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Effect of Anions on Amiloride-Sensitive, Active Sodium Transport across Rabbit Colon, *in Vitro*

Evidence for "Trans-inhibition" of the Na Entry Mechanism

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Summary. Replacement of Cl in the solutions bathing partial mucosal strips of rabbit descending colon with sulfate, isethionate, hydroxypropane-sulfonate and, to a lesser degree, ethanesulfonate stimulates active Na absorption (J_{net}^{Na}) when the baso-lateral pump mechanism is not saturated. These effects are rapid in onset and are readily reversible. Our findings indicate that these stimulatory anions decrease the resistance of the amiloride-sensitive Na entry step at the mucosal membrane (R_{Na}^{m}). However, when the active Na pump mechanism at the baso-lateral membrane is saturated these stimulatory anions do not decrease the resistance of the Na entry process. These findings suggest the presence of a negative feedback between the activity of the pump mechanism and the resistance of the Na entry step which may be mediated by the size of the intracellular Na transport pool. In other words, it seems that when the baso-lateral pump is operating at its maximal rate the resistance to Na entry across the mucosal membrane through the amiloride-sensitive pathway is at a minimum and cannot be further decreased.

Previous studies on Na and Cl transport by an in vitro preparation of rabbit descending colon have demonstrated that (i) the transepithelial electrical potential difference (ψ_{ms}) and the short-circuit current (I_{sc}) are entirely attributable to active Na transport from the mucosal solution to the serosal solution; (ii) amiloride inhibits active Na transport by blocking Na entry into the cell from the mucosal solution; (iii) although Cl is also actively absorbed, this process is electrically silent and probably involves a one-for-one exchange with HCO_3; and, (iv) there appears to be no interaction between the transport processes responsible for active Na and Cl absorption inasmuch as Cl absorption is not affected when Na in the bathing media is replaced with choline or when active

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Na transport is blocked with amiloride (Frizzell *et al.*, 1975; Frizzell, Koch & Schultz, 1976; Schultz, Frizzell & Nellans, 1977).

In our earlier studies we observed that when Cl in both bathing media was replaced with isethionate, active transport (J_{net}^{Na}) was markedly stimulated as a result of an increase in the unidirectional flux of Na from mucosa-to-serosa (J_{ms}^{Na}) alone; excellent agreement between J_{net}^{Na} and the I_{sc} persisted under these conditions. Although we pointed out that in these experiments the control values (i.e., in the presence of Cl) were significantly lower than those generally observed, we could offer no explanation for the apparent stimulatory effect of isethionate (Frizzell *et al.*, 1976) and the observation was "shelved". However, on a number of occasions during the past two years the same phenomenon was noted and, again only when the initial values of J_{net}^{Na} were low. These recurrences prompted the present investigation dealing with the effect of replacing Cl with various anions on J_{net}^{Na}. The results indicate that (i) replacement of Cl in the bathing media with several anions rapidly stimulates active Na absorption through the amiloride-sensitive pathway; (ii) this effect appears to be related to the presence of the replacement anion in the mucosal solution and is associated with an increase in the conductance of the active Na transport pathway; and (iii) the magnitude of the effect is inversely related to the rate of active Na transport prior to the replacement of Cl. These observations suggest that when the rate of active Na transport is low, Na entry across the mucosal membrane via an amiloride-sensitive pathway is rate-limiting and that certain anions enhance this entry process. At high spontaneous or induced rates of Na entry, the pump mechanism at the baso-lateral membrane is saturated (i.e., it is operating at its maximal rate in the presence of a normal Ringer's solution containing 10 mM glucose) and becomes the rate limiting step for transcellular Na movements. However, our data also indicate that when the pump mechanism at the baso-lateral membrane is saturated, the Na-conductance of the amiloride-sensitive entry step cannot be further increased. These findings suggest the presence of a negative feedback between the size of the intracellular Na transport pool and the permeability of the amiloride-sensitive entry pathway at the mucosal membrane.

Materials and Methods

White rabbits of either sex, weighing 2–3 kg, were sacrificed with intravenously administered pentobarbital. "Partial mucosal strips" of segments of descending colon were ob-

tained as described previously (Frizzell *et al.*, 1976) and mounted in the short-circuit apparatus described by Schultz and Zalusky (1964). Briefly, 1.13 cm^2 of tissue was held between two halves of a plexiglass chamber and exposed on each surface to 10 ml of a buffered electrolyte solution at 37 °C. The composition of the normal electrolyte solution (NSR) was (in mM): 140 Na; 124 Cl; 21 HCO_3; 5.4 K; 2.4 HPO_4; 0.6 H_2PO_4; 1.2 Mg; 1.2 Ca; and 10 glucose. Chloride-free solutions were prepared by isosmotic replacement of NaCl with the sodium salts of isethionic acid (IE), ethanesulfonic acid (ESA), *p*-phenol-sulfonic acid (PSA) (Eastman Kodak Co.); 3-hydroxypropane sulfonic acid (HPSA) (Aldrich Chemical Co.); or sulfate, (Fisher Scientific Co.). The sulfate solution was prepared by replacement of NaCl with 50% Na_2SO_4 and 50% mannitol to maintain isosmolarity. In the chloride-free solutions $MgCl_2$ and $CaCl_2$ were replaced with $MgSO_4$ and $CaSO_4$, respectively. The individual bathing solutions were perfused through each half chamber by means of a gas-lift circulating system driven by a water-saturated gas mixture of 95% O_2 and 5% CO_2; all solutions had a pH of 7.4.

All experiments were carried out under short-circuit conditions. Tissue resistance (R_t) was calculated from the ratio of the open-circuit potential difference, ψ_{ms}, determined briefly at 10-min intervals, to the short-circuit current, I_{sc}. In cases with low I_{sc}, for example in the presence of amiloride, a short (1-sec) pulse of direct current was passed across the preparation and the resulting potential difference was used to calculate the tissue resistance. As pointed out earlier (Schultz *et al.*, 1977) isolated rabbit colon behaves as an ohmic resistor over the range ± 50 mV.

After the electrical parameters had reached a steady-state with the normal saline Ringer's (NSR) bathing both sides of the preparation, 4–5 determinations of I_{sc} and ψ_{ms} were recorded (control period). Then the solution bathing either one or both sides of the tissue was changed to another in which Cl had been replaced by one of the test anions, and the I_{sc} and ψ_{ms} were monitored for at least 40 to 50 min. Finally, either amiloride (Merck, Sharp & Dome) or amphotericin B (Squibb) was added to the mucosal solution. In some experiments the reversibility of the anion-effect was examined by replacing the experimental solution with the normal Cl-containing buffer (NSR).

Results are expressed as the mean $\pm$ SEM based on the number of experiments performed. Differences between means were evaluated using the paired *t*-test; a value of $p < 0.05$ is considered significant.

