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Current-Voltage Relations of the Basolateral Membrane in Tight Amphibian Epithelia: Use of Nystatin to Depolarize the Apical Membrane

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Summary. Exposure of the mucosal side of toad (*Bufo bufo*) urinary bladder and frog (*Rana ridibunda*) skin to the polyene ionophore nystatin, resulted in stable preparations in which the apical resistance was negligible compared to the basolateral resistance. The preparations support passive K currents in both directions and an amiloride-insensitive Na current in the apical-serosal direction which is blocked by ouabain. The nystatin-treated toad bladder was used to study the electrical properties of the basolateral membrane by means of current-voltage curves recorded transepithelially. The K current showed strong rectification at cellular potentials negative with respect to the interstitial space. The ouabain-sensitive current increased with membrane voltage at negative voltages but saturated above +20 mV.

Key Words basolateral membrane · current-voltage relations · nystatin · potassium transport · tight epithelia

Introduction

According to the Koefoed-Johnsen and Ussing model (Koefoed-Johnsen & Ussing, 1958) amphibian epithelia like frog skin and toad urinary bladder are composed of two functional membranes in series: the sodium-specific apical membrane, and the basolateral membrane which contains the ouabain-blockable Na pump, and is also permeable to K and Cl (for review *see* Macknight, Di Bona & Leaf, 1980). Due to the relative simplicity of transepithelial measurements these systems were widely used to study Na transport and its regulation by hormones, as well as the physical properties of the various transport pathways.

A common problem in such studies is the difficulty to distinguish between effects on the apical membrane, basolateral membrane and tight junction. One possible approach to overcome this difficulty is to treat one of the membranes in series with an unspecific ionophore, assuming that in the ionophore-treated preparation the transepithelial current and resistance are governed by the other, unmodified membrane. Lichtenstein and Leaf (1965) and Sharp et al. (1966) used Amphotericin B to investigate the steps involved in stimulating Na transport in toad bladder by ADH and metabolic substrates, respectively. Nielsen (1977, 1979) examined the coupling between Na and K in filipin-treated frog skin. In a number of studies the polyene antibiotic nystatin was used as a tool to 'eliminate' the apical resistance in mammalian epithelia. Lewis et al. (1977) showed that mucosal addition of nystatin to rabbit urinary bladder increased several-fold the total conductance, but had no direct effect on the basolateral or junctional conductances. Active and passive Na fluxes were measured across the basolateral membrane of nystatin-treated rabbit urinary bladder by Eaton, Frace and Silverthorn (1982). Similar studies were performed in rabbit descending colon in which nystatin was used to measure the basolateral I–V curves (Wills, 1981; Wills et al., 1979).

In the present study we describe the electrical properties of the basolateral membrane in two amphibian epithelia: toad (*Bufo bufo*) bladder and frog (*Rana ridibunda*) skin. Unlike previous cases, steady nystatin-induced currents could be measured for extended periods of time. This was achieved by the use of chloride-free solutions both in the mucosal and serosal compartments. The high stability of the nystatin-treated epithelial enabled us to measure conveniently the effects of the internal ion composition on the basolateral membrane. Near instantaneous current-voltage relations were measured in nystatin-treated toad bladder under various conditions. The K-specific current showed strong rectification at cellular potentials negative with respect to the interstitial space, suggesting voltage-dependent gating of the basolateral K pathway. On the other hand, the ouabain-sensitive current was linearly dependent on the membrane potential in this range but appeared to saturate above +20 mV.

Abbreviations: G, transepithelial slope conductance; ΔG_{ATP}, free energy of ATP hydrolysis; I_{sc}, transepithelial short-circuit current; I–V curve, current-voltage relations; P_i, permeability of ion i; $V_{I=0}$, transepithelial open-circuit potential.

Materials and Methods

Animals

Toads (*Bufo bufo*) and frogs (*Rana ridibunda*) obtained from F. Stein, F.R.G., were kept at room temperature with free access to tap water. The animals were double-pithed and the epithelial tissue (urinary bladder or skin) was removed and mounted as a flat sheet (nominal surface area 3 cm^2) in a modified Ussing-type chamber. The serosal compartment of this chamber was an 18-ml volume open to the atmosphere and stirred by aeration. The mucosal compartment was of 1 ml volume through which solution was continuously flowing at a rate of 1 to 50 ml/min. For further details see Gebhardt and Lindemann (1974) and Palmer, Edelman and Lindemann (1980).

Solutions

Most of the experiments were carried out using two types of sulfate Ringer's. The first, denoted Na60 (60 mM Na activity), contained (in mM) 54.5 Na_2SO_4, 4 K_2HPO_4, 1 KH_2PO_4 and 1 Ca-gluconate (pH = 7.5). The second, denoted NaO (Na-free solution) had a similar composition to Na60 except that Na_2SO_4 was replaced by an equal amount of K_2SO_4. In addition 5 mM glucose were present in all serosal solutions and 80 μM amiloride and nystatin in all mucosal solutions.

