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Electrical Properties of the Rabbit Urinary Bladder Assessed Using Gramicidin D

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Summary. Recently, antibiotics have enjoyed widespread usage as tools in studies of epithelial transport. In the present study we assess the usefulness of the pore-forming antibiotic gramicidin D as a means for probing the electrical properties of the tight epithelium rabbit urinary bladder. Addition of 50 μM gramicidin to the mucosal bath (either a NaCl or KCl Ringer's solution) led to a large irreversible increase in the transepithelial conductance (G_T) within 800 sec. G_T increased by approximately 1200% and 500% in KCl and NaCl Ringer's solutions, respectively. Microelectrode measurements of the resistance ratio (the ratio of apical membrane resitance to basolateral membrane resistance) showed that apical membrane resistance is decreased by the drug. Measurements of the basolateral membrane resistance (R_{bl}) and tight junctional resistance (R_j) using a new and independent method (based on the perturbation of basolateral membrane electrogenic Na^+ pump) demonstrated that R_{bl} and R_j were unaffected, suggesting that the effects of gramicidin are restricted to the apical membrane for periods of at least 2 hours after drug addition. The selectivity of the gramicidin-induced permeability in the apical membrane was calculated from measurements of the apical membrane potential after ion substitutions using a modified version of the constant field equation. The selectivity sequence for cations was $Cs^+ > K^+ > Na^+ > Li^+ >$ choline. Unlike the commonly used polyene antibiotics nystatin and amphotericin B, gramicidin did not induce a significant Cl^- permeability. In addition, the dose-response curve had a slope of 1. A method is described for calculating membrane resistances directly from transepithelial measurements under some conditions of gramicidin use, without requiring the use of microelectrode measurements.

Key words antibiotics · gramicidin D · tight epithelium · membrane resistances · electrogenic pump · microelectrodes

Introduction

Active sodium transport across epithelial cells is a two-step process involving a Na^+ entry step across the apical membrane down a net electrochemical gradient, and a Na^+ extrusion step against a net electrochemical gradient across the basolateral membrane by the $(Na^+ + K^+)$-ATPase. The apical membrane entry step has received greater attention for several reasons. First it is more readily accessible to external experimental perturbations and second, the apical membrane is commonly considered to be the rate-limiting barrier to ion movement. In contrast relatively little is known concerning the transport properties of the basolateral membrane. This lack of information is due in part to the difficult task of perturbing the cell ionic composition (in a controlled manner) while simultaneously performing quantitative measurements of the properties of the basolateral membrane. Recently, the polyene antibiotics nystatin and amphotericin B have been employed to access the properties of the basolateral membrane and the $(Na^+ + K^+)$-ATPase. These substances have been used by several investigators to reduce (if not eliminate) the apical membrane as a limiting barrier to ion movement and thus expose the basolateral membrane to electrophysiological and radio-tracer experiments (Lewis, Eaton, Clausen & Diamond, 1977; Lewis, Wills & Eaton, 1978; Nielsen, 1979; Wills, Lewis & Eaton, 1979; Graf & Giebisch, 1980; Kirk, Halm & Dawson, 1980). These polyene antibiotics do however create some potential problems. First the drugs can cause cell swelling because they are nonselective for small monovalent cations and anions. Thus the apical (or mucosal) bathing solution must be designed so as to reduce the possibility of cell swelling by a rapid influx of anions and cations. In addition, the polyenes do not bind irreversibly. For example, the action of the drugs can be easily eliminated by washing the tissue with an antibiotic-free solution. This feature raises the possibility (over a long period of time) that the polyenes could enter the cell and disrupt normal metabolism. Similarly it is conceivable that the drugs might reach the basolateral membrane and alter its permeability properties.

For these reasons we investigated the action of another pore-forming antibiotic, gramicidin D. Gramicidin D offers the following advantages: (1) it has been extensively studied in artificial membrane

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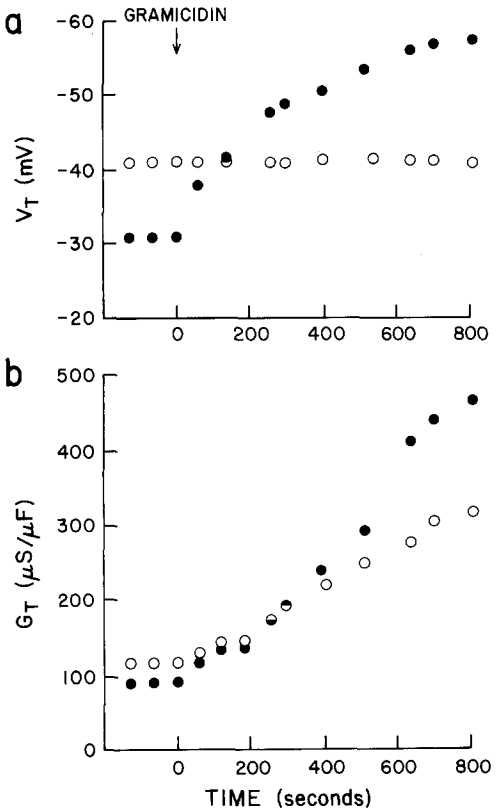

Fig. 1. Response of the transepithelial potential (V_T) (upper frame) and transepithelial conductance (G_T) (lower frame) to addition of gramicidin D (arrow) to the mucosal solution at a final concentration of 50 μM. Open circles (○) show the case in which gramicidin D is added to a NaCl mucosal Ringer's solution and closed circles (●) gramicidin D added to a KCl mucosal Ringer's solution. In both cases NaCl Ringer's solution is in the serosal chamber. The smaller change in potential and conductance when gramicidin is added to a NaCl mucosal bath as compared to a KCl bath reflects the greater selectivity of gramicidin to K^+ over Na^+

systems and (2) it is thought to be relatively impermeable to anions. Therefore in this paper we report the effects of this drug on the electrical properties of the apical membrane of the mammalian tight epithelium, rabbit urinary bladder and evaluate the usefulness of this drug as a probe for characterizing basolateral membrane properties.