Results

Effect of Anions on I_{sc} and G_t

The effects of replacing 107 mM Cl in the solutions bathing both sides of the tissue with isosmolar isethionate (IE), hydroxypropane sulfonate (HPSA), ethane-sulfonate (ESA), *p*-phenolsulfonate (PSA), or sulfate (SO_4) on I_{sc} and total tissue conductance, G_t, are given in Table 1. IE, HPSA, and SO_4 caused statistically significant increases in I_{sc}, whereas the effect on G_t was apparently variable (*see below*). On the average, ESA had only a small and statistically insignificant effect on I_{sc} and decreased G_t, whereas PSA markedly decreased I_{sc} and increased G_t.

Examples of the time-courses of the effects of IE, HPSA, SO_4, and PSA on I_{sc} are shown in Figs. 1–4. The actions were rapid in onset

Table 1. Effect of various anions on short-circuit current (I_{sc}) and tissue conductance (G_t)[a]

Anion	n	I_{sc} (μEquiv/cm²hr)		G_t (mmhos/cm²)	
		Control	Test	Control	Test
IE	13	2.5±0.3	3.5±0.4*	4.2±0.3	4.0±0.3
HPSA	10	2.5±0.3	3.9±0.4*	3.9±0.3	3.8±0.2
SO_4	13	2.7±0.3	3.9±0.3*	4.3±0.4	4.2±0.3
ESA	7	2.6±0.5	2.8±0.4	4.7±0.5	3.9±0.4
PSA	12	2.4±0.2	1.2±0.2*	4.3±0.2	5.1±0.5*
CL	33	2.0±0.2	2.5±0.2	3.9±0.2	4.2±0.2

[a] 107 mM Cl in both bathing solutions was replaced isotonically with the *test* anion; the I_{sc} and G_t observed when a new steady state was established are compared with the values observed in control tissues. The row labelled "Cl" indicates the results of experiments in which 90% of the normal bathing medium was removed and replaced with the same medium. n designates the number of experiments; and asterisk (*) indicates a value which differs from the control value at $p<0.05$. The abbreviations are given in the text (*see* Methods).

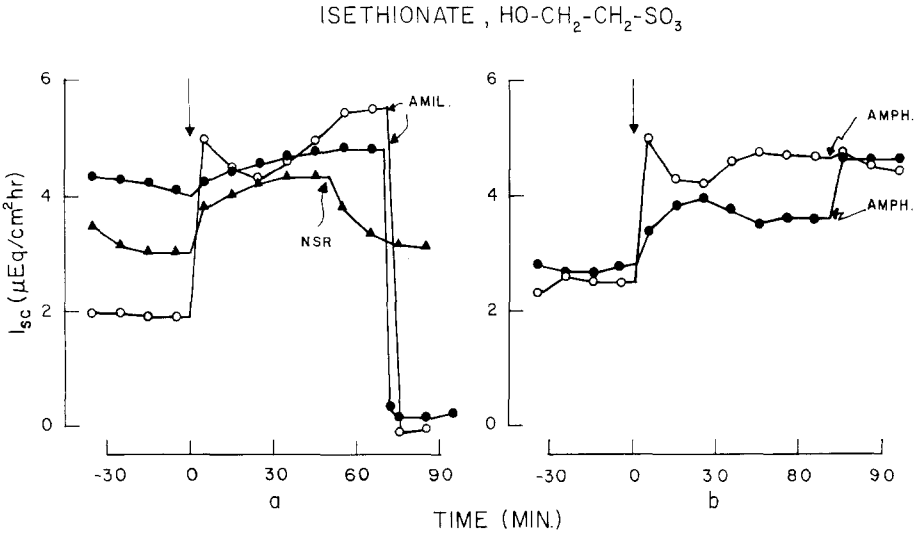

Fig. 1. Several examples of the effect of IE (added to both bathing solutions at the arrow) on the I_{sc}. *NSR* indicates the effect of replacing both bathing media with the Cl-containing buffer. *AMIL.* and *AMPH.* indicate the effects of amiloride (10^{-4} M) or amphotericin B (15 μg/ml) added to the mucosal solution. Note, in (*a*), the decreasing effectiveness of IE with increasing control I_{sc}. Note, in (*b*), the failure of amphotericin B to increase I_{sc} when the latter had already achieved its maximal value

with a tendency for a further small increase over a time period from 40 to 50 min. The effects of all anions tested were reversible; after replacing the bathing solutions on both sides of the tissue with the normal

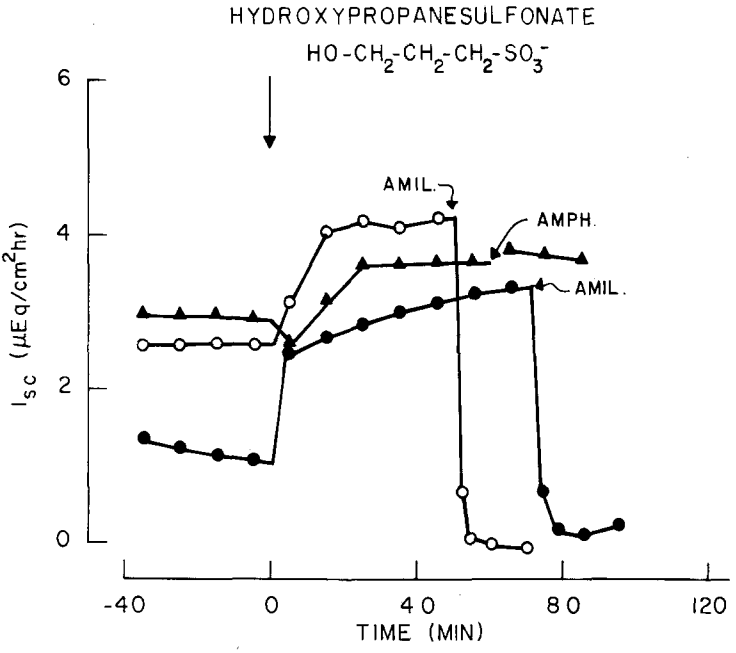

Fig. 2. Examples of the effect of HPSA (added to both solutions at the arrow) on the I_{sc}. (*See* legend to Fig. 1 for details)

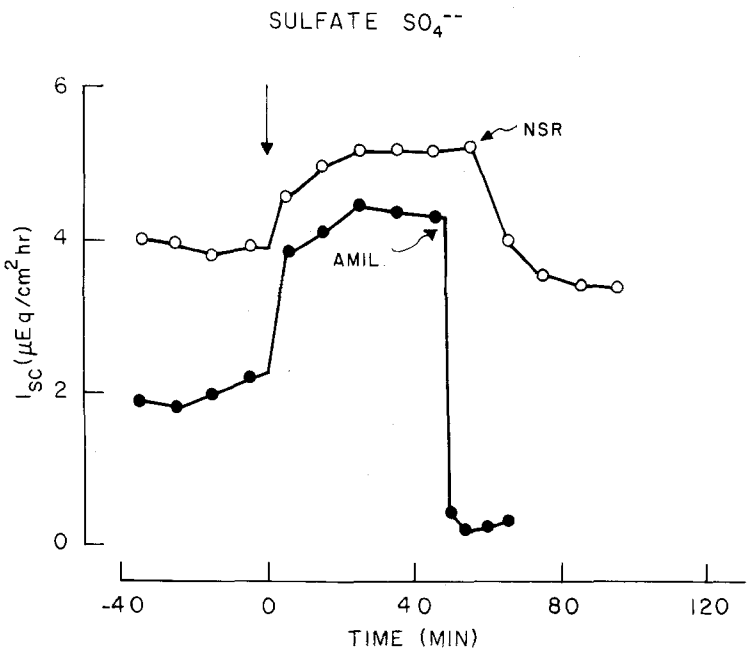

Fig. 3. Effect of SO_4 (added to both bathing solutions at the arrow) on the I_{sc}. (*See* legend to Fig. 1 for details)

