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Interaction between Cell Sodium and the Amiloride-Sensitive Sodium Entry Step in Rabbit Colon

Klaus Turnheim, Raymond A. Frizzell, and Stanley G. Schultz*

Department of Physiology, University of Pittsburgh School of Medicine, Pittsburgh, Pa. 15261

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Summary. Ouabain abolishes the short-circuit current (I_{sc}) and decreases the transepithelial conductance (G_t) of rabbit colon. In contrast, amphotericin B elicits a maximum I_{sc} and markedly increases G_t. However, in *both* instances the amiloride-sensitive Na entry step is completely blocked, presumably due to an increase in cell Na. Conversely, when Na-depleted tissues are suddenly exposed to 140 mM Na, the amiloride-sensitive I_{sc} and the amiloride-sensitive component of G_t ($_aG_{Na}$) increase abruptly to their maximum values and then decline to steady-state plateaus with a half time of ~6 min; throughout the decline $(I_{sc}/{}_aG_{Na})=E_{Na}$ is constant at a value of 95 mV. In the presence of amphotericin B, the I_{sc} abruptly rises to the same maximum but does not decline. These findings indicate that in the presence of 140 mM Na the conductance of the amiloride-sensitive Na entry step can vary from a maximum value of approximately 1.6 mmhos/cm^2 when cell Na is depleted, to zero when cell Na is abnormally elevated (e.g., in the presence of ouabain or amphotericin B). Our findings are consistent with a system in which the pathway responsible for transcellular Na transport parallels another cellular compartment with which it communicates. The Na capacity of the active transport pathway appears to be very small so that this compartment fills rapidly after exposure of Na-depleted cells to 140 mM Na, and active transepithelial Na transport is initiated and reaches steady-state levels quickly. The Na capacity of the second compartment is much larger; the Na content of this compartment appears to be responsible for the negative feedback effect on the permeability of the amiloride-sensitive entry step.

Recent studies (Turnheim, Frizzell & Schultz, 1977) dealing with the mechanism of active Na transport across descending rabbit colon, *in vitro*, have demonstrated that, when the rate of active Na transport is low, Na entry into the transporting cells via an amiloride-sensitive pathway is the rate-limiting step for transepithelial Na transport and that replacement of Cl in the mucosal solution with certain anions enhances this entry step. At high spontaneous or induced (e.g., amphotericin B) rates of Na entry the pump mechanism at the baso-lateral membrane is saturated and it becomes the rate-limiting step for transcellular Na

* To whom reprint requests should be made.

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movements. However, our data also indicate that when the pump rate is maximal, the conductance of the amiloride-sensitive entry step cannot be further increased. These findings suggested 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 which could serve to limit (protect) the size of the Na pool when active extrusion from the cell is rate limiting.

The purpose of the present study was to examine further the relation between the size of the intracellular Na transport pool and the conductance of the amiloride-sensitive Na entry step in *in vitro* descending rabbit colon.

Materials and Methods

White rabbits of either sex (2–4 kg) were sacrificed by intravenous injection of pentobarbital. A segment of descending colon was removed, opened, washed free of intestinal contents and the outer muscle layers were stripped off as described previously (Frizzell, Koch & Schultz, 1976). In some experiments, segments of tissue were mounted as flat sheets in the short-circuit apparatus described by Schultz and Zalusky (1964) in which 1.27 cm^2 (serosal surface) of tissue is bathed on each surface with oxygenated, buffered electrolyte solutions maintained at 37 °C; provisions are made for monitoring the open-circuit transepithelial electrical potential difference (with respect to the mucosal solution), ψ_{ms} and the short-circuit current, I_{sc}. Because the tissue behaves as an ohmic resistor over the range ± 50 mV (Schultz, Frizzell & Nellans, 1977), the tissue conductance, G_t, is simply I_{sc}/ψ_{ms}. Under conditions when the ψ_{ms} or I_{sc} are low, G_t was determined by passing a brief (1 sec) pulse of direct current (50 $\mu A/cm^2$) across the tissue and monitoring the resulting potential difference.

The undirectional influx of ^{22}Na from the mucosal solution into the epithelium (J_{mc}^{Na}) was determined under short-circuit conditions using a modification of the influx apparatus described by Frizzell and Schultz (1972) and the techniques described by Schultz *et al.* (1967). Briefly, 8 partial mucosal strips of colon from the same animal were mounted mucosal surface up in the chambers. After preincubating the tissues for 30–45 min in the presence of mucosal and serosal solutions of desired compositions, the mucosal solution is withdrawn and replaced with a solution containing ^{22}Na and ^{3}H-polyethylene glycol (PEG) (mol wt 1,000). The mucosal surface of the tissue is exposed to this solution for 40 sec; the radioactive solution is then withdrawn, the chamber is flushed (1–2 sec) with ice-cold isotonic mannitol solution, and the exposed tissue (1.13 cm^2) is punched-out, washed briefly (1–2 sec) in ice-cold mannitol solution, and extracted for at least 2 hr in 0.1 M HNO_3. The extracts are assayed for ^{22}Na and ^{3}H using a triple-channel liquid scintillation spectrometer. The uptake of ^{22}Na by the tissue is calculated after correction for the volume of adherent radioactive medium given by the ^{3}H-PEG space. Previous studies have shown that ^{22}Na uptake by the tissue, determined in this manner, (i) is a linear function of time for at least 60 sec which extrapolates through the origin and (ii) is not significantly overestimated by possible failure of ^{3}H-PEG to completely account for adherent extracellular ^{22}Na so that the uptake at 40 sec is a valid measure of the unidirectional influx into the epithelium (i.e., the zero-time rate of uptake) (R.A. Frizzell & K. Turnheim, *manuscript submitted*). The values of ψ_{ms} and I_{sc} reported are those

obtained immediately before determination of the unidirectional influx. All influx experiments were carried out in a warm room at 37 °C.

The normal electrolyte solution (NSR) contained (mM): Na, 140; Cl, 124; HCO_3, 21; K, 5.4; HPO_4, 2.4; H_2PO_4, 0.6; Mg, 1.2; Ca, 1.2; and glucose, 10. This solution has a pH of 7.4 at 37 °C when gassed with a mixture of 95% O_2 and 5% CO_2. Na-free media were prepared by isotonic replacement of Na with choline. Amphotericin B was obtained from Squibb (as Fungizone®); amiloride was a generous gift from Merck, Sharp and Dome; ^{22}Na was obtained from New England Nuclear Corp.

All errors are expressed as the standard error of the mean. A value of $p<0.05$ calculated using the two-tail t test is considered a statistically significant difference.

Results

Two approaches to the problem were taken. The first involved examining the effect of increasing the intracellular Na content on J_{mc}^{Na} and the amiloride-sensitive conductance pathway of the tissue; the second involved examining the effect of a decrease in cell Na content on these parameters.

