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Effects of Anions on Cellular Volume and Transepithelial Na^+ Transport across Toad Urinary Bladder

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Summary. The effects of complete substitution of gluconate for mucosal and/or serosal medium Cl^- on transepithelial Na^+ transport have been studied using toad urinary bladder. With mucosal gluconate, transepithelial potential difference (V_T) decreased rapidly, transepithelial resistance (R_T) increased, and calculated short-circuit current (I_{sc}) decreased. Calculated E_{Na} was unaffected, indicating that the inhibition of Na^+ transport was a consequence of a decreased apical membrane Na^+ conductance. This conclusion was supported by the finding that a higher amiloride concentration was required to inhibit the residual transport. With serosal gluconate V_T decreased, R_T increased and I_{sc} fell to a new steady-state value following an initial and variable transient increase in transport. Epithelial cells were shrunken markedly as judged histologically. Calculated E_{Na} fell substantially (from 130 to 68 mV on average). Ba^{2+} (3 mM) reduced calculated E_{Na} in Cl^- Ringer's but not in gluconate Ringer's. With replacement of serosal Cl^- by acetate, transepithelial transport was stimulated, the decrease in cellular volume was prevented and E_{Na} did not fall. Replacement of serosal isosmotic Cl^- medium by a hypo-osmotic gluconate medium (one-half normal) also prevented cell shrinkage and did not result in inhibition of Na^+ transport. Thus the inhibition of Na^+ transport can be correlated with changes in cell volume rather than with the change in Cl^- per se. Nystatin virtually abolished the resistance of the apical plasma membrane as judged by measurement of tissue capacitance. With K^+ gluconate mucosa, Na^+ gluconate serosa, calculated basolateral membrane resistance was much greater, estimated basolateral emf was much lower, and the Na^+/K^+ basolateral permeability ratio was much higher than with acetate media. It is concluded the decrease in cellular volume associated with substitution of serosal gluconate for Cl^- results in a loss of highly specific Ba^{2+}-sensitive K^+ conductance channels from the basolateral plasma membrane. It is possible that the number of Na^+ pump sites in this membrane is also decreased.

Key Words cell volume · anions · Na^+ transport · basolateral K^+ conductance · urinary bladder · Na^+ pump

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Introduction

The urinary bladder of the toad *Bufo marinus* has been used extensively in the study of Na^+ transport across 'tight' epithelia (Macknight, DiBona & Leaf, 1980). In most studies of Na^+ transport attention has focused on such transport with the tissue bathed in Cl^- Ringer's and few studies of the effects of medium anions have been published. Singer and Civan (1971), in a study in which a variety of anions replaced medium Cl^-, suggested that the effects of anion substitution on transepithelial Na^+ transport were mediated at one or more positively charged sites with relatively weak field strength.

Narvarte and Finn (1980, 1983) applied microelectrode techniques to study effects of anion substitution in the mucosal and serosal media. With gluconate or sulfate substituted partially or completely for mucosal Cl^- transepithelial potential difference decreased and resistance increased. They concluded that the primary effect of these anions was to decrease apical plasma membrane conductance (Narvarte & Finn, 1983). Their study of serosal substitution was limited to investigation of the transient stimulatory phase which follows replacement of medium chloride by gluconate, sulfate or iodide.

To further study effects of medium anion substitution on Na^+ transport across toad urinary bladders we have applied conventional techniques to determine open-circuit transepithelial potential difference, resistance, short-circuit current and capacitance, and have combined these with use of the ionophore nystatin to effectively remove the apical membrane resistance and study the properties of the basolateral membrane. Our results provide further evidence that mucosal gluconate decreases transepithelial Na^+ transport by reducing apical membrane Na^+ conductance. Rather than study the

transient stimulation of transport which follows serosal gluconate, we have focused on the inevitable inhibition which results. We have obtained evidence that this inhibition is primarily a consequence of a decreased basolateral membrane K^+ conductance resulting from the decreased cellular volume following the replacement of the permeant anion Cl^- by the nonpermeant anion gluconate.

Materials and Methods

Toads of the species *Bufo marinus*, obtained from the Dominican Republic (National Reagents Inc., Bridgeport, Conn.), were doubly pithed and their hemibladders removed and mounted in Ussing-type chambers giving an exposed surface area of 8.0 cm^2. The serosa was supported on a nylon mesh and a slight excess of mucosal medium held the tissue flat against the mesh. Initially hemibladders were bathed on both mucosal and serosal surfaces with NaCl Ringer's. Air bubbled through the chambers provided both oxygenation and stirring. All experiments were carried out at room temperature (15 to 20°C).

