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ANION TRANSPORT REGULATES INTRACELLULAR pH IN RENAL CORTICAL TISSUE

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The regulation of cell pH by anion transport was examined in suspensions of rabbit renal proximal tubules. Values for cell pH were derived from ^{14}C-labeled 5,5-dimethyloxazolidine-2,4-dione distribution. In buffer with 10 mM/l HCO_3^- and gassed with 95% O_2/5% CO_2, the anion transport inhibitors, 4-acetamido-4′-isothiocyano-2,2′-disulfonic stilbene and furosemide, raised the cell-to-extracellular pH gradient from 0.23 ± 0.02 to 0.31 ± 0.02 and 0.31 ± 0.03, respectively, but in combination their effects were not additive. Replacement of extracellular Cl^- by NO_3^- raised the pH gradient from 0.24 ± 0.04 to 0.37 ± 0.05. Neither inhibitor raised the pH gradient in Cl^--free media. Incubation of suspensions in HCO_3^-- and CO_2-free media raised the pH gradient from 0.18 ± 0.02 to 0.29 ± 0.03. Removal of Cl^- in addition to HCO_3^- and CO_2 raised the pH gradient still further, to 0.36 ± 0.02. The results demonstrate that two different anion transport inhibitors raise cell pH and the cell-to-extracellular pH gradient in proximal tubules and are consistent with the idea that the mechanism for this effect is inhibition of alkali anion exit from the tubule cell. This process appears to depend on extracellular Cl^- and probably occurs primarily by HCO_3^- transport. The results support the concept that alkali anion transport, most probably HCO_3^- exit from the peritubular cell border, is an important regulator of cell pH in renal proximal tubule.

Introduction

In renal tissue, cell pH and HCO_3^- activity have been found to be higher than values for other tissues measured in a similar way [1–8]. This has been demonstrated in suspensions of renal tubules from dog, rat and rabbit by indicator techniques and in *Necturus* proximal tubule in vivo by microelectrode. These values are far from electrochemical equilibrium and fall in response to deoxygenation or treatment of the tissue with ouabain [3,9]. The carbonic anhydrase inhibitor, acetazolamide, and the anion transport inhibitor, 4-acetamido-4′-isothiocyano-2,2′-disulfonic stilbene, both increase intracellular alkalinity [3].

We have interpreted the relative intracellular alkalinity in renal cortex to be a consequence of active secretion of H^+ by renal proximal tubule cells [3]. Furthermore, we have proposed that the maneuvers which decrease cellular alkalinity do so, at least in part, by inhibiting active H^+ secretion and that maneuvers which raise intracellular pH interfere with the disposition of intracellular alkali and bicarbonate.

The purpose of the current studies was to examine the role of anion transport in the regulation of cell pH with a view towards identifying those processes important in the exit of alkali from the cell. The results of these studies support the hypothesis that the transport of HCO_3^- out of the cell regulates cell pH to an important degree.

Methods

Cortical renal tubule fragments were prepared by enzymatic disaggregation of kidneys from New Zealand White rabbits as described previously [3].

Briefly, the kidneys were perfused in situ with phosphate-buffered saline to clear them of blood, removed from the animal, the cortex dissected out and incubated in the same buffered saline containing 1 mg/ml of Pronase E, a non-specific protease derived from *Streptomyces griseus* (EM Laboratories, Inc., Elmsford, NY). The mixture was bubbled with 95% O_2/5% CO_2 and mixed by magnetic stirrer at room temperature. At 20-min intervals the mixture was allowed to settle for 2–3 min. The material remaining in suspension was subjected to a shearing force by being drawn into and ejected from a syringe fitted with large bore plastic tubing. Tissue was removed from the enzyme by centrifugation and washing. This material and that accumulated from two additional 20-min incubations of the original mixture were combined and filtered through gauze to remove aggregates. The disaggregated tubules and cells were made up to a suspension with a packed cell volume of 6–9% with buffer of the following composition (mM): Na^+ (140); K^+ (5), Ca^{2+} (2.5), Mg^{2+} (1) HCO_3^- (10), Cl^- (134), $HPO_4^{2-}/H_2PO_4^-$ (3) and SO_4^{2-} (1). The enzyme solutions, washes, and final incubation medium contained 1 gm/dl albumin and 5.5 mM D-glucose. In Cl^--removal experiments, NO_3^- replaced the Cl^-, mequiv. for mequiv. The suspensions were incubated at 37°C and gassed with 95% O_2/5% CO_2, resulting in media pH values of 7.05–7.12. These conditions were chosen to provide reliably measurable cell-to-extracellular pH gradients [3].