Effect of Ouabain on Tissue Conductance and J_{mc}^{Na}

The results of a series of experiments in which the amiloride-sensitive component of the total tissue conductance was evaluated before and after treatment of the tissue with 10^{-4} M ouabain are summarized in Fig. 1

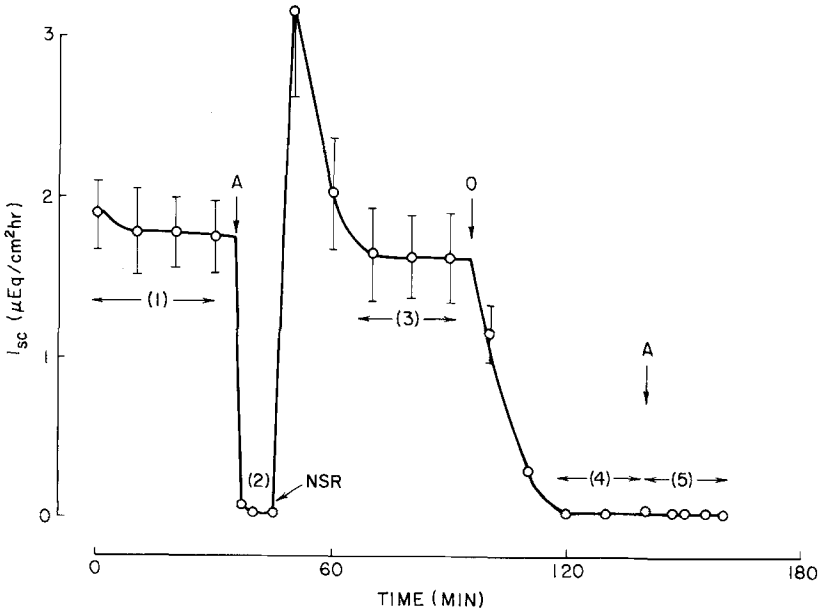

Fig. 1. Effect of amiloride (A) and ouabain (O) on the I_{sc}. Data represent the means and SEM based on 7 experiments

Table 1. Effect of amiloride and ouabain on tissue conductance[a]

	Phase	G_t (mmhos/cm^2)	${}_aG_{Na}$ (mmhos/cm^2)
Control	(1)	2.7 ± 0.2	
+ amiloride	(2)	2.3 ± 0.2	0.4
Control	(3)	2.8 ± 0.2	
+ ouabain	(4)	2.4 ± 0.1	0.4
+ amiloride	(5)	2.4 ± 0.1	

[a] Data from the experiments illustrated in Fig. 1; the numbers in parentheses refer to the time periods (phases) designated in Fig. 1.

and Table 1. After the I_{sc} stabilized (usually 30–40 min after mounting the tissue), the total tissue conductance, G_t, was determined 4 times over a period of 30 min; this period is designated as phase (*1*) in Fig. 1 and Table 1. Then amiloride (10^{-4} M) was added to the mucosal solution (*A*) and the I_{sc} was promptly abolished; G_t was determined 3 times during the next 10 min [phase (*2*)]. Amiloride was then washed-out by flushing the mucosal chamber and reservoir with 200 ml of NSR that did not contain amiloride over a period of 1–2 min.[1] As shown in Fig. 1, this resulted in a prompt restoration of the I_{sc} which, after a transient overshoot (which will be discussed below), reached levels that did not differ significantly from the pre-amiloride level; G_t was determined 3 times during the next 20 min [phase (*3*)]. Then, ouabain (10^{-4} M) was added to the serosal bathing solution. This resulted in a decline in the I_{sc}, which reached zero after approximately 25 min; G_t was then measured 3 times during the next 20 min [phase (*4*)]. Finally, amiloride was added to mucosal solution and G_t was determined 4 times during the ensuing 20 min [phase (*5*)]. The values of G_t during each of the five phases illustrated in Fig. 1 are given in Table 1. In this series of experiments, the amiloride-sensitive component of the total tissue conductance averaged 0.4 mmhos/cm^2; this component is designated ${}_aG_{Na}$ inasmuch as it represents the conductance of the active Na transport pathway (Schultz *et al.*, 1977). Ouabain significantly decreased G_t and when the I_{sc} was reduced to zero, the decrease in G_t is precisely equal to the amiloride-sensitive component. Finally, amiloride has no additional effect on G_t in the presence of a maximal response to ouabain.

1 Amiloride was flushed out of the mucosal solution by rapidly introducing amiloride-free NSR and simultaneously aspirating fluid so that the level in the mucosal reservoir was unchanged. In this way the mucosal solution was turned over 20 times within a brief period while maintaining the tissue under short-circuit conditions.

Table 2. Effect of ouabain on unidirectional sodium influx (J_{mc}^{Na})

	ψ_{ms} (mV)	G_t (mmhos/cm^2)	I_{sc} (μeq/cm^2 hr)	J_{mc}^{Na} (μeq/cm^2 hr)
A. Preincubation in presence of 140 mM Na ($n=20$)				
Control	9 ± 1	4.5 ± 0.2	1.5 ± 0.2	3.1 ± 0.2
+Ouabain	1 ± 1[a]	3.8 ± 0.2[a]	0.1 ± 0.1[a]	1.8 ± 0.1[a]
B. Preincubation in Na-free mucosal solution ($n=6$)				
Control	–	–	–	3.8 ± 0.3
+Ouabain	–	–	–	3.1 ± 0.4[b]

[a] Statistically different from control at $p<0.01$.
[b] Does not differ significantly from control ($p>0.1$).

Thus, the conductance of the active Na transport pathway is abolished by ouabain; this could be due to a decrease in the conductance of the entry step and/or a decrease in the conductance of the mechanism responsible for active extrusion of Na across the baso-lateral membranes. In order to determine whether the effect of ouabain on G_t (and the lack of an additive action by amiloride) is due to inhibition of the amiloride-sensitive entry step, the effect of 10^{-4} M ouabain on J_{mc}^{Na} was examined. The results of these experiments are given in Table 2*A*. As above, ouabain abolished ψ_{ms} and I_{sc} and brought about a significant decrease in G_t. Further, ouabain decreased J_{mc}^{Na} by 1.3 ± 0.2 μeq/cm^2 hr; this value does not differ significantly from the decrease in I_{sc}(1.4 ± 0.2 μeq/cm^2 hr). The residual J_{mc}^{Na} in the presence of ouabain represents ^{22}Na entry into the paracellular shunt pathway.[2] Thus, the effect of ouabain on J_{mc}^{Na} is precisely what we have previously observed when J_{mc}^{Na} is determined in the presence of amiloride, namely, equivalent decreases in J_{mc}^{Na} and I_{sc} consistent with complete inhibition of Na entry into the transporting cells (Frizzell *et al.*, 1975; R.A. Frizzell & K. Turnheim, *manuscript submitted*).

Several investigators have reported that ouabain inhibits unidirectional influx of Na across the outer membrane of isolated frog skin (Biber, 1971; Erlij & Smith, 1973; Moreno *et al.*, 1973; Rick, Dörge & Nagel, 1975), and in some instances the effect of ouabain in the serosal solution was equivalent to that observed when amiloride was present in the mucosal solution (Erlij & Smith, 1973; Moreno *et al.*, 1973). In addition, Erlij and Smith (1973) concluded that this was due to an increase in the

2 We have demonstrated that J_{mc}^{Na} is given by the sum of J_{net}^{Na} (or I_{sc}) plus entry into the paracellular shunt pathway (Frizzell & Schultz, 1978; Frizzell & Turnheim, *manuscript submitted*). Hence, under control conditions (Table 2), the latter amounts to 1.6 μeq/cm^2 hr. The residual flux in the presence of ouabain (1.8 μeq/cm^2 hr) does not differ significantly from this value.

intracellular Na content of the skin because when skins were preincubated with ouabain in a Na-free medium, the unidirectional influx of Na from a solution containing 10 mM Na was not inhibited. To examine this point for the case of rabbit colon, segments of tissue were preincubated for 30–45 min in the presence of a *mucosal* solution in which Na was completely replaced with choline; the serosal solutions consisted of NSR with or without 10^{-4} M ouabain. As shown in Table 2*B*, under these preincubation conditions, the presence of ouabain in the serosal solution did not significantly affect J_{mc}^{Na} from a solution containing 140 mM Na. These results are entirely consistent with the earlier observations of Erlij and Smith (1973) and, at face value, suggest that ouabain does not inhibit J_{mc}^{Na} when the tissue is preincubated in a Na-free mucosal solution, because under these conditions the size of the intracellular Na pool does not increase. However, an alternative interpretation is possible; namely, that when transepithelial Na transport is abolished by depletion of the intracellular transport pool, ouabain does not bind as readily to the pump sites at the basolateral membranes. This possibility was considered by Erlij and Smith (1973) but dismissed as unlikely; however, their interpretation of the experimental results that led to this conclusion is open to question. In any event, the following experiments were carried out to examine this possibility for the case of rabbit colon:

