Inhibition of the Bicarbonate Exit Step in Urinary Acidification by a Disulfonylic Stilbene

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A B S T R A C T Acidification of the luminal solution by the isolated turtle bladder involves H⁺ secretion by a pump at the luminal membrane. The OH⁻ dissociated in this process reacts with CO₂ and forms HCO₃⁻ which moves passively out of the cell across the serosal cell membrane. In the present study, this exit step for HCO₃⁻ was inhibited by serosal addition of the disulfonylic stilbene, SITS, an agent which is thought to bind to a transport protein at the serosal cell membrane. 90 min after serosal addition of 0.5 mM SITS, H⁺ secretion decreased by > 80%. In contrast, luminal addition of SITS had no effect. During inhibition of H⁺ secretion by serosal SITS, overall cell pH, measured by the 5,5-dimethyl-2,3-oxazolidinedione method, increased from 7.48±0.03 to 7.61±0.02. This increase of 0.13±0.02 pH U was associated with a much larger regional pH increase as judged from the decrement in the attainable pH gradient across the epithelium. After serosal SITS, this gradient was reduced from 2.88±0.06 to 2.09±0.11 pH U. In the absence of evidence for increased H⁺ permeability or a change in the force of the H⁺ pump, the gradient decrement of 0.79±0.08 U reflects a similar pH increment on the cytoplasmic side of the pump.

SITS inhibits the exit of bicarbonate across the serosal cell membrane and, thereby, creates a compartment of high alkalinity in series with the pump. The increased electrochemical gradient across the active transport pathway is the primary factor in the inhibition of urinary acidification.

INTRODUCTION

Several lines of evidence suggest that the isolated turtle bladder acidifies the solution bathing its luminal surface by a process of active H⁺ secretion located at the luminal membrane (3–6). The solution bathing the serosal surface of the epithelium is alkalinized at almost the same rate (3, 6). From what is known about the electrochemical profile across the epithelium (4, 5), the movement of alkali from the cell interior to the serosal solution occurs passively down an electrochemical gradient. The hydroxyl ions generated behind the pump are buffered by CO₂ and rapidly form HCO₃⁻ in a carbonic anhydrase catalyzed reaction. The HCO₃⁻ formed this way is thought to move across the basolateral membrane with little resistance, the luminal cell membrane being the major acid-base barrier.

The present investigation deals with the effects of a disulfonylic stilbene, SITS, on the two cell membranes across which the transport processes of urinary acidification take place. It is shown that SITS, an agent which inhibits anion transport in a variety of tissues, inhibits acidification when added to the serosal but not to the luminal side of the epithelium. The mechanism by which serosal addition of SITS inhibits acidification is explored. These studies indicate that SITS inhibits the exit step for HCO₃⁻ transport into the serosal solution and that this inhibition leads to increased alkalinity in a cellular compartment in series with the active transport pathway.

METHODS

Urinary bladders from the freshwater turtle, Pseudemys scripta, were mounted in lucite chambers as previously described (3). All experiments were performed in the short-circuited state by voltage clamping except for brief periods when the open-circuit potential difference was recorded. Bladders were bathed on both sides with a NaCl Ringer's solution of the following composition in millimoles per liter:

1Abbreviations used in this paper: DMO, 5,5-dimethyl-2,3-oxazolidinedione; Jₘ, rate of H⁺ secretion; SITS, a disulfonylic stilbene.

2 For the purposes of this study, we refer to the anion moving out of the cell as HCO₃⁻. It is possible that some OH⁻ dissociated in the process of acidification is transported as such from cell to serosal solution.
NaCl, 114.4; KCl, 3.5; MgCl₂, 0.5; Na₂HPO₄, 2.0; CaCl₂, 1.8. In most experiments, the solutions were bubbled with air which had passed through 3-M KOH traps to remove all ambient CO₂, and the rate of H⁺ secretion (Jₜₜ) was measured by pH stat titration (3) with both media maintained at pH 7.0. In some experiments in which sodium transport was abolished by 0.5 mM ouabain, the serosal gas phase was 5% CO₂ in air, and Jₜₜ was measured as the short-circuit current. In these experiments, the serosal pH was maintained at 5.8 to keep the HCO₃⁻ concentration low. Previous studies have shown that the short-circuit current measures Jₜₜ in bladders in which sodium transport is abolished by ouabain both in the absence (6) and presence (7) of exogenous CO₂.