Solutions

Solutions were prepared fresh as required from analytical grade reagents. Sodium chloride Ringer's contained (in mM) Na, 116; K, 3.5; Ca, 1.0; Mg, 1.0; Cl, 122.5; SO_4, 1.0; glucose, 10, buffered at 7.8 by HPO_4, 2. Chloride-free Ringers' were prepared from either the gluconate or acetate salts of Na, K and Ca, and contained (in mM) Na, 116; K, 3.5; Ca, 1.0; Mg, 1.0; SO_4, 1.0; either acetate or gluconate, 122.5; glucose, 10; buffered at pH 7.8 with HPO_4, 2. Sodium-free Ringer's was prepared by equimolar replacement of Na with K, and Ca was omitted when this solution bathed the apical membrane. Hypotonic gluconate Ringer's had the following composition, (in mM): Na, 58; K, 3.5; Ca, 1.0; Mg, 0.5; SO_4, 0.5; gluconate, 61.5; glucose, 5; buffered at pH 7.8 with HPO_4, 1. Though gluconate reduces medium Ca^{2+} activity (Christoffersen & Skibsted, 1975), we did not increase Ca^{2+} concentration in the media as preliminary studies confirmed the findings of Narvarte and Finn (1980, 1983), who found no difference when Ca^{2+} was increased to 4.5 mM.

Nystatin (Sigma Chemicals Co., St. Louis, Mo.) was dissolved in methanol at a concentration of 5 mg ml^{-1} (29,235 U · ml^{-1}) and was added to a mucosal solution to give a final activity of 730 U · ml^{-1}. A range of stock solutions of amiloride (a gift from Merck, Sharp & Dohme, N.Z. Ltd.) in distilled water were prepared, and microliter quantities were added to the mucosal solutions. ADH (pitressin) was obtained from Parke Davis & Co., Sydney.

Electrical Measurements

All experiments were carried out under open-circuit conditions. Spontaneous transepithelial voltage (V_T) was monitored via 3 M KCl agar bridges by calomel electrodes and recorded on a Rikadenki paper-chart recorder. To measure transepithelial resistance (R_T) and its variation with time, square current pulses of 0.5- or 5-sec duration and 40 μA magnitude were passed across the tissue at 1-min intervals via Ag/AgCl electrodes and the resistance was calculated from the resultant steady-state voltage using Ohm's law. All values of R_T were corrected for the solution series resistance. Short-circuit current (I_{sc}) was calculated from R_T and V_T using Ohm's law.

Capacitance (C_T) was determined from the relationship $\tau = R_T C_T$ (where τ = the time constant, R_T = tissue resistance, C_T = tissue capacitance). Individual square current pulses of 0.5- or 5-sec duration and 40 μA magnitude were passed across the tissue and the resultant time-dependent voltage change displayed on a Tekronix storage oscilloscope.

I_{sc} and R_T were normalized with respect to C_T measured in the presence of amiloride (10^{-4} M). Under these circumstances, a value for C_T of 1 μF corresponds approximately to an apical surface area of 1 cm^2, and, as demonstrated by Lewis and Diamond (1976), such estimates of surface area compensate for variations between preparations that arise from different degrees of stretch and folding of the tissue within the chambers.

Capacitance measurements were also used to confirm the action of nystatin. The time course of the voltage following a square current pulse across a tight epithelium conforms to the following equation (Lewis & Diamond, 1976):

$$V_T(t) = V_b e^{-t/R_b C_b} + V_a e^{-t/R_a C_a} \quad (1)$$

where V_T, V_b, and V_a are time-dependent voltages across the whole epithelium, basolateral plasma membranes and apical plasma membranes, respectively, and R_a, R_b and C_a, C_b are the respective resistances and capacitances of the plasma membranes. When R_a is much greater than R_b (e.g. after the addition of amiloride) Eq. (1) reduces to

$$V_T(t) = V_a e^{-t/R_a C_a}. \quad (2)$$

Under these circumstances $V_T(t)$ is expected to follow a single exponential and the effective capacitance calculated from this exponential as τ/R approximates the capacitance of the apical membrane (Lewis & Diamond, 1976). Conversely, after the addition of nystatin, R_b will greatly exceed R_a (Lewis et al., 1977) and so Eq. (1) reduces to

$$V_T(t) = V_b e^{-t/R_b C_b}$$

and calculated tissue capacitance in this instance approximates the capacitance of the basolateral membrane.