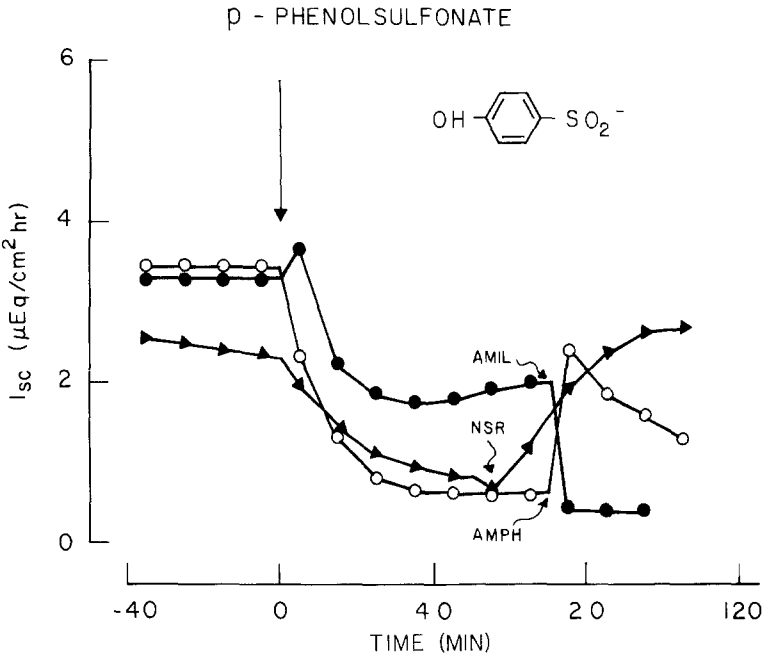

Fig. 4. Effect of PSA (added to both bathing solutions at the arrow) on the I_{sc}. Note effect of amiloride (*AMIL*), reversibility (*NSR*) and transient effect of amphotericin B (*AMPH*)

electrolyte solution (NSR), the I_{sc} slowly returned to control values (Figs. 1, 3, 4).

The magnitude of the increase in I_{sc} in the presence of IE, HPSA, and SO_4 was inversely related to the control I_{sc}; in tissues characterized by a low spontaneous I_{sc}, stimulation of I_{sc} was large, whereas the effect was comparatively small if I_{sc} was spontaneously high (Figs. 1, 3). A maximal I_{sc} of about 5–6 μEquiv/cm^2 hr was never surpassed. Similarly, addition of amphotericin B to the mucosal bathing solution (15 μg/ml) brought about a further increase in the I_{sc} only when IE, HPSA, or SO_4 had not already increased I_{sc} to its maximal value (Figs. 1 and 2). In all instances addition of amiloride to the mucosal solution (10^{-4} M) promptly abolished the I_{sc} (Figs. 1–4).

The effect of replacement anions on the I_{sc} is plotted as a function of the control I_{sc} in Fig. 5. Despite the scatter, there is a clear inverse relation ($p<0.01$) such that $\Delta I_{sc}=0$ when the control I_{sc} is 5.7 μEquiv/cm^{2}hr. The effect of addition of amphotericin B to the mucosal solution in the presence of Cl or replacement anions is illustrated in Fig. 6. There is a clear inverse relation between the I_{sc} observed prior to the addition

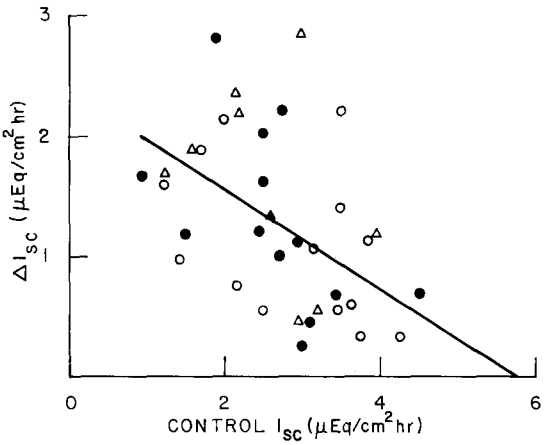

Fig. 5. Relation between the increase in I_{sc} (ΔI_{sc}) elicited by SO_4 (○), IE (●) HPSA (△) and ESA (▲) and the control I_{sc}

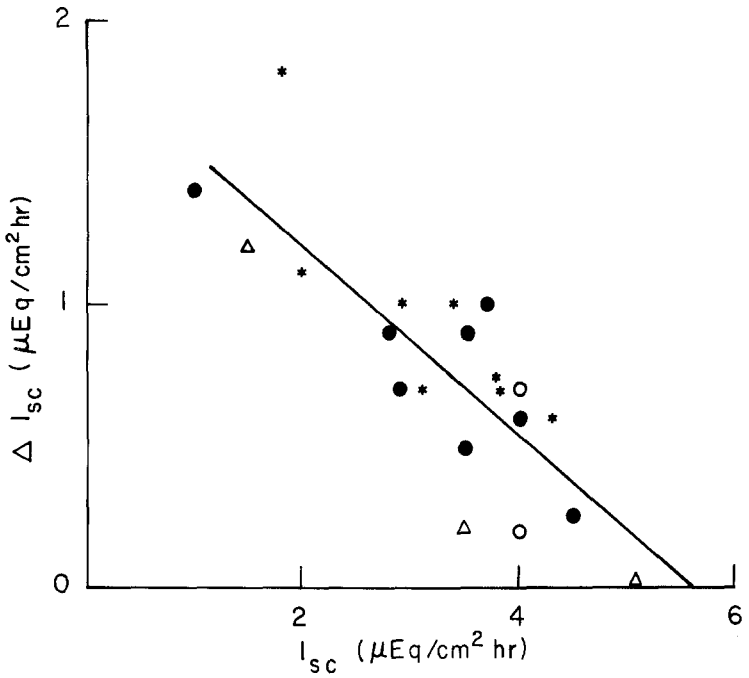

Fig. 6. Relation between the increase in I_{sc} (ΔI_{sc}) elicited by amphotericin B in the presence of Cl (*), SO_4 (○), IE (●) and HPSA (△) and the I_{sc} prior to addition of amphotericin B to the mucosal solution

of amphotericin B and the magnitude of the response (ΔI_{sc}) and as in Fig. 5, $\Delta I_{sc}=0$ when control $I_{sc}=5.7$ μEquiv/cm^{2}hr. A similar relation between the effect of amphotericin B on the I_{sc} in tissues bathed by