In a few experiments various chloride Ringer's solutions were used. All solutions contained 1 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 3.5 mM K-phosphate buffer (pH = 7.5). In addition they contained one of the following mixtures: 1) 100 mM NaCl (Na-Ringer's); 2) 100 mM choline chloride (choline Ringer's); 3) 85 mM KCl + 15 mM choline chloride (K-Ringer's); 4) 85 mM KCl + 50 mM sucrose (K-sucrose-Ringer's). The sucrose used in the last Ringer's was required to prevent cell swelling when high K activity was applied on the serosal side (Palmer et al., 1980). As in the sulfate Ringer's amiloride + nystatin and glucose were added to mucosal and serosal Ringer's, respectively.

Nystatin (Sigma, 5400 units/mg) was added under vigorous stirring from a freshly made solution of 60 mg/ml in dimethylsulfoxide which was protected from light. The final concentration of this antibiotic in the mucosal compartment was 10 to 20 μg/ml (10.7 to 21.4 μM) for toad bladder, and 50 μg/ml for frog skin. Mucosal addition of dimethylsulfoxide alone (0.03%) had no effect on the electrical properties of these epithelial tissues. Amiloride was applied from an aqueous 8 mM solution. Ouabain (Sigma) was dissolved in the appropriate Ringer's to a final concentration of 5 mM.

Electrical Measurements

The mounted tissue was clamped to zero voltage and I_{sc} was continuously recorded. Electrical slope conductance (G) was measured by recording the current deflections in response to brief (1 sec) 10-mV displacements of the clamping potential. $V_{I=0}$ was measured by clamping the current to zero for a short period (~10 sec).

I–V curves were obtained by generating a stepwise voltage change and monitoring the corresponding currents. Two patterns of voltage change were applied. In the first the voltage was changed in 5-mV steps from −10 to +100 mV.[1] In the second, −5-mV steps from +10 to −100 mV were applied. The step duration was 20 msec, a period long enough to establish a steady current in bladders, but not in skin. This observation was frequently verified by displaying the current transients on an oscilloscope. In chloride Ringer's (Fig. 6) the measuring range was extended to ±150 mV. No long-term effects of the applied voltages on the electrical properties of the tissue could be detected; repeated measurements over a short period (< 5 min) yielded identical I–V curves.

Control of the clamping voltage, storage of the data and subtraction of I–V curves were done with a Data General Nova 1230 computer.

Statistics: Data are expressed as mean ± SEM

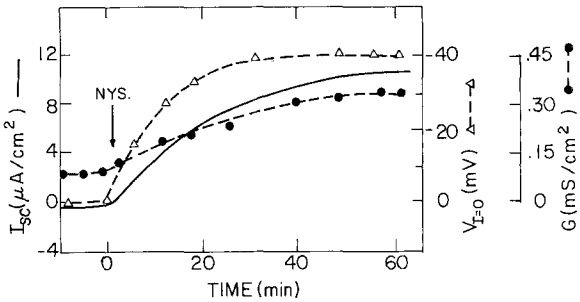

Fig. 1. Time course of nystatin action. A toad (*Bufo bufo*) bladder was pre-equilibrated in Na0 + 80 μM amiloride (mucosal) and Na60 (serosal). At the time indicated by arrow, 10 μg/ml nystatin (abbreviated NYS.) were added to the mucosal side. The changes in I_{sc} (—), $V_{I=0}$ (Δ--Δ) and G (●--●) with time were measured. *See* footnote for polarity definitions

Results

Nystatin Incorporation into the Apical Membrane

Figure 1 shows the increase in I_{sc}, G and $V_{I=0}$ which follow the application of the 10 μg/ml (54 units/ml) of nystatin to the solution perfusing the mucosal side of a mounted urinary bladder. The mucosal Ringer's in this experiment was Na0 and the serosal Ringer's was Na60. Under these conditions the cells are depleted of Na and no active transport takes place. Therefore, the observed current is the net result of passive K and Na fluxes driven by the concentration gradients. Since the basolateral membrane is essentially impermeable to Na (Macknight et al., 1980), it is likely that serosal-to-mucosal Na flux is limited to the paracellular shunt, while K flows mainly through the apical nystatin channels and the basolateral K-specific pathway. Usually a steady (maximal) potential was

[1] Polarity definitions: The mucosal potential is negative with respect to the serosal. The positive direction of I_{sc} is mucosa to serosa.

established ~60 min after the application of the ionophore. It could be maintained for at least one additional hour (*see below*).

