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Effect of Amiloride on Conductance of Toad Urinary Bladder

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Summary. The transepithelial conductance of toad bladder epithelia and the amplitude of the fluctuations of this conductance caused by the action of the underlying smooth muscle have been further investigated. In particular, amiloride was found to reduce both tissue conductance and its fluctuating component to the same extent. Analysis suggests that the steady-state conductance of the toad urinary bladder may be associated only with the paracellular pathway for ions.

The epithelium of the toad urinary bladder is composed of a layer of polarized cells separated by highly tortuous intercellular spaces. These make an extremely small contribution to the total volume of the layer in the absence of transepithelial water flow. Each space narrows considerably in the region adjacent to the urinary surface, from which it is separated by a "tight junction" (zonula occludens), which was originally considered to be impermeable to ions and water (Farquhar & Palade, 1963). However, more recently several studies (Reuss & Finn, 1974; Civan & DiBona, 1978) have indicated that ions can move through these regions, and the term "limiting junctions" (DiBona, 1972) has been given appropriately to the apical zone.

There are, therefore, potentially two pathways, paracellular and cellular, by which ions might cross the epithelial cell layer. The estimation of the partial conductances, G_P (paracellular) and G_C (cellular) of each pathway has depended on the inhibition of ion-transport through the cells (Yonath & Civan, 1971; Hong & Essig, 1976). Such inhibition has been presumed to be without effect on the paracellular conductance. As the luminal membrane is much more permeable to Na than to either Cl^- of K^+ (Robinson & Macknight, 1976; Macknight, 1977) the cellular conductance in the steady state is believed to reflect Na movement alone.

Recently it has been shown that the movement of the smooth muscle in the subepithelial tissue of the toad bladder results in fluctuations in the conductance of the paracellular pathway but is without influence on the active transport of Na measured under short-circuit conditions (i.e., short-circuit current, I_{sc}, is unaffected by smooth-muscle activity (Gordon, 1978*b*)). Changes in epithelial conductance with changes in smooth muscle activity thus allow one to distinguish between the "active" and "passive" conductances. In the conventional model the conductance of the active cellular pathway is reduced by amiloride and enhanced by vasopressin. In previous experiments (Gordon, 1978*b*), however, amiloride seemed to reduce the magnitude of the fluctuating component of the conductance, indicating an effect of this drug on the paracellular conductance. However, because the total tissue conductance, G_t, was not affected significantly by amiloride in that series of experiments, further experiments have been performed to investigate the changes in conductance during inhibition of active sodium transport by amiloride. These reveal that amiloride affects the paracellular conductance of the toad bladder epithelium. Such an effect is not predicted by the conventional model of two parallel conductive pathways.

Materials and Methods

Large female toads of the species *Bufo marinus* were obtained from the Dominican Republic (National Reagents, Bridgeport, Conn.) and were kept on wood shavings with free access to water. The toads were doubly pithed and the hemibladders dissected from the abdominal cavity and mounted between the two identical halves of Ussing-type chambers (exposed surface area 10.0 cm^2). Both chambers were filled with sodium Ringer's solution (in mmol/liter): Na, 115.6; K, 3.0; Ca, 1.0; Mg, 1.0; Cl, 119; HPO_4, 1.8; glucose, 10. A

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Table 1. Ratios of conductances measured before and during inhibition by amiloride[a]

	G_t ($10^4\,\Omega^{-1}\cdot cm^{-2}$)	G_t^a ($10^4\,\Omega^{-1}\cdot cm^{-2}$)	$\frac{G_t}{G_t^a}$	$\frac{\Delta G_t}{\Delta G_t^a}$	$\frac{\Delta G_t/\Delta G_t^a}{G_t/G_t^a}$
	1.24	0.50	2.48	2.60	1.05
	2.48	1.08	2.30	2.70	1.17
	0.77	0.43	1.79	1.45	0.81
	2.00	1.11	1.80	1.43	0.79
	1.20	0.90	1.33	1.26	0.95
	1.31	0.92	1.42	1.44	1.01
	2.96	2.14	1.38	1.31	0.95
	1.67	1.24	1.35	1.66	1.23
	1.00	0.88	1.14	1.26	1.11
	1.00	0.91	1.10	1.66	1.51
Mean	1.56	1.01	1.61	1.68	1.06
±SEM	±0.23	±0.15	±0.15	±0.17	±0.07

[a] The latter is indicated by the superscript "a". G_t is the transepithelial conductance and ΔG_t is the amplitude of the conductance fluctuations caused by the contractions of the smooth muscle.

continuous stream of fine bubbles of air provided oxygenation and stirring throughout the experiments. All experiments were performed at room temperature (18–22 °C).