From estimates of the relative areas of the apical and basolateral membranes made from histological sections of the epithelial cells of toad bladder, one would predict that the capacitance of the basolateral membrane would exceed that of the apical membrane by a factor of five, approximately. Thus the application of nystatin to the apical membrane and the concomitant marked reduction in apical resistance should result in an appreciable increase in measured tissue capacitance. This effect is demonstrated graphically in Fig. 10.

Histology

At the end of representative experiments, the tissue was fixed in the chambers by the addition of 25% glutaraldehyde to give a final concentration of 1% in both the serosal and mucosal media. After 15 min, the tissue was removed from the chambers and placed in a similar solution for a further 45 min. Tissue was

processed for electron microscopy by postfixation in OsO_4, alcohol dehydration and embedding in resin. Sections were cut for light microscopy and transmission electron microscopy on a LKB III or a Reichert-Jung ultracut E ultramicrotome. Semi-thin sections (1 μm) for light microscopy were stained with methylene blue. Ultrathin sections for electron microscopy were stained with uranyl acetate and lead citrate. They were examined using a Philips EM 3000 electron microscope.

Calculation of Electrical Parameters

A simple electrical equivalent circuit of the toad bladder epithelium can be drawn for each protocol used (Fig. 1), and, by means of circuit analysis, values for each of the circuit components can be determined.

Solutions of Similar Composition on Both the Serosal and Mucosal Surfaces

The electrical equivalent of the epithelium during this protocol is shown in Fig. 1*a* and the transepithelial voltage described by

$$V_T = E_c \frac{R_j}{(R_c + R_j)}$$

where R_c = transcellular resistance, which is equivalent to the sum of the apical membrane resistance (R_a) and the basolateral membrane resistance (R_b), E_c = the cellular electromotive force (emf) which is equivalent to the sum of the apical emf (E_a) and basolateral emf (E_b). Under steady-state conditions, $E_c = E_{Na}$ (Schultz, Frizzell & Nellans, 1977; Lewis, Wills & Eaton, 1978). R_j is the junctional resistance.

An estimate of the values of E_c and R_j can be obtained by the method of Yonath and Civan (1971) as applied by Wills, Lewis and Eaton (1979). In brief, this entails selectively altering the apical membrane resistance in the absence of any alterations in the electromotive forces across either the apical or basolateral membranes. Under these conditions a graph of V_T against R_T gives a linear double intercept plot described by the following equation,

$$\frac{V_T}{E_c} + \frac{R_t}{R_j} = 1.$$

The V_T intercept of such a plot is equivalent to the cellular emf (E_c) and the R_T intercept to the junctional resistance R_j. The value of R_c can then be obtained since

$$\frac{1}{R_c} = \frac{1}{R_T} - \frac{1}{R_j}.$$

Solutions of Different Composition on the Apical and Basolateral Surfaces

Because of the differences in the junctional permeability to the various ions on either side of the epithelium, a junctional potential (E_j) is generated (Fig. 1*b*) and, in this instance, the transepithelial potential (V_T) is given by

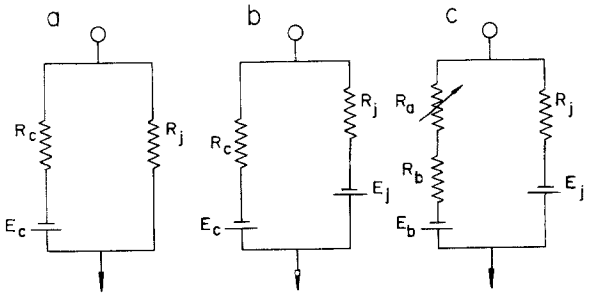


Fig. 1. Equivalent electrical circuits for the toad bladder epithelium under the various conditions reported in the Results. (*a*) Similar mucosal and serosal solutions. (*b*) Mucosal and serosal solutions of different composition. (*c*) Nystatin added to the mucosal chamber at final activity of 730 units/ml. Note, in this instance solutions of different composition are present in the serosal and mucosal chambers. E_c = transcellular emf, equivalent to sum of apical (E_a) and basolateral (E_b) emf. R_c, transcellular resistance, equivalent to sum of apical (R_a) and basolateral (R_b) resistances. R_j = junctional resistance; E_j = junctional emf arising from selective ionic junctional permeability

$$V_T = \left(\frac{E_c}{R_c} + \frac{E_j}{R_j}\right)\left[\frac{R_c R_j}{R_c + R_j}\right].$$