In contrast to previous studies (Lewis et al., 1977; Russel, Eaton & Brodwick, 1977) prolonged exposure to nystatin (both in Na- and K-mucosal solutions) did not cause large changes in the transepithelial conductance nor cell swelling and tissue disintegration. This improvement is probably due to the use of sulfate Ringer's on both sides of the tissue. The absence of Cl in the mucosal Ringer's will prevent net salt uptake through the nystatin channels which would cause swelling, and the absence of Cl in the serosal Ringer's decreases the relative nystatin effect on the transepithelial conductance by increasing the basolateral resistance.

In 13 bladders treated as shown in Fig. 1 a steady potential of -37.3 ± 1.6 mV (mean ± SEM) was established. Substituting this value and the Na and K activities of the solutions used in the Goldman-Hodgkin-Katz equation assuming negligible permeability of sulfate, yields a ratio of $P_K/P_{Na} = 6.1$. If anions do have significant permeabilities, the observed potential will represent a higher K to Na permeability ratio. The high permeability ratio (which also involves the contribution of the nonspecific paracellular pathway) indicates that the nystatin-treated bladder in the absence of mucosal Na, is essentially a K-specific barrier; i.e., nystatin 'opens' the apical membrane to K without affecting the basolateral membrane permeability to Na significantly.

Incorporation of nystatin into the apical membrane of frog skin followed a similar time course. In this case higher nystatin concentrations were required (50 μg/ml), and the steady-state open-circuit potential was slightly lower: -32 ± 2 mV ($n = 7$).

A major question is whether the amount of nystatin incorporated into the apical membrane is sufficient to make its resistance and potential negligible compared to the basolateral one. Direct answer to this question will require the estimation of the voltage divider ratio as done for rabbit bladder (Lewis et al., 1977). We could not perform such measurements; however, the following findings suggest that in toad bladder, too, nystatin reduced the mucosal resistance and potential to negligible values. (1) I_{sc} and $V_{I=0}$ reached steady values although the mucosal surface was continuously perfused with fresh ionophore solution (Fig. 1). (2) The steady I_{sc} was not affected by raising the ionophore concentration from 10 to 20 μg/ml (not shown). (3) The transcellular potential, obtained from *I–V* curves corrected for the paracellular contribution, is equal to the K equilibrium potential (*see below*).

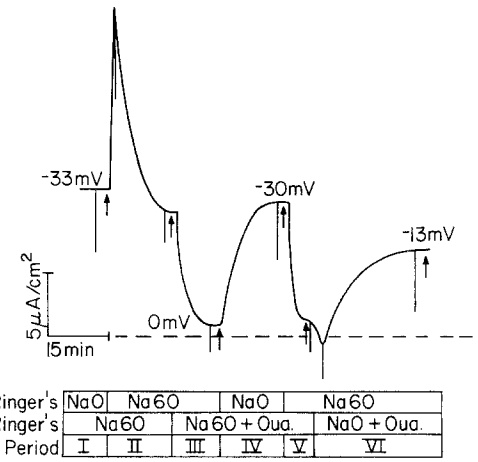

Fig. 2. Effect of medium composition on the transepithelial current and conductance in toad bladder. A bladder, pretreated for 1 hr with 10 μg/ml nystatin, was exposed to different mucosal and serosal solutions, listed in at the bottom of the Figure. All mucosal solutions contained in addition 10 μg/ml nystatin + 80 μM amiloride and the serosal solutions contained 5 mM glucose. I_{sc} was continuously measured except for brief interruptions, at the times marked by arrows, to record *I–V* curves. The vertical spikes are the current deflections in response to 10 mV displacements of the clamping voltage (used for slope conductance measurements). The dashed line is the zero current level

In conclusion, incubating the mucosal surface of toad bladder with nystatin in sulfate Ringer's results in a stable preparation in which the apical resistance and potential are likely to be negligibly small compared to the basolateral values. In such preparations transepithelial current-voltage measurements may be performed to study the electrical properties of the basolateral membrane.

Effects of Varying Ion Composition on the Electrical Parameters of Nystatin-Treated Epithelia

Figure 2 shows a typical protocol of changes made in the composition of the bathing solutions and their effects on I_{sc}, $V_{I=0}$ and G. The initial state (period I) was the one obtained 60 min after adding nystatin (Fig. 1), i.e. a steady nystatin-induced current measured in the presence of Na0 in the mucosal and Na60 in the serosal compartment. Substituting the mucosal K by Na gave rise to a transient increase in I_{sc} followed by a slow decay to a new steady value somewhat lower than the previous one (period II, Fig. 2). Now both bathing solutions are identical Na Ringer's; thus, the steady-state current measured under these conditions represents active transport only, and is the

sum of transcellular flows of Na (mucosal to serosal) and K (serosal to mucosal). Both ions are transported across the basolateral and apical barriers by the Na/K pump and the nystatin channels, respectively. The large, transient increase in current observed at the beginning of period II may be accounted for by a difference in the kinetics of the two processes involved, i.e. fast activation of the electrogenic pump by the entry of Na into the cell and slower dissipation of the K gradient across the apical membrane.