After a stable Jₜₜ was established by either of the two methods, 4-acetamido-4'-isothiocyanato-2,2'-disulfonic stilbene, SITS (Polysciences, Inc., Warrington, Pa.), was added to the serosal or luminal solution in the dosage indicated. The SITS was freshly dissolved in the Ringer's solutions after the control periods.

In another series of bladders, the effect of SITS was examined from the distribution of the weak acid [¹⁴C]5,5-dimethyl-2,3-oxazolidinedione ([¹⁴C]DMO). Isotopic compounds [¹⁴C]DMO and [¹⁴C]inulin were obtained from New England Nuclear, Boston, Mass., and were counted in a Beckman liquid scintillation counter, model LS-250 (Beckman Instruments, Inc., Fullerton, Calif.). Hemibladders from the same turtle were bathed in NaCl Ringer's to which [¹⁴C]DMO was added. Serosal pH was held at 7.40±0.01 by hand titration with 0.01 N HCl or 0.01 N NaOH. Luminal pH was kept at 7.40 by pH stat titration. After a control Jₜₜ was established for a minimum of 2 h, SITS was added to the serosal solution of one hemibladder, the other serving as a control. The bladders were removed from the chambers and blotted dry with filter paper 30 min after the addition of SITS. They were weighed before and after drying at 100°C in a Precision Thelco oven (Scientific Company, Chicago, Ill.), for at least 3 h to determine tissue water. With this oven, dry weight was achieved by 90 min. Tissue counts were extracted in 0.01 N HNO₃ for 15 h. To insure that the extraction was complete, a series of bladders was solubilized with 1 M KOH, and the residual tissue counts were measured. A quench correction was made by adding a known number of counts to the dis- solved tissue. This maneuver verified that the extraction from the tissue was complete.

For measurement of the extracellular space, [¹⁴C]inulin was used in a separate series of bladders handled in an identical manner. The inulin space in control halves was not different from SITS-treated bladder halves, 41.7±4.7 and 43.0±2.8%, respectively.

The intracellular pH was calculated according to Waddell and Butler (8) with a DMO pH extrapolated to 6.27 at 23°C and an inulin space of 42.4%. A validation of the DMO technique for the estimation of overall intracellular pH in the turtle bladder has been given (4). Evidence was presented that DMO is not actively transported nor significantly bound to cell constituents in the tissue. As has been considered previously, in a multicompartment system with a heterogenous pH (4) the distribution of a weak acid is determined by the mean OH⁻ concentration. All values are given as mean±SEM for a surface area of 8 cm² of exposed bladder.

**RESULTS**

**Effect of serosal and luminal addition of SITS on H⁺ secretion (Jₜₜ) in the absence of exogenous CO₂ and HCO₃⁻.** Table I shows how the serosal addition of 0.5 mM SITS causes a progressive inhibition of Jₜₜ as measured by pH stat titration. Jₜₜ was inhibited by 38% at 30 min and by 84% at 90 min in six experiments carried out in the absence of exogenous CO₂.

In contrast to the inhibition produced by serosal SITS, luminal addition had no effect on the rate of pH stat titration. In five bladders, the control Jₜₜ was 0.76±0.06 μmol/h. 90 min after luminal addition of 0.5 mM SITS, the Jₜₜ was 0.82±0.10 μmol/h.

