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Acid pH and Weak Acids Induce Na – Cl Cotransport in the Rabbit Urinary Bladder

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Summary. We have described a coupled Na – Cl entry step at the apical membrane of a tight epithelium, the rabbit urinary bladder. Mucosal pH values, more acid than 4.6, stimulate a 20 to 40-fold increase in mucosal-to-serosal Na^+ and Cl^- flux. The flux increase is almost completely blocked by low concentrations of bumetanide. The transepithelial movement of Na^+ and Cl^- is normally electroneutral; however, when weak acids (such as acetate) are present in the mucosal solution, the acid-induced increase in flux is accompanied by a large increase in short-circuit current. Besides blockage by bumetanide, both the increase in flux and short-circuit current are blocked by: (1) Na^+-free solutions on the mucosa; (2) Cl^--free solutions on the mucosa; (3) phosphodiesterase inhibitors; (4) ouabain in the serosal solution; (5) K^+-free solutions on the serosa; and (6) HCO_3^--free solutions on the serosa. The increase in the fluxes and the short-circuit current is unaffected by: (1) amiloride application in the mucosal solution; (2) mucosally applied stilbene derivatives which block Cl^-/HCO_3^- exchange (SITS); and (3) Cl^--free solutions applied to the serosa. We interpret these results to imply a coupled Na – Cl uptake step at the apical membrane which is stimulated by intracellular acetate (or pH). The uptake step leads to a movement of Na^+ and Cl^- across the basolateral membrane, which is mediated by the Na^+, K^+-ATPase and a $Na/Cl/HCO_3^-$ exchange mechanism. Our results demonstrate that "tight" epithelia may, under appropriate circumstances, demonstrate mechanisms of ion movement which are similar to "leaky" epithelia.

Key Words rabbit urinary bladder · tight epithelium · Na – Cl cotransport · weak acids · anion transport · basolateral membrane

Introduction

To individuals interested in the transport processes of epithelial tissue, there has, for many years, been a dichotomy between the so-called "tight" and "leaky" epithelia. The initial division was based primarily on the very large differences in transepithelial conductance observed in different tissues. This difference can be attributed to a large variation in the resistance of the paracellular pathway in different epithelia (Diamond, 1974). This variation of paracellular conductance would be, in itself, enough justification for the classification of "tight" and "leaky" tissues; however, as the properties of the two epithelial types were more carefully examined, other more striking differences were noted. In particular, all truly "tight" epithelia appeared to have an apical Na^+ conductance which was sensitive to amiloride (for a review, *see* Benos, 1982). In the absence of this Na^+ conductance, the conductance of the apical membrane was very low. In contrast, "leaky" epithelia were not sensitive to the same low concentrations of amiloride. Nonetheless, Na^+ did cross the apical membrane and this movement contributed to volume movements of water. Much of the apical Na^+ entry in "leaky" tissues does not appear to be via conductive pathways, and a variety of electroneutral transport processes have been described which probably contribute to apical Na^+ entry. Among these are Na^+/H^+ exchange systems (Kinsella & Aronson, 1980; Weinman & Reuss, 1982), and Na^+-anion cotransport systems (Frizzell, Field & Schultz, 1979; Spring & Ericson, 1982; Warnock & Eveloff, 1982). Apparently, none of these systems is normally present in "tight" epithelia, which would seem a further justification for the dichotomous classification of epithelial tissue.

What we report here is that, at least under certain conditions, a Na^+-anion cotransport system can be demonstrated in the apical membrane of a tight epithelium. This suggests to us that the membranes of epithelial cells may be more closely related than their classifications imply.

Lewis and Diamond (1976) showed that when rabbit urinary bladder was bathed in normal rabbit saline, varying the mucosal pH between 7.4 and 3.8 had little effect on the electrical parameters of the bladder. Despite these observations, we have shown that when both acetate (or another weak acid) is present in the bathing solution, and the mucosal pH is acidified, there is a large transient

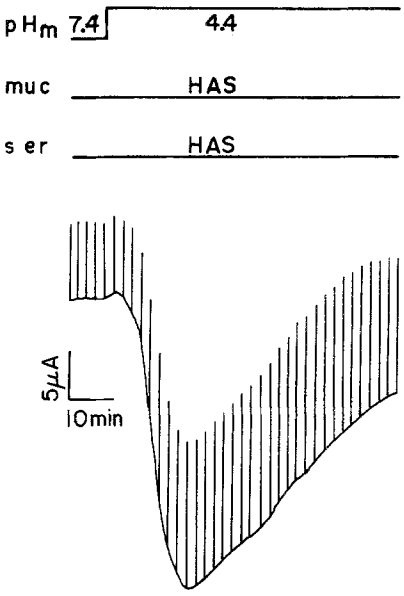

Fig. 1. The response of urinary bladder to acid mucosal solutions in the presence of acetate. In the Figure, positive current is downward. The tissue is initially bathed in a saline buffered with both HEPES and acetate (HAS). As indicated by the upper bar, the mucosal solution is rapidly acidified from pH 7.4 to pH 4.4. This acidification produces a large increase in short-circuit current (lower trace) from an initial value of 0.4 μA, to a final value of 31 μA. The vertical displacements of the current record are the current responses to a 5-mV stimulus

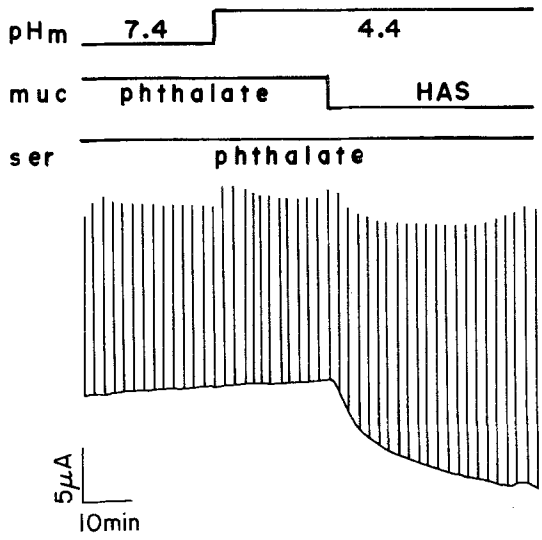

Fig. 2. Acetate is a necessary requirement for an acid-induced increase in short-circuit current. When the mucosal solution is acidified (indicated by the upper bar) in the presence of phthalate, there is no change in short-circuit current. When the mucosal solution is subsequently replaced by Hepes-acetate saline, the short-circuit current increases in a manner similar to Fig. 1. The initial short-circuit current was 2.0 μA, which increased to 12.5 μA after addition of acetate. The vertical steps in I_{sc} are the current responses to a 10-mV stimulus

increase in the short-circuit current, a substantial increase in transepithelial conductance, and a decrease in the ratio of apical-to-basolateral membrane resistance. Moreover, even in the absence of acetate, when the mucosal solution is acidified, there is a large increase in both Na^+ and Cl^- flux, even though there is little or no alteration in membrane electrical parameters and short-circuit current. (Preliminary reports have already been published: Ifshin & Eaton, 1979; 1981.)

Materials and Methods

The preparation of the urinary bladder and the method for mounting the preparation has been previously described (Lewis, Wills & Eaton, 1978).