Next, the serosal medium was replaced by the same solution containing 5 mM ouabain (period III). As a result the current and potential dropped to nearly zero values. The slope conductance measured at this state will essentially represent the paracellular conductivity, but a parallel ouabain-insensitive sodium-conducting pathway in the basolateral membrane may also contribute to this value (Chase & Al-Awqati, 1981). When the mucosal Ringer's was changed back to Na0 (period IV), almost all the original current could be restored. In 13 bladders subjected to these maneuvers the average potential measured in this state was 95% ± 2.9% of the original value (measured during period I). The high degree of reversibility favored the comparison of electrical properties measured with the different ion compositions and indicates that; (1) the ionic substitutions did not affect the preparation's integrity, (2) there was no significant incorporation of nystatin into the basolateral membrane during the experiment.

Changing the mucosal solution back to Na60 (period V) decreased the current again to near zero values. Note that this process was much faster than the opposite one (period IV). The observed difference in rate can be explained as follows: in the presence of mucosal amiloride the exchange of cellular Na with mucosal K can occur only through the nystatin channels. This exchange will be slower than the replacement of cellular K by mucosal Na, where potassium can, in addition, leave the cell through the basolateral K-specific pathway.

The last change was to substitute the serosal Ringer's by ouabain containing Na0 Ringer's (period VI). This substitution gave rise to a small and transient negative current followed by a slowly developing positive current. The initial current change to negative values is in the direction expected for serosal-to-mucosal K flow. The subsequent positive current, however, is more difficult to interpret, and seems to indicate that under these conditions the basolateral membrane conducts more Na than K. Possible explanations for this unexpected finding are discussed below.

Table 1. Electrical parameters before and after nystatin treatment of toad bladder[a]

Solutions		I_{sc} ($\mu A/cm^2$)	G (mS/cm^2)	Number of bladders
Mucosal	Serosal			
Na20[b]	Na60	4.50 ± 0.33	0.10 ± 0.02	13
Na0 + ami.	Na60	0.30 ± 0.17	0.06 ± 0.01	13
Na0 + ami. + nys.	Na60	12.60 ± 1.15[c]	0.40 ± 0.03[c]	13
Na60 + ami. + nys.	Na60	7.00 ± 1.02	0.28 ± 0.02	9
Na60 + ami. + nys.	Na60 + oua.	2.00 ± 0.63	0.20 ± 0.02	11
Na60 + ami. + nys.	Na0 + oua.	6.70 ± 0.41	0.50 ± 0.06	7

[a] Data expressed as means ± SEM.
[b] A 2:1 mixture of Na0 and Na60.
[c] Measured after the nystatin-induced effects reached steady values.

Data from 13 toads were averaged, but only in 7 cases was the whole sequence shown in Fig. 2 completed.

The Table summarizes the effects of nystatin, ouabain and cation composition on I_{sc} and G. Nystatin increased the amiloride-insensitive conductance by 6.7-fold (from 0.06 mS/cm^2 to 0.4 mS/cm^2), but only 50% of the nystatin-induced conductivity reversed when K was eliminated from the mucosal compartment and the pump was blocked by ouabain (drop from 0.4 mS/cm^2 to 0.2 mS/cm^2). This can be accounted for either by assuming nystatin effects on the paracellular pathway, or by assuming significant ouabain-insensitive Na conductivity in the basolateral membrane. Blocking the pump with ouabain in the presence of Na60 on both sides of the tissue caused a 30% drop in G. This, however, does not necessarily mean that 30% of the current flows through the Na pump, and it can be accounted for by a decrease in the cellular K activity induced by ouabain (*see below*).

Similar experiments to those summarized in Fig. 2 and the Table were carried out with frog skin. A typical response pattern is shown in Fig. 3. The skin response differed from that of the urinary bladder in the following way: (1) No overshoot was observed when Na0 was replaced by Na60 on the mucosal side (period II). (2) When the preparation was exposed to Na60 (mucosal) and Na0 + ouabain (serosal), a steady negative current could be demonstrated (period IV). However, both current and conductance were substantially smaller than those observed for the reverse situation (pe-

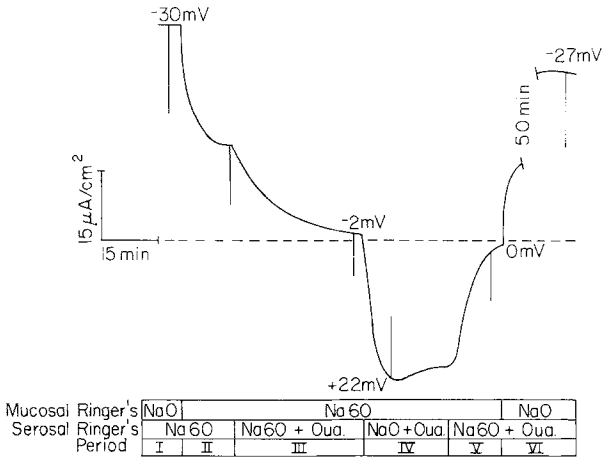

Fig. 3. Effect of medium composition on the transepithelial current and conductance in frog skin. Details are as in the legend to Fig. 2. Nystatin concentration was 50 μg/ml