Table 2. Effect of unilateral replacement of chloride with isethionate on the short-circuit current[a]

Initial	n	Control	36 mM IE	107 mM IE	Final
M	6	2.8±0.3	2.8±0.3	3.8±0.4*	3.7±0.4
S	5	2.0±0.5	1.9±0.5	1.8±0.4	2.8±0.5*

[a] 36 mM Cl was replaced in *either* the mucosal (*M*) or the serosal (*S*) solution with isethionate (IE); after a new steady state was achieved, 107 mM Cl was replaced in the same solution with IE. The column labelled "Final" gives the results of replacing 107 mM Cl with IE in the *opposite* solution. n designates the number of experiments; all other values give the I_{sc} in μEquiv/cm^{2}hr. An asterisk (*) indicates a value that differs at $p < 0.05$ from the previous value.

NSR has been noted previously in a much larger series of studies (Frizzell & Schultz, 1976; Frizzell, *in preparation*).

The results of experiments designed to determine the concentration-dependence of the effect of IE and whether this anion is more effective when added to one or both bathing solutions are summarized in Table 2. In these experiments 36 mM Cl in the solution bathing either the mucosal (*M*) or the serosal (*S*) surface of the tissue was replaced with 36 mM IE. After a steady state was achieved, 107 mM Cl in the *same* bathing solution was replaced with 107 mM IE. Finally, when a new steady state was achieved, 107 mM Cl in the *opposite* bathing solution was replaced with 107 mM IE. It is apparent from the data given in Table 2 that 36 mM IE in either the mucosal or serosal solution did not significantly affect the I_{sc} while 107 mM IE significantly increased the I_{sc} but only when present in the mucosal solution. However, the results of these experiments must be interpreted with some caution inasmuch as replacement of Cl in only one bathing solution with a less permeant anion (*see below*) would be expected to give rise to a diffusion potential across this moderately leaky epithelium. The direction of this diffusion potential would be such that (i) replacement of Cl in the mucosal solution alone would tend to increase the I_{sc} whereas (ii) replacement of Cl in the serosal solution alone would tend to decrease the I_{sc}. Thus, conceivably, the increase in I_{sc} observed when IE is present in the mucosal solution alone could be entirely due to a diffusion potential and the failure to see any change in I_{sc} when IE is present in the serosal solution alone could be due to a diffusion potential which opposes the anion-stimulated increase in I_{sc}. This explanation is unlikely for two reasons. First, if the effect of IE is exerted from the serosal solution then, in both sets

of experiments shown in Table 2, the diffusion potential would have to almost precisely oppose the anion-induced increase in I_{sc}. Second, the increase in I_{sc} elicited when 107 mM IE is present in the mucosal solution alone is almost entirely inhibited by amiloride. Since amiloride only blocks Na entry into the cell and has no effect on the passive transepithelial conductance properties of the tissue (Frizzell *et al.*, 1976; Schultz *et al.*, 1977), it seems reasonable to conclude that the increase in I_{sc} elicited by mucosal IE is almost entirely due to an increase in J_{net}^{Na} and is not significantly affected by diffusion potentials. A possible explanation for the absence of large diffusion potentials when Cl is replaced in only one bathing solution will be offered below.

Effect of Amiloride on Anion-Induced Increases in I_{sc} and G_t

The effects of addition of 10^{-4} M amiloride to the mucosal solution on the I_{sc} and G_t in the presence of several replacement anions are illustrated in Figs. 1–3 and summarized in Table 3. We see that the average I_{sc} in the presence of 107 mM IE, HPSA and SO_4 is considerably greater than that in the presence of 107 mM ESA, a finding that is consistent with the data given in Table 1 indicating that, on the average, ESA does not markedly stimulate the I_{sc}. In all instances, amiloride rapidly abolished the I_{sc} and brought about a significant decrease in tissue conductance (G_t). As discussed previously (Frizzell *et al.*, 1975;

Table 3. Effect of amiloride on the short-circuit current (I_{sc}) and tissue conductance (G_t) in the presence of various replacement anions[a]

Test anion	n	I_{sc} (μEquiv/cm²hr)		G_t (mmhos/cm²)		"Calculated" $_aG_{Na}$
		Control	+Amiloride	Control	+Amiloride	
IE	5	3.6±0.1	0.1±0.1	4.0±0.6	2.8±0.5	1.2
HPSA	5	4.2±0.5	0.1±0.1	3.7±0.4	2.5±0.4	1.2
SO_4	4	4.3±0.7	0.3±0.1	3.6±0.3	2.4±0.2	1.2
ESA	5	2.9±0.7	0.1±0.1	3.5±0.6	2.8±0.4	0.7
Cl	10	1.8±0.2	0.0±0.0	3.1±0.4	2.6±0.3	0.5

[a] Control values are the steady-state data obtained in the presence of 107 mM of the test anion in both bathing solutions. The values given in the columns labelled "+Amiloride" are obtained within 1 min following the addition of 10^{-4} M amiloride to the mucosal solutions. The values of $_aG_{Na}$ (in mmhos/cm²) were calculated as described in the text. n designates the number of experiments.

Frizzell *et al.*, 1976; Schultz *et al.*, 1977) the decrease in G_t in the presence of amiloride is entirely attributable to a decrease in the conductance of the active Na transport pathway ($_aG_{Na}$) due to an increase in the resistance of the mucosal membrane to Na entry. The calculated values of $_aG_{Na}$ in the presence of the various anions are given in Table 3. It should be noted that in the presence of anions which stimulated the I_{sc}, $_aG_{Na}$ averaged 1.2 mmhos/cm^2. However, in the presence of ESA which only slightly stimulates I_{sc}, $_aG_{Na}$ averaged 0.7 mmhos/cm^2; this value is in excellent agreement with those previously observed in the presence of the normal, Cl-containing buffer (0.6–0.7 mmhos/cm^2) (Frizzell *et al.*, 1976; Schultz *et al.*, 1977). As shown in Table 3, in the present studies, $_aG_{Na}$ in the presence of Cl averaged only 0.5 mmhos/cm^2; this lower value is consistent with the lower control values of I_{sc} observed in this series[1].

Effect of p-Phenolsulfonic Acid

As shown in Table 1 and Fig. 4, the presence of 107 mM PSA in both bathing solutions resulted in a decline in I_{sc}. The data given in Table 4 indicate that this decrease was more marked when PSA was added to the mucosal solution alone than when added to the serosal

Table 4. Effect of unilateral presence of *p*-phenylsulfonnic acid on the short-circuit current (I_{sc}) and tissue conductance (G_t)[a]

Side	n	I_{sc} (μEquiv/cm^{2}hr)		G_t (mmhos/cm^2)	
		Control	PSA	Control	PSA
M	5	3.5±0.6	1.2±0.4*	5.0±0.3	5.2±0.5
S	5	3.5±0.7	2.4±0.6*	5.1±0.4	4.2±0.4*

[a] 107 mM Cl was replaced with *p*-phenylsulfonic acid (PSA) in either the mucosal (*M*) or serosal (*S*) solution. n designates the number of experiments; an asterisk (*) indicates a significant difference from control at $p<0.05$.