First, the reversibility of the ouabain-effect was examined using tissues bathed by NSR. After the I_{sc} had stabilized, 10^{-4} M ouabain was added to the serosal solution; then, after the I_{sc} reached zero (about 25 min later; *see* Fig. 1) the serosal solution was replaced repeatedly with a ouabain-free NSR over a period of 1 hr. There was only a partial recovery of the I_{sc}, which reached a value less than 30% of the initial I_{sc}, indicating that the action of ouabain is only slowly reversible during the time-frame of these studies. Then, both surfaces of tissues from the same animals were bathed in Na-free, choline-Ringer's with 10^{-4} ouabain in the serosal solution for a period of 1 hr; this is more than sufficient time for a complete ouabain-effect in the presence of Na. Ouabain was then washed out of the serosal solution as described above and the Na-free media were replaced with NSR. This resulted in a prompt and sustained restoration of the I_{sc}; the steady-state value reached averaged 2.5 μeq/cm^2 hr and did not differ significantly from the control values. Thus, these tissues behaved as if they had never been exposed to ouabain, strongly suggesting that ouabain binding is either abolished or markedly slowed in the absence of Na. These findings will be discussed further below.

Effect of Amphotericin B on I_{sc}, G_t and ${}_aG_{Na}$

Ouabain brings about an increase in cell Na by inhibiting the Na pump mechanism located at the baso-lateral membrane; results obtained on toad urinary bladder suggest that most of the Na gained by the cells is derived from the mucosal solution (Macknight, Civan & Leaf, 1975*a*). Another way to increase the size of the Na transport pool is by exposing the mucosal membrane to amphotericin B. This polyene antibiotic increases the permeability of artificial and biological membranes to small ions and nonelectrolytes, presumably by forming transmembrane pores or channels having a diameter of approximately 7–8 Å (Finkelstein & Holz, 1973). In rabbit colon, the addition of amphotericin B to the mucosal solution brings about prompt and sustained increases in I_{sc}, ψ_{ms}, J^{Na}_{net}, J^{Na}_{mc} and G_t, and elicits K secretion. The rate of K secretion is less than 10% of the rate of active Na absorption, and there is still good agreement between the I_{sc} and J^{Na}_{net} (Frizzell & Schultz, 1976; Turnheim *et al.*, 1977; R.A. Frizzell & K. Turnheim, *manuscript submitted*). It should be stressed that amphotericin B increases I_{sc} and J^{Na}_{net} *only* when the control values (prior to the addition of the antibiotic) are below the "maximal rate" of the baso-lateral pump mechanism, which is 4–6 μeq/cm^2 hr; under these conditions the addition of amphotericin B to the mucosal solution increases the I_{sc} to the maximal rate. When the I_{sc} is already at the maximal rate (e.g., spontaneously, in the presence of aldosterone or in the presence of certain anions), amphotericin B does not bring about a further increase in I_{sc} even though J^{Na}_{mc} and G_t increase and K secretion is elicited (Turnheim *et al.*, 1977; Frizzell & Schultz, 1978; R.A. Frizzell & K. Turnheim, *manuscript submitted*). There is no evidence that this antibiotic has any effect on the basolateral membrane; its entire effect can be attributed to an alteration in the normal perm-selective properties of the mucosal membrane.

The relations between I_{sc} and the Na concentration in the bathing media, $[Na]_m$, in the absence and presence of a maximally effective concentration of amphotericin B (15 μg/ml) are shown in Fig. 2. In the absence of amphotericin B, the maximum I_{sc} in this series of experiments was approximately 2 μeq/cm^2 hr and a half-maximal I_{sc} is observed when $[Na]_m \cong 10$ mM. In the presence of amphotericin B and 140 mM Na, the I_{sc} was at its maximal level, which in this series of experiments averaged 5 μeq/cm^2 hr. Several points should be noted: First, amphotericin B does not significantly stimulate the I_{sc} when $[Na]_m$ is less than 10 mM; for example, when $[Na]_m = 4$ mM the I_{sc} was 0.72 ± 0.13 μeq/cm^2 hr

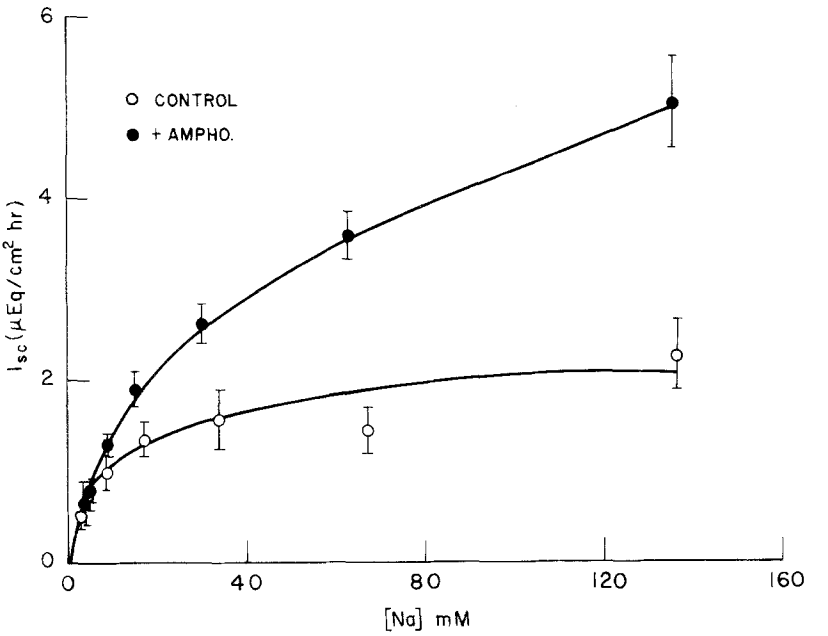

Fig. 2. Steady-state relation between I_{sc} and the Na concentration in the bathing media in the absence (○) and presence (●) of amphotericin B (15 μg/ml). Data represent means and SEM based on 8 experiments

under control conditions and 0.79 ± 0.09 μeq/cm² hr in the presence of amphotericin B. Thus, in the presence of low Na concentrations, Na entry into the cell *does not* appear to be rate limiting, whereas this step *is* rate limiting at concentrations of Na greater than 15–20 mM. Second, in the absence of amphotericin B the addition of amiloride to the mucosal solution rapidly abolishes the I_{sc}, but in the presence of amphotericin B, amiloride has no effect on the I_{sc} (not shown). Bentley (1968) and Ehrlich and Crabbe (1968) noted that amiloride had no effect on the I_{sc} across toad urinary bladder in the presence of amphotericin B, but did not elaborate upon these observations. Finally, unlike the case for control tissues, the I_{sc} across amphotericin B-treated tissues does not clearly saturate with increasing $[Na]_m$.

To explore these observations further, the effect of amiloride on J_{mc}^{Na} in the presence of amphotericin B was examined; the results of these studies are summarized in Table 3. In this series of studies the maximum I_{sc} was approximately 4 μeq/cm² hr. Of major interest is the finding that amiloride has no effect on J_{mc}^{Na}, I_{sc}, or G_t. Thus, in the presence of amphotericin B, there is no detectable component of the G_t or J_{mc}^{Na} that is inhibitable by amiloride.

Table 3. Effect of amiloride on unidirectional influx of sodium (J_{mc}^{Na}) in the presence of amphotericin B

	ψ_{ms} (mV)	G_t (mmhos/cm^2)	I_{sc} (μeq/cm^2 hr)	J_{mc}^{Na} (μeq/cm^2 hr)
Control	27 ± 2	4.8 ± 0.4	4.0 ± 0.2	6.3 ± 0.2
+ Amiloride	24 ± 1	5.1 ± 0.4	4.0 ± 0.3	6.3 ± 0.4

Average values of 16 determinations on paired tissues from 4 animals.