Materials and Methods

Rabbit urinary bladders were dissected and mounted in an *in vitro* chamber as described by Lewis et al. (1977). Ag–AgCl electrodes placed approximately 5 mm on either side of the epithelium were used to monitor transepithelial voltage (V_T). Another pair of Ag–AgCl wires were placed in the rear of each half-chamber and were used to pass current. Both sets of Ag–AgCl wires were connected to an automatic voltage clamp which measured V_T, short-circuit current (I_{sc}), transepithelial conductance (G_T) and the capacitance of the epithelium (C_T; *see* Lewis & Diamond, 1976).

Microelectrodes were fabricated as previously described (Lewis et al., 1978) and were rejected if their resistance was less than 20 MΩ. Remote fine positioning of the microelectrode in the mucosal solution was achieved through a hydraulic microdrive (Stoelting Co., Chicago, IL). The microelectrode was connected to one of the inputs of a differential electrometer (M750, WP Instruments, Hamden, CT), while the other input was connected to the voltage-measuring Ag–AgCl wire in the serosal chamber. Both the chambers and hydraulic drive rested on an antivibration table (Barry Control, Mass.). The resistance ratio (α = ratio of apical to basolateral membrane resistance, R_a/R_{bl}) was measured by applying a computer-regulated transepithelial current pulse and simultaneously recording V_T and the voltage between cell interior and the serosal solution (i.e. the basolateral membrane potential V_{bl}). All voltages and the current were digitized by an analog-to-digital converter to an accuracy of ±0.05 mV. The digitized signals along with time were accessed by a small computer (North Star Co.) and stored on disk for future analysis. In addition the baseline and pulse voltages and currents were printed on a Decwriter II along with the calculated conductance, α and I_{sc}. This hard copy allowed continuous monitoring of the viability of the preparation.

V_T is given as the potential of the mucosal solution with respect to that of the serosal solution, and the apical and basolateral membrane potentials V_a and V_{bl} as the potential of the cell interior with respect to those of the mucosal and serosal solution, respectively.

The composition (mM) of the usual bathing solution (Na^+ solution) was: 110.0 NaCl; 25 $NaHCO_3$; 7 KCl; 2.0 $CaCl_2$; 1.2 $MgSO_4$; 1.2 NaH_2PO_4; and 11.1 glucose buffered at pH 7.4 and gassed with 95% O_2 – 5% CO_2. With the exception of the experiment described in Results (p. 49) all serosal solutions had this composition. In this experiment all K^+ was omitted from the bathing solution.

In some experiments the mucosal solution Na^+ was replaced equimolarly for choline, Li^+, K^+ or Cs^+; all other ions remained constant. In another experiment the Cl^- in the mucosal K^+ solution was replaced with methanesulfonate, and the voltage-measuring Ag–AgCl electrodes were replaced with 1 M KCl agar bridges.

Gramicidin D (Sigma Chemical Co., St. Louis, MO), dissolved in methanol at a concentration of 5 mg/ml, was added to the mucosal solution to a final concentation of 50 μM except for the experiments for determining the dose response curve of gramicidin D. Addition of similar amounts of methanol alone (200 λ in 15 ml) had no measureable effect on the electrical properties of the bladder.

Bathing solution temperature was maintained at 37 °C. Errors are given as standard errors of the mean.

Results

Transepithelial Effects of Gramicidin D

Mucosal addition of gramicidin D to a final concentration of 50 μM, caused G_t to increase in either a NaCl or KCl mucosal bathing solution over a period of 25 min. In KCl Ringer's V_T increased from -31 ± 2.9 mV to -55 ± 2.0 mV ($n = 5$). Although V_T had a wide range of values in a NaCl Ringer's before gramicidin ($V_T = -44 \pm 5.4$ mV, $n = 5$), after mucosal addition V_T reached a value of -39 ± 3.0 mV. An example of the time-dependent V_T and G_T responses to gramicidin D is shown in Fig. 1. In Na^+

Table 1. Estimated resistance values of the rabbit urinary bladder in NaCl Ringer's solution before and after mucosal addition of gramicidin D

	α	R_T (KΩ μF)	R_a (KΩ μF)	R_{bl} (KΩ μF)	R_j (KΩ μF)
Control	32 ±4.5	31 ±3.3	47 ±9.9	1.6±0.3	97±23
Time after gramidicin D exposure:					
800 sec (Method I)	1.9±0.5	4.7±1.4	3.6±1.4	[a]	[a]
2 hr (Method II)	2.7±0.7	5.2±1.5	3.8±0.6	1.6±0.4	>100
$n=5$					

[a] Method I is based on microelectrode measurements of the resistance ratio and assumes that R_{bl} and R_j are unaffected by gramicidin.
(1 μF ≅ 1 cm² apical membrane area.)

solutions G_T increased from a value of 44 ±10.3 μS/μF to 218±29.0 μS/μF after 800 sec of exposure to gramicidin D. Similarly after addition of gramicidin D to a KCl mucosal Ringer's G_T increased from 34±3.5 μS/μF to 423±120.1 μS/μF 800 sec after antibiotic addition. Cell viability (defined as a constant V_T and G_T) was maintained in either Na^+ or K^+ bathing solutions for periods up to 3 hr with no visual evidence of cell swelling. Unlike nystatin (*see* Lewis et al., 1977) gramicidin D is poorly reversible in the rabbit urinary bladder.