A nylon mesh was used to support the tissue. The subepithelial tissue faced the nylon mesh and the serosal medium was introduced after the mucosal chamber was filled. The level of the fluid in the serosal chamber was adjusted so that there was no hydrostatic pressure gradient across the hemibladder. Tissue was compressed at the chamber edge by a rubber 0 ring. No grease or tissue adhesive was applied to minimize "edge-damage," since preliminary studies using these chambers did not reveal appreciable alterations in tissue conductance with high vacuum silicon grease coating the chamber margins. The absence of appreciable "edge-damage" is indicated by the relatively low values obtained for tissue conductance, G_t, given in Table 1.

Tissue was voltage-clamped in the chambers at 0 mV (short-circuit condition) for 1 hr before being clamped at a voltage, V (serosa positive with respect to mucosa), somewhere between 0 mV and its initial open-circuit PD. This allowed an accurate measurement of the fluctuating component of the current, ΔI, arising from the effect of the smooth-muscle activity. Usually a PD of 10 mV was sufficient for this purpose. This voltage remained across the tissue for the rest of the experiment except for brief periods, during which the conductance of the tissue, G_t, was estimated. G_t was determined by changing the clamp-voltage by 10 mV for 5 sec and measuring the change in current. When the fluctuations in current had remained constant in magnitude and periodicity over a 20-min period, a 100 µl aliquot of amiloride solution (10 mmol/liter) was introduced into the mucosal chamber (containing 10 ml) giving a final amiloride concentration of 10^{-4} M. Simultaneously, 100 µl sodium Ringer's solution was added to the serosal chamber. The amiloride was a gift of Merck, Sharp and Dohme (N.Z.) Ltd.

Symbols

G_t, G_c, G_p	Transepithelial, cellular, and paracellular conductances.
ΔG_t or ΔG_p	Amplitude of the variation of G_t caused by the movement of the underlying smooth muscle.
Superscript "a"	Indicates the parameter's value during total inhibition of active transport produced by amiloride.
I_{sc}	Short-circuit current.
ΔI	Amplitude of the variation of current due to transepithelial voltage and contractions of smooth muscle.

Results

From the experiments the following data were derived: (i) total tissue conductance before amiloride, G_t, and after amiloride, G_t^a was derived from the change in transepithelial current when the tissue was briefly clamped at regular intervals for 5 sec at a PD (ΔV) 10 mV greater than the imposed clamping voltage; (ii) the ratio $\Delta G_t/\Delta G_t^a$, of the fluctuations in conductance induced by the smooth-muscle activity before and after amiloride, was derived from the magnitude of the fluctuations in transepithelial current observed with tissue voltage-clamped for a prolonged period at other than zero mV. Note that the absolute value of the conductance which fluctuates could not be measured, as only the amplitude of the fluctuations was observable.

The effect of amiloride on ΔG_t can be measured directly from ΔI if the contractions of the smooth muscle of the underlying tissue do not change in amplitude or in frequency during the experiment. If the pattern of the fluctuating current, which can be composed of several components varying in amplitude, altered over the experimental period, then the following procedure was adopted as illustrated in Fig. 2. An envelope around the trace was constructed by joining the peaks with one set of straight lines and the troughs with a second set. The areas within the envelope of the traces recorded over the same time period, before and after the addition of amiloride, allowed estimation of the magnitude of the current fluctuations which is proportional to ΔG_t.

Figure 1 shows tracing in which the pattern of fluctuations remained simple and unchanged during the experiments. The values of $\Delta G_t/\Delta G_t^a$ are identical with G_t/G_t^a. Similar tracings are shown in Fig. 2 where the patterns of current fluctuations are more complex. The areas between the dotted lines are considered to be proportional to ΔG_t.

