Nystatin as a Probe for Investigating the Electrical Properties of a Tight Epithelium

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ABSTRACT We show how the antibiotic nystatin may be used in conjunction with microelectrodes to resolve transepithelial conductance G_t into its components: G_a, apical membrane conductance; G_{bl}, basolateral membrane conductance; and G_j, junctional conductance. Mucosal addition of nystatin to rabbit urinary bladder in Na^+-containing solutions caused G_t to increase severalfold to ca. 460 μmho/μF, and caused the transepithelial voltage V_t to approach +50 mV regardless of its initial value. From measurements of G_t and the voltage-divider ratio as a function of time after addition or removal of nystatin, values for G_a, G_{bl}, and G_j of untreated bladder could be obtained. Nystatin proved to have no direct effect on G_{bl} or G_j but to increase G_a by about two orders of magnitude, so that the basolateral membrane then provided almost all of the electrical resistance in the transcellular pathway. The nystatin channel in the apical membrane was more permeable to cations than to anions. The dose-response curve for nystatin had a slope of 4.6. Use of nystatin permitted assessment of whether microelectrode impalement introduced a significant shunt conductance into the untreated apical membrane, with the conclusion that such a shunt was negligible in the present experiments. Nystatin caused a hyperpolarization of the basolateral membrane potential in Na^+-containing solutions. This may indicate that the Na^+ pump in this membrane is electrogenic.

INTRODUCTION

Even the simplest epithelium consists of three types of membranes that generally differ in resistance and in relative permeability characteristics: apical and basolateral cell membranes and junctions. Several studies have sought to resolve the properties of these three membranes by cable analysis and measurement of voltage divider ratios (Frömter and Diamond, 1972; Reuss and Finn, 1974; Lewis et al., 1976), by analysis of transepithelial diffusion potentials (Koefoed-Johnsen and Ussing, 1958; Sachs et al., 1970; Barry et al., 1971), or by use of amiloride to vary apical membrane conductance alone (Reuss and Finn, 1974; Lewis et al., 1976). However, the validity of each of these three methods rests on assumptions. In particular, the first and third methods assume that apical membrane conductance is unaffected by impalement with a microelectrode, an assumption that has been questioned by Lindemann (1975).

The present paper describes a simple new method for resolving the three membrane conductances as well as for assessing Lindemann's objection. The method involves using the polyene antibiotic nystatin to decrease the apical membrane resistance to a very low value.

Our experiments have been carried out in rabbit urinary bladder, a tight epithelium whose properties were described by Lewis and Diamond (1976) and by Lewis et al. (1976). Briefly, this preparation has three cell layers, of which the one nearest the mucosal solution generates all the short-circuit current (I_{sc}) and is the site of virtually all the transepithelial resistance. Throughout this paper the terms "apical membrane" and "basolateral membrane" will be understood to refer only to the cell membranes of this transporting layer. I_{sc} arises entirely from active Na^+ transport. Junctional resistance is much higher than transcellular resistance. Transepithelial conductance increases with I_{sc} because of a transport-related conductance channel in the apical membrane.

MATERIALS AND METHODS

Rabbit urinary bladders were dissected and mounted as described by Lewis and Diamond (1976). The chamber used by Lewis et al. (1976) for microelectrode studies differed from that used by Lewis and Diamond (1976) for gross electrode studies in ways that introduced some edge damage. Therefore, we used a new microelectrode chamber design (Fig. 1, from Lewis, 1977) that eliminates edge damage and harmful hydrostatic pressure differences across the membrane while facilitating solution changes and temperature regulation. Ag-AgCl electrodes on either side of the preparation were used for continuous

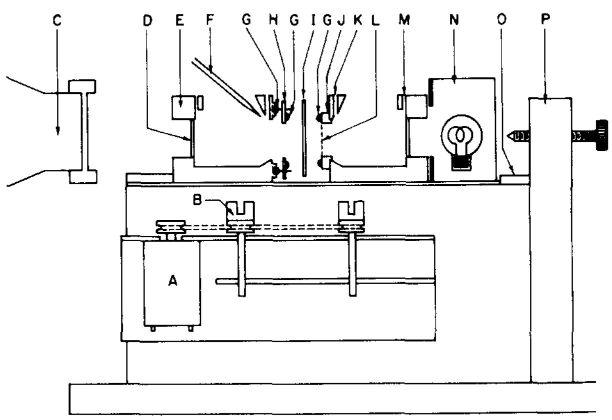

FIGURE 1. Schematic cross-section of chambers. A = motor to drive external magnet B (internal magnetic spin bars not shown); C = dissecting microscope; D = glass window; E = chamber (water jacket is omitted for clarity); F = microelectrode driven by a hydraulic drive micromanipulator; G = silicone sealant layer; H, J = plastic ring with 20 pins (H) around circumference; I = piece of bladder; K, M = ports for voltage electrode (K) and current passing electrode (M); L = nylon mesh for supporting membrane during microelectrode impalements; N = light source; O = grooved platform into which ridge on bottom of chamber inserts to prevent lateral movement of chambers; P = vise.

monitoring of transepithelial voltage (V_t), I_{sc}, transepithelial conductance (G_t), and effective transepithelial capacitance (C_t; see Lewis and Diamond, 1976). The open top of the chambers allowed ready access of glass microelectrodes to the mucosal (apical) surface of the epithelium. A light source and Wild dissecting microscope placed at opposite ends of the chamber facilitated positioning the microelectrodes and monitoring cell viability.