Localization of the Gramicidin Effect

It is necessary to determine whether gramicidin alters the conductance of only the apical membrane or whether other membranes (basolateral and tight junctions) are also influenced. This question was answered using the procedure of Lewis et al. (1978). In brief, a conventional microelectrode was placed into a cell and the resistance ratio (α) monitored during mucosal gramicidin action. Table 1 summarizes the values for α and R_T before and 800 sec after addition of mucosal gramicidin D in five preparations. Using the equation of Lewis, Eaton and Diamond (1976) in conjunction with measured values for α and R_T (before and during gramicidin D) one can calculate the individual resistances. Given that R_T for the untreated condition is described by the following equation:

$$R_T = R_j R_b(\alpha+1)/[R_j + R_b(\alpha+1)] \quad (1)$$

and *assuming* that gramicidin affects only the apical membrane resistance, then after drug addition the transepithelial resistance (now R'_T) will similarly be a function of the new resistance ratio (α') or

$$R'_T = R_j R_b(\alpha'+1)/[R_j + R_b(\alpha'+1)]. \quad (2)$$

Calculations of the membrane resistances using this technique (Method I) are presented in Table 1. Initially, these estimates appear to be in excellent agreement with the results of previous experiments using nystatin (Lewis et al., 1977) and impedance analysis (Clausen, Lewis & Diamond, 1979).

Unfortunately, because of the prolonged response time for gramicidin (25 min as compared to <2 min for nystatin) it was not possible to measure the action of the drug within the same cell. This prevented a comparison of G_T and α as was performed in our previous nystatin study. For this reason we sought another means for evaluating membrane and junctional resistances after gramicidin D. The technique which we developed is based on the ability to alter the basolateral resistance, electromotive force or both without causing changes in the apical membrane or junctional resistances at least for short periods of time. There are a number of mechanisms available for changing either the basolateral resistance and/or electromotive force. One such mechanism exploits the selective permeability of the basolateral membrane. In the absence of active Na^+ transport the basolateral membrane potential can be described by the constant field equation where K^+ is the most permeable ion (Lewis et al., 1978). Thus a maneuver designed to either increase or decrease serosal K^+ activity will result in a depolarization of hyperpolarization of the basolateral membrane and in addition a change in the membrane resistance. A second approach is to either stimulate or inhibit a current source in the basolateral membrane. A possible current source is the $(Na^+ + K^+)$-ATPase. This ATPase has been demonstrated in the bladder to be electrogenic or charge (current)-carrying (Lewis et al., 1978). Stimulation of the pump can be accomplished by increasing either intracellular Na^+ activity or Na^+ entry rate, or restoration of K^+ to a K^+-free serosal solution (*see* Wills & Lewis, 1980). Removal

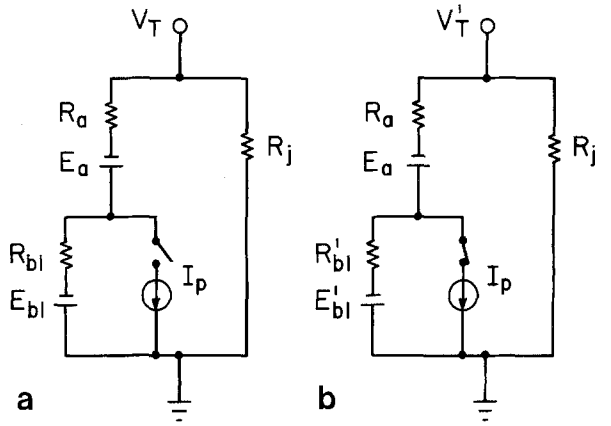

Fig. 2. Electrical equivalent circuit for the rabbit urinary bladder (*a*) with an inhibited current source (*I*) in the basolateral membrane and (*b*) with an activated current source. V_T, V_T' and V_{bl}, V_{bl}' are the transepithelial and basolateral membrane potentials when the pump is inhibited and activated, respectively. E_a and E_{bl} (or E_{bl}') are the apical and basolateral membrane electromotive forces, respectively, and R_a, R_{bl} (or R_{bl}') and R_j are the apical and basolateral membrane and junctional resistances, respectively

of K^+ from the serosal solution will allow a reversible inhibition of the pump while 10^{-4} M serosal ouabain will irreversibly inhibit the pump (Wills & Lewis, 1980).

Our protocol was first to increase Na^+ entry across the apical membrane using gramicidin D. After the tissue reached a stable plateau level for G_T we next inhibited the pump by bathing the basolateral membrane in a K^+-free NaCl Ringer's for approximately 1 hr. This pump inhibition allows an increase in intracelluar Na^+ activity. The last step was a rapid return of K^+ to the serosal solution. Restoration of K^+ (7 mM) elicits a rapid and transient hyperpolarization of both the basolateral membrane potential (V_{bl}) and transepithelial potential (V_T). (An alternate approach would be to increase Na^+ entry and consequently intracellular Na^+ activity using gramicidin D and then inhibit the pump using ouabain. Such a sequence will cause a rapid depolarization of both the basolateral membrane and transepithelial potentials.) Figure 2 is an electrical equivalent circuit which describes in terms of resistors, batteries and a current source the activation and inactivation of the pump. The ratio of the change in the basolateral membrane potential to transepithelial potential. ($\Delta V_{bl}/\Delta V_T$) yields the following equation (*see* Appendix for derivation and assumptions behind this method):

$$\frac{\Delta V_{bl}}{\Delta V_T}=1+\frac{R_a}{R_j}=1+\gamma; \qquad \gamma\equiv\frac{R_a}{R_j}.$$