1 In most previous studies I_{sc} averaged 2.6 μEquiv/cm^{2}hr and $_aG_{Na}$ averaged 0.6–0.7 mmhos/cm^2. In one series of studies reported previously (Schultz *et al.*, 1977; Fig. 5), I_{sc} averaged 1.7 μEquiv/cm^{2}hr and $_aG_{Na}$ was 0.45 mmhos/cm^2. These findings together with those reported in the present paper are consistent with the notion that spontaneous variations in I_{sc} are due to differences in the resistance of the amiloride-sensitive Na entry step which appears to be rate limiting when I_{sc} is submaximal.

solution alone. Several other points are of interest. First, the effect of PSA in the mucosal solution or both solutions is reversed following replacement with the normal Cl-containing buffer (Fig. 4). Second, in the presence of PSA, the addition of amphotericin B to the mucosal solution (15 μg/ml) only resulted in a transient increase in I_{sc}.

Discussion

Effect of Stimulatory Anions on I_{sc}, G_t, ${}_aG_{Na}$ and the Electromotive Force of the Active Na Transport Pathway

These studies demonstrate that replacement of Cl in both bathing media with some anions results in prompt increases in I_{sc} only when the initial (control) I_{sc} is "submaximal" (*see* Fig. 5). The finding that the I_{sc} in the presence of these anions is completely inhibited by amiloride, together with the previous finding that $I_{sc}=J_{net}^{Na}$ in the presence of isethionate (Frizzell *et al.*, 1976), indicate that these anions stimulate active Na transport through the "physiological" pathway. In addition, it appears that this effect is dependent upon the presence of the replacement anions in the mucosal solution and is entirely reversible.

The relation between the change in I_{sc} and the accompanying change in G_t for the *individual* experiments whose *average* results are reported in Table 1, is given in Fig. 7. Clearly, there is a highly significant ($p<0.01$) linear relation between ΔI_{sc} and ΔG_t; and when $\Delta I_{sc}=0$, $\Delta G_t=-1.1$ mmhos/cm^2. The results of previous studies have shown that in the presence of 124 mM Cl the partial ionic conductance of Cl across this tissue, due to diffusional movements through paracellular pathways, is 1.5 mmhos/cm^2 (Frizzell *et al.*, 1976). Thus a decrease in G_t of approximately 1.3 mmhos/cm^2 would be expected when 107 mM Cl is replaced with an *inert,* impermeant anion; this value is in reasonable agreement with the value of $\Delta G_t=-1.1$ mmhos/cm^2 when $\Delta I_{sc}=0$ suggesting that SO_4, IE, HPSA and ESA are relatively impermeant anions compared to Cl.

An explanation for the increase in G_t with increasing ΔI_{sc} (above the decrease that would be expected when Cl is replaced with an inert, impermeant anion) emerges, at least in part, from the relation between ΔI_{sc} and ΔG_t or ${}_aG_{Na}$ for the *individual* experiments summarized in Table 3. A plot of ΔI_{sc} *vs.* ΔG_t or ${}_aG_{Na}$ following addition of amiloride to the solution bathing the mucosal surface of tissues exposed to SO_4, IE,

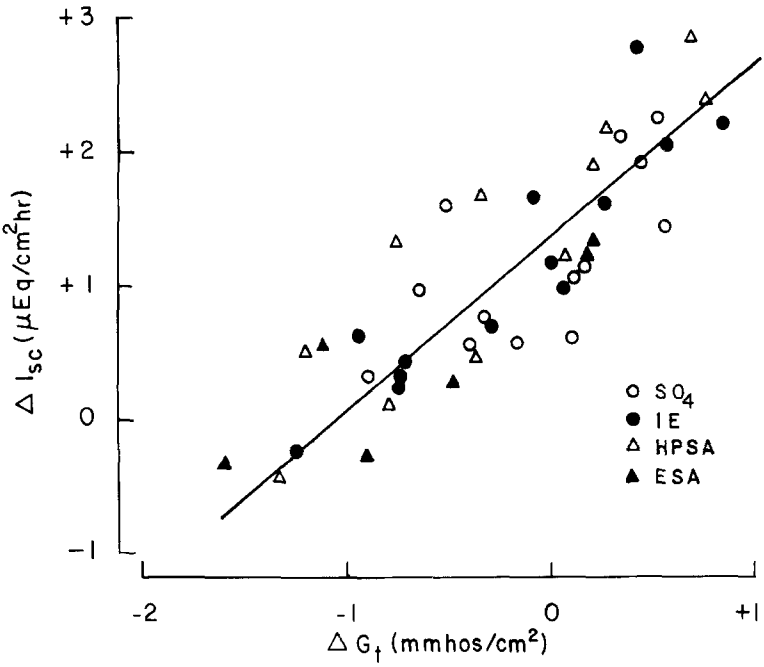

Fig. 7. Relation between increase in I_{sc} (ΔI_{sc}) elicited by stimulatory anions and change in tissue conductance (ΔG_t). Data are from the individual experiments summarized in Table 1

HPSA or ESA is given in Fig. 8. Clearly, there is a linear relation between the I_{sc} prior to the addition of amiloride to the mucosal solution and ${}_aG_{Na}$ that extrapolates to the origin. Schultz *et al.* (1977) demonstrated that the active Na transport pathway across rabbit colon displays ohmic behavior so that

$$I_{sc} = {}_aG_{Na} \cdot E_{Na} \tag{1}$$

where E_{Na} is the "overall electromotive force" of the pathway (Ussing & Zerahn, 1951). The slope of the line shown in Fig. 8, or E_{Na}, is equal to 95 mV; this value is in excellent agreement with the E_{Na} of 100–117 mV estimated previously by two independent means for rabbit colon under control conditions (Schultz *et al.*, 1977).