Effect of Sudden Increases of $[Na]_m$ *on* I_{sc} *and* G_t

Figure 3 illustrates typical results of experiments in which $[Na]_m$ was increased from 0 to 140 mM by sequential addition of NSR to media which were initially Na-free. When $[Na]_m$ is incremented, there is an over-shoot in the I_{sc} which then declines to a steady-state value; this over-shoot is not observed when amphotericin B is present in the mucosal solution. Note that the steady-state values achieved in the absence or presence of amphotericin B correspond reasonably well with the data illustrated in Fig. 2. Further, when $[Na]_m$ is decreased in a stepwise fashion by sequential additions of a Na-free solution to both bathing media which initially contained NSR, there is a small transient

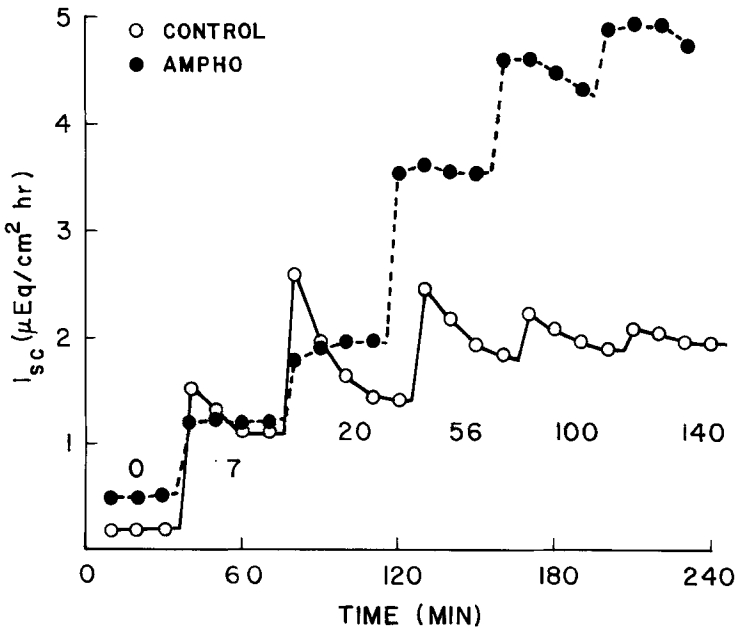

Fig. 3. Typical responses to sequential increments in Na_m on the I_{sc} in the absence (○) and presence (●) of amphotericin B. Numbers under the curves indicate the Na concentration in both bathing media during each time period

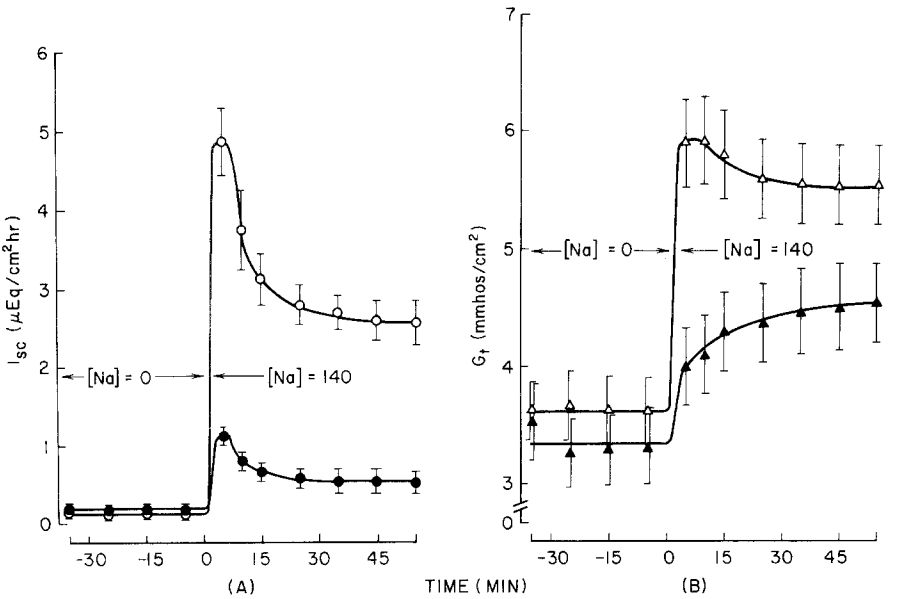

Fig. 4. Effects of suddenly increasing the Na concentration from zero to 140 mM on I_{sc} and G_t in the absence (○, △) and presence (●, ▲) of amiloride. Data derived from 11 experiments

under-shoot of the I_{sc}, followed by an increase to a steady-state value; this is not observed in the presence of amphotericin B (data not shown).

The effects of suddenly increasing $[Na]_m$ from 0 to 140 mM on I_{sc} and G_t in the absence or presence of amiloride are summarized in Fig. 4. In the absence of Na, $I_{sc} \cong 0$ and $G_t = 3.5$ mmhos/cm^2. Replacement of the Na-free, choline-Ringer's with NSR ($[Na] = 140$ mM) results in a rapid increase in I_{sc} which, after reaching a value of 5 μeq/cm^2 hr, declines to a steady-state value of approximately 2.5 μeq/cm^2 hr; at the same time G_t increases to a peak value of 6 mmhos/cm^2 hr and then declines to a steady-state value of 5.5 mmhos/cm^2. In the presence of amiloride, I_{sc} increases to a peak value of 1.1 μeq/cm^2 hr and declines to a steady-state value of 0.5 μeq/cm^2 hr; and, G_t increases from 3.3 mmhos/cm^2 to 4.5 mmhos/cm^2. The increase in G_t in the presence of amiloride is equal to 1.2 mmhos/cm^2; this value is in excellent agreement with the value of 1.1 mmhos/cm^2 which was previously estimated for the Na conductance of the paracellular shunt pathway (Frizzell *et al.*, 1976). Thus, the increase in transepithelial conductance in the presence of amiloride can be entirely attributed to the fact that the paracellular pathway is far more permeable to Na than to choline.

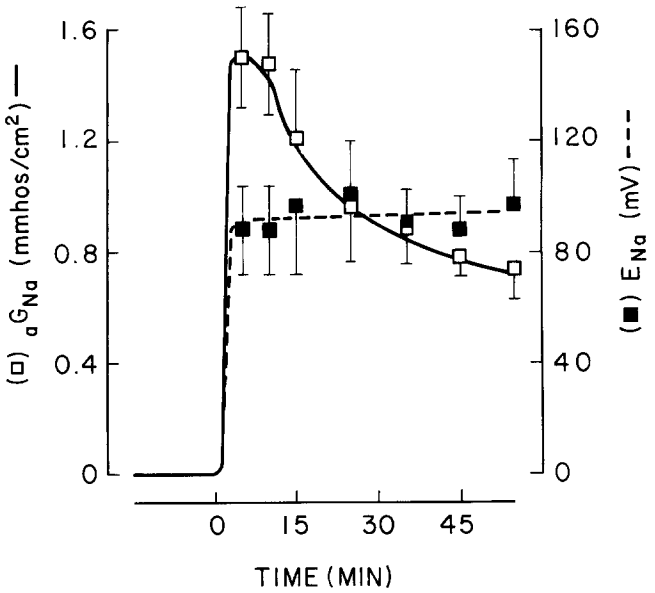

Fig. 5. $_aG_{Na}$ and E_{Na} calculated from the data given in Fig. 4

The time-course of the change in the *amiloride-sensitive component* of G_t ($_aG_{Na}$) following a rapid increase in $[Na]_m$ from 0 to 140 mM, calculated from the data given in Fig. 4*B*, is illustrated in Fig. 5 (open squares). After increasing $[Na]_m$ from 0 to 140 mM, $_aG_{Na}$ increases from 0 to a maximum value of 1.5 mmhos/cm^2 and then declines to a steady-state value of 0.7 mmhos/cm^2. The data given by the filled squares (■) represent the ratios of the amiloride-sensitive I_{sc} (in μA/cm^2) to $_aG_{Na}$; the significance of these data will be discussed below.