Solutions

In all salines except the dissection saline, 11 mM glucose was added. 10 mM HEPES (pK 7.55) was also added for buffering at pH 7.4. Other salines used in determining the pH range of the response were buffered with 10 mM MES (pK 6.15), 10 mM *o*-phthalate (pK 5.51), acetate, or other weak acids (approximate pK 4.7). The dissecting saline had 22 mM glucose. The composition of the sulfate saline in mM was Na_2SO_4 (56.7), K_2SO_4 (3.5), $NaHCO_3$ (25), Na_2HPO_4 (1.2), $MgSO_4$ (1.2), Ca methane sulfonate (10). Approximately 85 mM of sucrose was added to it to maintain normal osmolarity. Occasionally, a saline in which methane sulfonate replaced Cl^- on a one-for-one basis was also used. In general, the basic composition of all salines in mM was NaCl (112), $NaHCO_3$ (25), KCl (5.8), K_2HPO_4 (1.2), $CaCl_2$ (2.0), and $MgSO_4$ (1.2). To this solution was added 10 mM of the Na^+ salt of one of the above buffers (e.g., 10 mM Na^+-acetate or Na^+-formate) + HCl to adjust the pH to the desired level. Modifications of these salines for specific experiments are described in the text.

Electrodes

Two Ag/AgCl electrodes placed at the far ends of the chambers were used for current passing across the epithelium. The mucosal current electrode was connected to a virtual ground which allowed monitoring of the transepithelial current. The serosal electrode was connected to the output of the voltage clamp. The transepithelial potential was measured by two calomel electrodes placed close to the tissue. On the serosal side, a simple calomel electrode (Fisher) was used. The mucosal potential electrode was the calomel half of a pH-sensitive electrode-calomel reference combination (Fisher). The calomel electrodes were connected to unity gain amplifiers which then fed into the voltage clamp. Since there was a difference of resistance of several orders of magnitude between the tissue and that of the solutions, no corrections were made for the small voltage drop across the solutions. Since calomel electrodes are pH-insensitive, this arrangement allowed measurements during pH changes without corrections.

The pH of the mucosal solution was constantly monitored by a pH-calomel combination electrode. The pH electrode was connected to a high impedance amplifier and referenced to the output of the unity gain amplifier for mucosal potential. This allowed current stimulation without disturbing the pH monitoring.

Carrier-free isotope fluxes ^{24}Na, ^{36}Cl, and/or ^{3}H-acetic acid were added to the mucosal solution to a final concentration

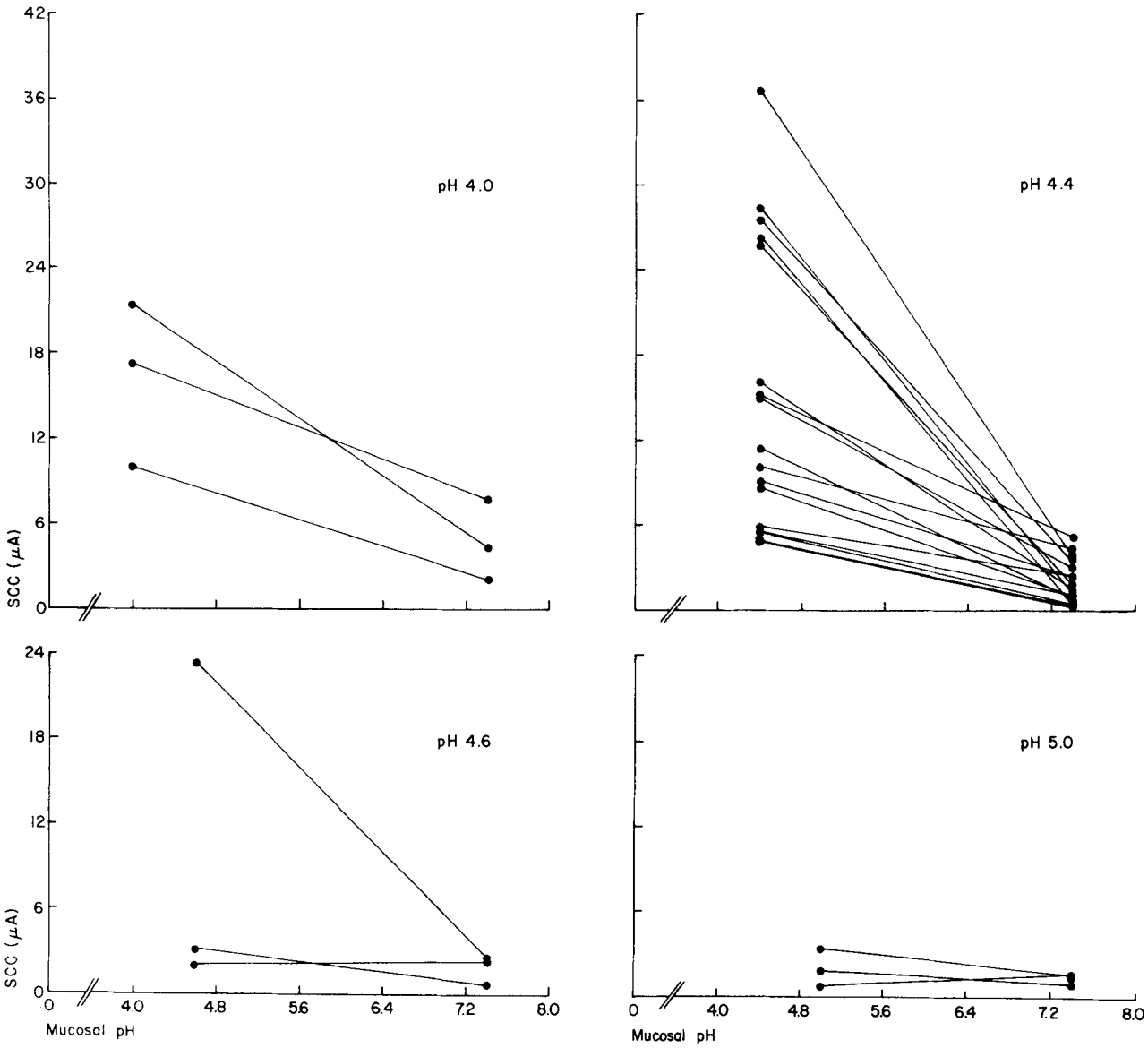

Fig. 3. Increase in short-circuit current produced by different levels of acidification in the presence of acetate. The points on the right represent control levels, while the points on the left represent the corresponding responses of I_{sc} to acidification. Although there was a substantial variability in the magnitude of the response to acidification, at all pH levels more acid than pH 4.6, there is always an increase in I_{sc} upon acidification

of 5, 2 and 3 μCi/ml, respectively. 100-μl samples were removed from the serosal solution at 10-min intervals, and the volume replaced with cold serosal saline. The samples were placed into 7.5 ml of toluene-triton-X cocktail and were counted immediately, and then again 14 days later. The difference in counts was taken as the ^{24}Na counts. In those few instances when ^{3}H and ^{36}Cl were measured simultaneously, the samples were counted with both a narrow tritium window and a window which extended from a bottom of 0.4 to a top of 0.9 of the total energy window for the counter. In this configuration, less than 5% of the counts in the tritium channel could be attributed to ^{36}Cl, and virtually none of the counts in the upper channel could be attributed to ^{3}H. In cases where there was more than a twofold change in the ^{36}Cl flux, the ^{3}H counts were corrected for ^{36}Cl contamination. The methods were otherwise similar to those of Eaton, Frace and Silverthorn (1982).

Results

Organic Acids and Acid pH Produce Increased I_{sc}

We observed that after 10 mM acetate had been applied to the mucosal surface of the rabbit urinary bladder, a rapid acidification of the mucosal solution to pH 4.4 produced a large transient increase in short-circuit current (I_{sc}) (Fig. 1). On the other hand, if the mucosal solution did not contain acetate, acidification produced no alteration in short-circuit current (Fig. 2). The increase in I_{sc} could not be due merely to the production of acetic acid

from acetate ion in acid solutions, since application of an acetate saline at pH 5.4, with the same concentration of free acetic acid as the saline at pH 4.4, produced no change in I_{sc}. Thus, to elicit an increase in I_{sc} required both a mucosal acid pH and mucosal acetate.