This analysis indicates that the anion-induced increase in I_{sc} can be attributed to an increase in the overall conductance of the active Na transport pathway with no change in the overall electromotive force of this pathway. In this respect the anion-induced increase in I_{sc} resembles

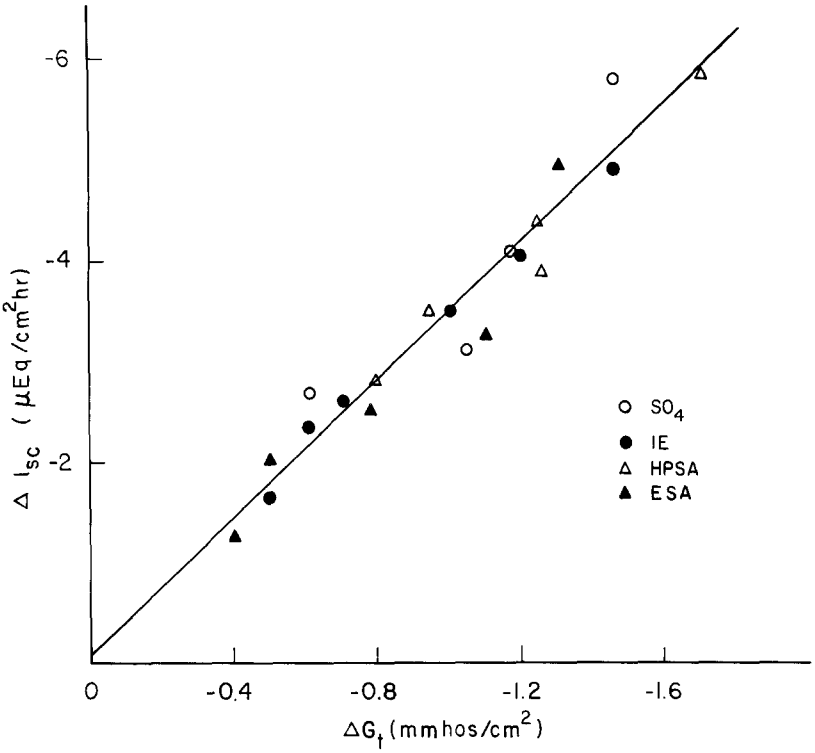

Fig. 8. Relation between decrease in I_{sc} (ΔI_{sc}) and decrease in tissue conductance (ΔG_t) following the additon of amiloride to the mucosal solution. Data are from the individual experiments summarized in Table 3

the increase in I_{sc} elicited by vasopressin in toad urinary bladder (Yonath & Civan, 1971) and the early increase in I_{sc} elicited by aldosterone in toad bladder (Spooner & Edelman, 1975; Siegel & Civan, 1976; Saito & Essig, 1973) and frog skin (Lang, Caplan & Essig, 1975).

Examination of the data given in Fig. 7 indicates that the increase in $G_t(\Delta G_t)$ (above the level expected when Cl is replaced with an inert, impermeant anion), associated with an anion-induced increase in I_{sc} (ΔI_{sc}) is approximately 3 times greater than that which can be accounted for by an increase in ${}_aG_{Na}$ alone. That is, the slope of the line is equal to 35 mV whereas if ΔG_t were due entirely to an increase in ${}_aG_{Na}$, the slope of the line should be $E_{Na} \cong 100$ mV. Thus, the increase in I_{sc} is also associated with an increase in the conductance of some other transepithelial pathway(s). If the conclusions of Schultz *et al.* (1977) are correct, this must represent an increase in the conductance of the paracellular

pathway to ions other than Cl in response to an increase in J_{net}^{Na}; however, further study is necessary to verify this point.[2]

Site of Action of Stimulatory Anions: Evidence for "Feedback" Between the Na Exit (Pump) and Entry Mechanisms

According to the classical "double membrane" representation of epithelial cells, an agent can stimulate active Na transport by (i) decreasing the resistance of the mucosal membrane to Na entry; (ii) increasing the activity of the active Na extrusion mechanism (pump) at the basolateral membrane; or (iii) some combination of these actions.[3] If this simple notion is correct, the site of action of the stimulatory anions can be inferred from the following line of reasoning:

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2 An increase in the conductance of the paracellular pathways to cations (particularly Na) and possibly even to the stimulatory anions associated with an increase in J_{net}^{Na} would reduce the magnitude of diffusion potentials that should arise when Cl in the mucosal solution alone is replaced with a less permeant anion. This may be, at least in part, an explanation for the finding that amiloride almost entirely abolished the I_{sc} when isethionate was present in the mucosal solution alone. The failure to observe significant diffusion potentials when isethionate replaces 107 mM Cl in the serosal solution alone may be due to the fact that under these conditions the tissue is actively absorbing Cl at the normal rate and that, as a result, the Cl concentration in the unstirred or poorly stirred subepithelial spaces may be much higher than 17 mM. Indeed, as the result of active Cl transport there may be no significant Cl asymmetry across the major resistive elements of the paracellular route even when the bulk serosal solution contains 107 mM isethionate.

3 The relations among the resistance of the mucosal entry step, pump activity and the rate of transepithelial Na transport emerge from the following formal considerations:

Under steady-state conditions the I_{sc} must be equal to the rate of Na entry into the cell across the mucosal membrane, I_{Na}^{m}. Further, inasmuch as Na entry into the cell appears to be a passive ("downhill"), conductive (rheogenic) process, it can be explicitly described in terms of equivalent electrical analogues as follows (Schultz *et al.*, 1977):

$$I_{Na}^{m} = (E_{Na}^{m} - \psi_{mc})/R_{Na}^{m}$$

Where, E_{Na}^{m} is the chemical driving force across the mucosal membrane and is equal to $(RT/\mathscr{F})$ ln $([Na]_m/[Na]_c)$ where [Na] is the Na activity and the subscripts m and c designate the mucosal solution and the intracellular transport pool respectively; ψ_{mc} is the electrical potential difference across the mucosal membrane with reference to the mucosal solution and represents the electrical driving force for entry; and, R_{Na}^{m} is the phenomenologic resistance of the mucosal membrane to the Na current. If entry is diffusional, R_{Na}^{m} is the so-called "integral resistance" given by Finkelstein and Mauro (1963); if entry is carrier-mediated, R_{Na}^{m} is more complex and depends upon the particulars of the carrier mechanism.

Thus, when $[Na]_m$ is constant, I_{Na}^{m} will increase when:

a) Increased pump activity leads to a decrease in $[Na]_c$ and an increase in E_{Na}^{m}.

b) Increased pump activity brings about a hyperpolarization of ψ_{mc} (cell interior more negative) (Schultz *et al.*, 1977; Frizzell & Schultz, 1978). [Note: Hyperpolarization of

Our results indicate that the magnitude of the stimulatory effect is inversely related to the I_{sc} under control conditions and that when the control I_{sc} is in the range of 5–6 μEquiv/cm^{2}hr little or no further increase can be elicited; this "maximum rate" of transport for this preparation has been noted previously (Frizzell & Schultz, 1976; Frizzell, *in preparation*; Schultz & Frizzell, 1978). In principle, this could be due to the fact the Na entry rate is maximal (i.e., the resistance to Na entry cannot be further reduced) or to the fact that the pump mechanism is saturated. The results of our studies with amphotericin B permit us to distinguish between these alternatives. We have shown that when the I_{sc} is "submaximal" the addition of this polyene antibiotic to the mucosal solution brings about (i) equivalent increases in I_{sc} and J_{net}^{Na}; (ii) an increase in the unidirectional influx of Na from the mucosal solution into the cell (J_{mc}^{Na}); (iii) an increase in tissue conductance; and (iv) K secretion into the mucosal solution. However, when the I_{sc} is "maximal", amphotericin B does not bring about a further increase in I_{sc} or J_{net}^{Na} in spite of the fact that J_{mc}^{Na} and G_t are increased and K secretion is elicited (Frizzell & Schultz, 1976; Frizzell, *in preparation*; Schultz & Frizzell, 1978); the relation we observed between ΔI_{sc} in response to amphotericin B and the control (pre-amphotericin B) I_{sc} is essentially identical to that illustrated in Fig. 6. We interpret these findings to indicate that *under all conditions,* amphotericin B disrupts the normal permselective properties of the mucosal membrane through a strictly physico-chemical interaction and thereby decreases the resistance of this barrier to the diffusional movements of Na and K. The failure of amphotericin B to increase I_{sc} and J_{net}^{Na} when these values are in the range of 5–6 μEquiv/cm^{2}hr must mean that the pump mechanism is saturated at this rate of active Na transport.

