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An Amiloride-Sensitive Na^+ Conductance in the Basolateral Membrane of Toad Urinary Bladder

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Summary. Exposing the apical membrane of toad urinary bladder to the ionophore nystatin lowers its resistance to less than 100 Ω cm^2. The basolateral membrane can then be studied by means of transepithelial measurements. If the mucosal solution contains more than 5 mM Na^+, and serosal Na^+ is substituted by K^+, Cs^+, or N-methyl-D-glucamine, the basolateral membrane expresses what appears to be a large Na^+ conductance, passing strong currents out of the cell. This pathway is insensitive to ouabain or vanadate and does not require serosal or mucosal Ca^{2+}. In Cl - free SO_4^{2-} Ringer's solution it is the major conductive pathway in the basolateral membrane even though the serosal side has 60 mM K^+. This pathway can be blocked by serosal amiloride (K_i = 13.1 μM) or serosal Na^+ ions ($K_i \sim 10$ to 20 mM). It also conducts Li^+ and shows a voltage-dependent relaxation with characteristic rates of 10 to 20 rad sec^{-1} at 0 mV.

Key Words nystatin · amiloride · basolateral membrane · toad urinary bladder · Na^+ transport · epithelial impedance

Introduction

The urinary bladder of the *Bufo marinus* toad has been extensively used as a model system for mammalian tight epithelia, and its transport characteristics were examined by a variety of methods. It is generally accepted that while the luminal surface of this tissue conducts only Na^+, the basolateral side is highly permeable to K^+ and does not passively transport Na^+ ions (for review *see* Macknight et al., 1980).

An approach used by several groups to investigate permeability characteristics of the basolateral membrane of toad bladder and other tight epithelia is to incorporate a nonspecific ionophore in the apical membrane. The resistance of the ionophore-invaded membrane becomes negligibly small and the whole tissue can be considered to be a single barrier. Thus, Lichtenstein and Leaf (1965) and Sharp et al. (1966) used amphotericin B-treated bladders to study the regulation of Na^+ transport by ADH and metabolism, respectively. Nystatin and gramicidin D were employed to study the electrical properties of the rabbit urinary bladder (Lewis et al., 1977; Eaton, Frace & Silverthorn, 1982; Lewis & Wills, 1982), and similar measurements were also carried out on the rabbit descending colon (Wills et al., 1977; Wills, 1981) and on frog skin (Nielsen, 1979).

Recently, measurements of K^+ and Na^+ currents across the basolateral membrane of nystatin-treated *Bufo bufo* toad bladder bathed in sulfate Ringer's solutions were reported (Garty, 1984*a*). An unexpected observation in this study was a ouabain-insensitive Na^+ current obtained for high mucosal Na^+ and high serosal K^+ activities. In the present paper we further examine this phenomenon using the more commonly studied *Bufo marinus* bladder, and report that under specific conditions the basolateral membrane expresses what appears to be a large Na^+- and Li^+-specific conductance. This pathway can be blocked either by serosal amiloride or high serosal Na^+. It is insensitive to ouabain or vanadate and does not depend on the presence of K^+, Ca^{2+} or Cl^- in the serosal solution.

Part of these data were reported in the 62nd meeting of the Deutsche Physiologische Gesellschaft (Garty, Warncke & Lindemann, 1985).

Symbols and Abbreviations

C_a, C_b	The apical and basolateral electrical capacitance, respectively
R_a, R_b, R_p, R_s	The apical, basolateral, paracellular and solution electrical resistance, respectively
X_m, X_c, X_s	Mucosal, cellular and serosal activity of ion X, respectively
I_{sc}	The transepithelial short-circuit current
G	The low frequency slope conductance
$J_{^{22}Na^+}$	The mucosal-to-serosal $^{22}Na^+$ flux
$NMDG^+$	N-methyl-D-glucamine.

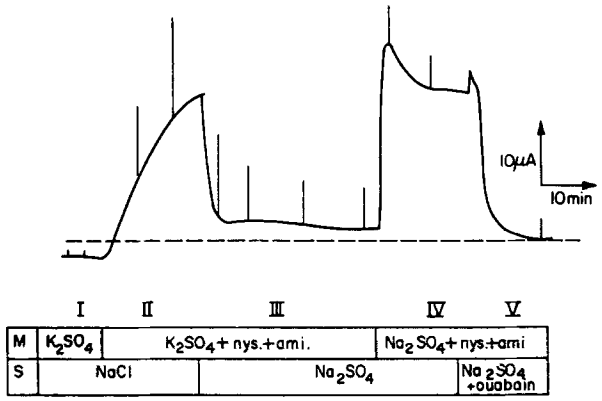

Fig. 1. Effects of nystatin and ouabain on I_{sc} and G. A mounted toad bladder was incubated with various mucosal (denoted M) and serosal (denoted S) solutions as indicated in the bottom of the Figure and in the text. The continuous trace is the short-circuit current and the vertical spikes are the current deflections in response to 5-mV displacements of the clamping potential. The dashed line is the zero current level

Materials and Methods

Animals

Bufo marinus toads were obtained from Lemberger (Germantown, Wisconsin) and kept at room temperature with free access to tap water. The animals were double-pithed, and the urinary bladders were removed and washed in NaCl Ringer's. The washed bladders were slightly stretched over a plastic ring (nominal surface area 3.14 cm^2) with the serosal side supported by a piece of filter paper, and the whole assembly was mounted in a Lucite® chamber described before (Palmer, Edelman & Lindemann, 1980; Garty, Edelman & Lindemann, 1983). The serosal compartment of this chamber had an 18-ml volume open to the atmosphere and was stirred by aeration, and the mucosal compartment had a closed 1-ml volume through which Ringer's solution was continuously perfused at rates of 1 to 50 ml/min.

Current Measurements

The mounted tissues were clamped to zero voltage and the short-circuit current was continuously recorded. The low-frequency slope conductance was evaluated during brief (1 sec) displacements of the clamping potential by 5 mV. Transcellular inward current (mucosal compartment to cell and cell to interstitial space) is designated as positive. Thus the basolateral membrane potential is negative when the cell is negative with respect to the interstitial space.

Admittance Measurements

The method used in the admittance measurements was as described by Warncke and Lindemann (1985). In brief, the holding voltage of 0 mV applied to the clamp was superimposed with sinusoidal voltages of 8 to 2 mV amplitude (depending on the frequency), generated digitally and stored in a static ROM. The sinewave frequencies were automatically decreased from 1600 to 0.2 Hz (4 steps per octave) with 5 periods at each frequency. The sinusoidal current response was amplified and sampled in synchrony with the ROM readout. The current time course was drift-corrected for each frequency, averaged and evaluated for amplitude and phase. The resulting spectra were analyzed in terms of coefficients of complex rational functions as described by Strobel (1968). This method has a larger range of convergence than the least-squares regression. The polynomial coefficients were rearranged into equations which could be solved for the parameters of the equivalent circuits used to model the epithelium (Figs. 3*A* and 12).

Tracer Flux Measurements

Mucosal-to-serosal $^{22}Na^+$ fluxes were measured by incubating the mucosal surface of a mounted bladder with a Ringer's solution containing 5 μCi/ml ^{22}NaCl, and removing 750-μl aliquots from the serosal compartment at 10-min intervals. The radioactive samples were mixed with 10 ml scintillation fluid (dioxane-based) and counted in a β-scintillation counter. After each sampling, 750 μl of nonradioactive solution were added to the serosal compartment. The calculated fluxes were corrected for this dilution.