Although the magnitude of the increase in short-circuit current was somewhat variable, there was always some increase when acetate was applied at pH 4.6 or below (*see* Fig. 3). In addition to the effect of acetate and acid pH on I_{sc}, the same treatment also produced a substantial increase in transepithelial conductance, and a decrease in the ratio of apical-to-basolateral membrane resistance (R_a/R_b) as measured with intracellular microelectrodes (Table 2).

Certain other organic acids, besides acetate, could also evoke an increase in I_{sc} and a decrease in transepithelial conductance when applied to the mucosa in acid solutions (Table 1). Propionate and butyrate produce responses similar to acetate. Of the various short-chain fatty acids, only formate failed to produce an increase in I_{sc}. Even succinate, applied in acidified mucosal solutions, was capable of eliciting a transient increase in short-circuit current, although the increase was only about one-tenth as large as the acetate response.

Table 1. Effects of various mucosally applied weak acids

Acid[a]	I_{sc} mucosal pH = 7.4	I_{sc} acid mucosal pH[b]	n
Formate	1.9±0.5	2.0±0.3	3
Acetate	2.2±1.5	15.8±9.8	16
Propionate	1.8±1.2	12.2±3.6	3
Butyrate	1.5±1.0	13.1±5.8	3
Succinate	2.3±0.2	2.3±0.5	2
	1.8±1.0	5.2±2.8*	3
	9.6±2.3	21.0±6.8**	2
Glutamate	2.2±1.0	1.5±0.8	3
Phthalate	2.2±1.4	1.2±0.9	3

[a] All acids (free acid and anionic form) were at a mucosal concentration of 10 mM except the third value for succinate (marked by **), which was 30 mM.
[b] The pH of the test solutions were 4.4 except for the succinate solutions (marked by * and **), which were pH 3.8.

Is the Increased I_{sc} Due Only to Increased Intracellular Acidity?

Although there was some variation in the magnitude of the increase in I_{sc} from one weak acid to the next, all of the substances we tested which were substantially uncharged at pH 4.0 (with the exception of formate), produced a response. All other weak acids tested, which were charged at pH 4.0, produced either no change in I_{sc} or a reduction consistent with the effect of pH alone. A summary of these results is presented in Table 1.

Thus, it appeared that the increased short-circuit current we observed might be due exclusively to an increase in intracellular acidity associated

Table 2. Membrane electrical parameters

Treatment	Mucosal pH	V_t(mV)[a]	I_{sc}(μA/cm²)[b]	R_t(Ωcm²)	R_a/R_b	V_a(mV)	V_b(mV)[c]	n[d]
Normal	7.4	−12.8	1.6	8000	9.2	−48±2.4	−60	9
Saline	4.4	−10.6	1.3	8200	9.3	−56±2.8	−66	10
Normal saline + 10 mM formate on mucosa	7.4	−26.8	2.4	11000	9.1	−36±2.0	−63	12
	4.1	−24.3	2.2	10800	7.0	−60±3.2	−84	12
Normal saline + 10 mM acetate on mucosa	7.4	−10.7	1.4	7600	8.6	−49±2.3	−60	11
	4.4	−30.4	8.7	3500	2.2	−50±2.1	−81	12
Normal saline + 10 mM acetate on mucosa/Na^+-free on serosa	7.4	− 9.8	1.2	8200	8.9	−52±2.2	−62	8
	4.4	−14.3	3.2	4500	1.2	−53±2.4	−67	10
Normal saline + 10 mM acetate on mucosa/HCO_3^--free on serosa	7.4	− 2.3	0.2	10900	18.7	−43±3.4	−46	6
	4.4	− 3.2	0.4	8700	16.2	−46±3.2	−49	5

[a] Transepithelial voltage with reference to serosal ground.
[b] Calculated from the transepithelial resistance and transepithelial voltage.
[c] Calculated from the transepithelial potential and the mean apical membrane potential.
[d] The number of impalements of the apical membrane while the transepithelial potential was stable at the value given in the third column.

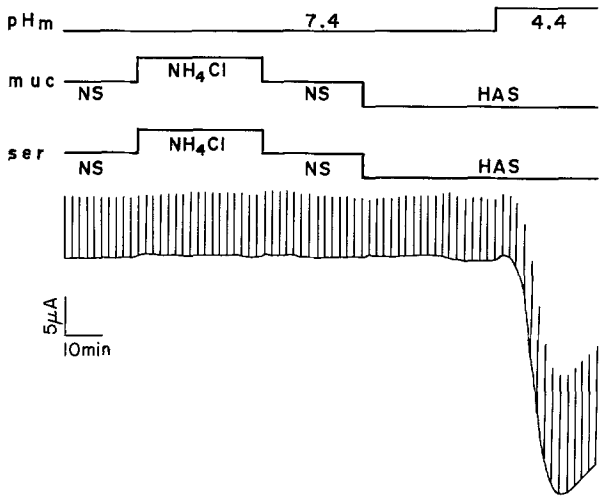

Fig. 4. The effect of intracellular acidification in the absence of acetate and acid mucosal solutions. The tissue was initially bathed symmetrically with normal saline without acetate. The tissue was then exposed to 20 mM ammonium chloride for 30 min. The saline containing NH_4Cl was then rapidly replaced with normal saline at pH 7.4, without acetate or ammonium. This should lead to rapid acidification of the intracellular compartment. The intracellular acidification does not lead to a change in I_{sc}. Even when acetate is subsequently added, there is no alteration in I_{sc}. Only when the mucosal solution is acidified to pH 4.4 in the presence of acetate, is there an increase in I_{sc} from an initial value of 0.4 μA to a maxiumum value of 31.0 μA. The vertical deflections in the I_{sc} trace are the current responses to a 5-mV stimulus

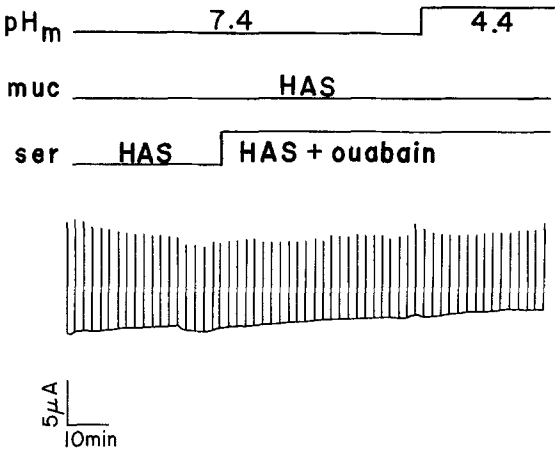

Fig. 5. Ouabain in the serosal solution blocks the acid-induced increase in I_{sc}. Initially the bladder was bathed symmetrically in hepes-acetate saline (HAS). (Initial $I_{sc}=0.5$ μA.) As indicated, 10^{-4} M ouabain was added to the serosal surface. Subsequently, when the mucosal solution was acidified to pH 4.4, there was no change in I_{sc} as there was in the case when ouabain had not been added (Fig. 1). The vertical deflections in the short-circuit current are the responses to a voltage stimulus of 5 mV

with the movement of free acid across the apical membrane with its subsequent dissociation inside the cell. If intracellular acidity were the primary causative agent producing the increase in I_{sc}, then any treatment which increased intracellular pH should produce a similar response. To test this possibility, we applied methods known to alter the intracellular pH. In the first case, we exposed the tissue to a gas mixture with a partial pressure of CO_2 six times higher than the normal gas mixture (95% O_2 − 5% CO_2). This exposure, which should produce prompt intracellular acidification (Boron & DeWeer, 1976; Thomas, 1976*b*), had no effect on I_{sc}. Similarly, application of ammonium salts will cause an initial alkalinization of the cell interior and a strong acidification on removal of extracellular NH_4^+ (Boron & DeWeer, 1976). Figure 4 shows that after equilibration for 30 min with NH_4^+ Cl, there was no effect on I_{sc} when the NH_4^+ saline was rapidly replaced with normal saline. Moreover, application of acetate under these conditions of presumptive intracellular acidification also produced no alteration in short-circuit current, even though the tissue was still responsive to the concomitant exposure to mucosal acidity *and* acetate.