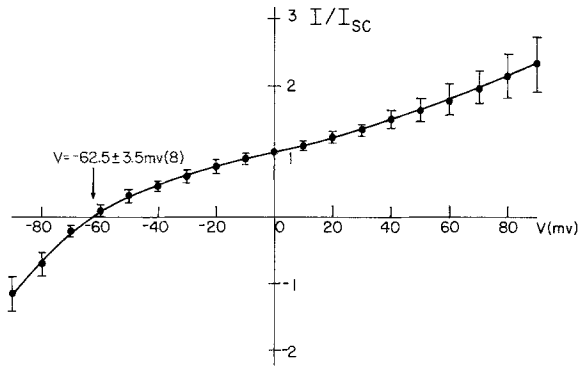

Fig. 4. *I–V* curve of the K-specific pathway. *I–V* curves were measured under different conditions and the difference, K-specific, curves were calculated as described in the text. Each current value was normalized to I_{sc} and data from 8 bladders were pooled together. The vertical bars are standard errors from the mean values of I/I_{sc}

riod I). (3) Exchange of cellular Na with mucosal K was very slow (period VI). Nevertheless, no significant loss of tissue integrity was detected and $V_{I=0}$ monitored at the end of period VI was only 10% lower than the original value measured more than 2 hours earlier.

Unfortunately, the nystatin-treated frog skin was not suitable for near instantaneous current-voltage measurements. After exposure of this preparation to nystatin the current response to voltage changes was relatively slow and did not reach steady values within 20 msec. For this reason the *I–V* curves described in the following section were measured in bladder preparations only.

Current-Voltage Relations of the Basolateral Membrane of Toad Bladder

Near instantaneous *I–V* curves were recorded in the nystatin-treated bladders as described under Materials and Methods. The measurements were done in the various steady states marked in Fig. 2 by arrows. The curves measured in the presence of Na0 mucosal and Na60 serosal (period I or in the end of period IV) will essentially represent current through the K-specific basolateral pathway and those measured in the presence of Na60 on both sides (in the end of period II) are expected to reflect current through the Na pump. However, both curves contain contributions from the paracellular shunt and possibly other, cellular pathways. In order to correct for such contributions we subtracted from each measurement a reference curve measured in Na60 plus ouabain (end of period III or V) assuming that it represents the contribution of all transepithelial pathways besides the K-specific basolateral pathway, and the Na pump. The validity of this assumption is discussed below.

Figure 4 shows the mean I_K-V (K-specific) curve calculated as the difference between two measurements taken either immediately before and in the end of period V, or in the end of periods I and III (Fig. 2). For positive and small negative potentials the current appeared to be a linear function of the transepithelial potential but strong rectification is evident for $V < -50$ mV. According to this curve the mean reverse potential of the K-specific current (i.e. the potential value at which the measured current reverses its sign) is -62.5 ± 1.3 mV (8) (mean ±SEM). This value is in good agreement with the transepithelial K equilibrium potential of -64.8 mV (K activities of 65 and 5 mM in the mucosal and serosal sides, respectively). The close resemblance between the measured reverse potential and the K equilibrium potential suggests that the shunt subtraction introduced in calculating this curve is probably correct and only a K-specific pathway contributes to the difference curve.

The change in slope conductance obtained for large negative potential values is in the opposite direction than expected according to the constant field equation (*see below*) and can be accounted for by one of two types of behavior: (1) voltage-dependent gating of the K-conducting pathway, (2) asymmetry of this pathway, i.e. it is more permeable to potassium flowing into the cell (negative current). To discriminate between these possibilities, we changed concentrations such that the re-

verse potential assumed opposite polarities and tried to see whether this will shift the break-point of the I–V curve.

At high cellular Na the expected dominance of serosal-to-mucosal passive K current could not be demonstrated in toad bladder (period VI, Fig. 2). We were, however, able to measure such a current when the mucosal Na Ringer's was replaced by a Na-free choline solution. The experimental protocol and the results obtained are summarized in Fig. 5. Since choline sulfate was not available, this rather short experiment, was done in chloride solutions.

When the mucosal surface was equilibrated with K-Ringer's containing nystatin, and the serosal compartment with Na-Ringer's + ouabain, a fairly steady positive current was recorded (mucosal-to-serosal K flow, period I, Fig. 5). Substituting the mucosal K by choline produced a sharp drop of current and conductance (period II, Fig. 5). The small negative current observed at the end of this period can be accounted for by serosal-to-mucosal Na flow through the paracellular pathway. From this current and the associated conductance, one can assess the tightness of the nystatin-treated bladder. In a number of instances prolonged exposure to nystatin in chloride Ringer's produced a leaky preparation characterized by much larger negative current and practically no choline-induced change of conductance.