Solutions

Several types of sulfate Ringer's solutions were used. They all contained 4 mM K_2HPO_4 + 1 mM KH_2PO_4 (pH 7.5), 1 mM Ca gluconate (unless otherwise indicated) and 54.5 mM of one of the following salts: K_2SO_4, Na_2SO_4, Cs_2SO_4, Li_2SO_4 and $(NMDG)_2SO_4$ (60 mM activity of Na^+, K^+, Li^+ or Cs^+). $(NMDG)_2SO_4$ was prepared by dissolving the amine base in water and titrating it to pH 7.5 with H_2SO_4. In testing the dependence of I_{sc} and G on the serosal and mucosal Na^+ activities, various mixtures of the above sulfate salts whose combined concentration was 54.5 mM were employed. In addition, several Cl^- Ringer's solutions were used. They contained 3.5 mM phosphate buffer (pH 7.3), 0.1 mM $CaCl_2$, 0.5 mM $MgCl_2$ and either 110 mM NaCl, or 85 mM NaCl + 50 mM sucrose or 85 mM KCl + 50 mM sucrose. All mucosal solutions contained 30 μg/ml nystatin and 80 μM amiloride. The ionophore was added from a freshly made stock solution of 60 mg/ml (in dimethylsulfoxide) under vigorous stirring. In most parts of the experiment the serosal solution contained 2 to 5 mM ouabain.

Statistics

Unless otherwise indicated data are expressed as mean ± SEM.

Materials

Nystatin (5260 ups units/mg), cytochalasin B, colchicine, and N-methyl-D-glucamine were obtained from Sigma; ^{22}NaCl (carrier-free 200 μCi/ml) from Amersham Corp.; and amiloride was a gift from Merck, Sharp and Dohme GmbH, Munich, FRG. All the conventional chemicals were analytical grade.

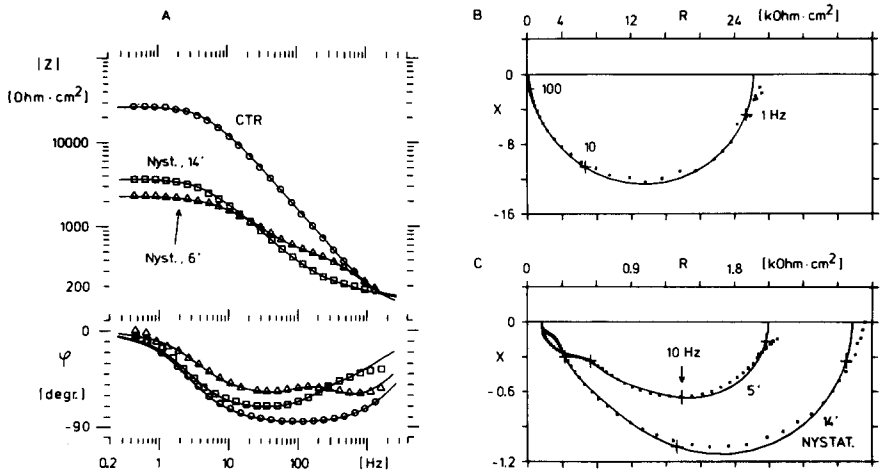

Fig. 2. Impedance spectra in the presence and absence of nystatin. (*A*) Bode diagram of the impedance magnitude $|Z|$ (shown in the upper traces) and the phase angle ρ (shown in the lower traces), measured between 0.35 and 1600 Hz and referred to chamber area of 1 cm^2. The measurement was performed 3 times: Before the addition of nystatin (○, CTR), 6 min after the addition (△) and 14 min after the addition (□). In each measurement the holding voltage was 0 mV. Only every second data point is shown. The solid lines are the best fits of a broken rational polynomial of third degree to the data points. The mucosal solution contained Na^+ activity of 60 mM and 100 μM amiloride, the principal anion being sulfate. The serosal solution was NaCl Ringer's with a nominal Ca^{2+} concentration of 100 μM. (*B,C*) Nyquist plots (real versus imaginary component (*X*)) of the impedance before, 5 min after, and 14 min after, the addition of nystatin. Frequencies of 1, 10, and 100 Hz are marked by crosses

Results

Nystatin Incorporation into the Apical Membrane

Accurate determination of the basolateral cationic conductances, and the prevention of nystatin-induced cell bursting, require the substitution of both mucosal and serosal Cl^- by an impermeable anion such as SO_4^{2-} (Lewis et al., 1977; Eaton et al., 1982; Garty, 1984*a*). In toad bladder, the substitution of mucosal Cl^- by SO_4^{2-} is tolerated by the tissue (e.g. Palmer et al., 1980); however, the removal of serosal Cl^- was reported to decrease I_{sc} and induce cell shrinkage (Lewis et al., 1985). We therefore initiated the experiment by incubating the serosal side of the tissue with Cl^--Ringer's solution, and replaced it by a SO_4^{2-}-Ringer's only after nystatin had lowered the apical resistance to a negligible value.

Figure 1 depicts the experimental protocol used to incorporate nystatin into the apical membrane and record the basolateral currents. The mounted bladder is first equilibrated with K_2SO_4- and NaCl-Ringer's solutions in mucosal and serosal sides, respectively (period I in Fig. 1). After the current and conductance reach steady low values, K_2SO_4 solution containing nystatin and amiloride is perfused through the mucosal compartment. This substitution brings about a large increase of I_{sc} and G, presumably due to a mucosal-to-serosal K^+ flux and a serosal-to-mucosal Cl^- flux (period II). At this point the serosal NaCl Ringer's is substituted by a Na_2SO_4 solution, causing an immediate drop in the current followed by a much slower decrease of conductance (period III). The magnitude of the remaining positive current (a mucosal-to-serosal K^+ flow) was subject to large experimental variations. In some cases it was very small (e.g. Fig. 1), but in others, values of more than 10 $\mu A/cm^2$ were recorded. When the mucosal solution is replaced by a Na_2SO_4-Ringer's solution (containing nystatin and amiloride) a much larger I_{sc} is observed. This current reaches a maximal value a few seconds after the mucosal substitution, and then slowly decays to a lower steady value (period IV). Adding ouabain to the serosal compartment blocks the current completely (period V) indicating that it is mediated by the Na^+,K^+-ATPase. Ouabain also significantly increases the membrane resistance.

Nystatin effects on the apical resistance and conductance were evaluated by measuring the tissue impedance during the various phases of the experiment in Fig. 1. Before the ionophore addition (period I), the impedance was essentially a single dispersion with a time constant of 23.4 msec (Fig. 2*A*). This component is made up of an ohmic resistance of 24.5 kΩ cm^2, essentially R_p, in parallel with a capacitance $C = (1/C_a + 1/C_b)^{-1}$, whose value is 0.956 $\mu F/cm^2$ (*see* the equivalent network in Fig. 3*A*). Six minutes after adding nystatin the impe-