Estimates of E_j and R_j were obtained by adding amiloride to the mucosal surface at a concentration of 10^{-4} M. Under these conditions R_c approaches infinity and the measured resistance and potential across the epithelium will approximate the values of R_j and E_j. Assuming E_j and R_j are the same during and after the addition of amiloride, a measure of R_c in the absence of amiloride can be obtained using

$$g_T = g_c + g_j$$

when g_j = junctional conductance measured in the presence of amiloride and g_T = transepithelial conductance in the absence of amiloride. Furthermore, knowing R_c, R_j and E_j for various conditions, measurement of V_T under similar conditions allows the calculation of E_c

$$E_c = \frac{V_T g_T - E_j g_j}{g_c}.$$

Nystatin-Treated Bladders with Different Solutions on Either Side

The addition of nystatin to the mucosal solution nonselectively reduces the apical membrane resistance. Furthermore, the emf of the apical membrane (E_a) is reduced to approximately zero and is thus eliminated from the electrical circuit (Fig. 1*c*). Thus, in the presence of nystatin, the transepithelial voltage is given by

$$V_T = \frac{E_b}{R_b} + \frac{E_j}{R_j}\left[\frac{R_b R_j}{R_b + R_j}\right].$$

Estimates of E_j and R_j were obtained prior to the addition of nystatin by the use of 10^{-4} M amiloride (*see above*). Assuming

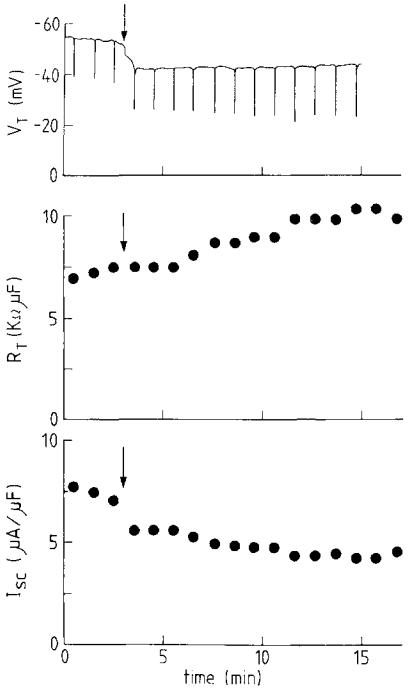


Fig. 2. Effect of mucosal Cl^- substitution on transepithelial voltage (V_t), transepithelial resistance (R_T) and calculated short-circuit current (I_{sc}). Initially the hemibladder was bathed with NaCl Ringer's on both the serosal and mucosal surfaces. Replacement of mucosal Cl by gluconate is indicated by the arrow

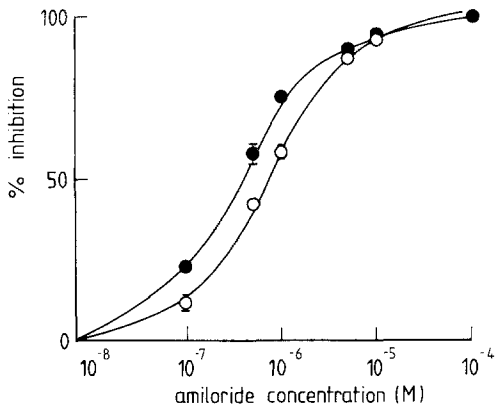


Fig. 3. Dose-response curve for the inhibitory effect of amiloride, added to the mucosal solution at the indicated concentration, on the calculated short-circuit current (I_{sc}). Closed circles (●) indicate values for hemibladders bathed with mucosal Cl Ringer's; open circles (○), values for hemibladders in which the mucosal Cl^- was completely replaced with mucosal gluconate. Vertical bars indicate SEM when greater than the symbol (n = 6)

these change very little following the addition of nystatin, the transcellular resistance, which is now equivalent to the basolateral resistance (R_b), can be determined from the following

$$g_T = g_b + g_j$$

and E_b can be calculated from the original equation describing V_T in the presence of nystatin, since R_j, E_j and R_b are now known.

$$E_b = \frac{V_T g_T - E_j g_j}{g_b}.$$

Values for the electrical parameters given in the Results section and in the Tables are means ± SEM of the number of observations shown in parentheses. Comparison between means were made using Students 't'-test.

Results

First, the effect on the magnitude of the net Na^+ transport across the toad urinary bladder of replacement of mucosal Cl^- with a relatively impermeant anion is described. Next, we demonstrate that changes in transepithelial Na^+ transport that follow serosal Cl^- replacement depend upon the substitute anion and can be correlated with changes in cell volume. To determine whether the stimulatory or inhibitory influence of Cl^- replacement is localized to the apical or basolateral membrane, nystatin, a pore-forming antibiotic, was used to decrease the apical membrane resistance rapidly. We could now investigate the influence of serosal anion substitution on the electrical properties of a preparation which is essentially a basolateral membrane in parallel with the tight junctions. We examined the passive permeability properties of the basolateral membrane and some characteristics of Na^+ activation of the rheogenic Na^+ pump.