Discussion

Effects of Increasing or Decreasing Cell Na on Amiloride-Sensitive Na Entry

MacRobbie and Ussing (1961) concluded that inhibition of the Na pump of frog skin with ouabain or by lowering the pH of the inner bathing solution resulted in a decrease in the permeability of the outer membrane to Na and that of the inner membrane to KCl and stated: "... it may be that any interference with the pump mechanism is accompanied by a decrease in the passive permeabilities to ions and that the active ion transport and the passive fluxes are not entirely independent".

Subsequent studies on Na-transporting epithelia such as frog skin, toad urinary bladder, and, most recently, rabbit urinary bladder have shown that inhibition of pump activity (with ouabain, metabolic inhibitors, deletion of K in the serosal solution, reduction of the temperature of the bathing media) decreases the rate of uptake of Na across the outer or mucosal membrane (Essig & Leaf, 1963; Biber, 1971; Erlij & Smith, 1973; Moreno *et al.*, 1973; Finn, 1975; Rick *et al.*, 1975) and increases the electrical resistance of these barriers (Larsen, 1973; Lewis, Eaton & Diamond, 1976; Helman & Nagel, 1977). The most reasonable interpretation of these findings is that an increase in cell Na (or a decrease in cell K)[3] resulting from inhibition of the pump, decreases the permeability of the outer or mucosal membrane to Na. Chen and Walser (1975) and Hong and Essig (1976) also found that inhibition of pump activity in toad urinary bladder (ouabain, 2-deoxyglucose) increased the overall resistance of the active Na transport pathway ($_aR_{Na}$), but their studies do not permit distinction between possible effects of these agents at the mucosal or baso-lateral membranes.

Our results with ouabain and amphotericin B confirm and extend some of these earlier findings. Both of these agents presumably bring about an increase in the size of the intracellular Na pool but for opposite reasons; the former by inhibiting the pump mechanism responsible for Na extrusion from the cell and the latter by increasing the rate of Na entry across the mucosal membrane, thereby eliciting a maximal pump rate. Ouabain decreases G_t and inhibits J_{mc}^{Na}, whereas amphotericin B increases G_t and stimulates J_{mc}^{Na}; however, in both instances the amiloride-sensitive components of G_t (i.e., $_aG_{Na}$) and J_{mc}^{Na} are abolished. In the case of amphotericin B one might expect that the increases of J_{mc}^{Na} and G_t are simply due to the formation of channels for Na entry into the cell in parallel with the physiological, amiloride-sensitive entry pathway, and that amiloride should decrease the G_t and, particularly, J_{mc}^{Na} in the presence of amphotericin B by blocking that component of influx that takes place via the "normal" entry step. But this is not observed; in the presence of amphotericin B the amiloride-sensitive entry step appears to have vanished. A possible explanation for the inability of amiloride to

3 In some of our studies and in many of the studies cited above there is almost certainly an inverse relation between cell Na content and cell K content so that, at present, one cannot be certain whether the effects noted are due to an increase in cell Na or a decrease in cell K (*c.f.* Robinson & Macknight, 1976). Throughout this paper we will interpret our observations in terms of changes in cell Na content without excluding the possibility that the effects noted may be, in part or entirely, due to changes in cell K content or in the activity of other intracellular ions, e.g., Ca, H.

inhibit J_{mc}^{Na} in the presence of amphotericin B is that the interaction between the antibiotic and the mucosal membrane destroys the normal Na entry sites or the amiloride binding sites. Although this possibility cannot be ruled out, an alternate explanation that is consistent with our observations is that an amphotericin-induced increase in the intracellular Na pool "blocks" (abolishes the conductance) of the normal entry step.

Cuthbert and Shum (1977) have published findings that may provide a "biochemical correlate" for some of our findings and some of the earlier observations cited above. These investigators found that inhibition of pump activity in frog skin by exposure to ouabain or a K-free inner solution decreases the number of specific amiloride-binding sites at the outer membrane in the presence of a high Na concentration, but not when the tissue is bathed by a solution containing only 1 mM Na. Likewise, our data (Table 2) indicate that ouabain completely inhibits the amiloride-sensitive J_{mc}^{Na} when the tissue is preincubated in the presence of 140 mM Na, but not when the preincubation solution bathing the mucosal surface of the tissue alone is Na-free; as noted above, similar observations were reported by Erlij and Smith (1973) for the case of isolated frog skin.

Although these observations are consistent with the notion that the inhibition of the amiloride-sensitive influx by ouabain is the result of an increase in intracellular Na (with Na derived from the mucosal solution) our findings suggest that the failure of ouabain to inhibit J_{mc}^{Na} when rabbit colon is preincubated in the presence of a Na-free mucosal solution *could* be due to the fact that ouabain is not as readily bound by the basolateral pump mechanism under these conditions. These findings are entirely consistent with the observations of Dunham and Hoffman (1971) and Joiner and Lauf (1977) that the rate of ouabain binding to the Na-K ATPase of erythrocytes at a given concentration of the glycoside is directly related to the pump rate. These findings are also entirely in accord with the evidence that ouabain only binds to the E_2 conformation of the Na-K-ATPase, which is generated in the presence of intracellular Na, Mg and ATP (Whittam & Chipperfield, 1975; Dahl & Hokin, 1974; Glynn & Karlish, 1975). Thus, it is entirely possible that when the intracellular Na pool accessible to the mucosal solution is depleted, the high-energy E_2 form of the ATPase is not generated (or is generated at a slow rate) and that ouabain binding is either abolished or the rate of binding is markedly decreased.

The results of the experiments summarized in Figs. 3–5 are also in accord with the notion that the size of the intracellular Na pool exerts a

negative feedback on the permeability of the amiloride-sensitive entry step. Thus, a sudden increase in the Na concentration of the bathing media brings about a sudden increase in the I_{sc} that is characterized by an overshoot which subsequently declines to a new steady-state level. This is most marked when the tissue is initially exposed to low Na concentrations (e.g., in incrementing from 0 Na to 7 mM Na or from 7 mM Na to 20 mM Na); the overshoot is much less marked when the tissue is initially exposed to relatively high Na concentrations (e.g., when incrementing from 100 to 140 mM) (Fig. 3). These findings are consistent with the notion that in the presence of high Na concentrations the size of the intracellular Na pool is near maximal and that, in turn, its negative influence on the entry step is near maximal. Two additional points should be stressed. First, an overshoot is *never* observed when amphotericin B is present in the mucosal solution. Second, as shown in Fig. 2, amphotericin B does not affect the steady-state I_{sc} when the Na concentration in the bathing solutions is less than 10 mM, suggesting that in the presence of low external Na concentrations and, presumably, a low intracellular Na pool entry is no longer rate limiting for transcellular transport. All of these findings are consistent with the notion that the size of the intracellular Na pool plays an important role in determining the resistance to Na movement across the mucosal membrane via the amiloride-sensitive route.

Further insight into this phenomenon may be gained from an analysis of the data summarized in Figs. 4 and 5. We have previously demonstrated (Schultz *et al.*, 1977) that the active Na transport pathway displays ohmic behavior over the range $\psi_{ms} = \pm 50$ mV, so that

$$I_{Na} = {}_aG_{Na}(E_{Na} - \psi_{ms}) \tag{1}$$

where I_{Na} is the rate of active Na transport expressed in $\mu A/cm^2$; $E_{Na} = E^m_{Na} + E^s_{Na}$ where E^m_{Na} is the electromotive force for Na entry across the mucosal membrane and E^s_{Na} is the electromotive force of the mechanism responsible for Na extrusion from the cell across the baso-lateral membrane; and $(1/{}_aG_{Na}) = {}_aR_{Na} = R^m_{Na} + R^s_{Na}$ where R^m_{Na} is the resistance of the amiloride-sensitive entry step and R^s_{Na} is the phenomenologic resistance of the exit process. The fact that E_{Na} and ${}_aG_{Na}$ are independent of ψ_{ms} permits us to write the familiar expression (Ussing & Zerahn, 1951)

$$I_{sc} = {}_aG_{Na} \cdot E_{Na} \tag{2}$$

when $\psi_{ms} = 0$.