Microelectrodes were of the fiber-filled type, pulled on an Industrial Associates horizontal pipet puller. They were filled with 3 M KCl and were rejected if their resistance was below 20 MΩ. Remote, fine positioning of microelectrodes within an accuracy of ±1 μm was achieved through a Stoelting hydraulic microdrive (Stoelting Co., Chicago, Ill.). The preparation rested on a steel plate, shock-mounted on tennis balls on a heavy table.

The voltage-divider ratio α (ratio of apical to basolateral membrane resistance, R_a/R_{bl}) was measured by applying a transepithelial current pulse (Anapulse stimulator, W-P Instruments, Inc., New Haven, Conn.) and simultaneously recording V_t and the voltage between the cell interior and the serosal solution (i.e. the basolateral membrane potential V_{bl}) on a Newport two-channel digital printer (Newport of North America, Inc., Villanova, Pa.) interfaced with two Weston digital voltmeters (Weston Instruments, Newark, N. J.). The printer was triggered by the stimulator to print V_t and V_{bl} during and after an applied current pulse. The lapsed time between measuring the voltage deflections and the base-line values was 600 ms. A 2-s transepithelial current pulse was applied at 6-s intervals during addition of nystatin to the mucosal solution, and at 30-s intervals during wash-off of nystatin. All voltages were measured to ±100 μV. This protocol permits accurate monitoring of spontaneous V_t, spontaneous V_{bl}, G_t, and α as a function of time.

For reasons discussed by Lewis and Diamond (1976), G_t was normalized not to chamber area but to C_t measured in the presence of amiloride, so that units of conductance are μmho/μF. Under these circumstances a C_t value of 1 μF corresponds approximately to an apical surface area of 1 cm^2. V_t is given as the potential of the serosal solution with respect to that of the mucosal solution, the apical and basolateral membrane potentials V_a and V_{bl} as the potential of the cell interior with respect to that of the mucosal and serosal solution, respectively.

The composition (mM) of the usual bathing solution ("Na^+ solution") was: 111.2 NaCl; 25 $NaHCO_3$; 5.8 KCl; 2.0 $CaCl_2$; 1.2 $MgSO_4$; 1.2 KH_2PO_4; and 11.1 glucose, buffered at pH 7.4 and gassed with 95% O_2-5% CO_2. The serosal solution always had this composition. The mucosal solution was sometimes changed to "K^+ solution" or "choline solution," which differed by equimolar replacement of NaCl-$NaHCO_3$ with these salts, all other solutes remaining at the same concentration. Under some circumstances, in an attempt to mimic the intracellular solution, a "K-sulfate solution" was used. Its composition (mM) was: 58.5 K_2SO_4; 25 $KHCO_3$; 10 Ca (methanesulfonate)$_2$; 1.2 $MgSO_4$; 1.2 KH_2PO_4; 11.1 glucose; 80 sucrose. Nystatin (Sigma Chemical Co., St. Louis, Mo.) dissolved in methanol at a concentration of 5 mg/ml (88,000 U/ml) was added in 1-μl portions to the mucosal solution. The final nystatin activity was 120 U/ml except in experiments to determine the dose-response curve. Except in the experiments described in Results (*Dose-Response Curve* and *Effect of Nystatin Added to the Serosal Solution*), nystatin was added only to the mucosal solution. Bathing solution temperature was maintained at 37°C. Errors are given as standard errors of the mean.

RESULTS

Nystatin Effect on G_t and V_t

Mucosal addition of nystatin to a final activity of 120 U/ml rapidly caused G_t to increase in either Na^+ or K^+ mucosal solutions, V_t to decrease in Na^+ solutions, and V_t to increase in K^+ solutions. For example, after 160 s in the experiment of Fig. 2, nystatin in Na^+ solution caused V_t to decrease from +81 to +49 mV (i.e. serosal solution positive) and G_t increase from 250 to 470 μmho/μF, but in K^+ solution caused V_t to increase from +13 to +38 mV and G_t to increase from 49 to 400 μmho/μF. If bladders were exposed to mucosal nystatin in Na^+ solutions for over 200 s, V_t continued to decrease, G_t continued to increase, and microscopic observation revealed cell swelling and desquamation. With nystatin treatment in K^+ solutions, V_t and G_t reached plateau values, and cell swelling was not seen.