In the next step, the serosal Na was substituted by K (period III, Fig. 5). As a result a steady negative current, whose magnitude is comparable to the initial I_{sc}, developed. This current is apparently due to serosal-to-mucosal K flow. The above protocol enabled us to compare I_K-V curves measured for two equal, oppositely oriented, gradients of K. I–V curves were recorded in the 3 steady states marked in Fig. 5 by arrows. The middle curve (choline mucosal, Na serosal) was assumed to represent the paracellular I–V curve and was subtracted from the other two in order to calculate the K-specific relations. The difference curves obtained for a single but typical experiment are shown in Fig. 6. The two curves appeared to be parallel vertically displaced lines, and exhibit similar rectification for large negative potential values. This observation supports the possibility of voltage-dependent gating and argues against possible asymmetry of P_K (in the latter case the I_K-V curve measured in period III should have exhibited decreased slope for $V > +100$ mV). Again the reverse potentials obtained after subtracting the reference curve (+100 and −85 mV) are in fairly good agreement with the K equilibrium potential

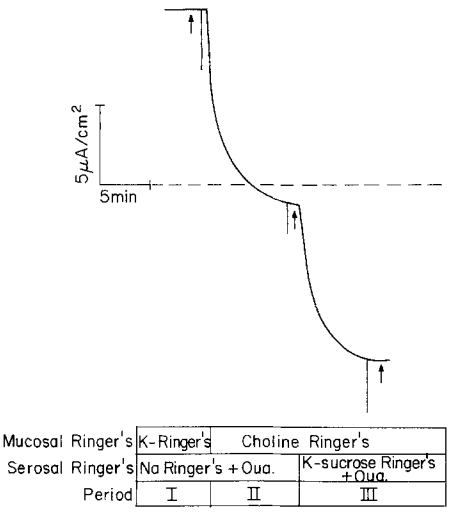

Fig. 5. Effects of various chloride Ringer's on the current and conductance of nystatin-treated bladder. The bladder was preincubated for one hour in KCl Ringer's containing nystatin (10 μg/ml) and amiloride (80 μM) in the mucosal side, and NaCl Ringer's + ouabain (5 mM) in the serosal side. Various substitutions, summarized in the bottom of the Figure, were made. All mucosal and serosal solutions contained nystatin + amiloride and ouabain, respecitively. The dashed line is the zero current level

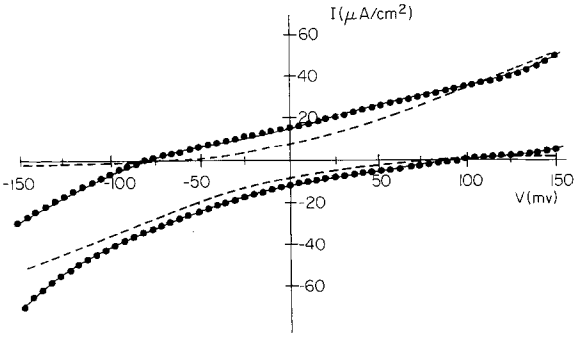

Fig. 6. I_K–V curves in chloride Ringer's. Two K-specific I–V curves, calculated as described in the text from measurements done during the experiment shown in Fig. 5, are plotted. The dashed lines are the K-specific I–V relations predicted by the constant field equation for $P_K = 1 \times 10^{-6}$ cm/sec and the K concentrations used in this experiment

(±81.4 mV, calculated for K activities of 88.5 and 3.5 mM). This reasonably good fit suggests that the correction made for the paracellular pathway is sufficiently accurate. The dashed lines in Fig. 6 are theoretical calculations of the K-specific I–V curves based on the constant field equation (Goldman, 1943), assuming a value of 1×10^{-6} cm/sec for P_K. The two experimental lines clearly deviate from I–V relations predicted for simple diffusion in a constant electric field, and this deviation is particularly large at high negative potentials.

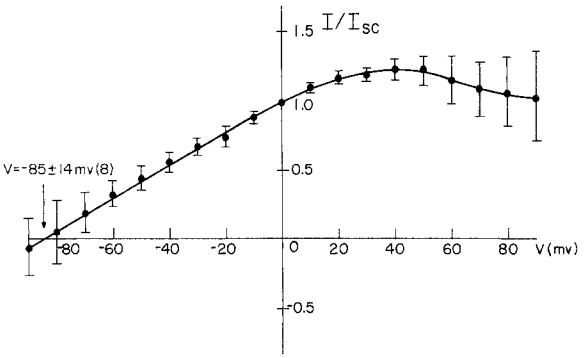

Fig. 7. Current-voltage relations of the ouabain-sensitive pathway. A difference curve was calculated for 8 bladders as described in the text; current values were normalized to I_{sc}. The vertical bars are standard errors from the mean values of I/I_{sc}

In an attempt to measure the current-voltage relations of the Na pump we calculated the ouabain-dependent line by subtracting the I–V curve measured at the end of period III (Fig. 2) from the one obtained immediately before the application of ouabain (end of period II, Fig. 2). Since in the two states identical solutions are used there is little doubt that measurements at the end of period III represent the paracellular contribution to the curve obtained at the end of period II. However, the calculated curve will represent the 'true' pump I–V curve only if the intracellular ion activities are the same in the two states, i.e. K and Na fluxes, though the nystatin channels, are high enough to 'clamp' the cellular activities of these ions to the mucosal values, irrespective of the pump activity.