We have also demonstrated that:

1) The maximal rate of the baso-lateral pump mechanism is 4–6 μeq/cm^2 hr (more often 5–6 μeq/cm^2 hr). This is sometimes observed spontaneously but can be readily induced by (i) addition of amphotericin B to the mucosal solution (Frizzell & Schultz, 1976; R.A. Frizzell & K. Turnheim, *manuscript submitted*) (Fig. 2); (ii) replacement of Cl in the bathing media with some anions such as isethionate (Turnheim *et al.*, 1977); or (iii) treatment of the tissue with aldosterone *in vitro* (Frizzell & Schultz, 1978).

2) E_{Na} is approximately 100 mV and is not only independent of ψ_{ms} but is also independent of I_{sc} and ${}_aG_{Na}$; that is, the same E_{Na} is calculated under control conditions and when the I_{sc} is partially or maximally stimulated by anions (Schultz *et al.*, 1977; Turnheim *et al.*, 1977). Thus, when only the entry step is affected, changes in I_{sc} are closely paralleled by changes in ${}_aG_{Na}$.

Thus, from Eq. (1), the *maximum* value of ${}_aG_{Na}$ consistent with a *maximum* I_{sc} of 4–6 μeq/cm^2 hr (110–160 μA/cm^2) and a *constant* E_{Na} of 100 mV is 1.1–1.6 mmhos/cm^2.

As shown in Fig. 4, when the Na concentration in the bathing media is suddenly increased from zero to 140 mM, the amiloride-sensitive portion of the I_{sc}, which must represent the Na current across the mucosal membrane[4] (I^m_{Na}), increases from zero to approximately 4 μeq/cm^2 hr at 5 min. The amiloride-sensitive I_{sc} then declines and reaches a steady-state value of approximately 2 μeq/cm^2 hr within 30–40 min. This decline conforms closely to a single exponential function of time with a halftime of ~6 min, and extrapolation to zerotime yields an amiloride-sensitive I_{sc} of 5 μeq/cm^2 hr (Fig. 6). Thus, the *initial rate* of Na entry from a mucosal solution containing 140 mM Na into Na-depleted cells is in

4 Under short-circuit conditions, the transepithelial current (I_{sc}) is entirely transcellular and the current flow across the mucosal membrane must equal that across the baso-lateral membrane. However, during the transient the ionic species responsible for the currents across these two membranes need not be the same. The amiloride-sensitive component of the I_{sc} can certainly be identified with the net flow of Na across the mucosal membrane (I^m_{Na}); movements of Na, K and/or Cl may contribute to the current across the baso-lateral membrane. Data obtained by Frizzell *(manuscript in preparation)* and Frizzell & Jennings (1977) indicate that exposure of the tissue to a Na-free mucosal solution leads to a decrease in cell Na and Cl (accompanied by cell H_2O) with no change in cell K content. Thus, the transient transepithelial current is almost certainly the result of Na entry into the cells across the mucosal membrane and an equivalent Cl entry into the cells across the baso-lateral membranes. When a true steady state is achieved

$$I_{sc} = I^m_{Na} = I^s_{Na}.$$

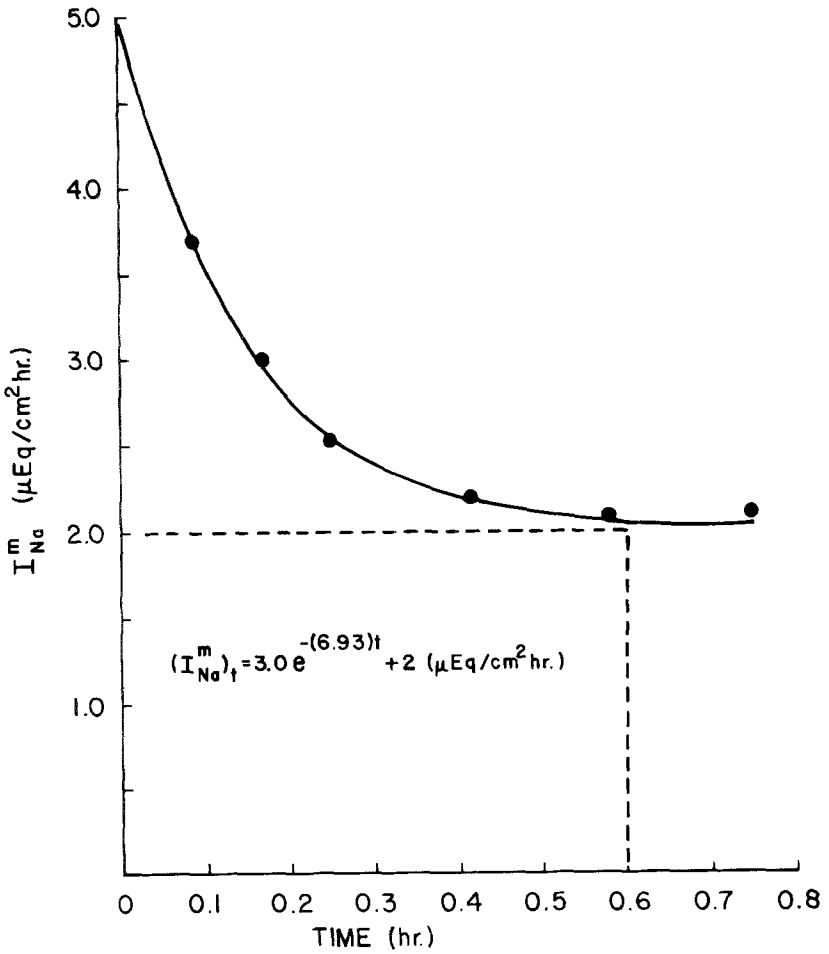

Fig. 6. I^m_{Na} as a function of time after suddenly increasing $[Na]_m$ from zero to 140 mM. The points are the mean values of the amiloride sensitive I_{sc} determined from Fig. 4*A*. The curve describes the equation shown

excellent agreement with the *maximal rate* of the baso-lateral pump mechanism in the presence of 140 mM Na.

As shown in Fig. 5, after suddenly increasing the Na concentration in both bathing media from zero to 140 mM, ${}_aG_{Na}$ averages 1.5 mmhos/cm^2 and then declines to 0.7 mmhos/cm^2. The former value is in excellent agreement with the "maximum" ${}_aG_{Na}$ calculated above, and the latter value is in excellent agreement with the value of the amiloride-sensitive conductance observed under control conditions in previous studies (Frizzell *et al.*, 1976; Schultz *et al.*, 1977; Turnheim *et al.*, 1977). The timecourse of the decline of ${}_aG_{Na}$ also conforms to a single exponential

function, and extrapolation to zerotime yields a value of 1.7 ± 0.2 mmhos/cm^2. Further, we have previously shown that under control conditions ${}_aR_{Na} = 1{,}400$–$2{,}000$ ohm cm^2 and that $R^m_{Na} \cong 10 R^s_{Na}$; thus R^s_{Na} is approximately 100–200 ohm cm^2. In the present studies, 5 min after increasing the external Na from zero to 140 mM, ${}_aR_{Na} = 667$ ohm cm^2 and subsequently increases to a value of 1,400 ohm cm^2. Therefore, if our previous estimates of R^m_{Na}/R^s_{Na} are correct, the increase in ${}_aR_{Na}$ (or decrease in ${}_aG_{Na}$) with time *must be almost entirely* due to an increase in R^m_{Na}.