These results suggest that intracellular acidification alone is not sufficient to produce the alteration of I_{sc} observed when weak acid salts in acid salines are applied to the mucosal surface of the tissue.

Is the Na^+ Transport System Involved?

In rabbit urinary bladder, virtually all of the short-circuit current can be attributed to Na^+ transport (Lewis & Diamond, 1976). Consequently, a large increase in I_{sc} associated with acidic-acetate applications suggested an activation of the transport system. If this were the case, blockers of the active extrusion of Na^+ by the basolateral Na^+, K^+-ATPase should reduce or eliminate the acidic-acetate response. We applied three known blockers of the basolateral Na pump: Ouabain (10^{-4} M in the serosal chamber; *see* Fig. 5); K^+-free saline on the serosal surface; or Na^+-free saline to the mucosal surface (Lewis & Diamond, 1976; Lewis et al., 1978; Eaton, 1981). In each case, the increase in I_{sc} produced by mucosal application of acidic-acetate salines was eliminated. An example of the blocking ability of mucosally applied Na^+-free saline is shown in Fig. 6.

Thus, the increase in short-circuit current was in some way due to an activation of the basolateral transport processes and increased Na^+ transport. The additional transported Na^+ must be entering

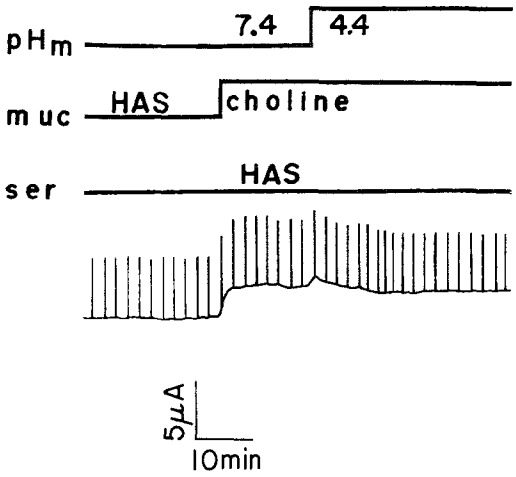

Fig. 6. Na^+-free saline in the mucosal solution blocks the acid-induced increase in I_{sc}. Initially the bladder was symmetrically bathed in hepes-acetate saline (HAS) with an initial short-circuit current of 2.9 μA. As indicated, the mucosal solution was replaced with a hepes-acetate saline in which all of the Na^+ was replaced with choline. Subsequent to this replacement, the mucosal solution was acidified to pH 4.4 without the change in short-circuit current which acidification normally produced (Fig. 1). The vertical deflections represent the current responses to voltage steps of 5 mV

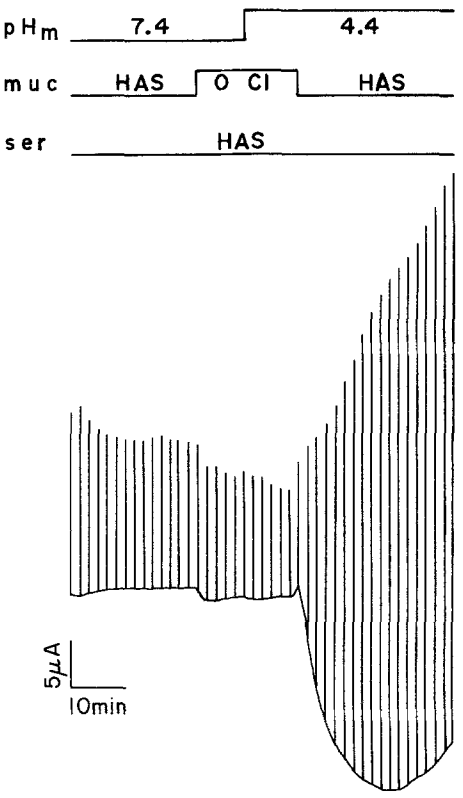

Fig. 7. Cl^--free saline in the mucosal solution blocks the acid-induced increase in I_{sc}. The bladder was initially bathed symmetrically in hepes-acetate saline (HAS) with an initial I_{sc} of 2.0 μA. As indicated by the second bar, the mucosal solution is replaced with methanesulfonate. Because of the asymmetry of the mucosal and serosal solutions, there is a small alteration in I_{sc}, but there is no additional increase in I_{sc} when the mucosal solution is acidified. When the acidified Cl^--free solution is replaced by an acidified Cl^--containing solution, there is a large, rapid increase in I_{sc}. The vertical deflections in the I_{sc} trace are the current responses to a voltage stimulus of 10 mV

from the mucosal solution across the apical membrane to produce short-circuit current. The major pathway for apical Na^+ entry with normal rabbit saline on the mucosal surface is via the amiloride-sensitive Na^+ conductance (Lewis & Diamond, 1976). However, the conductive pathway did not appear to be the mechanism by which Na^+ crossed the mucosa after stimulation by acidic-acetate salines. We felt this was true because amiloride (10^{-4} M) blocked all short-circuit current both when applied to the mucosal surface in normal rabbit saline or in rabbit saline at pH 4.4 (although the initial I_{sc} was reduced from that at pH 7.4), but amiloride was unable to reduce the increase in I_{sc} that accompanied application to the mucosa of acetate in pH 4.4 saline. This result implied that the increase in I_{sc} associated with acidic-acetate saline was due to increased Na^+ transport, and that at least one of the mechanisms for Na^+ extrusion into the serosal solution was the normal Na^+, K^+-ATPase, but the mechanism for increased Na^+ entry at the apical membrane did not display the normal characteristics of the amiloride-sensitive Na^+ permeability.

Other Blockers of the Acidic-Acetate-Induced Increase in I_{sc}

Since the Na^+ entry implicated by the increased I_{sc} associated with mucosal application of acidic-acetate salines was not via the amiloride-sensitive conductance pathway, we considered the possibility that we might be activating a Na^+-anion cotransport system (Argenzio & Whipp, 1979; Frizzell et al., 1979). To examine this possibility, we applied a Cl^--free saline to the mucosal surface. Although there was a small bi-ionic potential due to the anionic asymmetry between the mucosal and serosal solutions, it was no longer possible to produce an increase in short-circuit current when the mucosal solution was acidified in the presence of acetate (Fig. 7). A similar application of Cl^--free to the serosal surface had no effect on the acid-acetate-induced increase in I_{sc}, while application of Cl^--free symmetrically again blocked the increase in I_{sc}.

We reasoned that if Na^+-anion transport was involved in the response to mucosally applied acid-

acetate solutions, then Cl^- transport blockers might inhibit the increased I_{sc}. We first tried SITS (4-acetamido-4′-isothiocyano-stilbene-2,2′-disulfonic acid), a powerful blocker of Cl^- transport in red blood cells (Cabantchik, Knauf & Rothstein, 1978), and some Cl^- transport in invertebrate excitable tissue (Russell & Boron, 1976; Thomas, 1976*a*; Boron, 1977). Unfortunately, SITS is poorly soluble, and may also be unreactive at pH 4.4. Consequently, we reacted a freshly prepared saline containing 1 mM SITS with the apical membrane prior to acidification in the hope that it would irreversibly bind to the anion transport protein (Boron, 1977; Roos & Boron, 1981). However, even when SITS was allowed to react for one hour prior to the mucosal acid-acetate challenge, there was no reduction in the short-circuit current response. We could, therefore, only conclude that SITS was ineffective as a blocker, but the lack of effect could be due either to the reaction conditions or an insensitivity of the system to normal anion transport blockers. In *Necturus* gallbladder, SITS is incapable of blocking one form of coupled NaCl entry (Larson & Spring, 1982; Spring & Ericson, 1982). This NaCl entry at the apical membrane is insensitive to mucosal HCO_3^-, SITS, or amiloride, but was blocked by bumetanide (Spring & Ericson, 1982). Because of these results, we attempted to block, with bumetanide, the Na – Cl-dependent increase in I_{sc} which we had observed. Unfortunately, bumetanide is poorly soluble in acidic solutions; however, its blocking ability is sufficiently potent that we hoped, if it were an effective blocker, that it would at least partially block the increase in I_{sc}. Figure 8 shows that 10^{-6} M bumetanide, applied to the mucosa, is capable of blocking approximately 50% of the increase in short-circuit current. In contrast, the carbonic anhydrase inhibitors, ethoxzolamide (1 mM) and acetazolamide (10 mM), produced no effect.