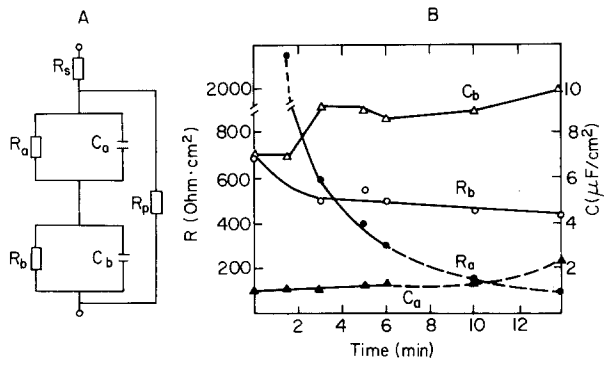

Fig. 3. Time course of the nystatin-induced changes in the tissues electrical parameters. (*A*) The electrical equivalent network of the epithelium used for the impedance analysis. The two membranes in series (apical, *a* and basolateral, *b*) are represented by resistance (R_a, R_b) and capacitance (C_a, C_b) elements. R_p and R_s are the paracellular and solution resistances, respectively. (*B*) Time course of changes in R_a, R_b, C_a, and C_b following the addition of 30 μg/ml nystatin (time zero) to the mucosal solution of a bladder. The solution composition is as in Fig. 2. At time zero R_a was larger than 24.5 kΩ cm² and it had dropped to 2150 Ω cm² 1.5 min after adding nystatin. At $t > 6$ min C_a and R_a could only be roughly estimated (dashed lines in Figure). During the 14-min period shown in the Figure I_{sc} increased from −1.4 to 9.2 μA/cm²

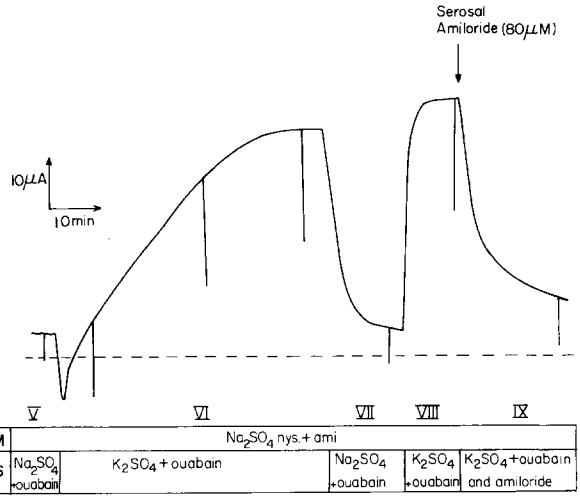

Fig. 4. Effects of serosal Na^+/K^+ exchange and amiloride on I_{sc} in a nystatin-treated bladder. I_{sc} and G were recorded for the different compositions of the serosal medium indicated in the bottom of the Figure. The mucosal solution was a Na_2SO_4 Ringer's + 30 μg/ml nystatin and 100 μM amiloride. All serosal solutions contained 5 mM ouabain. Amiloride was added to the serosal compartment at the time indicated by an arrow to a final concentration of 80 μM from a stock 8 mM solution. The dashed line and vertical spikes are as described in the legend to Fig. 1

dance is decreased, and a double dispersion, attributed to the apical and basolateral membranes, becomes obvious (triangles in Fig. 2*A*). After an additional 8 min the apical resistance becomes even smaller and the impedance is now dominated by the basolateral membrane. Nyquist diagrams of the impedance before and after the addition of nystatin are shown in Fig. 2*B*,*C*. The low frequency deviations of the data points from the fitted lines observed in these plots may be attributed to small drifts of R_b with time.

The equivalent network shown in Fig. 3*A* was used to calculate the resistances and capacitances before and after nystatin treatment. This minimal network has six components, only five of which can be determined by a single transepithelial measurement. This difficulty was overcome as follows: we assumed that before adding nystatin, in the presence of mucosal amiloride, R_a is very large and the total tissue resistance is therefore close to R_p. The value of R_p estimated in this way was assumed to remain constant during the ionophore incorporation. Even if changes in R_p do take place, they are not expected to significantly affect the other parameters since after nystatin treatment the cellular (basolateral) conductance is much larger than the parallel shunt conductance. The changes in R_a, R_b, C_a and C_b during the incorporation of nystatin in the apical membrane were calculated from the impedance data, using the above assumption, and are illustrated in Fig. 3*B*. The main feature in this Figure is a dramatic drop of R_a from 2150 Ω cm², 1.5 min after adding the ionophore, to less than 100 Ω cm² 12.5 min later. In addition, a small decrease in R_b and an increase in C_b were observed. At $t > 6$, R_a and C_a could not be accurately determined due to drifts in R_b (dashed lines in Fig. 3*B*). Nevertheless, the data clearly demonstrate that in the nystatin-treated preparation the apical membrane is not any more a significant barrier to cation movement. Thus, the electrical properties of the basolateral membrane can now be studied by means of transepithelial measurements. Moreover, the later substitution of serosal Cl^- by SO_4^{2-} (period III of Fig. 1, *not shown* in Figs. 2 and 3) increases the basolateral resistance and makes this approximation even more valid.

Noteworthy is the moderate decrease in R_b shown in Fig. 3*B*. We cannot exclude the possibility that it was due to an invasion of the basolateral membrane by a small amount of nystatin. If so, it was an exceptional occurrence: in the majority of experiments R_b increased when nystatin was added to a Na^+-containing mucosal solution. Figure 2 shows the more typical increase in R_b between the 6th and 14th minute of exposure to mucosal nys-

Table 1. Effects of substituting the serosal Na^+ on I_{sc} and G[a]

Substituting ion	I_{sc} ($\mu A/cm^2$)		G (mS/cm^2)		n
	before	after	before	after	
K^+	1.0 ± 0.20	15.8 ± 0.3	0.22 ± 0.05	0.63 ± 0.07	9
Cs^+	0.3 ± 0.20	16.4 ± 0.5	0.23 ± 0.08	0.82 ± 0.09	9
$NMDG^+$	0.0 ± 0.25	14.6 ± 0.5	0.24 ± 0.07	0.62 ± 0.09	6

[a] Bladders were pre-equilibrated in Na_2SO_4 Ringer's solution containing 80 μM amiloride + 30 μg/ml nystatin (mucosal) and 5 mM ouabain (serosal). The serosal solution was then substituted by either K_2SO_4 or Cs_2SO_4 or $(NMDG)_2SO_4$ Ringer's solution (containing 5 mM ouabain). The electrical parameters were measured before, and 45 min after, the substitution. Data are expressed as mean ± SEM, and n is the number of bladders.

tatin. The increase may be explained by a drop in basolateral K^+ conductance due to partial replacement of cellular K^+ by mucosal Na^+.

The Amiloride-Blockable Basolateral Pathway

When the bladders were maintained in Na_2SO_4 Ringer's solutions containing amiloride + nystatin (mucosal) and ouabain (serosal), a steady, nearly zero I_{sc} and relatively high resistances were recorded (period V, Figs. 1 and 4). Substituting the serosal solution by a ouabain-containing K_2SO_4 solution, induced a small and transient negative current, followed by a slowly developing large positive current (period VI, Fig. 4). It is expected that under the experimental conditions of period VI the major ion flux would be a serosal-to-mucosal K^+ flow, i.e., negative I_{sc}. Thus it is surprising that the negative current observed is transient and gives way to a large, slowly developing positive I_{sc}, paralleled by an increase of G. This unexpected phenomenon was highly reproducible, and could also be observed if serosal Na^+ was substituted by Cs^+ or $NMDG^+$ rather than K^+ (Figs. 5, 8, and Table 1). Each of these substitutions led to the slow development of a positive I_{sc} and a substantial increase of G. Thus, it appears that under the experimental conditions of Fig. 4 a major conductive pathway which passes Na^+ ions is activated or induced in the basolateral membrane.