Finally, as shown in Fig. 5, E_{Na} remains essentially constant throughout the transients in I_{sc} and ${}_aG_{Na}$ and averages ~ 95 mV; this value is in excellent agreement with those observed previously (Schultz *et al.*, 1977; Turnheim *et al.*, 1977). Thus, the decline in I_{sc} is almost precisely paralleled by the decline in ${}_aG_{Na}$.

The results of these and previous investigations are illustrated "schematically" in Fig. 7; the numbers employed are "typical values" drawn from numerous studies. The most reasonable explanation for some of these findings is that there is a positive relation between cell Na content and the resistance of the amiloride-sensitive entry step. Thus, when Na-depleted cells are suddenly exposed to bathing solutions containing 140 mM Na, R^m_{Na} is initially minimal and I^m_{Na} is maximal. As the cell Na content is repleted, R^m_{Na} increases and I^m_{Na} decreases until a steady-state value of R^m_{Na} is achieved. Throughout the transient, the increase in R^m_{Na} is closely paralleled by the decrease in I^m_{Na} so that E_{Na} remains essentially constant[5]. The steady-state value of R^m_{Na} is dependent, at least in part, on the size of the intracellular pool ($[Na]_c$) and, when $[Na]_m$ exceeds 20 mM, Na entry becomes rate limiting (i.e., the rate of entry is well below the maximal level of pump activity.) When $[Na]_m < 10$ mM, the combined effects of $[Na]_m$ and $[Na]_c$ on R^m_{Na} are such that it is not rate limiting. In the presence of 140 mM Na, a further increase in $[Na]_c$ elicited by addition of amphotericin B to the mucosal solution or ouabain to the serosal solution leads to an increase in R^m_{Na} and, ultimately, complete block of the amiloride-sensitive entry step. Finally, the overshoot observed after amiloride is washed out of the mucosal solution (Fig. 1) can probably be attributed to the fact that treatment of the tissue with amiloride results in a decrease in the Na pool derived from the mucosal solution (Dörge & Nagel, 1970; Nagel & Dörge, 1970; Macknight *et al.*, 1975*b*). Thus, following washout of

5 Since $I_{sc}(R^m_{Na} + R^{Na}) = E_{Na}$ and $R^m_{Na} \gg R^s_{Na}$, the notion that $I^m_{Na} R^m_{Na} = \text{constant} \cong E_{Na}$ is consistent with our findings within experimental error.

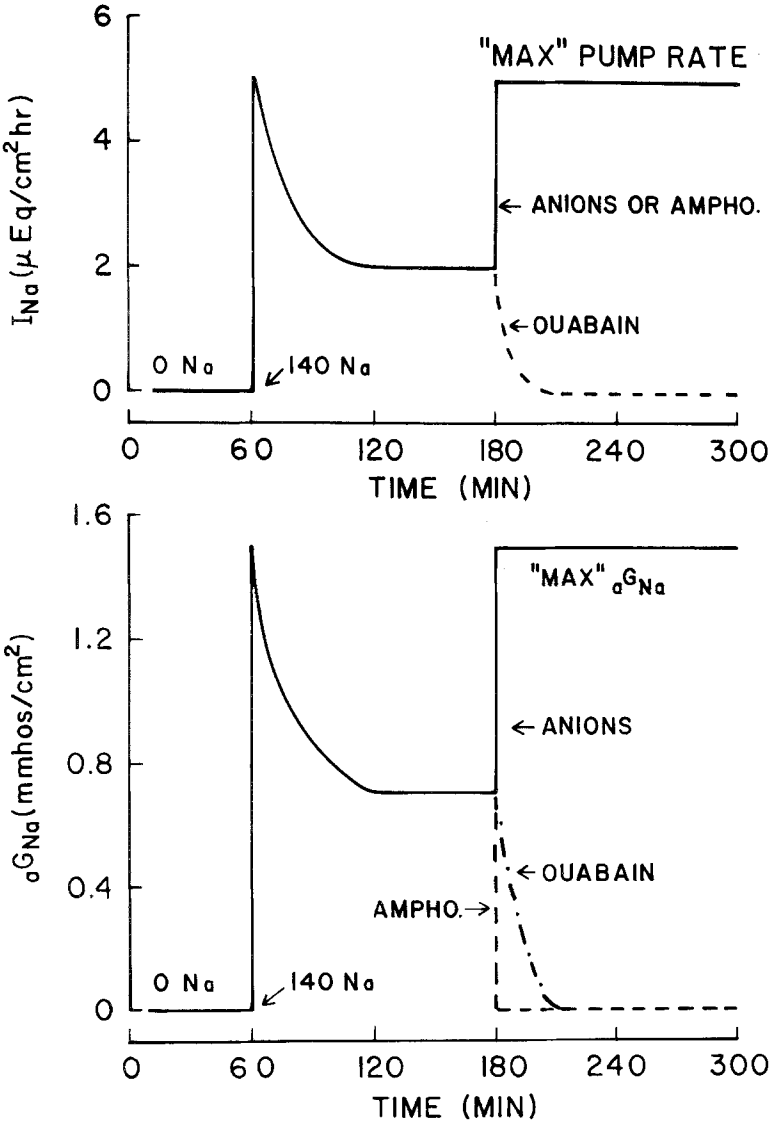

Fig. 7. Summary of typical results of these and previous studies (Turnheim *et al.*, 1977)

amiloride, Na-poor cells are suddenly exposed to 140 mM Na in the mucosal solution.

Since stimulatory anions elicit the maximal I_{sc} and $_aG_{Na}$ in the presence of 140 mM Na, the effect of these anions may be to block the interaction between $[Na]_c$ and R^m_{Na} so that the system behaves as if $[Na]_c \cong 0$. If this admittedly speculative inference is correct, it could have important implications regarding the mechanism of this interaction.

However, there are a number of puzzling observations that require explanations: One is the rather close agreement between the maximum I^m_{Na} observed immediately after exposure of Na-depleted cells to 140 mM Na and the maximum steady-state pump rate elicited by amphotericin B. Why should the maximum rate of net Na entry across the mucosal membrane during this *transient response* correspond so closely to the maximum *steady-state rate* of pump activity that can be achieved by this system? Why is the maximum ${}_aG_{Na}$ observed during the transient equal to that observed under steady-state conditions when the pump rate is spontaneously maximal? And, why is the value of E_{Na} (the value of ψ_{ms} necessary to abolish Na transport through the active pathway) preserved throughout? In short, why does Eq. (2), which describes the steady-state behavior of the system, apply at zerotime and throughout the transient? There are several possible explanations. The first is that there is (are) some mechanism(s), other than $[Na]_c$, that prevents the maximum rate of Na entry from exceeding the maximum pump rate.[6] Second, it is possible that the maximum conductance of the amphotericin B channels is equal to the maximal conductance of the amiloride-sensitive entry pathway. If so, since treatment with amphotericin B abolishes the amiloride-sensitive pathway, the maximum conductance of the entry step would not be affected by this agent (i.e., the "normal" entry pathway would simply be replaced by an artificial pathway with no change in maximum conductance) and the value of 4–6 $\mu eq/cm^2$ hr would not represent the maximum pump rate but simply the maximum entry rate. This would seem to be an extremely unlikely happenstance but one that must be ruled out by direct measurements of the Na conductance of the mucosal membrane in the presence of amphotericin B. In rabbit urinary bladder, nystatin (a polyene antibiotic whose effects on sterol-containing membranes closely resemble those of amphotericin B) increases the conductance of the mucosal membrane 100-fold and essentially eliminates the mucosal membrane as the rate-limiting step for ion movements (Lewis *et al.*, 1977).

A possible explanation for some of these observations stems from an analysis of the curve shown in Fig. 6. Frizzell (*manuscript in preparation*) and Frizzell and Jennings (1977) have shown that the intracellular Na content of colonic mucosa in the presence of 140 mM Na is approximately

6 One possibility is that active Na transport is the result of asymmetric movements across the equivalent of a single barrier that does not have a significant capacity for Na; e.g., the flow of current across a pure resistor. In this type of continuum, entry and exit must always be equal (no capacitative lag). Cereijido *et al.* (1974) have proposed such a system to account for their findings on frog skin.