Table 1 presents data from 10 experiments in which the changes in transepithelial conductance before and after the addition of amiloride were compared with the corresponding changes in the amplitude of the fluctuations in conductance induced by the smooth-muscle activity. Column 1 shows total tissue conductance, G_t. These values are of similar

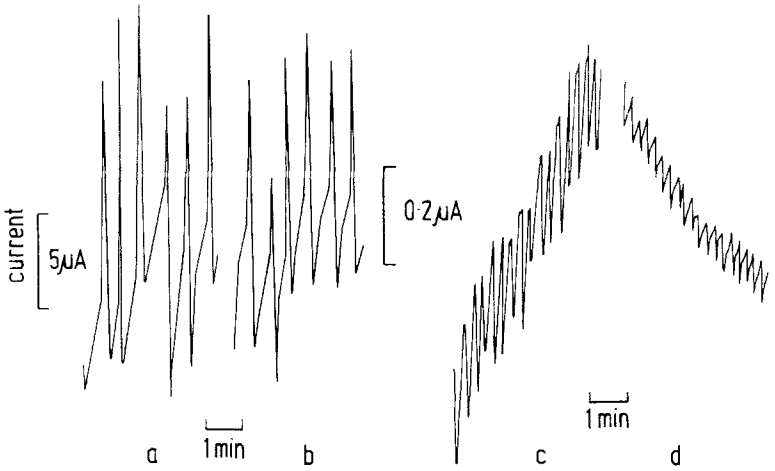

Fig. 1. Two examples of regular patterns of current fluctuations from different toad bladders before (*a*, *c*) and during (*b*, *d*) the addition of amiloride. In *a*, $I_{sc} = 20\,\mu A/cm^2$, the concentration of amiloride (*b*) was 5×10^{-5} mole/liter and $G_t/G_t^a = 1.15$ and $\Delta G_t/\Delta G_t^a = 1.16$ (*see* text for definition). In (*c*) $I_{sc} = 12\,\mu A/cm^2$, the concentration of amiloride (*d*) was 3×10^{-4} mole/liter and $G_t/G_t^a = 2.50$ (and $\Delta G_t/\Delta G_t^a = 2.60$). The applied voltage in both experiments was 20 mV (serosa positive). The angle of the traces to the horizontal in (*c*) and (*d*), indicate gradual changes in either the active or passive pathways with time (note high magnification)