The suspensions used in the cell pH studies were allowed to equilibrate for 30 min, during which large cell aggregates were removed. In the Cl^--free studies aliquots of suspension were centrifuged, washed twice and resuspended in the Cl^--free medium. The remaining suspension was divided into equal aliquots. Thus, control and experimental samples were provided for each experiment. 2 μCi of ^{14}C-labeled 5,5-dimethyloxazolidine-2,4-dione and 5 μCi of ^{3}H-labeled poly(ethylene glycol) were added to each aliquot of suspension. The various pharmacologic agents studied were also added at this time and incubation proceeded for the indicated time periods.

At the end of the incubation period, multiple 1 ml portions of each suspension aliquot were rapidly centrifuged in the cold in capped tubes. The tissue plugs obtained were weighed rapidly, allowed to dry overnight at room temperature, then heated to 60°C for 2 h, and reweighed. Tissue samples were prepared for liquid scintillation counting by solubilization in 1 M NaOH and heating to 60°C for 1 h. Concentrations of ^{14}C and ^{3}H in tissue extracts and supernatant were determined by liquid scintillation counting. Quench correction was obtained by automatic counting of an external standard through each sample and a set of quenched standards of known activity. Cell pH was calculated from the distribution of ^{14}C-labeled 5,5-dimethyloxazolidine-2,4-dione according to the method of Waddell and Butler [10]. Cell water was derived by correction of the wet weight-dry weight difference (total tissue water) for ^{3}H content (trapped supernatant). External, pH, as used herein, is the pH of the suspension at the time of sampling determined by a pH electrode. Each value for cell pH is the mean of 5–8 samples. Results were analyzed by a paired Student's *t*-test, and statistical significance was taken as $P < 0.05$. Results are expressed as means ±1 S.E.

Cell pH values and gradients determined in this way varied somewhat from preparation to preparation, even under the same buffer and gassing conditions. The mean cell-to-extracellular pH gradient of the control points from all the experiments reported in this and a previously published study [3] was 0.2 units; the standard deviation, 0.05 units. However, in any given study, the standard deviation of the values in each experimental condition was generally less than 0.02 units. Thus, this method for determining cell pH would appear to be able to detect changes of as little as 0.05 pH units.

The suspensions used in these studies were composed predominantly of cells and tubule fragments derived from renal proximal tubules. This was assessed by phase-contrast microscopy and light microscopy of sections stained with periodic acid-Schiff reagent as reported previously [3]. This preparation retains its cellular integrity and is metabolically active for several hours.

Results

Similar to what we have reported previously [3], under the acid-base conditions used in the current experiments the interior of the cell is alkaline compared to the medium; the cell-to-extracellular pH gradient was approximately 0.2 pH units.

Previously, the anion transport inhibitor,

TABLE I

EFFECT OF ANION TRANSPORT INHIBITORS ON CELL pH

None of the experimental groups was statistically different from the others in any parameter. Control and experimental suspensions from the same animals were incubated in parallel with 5% CO_2 and 10 mM HCO_3^-; period of exposure to drugs was 60 min. Concentration of 4-acetamido-4′-isothiocyano-2,2′-disulfonic stilbene (SITS) was 0.2 mM; concentration of furosemide was 3 mM. pH_c, cell pH; pH_e, extracellular pH; ΔpH, $pH_c - pH_e$. Values are means ± S.E., $n = 5$.

	Control	SITS	Furosemide	SITS and furosemide
pH_c	7.34 ± 0.02	7.43 [a] ± 0.02	7.42 [a] ± 0.03	7.43 [a] ± 0.02
pH_e	7.11 ± 0.02	7.12 ± 0.02	7.11 ± 0.02	7.12 ± 0.02
ΔpH	0.23 ± 0.02	0.31 [a] ± 0.02	0.31 [a] ± 0.03	0.31 [a] ± 0.01

[a] Significantly different from control, $P < 0.05$.

4-acetamido-4′-isothiocyano-2,2′-disulfonic stilbene, has been shown to raise cell pH [3]. Table I shows the results of a group of experiments in which tubule suspensions were exposed to this agent and the anthranilic acid, furosemide, for 60 min. Confirming the results previously reported, the disulfonic stilbene alone raised cell pH, the cell-to-extracellular gradient increasing from 0.23 to 0.32 pH units. The results of these experiments also indicate that the chemically unrelated anion transport inhibitor, furosemide, raises cell pH to a similar degree. The last column of the table reports the effect on cell pH of the suspension of exposure to both agents simultaneously. The effects of these two transport inhibitors on cell pH were not additive; the values for cell pH and pH gradient were not significantly different from those with each agent alone.