On the other hand, several agents which increase levels of cyclic adenosine monophosphate (cAMP), and thereby inhibit Na-anion cotransport (Nellans, Frizzell & Schultz, 1973; Frizzell, Dugas & Schultz, 1975; Armstrong & Youmans, 1980; Berridge, 1980; Field, Smith & Bolton, 1980), inhibit the increase in I_{sc} associated with acidic-acetate salines.

Theophylline and isobutyl-methylxanthine (IBMX) are phosphodiesterase inhibitors whose addition to the bathing media should increase intracellular levels of cAMP (Field, 1978; Satake, Durham & Brodsky, 1981). Dibutyryl-cyclic-adenosine monophosphate (d-b-cAMP) can act like cAMP in other tissues, but is able to cross membranes more easily (Fenton, Gonzalez & Clancy, 1978). The addition of either d-b-cAMP, IBMX, or theophylline to both chambers either reduced or completely blocked the increase in I_{sc} after mucosal application of acidic-acetate saline. (Figure 9

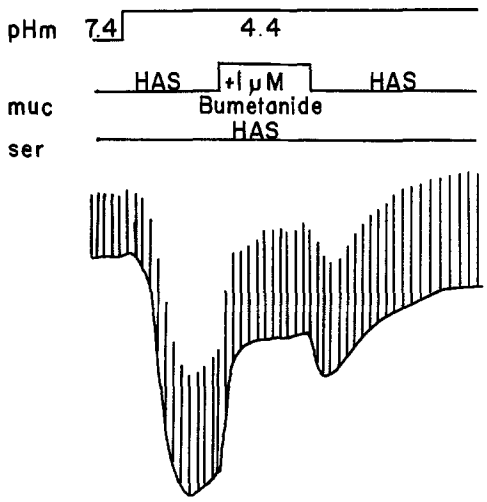

Fig. 8. The effect of mucosally applied bumetanide on the short-circuit current. The tissue is initially bathed in symmetric solutions of normal saline plus acetate. On acidification of the mucosal solution, there is a large increase in I_{sc} which can be partially blocked by mucosal application of 10^{-6} M bumetanide. The effect of bumetanide is, at least partially, reversible. The exact magnitude of the reversibility is difficult to assess, since the response of the short-circuit current to acidification is itself transient

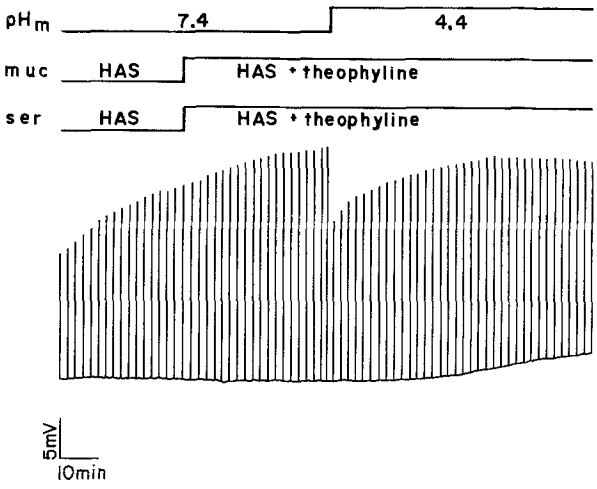

Fig. 9. Theophylline blocks the response to mucosal acidification. The lower trace shows the transepithelial potential in response to the treatments represented by the bars at the top of the Figure. Unlike the responses in previous Figures after exposure to 1 mM theophylline, there is no alteration in the transepithelial potential where the mucosa is acidified. The vertical deflections in the lower trace represent the voltage responses to a current stimulus of 10 μA

shows the effect of treatment with a saline containing 10 mM theophylline.)

Apical and Basolateral Membrane Potentials

The decrease in the voltage divider ratio, α, and the increase in transepithelial conductance suggest, at least, an increase in the conductance of the apical membrane of the cells. We hoped that a more careful examination of the individual membrane potentials might give some insight into the mechanism of the increase in I_{sc}. In Table 2 are representative potentials from several types of experiments. In the first, the tissue is first bathed in a normal saline at pH 7.4. The pH is subsequently reduced to 4.4. In the second experiment, the protocol is the same, except that 10 mM formate has been added to the mucosal solution. The results show that although formate applied at pH 4.4 has little effect on the transepithelial potential, the transepithelial resistance, the voltage divider ratio, or the short-circuit current, it does produce a substantial hyperpolarization of the basolateral membrane. When 10 mM acetate is applied under the same conditions, it causes large changes in all of these tissue properties, as well as a large increase in basolateral potential.

There are several possible sources for the increase in basolateral potential in the presence of mucosal formate and acetate. The obvious source is the electrogenicity of the basolateral Na^+-K^+ transport system (Lewis et al., 1978; Eaton et al., 1982). Ouabain and other Na^+, K^+-ATPase blockers are known to depolarize the basolateral membrane of rabbit urinary bladder (Eaton, 1981; Eaton et al., 1982). However, since intracellular ion activities are also altered by these treatments, other gradient-dependent exchange systems may also be blocked by these treatments. Consequently, we examined the possibility that the intracellular acidity, which is likely to be induced by mucosal formate and acetate, may have activated one of the pH regulatory mechanisms described in other tissues (Russell & Boron, 1976; Thomas, 1976*a*; Aickin & Thomas, 1977; Roos & Boron, 1981; Weinman & Reuss, 1982; Boron & Boulpaep, 1983). Both serosal Na^+-free solutions and nominally HCO_3^--free solutions block a substantial fraction of the increase in basolateral membrane potential and the transient increase in short-circuit current. Thus, there appears to be a Na^+ and HCO_3^--dependent mechanism at the basolateral membrane contributing to both the large alteration of basolateral potential and the transepithelial ion transport increase in I_{sc}.

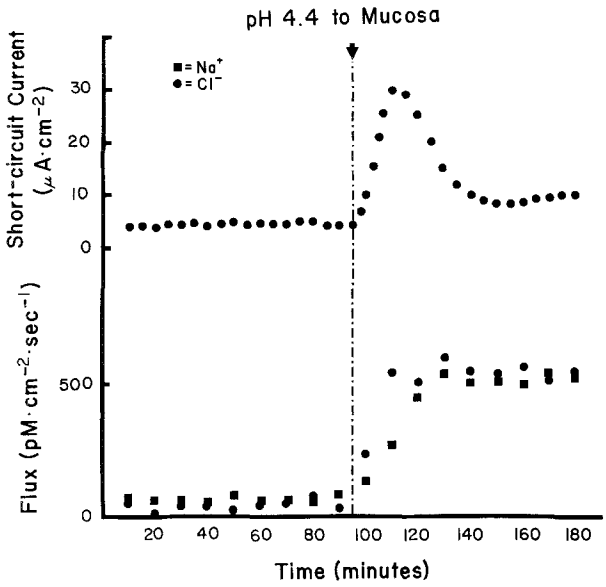

Fig. 10. Comparison of I_{sc} and Na^+ and Cl^- fluxes after mucosal acidification. In the upper record is the response of the short-circuit current to mucosal acidification (increasing I_{sc} is upward); in the lower record are the transepithelial Na^+ (■) and Cl^- (●) fluxes. Note that even though the short circuit returns close to the control level, the Na^+ and Cl^- fluxes remain elevated. Also, the charge movement implied by the maximal increase in short-circuit current is approximately one-half of the charge movement associated with the maximal Na^+ flux

Do Ion Fluxes, After Acidification, Correspond to Short-Circuit Current?