The current and conductance changes were fully reversible. Substituting K_s^+ by Na^+ lowered I_{sc} and G to their initial values (period VII), and a second substitution of Na_s^+ by K^+ increased them again (period VIII). Note, however, that the second induction of positive I_{sc} is much faster than the first

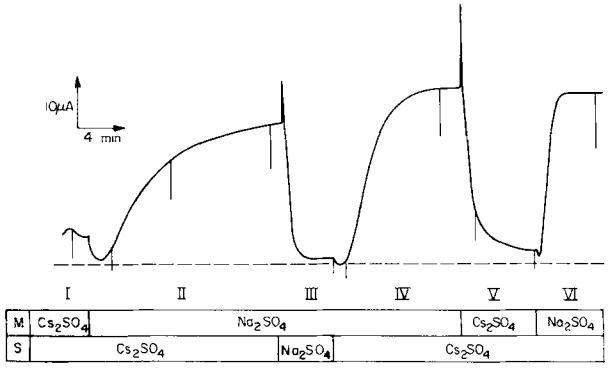

Fig. 5. Effects of mucosal and serosal Na^+/Cs^+ exchange on I_{sc} and G. I_{sc} and G were recorded for different mucosal (M) and serosal (S) media indicated at the bottom of the Figure. All the mucosal solutions included 30 μg/ml nystatin + 100 μM amiloride, and all the serosal solutions included 5 mM ouabain

one. This difference in rates was also observed if the tissue was pre-equilibrated in Na^+-free solutions, and the current changes were induced by mucosal substitutions (*cf.* periods II and VI in Fig. 5). A possible interpretation of these data is that the imposition of a large inverse Na^+ gradient across the basolateral membrane triggers events which make it more permeable to Na^+ than to K^+. The first appearance of positive I_{sc} (period VI, Fig. 4, period II, Fig. 5) is determined by the rate of these events and is relatively slow. The following substitutions produce current changes which are limited only by the diffusion of ions to and across the membrane and are therefore much faster. This hypothetical modification of basolateral characteristics may involve changes in cell volume, fusion of cytoplasmic vesicles and/or membrane cytoskeleton interactions. We therefore examined the possible effects of cytoskeleton disrupting agents on its rate. How-

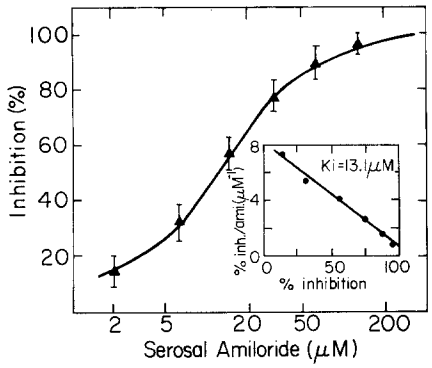

Fig. 6. The amiloride dose-response relationship. Bladders were first equilibrated in mucosal Na_2SO_4 Ringer's containing 80 μM amiloride and 30 μg/ml nystatin, and serosal K_2SO_4 Ringer's containing 5 mM ouabain. Increasing concentrations of amiloride were then added to the serosal solution and the % inhibition of the steady-state I_{sc} is plotted against the diuretic concentration. Mean ± SEM of three experiments are presented. Insert: An Eadie-Hofstee analysis of the amiloride inhibition curve. The experimental data could be fitted to a straight line ($R = -0.995$) and the amiloride inhibition constant derived from it is 13.1 ± 0.6 μM

ever, neither cytochalasin B (20 μg/ml, added 10 min before the ionic substitution), nor colchicine (10^{-4} M, added 4 hr before the experiment), seem to have an effect on the rate of the current changes.

The positive current developed by the removal of serosal Na^+ was found to be amiloride sensitive. Adding 80 μM amiloride to the *serosal* solution blocked it almost completely and lowered the transepithelial conductance significantly (period IX, Fig. 4). In five bladders the diuretic lowered I_{sc} from a mean value of 13.0 ± 1.1 to 1.6 ± 0.4 μA/cm^2 and reduced G from 0.8 ± 0.04 to 0.5 ± 0.03 mS/cm^2. The inhibitory effect of serosal amiloride was slower than the "normal" mucosal effect of the diuretic, but it was fully reversible. Substituting the serosal solution by an amiloride-free Ringer's restored the original current and conductance within 30 min. It should be emphasized that the diuretic was present in the mucosal solution for about 3 hr prior to its application to the serosal compartment. Thus, apical or intracellular effects of amiloride cannot account for the observed inhibition. Measuring the transepithelial potential and conductance before and after the addition of amiloride enables calculation of the electromotive force of the amiloride-blockable basolateral pathway (e.g. Lewis et al., 1985). For the above data, a mean value of 38 mV was obtained. Substituting this value plus the mucosal (cellular) and serosal activities of K^+ and Na^+ in the Goldman-Hodgkin-Katz equation yields a Na^+/K^+ permeability ratio of 4.65. This calculation assumes, however, that Na^+ and K^+ are the only ions conducted by the amiloride-sensitive pathway and that their cellular activities are equal to the mucosal concentrations. Thus the calculated permeability ratio should be viewed with some caution.

The amiloride dose-response relationship was evaluated by measuring the relative inhibition of I_{sc} induced by increasing concentrations of the diuretic. The data could be fitted by a Michaelis-Menten curve with a K_i of 13.1 μM (Fig. 6). This value is considerably higher than the inhibition constants measured for the apical Na^+ channels (Benos, 1982), but is sufficiently low to exclude nonspecific effects of the diuretic.

To further analyze the amiloride-blockable basolateral conductance we measured admittance spectra before and after the substitution of serosal Na^+ by K^+ and the application of amiloride. Figure 7*A* shows spectra measured either before or 2, 3.5, 5 and 15 min after substituting the serosal Na^+ by K^+. The main feature of the measured spectra is a gradual increase of the low frequency admittance and the appearance of a relaxation component. The corresponding Nyquist plots show a low frequency inductive component not observed before (*cf.* Fig. 11*B*) and indicate the slow appearance of a new voltage-dependent conductive pathway. This pathway is characterized by a frequency-independent conductance G_1, a voltage-dependent conductance ΔG and a relaxation rate coefficient λ (*see* Appendix). The appearance of a similar inductive component in the impedance spectra of the gallbladder epithelium, was attributed to polarization phenomena arising in boundaries adjacent to cell membranes (Gogelein & Van Driessche, 1981). This explanation cannot account for our data, since the inductive relaxation was observed only for a specific ionic composition and could not be seen with pump-mediated currents of comparable magnitude (e.g. periods II and V of Fig. 1).

The addition and removal of amiloride to and from the serosal compartment had complex effects on the admittance spectra (Fig. 7*B,C*). Analyzing the spectra in terms of Eq. (6) of the Appendix revealed that the diuretic decreased G_1, ΔG and λ. The serosal amiloride effects were fully reversible in that the original values of I_{sc}, G_1, ΔG and λ could be recovered upon the removal of the blocker (compare upper spectra in Figs. 7*B* and 7*C*).