The ouabain-dependent current, shown in Fig. 7, appeared to be independent of the transmembrane potential for $V > 20$ mV and decreased linearly for smaller values of V. The experimentally measured reverse potential is -85 ± 14 mV (8). Voltage-independent current is expected if the Na pump is a "constant current source" with nearly infinite resistance. This independence was experimentally observed for the giant neuron of the mollusc *Anisodoris* (Marmor, 1971). In our case, however, the voltage-independent range was observed for higher, "non physiologic," potential values. The mean reverse potential obtained, does not support the assumption that the above calculated curve is the Na pump I–V curve either. For equal cellular and serosal activities of K and Na and fully coupled system, the pump reverse potential should be equal to $\frac{1}{F}\Delta G_{ATP}$ (*cf.* Eq. 10 in Chapman and Johnson, 1978). Values of ΔG_{ATP} were estimated in different cells (e.g. Meer et al., 1978; Warncke & Slayman, 1980; Williamson et al., 1981; Erecińska & Wilson, 1982) and a reasonable number would be -11 kcal/mole, i.e. a predicted reverse potential of -477 mV for the isolated Na pump. The large difference between the predicted and experimental values of the reverse potential shows that at least one of the above discussed assumptions is incorrect (*see below*).

Discussion

We exposed the mucosal surface of toad bladder and frog skin to the polyene ionophore nystatin in order to increase the conductance of the apical membrane and study some of the electrical properties of the basolateral membrane by means of transepithelial measurements. This method requires that the following conditions are met:

1. Nystatin effect must be large enough so that the resistance and steady-state potential of the apical membrane become negligible relative to those of the basolateral membrane.
2. Ionophore incorporation must be restricted to the apical membrane, leaving the basolateral membrane unmodified.
3. Tissue integrity must be maintained, paracellular pathway should be small and excessive cellular volume changes avoided.

Our preparations appear to fulfill these conditions. As discussed above, the data suggest that the apical conductance in the nystatin-treated bladder is much larger than the basolateral conductance. Since the ionophore has similar Na and K permeabilities (Lewis et al., 1977; Russell et al., 1977; Wills, 1981) the increase in conductance will also abolish the apical membrane potential. The P_K/P_{Na} ratio in nystatin-treated bladders was larger than 6, indicating that nystatin did not affect the basolateral membrane or the paracellular shunt to a large extent. This permeability ratio could be maintained for at least one hour, during which the mucosal and cellular ion compositions were changed several times. Thus, the ionophore, probably by virtue of its hydrophobicity, was essentially restricted to the apical membrane. A similar observation was made in yeast (Von Hedenstrom & Joffer, 1979) where the effect of nystatin was limited to the plasma membrane and mitochondrial function was not affected.

Unlike previous studies in tight epithelia, most of the above experiments were carried out in Cl-free solutions (both mucosal and serosal). The substitution of Cl by the impermeable ion SO_4 had two major advantages: (1) The absence of Cl in the apical solution prevented excessive salt move-

ment through the nystatin channels, known to induce cell bursting (Lewis et al., 1977; Russell et al., 1977). This is probably the reason for the high stability achieved in these preparations. Steady currents in a nystatin-treated bladder were also measured by Eaton et al. (1982). In this study Ca was eliminated from the mucosal Ringer's in addition to the substitution of Cl by SO_4. Russell et al. (1977) showed that neither Ca nor Mg can readily move through the nystatin channels. Yet, the possibility of small leak of Ca that will raise the cellular Ca activity above the physiological value should be borne in mind.

An alternative way to overcome the problem of cell bursting is to use a cation-specific ionophore such as gramicidin D (Wills, 1981; Lewis & Wills, 1982). In the *Bufo bufo* urinary bladder mucosal addition of 1 μM gramicidin D had no effect on the amiloride-insensitive conductance. This, however, could be due to the fact that the ionophore concentration applied on rabbit bladder was much higher than that used by us.

Serosal Cl-free Ringer's decreased the basolateral conductance and thus increased the relative contributions of the transport systems of interest (the Na pump and the K-specific pathway) to the total basolateral I–V curve. The high permeability of this membrane to Cl (Macknight et al., 1980), made it almost impossible to determine accurately the ouabain-sensitive I–V curve in the presence of high serosal Cl.