Given this relationship and measurements of ΔV_{bl} and ΔV_T as well as simultaneous measurements of the transepithelial resistance (R_T) and resistance ratio (α) we can write three equations having three unknowns:

$$\gamma=R_a/R_j; \tag{3}$$

$$\alpha=R_a/R_{bl}; \tag{4}$$

$$R_T=\frac{(R_a+R_{bl})R_j}{R_a+R_{bl}+R_j}. \tag{5}$$

After re-arranging Eq. (5) and substituting in Eqs. (3) and (4) we can solve for R_j:

$$R_j=R_T\left(1+\frac{\alpha}{\gamma(1+\alpha)}\right). \tag{6}$$

Equation (3) is then used to solve for R_a and Eq. (4) for R_{bl}.

Figure 3 shows the rapid hyperpolarization of both basolateral membrane and epithelium in response to addition of K^+ to the serosal solution of a bladder whose apical membrane had been treated with gramicidin D. The calculated values for the three resistive elements are in Table 2. Again, the mean values are in excellent agreement with those determined by another method (*see* Table 1). These data indicate that gramicidin D affects only the apical membrane and does not measurably change the resistance of either the basolateral membrane or tight junctions.

Selectivity of Apical Membrane After Gramicidin D Treatment

We determined the ionic selectivity of the gramicidin channel in the apical membrane by measuring the influence of this antibiotic on the apical membrane potential (V_a) when the mucosal solution contained Na^+, K^+, Li^+, choline or Cs^+ chloride salts, or a K^+ methanesulfonate Ringer's.

i) Cations. As in the nystatin experiments of Lewis et al. (1977) gramicidin D in a NaCl Ringer's caused V_a to achieve a common value (-13.3 ± 1.5 mV; $n=5$; cell interior negative) independent of the initial (pre-gramicidin) potential. Consequently, gramicidin D makes insignificant the permeability characteristic of the natural membrane, and V_a reflects the selectivity properties of the gramicidin channel which is bathed on the mucosal side by NaCl and on the intracellular side by high K^+ (as measured by Wills and Lewis, 1980, and Lewis et al. 1978). The polarity of the apical membrane potential and the known ion gradients indicate that the gramicidin channel has greater selectivity for K^+ over Na^+. In mucosal K^+ Ringer's the apical membrane depolarized to -3.5

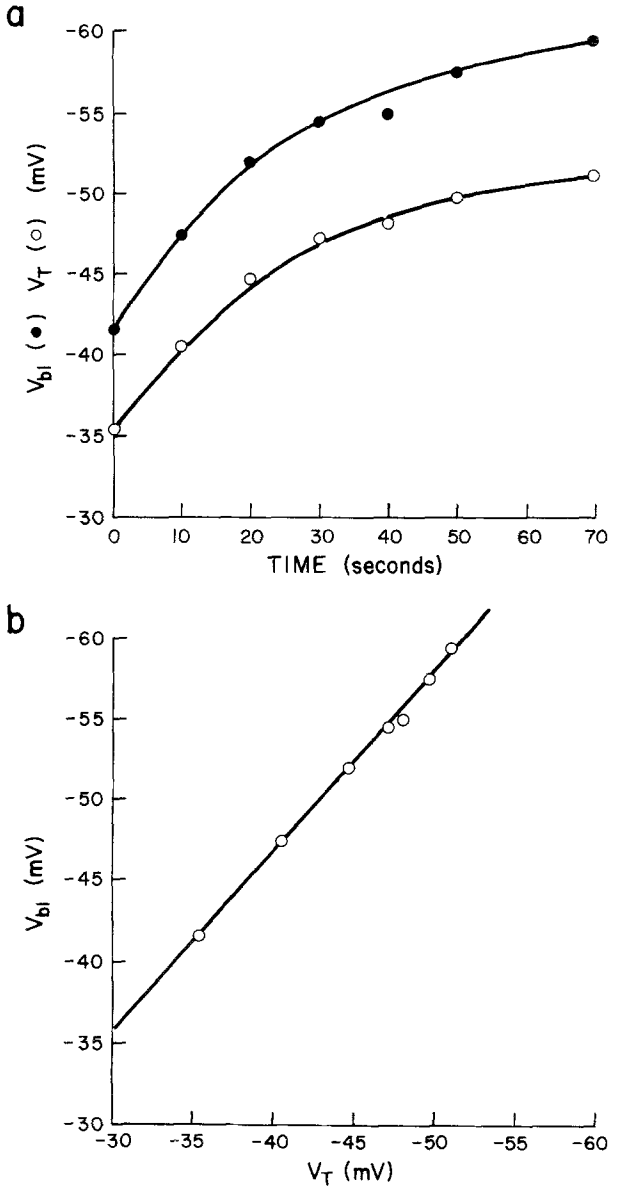

Fig. 3. Response of the basolateral membrane potential (V_{bl}) and transepithelial potential (V_T) to addition of KCl (7 mM) to a K^+-free serosal NaCl Ringer's. K^+ addition to the serosal solution stimulates the $(Na^+ + K^+)$-ATPase. The hyperpolarization is then caused by an *IR* drop across the basolateral membrane and also the junction. In the first 70 sec transepithelial resistance and fractional resistance do not change. Lower frame is a plot of paired measurements of V_{bl} and V_T. A linear regression yields a slope $(1+\gamma)$ of 1.085 ($r^2 = 0.998$) indicating that the junctional resistance is some 12 times greater than the apical resistance [*see* Eq. (3)]. From the transepithelial resistance of 6,100 Ω μF, α of 4.4 and γ of 0.085 we calculate $R_a = 5.5$ KΩ μF, $R_b = 1.3$ KΩ μF and $R_j = 65$ KΩ μF