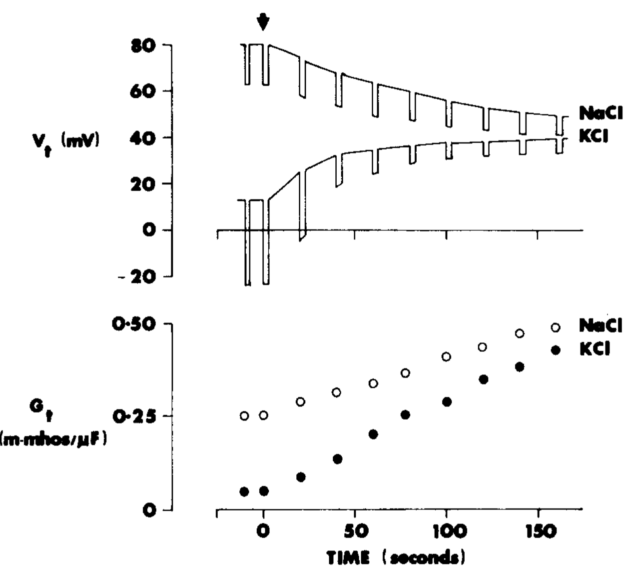

FIGURE 2. Time course of spontaneous transepithelial potential (V_t) and transepithelial conductance (G_t) after addition of 120 U/ml nystatin (at arrow) to the mucosal solution of rabbit bladder in Na^+ solution or in K^+ solution. Note that with time V_t and G_t for both conditions converge. Rectangular steps in V_t are caused by passing a brief transepithelial current pulse (9.29 μA and 3.93 μA for Na^+ and K^+ solutions, respectively), so that the height of the steps is proportional to transepithelial resistance = $1/G_t$.

Locus of the Nystatin Effect

Is the rapid increase in G_t upon mucosal addition of nystatin due solely to an increase in apical membrane conductance G_a, or does nystatin also increase basolateral membrane conductance G_{bl} and junctional conductance G_j? To answer this question, we used an intracellular microelectrode to monitor the voltage-divider ratio α after addition of nystatin to the mucosal solution. Fig. 3 A depicts the change in log $(1/\alpha)$ = log (G_a/G_{bl}) and in log G_t as a function of time after addition of nystatin (Fig. 3 A *left*) and after removal of nystatin (Fig. 3 A *right*). It is apparent that not only G_t but also $(1/\alpha)$ were increased by nystatin. These effects were reversed by removal of nystatin, but reversal of the effect was about 60 times slower than onset of the ffect (note difference in time scale of Fig. 3 A between left and right).

Since α measurements are independent of G_j, and since nystatin increases both G_t and $1/\alpha$, the nystatin effect must at least partly involve an increase in G_a. If nothing more than an increase in G_a were involved, then Fig. 3 A would imply that nystatin increased G_a 200-fold, since G_a/G_{bl} goes from 0.05 to 10. However, more complex interpretations are also possible: nystatin might also alter G_j, an effect that would not be detectable by α measurements; and a change in G_{bl} might be masked by a larger increase in G_a. For example, nystatin added to the mucosal solution could affect G_{bl} in two ways: nystatin might enter the cell, reach

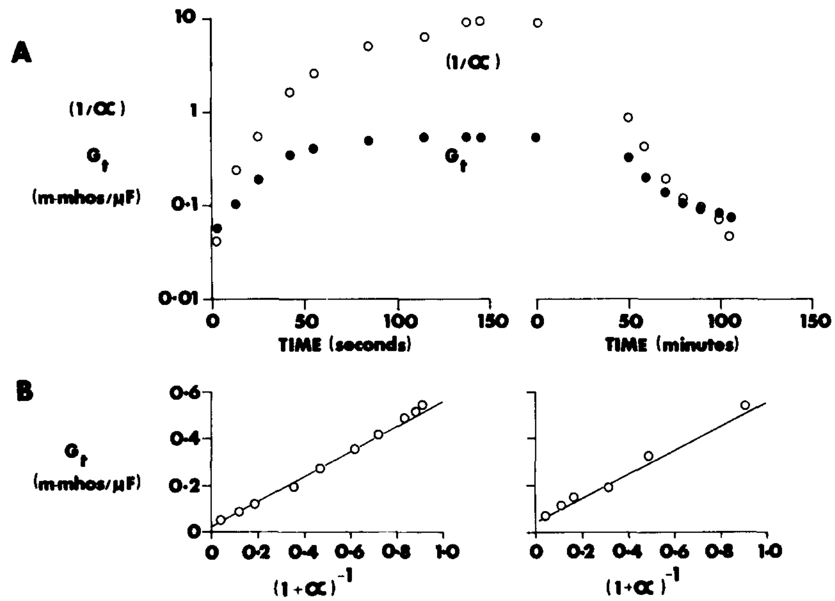

FIGURE 3 A. *Above*, Logarithms of the inverse voltage-divider ratio $1/\alpha$ (○) and of G_t(●) as a function of time after addition of 120 U/ml nystatin to the mucosal solution (*left*), or after removal of nystatin from the mucosal solution (*right*). Note different time scales on left and right. Fig. 3 B *below*, plots of G_t vs. $(1 + \alpha)^{-1}$ from the data of Fig. 3 A *left* and *right*. The straight lines were obtained from linear regression analysis and yield values of G_j and G_{bl}, as discussed in the text.