Replacement of Mucosal Chloride

When gluconate replaced mucosal Cl^- the transepithelial potential (V_T) decreased rapidly accompanied by an increase in transepithelial resistance. Calculated short-circuit current (I_{sc}) decreased (Fig. 2). Table 1 summarizes results from eight experiments. The decrease in V_T though rapid was relatively small and just failed to reach statistical significance. Changes in R_T and I_{sc} were significant, I_{sc} decreasing by some 35 ± 7%. This inhibition of current was rapidly reversed when Cl^- was restored to the mucosal medium. Since the apical plasma membrane of the epithelial cells in the urinary bladder of the Dominican toad is predominantly, if not exclusively, permeable to Na^+ (Macknight, 1977), these changes are not the direct consequence of the substitution of gluconate for Cl^-, but must result from a modification of Na^+ transport across the tissue.

There are a number of possible explanations for this inhibitory effect of gluconate. These include:

Table 1. Effects of gluconate substituted for mucosal Cl^- [a]

	V_T (mV)	R_T (kΩ · μF)	I_{sc} (μA/μF)	R_j (kΩ · μF)	E_{cell} (mV)
NaCl Ringer's	53 ± 6	10.5 ± 2.5	8.6 ± 2.8	26.4 ± 4.1	123 ± 4
Mucosal Na^+ Gluconate Serosal NaCl Ringer's	45 ± 7	13.7 ± 2.9	6.1 ± 2.5	29.5 ± 3.6	121 ± 11
Δ	8	3.2	2.5	3.1	2
P	>0.05	<0.001	<0.02	>0.50	>0.80

[a] Values are means ± SEM ($n = 8$).

first, a decrease in the net electrochemical driving force for Na^+ entry into the cell caused by either an increase in cell Na^+ or by a depolarization of the apical membrane potential (cell interior becoming more positive with respect to the mucosal solution); secondly, a decrease in the number of conducting Na^+ channels in the apical membrane, which could result from either an increased rate of Na^+ binding to an external inhibitory receptor or a decreased rate of Na^+ dissociation from the receptor (as proposed by Fuchs, Larsen & Lindemann, 1977); thirdly, a decrease in the permeability of a single Na^+ channel.

Although it is not possible from our measurements to differentiate between the second and third possibilities, we can determine whether the driving force for Na^+ entry has been decreased by calculating the E_{Na} with mucosal Cl^- and with mucosal gluconate. The parameter E_{Na}, as calculated, equals the sum of the electromotive forces, E_c, across the apical and basolateral membranes (*see* Materials and Methods; *also* Schultz et al., 1977; Lewis et al., 1978). A decrease in E_{Na} indicates a decrease in basolateral emf and/or a decrease in apical emf. Table 1 shows that E_{Na} did not change significantly, while junctional resistance increased slightly though not significantly. Since gluconate is less permeant than Cl^-, such an increase would be expected. Therefore, since E_{Na} was unchanged, the decrease in I_{sc} following gluconate did not result from an alteration of the cell Na^+ concentration nor from changes in apical (or basolateral) membrane potentials.

The concentration of amiloride required to halve the rate of Na^+ transport across toad urinary bladder is dependent on the Na^+ concentration in the mucosal solution, i.e. Na^+ and amiloride demonstrate competitive inhibition (Bentley, 1968). If gluconate alters the kinetics of Na^+ binding one might expect to measure a change in the dose-response curve for amiloride inhibition of current. Dose-response curves for Cl^- and for gluconate media are shown in Fig. 3. The best-fit values for the K_I (concentrations of amiloride required to decrease the current by half) are 0.3 μM for Cl^- Ringer's and 0.82 μM for mucosal gluconate Ringer's. Therefore, gluconate not only depressed Na^+ transport but a higher concentration of amiloride is required to inhibit the residual transport.

We conclude that mucosal gluconate decreases Na^+ transport by decreasing apical membrane Na^+ permeability and not by altering the net electrochemical gradient driving Na^+ entry across the apical membrane. Whether gluconate modifies single-channel permeability or the density of conducting Na^+ channels is unknown.