± 1.2 mV ($n = 5$) again supporting the proposal that the gramicidin channel is more K^+-selective than Na^+-selective. Similar measurements of the apical membrane potential as a function of mucosal cation composition allowed a determination of the selectivity sequence for the gramicidin channel. To estimate this sequence we measured the change in the apical membrane potential after replacing the K^+ in the mucosal bath (in this case a K^+ Ringer's solution) with another cation. Using a modified form of the constant field equation (*see* Lewis et al., 1978) we were able to calculate the relative selective permeability (P_i/P_K) of the gramicidin channel:

$$P_i/P_K = \frac{(K)_1}{(i)_2} \cdot \left(\exp\left(\Delta V_a \cdot \frac{zF}{RT}\right)\right)^{-1} \quad (7)$$

where $(K)_1$ and $(i)_2$ are the activities of K^+ or the replacement cation in the mucosal Ringer's solution, respectively. Table 2 lists the change in apical potential (ΔV_a) for the above cations relative to K^+. Also shown are the resistance ratios and calculated selective permeability.

Table 2. Selective permeability of gramicidin D

Ion	ΔV_a	α	P_i/P_K	P_i/P_K [a]
K^+	0	0.95	1	1
Cs	−2.0	0.82	1.1	1.2
Na	+10.3	1.54	0.67	0.29
Li	+22.4	1.8	0.43	0.08
Choline	+64.1	2.4	0.07	–
Cl^- [b]	+0.7	0.78	0.01	–

[a] Values from Meyers and Haydon (1973) for gramicidin A. (Gramicidin D is composed of approximately 87 % gramicidin A.)
[b] Replaced by $MeSO_4^-$ with K^+ as the counter-ion.

ii) Anions. To test for anion selectivity Cl^- was replaced with $MeSO_4^-$ where K^+ was the counter-ion. Performing a similar calculation as above we found that Cl^- was essentially impermeable through the gramicidin pathway ($P_{Cl}/P_K \cong 0.01$).

Concentration-Conductance Relationship

All results shown so far were obtained at a gramicidin D dose of 50 μM. Figure 4 is a dose-response curve log $1/\alpha$ *vs.* log gramicidin concentration in K^+ solutions. Because of the slow time course of gramicidin action, α was measured when there was negligible change in the transepithelial conductance (G_T). Since G_{bl} is not altered by gramicidin action, log $1/\alpha$ is proportional to log G_a. The lowest dose tried (0.5 μM) has a negligible effect on the measured resistance ratio while doses greater than 50 μM did not cause further increases in the term $(1/\alpha)$. A similar log-log plot of transepithelial conductance versus gramicidin concentration *(not shown)* is fitted well by a linear equation with a slope of 1.

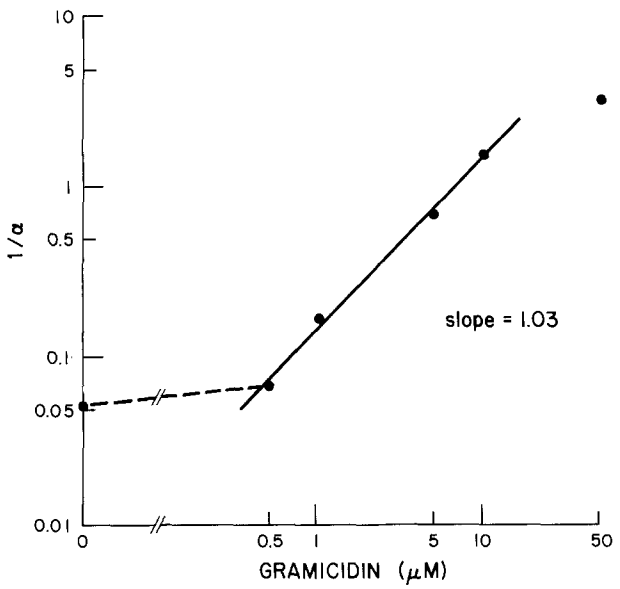

Fig. 4. Dose-response curve for gramicidin D effect on $(1/\alpha) = (G_a/G_{bl})$. Ordinate and abscissa are logarithmic. α was measured when G_T had reached a stable value after each dose ($\cong 800$ sec). Since gramicidin does not alter G_{bl} the ordinate is proportional to G_a. Pre-gramicidin α is represented by an arbitrary low value on the abscissa

Transepithelial Estimates of R_j

To determine junctional resistance (from transepithelial measurements) we used the method of Wills et al. (1979). In brief, this method consists of selectively decreasing apical resistance in the absence of alterations in the electromotive forces of either apical or basolateral membranes. A plot of G_T (transepithelial conductance) versus I_{sc} (short circuit current) will have a slope which is equal to the inverse emf of the cells (the sum of the apical and basolateral emf's; *see* Lewis et al., 1978) and an intercept equal to the paracellular or junctional conductance (*see* Yonath & Civan, 1971). An alternate and, in theory, equivalent plot is V_T versus R_T (Wills et al., 1979). This is a linear double-intercept plot where the V_T intercept is the cellular emf and the R_T intercept is equal to R_j.

For either of these methods to be valid only the cell resistance pathway can change and the cell emf must be constant over the measuring period, i.e., even though we are changing the apical conductance to Na^+ and K^+ (and perhaps Cl^-) the cell ion activities must remain constant.