The effect on cell pH of removal of the major extracellular anion, Cl^-, by substitution with NO_3^- is shown in Table II. In Cl^--free medium, cell pH was increased by approximately 0.13 units. The effect of each of the transport inhibitors, the disulfonic stilbene and furosemide, in the absence of media Cl^- is also shown in Table II. These agents both failed to raise cell pH in the Cl^--free medium.

In order to examine the effect of altering the alkali anion composition of the cell on cell pH, experiments were performed in which the regular medium was replaced by one containing no exogenous CO_2 or HCO_3^- (replaced with Cl^-), one containing no Cl^- (described previously) or one containing neither CO_2/HCO_3^- nor Cl^- (all replaced by NO_3^-). CO_2/HCO_3^--free incubations were gassed with 100% O_2. The results of these experiments are reported in Table III. Removal of exogenous CO_2 and HCO_3^- raised cell pH; Cl^- removal increased cell pH to a similar degree in this group of studies. The combination of HCO_3^-- and CO_2-free conditions and Cl^- removal were additive;

TABLE II

EFFECTS OF Cl^- REMOVAL AND ANION TRANSPORT INHIBITORS ON CELL pH

Control and experimental suspensions from the same animal were incubated in parallel with 5% CO_2 and 10 mM HCO_3^-; period of exposure to Cl^--free medium or drugs was 60 minutes. Concentration of 4-acetamido-4′-isothiocyano-2,2′-disulfonic stilbene (SITS) was 0.2 mM; concentration of furosemide was 3 mM. Values are mean ± S.E., $n = 5$.

	Control	Cl^--free	Cl^--free and SITS	Cl^--free and furosemide
pH_c	7.36 ± 0.02	7.47 [a] ± 0.03	7.45 [a] ± 0.02	7.37 [b] ± 0.02
pH_e	7.12 ± 0.02	7.10 ± 0.03	7.10 ± 0.03	7.07 [b] ± 0.03
ΔpH	0.24 ± 0.04	0.37 [a] ± 0.05	0.35 [a] ± 0.03	0.30 [a,b] ± 0.04

[a] Significantly different from control, $P < 0.05$.
[b] Significantly different from Cl^--free, $P < 0.05$.

TABLE III

EFFECT OF ANION SUBSTITUTIONS ON CELL pH

Control suspensions were incubated with 5% CO_2 and 10 mM HCO_3^-; CO_2/HCO_3^--free suspensions had HCO_3^- replaced by Cl^- and were gassed with 100% O_2; Cl^--free suspension had Cl^- replaced by NO_3^-; CO_2/HCO_3^-- and Cl^--free suspensions had both anions replaced by NO_3^-. The period of exposure to each condition was 60 min. Values are mean ± S.E., $n = 5$.

	Control	CO_2/HCO_3^--free	Cl^--free	CO_2/HCO_3^-- and Cl^--free
pH_c	7.28 ± 0.01	7.39 [a] ± 0.01	7.35 [a] ± 0.02	7.46 [a,b] ± 0.01
pH_e	7.10 ± 0.02	7.10 ± 0.02	7.11 ± 0.02	7.10 ± 0.02
δpH	0.18 ± 0.02	0.29 [a] ± 0.03	0.24 [a] ± 0.01	0.36 [a,b] ± 0.02

[a] Significantly different from control, $P < 0.05$.
[b] Significantly different from both Cl^--free and CO_2/HCO_3^--free, $P < 0.05$.

cell pH and the cell-to-extracellular pH gradient were higher than with either maneuver alone.