Replacement of Serosal Chloride

Replacement by Gluconate

Unlike mucosal Cl^- replacement, which caused a rapid decrease in Na^+ transport, replacement of serosal Cl^- with gluconate resulted in changes in Na^+ transport which were time dependent, and there was often an initial increase in Na^+ current followed by a decay to a current lower than control. Figure 4 illustrates one such response. The time required to reach a new (lower) steady-state value correlated directly with the control I_{sc} (Fig. 5). The delay between the addition of gluconate and the decrease in I_{sc} suggests that gluconate does not modify the passive or active properties of the basolateral membrane directly. Instead, it might initiate a chain of events which ultimately results in a modification of the permeability of either the apical or the basolateral membranes.

As with mucosal gluconate, serosal gluconate could inhibit transepithelial Na^+ transport in a number of different ways. Possibilities include: a decrease in apical membrane Na^+ permeability, an alteration in pump kinetics resulting in an increase in

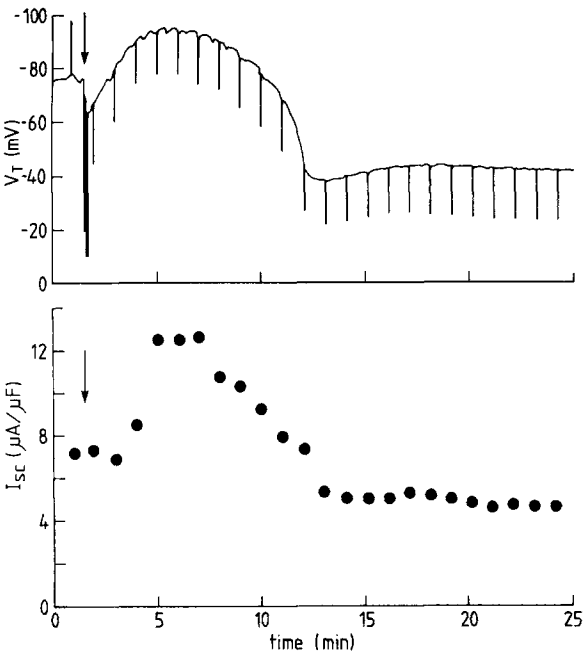


Fig. 4. Effect of substitution of gluconate for serosal Cl^- on transepithelial voltage (V_T) and calculated short-circuit current (I_{sc}). Initially the hemibladder was bathed with normal NaCl Ringer's on both the serosal and mucosal surfaces. The replacement of serosal Cl^- by gluconate is indicated by the arrow. Note that the response to serosal gluconate was time dependent. There was an initial increase in I_{sc} followed by a decrease to a current value lower than that in the control NaCl Ringer's

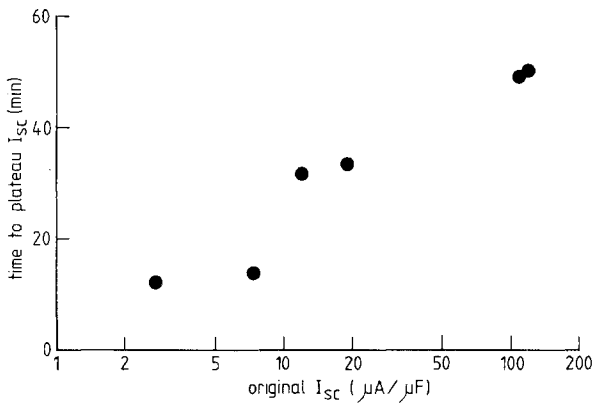


Fig. 5. Relation between the original I_{sc} and the time taken before the new lower plateau I_{sc} was achieved following the substitution of serosal Cl^- with gluconate

cell Na^+, a decrease in basolateral membrane passive permeability to K^+ (which would increase basolateral membrane resistance), or an increase in basolateral membrane permeability to Na^+ (which would decrease basolateral resistance).

One can differentiate between the first possibility and the latter three by calculating E_{Na}. This parameter will not change if apical membrane permeability is decreased, but will decrease if cell Na^+ increases or if the basolateral membrane Na^+ to K^+ permeability ratio increases (causing the basolateral membrane potential to depolarize).

Since E_c was not affected by mucosal gluconate alone (Table 1), experiments to estimate E_c with serosal gluconate were performed with gluconate replacing Cl^- in both serosal and mucosal media. This avoided the need to correct the measurements for E_j. Table 2 shows that, on average, the cell emf decreased from 130 to 68 mV. This decrease certainly rules out the possibility that decreased apical membrane Na^+ permeability is the sole cause of the decrease in Na^+ transport with gluconate as serosal anion. Furthermore, in four experiments serosal addition of ADH to a preparation bathed with serosal gluconate caused a 66 ± 13% increase in I_{sc} without any change in the calculated cell emf of 68 ± 6 mV. As expected, therefore, increasing apical membrane Na^+ permeability did not, of itself, affect cell emf. The fact that such a large increase in Na^+ transport can occur after ADH suggests that the pump itself is not inhibited directly by serosal gluconate.