On the basis of the effects of chloride-free mucosal solutions, bumetanide, and the agents which activate cAMP, it appeared that there might be a Na–Cl cotransport system at the mucosal membrane. If this were true, then one might expect increased transepithelial fluxes of Na^+ and Cl^-. To test this possibility, we measured the transepithelial fluxes of Na^+ and Cl^- under a variety of conditions. In Fig. 10, the fluxes of Na^+ and Cl^- are compared with the short-circuit current after mucosal acidification in the presence of acetate. Associated with the increase in I_{sc} is a large increase in both Na^+ and Cl^- flux. Interestingly, these fluxes continue at an elevated level despite the only transient alteration of short-circuit current.

The effect of a sudden increase in mucosal H^+ concentration on the transepithelial fluxes of Na^+, Cl^- and acetate is more completely presented in Fig. 11. After the acidification, there is a substantial increase in all three fluxes; however, the relative increase in the flux of the three ions is different. Acetate ion showed the largest increase. This increase is not entirely unexpected, since the permeability of the protonated form of acetate should be extremely high.

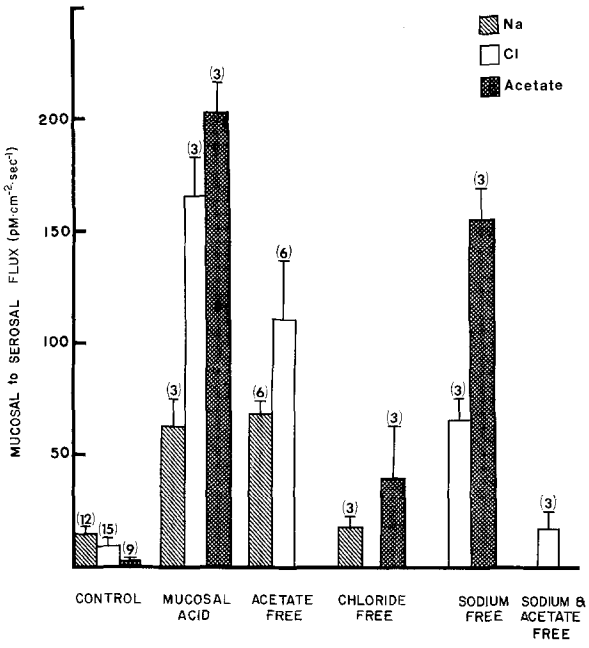

Fig. 11. Mucosal-to-serosal flux of Na^+, Cl^-, and acetate in response to various different conditions. The singly crosshatched bars represent Na^+ flux, the open bars, Cl^- flux, and the doubly crosshatched bars, acetate flux. The bars represent the mean value of the flux with the standard deviation of the mean indicated. Each bar represents no less than 3 experiments. The treatments indicated along the abscissa are, from left to right: normal rabbit saline + 10 mM acetate (control); the same solutions at pH values between 4.0 and 4.6 (mucosal acid); the remaining treatments are all under conditions of mucosal acidity, but with no acetate, methane-sulfonate replacing Cl^- (Cl^--free), or choline replacing Na^+ (Na^+-free and Na^+ and acetate-free)

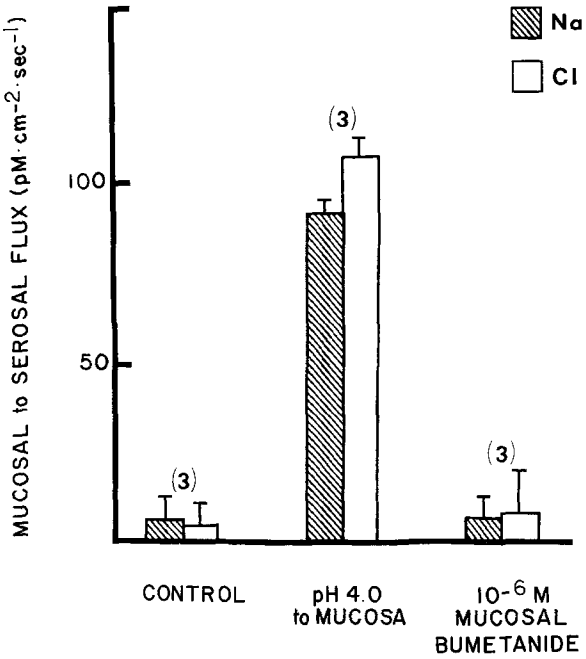

Fig. 12. The effect of mucosal addition of bumetanide on the mucosal-to-serosal Na^+ and Cl^- fluxes. No acetate is present in the mucosal saline. The Na^+ flux is represented by the crosshatched bars, and the Cl^- flux by the open bars. Although enough bumetanide was added to produce a 1 mM solution, the poor solubility of the substance will produce a final concentration of approximately 10^{-5} M

If the mechanism for increased apical Na^+ entry after mucosal acidification is via Na – Cl cotransport, then equal increases of Na^+ and Cl^- fluxes might be expected; however, the increase in Cl^- flux was always larger than the increase in Na^+ flux. This suggested that there might be both a Na^+-dependent and a Na^+-independent Cl^- flux after mucosal acidification (White & Imon, 1982). To clarify the mechanisms responsible for the fluxes of the three ions, we examined the dependence of each ion's flux on the presence of the other two ions.

Figure 11 also shows that without Cl^- in the mucosal solution, there is no increase in Na^+ flux on mucosal acidification, although there is still an increase in acetate flux, but at a level below that in the presence of Cl^-. In the absence of Na^+, there is still a substantial increase in Cl^- and acetate flux, although both are somewhat below the levels before the removal of Na^+. In the absence of acetate, there is still an increase in the flux of Na^+ and Cl^- despite the fact that there is no electrical response under these conditions. Finally, in the absence of sodium and acetate, Cl^- flux is not significantly different than the control flux prior to mucosal acidification.

The implication of these experiments appears to be that, in acid mucosal solutions, a large component of transepithelial mucosal-to-serosal Na^+ flux depends upon Cl^- in the mucosal solution. There also appears to be one component of the mucosal-to-serosal flux of Cl^- (from acid mucosal solutions) which depends upon Na^+, and another which depends upon mucosal acetate.

To further investigate the mechanisms of transepithelial ion flux, we applied several agents known to block various types of ion transport.

In two such experiments, 10^{-3} M amiloride was applied to the mucosal surface of the tissue after previous acidification. To insure that the amiloride blocked any Na^+/H^+ exchange that might be present, we reduced mucosal Na^+ to 20 mM by replacing Na^+ with choline. With lowered mucosal Na^+, the transepithelial fluxes of both Na^+ and Cl^- are substantially reduced (despite the normal mucosal concentration of Cl^-), both in the control at pH 7.4 and with the mucosal solution acidified (pH 4.0). Amiloride, however, does not reduce the acid-induced fluxes. On the other hand, 10^{-6} M

bumetanide applied to the mucosa is capable of reducing acid-induced Na^+ and Cl^- flux to control values (Fig. 12).