The need to carry out I–V measurements in SO_4 Ringer's forced us to prefer in this study *Bufo bufo* toads over the more commonly studied species *Bufo marinus*. We observed that substituting the serosal Cl by SO_4 in bladders originated from *Bufo marinus* (before the application of nystatin) caused a large increase in the paracellular conductance. This phenomenon was not observed in bladders removed from *Bufo bufo* toads or in frog skin.

The computation of basolateral I–V curves required the accurate determination of the paracellular I–V curves. We assumed that the measurements taken at the end of periods III and V of Fig. 2 reflect properties of the paracellular shunt plus those of cellular ouabain-insensitive Na pathways. This assumption is probably justified in constructing the ouabain-sensitive I–V curve, but is more problematic for the construction of the I_K-V curve, since the paracellular Na and K permeabilities might differ to some extent. However, the fact that the reverse potential of the computed I_K-V curve was in very good agreement with the expected K equilibrium potential indicates that in this case the subtraction is justified. Furthermore, we did not see consistent differences in the paracellular I–V curves measured prior to the addition of nystatin (in the presence of amilorde) with different mucosal Na concentrations. An alternative approach to obtain the paracellular I–V curve would be to use a specific blocker of the K pathway. Ba, known to block K channels in frog skin (Nagel, 1979) was precipitated by sulfate, and Cs which was reported to block apical K channels in frog skin (Zeiske & Van Driessche, 1979) had no effect in our preparation.

The K-specific I–V curves showed strong rectification at potentials more negative than -50 mV. The rectification did not shift with the reverse potential and, therefore, is probably based on voltage-controlled gating. It is interesting to note that the rectification occurs in the vicinity of the physiological basolateral potential (Fromter & Gebler, 1977; Helman & Fisher, 1977). Thus it may serve to limit cellular K loss in cases where the membrane potential deteriorates. This observation is specially interesting in the light of recent suggestions that the basolateral K conductance is subject to cellular regulation (Schultz, 1981; Davis & Finn, 1982). The ouabain-sensitive I–V curve had a reverse potential of -85 ± 14 mV. As discussed above this value is close to the basolateral membrane potential and much higher than the predicted reverse potential of the Na pump under normal conditions. This discrepancy can be accounted for in one of three ways: (1) The turnover rate of the pump is high enough to accumulate K in the cell although the apical P_K is high and K was removed from the mucosal medium. In this case the curve measured in the presence of ouabain will not account properly for the passive basolateral K fluxes, since it was measured for a lower cellular K activity. (2) The hydrolysis of ATP by the Na pump is not fully coupled to the active transport process and this system operates at an efficiency which is much lower than 100% (Kedem & Caplan, 1965). Passive ion leaks through the Na pump had been recently described in reconstituted vesicles (Karlish, Lieb & Stien, 1982). However, it is doubtful whether these small passive fluxes can account for the large discrepancy between the expected and observed reverse potentials. (3) In the nystatin-treated bladder the ATP/ADP ratio could be much lower than assumed (e.g. nystatin-induced impairment of mitochondrial function). Direct measurements of the cellular ATP, ADP and K activities at the different states will be required in order to distinguish between these possibilities. Nevertheless it is of interest to note that the ouabain-sensitive current was practically volt-

age independent above 20 mV. Errors in the ouabain-independent curve will be least likely to disturb the computed pump I–V curve in this range where the slope of the I_K–V curve is relatively small (Fig. 4).

A puzzling observation was the positive current developed after substituting the serosal Ringer's by Na0 + ouabain (period VI in Fig. 2). The same phenomenon was observed when chloride Ringer's was used (not shown), but not if mucosal Na was replaced by choline (Fig. 5). One possible explanation for this observation is that in the presence of high cellular Na and high serosal K, ouabain, although applied at very high concentration (5 mM), failed to block the Na pump. It is well known that a high external K activity can decrease the binding of ouabain to the Na, K-ATPase, and that such an effect is also induced by a high cellular Na activity (Glynn & Karlish, 1975). In the amphibian bladder, high serosal K alone did not prevent the binding of ouabain to the Na, K-ATPase (Palmer et al., 1980; H. Garty, *unpublished data*). However, the combination of high serosal K and high mucosal Na could bring about dissociation of ouabain from the pump. Alternatively, one may speculate that the unusual serosal and mucosal ion composition applied during this period decrease the basolateral value of P_K/P_{Na} by affecting other transport pathways besides the Na, K-ATPase.

In summary, equilibrating the mucosal surface of toad urinary bladder and frog skin, maintained in Cl-free solutions, with nystatin, enables us to examine the electrical properties of the basolateral membrane by means of transepithelial measurements. The high stability of the nystatin-treated preparations makes this method a convenient way to study effects of various reagents and ion compositions on the basolateral membrane.

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