**Effect of serosal SITS on the short-circuit current in ouabain-treated bladders in the presence of CO₂.** Since Jₜₜ was limited by the availability of CO₂ in the pH stat studies, the effects of serosal SITS were also examined at high rates of secretion in the presence of exogenous CO₂. Schwartz (7) has shown that Jₜₜ reaches near maximal levels when 5% CO₂ is added to the serosal gas phase. In six bladders in which sodium transport was inhibited by 0.5 mM ouabain Jₜₜ was measured as the short-circuit current with 5% CO₂ in the serosal gas phase. Serosal HCO₃⁻ concentrations were maintained low by keeping the serosal pH at 5.8. Serosal addition of 0.5 mM SITS reduced Jₜₜ from 1.82±0.30 to 0.35±0.07 μmol/h after 90 min. This 81% inhibition of Jₜₜ in the presence of CO₂ was close to the 84% inhibition observed in the CO₂-free system (Table I).

**Transient reversal of SITS inhibition by exogenous CO₂.** Since SITS inhibits Jₜₜ only from the serosal side and SITS is thought to inhibit anion transport by binding to a transport protein at the cell membrane, it is postulated that SITS interferes with the anion exit step of urinary acidification which consists of the movement of HCO₃⁻ from cell to serosal solution. The accumulation of HCO₃⁻ within the cell would inhibit the H⁺ pump. To explore this possibility, Jₜₜ was inhibited by serosal SITS in four ouabain-treated bladders in the absence of exogenous CO₂. When Jₜₜ was inhibited by

<table>
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<th>TABLE I</th>
<th>Inhibition of H⁺ Secretion by Serosal Addition of SITS</th>
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<td>Control</td>
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L. H. Cohen, A. Mueller, and P. R. Steinmetz
about 80%, the serosal gas phase was changed to 5% CO₂ in air, and serosal pH was maintained at 5.8 to avoid the introduction of HCO₃⁻. One of the experiments is shown in Fig. 1. The addition of CO₂ caused a partial but transient restoration of Jₜ. The initial stimulation of Jₜ suggests that the pump was capable of increasing its transport rate when the cellular alkalinity was reduced by CO₂. The failure of CO₂ to sustain Jₜ can be attributed to the accumulation of newly generated HCO₃⁻ which cannot pass through the exit sites blocked by SITS. If this interpretation is correct, cell pH should increase after SITS. Therefore, we estimated the apparent cell pH by means of the DMO technique.

Effect of inhibition of Jₜ on overall cell pH. In Table II, values for the apparent cell pH are given for seven experiments in which bladder halves exposed to SITS were compared with control halves of the same bladder. The apparent cell pH in the control halves was 7.48, a value comparable to that obtained in a previous study (4). In the bladder halves exposed to SITS, Jₜ was inhibited by about 60% after 30 min and in each case, the cell pH was increased over that of the control half. Although the average increment of 0.13 ±0.02 pH U was small, it was highly significant.

The DMO method provides an estimate of the average cellular OH⁻ concentration. The apparent pH value of 7.61±0.02 obtained during inhibition of Jₜ by SITS could reflect a larger increase in regional OH⁻ concentration.

Effect of serosal SITS on the maximal pH gradient. If SITS causes an accumulation of OH⁻ behind the pump, it would be expected to reduce the maximal concentration gradient for H⁺ that can be generated across the epithelium. In Table III, the maximal pH gradient, (ΔpH)ₐ=0, was measured before and after serosal addition of 0.2 mM SITS in six ouabain-treated bladders in the absence of exogenous CO₂. Jₜ was measured as the short-circuit current, and (ΔpH)ₐ=0 was taken as the difference between the luminal pH at which the current became zero and the serosal pH of 7.40. Control Jₜ was 0.64±0.08 μmol/h, and (ΔpH)ₐ=0 was 2.88±0.06 pH U. About 30 min after SITS addition, Jₜ was inhibited by 50% and (ΔpH)ₐ=0 was reduced to 2.09±0.11 pH U. The decrement in the achievable transepithelial pH gradient of 0.79±0.08 was large. If the force of the pump is unaffected by SITS, then this decrement reflects a comparable pH increase in a critical region on the cytoplasmic side of the active transport pathway.