the basolateral membrane, and increase G_{bl} directly; or a change in G_a might permit sufficient ion or water flow across the apical membrane to alter intracellular ion concentrations or cell volume, thereby indirectly increasing or decreasing G_{bl}. If these effects were significant, we might expect an initial rapid increase in G_t and G_a/G_{bl} as nystatin reached the apical membrane, followed by a slower increase in G_t and change in G_a/G_{bl} as nystatin began to affect G_{bl}. In fact, in nystatin-containing Na^+ solutions there is a decrease in G_a/G_{bl} at long times (>5 min) that could be interpreted as nystatin entry into the cell, causing a delayed increase in G_{bl}. This observation need not, however, indicate nystatin entry into the cell, since the increase in intracellular Na^+ expected in nystatin-containing Na^+ solutions could cause cell swelling or directly affect G_{bl} (Bezanilla and Armstrong, 1972). To assess the possibility of nystatin entry requires an experiment in which the ionic composition of the mucosal solution approximates the intracellular composition, so that an indirect effect of nystatin on G_{bl} due to changes in intracellular composition could be minimized. Treatment with nystatin in K^+ solutions is such an experiment. We found that in nystatin-containing K^+ solutions, G_{bl} and V_{bl} remained constant for at least 10 min, and G_a/G_{bl} did not change further during this time after reaching a plateau in about 2 min. This implies that nystatin does not reach the basolateral membrane.

A more quantitative assessment of any nystatin effects on G_{bl} and G_j is possible as follows. From a simplified equivalent circuit of an epithelium, where the junctional conductance G_j is in parallel with the series combination of G_a and G_{bl} (Lewis et al., 1976; Lewis, 1977), G_t may be related to the three individual conductances as:

$$G_t = G_j + G_a G_{bl}/(G_a + G_{bl}). \quad (1)$$

Substituting $G_a = G_{bl}/\alpha$ into Eq. (1) yields:

$$G_t = G_j + G_{bl}\,[1/(1 + \alpha)]. \quad (2)$$

If brief exposure to nystatin does not affect G_{bl} (as suggested by the preceding paragraph), and if G_j is also unaffected, then Eq. (2) means that a graph of G_t vs. $(1 + \alpha)^{-1}$ should yield a straight line with a slope G_{bl} and ordinate intercept G_j. From Fig. 3 A, the sets of $(G_t, 1/\alpha)$ values at different times have been replotted as graphs of G_t vs. $(1 + \alpha)^{-1}$ in Fig. 3 B. For addition of nystatin (*left*) the slope G_{bl} is 570 μmho/μF (R_{bl} = 1,800 Ω-μF), and the intercept G_j is 12 μmho/μF (R_j = 83,000 Ω-μF), with a correlation coefficient of 1.00. For wash-off of nystatin (Fig. 3 B *right*) G_{bl} is 550 μmho/μF (R_{bl} = 1,800 Ω-μF) and G_j is 46 μmho/μF (22,000 Ω-μF), with a correlation coefficient of 0.99. Table I summarizes the results of seven such experiments. These excellent fits to straight lines (in which the data points displayed no consistent deviation from linearity), and the agreement between the two G_{bl} values, mean that brief exposure to nystatin does not measurably affect G_{bl} or G_j but only G_a.[1] However, G_j estimated from wash-off of nystatin (Fig. 3 *right*) is greater than G_j estimated from addition of nystatin (Fig. 3 *left*). This discrepancy is probably caused by secondary delayed effects on G_j due to some cell swelling, lysis, or desquamation, rather than by incorporation of nystatin into junctional complexes.

Additional evidence that corroborates our hypotheses that (*a*). G_{bl} does not change significantly after addition of nystatin; (*b*). G_a and G_{bl} are not directly related; and (*c*) the mucosal solution composition does not directly effect the

[1] Estimates of G_{bl} in rabbit urinary bladder using AC analysis (670 ± 100 μmho/μF, n = 9, Clausen, Lewis and Diamond, personal communication) and nystatin (790 ± 70 μmho/μF, n = 7, Table I) are in good agreement (t-test yields $P > 0.2$). In another tissue, rabbit descending colon, the estimate for G_{bl} using nystatin (Dr. N. Wills, personal communication) and amiloride (Schultz, Frizzell and Nellans, 1977; Dr. N. Wills, personal communication) are likewise in good agreement. These two lines of evidence support our contention that mucosal nystatin does not alter the basolateral membrane conductance. However, if one considers the difference of the mean G_{bl} between the methods (18%), one can predict the effect that this difference will have on the I-V relationship and relative selective permeability of the basolateral membrane. The I-V relationship, if linear (see Results), will have a slope change of 18%. The relative selective permeability will: (*a*) approach unity if nystatin increases conductance in a nonselective manner; (*b*) increase, decrease, or remain unchanged if nystatin increases the conductance of only one ion; or (*c*) remain unchanged if nystatin increases the conductance of all ions proportionately.