$^{22}Na^+$ Flux Measurements

Figure 8 depicts parallel measurements of I_{sc} and the mucosal-to-serosal $^{22}Na^+$ flux ($J_{^{22}Na^+}$) in a nys-

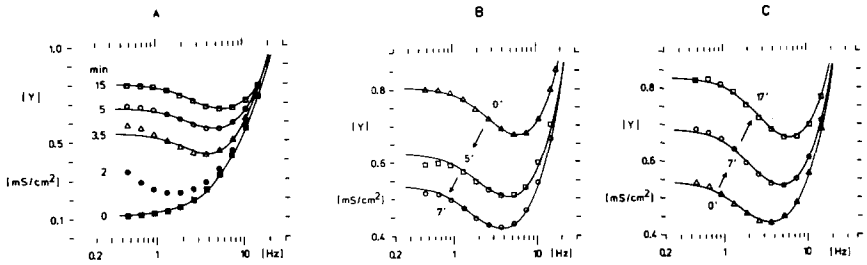

Fig. 7. Admittance changes during the evolution of the Na^+ inward current and the application of amiloride. (*A*) The admittance spectra were measured before (■), 2 min (●), 3.5 min (△), 5 min (○) and 15 min (□) after the substitution of serosal Na^+ by K^+. A holding voltage of 0 mV was used in all measurements. I_{sc} increased during this period from −0.9 to 9.6 $\mu A/cm^2$. The continuous lines are the best fits by the model discussed in the Appendix. The spectra obtained for $t = 2$ and 3.5 min could not be satisfactorily fitted by the model at low frequencies, presumably as a result of parameter drift during the scan. (*B*) Amiloride (50 μM) was added to the serosal solution at $t = 0$ and spectra were taken before (△), 5 min after (□) and 7 min after (○) its addition. I_{sc} decreased during this period from 9.6 to 0.7 $\mu A/cm^2$ and λ decreased from 15 to 9 rad sec^{-1}. (*C*) Serosal amiloride was removed at $t = 0$ and spectra were measured before (△), 7 min after (○) and 17 min after (□) its removal

tatin-treated ouabain-blocked bladder. At the initial state (similar Na_2SO_4 solutions on both compartments), I_{sc} was nearly zero and J_{22Na^+} had a significant, relatively constant value. The tracer flux at this stage was insensitive to serosal amiloride (*not shown*). Substituting serosal Na^+ by $NMDG^+$ induced more than a twofold increase in J_{22Na^+} which seemed to correlate in time with the development of I_{sc}. Amiloride, added to the serosal compartment inhibited both the electrical current and the tracer flux. In other experiments (*not shown* in the Figure) similar measurements were performed using mucosal K_2SO_4 rather than Na_2SO_4 Ringer's. The tracer Na^+ fluxes measured under these conditions were not influenced by the replacement of Na_s^+, and no sensitivity to serosal amiloride could be detected. Thus relatively high mucosal (cellular) Na^+ seems to be essential for the expression of the basolateral amiloride-sensitive pathway. The possible correlation between I_{sc} and J_{22Na^+} was examined by comparing the incremental changes induced in their values by the removal of serosal Na^+ and the addition of amiloride (insert in Fig. 8). It appears that amiloride induces similar changes in the current and tracer flux. The data points could be fitted to a straight line with a slope of 1.06 ± 0.2, intercept of 0.6 ± 2.0 nmol/min and a correlation coefficient of 0.95. Thus, the diuretic-induced decrease in I_{sc} is fully accounted for by an inhibition of a mucosal-to-serosal Na^+ flux. On the other hand, the increase in I_{sc} induced by substituting serosal Na^+ by $NMDG^+$ was much larger than the increase observed in J_{22Na^+}, and the data could not be fitted to a straight line ($R < 0.75$). This observation may be explained by assuming that the serosal substitution not only activates an amiloride-blockable Na^+ flux, but also inhibits other basolateral or paracellular electroneutral Na^+ exchange processes.

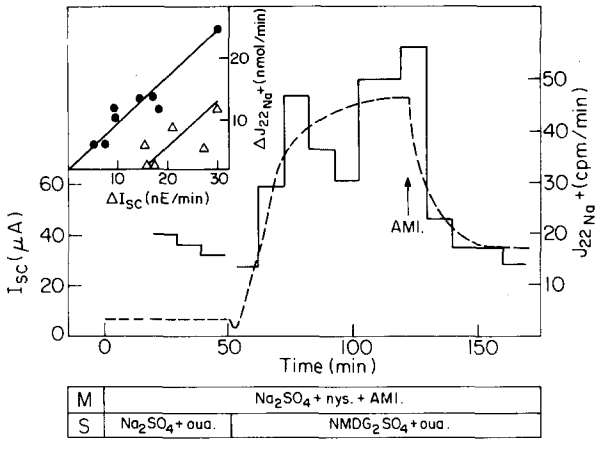

Fig. 8. Mucosal-to-serosal $^{22}Na^+$ fluxes. I_{sc} (dashed line) and J_{22Na^+} (stepwise line) were measured in parallel for the different mucosal and serosal solutions listed at the bottom of the Figure. Amiloride (110 μM) was added to the serosal solution at the time indicated by an arrow. Insert: The incremental change in short-circuit current (ΔI_{sc}) and mucosal-to-serosal $^{22}Na^+$ flux (ΔJ_{22Na^+}) are compared. (△) Increases in I_{sc} and J_{22Na^+} were induced by substituting the serosal Na^+ by $NMDG^+$. (●) Decreases in I_{sc} and J_{22Na^+} were induced by adding amiloride to the serosal solution ($(NMDG)_2SO_4$ Ringer's)

Potential Sources of the Amiloride-Blockable Current and Conductance

Although the experiments in Figs. 2–8 were all carried out in the presence of at least 2 mM ouabain, the possibility remains that the basolateral, amiloride-sensitive current observed is mediated by the Na^+ pump. According to this interpretation, the slow appearance of positive current upon the substitution of serosal Na^+ by K^+ reflects the release of the Na^+,K^+-ATPase from the ouabain block, in-

Table 2. Effects of various mucosal and serosal substitutions and additions on I_{sc} and G[a]

Treatment	Initial values		Fractional change		n
	I_{sc} (μA/cm^2)	G (mS/cm^2)	I_{sc}	G	
A. Adding 200 μM $NaVO_3$ to the serosal medium	14.4 ± 1.4	1.13 ± 0.14	0.97 ± 0.17	1.02 ± 0.10	5
B. Substituting Na_m^+ by Li^+	13.0 ± 1.13	0.76 ± 0.04	0.85 ± 0.10	0.90 ± 0.10	3
C. Substituting K_s^+ by $NMDG^+$	24.2 ± 1.20	0.91 ± 0.25	0.98 ± 0.13	0.81 ± 0.12	4
D. Lowering Ca_s^{2+} to 50 μM	11.2 ± 1.10	0.78 ± 0.20	1.02 ± 0.02	1.0 ± 0.05	4
Lowering Ca_m^{2+} to <10 μM	25.2 ± 1.43	1.25 ± 1.33	1.01 ± 0.01	0.99 ± 0.01	4

[a] Bladders were incubated in mucosal Na_2SO_4 Ringer's containing 30 μg/ml nystatin + 80 μM amiloride and serosal K_2SO_4 Ringer's containing 5 mM ouabain. After a steady current and conductance (denoted initial values) were established, the following additions or substitutions were made:
A. 200 μM $NaVO_3$ were added to the serosal medium.
B. The mucosal solution was substituted by a Li_2SO_4-Ringer's (plus nystatin and amiloride).
C. The serosal solution was substituted by a $(NMDG)_2SO_4$-Ringer's (plus ouabain).
D. The serosal Ringer's was replaced by a similar solution whose Ca^{2+} activity is 50 μM (upper row). In another set of experiments the mucosal Ringer's was replaced by a similar solution to which no Ca^{2+} was added ($Ca_m^{2+} < 10$ μM).
Data are expressed as fractional change in I_{sc} and G (mean ± SEM) and n is the number of experiments.

duced either by the high serosal K^+ and/or the high cellular Na^+ (Glynn & Karlish, 1975). Such a pump-mediated current could be amiloride sensitive too, although diuretic concentrations higher than 100 μM are normally required in order to inhibit the Na^+,K^+-ATPase (Ebel et al., 1972; Soltoff & Mandel, 1983). This simple explanation seems, however, to be excluded by at least six findings:

1. Ouabain, at a concentration of 1 mM, was shown to inhibit the pump-mediated I_{sc} in toad bladder, even when Na_s^+ was replaced by K^+ and Na_c^+ was high (Palmer et al., 1980).