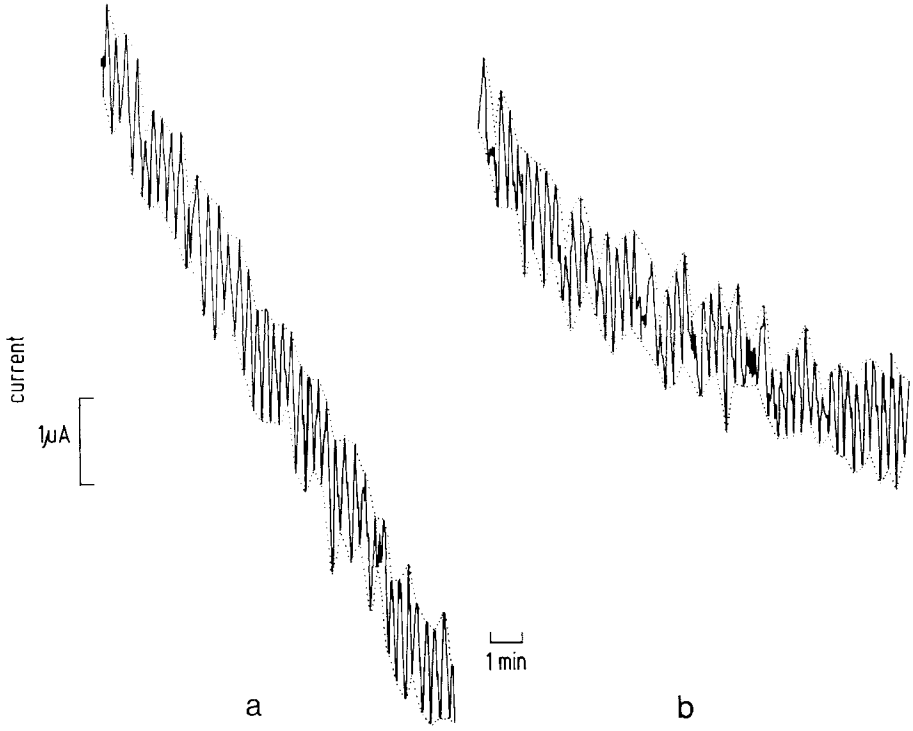

Fig. 2. The value of ΔG_t is defined as being proportional to the area of the envelope of the trace per unit time. $\Delta G_t/\Delta G_t^a = 1.2$; $G_t/G_t^a = 1.3$. Experimental conditions: 30 mV (serosa positive) applied, $I_{sc} = 16\,\mu A/cm^2$ and amiloride concentration (*b*) 10^{-4} mole/liter

magnitude to those observed recently by others in preparations mounted to avoid "edge damage" (Higgins *et al.* 1975; Erlij, 1976). The average G_t was 1.56 ± 0.23 ($10^4\,\Omega^{-1}\,cm^{-2}$). (For comparison with other published data, the average of individual tissue resistance was $7542 \pm 950\,\Omega\,cm^2$.) After amiloride (column 2), tissue conductance, G_t^a, fell to an average of 1.01 ± 0.15 ($10^4\,\Omega^{-1}\,cm^{-2}$), the difference in conductance of 0.55 ± 0.13 being highly significant in a paired *t* test ($P < 0.005$). (The average of individual tissue resistance after amiloride was $11{,}888 \pm 1780\,\Omega\,cm^2$.) Thus amiloride reduced tissue conductance markedly, as others have found (Bentley, 1968). This reduction is reflected in the ratio G_t/G_t^a (column 3) of 1.61 ± 0.15. However, as shown in column 4, amiloride also affected the ratio $\Delta G_t/\Delta G_t^a$ (which reflects the change in conductance of the paracellular pathway) to the same extent, the mean of 1.68 ± 0.17 not differing significantly ($P > 0.50$) from that of 1.61 ± 0.15 for G_t/G_t^a. Linear regression analysis confirms the essential quality of the two ratios with a correlation coefficient of 0.81 ($P < 0.005$) between $\Delta G_t/\Delta G_t^a$ and G_t/G_t^a. As discussed below, this effect of amiloride on $\Delta G_t/\Delta G_t^a$ is not consistent with the belief that amiloride affects only the conductance in the active cellular pathway, G_c. Finally, column 5 shows the ratio

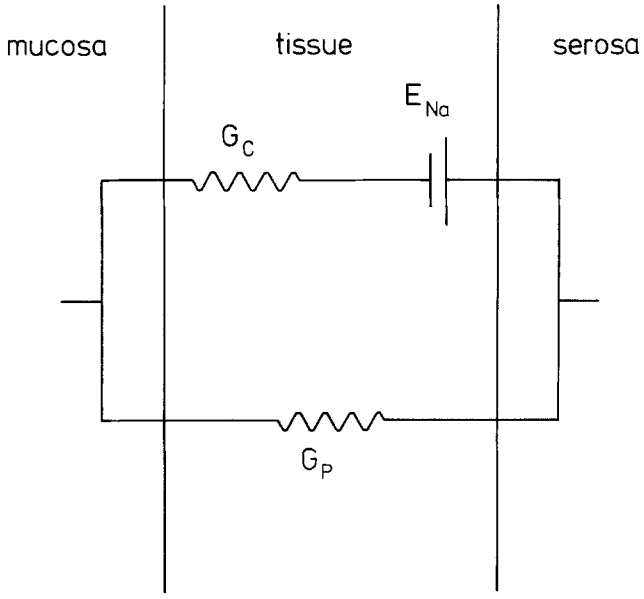

Fig. 3. Conventional equivalent circuit representing the electrical pathways for epithelial cells which actively transport sodium. E_{Na}: Electromotive force of the sodium transporting mechanism. G_c: conductance to the sodium current. G_p: conductance to passive ions. (Adapted from Ussing & Zerahn, 1951)

between the ratios of the two conductances. This does not differ significantly from unity.