basolateral potential or conductance can be obtained from the following experiment. By examining Fig. 4 the relationship between G_t and $(1 + \alpha)^{-1}$ for apical solutions containing high Na^+ ("Na^+ solution" in Materials and Methods) or high K^+ ("K-sulfate solution" in Materials and Methods), one finds that there is virtually no difference in G_{bl} for the two solutions and in both cases the

TABLE I

RESISTANCE VALUES IN RABBIT URINARY BLADDER

I_{sc}	R_a	R_{bl}	R_j	r
$\mu A/\mu F$	Ω-μF	Ω-μF	Ω-μF	
0.6	20,000	1,100	∞	0.98
0.8	43,000	1,400	∞	0.998
1.3	20,000	1,800	∞	0.998
1.4	22,000	1,800	80,000	0.998
2.4	21,000	1,100	31,000	0.999
3.0	14,000	1,000	∞	0.996
6.5	9,400	1,100	∞	0.99

Membrane resistance values in rabbit bladders in the absence of nystatin, calculated by extrapolation of the time course of nystatin action (addition of nystatin) as in Fig. 3 B. r is the correlation coefficient for straight-line plots as in Fig. 3 B. Each line is based on a different bladder, whose short-circuit current is given in the first column.

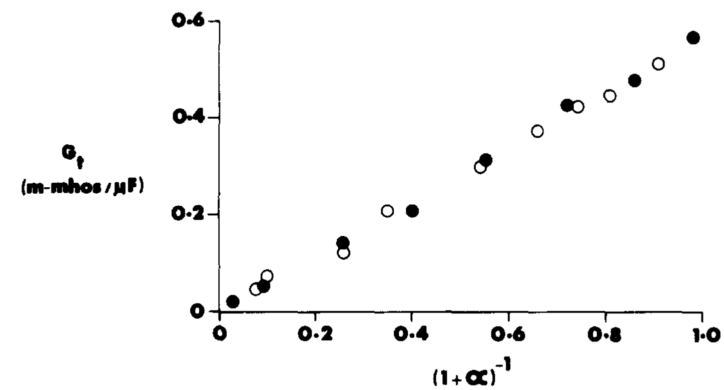

FIGURE 4. The relationship between G_t and $(1 + \alpha)^{-1}$ when nystatin is applied to the apical membrane in Na^+-containing solution (○) and when it is applied in a solution containing high K sulfate (●). The slope of the lines predicts a basolateral conductance in Na^+ solution of 0.57 mmho/μF and K sulfate solution of 0.59 mmho/μF. The intercepts imply a very small junctional conductance for both solutions.

intercepts suggest an infinitely small conductance for G_j. This demonstrates that the α values produced by nystatin are not dependent upon the apical solutions if they contain permeable cations and that, at least for low transport rates (<3 μA/μF), G_{bl} does not depend upon the composition of the apical solution.

Basolateral I-V Relation

Before or after nystatin treatment, R_j is greater than $(R_a + R_{bl})$, so that most applied current flows across the cells rather than across the junctions. However,

R_{bl} after nystatin is much greater than R_a, so that the transepithelial I-V relation of nystatin-treated bladders mainly reflects properties of the basolateral membrane. We measured this I-V relation in two bladders and found it to be linear over the range −155 mV to +40 mV (potential of cell interior with respect to serosal solution).

Selectivity of Nystatin-Doped Apical Membrane

We assessed the ionic selectivity of the nystatin channel in the apical membrane by comparing nystatin-induced changes in V_a and V_{bl} in Na^+, K^+, and choline mucosal solutions (Fig. 5).

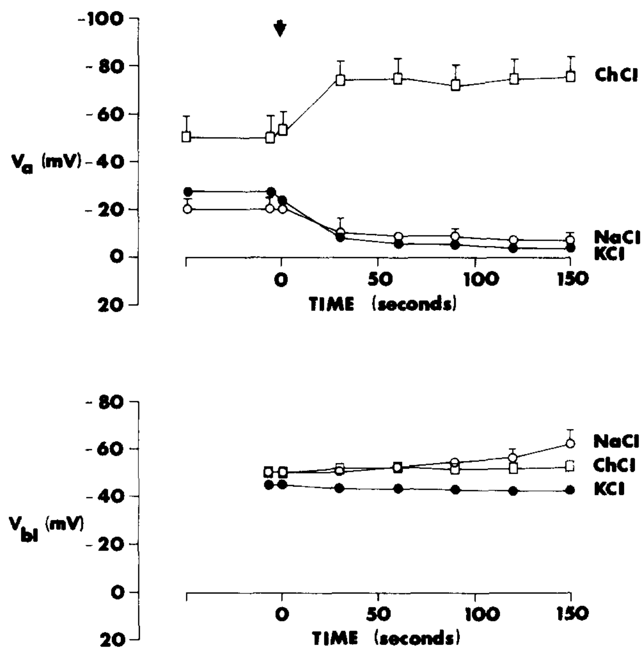

FIGURE 5. Effect of 120 U/ml nystatin (added at arrow) on the resting potentials of the apical membrane (V_a) and basolateral membrane (V_{bl}) in Na^+ (○), K^+ (●), or choline (□) solutions. Bars above points indicate average value ± SEM. Points without bars had SEM too small to be visible in the figure. See text for discussion.