To approximate this condition mucosal NaCl Ringer's was replaced with a KCl Ringer's (the K^+ activity in the cell and mucosal solution being near equal). Next gramicidin D was added to the mucosal solution and V_T, $R_T(1/G_T)$ and I_{sc} (calculated as

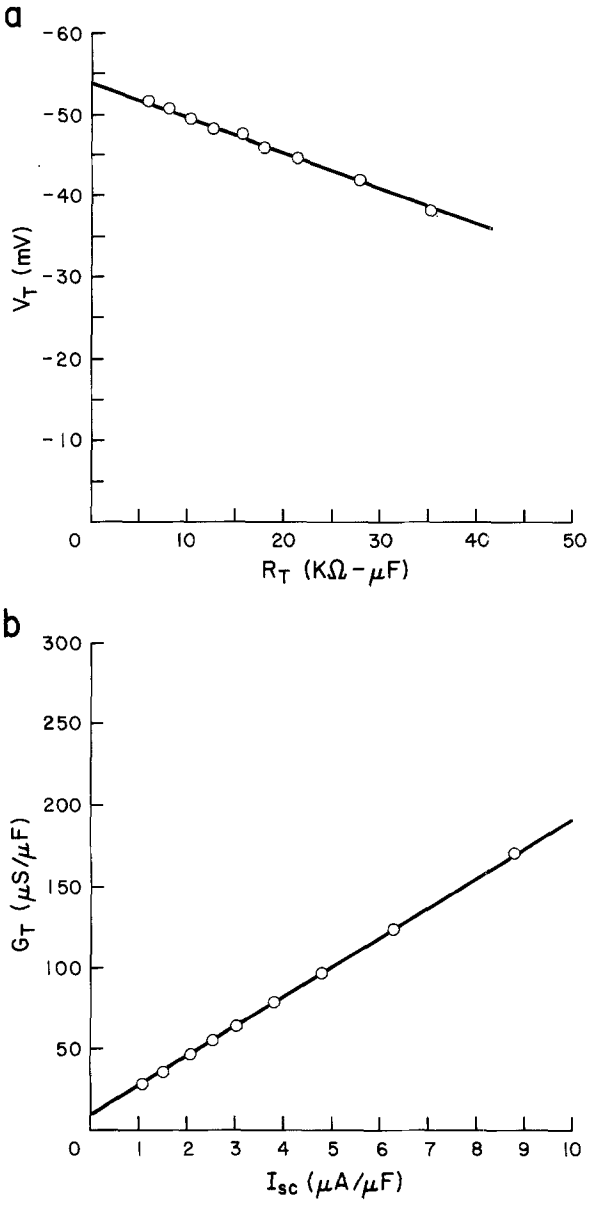

Fig. 5. (*a*) Plot of V_T versus R_T and (*b*) G_T versus I_{sc}. In this experiment gramicidin D was added to a KCl mucosal Ringer's solution. The calculated E_c and R_j for *a* and *b* are 54.2 and 54.1 mV and 120 and 121 KΩ μF, respectively. This example indicates that both systems yield values which are in reasonable agreement (*see* Table 3 for other estimates), with correlation coefficients of 0.998 and 1.000, respectively

V_T/R_T) were monitored.[1] Figure 5*a,b* are plots of V_T versus R_T and G_T versus I_{sc}, respectively. Note that

[1] It must be emphasized that the I_{sc}, in the case where the mucosa is bathed with a KCl Ringer's and the serosa by a NaCl Ringer's, is not a measure of Na^+ transport but rather is simply a measure of net ionic current across the epithelium. As a consequence a plot of G_T *vs.* I_{sc} will have an inverse slope which is equal to the sum of the apical and basolateral electromotive force (emf).

Table 3. Estimates of R_j and E_c from $G_T - I_{sc}$ and $V_T - R_T$

	$I_{sc}-G_T$		r^2	V_T-R_T		r^2	
	R_j (kΩ μF)	E_c (mV)		R_j (kΩ μF)	E_c (mV)		V_{bl} (mV)
KCl Ringer's mucosal solution							
1	130	−54.2	1.00	131	−54.2	0.997	−55
2	57	−57.4	0.999	49	−59.4	0.984	−63
3	65	−53	0.999	50	−55.9	0.954	−52
4	74	−66.8	0.999	70	−67.5	0.996	−64
NaCl Ringer's mucosal solution							
1	−88	−27.3	0.997	487	−29.6	0.01	
2	1,690	−47.5	0.99	119	−50.2	0.17	
3	−2.5	−32.2	0.995	−18	−29.7	0.94	
4	119	−35.2	0.999	356	−34.8	0.05	
5	288	−49	0.999	96	−49.8	0.31	

both plots are straight lines and yield nearly identical values for junctional conductance and cell emf. Table 3 lists the estimates thus obtained for junctional resistance, cell emf and the measured basolateral potential (using a microelectrode).

The previous experiment was repeated with a mucosal NaCl Ringer's. A comparison of the junctional conductance and cell emf determined from the two plots did not agree. An example of these plots is shown in Fig. 6*a*, *b*. This disagreement will be addressed in the Discussion.