The findings that (i) *both* the stimulatory anions and amphotericin B are effective when the I_{sc} is "submaximal"; (ii) *both* are ineffective when the I_{sc} is "maximal"; and (iii) the "maximum" I_{sc} is the same under *both* conditions (Figs. 5 and 6) strongly suggest that these anions act to decrease the resistance to Na movement through the amiloride-

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ψ_{mc} could result from transient diffusion potentials across the mucosal or serosal membranes when the ionic composition of the bathing media is altered. However, if Cl diffuses out of the cells when 90% of the Cl in both bathing media is replaced with a less permeant anion, the resulting diffusion potentials across the mucosal, serosal or both limiting membranes would, if anything, depolarize ψ_{mc}.]

c) R_{Na}^{m} decreases.

sensitive entry step; a stimulatory effect of these anions on the pump mechanism seems highly unlikely.

This conclusion is supported by the findings that:

a) The anions are rapidly effective when added to the mucosal solution alone but are apparently ineffective when added to the serosal solution alone. In view of the fact that these anions are not likely to enter the cells rapidly an intracellular site of action seems improbable.

b) The action of these anions can be attributed to an increase in ${}_aG_{Na}$ with no effect on the overall E_{Na}. We have previously demonstrated that the resistance of the active Na transport pathway ($1/{}_aG_{Na}$) is approximately 1700–2000 ohm cm^2 and that approximately 90% of this "overall" resistance is represented by the Na entry step at the mucosal membrane (R^m_{Na}) (Schultz *et al.*, 1977). As shown in Table 3, in the presence of the stimulatory anions the resistance of the active Na transport pathway declines to approximately 800 ohm cm^2. If our previous estimates are correct, this *cannot* be attributed entirely to a decrease in the resistance of the Na exit process but *must* involve a substantial decrease in the resistance of the amiloride-sensitive entry step.

Thus, all of our findings are consistent with the notion that the stimulatory anions act at the mucosal membrane to increase the ease of Na entry into the cell through the physiologically operant entry step. There is no evidence whatsoever for a primary action at the level of the pump mechanism.

If this is the case, the data presented in Fig. 7 raise a perplexing question, namely: Why do these anions fail to elicit an increase in G_t or ${}_aG_{Na}$ when the pump is already operating at its saturation level? That is, an increase in G_t, above the level expected when Cl is replaced with an inert, impermeant anion, is only observed in association with an increase in I_{sc}; but, when the I_{sc} is at the maximal level so that $\Delta I_{sc}=0$ there is no apparent increase in G_t. Thus, it appears that when the entry rate is sufficient to saturate the pump mechanism, the resistance to Na entry via the amiloride-sensitive path cannot be further decreased[4].

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4 As discussed below the effect of stimulatory anions could be the result of (i) increasing (unmasking) the number of accessible Na entry sites, and/or (ii) decreasing the resistance to Na movement across each site. Thus, the finding that the tissue conductance cannot be increased by these anions when the pump is saturated suggests that (i) the total number of accessible sites (with fixed resistance) is determined by the saturation level of the pump or (ii) the total number of sites is not determined by the saturation level of the pump mechanism but the *combined* conductance of the accessible sites is limited by the pump activity.

As noted above, this behavior is quite different from that observed with amphotericin B inasmuch as this agent *always* results in an increase in G_t regardless of the level of pump activity.

These observations raise the possibility of a "feedback" interaction between the level of pump activity and the entry step, possibly mediated by the size of the intracellular transport pool. Thus, it is possible that when the pump is operating at its saturation level, the size of the Na transport pool is maximal, and this pool size somehow prevents a further increase in the permeability of the amiloride-sensitive entry step. Such a negative feedback or "trans-inhibition" would serve to "protect" the size of the pool when pump activity is rate limiting.

Although this notion is admittedly speculative, there are a number of observations in the literature consistent with a feedback interaction between the basolateral pump and the Na entry step. For example, *inhibition* of pump activity (by removing K from the serosal bathing solution, ouabain, metabolic inhibitors) apparently increases the resistance of the mucosal or outer membrane to Na entry in frog skin (Mac Robbie & Ussing, 1961; Biber, 1971; Larsen, 1973; Erlij & Smith, 1973), toad urinary bladder (Essig & Leaf, 1963) and rabbit urinary bladder (Lewis *et al.*, 1976). In this respect it is of interest that Erlij and Smith (1973) reported that ouabain inhibits Na uptake across the outer membrane when the tissue was preincubated in a Na-containing buffer but has no effect on uptake when the tissue is preincubated in the absence of Na; these findings are consistent with the notion that the decrease in the permeability of the outer membrane to Na resulting from treatment with ouabain is due to an increase in cell Na concentration.[5]

Hong and Essig (1976) have recently demonstrated that treatment of toad urinary bladder with submaximal inhibitory concentrations of ouabain or the metabolic inhibitor, 2-deoxyglucose, depresses ${}_aG_{Na}$ deter-

5 Several points should be stressed. First, the inhibitions of net or unidirectional influx observed by Biber (1971), Erlij and Smith (1973), and Essig and Leaf (1963) could have been due to changes in ψ_{mc} (more positive) resulting from inhibition of the pump (*see* Footnote 3); however, the results reported by Larsen (1973) and Lewis *et al.* (1976) indicate that inhibition of the pump mechanism brings about an increase in the electrical resistance of the outer or mucosal membrane (our R^m_{Na}). Frizzell (*in preparation*) and Turnheim *et al.* (*in preparation*) have shown that treatment of rabbit colon with ouabain decreases the unidirectional influx of Na into the cells across the mucosal membrane and increases the electrical resistance of the tissue; these results are in accord with those cited above.