Discussion

In an earlier study of toad urinary bladder, Gordon (1978*b*) investigated the small spontaneous fluctuations in transepithelial current, and therefore, of tissue conductance, which are observed in tissue bathed on both surfaces with sodium Ringer's and voltage-clamped at potentials other than zero (i.e., not completely short circuited). These fluctuations seem to result from alterations in transepithelial ion movement rather than reflecting transient polarizations of the cells, for the latter are abolished by amiloride (L.G.M. Gordon, *unpublished*), whereas the fluctuations reported here were never abolished by amiloride, as shown in Table 1. They are caused by smooth-muscle activity, the contractions of which were shown to occur simultaneously with the fluctuations in conductance. Verapamil, a known inhibitor of smooth-muscle contractions, abolished the fluctuations in current and conductance.

In contrast to the findings with tissue clamped at voltages other than zero, no spontaneous fluctuations in current occurred when tissues were voltage clamped at zero (i.e., completely short circuited). Since, under these conditions, current can flow only through the active pathway, the absence of these fluctuations with voltage clamping at zero precludes the active pathway as the site of these fluctuations. This conclusion was supported by the finding that the fluctuations in current were exaggerated with hyperosmotic mucosal solutions which are known to open up the limiting junctions and, therefore, to affect the paracellular pathway (Urakabe, Handler & Orloff, 1970; DiBona & Civan, 1973; Wade, Revel & DiScala, 1973).

It was concluded (Gordon, 1978*b*) that these fluctuations in current and conductance were confined to the paracellular pathway. Analysis of these fluctuations, therefore, provides a method for determining the contribution of the paracellular pathway to the electrical parameters of the epithelium.

The amplitude of the fluctuations in transepithelial conductance, although related to the transepithelial conductance for different morphological conditions of the paracellular pathway produced by changes in the external media, are not necessarily in simple proportions, e.g., where the morphology is changed directly by hyperosmotic mucosal solutions or directly by agents such as oxytocin which affect the smooth-muscle tension. Previously, in an attempt to analyze quantitatively the results of experiments investigating the action of smooth muscle on transepithelial conductance, this proportionality was presumed to be correct (Eq. (2), Gordon, 1978*b*), but the results from the studies of amiloride inhibition of epithelial transport discussed below invalidate this assumption.

There is much evidence favoring the hypothesis that amiloride inhibits the entry of sodium to epithelial cells in "tight" epithelia. Not only does it virtually abolish transepithelial sodium transport at sufficiently high concentration (10^{-4} M) in the mucosal medium (Bentley, 1968), but microelectrode studies reveal an increased apical membrane resistance in epithelial cells in frog skin (Nagel, 1976; Helman & Fisher, 1977), toad and *Necturus* urinary bladder (Reuss & Finn, 1975*a*, *b*; Frömter & Gebler, 1977; Sudou & Hoshi, 1977) and rabbit colon (Schultz, Frizzell & Nellans, 1977). Furthermore, measurements of cellular ions in toad urinary bladder reveal a decrease in sodium of mucosal origin after amiloride (Macknight, Civan & Leaf, 1975). In contrast, it is generally accepted that amiloride is without effect on movements of ions through the paracellular pathway. Evidence which apparently supports this conclusion comes from isotopic flux studies in toad bladder (Larsen, 1973) and frog skin (O'Neil & Helman, 1976; Biber & Mullen, 1977). Amiloride has been used, therefore, to estimate the relative conductances of the two parallel pathways, where $G_t = G_p + G_c$. After amiloride (10^{-4} M) has abolished active transepithelial sodium transport, $G_c = 0$, and $G_t^a = G_p$,

as can be demonstrated in the "classic" equivalent circuit (Fig. 3).

From this simple relationship we would predict that the passive conductance estimated from the fluctuations in current induced by the activity of the smooth muscle would not be affected by amiloride, i.e., that $\Delta G_t/\Delta G_t^a = 1$. Furthermore, since amiloride decreases total tissue conductance, $G_t/G_t^a > 1$ (column 3, Table 1). But as shown in Table 1, the prediction concerning the fluctuating conductance is not fulfilled, for though $G_t/G_t^a > 1$, $\Delta G_t/\Delta G_t^a = G_t/G_t^a$, indicating that if the "classic" electrical circuit (Fig. 3) is correct, amiloride has decreased both G_p and G_t to the same extent.