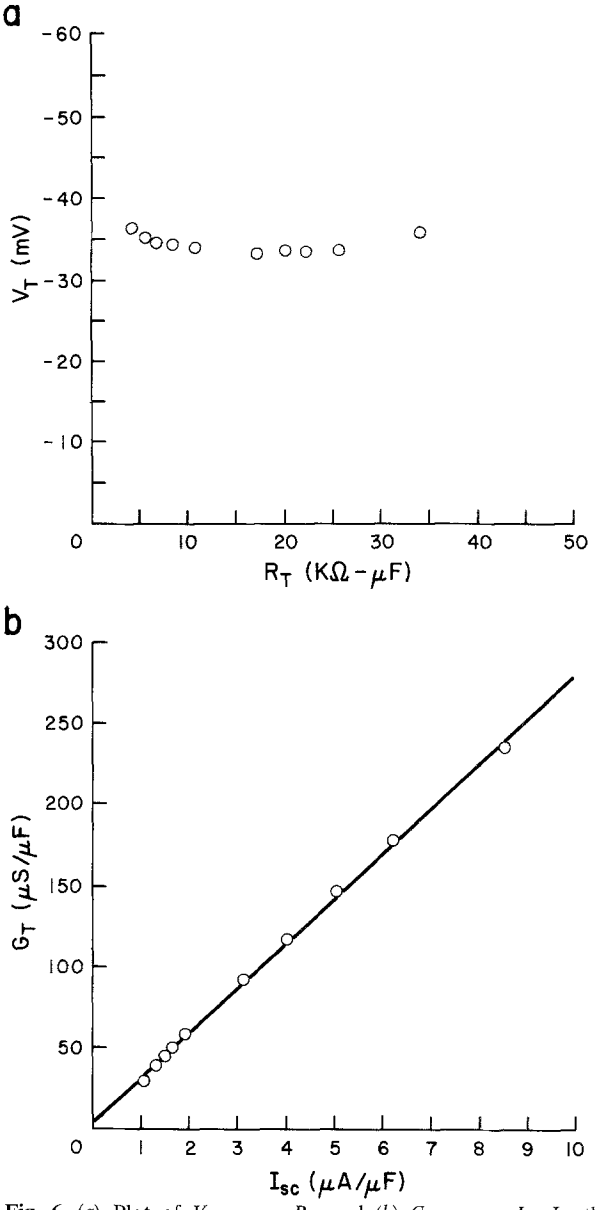

Fig. 6. (*a*) Plot of V_T versus R_T and (*b*) G_T versus I_{sc}. In this experiment gramicidin D was added to a NaCl mucosal Ringer's solution. Note the biphasic response of $V_T - R_T$ (*see a*), while $G_T - I_{sc}$ (*b*) shows a nearly linear response. The E_c and R_j values for *a* and *b* are −34.8 and −35.2 mV, and 356 and 119 KΩ μF, with a r^2 of 0.05 and 0.999, respectively. This disagreement of values indicates that the assumption of constancy of E_c and R_j is being violated

Discussion

In this section we will consider in turn a comparison of the action of gramicidin on the apical membrane of the urinary bladder to planar lipid bilayers, and a new method for determining membrane resistances of this epithelium.

Comparison of Actions

The first reported use of an antibiotic on an epithelium was by Lichenstein and Leaf in 1965. These authors used the polyene antibiotics nystatin and amphotericin B as agents which to a certain extent mimicked the hormonal action of aldosterone. These two antibiotics have recently gained in popularity as tools for studying ion transport across a number of epithelia (Lewis et al., 1977; Frizzell & Turnheim, 1978; Reuss, 1978; Clausen et al., 1979; Wills et al., 1979). As reported by Lewis et al. (1977) nystatin is not perfectly cation-selective ($P_{Na}/P_{Cl} \cong 3.0$) and as a consequence this antibiotic will allow an influx of anions which will result in cell swelling. Unlike nystatin, the gramicidin D channel has negligible anion permeability in both lipid bilayers and the apical membrane of the rabbit urinary bladder. Table 3 lists the relative permeabilities of gramicidin D in the rabbit urinary bladder and in a lipid bilayer indicating that for both preparations anion permeability (in this case Cl^-) is almost immeasurably small. Such a low anion permeability will allow the cells to maintain their volume without the necessity of using

impermeable anion replacements such as SO_4^{2-} which might by themselves alter membrane properties.

In lipid bilayers gramicidin D is known to open and close as two opposing monomers associate (open) and dissociate (close). This formation and degradation of a conductive unit leads, at the single channel level, to step changes of the membrane conductance. When many channels are in the membrane one can only record a mean conductance which reflects at any one time a statistical probability that a constant number of channels will be in the open or conductive configuration. The present finding of gramicidin-induced conductance in the urinary bladder raises the question do gramicidin channels in a biological preparation such as the bladder also "flicker"? Anderson (1977) demonstrated that 2.5×10^{-4} M phloretin increased the mean open or conductive time of a gramicidin channel in a planar lipid bilayer while a similar concentration of phloretin on just the bilayer caused insignificant changes. Therefore a preliminary answer as to whether the gramicidin channel flickers in the bladder membrane might be attained by adding phloretin to the mucosal solution after a stable gramicidin conductance has been reached. In preliminary experiments we found indeed that the apical conductance was increased twofold within 5 min after adding phloretin. Although this initially suggested that phoretin increases the open time of the gramicidin channels, we subsequently found that addition of phloretin to the mucosal solution in the *absence* of gramicidin also caused the apical membrane conductance to increase (deMoura, Wills & Lewis, *unpublished observations*) in a similar manner. Consequently, studies of the kinetic properties of gramicidin in this system appear to require the application of fluctuation analysis.

Membrane and Junctional Resistance Estimates

i) Microelectrodes. Two methods have been used for determining the individual membrane resistances of the rabbit urinary bladder. The first method is one that requires a perturbation of either of the cell membrane resistances. This method has been in use for a number of years, on a variety of tight or high resistance epithelia. Gramicidin D causes a decrease in the apical membrane resistance and the calculated resistances are in good agreement with those determined by other methods.