2. Vanadate, which unlike ouabain should effectively inhibit the Na^+,K^+-ATPase even at high K^+ activities (Cantley & Josephson, 1978), did not influence the observed current or conductance (Table 2A).

3. Substituting the mucosal Na^+ by Li^+ which is poorly pumped by the Na^+,K^+-ATPase (Dunham & Senyk, 1977) did not reduce the basolateral current or the ability of amiloride to block it (Table 2B).

4. Replacing the serosal Na^+ by $NMDG^+$, an ion which presumably does not interact with the Na^+,K^+-ATPase, produced similar increases in I_{sc} and G (Table 1). In addition the substitution of serosal K^+ by $NMDG^+$ did not lower the amiloride-blockable flux (Table 2C).

5. Association and dissociation of ouabain from the Na^+,K^+-ATPase will not explain the difference in rates observed between the first and second removal of serosal Na^+ (i.e. periods VI and VIII, Fig. 4, periods II and VI, Fig. 5).

6. The electric properties of this pathway and in particular its high electrical conductance are different from those expected in the case of a pump-mediated current.

Thus the observation that the current and conductance under question are insensitive to ouabain, vanadate, mucosal substitution of Na^+ by Li^+, and serosal substitution of K^+ by $NMDG^+$, but are blocked by amiloride at concentrations which are well below those reported to inhibit the Na^+,K^+-ATPase, make the possibility that they are pump-mediated very unlikely.

The only electrogenic Na^+-transporting pathway identified in the basolateral membrane of this epithelium (besides the Na^+,K^+-ATPase) is a Na^+/Ca^{2+} exchanger (Chase & Al-Awqati, 1981), and such an antiport could also be amiloride sensitive (Jurkowitz et al., 1982; Smith et al., 1982). To examine whether Na^+/Ca^{2+} exchange is responsible for the amiloride-blockable I_{sc} observed in the above experiments, we looked at the potential effects of the removal of mucosal Ca^{2+} and the lowering of its serosal activity from 1 mM to 50 μM.[1] As seen from Table 2D the large decrease in mucosal or serosal Ca^{2+} activity had no effect on I_{sc} and G. Similar lowering of serosal Ca^{2+} was shown to inhibit Na^+/Ca^{2+} exchange (Garty & Lindemann, 1984). The insensitivity of the amiloride-blockable I_{sc} to serosal Ca^{2+} excludes the possibility that basolateral Na^+/Ca^{2+} exchange is the source of this flux. Lack of mucosal Ca^{2+} effects indicate that active nystatin-mediated transepithelial Ca^{2+} transport cannot account for the data either.

The remaining possibility is therefore that under the experimental conditions of the present study

[1] Lower serosal Ca^{2+} activities were avoided in order to prevent the increase of paracellular conductance (Hays, Singer & Malamed, 1965).

a yet unknown Na^+ transporter is expressed in the basolateral membrane of toad bladder. The observed changes in current and conductance may in principle be accounted for either by assuming that this transporter is a rheogenic Na^+ pathway (channel or carrier), or an electroneutral Na^+/cation exchanger in conjunction with a conductive pathway for the exchanged cation. In the second case, the Na^+ gradient will drive a net transepithelial Na^+ flux and recycling of the exchanged cation across the basolateral membrane. The resulting steady-state accumulation of this cation in the cell could account for the observed increase in G. Since the amiloride-blockable I_{sc} is insensitive to the principal basolateral cation (K^+, Cs^+ or $NMDG^+$) and to Ca^{2+}, the only candidate for such an exchanger is a Na^+/H^+ antiport. Evidence for the existence of a Na^+/H^+ antiport in toad bladder plasma membrane was presented by LaBelle and Eaton (1983) and this transporter is, of course, amiloride blockable. However, it is hard to see how an increase in the cellular H^+ activity will account for the large conductance change observed.[2] Thus, although an electroneutral Na^+/H^+ exchange plus high H^+ conductance may in principle explain the above data, the conductance changes observed are far too large for such a mechanism. In addition, if the Na^+ flux and I_{sc} are mediated by different pathways the removal of Na^+ from the serosal compartment should induce a mucosal-to-serosal Na^+ flux which precedes the increase of I_{sc}. The tracer flux measurements in Fig. 8, although averaging J_{22Na^+} over 10-min intervals, clearly indicate that, if anything, changes in I_{sc} precede the Na^+ increase in J_{22Na^+}.

Finally, we examined the possibility that the development of amiloride-sensitive I_{sc} and G has to do with the fact that in our experiments Cl^- was replaced by SO_4^{2-}. The substitution of serosal Cl^- by gluconate was shown to cause changes in cell volume (Lewis et al., 1985) and such shrinkage might be relevant to the above observations. As discussed above and in previous publications (Eaton et al., 1982; Garty, 1984*a*), the nystatin-treated bladders are often unstable in Cl^- solutions. Nevertheless, in several experiments it was possible to demonstrate the development of amiloride-blockable I_{sc} in Cl^--Ringer's, too (Fig. 9). Thus it seems that the substitution of mucosal and/or serosal Cl^- by SO_4^{2-} is not a prerequisite for the expression of this new pathway. However, the typically high Cl^- conductance prevented detailed analysis of the amiloride-sensitive I_{sc} and G in Cl^- solutions.

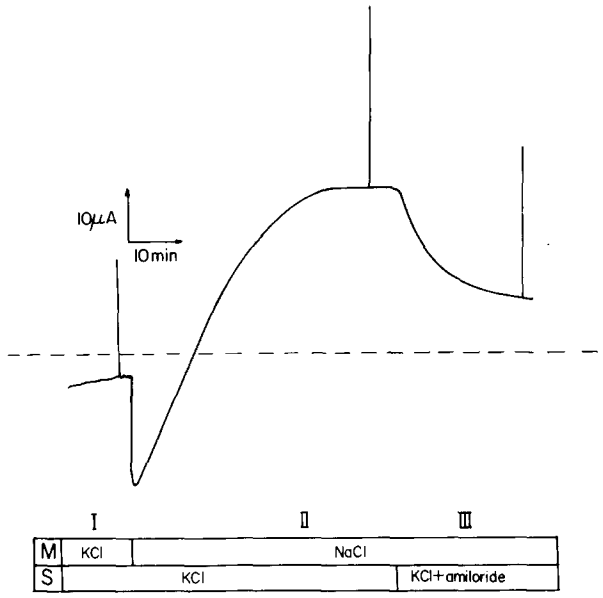

Fig. 9. Effects of serosal Na^+-free solution and amiloride on I_{sc} and G measured in Cl^--Ringer's solution. At the initial stage (period I) both mucosal and serosal solutions were KCl-Ringer's (85 mM K^+). In period II the mucosal K^+ was replaced by the same concentration of Na^+, and in period III amiloride (80 μM) was added to the serosal compartment. All mucosal and serosal solutions contained 30 μg/ml nystatin + 80 μM amiloride and 5 mM ouabain, respectively

The Influence of Mucosal and Serosal Na^+ Activities

The Na^+ dependence of the basolateral amiloride-blockable pathway was evaluated by measuring changes in I_{sc} and G induced by different mucosal and serosal Na^+ activities. In the first set of experiments the serosal Na^+ activity was zero and the mucosal Na^+ activity was varied from 0 to 60 mM by perfusing the apical surface with different $(NMDG)_2$

[2] If the potential across the basolateral membrane is clamped to zero and H^+ flow can be described by the constant field equation then:

$$G_H = \frac{F^2}{RT} P_H(H_s^+ + H_c^+)/2$$

when G_H and P_H are the proton conductance and permeability, and H_s^+ and H_c^+ are the H^+ activities in the serosal and cellular compartments, respectively (*cf.* Lindemann, 1977). The change in proton conductance ΔG_H induced by a given change in the cellular H^+ activity ΔH_c^+ (at constant P_H and H_s) will be:

$$\Delta G_H = \frac{1}{2}\frac{F^2}{RT} P_H \cdot \Delta H_c^+.$$

Substituting the observed amiloride-blockable change of the transepithelial conductance (~0.25 mS/cm²) in the above equation will yield a value of 1.3×10^{-7} M · cm · sec^{-1} for $P_H \cdot \Delta H_c^+$. Thus, one would have to assume an exceptionally high value for P_H and the Na^+-induced acidification (e.g. $P_H = 10^{-4}$ cm · sec^{-1} and ΔH_c = 1 mM) in order to account for the observed conductance change.