This unexpected result has important implications concerning the electrical properties of the pathway through which ions traverse the toad urinary bladder. But before accepting the conclusion that amiloride has affected the paracellular pathway, it is necessary to exclude other possible explanations for these results. Damage incurred in the mounting of the tissue could result in an increase in the value of G_p, as calculated after the addition of amiloride. However, this should have little effect on the present results since both $\Delta G_t/\Delta G_t^a$ and G_t/G_t^a should have been similarly overestimated. Alternatively, the fluctuations in current with smooth-muscle activity might not reflect changes only in the conductance of the paracellular pathway but might result from changes in amiloride-sensitive cellular transport pathways. But the absence of these fluctuations when tissue bathed on both surfaces by sodium Ringer's is short-circuited must exclude a contribution from an active transport of sodium through a fluctuating conductance in such a pathway. Passive movements of other ions through an amiloride-sensitive cellular pathway affected by smooth-muscle activity might result in small fluctuations in current in a preparation voltage-clamped at other than zero transepithelial PD. However, the apparent low permeability of the mucosal cellular membrane to both potassium (Robinson & Macknight, 1976) and chloride (Macknight, 1977) offers little support to this alternative.

The simplest explanation, therefore, is that amiloride has altered the conductance of the paracellular pathway. That the ratio $(\Delta G_t/\Delta G_t^a)/(G_t/G_t^a)$ is unity implies that the full effect of amiloride is exerted on the same conductance pathway as that affected by smooth-muscle activity, i.e., the paracellular pathway. As a corrolary, since amiloride does completely inhibit transepithelial sodium transport, one is also forced to conclude that there is no amiloride-sensitive electrical conductance through the cellular transport pathway.

This conclusion follows from the following considerations. Both G_p and ΔG_p will be proportional to the electrolyte concentration (i.e., to the number of charge carriers) in the paracellular pathway. Thus, under conditions in which only the electrolyte concentration in the paracellular pathway changes,

$$\Delta G_p = kG_p \tag{1}$$

where k is a constant. (Note that Eq. (1) will not necessarily hold for changes in G_p caused by hypertonic mucosal solutions or vasopressin, both of which are known to substantially change the morphology of the paracellular pathway.)

The transepithelial conductance, G_t, can be simply expressed in terms of the conventional model (Fig. 3) as

$$G_t = G_c + G_p. \tag{2}$$

In the presence of amiloride

$$G_t^a = 0 + G_p^a. \tag{3}$$

In the previous study (Gordon, 1978*b*) it was found that fluctuations in tissue conductance associated with contractions of the underlying smooth muscle (ΔG_t), were entirely due to changes in the conductance of the paracellular pathway. That is,

$$\Delta G_t \equiv \Delta G_p \tag{4}$$

and

$$\Delta G_t^a \equiv \Delta G_p^a. \tag{5}$$

Thus, using Eqs. (1) to (5) above we can show sequentially that

$$\frac{\Delta G_t}{\Delta G_t^a} = \frac{\Delta G_p}{\Delta G_p^a} = \frac{G_p}{G_p^a} = \frac{G_t - G_c}{G_t^a}, \tag{6}$$

but experimentally it is found that

$$\frac{\Delta G_t}{\Delta G_t^a} = \frac{G_t}{G_t^a} \tag{7}$$

and therefore, from Eqs. (6) and (7), $G_c = 0$.

This result does not mean that both cellular membranes offer infinite electrical resistance to sodium movement, but that the pathway as a whole, or one element of it, cannot be represented as a simple resistance. For example, there is evidence, cited above, from microelectrode studies, which indicates that amiloride increases the electrical resistance of the apical cellular membrane. In contrast, sodium crosses the basolateral cellular membrane predominantly through a mechanism activated by a metabolic process with little recycling of sodium across