In additional experiments, 10^{-3} M SITS applied to the mucosa, and 10^{-6} M bumetanide applied to the serosa, were ineffective in altering the acid-induced Na^+ or Cl^- fluxes. On the other hand, after a pH 4.0 mucosal solution had increased mucosal-to-serosal flux by a factor of 2.2 above the control, 10^{-3} M SITS applied to the serosa was capable of substantially reducing the transepithelial Cl^- fluxes to a value only 10% above control levels.

These results added to an already complicated picture, but seemed to support the concept of a Na–Cl cotransport system at the mucosal membrane (although there also appears to be some alternative entry mechanism for Cl^-). The results also seem to imply a SITS-sensitive process at least partially responsible for Cl^- exit at the basolateral membrane.

Table 3.

Treatment	Effect on acetate + pH-induced increase in I_{sc}	Effect on trans-epithelial Na^+ or Cl^- flux
1. Ouabain to serosa	Blocks	–
2. K^+-free to serosa	Blocks	–
3. Na^+-free to mucosa	Blocks	Cl^- flux reduced
4. Cl^--free to mucosa	Blocks	Na^+ flux reduced
5. Amiloride to mucosa	No effect	No effect
6. SITS to mucosa	No effect	–
7. Bumetanide to mucosa	Blocks	Na^+ and Cl^- flux reduced
8. cAMP mimics or phosphodiesterase blockers	Blocks	–
9. Cl^--free to serosa	No effect	–
10. HCO_3^--free to serosa	Blocks	–
11. Na^+-free to serosa	Blocks	–
12. SITS to serosa	–	Cl^- flux reduced

Discussion

Briefly, we have observed a large increase in short-circuit current and transepithelial Na^+ and Cl^- flux when an acetate-containing solution on the mucosal surface of rabbit urinary bladder is rapidly acidified. At the same time as the increase in I_{sc}, there is a substantial reduction in transepithelial resistance and the ratio of apical-to-basolateral membrane resistance, and a substantial increase in mucosal-to-serosal Na^+ and Cl^- fluxes. Various treatments affect these increases in both I_{sc} and isotopic flux. The effects of various treatments are summarized in Table 3. We feel that our results can be explained by a combination of several membrane mechanisms.

Mechanism for Increase in I_{sc}

Because both ouabain and K^+-free salines applied to the serosal surface of the tissue block the increase in short-circuit current, the basolateral Na^+, K^+-ATPase would appear to be one step in a sequence of events responsible for the pH and acetate-induced increase in I_{sc}. The involvement of the Na^+, K^+-ATPase implies at least a transient increase in intracellular Na^+, the substrate for the ATPase (Eaton, 1981; Eaton et al., 1982). If an increase in intracellular Na^+ is necessary for the response, then removal of mucosal Na^+ would be expected to block the increase in I_{sc}, as it does.

However, the Na^+, K^+-ATPase cannot be the only mechanism for ion movement across the basolateral membrane, since there is substantial mucosal-to-serosal Cl^- flux in addition to the Na^+ flux. Both fluxes persist even after the initial increase and subsequent reduction in I_{sc}. This implies that, in the long term, the net movement of Na^+ and Cl^- across the tissue in response to mucosal acid loads is electroneutral.

Mechanism for Mucosal Entry of Na^+

As pointed out above, the increase in I_{sc} associated with mucosal acidification in the presence of acetate requires increased entry of Na^+ at the mucosal surface, with its subsequent transport across the basolateral membrane by the Na^+, K^+-ATPase. This view is also supported by the transepithelial flux increase after mucosal acidification. However, the mechanism for the Na^+ entry after acidification is not the normal entry pathway for Na^+ present in bladders exposed to symmetrical pH 7.4 salines, since Na^+ entry is not blocked by low concentrations of amiloride (Lewis & Diamond, 1976; Lewis, Eaton & Diamond, 1976). Moreover, that high concentrations of amiloride do not block mucosal-to-serosal Na^+ flux also suggest that Na^+/H^+ exchange at the mucosal membrane is probably not the mechanism of Na^+ entry as it is in several other epithelial tissues (Kinsella & Aronson, 1980; Weinman & Reuss, 1982). In particular, our results seem to preclude a mechanism similar to that described in gallbladder (Peterson, Wood, Schulze & Heintze, 1981), where butyrate (and

other short-chain fatty acids) is capable of strongly stimulating Na and Cl uptake at the mucosal membrane. The stimulation is accomplished by entry of the free acids, with subsequent dissociation into the weak acid anion and hydrogen ion. The hydrogen ion stimulates Na^+/H^+ exchange, and the weak acid anion (particularly butyrate) substitutes for bicarbonate in Cl^--bicarbonate exchange mechanism. The net effect is Na−Cl entry which is coupled via the weak acid entry.

In our experiments, besides the lack of effect of amiloride, Na/H exchange seems an unlikely candidate to promote Na^+ entry across the apical membrane, since this would also require movement of H^+ ion uphill into the mucosal solution in the face of an extremely adverse concentration gradient. On the other hand, our experiments cannot rule out the presence of such an exchange mechanism under more normal conditions, when the Na^+ and H^+ gradients are more favorable to Na/H exchange activity. Although Na^+/H^+ exchange is probably not responsible for Na^+ entry from the mucosal solution into the cell, the observation that there is a substantial chloride flux which is stimulated by mucosal acetate suggests that, as in the gallbladder model of Petersen et al. (1981), there may be substantial Cl^-/HCO_3^- or Cl^-/acetate exchange at the apical membrane which favors Cl^- entry from the mucosa to the cell and HCO_3^- or acetate exit. Gradients which would favor such movement are established by the acidification of the mucosal solution, which dramatically lowers the free HCO_3^- and favors low acetate anion in the mucosal solution and high acetate in the cell interior.

The blockage of the acid-induced short-circuit current and acid-enhanced Na^+ and Cl^- flux by mucosal application of bumetanide, strongly suggests that the primary mechanism for apical Na^+ entry, after acidification of the mucosal solution, is via a Na^+-anion cotransport system similar to that previously described in *Necturus* gallbladder (Spring & Ericson, 1982). The system may also be similar to the Na−Cl cotransport described in squid axon (Russell, 1981), and in flounder intestine (Musch et al., 1982). Both of these systems are bumetanide-sensitive, and require Na and Cl. They also require K^+. Unfortunately, we have not tested the effect of mucosal K^+ removal on the increased short-circuit current or increased Na^+ and Cl^- fluxes produced by mucosal acidification. The most interesting aspect of coupled Na and Cl uptake is the observation that the mucosal-to-serosal fluxes of Na^+, Cl^- and acetate persist for as long as the mucosal solution is acidified, but the increase in short-circuit current after mucosal acidification is a transient event. Moreover, even in the absence of mucosal acetate, there is a large increase in mucosal-to-serosal Na^+ and Cl^- flux after mucosal acidification, even though there is no change in I_{sc} associated with acidification. The implication of these results appears to be that mucosal acidity stimulates Na^+-anion cotransport. In the presence of chloride, Na^+ and Cl^- enter the cell across the apical membrane, after which the ions move across the basolateral membrane to produce an essentially electroneutral movement of Na^+ and Cl^- across the epithelium. This produces net mucosal-to-serosal flux of Na^+ and Cl^- with little or no change in I_{sc}.

In the presence of acetate immediately after mucosal acidification, besides Na−Cl movement, there may also be a similar movement of Na^+ and acetate anion across the apical membrane. As with Cl^-, some of the acetate may move across the basolateral membrane; however, some would be recycled across the apical membrane as the protonated form. This would leave an excess of Na^+ in the cell to produce an increase in I_{sc}. With continued mucosal acidification, the intracellular acetate concentration would rapidly reach an equilibrium essentially independent of the rate of Na^+-acetate entry. Thus, the large increase in intracellular acetate would produce a restoration of the essentially electroneutral movement of Na^+ and chloride across the basolateral membrane, and a reduction of the transient increase in I_{sc}.