As discussed by Lewis et al. (1976), V_a in Na^+ solutions in the absence of nystatin varies greatly with changes in I_{sc} and with changes in the Na^+ conductance G_{Na} of the apical membrane. Lewis et al. (1976) found a range of V_a from a negative value of −40 mV in bladders with low transport rates to values near zero in bladders with high transport rates. Subsequently, we have found reversed V_a values as high as +50 mV in bladders with very high I_{sc}. Nevertheless, Fig. 6 shows that, independent of the initial value and even initial sign of V_a (and hence independent of G_{Na}), nystatin changed V_a in Na^+ solutions to approximately the same value: on the average, -6.7 ± 2.5 mV ($n = 5$, cell interior negative). Evidently, nystatin completely masks the permeability characteristics of untreated membrane, and V_a reflects only the properties of the nystatin channel. This is as expected from the great increase in G_a caused by nystatin.

Nystatin caused V_a in K^+ solutions to depolarize to -5.8 ± 3.2 mV ($n = 3$; Fig. 5), a value essentially the same as that in Na^+ solutions, suggesting that the nystatin channel has similar permeability to Na^+ and K^+. In choline solutions V_a of untreated bladders was larger than in Na^+ or K^+ solutions, and V_a hyperpolarized rather than depolarized with nystatin to -75 ± 9 mV ($n = 3$). This suggests that the native apical membrane has low permeability to choline, and that the permeability of the nystatin channel is much lower to choline than to K^+, Na^+, and possibly Cl^-. The large V_a of nystatin-treated bladder in choline solutions is presumably a K^+ and/or Cl^- diffusion potential. Independent of mucosal solution composition, G_a increased to approximately 7 mmho/μF in the first 2 min after exposure to nystatin. Thereafter, however, G_a in choline

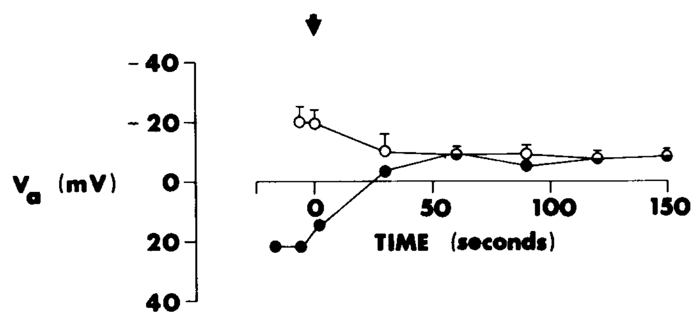

FIGURE 6. Apical membrane potential V_a as a function of time after addition of 120 U/ml nystatin at zero time (arrow). Points ○ are average values from bladders with low I_{sc} (average of 1 μA/μF; bars above points = average value ± SEM: points ●, from a bladder with high I_{sc} (5.6 μA/μF). Note that nystatin causes V_a values for the two bladders to converge.

solutions began to decrease, due possibly to a decrease in intracellular (K^+) and hence in extracellular (K^+) in the unstirred layer immediately adjacent to the apical membrane. After 10 min in nystatin-containing choline solutions, V_a depolarized towards zero.

In contrast to the large effects of solution composition and nystatin on V_a, effects on V_{bl} were much slighter (Fig. 5). V_{bl} was initially -53 ± 3.1 mV ($n = 5$) in Na^+ solutions, -53 ± 6 mV ($n = 3$) in choline solutions, and -45 ± 5 mV ($n = 3$) in K^+ solutions. Nystatin caused no immediate change in V_{bl} in choline solutions (but a depolarization towards zero after 10 min), a slight depolarization of a few millivolts in K^+ solutions, and invariably a hyperpolarization of 10 mV in Na^+ solutions. The significance of these observations is considered in the Discussion.

Dose-Response Curve

All results discussed so far were obtained at a nystatin dose of 120 U/ml. Fig. 7 is a dose-response curve of log ($1/\alpha$) against log nystatin activity, in Na^+ solutions. α was measured 2 min after addition of nystatin, because this period produced the maximum reversible response for the maximum nystatin dose of 120 U/ml in Na^+ solutions. Since G_{bl} is unaffected by brief exposure to nystatin (see above), the ordinate of Fig. 7 is actually proportional to log G_a. It is apparent that the lowest dose tested, 40 U/ml, scarcely affected log G_a, but that for higher doses the curve rises steeply with a slope of 4.6.