A number of epithelia contain Ba^{2+}-sensitive K^+ channels in their basolateral membranes (Lewis, Hanrahan & Van Driessche, 1984). In four experiments addition of 3 mM Ba^{2+} to the serosal solution of bladders bathed in Cl^- Ringer's caused a 27 ± 2% decrease in I_{sc} and a decrease in cell emf from 122 ± 4 mV to 79 ± 5 mV. In contrast, the addition of 3 mM Ba^{2+} to the serosal solution of bladders bathed in gluconate Ringer's with a mean cell emf of 55 ± 3 mV did not decrease I_{sc} or cell emf further. These results suggest that serosal gluconate in some way inactivates Ba^{2+}-sensitive K^+ pathways in the basolateral membranes. Thus they indicate that the decrease in cell emf and in I_{sc} with serosal gluconate is a consequence of an increase in basolateral membrane resistance reflecting loss of specific K^+ channels associated with a decrease in basolateral membrane emf.

How does gluconate elicit this change in basolateral membrane properties and in transepithelial Na^+ transport? Hughes and Macknight (*unpublished observations*), analyzed epithelial cells scraped from toad urinary bladders after 120-min incubation in Cl^--free gluconate Ringer's. Cellular water content had decreased by 28% and cell Cl^- content was essentially zero. This raises the possibility that the decrease in current and in basolateral membrane K^+ permeability may be correlated directly with a decrease in cell volume.

This possibility was investigated by studying light- and electron-micrographs of paired hemiblad-

Table 2. Effects of gluconate substituted for mucosal and serosal Cl^- [a]

	V_T (mV)	R_t (kΩ · μF)	I_{sc} (μA/μF)	R_i (kΩ · μF)	E_{cell} (mV)
NaCl Ringer's	57 ± 6	7.7 ± 0.9	8.2 ± 1.2	14.4 ± 1.7	130 ± 6
Na Gluconate Ringer's	16 ± 3	12.8 ± 1.5	1.3 ± 0.2	17.3 ± 2.0	68 ± 4
Δ	45	5.1	6.9	2.9	63
P	<0.001	<0.001	<0.001	>0.05	<0.001

[a] Values are means ± SEM (n = 12).

ders, one incubated in Cl^- Ringer's, the other in gluconate Ringer's. As can be observed readily, tissue incubated in gluconate Ringer's had a shrunken appearance when compared to tissue incubated in Cl^- Ringer's (Fig. 6). This supports the hypothesis that a decrease in cell volume was associated, directly or indirectly, with the decrease in basolateral membrane permeability.

Replacement by Acetate

Even though a decrease in cell volume accompanied the decreases in I_{sc} and in cell emf with serosal gluconate, one can speculate that these changes in electrical parameters resulted from the loss of cell Cl^- content rather than from the decreased cell volume. To test this hypothesis we selected acetate as a Cl^- replacement. In another epithelial tissue (mammalian renal cortex, Cooke & Macknight, 1984), substitution of acetate for medium Cl^- results in an appreciable cellular swelling and replacement of cellular Cl^- with acetate. Light- and electron-microscopy (Fig. 7*a,b*) demonstrate that in toad bladder epithelial cells acetate prevents the shrinkage of the cells associated with gluconate. Indeed some cells appear to have swollen somewhat under these conditions.

Replacing serosal Cl^- with acetate caused a time-dependent increase in short-circuit current (Fig. 8). This increase averaged 25 ± 7% in eight experiments. Cell emf averaged 124 ± 6 mV (n = 4) under these conditions. As in tissues bathed with chloride, addition of 3 mM Ba^{2+} to the serosal medium reduced cell emf appreciably. Since the loss of cell Cl^- without cell shrinkage did not decrease I_{sc} or cell emf, the decreases in these parameters caused by gluconate are more likely to be a direct consequence of the decreased cell volume than of the decreased cell Cl^-. This conclusion is supported by the effects of hypo-osmotic gluconate Ringer's, which also prevented cellular shrinkage (Fig. 7*c*) and in which transepithelial Na^+ transport was maintained at values comparable to those in Cl^- Ringer's.