The Induction of Apical Na−Cl Cotransport

The demonstration of a Na−Cl cotransport mechanism in the apical membrane prompts the question of how the mechanism is induced. Our initial interpretation was that the transport mechanism was always present, but in a form incapable of producing coupled Na−Cl movements. The acidification of the mucosal solution with some concomitant acidification of the intracellular compartment, altered the conformation of the latent transporter to allow Na−Cl uptake. This explanation of our results is a possibility, but the provocative work of Lewis and de Moura (1982) suggests another possibility. In their work, they demonstrated an incorporation of new amiloride-sensitive Na^+ channels into the apical membrane in response to a variety of mechanical or osmotic stimuli. They attribute the source of these channels to fusion of channel-containing intracellular vesicles with the apical membrane. It seems perfectly reasonable to

us that the increase in Na−Cl cotransport could be the result of a similar fusion with the following qualification.

In our experiments, we see no increase in the amiloride-sensitive Na^+ conductance. This implies that either different vesicles than those which contain amiloride-sensitive channels are fusing with the apical membrane (ones which only contain Na−Cl transporters), or the amiloride-sensitive channels have themselves been modified.

Alterations in Membrane Potentials and Membrane Conductances

Microelectrode recording revealed that when the mucosal solution was acidified, there was a substantial hyperpolarization of the basolateral membrane. There are two possible sources for the hyperpolarization. First, since the Na^+, K^+-ATPase seems to be responsible for at least part of the Na^+ movement across the basolateral membrane, the electrogenicity of this system may contribute to the potential (Rose & Narhwold, 1976; Lewis et al., 1978; Helman, Nagel & Fisher, 1979; Kirk, Halm & Dawson, 1980; Eaton et al., 1982). But the potential generated by the ATPase may not be large enough to explain the magnitude of the basolateral potential (Eaton et al., 1982). A second possible source of a portion of the basolateral potential may be a cotransport system which moves Na^+ and HCO_3^- from the serosal solution into the cell, with a concomitant movement of Cl^- (possibly conductive) out of the cell into the serosal solution. The four results which suggest the presence of this $Na^+/HCO_3^-/Cl^-$ transport system are: (1) a large negative basolateral membrane potential which is made more positive by HCO_3^--free serosal solutions; (2) a basolateral potential which is also depolarized by Na^+-free serosal solutions; (3) in both instances, a concomitant blockage by Na^+-free and HCO_3^--free serosal solutions of the transient increase of I_{sc} associated with mucosal acidification in the presence of acetate; and (4) the observation that SITS is capable of reducing a portion of the transepithelial Cl^- flux.

There are several possible mechanisms for this cotransport system, any of which, if present, could contribute to the basolateral hyperpolarization in a different manner. One simple possibility is that the basolateral transport system is electroneutral, transporting one Na^+ and one HCO_3^- from the serosa into the cell, while one Cl^- and one other cation are transported out. Several systems of this sort have been postulated or observed (Thomas, 1977; Boron & Russell, 1983). Under these conditions, all of the basolateral hyperpolarization would come from the enhanced activity of the Na^+, K^+-ATPase caused by the increased intracellular Na^+.

An alternative possibility which cannot be logically distinguished on the basis of our results, would be a cotransport system similar to that described by Boron and Boulpaep (1983), in which one Na^+ and two HCO_3^- are transported from the serosa to the cell interior. This system is itself electrogenic and enhances Cl^- movement from the cell interior to the serosal solution by promoting a favorable electrical gradient for Cl^- movement through conductive pathways. This system is attractive in several ways. A substantial Cl^- conductance of the basolateral membrane has been previously described (Lewis et al., 1978). Also, the system is activated by intracellular acidification, and is enhanced by the large HCO_3^- gradient produced by intracellular acidification. This system is capable of generating very large hyperpolarizing potentials at the basolateral membrane of *Ambystoma*, the tiger salamander (Boron & Boulpaep, 1983). Thus, the presence of a similar system in rabbit urinary bladder would make it unnecessary to postulate extraordinarily large electrogenic responses from the Na^+, Ka^+-ATPase: responses larger than any heretofore observed (Lewis et al., 1978; Eaton et al., 1982). However, despite the attractiveness of the system in explaining the large basolateral hyperpolarization, we have no fundamental result which would allow us to favor the one possibility of an electrogenic $Na^+-HCO_3^-$ system and electrogenic Na^+, K^+-ATPase operating in concert, as opposed to the alternative possibility of an electroneutral Na^+-anion transport with an extremely active Na^+, K^+-ATPase. Our results are summarized in the following diagrammatic model (Fig. 13).

The fundamental steps depicted in Fig. 13 are: (1) Acidification of the mucosal solution at constant CO_2 tension, or in the presence of the anion of a weak acid (such as acetate), leads to the formation of carbonic acid or the protonated form of the weak acid (acetic acid); (2) The protonated form of the weak acid enters the cell and dissociates to acidify the intracellular compartment; (3) The mucosal acidity (and possibly the increased intracellular acidity) activates a Na−Cl cotransport system at the mucosal membrane, which allows downhill movement of Na^+ and Cl^- into the cell. The cotransport can be blocked by bumetanide; (4) The increased intracellular acidity also activates a $Na^+-HCO_3^-$ cotransport system which moves Na^+ and HCO_3^- from the serosal solution

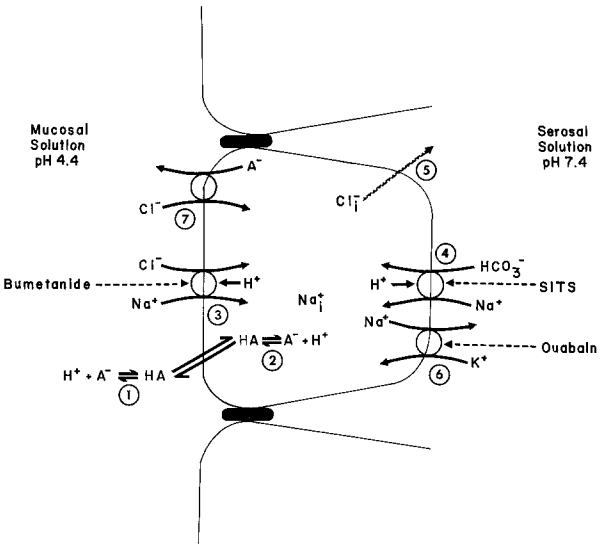

Fig. 13. Possible model for the movement of Na^+, Cl^-, and weak acids after mucosal acidification. The significant steps in the model are: (1) Mucosal acidification promotes formation of free weak acid which enters the cell and (2) dissociates; (3) The mucosal acidification and intracellular acidification appears to promote coupled Na−Cl uptake, which is blockable by mucosal bumetanide; (4) The intracellular acidity also stimulates a $Na^+ - HCO_3^-$ uptake mechanism at the serosal membrane. This system is inhibited by SITS, and may involve cotransport of Cl^-; alternatively, (5) Cl^- may leave the cell conductively; (6) The Na^+ which enters the cell is removed by the Na^+, K^+-ATPase, which is blockable by ouabain; (7) Finally, there may be an alternative mechanism for Cl^- entry which involves exchange for intracellular HCO_3^- or the anion of another weak acid

to the cell interior; (5) At the same time, it directly or indirectly promotes movement of Cl^- from the cell to the serosal medium; (6) Increased intracellular Na^+ activates the Na^+, K^+-ATPase, which moves Na^+ from the cell interior to the serosal solution; (7) Chloride entry may also take place via an independent pathway which is stimulated by acetate.

The primary significance of this lies in the uncovering of a mechanism for Na^+ uptake in a tight epithelium, which has been previously described only in leaky epithelia. This suggests that latent mechanisms may be present in all epithelia which are normally only expressed in a few, but which can be revealed in other epithelia by appropriate alterations in their environment.

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