Effects of Nystatin Added to the Serosal Solution

All results discussed so far were obtained with nystatin added only to the mucosal solution. The effects of nystatin added only to the serosal solution differed in four respects.

1. The nystatin-dependent increase in G_t was much smaller. This is expected, since G_t of untreated bladder is primarily determined by R_a rather than by R_{bl}, and the apical membrane is not accessible to serosally added nystatin.
2. α ($= G_{bl}/G_a$) increased rather than decreased, because serosally and mucosally added nystatin increase G_{bl} and G_a, respectively.

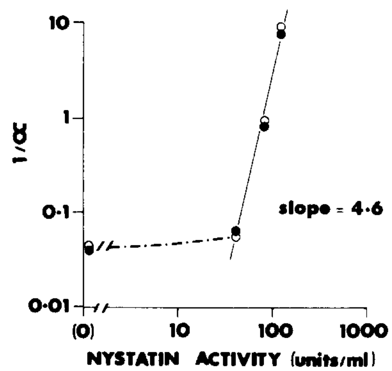

FIGURE 7. Dose-response curve for nystatin effect on $(1/\alpha) = (G_a/G_{bl})$. Scales are double logarithmic. α was measured 2 min after addition of nystatin to the mucosal solution of a bladder bathed in Na^+ solutions. Since nystatin does not affect G_{bl} within 2 min, the ordinate is proportional to log G_a. Points ○ and ● are from two different bladders. The measurement in the absence of nystatin is depicted at an arbitrary low abscissa value.

3. The time course of nystatin action was up to 60 times longer, because the connective tissue and two cell layers on the serosal surface constitute a significant unstirred layer and perhaps a nystatin sink, whereas the transporting layer is in direct contact with the mucosal solution.
4. Effects of serosally added nystatin were poorly reversible, whereas those of mucosally added nystatin were readily reversible. Unstirred layers and nystatin sinks on the serosal surface are again the probable explanation.

DISCUSSION

We consider four problems: comparison of nystatin effects on urinary bladder and on bilayers; reassessment of microelectrode impalement artifacts; estimates of R_a, R_{bl}, and R_j; and reassessment of basolateral membrane properties.

Comparison with Bilayers

The epithelium on which the polyene antibiotics, nystatin and amphotericin B, were first tested was toad urinary bladder (Lichenstein and Leaf, 1965; Sharp et al., 1966). The effects of these antibiotics on artificial bilayers were subsequently studied in detail. The effects of nystatin on apical membrane of mammalian urinary bladder resemble those of nystatin and amphotericin B on bilayers.

i. Bilayers must contain sterols for a maximal antibiotic-induced conductance change (Andreoli, 1973; Finkelstein and Holz, 1973). This requirement is satisfied by mammalian bladder cell membranes, which have a molar cholesterol:phospholipid ratio of 0.6 (Ketterer, et al., 1973).

ii. Nystatin added to one side of a bilayer imparts cation selectivity, while nystatin added to both sides imparts anion selectivity (Marty and Finkelstein, 1975). In our experiments nystatin could be added only to the extracellular side of the apical membrane, and the resulting selectivity was probably cationic (estimated $P_{Cl}/P_K = 0.28$; see below).

iii. The pore size of amphotericin B in layers is ca. 7 Å, and that of nystatin is similar. Molecules with a Stokes-Einstein radius greater than 4 Å are virtually impermeant (Holz and Finkelstein, 1970). The low permeability of nystatin-treated rabbit bladder to the large cation choline fits this pattern.

iv. The dose-response curve of nystatin has as steep a slope in bilayers (Finkelstein and Holz, 1973) as in rabbit bladder and red cells (Cass and Dalmark, 1973). This slope may indicate that several nystatin molecules are required to form one channel.

v. The steady-state conductance produced in bilayers by a given nystatin dose is not reproducible among nystatin preparations (Marty and Finkelstein, 1975). We found that effects on rabbit bladder conductance were consistent within a batch of nystatin but not among batches.

vi. Nystatin-induced conductance in bilayers varies inversely with temperature (Cass et al., 1970). We did not investigate the question for two reasons: rabbit urinary bladder is very sensitive to any temperature shift from 37°C; and nystatin-induced apical conductance at 37°C is already so high (7 mmho/μF) that any increase in conductance at lower temperatures would be difficult to quantitate.

