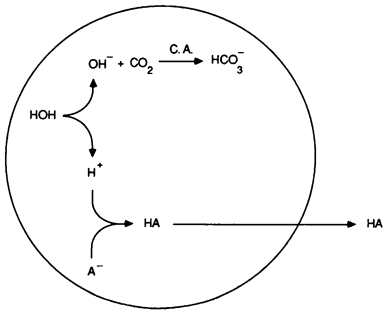

Figure 7. Use of an outward acetate gradient to collapse an inward HCO_3^- gradient.

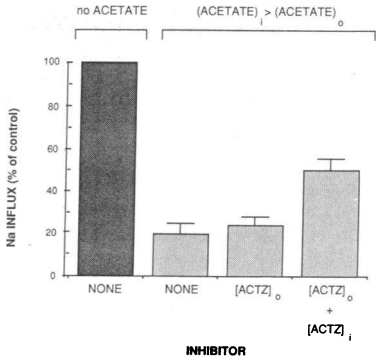

Figure 8. Effect of acetazolamide on Na^+ uptake in the presence of an outward acetate gradient. Basolateral membrane vesicles were preequilibrated for 120 min in a medium consisting of 31 mM potassium-gluconate, 72 mM TMA-gluconate, 30 mM mannitol, 6 mM Mes, 5 mM Hepes, 2.5 mM TMA-OH, pH 6.0, that contained in addition either 121 mM potassium-gluconate (no ACETATE), or 60 mM potassium-acetate and 61 mM potassium-gluconate [$(Acetate)_i > (Acetate)_o$]. The uptake of 1 mM ^{22}Na was assayed after 2 s incubation in a medium of final composition 97 mM potassium-gluconate, 28 mM $KHCO_3$, 72 mM TMA-gluconate, 37 mM mannitol, 0.6 mM Mes, 5 mM Hepes, 3.1 mM TMA-OH, pH 7.5, that contained in addition either 6 mM potassium gluconate (no Acetate), or 6 mM potassium-acetate [$(Acetate)_i > (Acetate)_o$]. All media were gassed with 95% N_2, 5% CO_2. Acetazolamide (0.6 mM) was added either to the incubation medium only ($[ACTZ]_o$) or to both the preequilibration medium and the incubation medium ($[ACTZ]_o + [ACTZ]_i$). Values shown represent means±SE for three separate experiments performed in triplicate on different membrane preparations, and are expressed as percentage of the control measured in the absence of acetate. For the condition $(Acetate)_i > (Acetate)_o$, $P < 0.03$ for $[ACTZ]_o + [ACTZ]_i$ vs. None or $[ACTZ]_o$ by use of the Scheffe F test.

However, histochemical and immunocytochemical studies have demonstrated that carbonic anhydrase is localized not only at the luminal membrane but also at the basolateral membrane and in the cytoplasm of the proximal tubule cell (see reference 31 for review). Moreover, carbonic anhydrase activity can be measured in both luminal and basolateral membrane vesicles isolated from the renal cortex (32). At least three sets of observations suggest that cytoplasmic and/or basolateral membrane carbonic anhydrase plays an important functional role in proximal tubule acid/base transport. First, acetazolamide substantially inhibits acid secretion even when the proximal tubule is microperfused with nonbicarbonate buffers (33). In this circumstance, breakdown of intratubular carbonic acid cannot be rate-limiting for acid secretion, suggesting that the inhibitory effect of acetazolamide must arise at a site other than the external face of the luminal membrane. Second, in contrast to the acid disequilibrium pH that results from an impermeant carbonic anhydrase inhibitor, a permeant carbonic anhydrase inhibitor inhibits HCO_3^- reabsorption without generating an acid disequilibrium pH (30). This indicates that the permeant inhibitor must substantially block the process of H^+ secretion across the luminal membrane, thereby preventing the accumulation of intratubular carbonic acid. Third, acetazolamide added to the capillary or peritubular bath inhibits the electrogenic transfer of HCO_3^- across the basolateral membrane (11, 12).

In this study, we examined the effects of acetazolamide on Na^+-HCO_3^- cotransport in renal cortical basolateral membrane vesicles. We found no appreciable direct effect of acetazolamide on this transport system. An important observation, however, was that the DIDS-sensitive transport of Na^+ via this system was absolutely CO_2/HCO_3^--dependent. Similar CO_2-dependence of basolateral Na^+-base cotransport has also been observed in the intact proximal tubule (34). Based on this observation, we tested whether acetazolamide could indirectly affect the rate of Na^+-HCO_3^- cotransport by virtue of its ability to inhibit the carbonic anhydrase-mediated generation or consumption of HCO_3^- at the basolateral membrane. This was indeed the case, as the drug caused either inhibition or stimulation of Na^+-HCO_3^- cotransport depending on the conditions with respect to pH and HCO_3^- gradients.

In our experiments, the effects of acetazolamide on Na^+-HCO_3^- cotransport were evident only when the vesicles were preloaded with acetazolamide and not when acetazolamide was present exclusively in the extravesicular medium. Based on the fact that the basolateral membrane vesicle preparation was found to have a predominantly right-side-out orientation, one interpretation of these findings is that the effects of acetazolamide resulted from inhibition of either cytoplasmic carbonic anhydrase trapped within the vesicles or carbonic anhydrase located on the internal surface of the basolateral membrane. However, the lack of effect of extravesicular acetazolamide does not necessarily indicate that there is no carbonic anhydrase present on the external surface of the basolateral membrane of the proximal tubule. Under the conditions of the experiments reported here, the intravesicular volume is < 1% of the extravesicular volume. Accordingly, transmembrane gradients of NH_4^+ or acetate would be expected to cause far larger changes in pH and $[HCO_3^-]$ in the intravesicular compartment than in the extravesicular compartment. Thus, our experiments would only have detected effects of carbonic anhydrase within the intravesicular compartment.

Under physiologic conditions, the likely function of carbonic anhydrase at the inner surface of the basolateral membrane is to facilitate the conversion of base from OH^-, which cannot be transported via the Na^+-HCO_3^- cotransport system, to HCO_3^-, which is readily transported. In addition, any carbonic anhydrase present on the external surface of the basolateral membrane may also play a functional role in facilitating the dissipation of the HCO_3^- accumulated outside the basolateral membrane, particularly if the peritubular interstitium is a poorly mixed compartment. By virtue of these actions, carbonic anhydrase would greatly facilitate the exit of base from the proximal tubule cell via the Na^+-HCO_3^- cotransport system. Inhibition of this process by acetazolamide would be expected to alkalinize the proximal tubule cell, as has actually been observed (35, 36). This in turn would result in inhibition of luminal membrane H^+ secretion. Thus, the indirect interaction of acetazolamide with the basolateral membrane Na^+-HCO_3^- cotransport system may be an important mechanism underlying inhibition of proximal tubule acid secretion by this agent. Similar effects may explain the inhibitory action of acetazolamide on acid/base transport in other epithelia in which Na^+-HCO_3^- cotransport has been described (24–26).

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