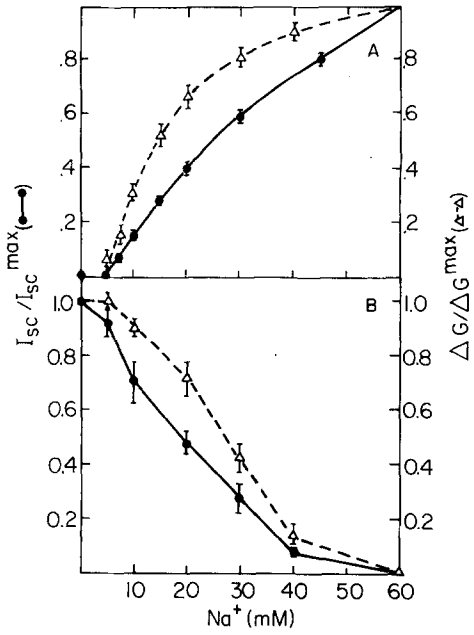

Fig. 10. Na^+ dependence of the amiloride-blockable basolateral pathway. I_{sc} and G were measured in bladders exposed to: (*A*) Serosal $(NMDG)_2SO_4$ Ringer's and various mucosal $Na_2SO_4/(NMDG)_2SO_4$ mixtures (Na_m^+ varying from 0 to 60 mM). (*B*) Mucosal Na_2SO_4 Ringer's and various serosal $Na_2SO_4/(NMDG)_2SO_4$ mixtures (Na_s^+ varying from 0 to 60 mM). All mucosal and serosal solutions included nystatin + amiloride and ouabain, respectively. I_{sc}^{max} is the current for $Na_m^+ = 60$ and $Na_s^+ = 0$ mM. ΔG^{max} is the difference in conductance between $Na_m^+ = 60$ mM and $Na_m^+ = 0$ (*A*) or between $Na_s^+ = 0$ and $Na_s^+ = 60$ mM (*B*). Data are expressed as fractional changes in current (I_{sc}/I_{sc}^{max}) and fractional Na^+-induced change in conductance ($\Delta G/\Delta G^{max}$) as function of Na_m^+ and Na_s^+. Means ± SEM of three experiments are presented. The initial currents and conductances were: (*A*) 1.0 ± 0.5 μA/cm² and 0.17 ± 0.07 mS/cm². (*B*) 13.0 ± 0.4 μA/cm² and 0.7 ± 0.1 mS/cm²

SO_4/Na_2SO_4 mixtures (Fig. 10*A*). Both I_{sc} and G increased linearly with Na_m^+ at low Na^+ activity, and seem to approach saturation at higher concentrations. However, the fractional current and conductance changes extrapolated to zero for Na_m^+ of about 5 mM. Thus, it seems that the basolateral pathway is operative only above a threshold mucosal Na^+ activity of about 5 mM; i.e., it requires the presence of Na^+ in the cell. Next we studied the influence of different serosal Na^+ activities in bladders maintained at constant (maximal) Na_m^+. Increasing Na_s^+ from 0 to 60 mM by incubating the serosal compartment with different $Na_2SO_4/(NMDG)_2SO_4$ mixtures led to a gradual decrease in I_{sc}/I_{sc}^{max} and $\Delta G/\Delta G^{max}$ from 1 to zero (Fig. 10*B*). Similar and even steeper changes in I_{sc} and G were obtained if K^+ or Cs^+ were used to substitute for Na_s^+ rather than $NMDG^+$. A fall in I_{sc} is expected from the decrease in the Na^+ driving force. However, a decrease in G upon the substitution of an impermeable ion ($NMDG^+$) by the transported ion (Na^+) is surprising. A possible explanation for this behavior is that high Na_s^+ can block the basolateral amiloride-sensitive pathway and therefore lower the membrane conductance. This possibility was further explored by measuring admittance spectra at several serosal Na^+ activities (Fig. 11). The increase in Na_s^+ was found to decrease the basolateral admittance in a complex way and reductions in G_1, ΔG and λ were observed. Na_s^+ effects on the admittance spectra are similar to changes induced by amiloride (Fig. 7*B*) suggesting that serosal Na^+ does indeed block the Na^+ pathway.

Discussion

The present study describes measurements of the transepithelial current, conductance, admittance and $^{22}Na^+$ fluxes in bladders whose apical membrane was shunted by the nonspecific cationic ionophore nystatin. Incorporating the ionophore into the apical membrane lowered its resistance to negligible values but had only a minor effect on the basolateral resistance. Thus, the nystatin-treated tissue could be considered to be a "single-barrier preparation" and used to study the basolateral characteristics by transepithelial measurements. When the serosal Na^+ activity of a ouabain-blocked bladder was lowered to zero a large, unexpected, positive current and an increase in the transepithelial conductance were obtained. This current is apparently a mucosal-to-serosal Na^+ flux. It was proportionate to the mucosal Na^+ (or Li^+) activity, insensitive to the principal serosal cation (K^+, Cs^+ or $NMDG^+$), and blocked by serosal amiloride (K_i = 13.1 μM). This Na^+ flux was mainly studied in SO_4^{2-} solutions but could also be demonstrated in Cl^- Ringer's. Possible explanations for this phenomenon such as ouabain-insensitive Na^+ pumping, electrogenic Na^+/Ca^{2+} exchange and an electroneutral Na^+/H^+ exchange in parallel with a H^+ conductive pathway, were tested and excluded. The high Na^+/K^+ selectivity ratio observed, and the sensitivity to amiloride also exclude the possibility that the current is mediated by nystatin channels which invade the basolateral membrane, or by a paracellular pathway. One therefore has to assume that under our experimental conditions a yet unknown Na^+ transporter is expressed in the basolateral membrane of toad bladder and becomes the major conductive pathway in this membrane. This transporter is Na^+ specific, electrogenic, and can be blocked either by serosal amiloride or high Na_s^+. It also shows a voltage-dependent relaxation with characteristic rates of 10 to 20 rad sec^{-1} at 0 mV.