Estimates of Chloride Permeability Induced by Nystatin

Because of the lack of information concerning the intracellular ion concentrations, quantitating the anionic permeability of the nystatin channels in the apical membrane is extremely difficult. However, by evoking certain assumptions we can calculate an approximate value for P_{Cl}/P_K as well as an estimate for intracellular $[Cl^-]$. The value for intracellular $[K^+]$ of 150 mM was estimated from the zero potential intercept of a plot of the serosal potassium concentration vs. basolateral membrane potential if one assumes that the basolateral membrane potential is close to the potassium diffusion potential; (Lewis and Eaton, unpublished observations). The potential for nystatin-treated apical membrane was -5.8 ± 3.2 mV and -74.4 ± 9.2 mV (cell interior negative) when the mucosal solution was KCl-$KHCO_3$ Ringer's and choline Cl-choline HCO_3 Ringer's, respectively. Since R_j has a value much greater than R_a or R_{bl}, we have not corrected for the influence of R_j on V_a or V_{bl}. If one assumes $P_{choline}$ to be negligible compared to P_K, and intracellular $[Na^+]$ to be much lower than intracellular $[K^+]$ in choline or K^+ solutions (which are Na^+-free), the Goldman-Hodgkin-Katz equation for the apical membranes becomes

$$V_a = \frac{RT}{zF} \ln \frac{[K]_o + (P_{Cl}/P_K)\,[Cl]_i}{[K]_i + (P_{Cl}/P_K)\,[Cl]_o} \tag{3}$$

Substituting the measured values for V_a in K^+ and choline solutions (−5.8 and −75 mV, respectively), the values for $[K]_o$ and $[Cl]_o$ in these solutions (see Materials and Methods), and the estimate $[K]_i = 150$ mM into Eq. (3) yields two equations in the two unknowns P_{Cl}/P_K and $[Cl]_i$. The result is $P_{Cl}/P_K = 0.28$, $[Cl]_i = 15$ mM. This P_{Cl}/P_K value agrees well with the value of 0.15 obtained for nystatin-treated *Aplysia* cell bodies (Russell et al., 1976). The value $[Cl]_i = 15$ mM agrees well with the value of 16 mM calculated from the spontaneous V_{bl} value of −53 mV on the assumption that Cl^- is in electrochemical equilibrium across the basolateral membrane.

Microelectrode Impalement Artifacts

Lindemann (1975) has stressed the risk of errors in microelectrode study of epithelial cells. If the cell is small, microelectrode impalement may introduce a significant shunt conductance in parallel with the native membrane conductance. Such a shunt conductance in one cell will scarcely alter the conductance of the whole epithelial sheet but may seriously underestimate the voltage and resistance of the impaled membrane, and hence may cause to be in error the voltage-divider ratios and resistance values derived from cable analysis. How significant is this effect in our experiments?

This question can be answered by examining the graphs of G_t vs. $(1 + \alpha)^{-1}$ in Fig. 3 B. In native membrane or just after addition of nystatin, R_a and α are high. A significant shunt in the apical membrane caused by a microelectrode would cause the measured α to be artifactually low and the measured $(1 + \alpha)^{-1}$ to be artifactually high. As R_a and α rapidly decrease with time after addition of nystatin (R_a decreasing to ~1% of its initial value), G_a becomes completely dominated by the nystatin channel, and any contribution from a microelectrode shunt is negligible. Thus, a microelectrode shunt would cause the graphs of Fig. 3 B to deviate below linearity at low $(1 + \alpha)^{-1}$ values (because the plotted abscissa values would exceed the true values for low $[1 + \alpha]^{-1}$). In fact, no such deviation from linearity is apparent in Fig. 3 B. Any microelectrode shunt conductance must be considerably less than the native membrane conductance. Naturally, this conclusion applies only to the present experiments and gives no assurance that microelectrode shunt conductance is negligible in other epithelia impaled with other microelectrodes.

Values for R_a, R_{bl}, and R_j

Table I summarizes values for R_a, R_{bl}, and R_j in seven untreated rabbit bladders, determined by extrapolation of G_t and α values after addition of nystatin as in Fig. 3 B. R_j values are consistently very high, as found by Lewis et al. (1976). R_a values vary inversely with I_{sc}, also as found by Lewis et al. (1976). However, our R_{bl} values are consistently several times lower than those of these investigators just as our α values are consistently higher than theirs. We attribute this difference to our improved chamber design and microelectrode techniques, hence more accurate measurements of α.

Reassessment of Basolateral Membrane Function

By reducing apical membrane resistance to a low value, nystatin may offer a powerful tool for studying the basolateral membrane. Fig. 5 illustrates such a use. Nystatin consistently causes V_{bl} to hyperpolarize by 10 mV in Na^+ solutions but not in K^+ or choline solutions. This hyperpolarization must somehow result from the increase in $[Na]_i$ expected after addition of nystatin in Na^+ solutions (Cass and Dalmark, 1973; Russell et al., 1976). V_{bl} appears in large part to be a K^+ diffusion potential. The hyperpolarization actually observed cannot be an Na^+ diffusion potential since the Na^+ gradient is in the wrong direction, but could arise from either the Na^+ pump in the basolateral membrane being electrogenic or an increased K^+ gradient across the basolateral membrane formed by an electrically silent Na^+-K^+ exchange pump. Further work employing nystatin is required to differentiate between these possibilities.

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