To exclude the possibility that the decrease in (ΔpH)ₐ=0 was caused by an increase in the passive

| Table II |
| Effect of Serosal Addition of SITS* on Jₜ and Apparent Cell pH |
| Control halves | SITS halves |
| Jₜ Cell pH' | μmol/h | Jₜ Cell pH' | ΔpH |
| 1 1.82 7.48 | 0.55 7.60 | 0.12 |
| 2 1.04 7.59 | 0.00 7.72 | 0.13 |
| 3 0.70 7.35 | 0.32 7.60 | 0.25 |
| 4 1.42 7.54 | 0.77 7.60 | 0.06 |
| 5 1.36 7.50 | 0.54 7.62 | 0.12 |
| 6 0.66 7.46 | 0.40 7.60 | 0.14 |
| 7 1.20 7.44 | 0.66 7.51 | 0.07 |
| Mean 1.36 7.48 | 0.54 7.61 | 0.13† |
| ±SE 0.14 0.03 | 0.07 0.02 | 0.02 |

Control halves are compared to their respective SITS-treated halves. Jₜ was measured by the pH stat method in the absence of exogenous CO₂. Apparent cell pH (pH') was determined 30 min after addition of SITS.

* 0.2 mM.
† P < 0.005.

**TABLE III**

| Effect of SITS on the Maximal Transepithelial pH Gradient, (ΔpH)ₐ=0 |
| Control | Jₜ | Serosal pH | (Luminal pH)ₐ=0 | (ΔpH)ₐ=0 |
| μmol/h | 7.40 | 4.52±0.06 | 2.88±0.06 |
| SITS (0.2 mM) | 0.32±0.03 | 7.40 | 5.31±0.11 | 2.09±0.11 |
| (Control-SITS) | | | | 0.79±0.08 |

Mean values±SE are given for six ouabain-treated bladders. The gas phase was CO₂-free air. (ΔpH)ₐ=0 was determined 30 min after serosal addition of SITS.

Anion Exit Step in Urinary Acidification In Vitro
H⁺ permeability of the bladder, the luminal pH was lowered 0.30 pH U below the pH at which net H⁺ secretion was zero. The rate of loss of H⁺ (−JH⁺) from the luminal compartment was not altered by SITS (n = 4), hence, there was no evidence that the reduced gradient was caused by back diffusion of H⁺ across the luminal membrane.

Alternatively, (ΔpH)H⁺ was might have been reduced by movement of HCO₃⁻ from the cell into the luminal solution instead of the serosal solution. Since (ΔpH)H⁺ was determined as the condition of zero net current in ouabain-treated bladders, only a charge carrying HCO₃⁻ flow would affect this value. The available evidence (4, 6, 9, 10), however, suggests that the luminal membrane is virtually impermeable to the diffusional flows not only of H⁺ but also of HCO₃⁻. Any electroneutral HCO₃⁻ secretion (see below paragraph) would not have been recorded by the current technique for the measurement of (ΔpH)H⁺.

**Effects of SITS on the transport of other ions.** In all of the studies presented, the serosal HCO₃⁻ concentration was kept low either by the use of a solution free of exogenous CO₂ or HCO₃⁻ at pH 7.0 or 7.4, or by lowering the serosal pH to 5.8 when the serosal gas phase was 5% CO₂. This design allowed us to focus on the disposition of alkali generated in series with the H⁺ pump. Leslie et al. (11) and Oliver et al. (12) have described a separate transport system which is involved in the absorption of Cl⁻ in exchange for secreted HCO₃⁻. This anion-exchange system is electroneutral and requires the presence of HCO₃⁻ in the serosal solution. The effect of SITS on the transcellular HCO₃⁻ movement from the serosal to the luminal side of the bladder has been explored in another study in which it was shown that HCO₃⁻ secretion and Cl⁻ absorption are not affected by serosal SITS (13), a result suggesting that there are separate sites for the exit of HCO₃⁻ and the oppositely directed electroneutral HCO₃⁻ flow.