0.55 μeq/cm^2. When the tissue is exposed to a Na-free *mucosal* solution for 30–45 min, cell Na content declines to 0.15 μeq/cm^2, and when it is exposed to a Na-free *serosal* solution for the same period of time, cell Na content declines to 0.40 μeq/cm^2; thus, approximately 75% of the total intracellular Na is derived from the mucosal solution. Integration of the transient portion of the curve shown in Fig. 6 from zero to 0.6 hr indicates that the total amount of Na that enters the cell via the amiloride-sensitive pathway is 1.63 μeq/cm^2; the area under the transient overshoot (i.e., above the dashed line) is equal to 0.43 μeq/cm^2. Thus, the *total* amount of Na that enters the cells in 0.6 hr from the mucosal solution is sufficient to replete the compartment accessible to the mucosal solution *and* to permit Na extrusion from the cell at the rate of 2 μeq/cm^2 hr from zero time. This analysis indicates that (i) all of the intracellular Na exchangeable from the mucosal solution enters the cell via the amiloride-sensitive pathway (a conclusion that is consistent with the results of R.A. Frizzell & K. Turnheim, *manuscript submitted*); (ii) transcellular Na transport proceeds at or very close to the "final" steady-state level from the outset; and, (iii) the negative feedback on Na entry is exerted by a cellular compartment that is only indirectly related to transcellular transport.

A system that is compatible with our results and that may explain some of the observations cited above is illustrated in Fig. 8. The pathway responsible for active Na transport is presumed to run parallel to another cellular Na compartment with which it communicates; the latter compartment exerts a negative feedback on the amiloride-sensitive entry step. The Na content (capacity) of the active transport pathway is presumed to be very small so that this route (compartment) is filled rapidly after exposure of Na-depleted cells to Na and active transcellular transport is initiated and reaches steady-state levels quickly. Accordingly, at $t=0$, $[Na]_c \cong 0$ and entry is maximal; however, because some of the Na that enters is diverted from the transport pathway into the cellular compartment, the rate of active Na transport is less than I^m_{Na}. When $[Na]_c$ is repleted ($t=0.6$ hr) the permeability of the entry step is reduced and a steady state in which $I^m_{Na}=J^N_{net}$ is achieved. As mentioned above, stimulatory anions may exert their effects by blocking the negative feedback; and the actions of ouabain and amphotericin B can be accomodated by the system as illustrated in Fig. 8.

It should be stressed that the experimental basis for this admittedly speculative notion is the excellent agreement between the area under the exponential portion of the transient (the overshoot) (0.43 μeq/cm^2) and

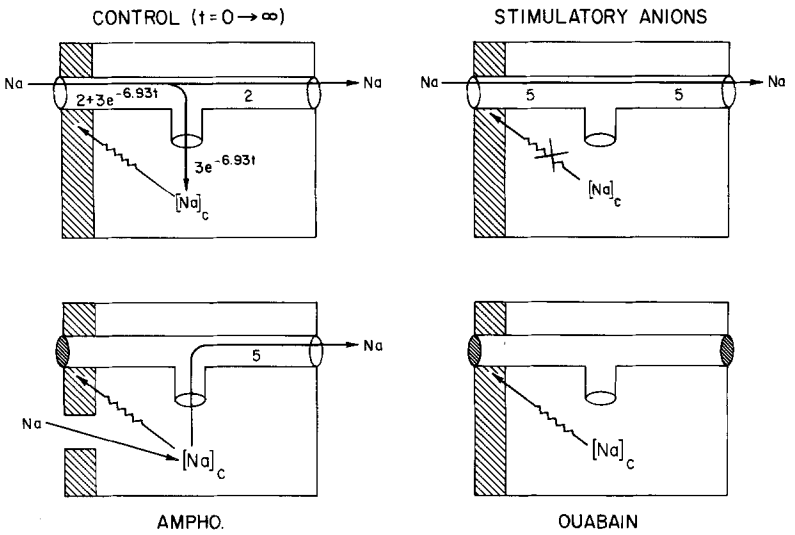

Fig. 8. Illustration of a "system" that is consistent with the present findings. The broken arrow signifies the negative interaction between $[Na]_c$ and the permeability of the entry step. Blockage of the entry or exit of the active transport pathway (the "tube") are indicated by hatching. It should be stressed that the proposed mechanism of action of amphotericin B is consistent with our other findings, but that the possibility that this antibiotic directly destroys the amiloride-sensitive entry step cannot be excluded

the amount of cell Na derived from the mucosal solution (0.40 μeq/cm^2). This finding leads, inescapably, to the conclusion that transcellular Na transport must be proceeding at a rate very close to 2 μeq/cm^2 hr (i.e., the steady-state rate) at a time very close to $t = 0$. This conclusion would not be seriously affected even if the area under the overshoot is overestimated by as much as 30%. In short, it seems quite clear that transcellular Na transport must be proceeding at or very close to the steady-state rate long before $[Na]_c$ is repleted and that the increase in R^m_{Na} is related to the repletion of $[Na]_c$ and not the size of the Na transport pool. The negative feedback would serve to preserve cell Na content (concentration) and in addition could provide a mechanism for homocellular regulation of transcellular Na transport under physiological conditions.

Related Findings on Other Epithelia

Morel and Leblanc (1975) and Leblanc and Morel (1975) have reported findings on isolated frog skin that resemble the present results

and are in accord with the notion of a negative feedback between cell Na and the mechanism responsible for Na entry into the cells across the outer membrane. Further, the findings of Cereijido *et al.* (1974) on isolated frog skin are entirely compatible with our conclusions and the system illustrated in Fig. 8 (top right).

Our findings and interpretations may provide at least partial explanations for several earlier, somewhat puzzling, observations on toad urinary bladder. Finn (1974), in an effort to test the notion originally proposed by Koefoed-Johnsen and Ussing (1958) that the outer or mucosal membrane of Na-transporting epithelia behaves as a Na-electrode, found that when active Na transport across toad urinary bladder was abolished by ouabain or reduction of the temperature of the bathing solutions to 5 °C, an abrupt decrease in the Na concentration of the mucosal solution from 112 to 2.4 mM did not significantly affect the transepithelial *PD* (ψ_{ms}). Further, when the initial ψ_{ms} was partially inhibited by graded doses of ouabain or amiloride or graded reductions in temperature, the changes in ψ_{ms} resulting from an abrupt reduction in the Na concentration of the mucosal solution were linearly related to the initial ψ_{ms}. The results obtained by Finn when active Na transport was completely inhibited with ouabain are entirely consistent with our finding that under these conditions the amiloride-sensitive Na entry step is blocked, probably due to an increase in cell Na; in all likelihood the same explanation can account for the results obtained when the temperature of the bathing media was reduced to 5 °C. In addition, the graded responses observed by Finn suggest that the Na conductance and the degree to which the outer or mucosal membrane approaches the behavior of a pure Na-electrode varies directly with the rate of active Na transport (pump activity).[7] A similar explanation may apply to the findings of Leb *et al.* (1965) and Snell and Chowdhury (1965).

Finally, it should be clear that the findings that metabolic inhibitors decrease the rate of Na entry across the outer barrier of frog skin cannot be construed as evidence for an active entry step (*c.f.* Biber, 1971; Moreno *et al.*, 1973); all of these findings can be readily accounted for by an increase in the resistance of the entry step in response to an increase in cell Na content.

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7 According to the equivalent electrical circuit model discussed by Schultz *et al.* (1977), an increase in R^m_{Na} would lead to a decrease in ψ_{ms} and a near parallel decrease in $\Delta\psi_{ms}$ in response to an abrupt change in $[Na]_m$. The "graded" responses noted by Finn (1974) are entirely consistent with this model.

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