A second method is to increase or decrease the basolateral membrane emf. If this maneuver can be performed in the absence of changes in the apical membrane or junctional resistances then one can easily calculate the apical, basolateral and junctional resistances by measuring the changes in 1) basolateral membrane potential and transepithelial potential, and 2) the transepithelial resistance and the resistance ratio. These measurements and calculations have been performed for the rabbit urinary bladder (*see* Results) and the values are in excellent agreement with those obtained with other methods.

ii) Transepithelial Measurements. In Results we showed that under the appropriate conditions gramicidin D could be gainfully employed to estimate junctional resistance using two different graphical approaches. When a KCl Ringer's was employed in the mucosal chamber the two approaches yielded nearly identical results for the junctional resistance and cell emf as shown in Table 3. If, however, a NaCl Ringer's was used the two approaches resulted in widely different values. Plots of G_T *vs.* I_{sc} were reasonably linear while V_T *vs.* R_T were definitely nonlinear and in some instances had slopes with sign opposite to that predicted. This discrepancy was obviously caused by either a change in the cell emf or junctional resistance. Microelectrode measurements of the membrane potentials and α indicated that it was a change in emf (both apical and basolateral) and not junctional resistance. This is not surprising since gramicidin will allow an increased influx of Na^+ (mucosa to cell) and also an increased efflux of K^+. This efflux will result in a decrease in cell K^+ activity (this has been measured using a K^+-selective microelectrode) and a decrease in basolateral membrane emf since this membrane potential is a K^+ diffusion potential attenuated by a finite Na^+ permeability (Lewis et al., 1978).

The discrepancy between the two plots is then a problem of the sensitivity of I_{sc} and V_T to changes in cell emf (E_c). I_{sc} is equal to the term $(E_c \cdot G_c)$ while V_T is equal to $(E_c \cdot G_c/(G_c+G_j))$. As long as E_c decreases at a slower rate when compared to G_c then I_{sc} will increase as G_T increases $(G_T = G_c + G_j)$. This, however, is not the case for V_T. V_T will closely follow E_c when cell conductance is much larger than shunt conductance, i.e., V_T will decrease as E_c decreases even though $R_T(1/G_T)$ is increasing.

Therefore, we conclude, when shunt resistance and cell emf are determined from transepithelial measurements a comparison of values from both equations is essential to validate the assumptions of invariant emf and shunt resistance.

In summary, we investigated the effects of the pore-forming antibiotic gramicidin D on the rabbit urinary bladder. This drug seems in many respects to be superior to nystatin, the greatest advantage of this antibiotic being that it is relatively impermeable

to anions. In addition to increasing the apical conductance using this drug we have also indicated how this drug can be easily used to obtain reliable estimates of individual membrane resistances. Future exploitation of the antibiotic will result in a greater understanding of how ions are transported across epithelia.

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Appendix

Derivation of the Equation for Estimating Membrane Resistances by Activation or Inhibition of a Basolateral Membrane Current Source

This method assumes that under open-circuit conditions at *short* time periods E_a, R_a and R_j are not affected by changes in the electrogenic pump or basolateral membrane potential. From the equivalent circuit given in Fig. 2 we can write equations for the basolateral membrane potential and transepithelial potential when the current source is disconnected (pump inactivated):

$$V_{bl}=\left(\frac{E_{bl}}{R_{bl}}-\frac{E_a}{R_a+R_j}\right)\frac{R_{bl}(R_a+R_j)}{R_a+R_{bl}+R_j} \tag{A1}$$

$$V_T=\left(\frac{E_{bl}+E_a}{R_a+R_{bl}}\right)\frac{R_j(R_a+R_{bl})}{R_a+R_{bl}+R_j} \tag{A2}$$

and when the current source is connected in parallel with basolateral membrane electromotive force and resistance (pump activation):

$$V'_{bl}=\left(\frac{E'_{bl}}{R'_{bl}}+I-\frac{E_a}{R_a+R_j}\right)\cdot\frac{R'_{bl}(R_a+R_j)}{R_a+R'_{bl}+R_j} \tag{A3}$$

$$V'_T=\left(\frac{E'_{bl}+IR'_b+E_a}{R_a+R'_{bl}}\right)\frac{R_j(R_a+R'_{bl})}{R_a+R'_{bl}+R_j}. \tag{A4}$$

The change in the basolateral membrane potential upon pump activation is the difference between Eqs. (A3) and (A1):

$$V_{bl}-V'_{bl}=\Delta V_{bl}$$

$$=\frac{(R_a+R_j)[E_{bl}(R_a+R'_{bl}+R_j)-(E'_{bl}+IR'_{bl})(R_a+R_{bl}+R_j)-E_aR_{bl}+E_aR'_{bl}]}{(R_a+R_{bl}+R_j)(R_a+R'_{bl}+R_j)}. \tag{A5}$$

Similarly the change in the transepithelial potential during pump activation is the difference between Eqs. (A4) and (A2):

$$V_T-V'_T=\Delta V_T$$

$$=\frac{(R_j)(E_{bl}(R_a+R'_{bl}+R_j)-(E'_{bl}+IR'_{bl})(R_a+R_{bl}+R_j)-E_aR_{bl}+E_aR'_{bl})}{(R_a+R_{bl}+R_j)(R_a+R'_{bl}+R_j)}. \tag{A6}$$

The ratio of the change in basolateral membrane potential to transepithelial potential [Eqs. (A5) and (A6)] yields:

$$\Delta V_{bl}/\Delta V_T=\frac{(R_a+R_j)}{R_j}=\frac{R_a}{R_j}+1. \tag{A7}$$

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