Sodium fluxes were not measured in this study. An estimate of the net sodium absorption, however, was obtained from the sum of the short-circuit current and H⁺ secretion measured simultaneously by pH stat titration (6). In five pairs of hemibladders, the net sodium absorption was 347±28, 352±24, and 347±24 μA in the control halves at 0, 60, and 90 min, respectively, and 355±29, 284±18 and 250±16 μA in the halves exposed to 0.5 mM serosal SITS. In SITS-treated halves the inhibition of the net sodium absorption was significant (P < 0.01) and increased from 20% at 60 min to 30% at 90 min. Ehrenspeck and Brodsky (14) found no significant inhibition of the net sodium absorption under somewhat different conditions.

**DISCUSSION**

Studies by Rothstein, Cabanthich, and Knauf (15–17) in erythrocytes, indicate that the disulfonic stilbenes inhibit anion transport by binding to a membrane protein, and that they appear to react with a site near the outside of the cell membrane (15–18). Inhibition of anion transport by SITS has been demonstrated in a variety of other tissues as well (19–21). Ehrenspeck and Brodsky (14) reported that SITS added to the serosal side of the turtle bladder inhibits anion transport as judged from the short-circuit current in sodium-free media. Furthermore, a preliminary report by Ehrenspeck et al. (22) suggests that the stilbenes bind to a protein located in the serosal cell membrane.

Our studies confirm the observation that in the absence of sodium transport the short-circuit current in turtle bladder is inhibited by SITS and specifically explore the mechanisms by which SITS inhibits urinary acidification. The results show that urinary acidification is inhibited by serosal but not by luminal addition of SITS. Since the H⁺ pump is located at the luminal membrane, it is likely that the inhibition is exerted at a transport site at the serosal membrane, presumably the SITS binding protein. Previous studies have shown that the rate of alkalization of the serosal compartment is about the same as the rate of H⁺ secretion into the luminal compartment when the small rate of lactate production, about 0.1 μmol/h, is taken into account (3, 6, 23). It is likely, therefore, that SITS inhibits the exit of HCO₃⁻ from cell interior to the serosal compartment and that SITS does not inhibit the H⁺ pump directly. Similarly, the observation that the H⁺ pump can be stimulated transiently by exogenous CO₂ suggests that the transport system at the luminal membrane is not the primary site of inhibition.

Most of the factors regulating the rate of H⁺ secretion are thought to operate at the active transport system for H⁺. They are the electrochemical gradient across the active transport pathway, the supply of metabolic energy to the pump system and the availability of carbonic anhydrase and CO₂ for the buffering of OH⁻ generated behind the pump (6). Once the dissociated OH⁻ ions have reacted with CO₂, the HCO₃⁻ ions formed this way are thought to freely move down an electrochemical gradient from the cytoplasm across the basolateral cell membrane into the serosal solution (3–6). Under the usual conditions, this exit step for HCO₃⁻ has not been considered rate-limiting for urinary acidification.

The observed inhibition of JH⁺ after serosal addition of SITS, however, is most easily explained by a marked reduction in the conductance for HCO₃⁻ across the basolateral cell membrane. As a consequence of the impaired exit of HCO₃⁻, the epithelial cells would become more alkaline. By means of the DMO technique, a small but significant increase in the overall cell pH was indeed demonstrated. The measured increase in the mean cellular OH⁻ concentration, however, provides no information on the distribution of the increased OH⁻.
concentration within the cells. The increase could be caused by a very high OH\(^-\) concentration in a small region or by a small increase in the bulk of the epithelium. The possibility of a large concentration change in a small region was approached by studying the trans-epithelial pH gradient that stops net H\(^+\) secretion, \((\Delta pH)_{\text{H}=0}\).