Second, the results reported by Robinson and Macknight (1976) suggest that the decrease in Na entry into toad urinary bladder observed by Essig and Leaf (1963) when the serosal surface of the tissue was bathed with a K-free solution is due to a decrease in cell K (not an increase in cell Na).

mined using an approach similar to that employed in the present studies. These observations, together with those cited above, are consistent with the notion that *limitation* of pump activity inhibits the ease of Na entry. The reason(s) for the saturation of pump activity in rabbit colon is unknown and is under investigation at this time. Two possibilities that immediately come to mind are (i) energy-limitation to a surfeit of pump sites or (ii) a limited number of pump sites (with a maximal turn-over rate). Each of these possibilities is apparently mimicked by treatment of the tissue with suboptimal concentrations of 2-deoxyglucose (limitation of energy) or ouabain (which may result in a reduction in number of operant transport sites). Thus the results of Hong and Essig (1976) suggest that when the pump is *forced* to saturate at a lower level of activity, ${}_aG_{Na}$ decreases.

It is well established that Na entry across the outer or mucosal membranes of several Na-transporting epithelia is a saturable function of the external Na concentration which parallels the rate of transepithelial Na transport (*c.f.* Lewis & Diamond, 1976; Lindemann & Voute, 1976, for reviews); that is, the "permeability" of the entry process decreases with increasing external Na concentration. However, in most of these studies the tissues were exposed to the external Na concentrations for relatively long periods so that it is not clear whether the decrease in unidirectional influx or net entry of Na is due to an increase in the external Na concentration *per se* or to a secondary increase in the intracellular Na concentration.[6] Recently Lindemann and his collaborators, employing a technique that permits estimation of the Na-current across the outer membrane of frog skin following rapid (less than 10 sec) changes in the outer Na concentration have concluded that the resistance to Na entry is a saturable function of external concentration; their data suggest that the internal concentration is only minimally affected during these brief exposure periods (Lindemann & Gebhardt, 1973; Lindemann & Voute, 1976; Fuchs, Larsen & Lindemann, 1977). Using this technique, it would be of interest to determine how the resistance to Na entry is affected when intracellular Na concentration is varied in the presence of a constant external concentration.

Finally, both "carrier" models and "pore" models have been proposed to account for the saturating behavior of the Na entry step (*c.f.* Lindemann & Voute, 1976; Lindemann & Van Driessche, 1977). It can

6 In addition, since the electrical potential difference across the outer or mucosal membrane was not monitored in these studies its role in the saturation process cannot be assessed.

be readily shown that both types of models can exhibit "trans-inhibitory" effects (i.e., a decrease in unidirectional influx or net entry with increasing internal concentration) as well as *cis*-inhibition (i.e., saturating behavior with increasing external concentration).

Possible Mechanism(s) of Anion Action

The mechanism(s) by which the stimulatory anions could decrease the apparent resistance of the amiloride-sensitive Na entry step (R^m_{Na}) depends to some degree upon the nature of this entry step. If Na entry is the result of diffusion through "pores" (*c.f.* Fuchs *et al.*, 1977; Lindemann & Voute, 1976; Lindemann & Van Driessche, 1977) a decrease in R^m_{Na} could result from (i) increasing the number of accessible pores; (ii) an increased ability of Na to enter the pores (increased "partition coefficient") and/or (iii) increased mobility of Na through the pores (Finkelstein & Mauro, 1963; Sandblom & Eisenman, 1967). Alternatively, if Na entry is "carrier-mediated" a decrease in R^m_{Na} could result from (i) increasing the number of accessible carriers; (ii) an increase in the ability of Na to bind to the carrier sites; and/or (iii) an increased turn-over rate of the carriers.

The simplest explanation for the observed stimulatory effects is that these anions interact with positive charges at the outer surface of the mucosal membrane. These interactions could be relatively uniform along the entire membrane surface, or the interactions could be localized to sites on or near the Na entry mechanism. These interactions could increase the number (unmask) of accessible entry sites or increase the ability of Na to interact with or pass through these sites.[7]

Stimulatory and inhibitory effects of anionic replacements of Cl on active Na transport have been noted in frog skin (Ferriera, 1968; Cuthbert, Painter & Prince, 1969; Fischbarg, Zadunaisky & DeFisch, 1967) and toad urinary bladder (Singer & Civan, 1971). Singer and Civan (1971) reported that the relative abilities of anions (in the mucosal solution) to stimulate active Na transport across toad urinary bladder paralleled the "lyotropic series" (or "Hofmeister series") suggesting that the effect was due to interaction of these anions with relatively weak

7 The possibility that the anions act by complexing Ca and reducing the free Ca concentration of the mucosal solution can be excluded by the finding that Na transport by rabbit colon in the presence of 10^{-6} M Ca does not differ from that observed in the presence of 1.2 mM Ca (Frizzell, 1977).

cationic sites. Further, the results of studies on erythrocytes indicate that exposure of the membrane to a variety of anions can bring about an increase in the passive permeability to cations and a concomitant decrease in the permeability to anions (*c.f.* Wieth, 1970a, b; Gunn & Tosteson, 1971); these findings suggest that the passive permeation pathways through the erythrocyte membrane respond to changes in charge density in a fashion similar to that of a simple ion-exchange membrane. The results of the present studies are consistent with this notion.

Dick and Lindemann (1975) demonstrated that *p*-chloromercuribenzoate (PCMB) and PCMB-sulfonate (PCMBS) stimulate Na transport by frog skin at concentrations of approximately 10 mM. PCMBS also stimulates Na transport by toad urinary bladder (Spooner & Edelman, 1976). These mercurial anions have a predilection for sulfhydryl groups; however, their specificity is not absolute. Some of the stimulatory anions used in the present study possess hydroxyl groups that are potentially capable of forming hydrogen bonds with sulfhydryl groups. Thus, it is of interest that isethionate (IE) is a potent stimulator of the I_{sc}, whereas ethanesulfonate (ESA), which differs from IE only in that it lacks a terminal hydroxyl group, was relatively ineffective as a stimulant of the I_{sc}. These observations raise the possibility that interaction of an anion with sulfhydryl groups at or near the Na entry site may increase the local Na activity, enhance partition of Na into a pore, increase the affinity of a binding site, etc.

Finally, we are presently unable to offer any explanation for the inhibitory effect of *p*-phenolsulfonic acid (PSA) which has a rapid onset and appears to be most effective when added to the mucosal solution. The finding that the effect of PSA is readily reversible suggests that it does not simply nonspecifically disrupt the transporting cells, as might be expected from some benzoic or phenolic compounds. The clarification of this problem will be the subject of future investigations.

In summary, our findings strongly indicate that the stimulatory anions act by increasing the permeability of the amiloride-sensitive entry step at the mucosal membrane. They further suggest that the degree to which this permeability is increased (i.e., $\Delta_a G_{Na}$) is directly proportional to the difference between the spontaneous or control I_{sc} and the "maximal" I_{sc}. When the spontaneous I_{sc} is low, these anions bring about a large increase in ${}_aG_{Na}$ and I_{sc} at constant proportion (i.e., E_{Na} is constant); when the spontaneous I_{sc} is at its maximal value, these anions do not increase ${}_aG_{Na}$ or the I_{sc}. These results suggest that the ability to decrease

the resistance of the Na entry step is influenced by the level of pump activity and is perhaps mediated by the size of the intracellular Na transport pool.

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