Discussion

We have previously utilized 5,5-dimethyloxazolidine-2,4-dione distribution to assess directional changes in overall cell pH in suspensions of rabbit renal proximal tubules [3]. The anion transport inhibitor 4-acetamido-4′-isothiocyano-2,2′-disulfonic stilbene was shown to increase cell pH. We proposed that this agent increased cell pH by blocking cellular exit of alkali anion, specifically HCO_3^-. This agent and related compounds have been demonstrated to block SO_4^{2-}, Cl^- and HCO_3^- exchange in red blood cells [5,11–13]. In renal proximal tubule, the peritubular application of the disulfonic stilbene has been reported to inhibit reabsorption of glycodiazine and decrease permeability to HCO_3^- [14,15]. In turtle urinary bladder this substance has been shown to decrease H^+ secretion and increase cell pH [16]. Of interest is that in this tissue 4-acetamido-4′-isothiocyano-2,2′-disulfonic stilbene appears to specifically inhibit the exit of alkali generated in series with the H^+ pump [17]. Cation transport, specifically Na^+-H^+ exchange in renal brush border membrane vesicles, is not affected by this compound [18]. To further confirm that inhibition of anion transport can increase cell pH, we examined the effect of furosemide. This agent has been shown to inhibit Cl^- transport in thick ascending loop of Henle and fluid absorption in proximal tubule and intestine [19–22]. 1–5-mM furosemide also inhibits Cl^- and SO_4^{2-} transport in red cells and Ehrlich ascites cells [23,24]. This agent is also chemically dissimilar to the disulfonic stilbene, has a number of metabolic effects in renal tubule cells (which may be secondary to its effects on ion transport) and is a weak carbonic anhydrase inhibitor [6,25–28]. These facts notwithstanding, it is apparent that both agents raise cell pH and that their effects are not additive. This is consistent with the idea that a common property, such as inhibition of anion transport, is responsible for the increase in cell pH produced by them.

Several facilitated diffusion systems for OH^- or HCO_3^- exit from a number of cell types have been identified [13,14,16,17,29]. In some cases these mechanisms have been shown to involve identifiable membrane protein components [5,30]. Both anion exchange and electrogenic alkali transfer have been described, sometimes in the same tissue, as in the case of turtle urinary bladder. Considering these systems, replacement of media Cl^- by NO_3^- in the current studies could raise renal tubule cell pH by either of two mechanisms. Substitution of an anion that is a less satisfactory substrate for a Cl^--HCO_3^- exchange mechanism could lead to alkali accumulation within the cell and thus raise cell pH. Such an anion-exchange system has been postulated along the peritubular cell borders of *Necturus* proximal tubules [31]. Alternatively, provision of an anion to which renal proximal tubule cells exhibit lower permeability compared to Cl^- can result in a less highly negative cell membrane potential and thus decrease the driving force for HCO_3^- exit. Electrophysiological measurements have suggested that peritubular cell membrane

of proximal tubule cells are less permeable to NO_3^- than to Cl^- [32]. The results of the present experiments do not allow for a choice between these two hypotheses. Whatever the mechanism involved, however, alkali exit appears to depend on extracellular Cl^-.

Since both the anion transport inhibitors used in these studies are also known to inhibit anion transport processes of several cell types [6,11–14,23,24, 31], we examined their effects on cell pH in the absence of media Cl^-. Since these agents failed to raise cell pH in Cl^--deficient media, we conclude that they and Cl^- removal act on a common locus to inhibit alkali exit. Cell pH in suspensions exposed to furosemide in the Cl^--free medium was actually lower than with Cl^- removal alone (Table II, second and fourth columns). We would suggest that, in a situation where alkali exit is already inhibited by Cl^- removal, lowering of cell pH may be due to the inhibition of renal cortical oxidative metabolism observed with this agent [25]. Indeed, inhibition of oxidative metabolism by deoxygenation has been demonstrated to lower cell pH in rabbit renal tubule suspensions [3].

It is difficult to determine whether alkali exit from the renal tubule cells occurs by OH^- or HCO_3^- transport since CO_2 is always present in an aqueous medium. If OH^- could exit the cell as easily as HCO_3^-, however, then the shift in the relative rates of production of OH^- and HCO_3^- within the cell resulting from the removal of HCO_3^- from the medium and gassing with 100% O_2 would not be expected to alter cell pH. In fact, cell pH rose with this maneuver, consistent with the idea that alkali exits predominantly in the HCO_3^- form. Replacement of Cl^- with NO_3^- in addition to the HCO_3^- and CO_2-free condition produced an additional alkalinization, presumably by interference with residual HCO_3^- exit. The results are consistent with the idea that the primary substrate for the alkali exit mechanism is HCO_3^- rather than OH^-. We have no direct evidence for the site of this process. However, since luminal surface HCO_3^- permeability is quite low [33], we suggest that it probably takes place along the peritubular cell border.

In summary, the results demonstrate that anion transport inhibitors of diverse properties raise cell pH in renal proximal tubules, probably by inhibition alkali exit out of cells. Alkali exit, furthermore, appears to depend on extracellular Cl^-, and appears to occur primarily by HCO_3^- transport. The results of these studies support the concept that cell pH in renal proximal tubules is regulated to a measurable degree by alkali anion transport, most probably the exit of HCO_3^- from the peritubular cell surface.

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