this membrane (Beauwens & Al-Awqati, 1976; Canessa, Labarca & Leaf, 1976; Macknight & McLaughlin, 1977), and because there appears to be no other resistive element for sodium movement through the active pathway, it must be inferred that the basolateral membrane is the site of negligible sodium conductance. Therefore, though a voltage-dependent ion movement through the active pathway has been implied in representations of the sodium pump as a constant voltage generator (E_{Na}) (Ussing & Zerahn, 1951) the present results argue against the suitability of this representation. Instead, it may be more appropriate to regard the sodium pump as a current source (Boulpaep, 1976; Fuchs, Larsen & Lindemann, 1977). The variation in current which follows an imposed voltage step would then be due to (i) ions moving through the paracellular pathway, (ii) changing ionic distributions in the double layers at the membrane surfaces, and (iii) the change of rate of sodium entry into the cell with concomitant potassium or chloride fluxes to or from the serosal solution for the preservation of electroneutrality of the intracellular fluid. Only capacitance transients would arise from (ii) and (iii) since the basolateral membrane is practically impermeable to Na (Beauwens & Al-Awqati, 1976; Canessa et al., 1976; Macknight & McLaughlin, 1977) so that no ions of the bathing media can permeate both mucosal and serosal membranes by passive processes (Robinson & Macknight, 1976; Macknight, 1977).

Transepithelial sodium transport and the metabolic energy utilized by this process both vary with changes in transepithelial voltage (Vieira, Caplan & Essig, 1972; Labarca, Canessa & Leaf, 1977). This is readily explained in the conventional model where the sodium pump is represented as a voltage source (Fig. 3). If the pump is regarded as a current source, this dependency of transepithelial sodium transport on transepithelial potential difference is also readily explained. When the transepithelial potential difference is altered, the ionic composition of the cells must change to reach the new steady state (process iii described in the paragraph above). Cellular sodium concentration will be altered and, as a consequence, the rate of pump activity will change. For example, when the open-circuited epithelium is short circuited, the movement of sodium ions into the cell from the mucosal solution will be facilitated, since only sodium carries appreciable current across this membrane, accompanied by a current flow across the basolateral membrane carried by chloride and potassium. The increased cellular sodium will stimulate pump activity and transepithelial sodium transport will be increased.

There is no doubt that inhibition of active sodium transport by amiloride decreases G_t, whereas stimulation of transport by vasopressin, for example, increases G_t. Thus any alternative model must account for this dependence of G_t on the rate of active sodium transport. One possibility (*A*) is that it reflects local alterations in ion concentrations in the lateral intercellular spaces or some part or parts thereof, thereby increasing the electrolyte concentration in the limiting junction (Gordon, 1978*a*). The structural organization of the epithelia, together with the localization of sodium pump sites along the basolateral membranes and the coupling of salt and water movements across "leaky" epithelia, has led to the suggestion that local osmotic gradients are generated within these lateral intercellular spaces (Diamond & Bossert, 1967). The magnitudes of these gradients are unknown but are usually estimated, in "leaky" epithelia, at no more than 10 to 20 mosM at most (Machen & Diamond, 1969). It is possible that in "tight" epithelia greater osmotic gradients could be generated since their hyrdraulic conductivity is so much lower. But, for total tissue conductance to change by, on average, as much as 60% with inhibition of sodium transport, a considerable change in ionic concentration would be required. It seems unlikely that the sodium concentration in the lateral intercellular spaces during transport could rise above medium concentration to this extent. An alternative explanation (*B*) of the inter-relationship between the rate of active sodium transport and the conductance of the paracellular pathway is based on the possibility of an accumulation, within the junctional complex, of sodium ions ejected directly from the cells through the membranes lining the limiting junction. This mechanism does not require that the lateral intercellular spaces be hyperosmotic.

Finally, the lack of effect of amiloride on sodium fluxes through the paracellular pathway (Hong & Essig, 1976) should be contrasted with the effect of this agent on paracellular electrical conductance detected in the present study. The serosal to mucosal sodium flux, which is taken as representing the movement of sodium through the paracellular pathway, will be influenced by the morphology of this pathway but not by an excess concentration of ions contributed by the active process of sodium transport into this region. Thus, unless amiloride altered the morphology of the pathway, it should not affect the passive sodium flux across the tissue even though, by inhibiting cellular sodium transport, it may have altered the concentration of sodium within some part of the pathway and thereby affected the electrical conductances across the tissue.

In summary, the present results, which reveal an equality between G_t/G_t^a and $\Delta G_t/\Delta G_t^a$, suggest that the

steady-state conductance of the toad urinary bladder reflects the properties of the paracellular pathway alone with the active transport of sodium across the cells modifying tissue conductance through its effects on the ionic concentration in some element or elements of the paracellular pathway. Additional experimental work will be required to assess the validity of this interpretation.

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