If the proton motive force of the pump and the passive H\(^+\) permeability are unaffected, then any change in \((\Delta pH)_{\text{H}=0}\) would be a function of a change in the electrochemical activity of H\(^+\) on the cytoplasmic side of the active transport pathway. The value \((\Delta pH)_{\text{H}=0}\) would serve as a probe of this activity on the inside of the pump pathway. According to such an analysis,\(^3\) the cellular compartment adjacent to and in series with the pump increased its pH by 0.79 U from 7.48 to 8.27 during SITS inhibition of acidification. Steinmetz and Lawson (24) and Beauwens and Al-Awqati (10) have shown that the luminal membrane has a very low passive H\(^+\) permeability, and that J\(_{\text{H}}\) is closely controlled by the electrochemical gradient across the luminal membrane (9). In these previous studies, however, the luminal pH was varied, and the cell pH remained near 7.4 or 7.5, as judged from DMO studies (4) and the constancy of \((\Delta pH)_{\text{H}=0}\). If we assume that concentration changes across the active transport pathway have similar effects on J\(_{\text{H}}\), whether they are applied from the luminal or from the cytoplasmic side of the membrane, then the accumulation of OH\(^-\) and HCO\(_3\) on the cytoplasmic side of the pump in SITS-treated bladders could account for much of the observed inhibition of J\(_{\text{H}}\).

For several reasons it is difficult to quantitate the inhibitory effect of a \(\Delta pH\) at the cytoplasmic side of the active transport pathway. First of all, \(\Delta pH\) effects on the two sides of the membrane are not necessarily symmetrical. The pH of the cytoplasmic side of the pump is probably regulated in a much narrower range than that of the luminal side. The increased alkalinity on the inside of the transport pathway would be expected to decrease the supply of protons to the pump site and also might inhibit certain metabolic reactions supplying energy for the translocation of protons.

It should be noted that the evidence for increased alkalinity was obtained at 50–60% inhibition of J\(_{\text{H}}\). Prolonged inhibition of J\(_{\text{H}}\) by SITS resulted in a decrease in the cellular alkalinity, as judged from \((\Delta pH)_{\text{H}=0}\), a result consistent with the interpretation that factors other than the local gradient come into play with time. Such secondary factors might also account for the modest reduction in the estimated sodium current observed after 1 h of exposure to serosal SITS when J\(_{\text{H}}\) was inhibited by 70%.

Relatively little is known about the role of the exit step for HCO\(_3\) in the regulation of urinary acidification. In the turtle bladder, as shown in this study, the high permeability of the basolateral cell membrane for HCO\(_3\) is dramatically reduced by exposure to the disulfonic stilbene, SITS. As a result, the exit step for HCO\(_3\) becomes rate-determining for H\(^+\) secretion. Studies by Frömter et al. (25) and Filho and Malnic (26) suggest that the peritubular cell membrane of the rat proximal tubule is also freely permeable to HCO\(_3\) and that carbonic anhydrase inhibitors reduce this permeability. Ullrich et al. (27) recently reported that SITS causes a marked inhibition of H\(^+\) secretion by the proximal tubule only when applied to the peritubular side. It appears, therefore, that the exit step for HCO\(_3\) is sensitive to SITS in different urinary epithelia which are capable of acidification. The results in turtle bladder indicate that the serosal addition of SITS creates a compartment\(^4\) of high alkalinity in series with the H\(^+\) pump. The increased concentration gradient across the active transport pathway is the primary factor in the inhibition of urinary acidification.

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\(^3\)Previous studies (9) have shown that the imposition of an electrical gradient \((\Delta \phi)\) reduces J\(_{\text{H}}\) in the same manner as a concentration gradient \((\Delta pH)\). For the purposes of this analysis the changes are expressed in pH units.

\(^4\)This physiologic analysis pertains to the epithelial cells involved in H\(^+\) secretion but does not provide information on whether H\(^+\) secretion is the function of all cells or of a population of carbonic anhydrase-rich cells (28).