Of potential significance is the observation that

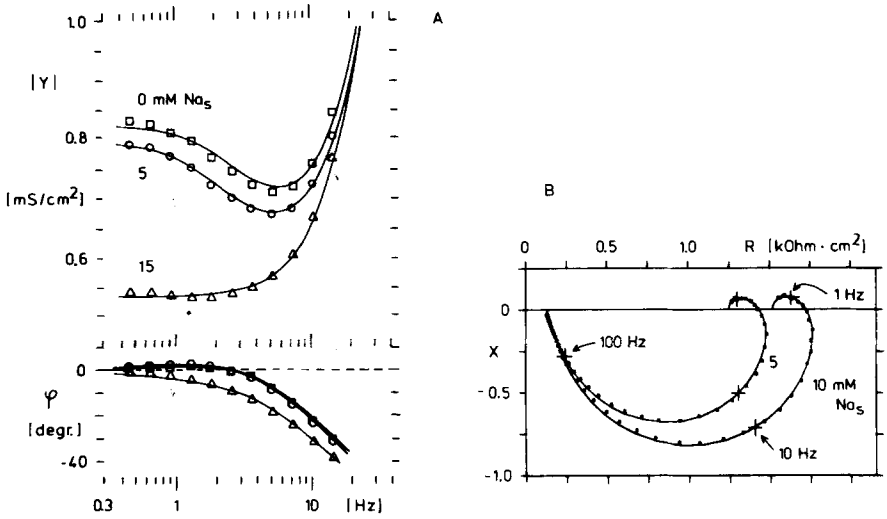

Fig. 11. Admittance spectra at different Na_s^+ activities. (*A*) Steady-state admittance magnitude ($|Y|$, upper trace) and phase angle (ρ, lower trace) were measured for serosal Na^+ activities of 0 (□), 5 mM (○) and 15 mM (△) at a holding voltage of 0 mV. The continuous lines are the best fits by the model discussed in the Appendix. Increasing Na_s from 0 to 15 mM lowered both G_1 and ΔG. (*B*) Nyquist diagrams measured for Na_s of 5 and 10 mM. In contrast to Fig. 2*C* both diagrams show pronounced inductive behavior at low frequencies. The inductive behavior is also evidenced by the positive phase angles on panel *A* and attributed to the basolateral Na conductance. At high frequencies the abscissa is approached at an angle smaller than 90 degrees. This may be indicative of collapsed lateral intercellular spaces (Clausen et al., 1979). Electron-microscopic controls have shown that the cells are shrunken in period VI of Fig. 4

the new basolateral Na^+ conductance develops quite slowly and reaches its maximal value at least 30 min after the first substitution of Na_s by K^+, Cs^+ or $NMDG^+$. Subsequent substitutions produce much faster current changes. This finding may suggest that establishing an inverse Na^+ gradient across the basolateral membrane triggers cellular events which slowly change its permeability characteristics. This hypothetical event seems, however, to be insensitive to cytoskeleton disrupting agents.

Not less surprising than the appearance of a basolateral amiloride-sensitive Na^+ transporter is the fact that in Cl^--free solutions it accounts for most of the basolateral conductance; i.e., the membrane is more permeable to Na^+ than to K^+. Thus, the events induced by the removal of serosal Na^+ may involve the inactivation of K^+ channels as well as the activation of Na^+ transporters. It is also possible that the ionic substitution triggers the conversion of K^+-specific channels to Na^+-conducting channels.

Effects of amiloride other than blocking the apical channels were in fact reported for toad bladder in the past. Bentley (1968) observed that adding amiloride to the serosal side of mounted toad bladder lowers I_{sc}. The diuretic concentrations required to produce this inhibition were much higher than those required to inhibit I_{sc} from the apical side. Measurements in toad bladder vesicles (Garty, 1984*b*; Garty & Asher, 1985) provided evidence for the existence of a Na^+-conductive pathway, different from the predominant apical channels, which can be blocked only by a relatively high concentration of amiloride ($K_i \sim 2$ to 6 μM). It is possible that the $^{22}Na^+$ flux with low affinity to amiloride measured in vesicles, is mediated by the conductive pathway studied in the present paper.

The current data do not provide any clues to the mechanism by which the basolateral membrane changes its permeability characteristics or the physiological significance (if any) of this process. These questions await future studies. It appears, however, that after decades of intensive research, the toad bladder epithelium still holds surprises.

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Appendix

The admittance spectra measured during the evolution of the basolateral amiloride-sensitive pathway (e.g. Fig. 4) were modelled using the equivalent network shown in Fig. 12. The pathway induced by the serosal substitution is described as a conductance (ΔG) in series with an inductance ($L = \Delta G^{-1}\lambda^{-1}$). Such an element will give rise to a delayed increase of conductance with voltage (V).

Suppose that the inward Na^+ current (I) flows via a basolateral ion channel characterized by density N_o and a single-channel current i. Thus

$$I(V, t) = i(V) \cdot N_o(V, t). \quad (1)$$

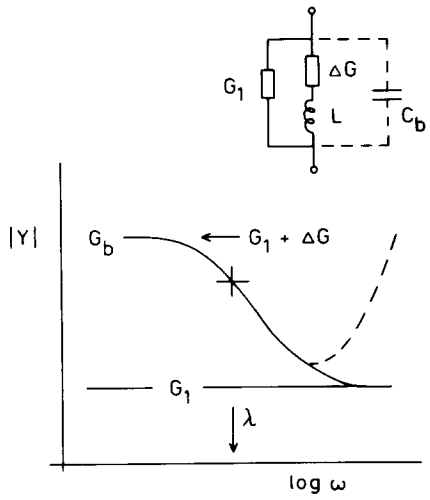

Fig. 12. A suggested equivalent network for the basolateral membrane in nystatin-treated ouabain-blocked bladder. The membrane conductance (G_b) is modelled as a frequency-independent term (G_1) plus another conductance (ΔG) in series with a large inductance (L). The expected admittance spectrum is shown below. Due to the membrane capacitance (C_b) the total admittance will increase at high frequencies (dashed line)

In response to a change in voltage, i will change instantly and only $N_0(t)$ will account for the relaxation. The rate will in simple cases be of pseudo-first order:

$$dN_0/dt = \lambda \cdot (N_0^*(V) - N_0(t)) \tag{2}$$

where N_0^* is the equilibrium value of N_0 at any V, and λ the relaxation rate coefficient in rad sec^{-1}. N_0^* will and λ may change instantly with voltage, but both are considered to be constant for the small sinusoidal voltage excursions used in the impedance measurements. Following the derivation of Mauro et al. (1970) and Warncke and Lindemann (1985), we obtain for the relaxation amplitude

$$\Delta G = i \cdot dN_0^*/dV \tag{3}$$

with dN_0^*/dV being positive, and for the conductance at high frequencies

$$G_1 = N_0^* \cdot di/dV. \tag{4}$$

The low-frequency conductance of the basolateral membrane will then be

$$G_b = (dI/dV)_{t=\infty} = N_0^* \cdot di/dV + i \cdot dN_0^*/dV = G_1 + \Delta G. \tag{5}$$

With dN_0^*/dV being positive, the delayed change of conductance with voltage is such that the conductance increases in response to a voltage change which makes the cellular potential more positive.

After elimination of capacitive effects from the data (dashed line in Fig. 12) the remaining admittance magnitude will be

$$|Y(\omega)| = \left[\frac{(G_1 + \Delta G)^2 + G_1^2\omega^2/\lambda^2}{1 + \omega^2/\lambda^2}\right]^{1/2} \tag{6}$$

where ($\omega = 2\pi f$) is the angular frequency. The resulting admittance spectrum is shown as an S-shaped curve in Fig. 12. Equation (6) was used to extract the values of G_1, ΔG and λ from the experimental data. For the curve labeled 15 min in Fig. 7*A* we obtained at a holding voltage of 0 mV: $G_1 = 0.6$ mS/cm^2, $\Delta G = 0.2$ mS/cm^2, $\lambda = 15$ rad sec^{-1}, $L = 330$ H, and $C_b = 6.2$ μF/cm^2.