Isolation of the Basolateral Membranes

The data presented so far suggest that serosal gluconate exerts its effect by decreasing cell volume. This then results in a decrease in basolateral membrane K^+ permeability. Because of the series arrangement of the apical and basolateral membranes, it is difficult using only transepithelial techniques to study the response to serosal gluconate or to acetate of the basolateral membrane alone. However, the antibiotic nystatin virtually abolishes the apical membrane resistance in rabbit urinary bladder (Lewis et al., 1977) and descending colon (Wills et al., 1979). In this section we will demonstrate *i*) that nystatin is equally rapid and potent in reducing the apical membrane resistance of toad urinary bladder, *ii*) that the change in transepithelial voltage following nystatin is greatly diminished in gluconate Ringer's, and *iii*) that basolateral K^+ permeability can be switched on by acetate and by half osmotic strength gluconate Ringer's and off again by changing from a dilute gluconate medium to an isosmotic gluconate Ringer's. Using nystatin, we estimated the basolateral membrane Na^+ and K^+ permeabilities and the concentration dependence of Na^+ activation of the rheogenic Na^+ pump.

i) Characteristics of the Action of Nystatin

Addition of nystatin at a final activity of 730 units/ml to the mucosal chamber of a toad urinary bladder bathed in K^+-gluconate mucosa and Na^+-gluconate serosa, in which there can be only residual Na^+ transport, rapidly decreased resistance and increased potential, both of which plateaued (in 14 out of 18 preparations) within 4 min to new steady-state values (Fig. 9). The remaining four preparations demonstrated a slow increase in voltage and decrease in resistance. On first inspection, one might

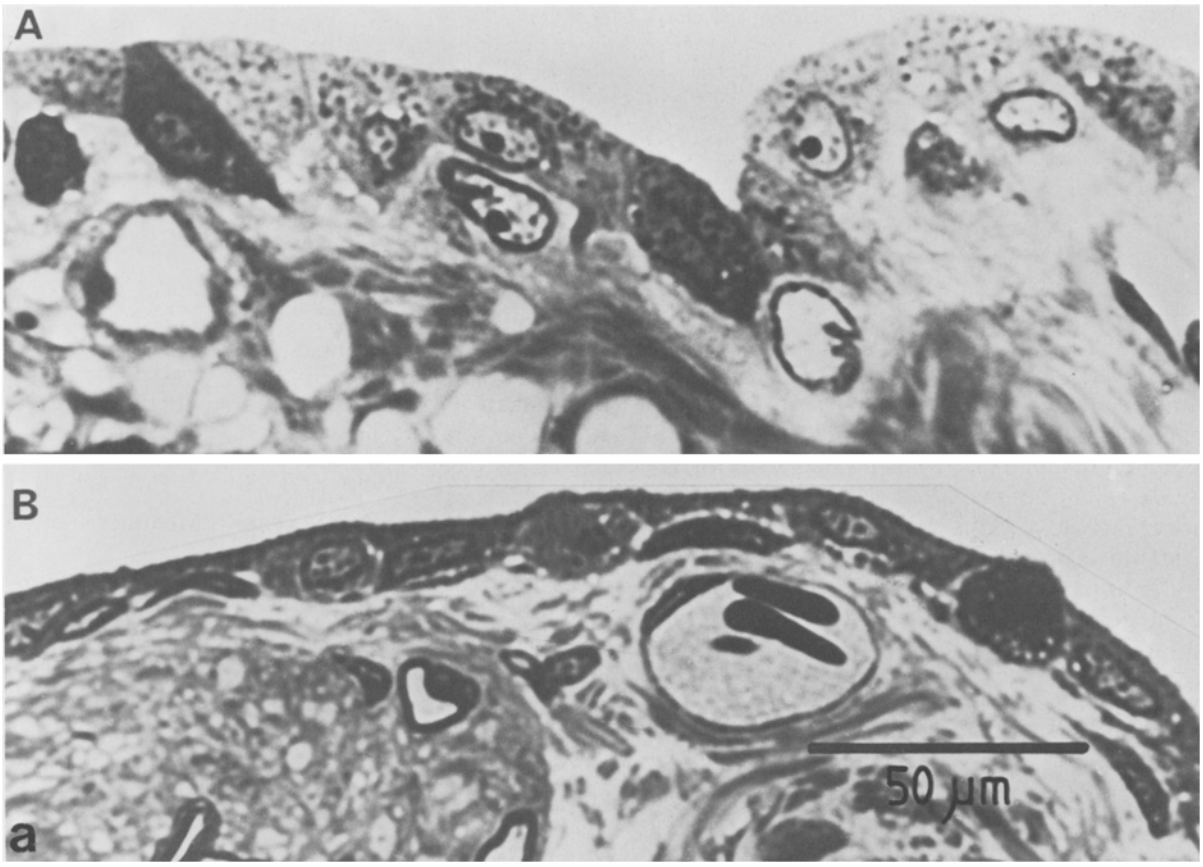
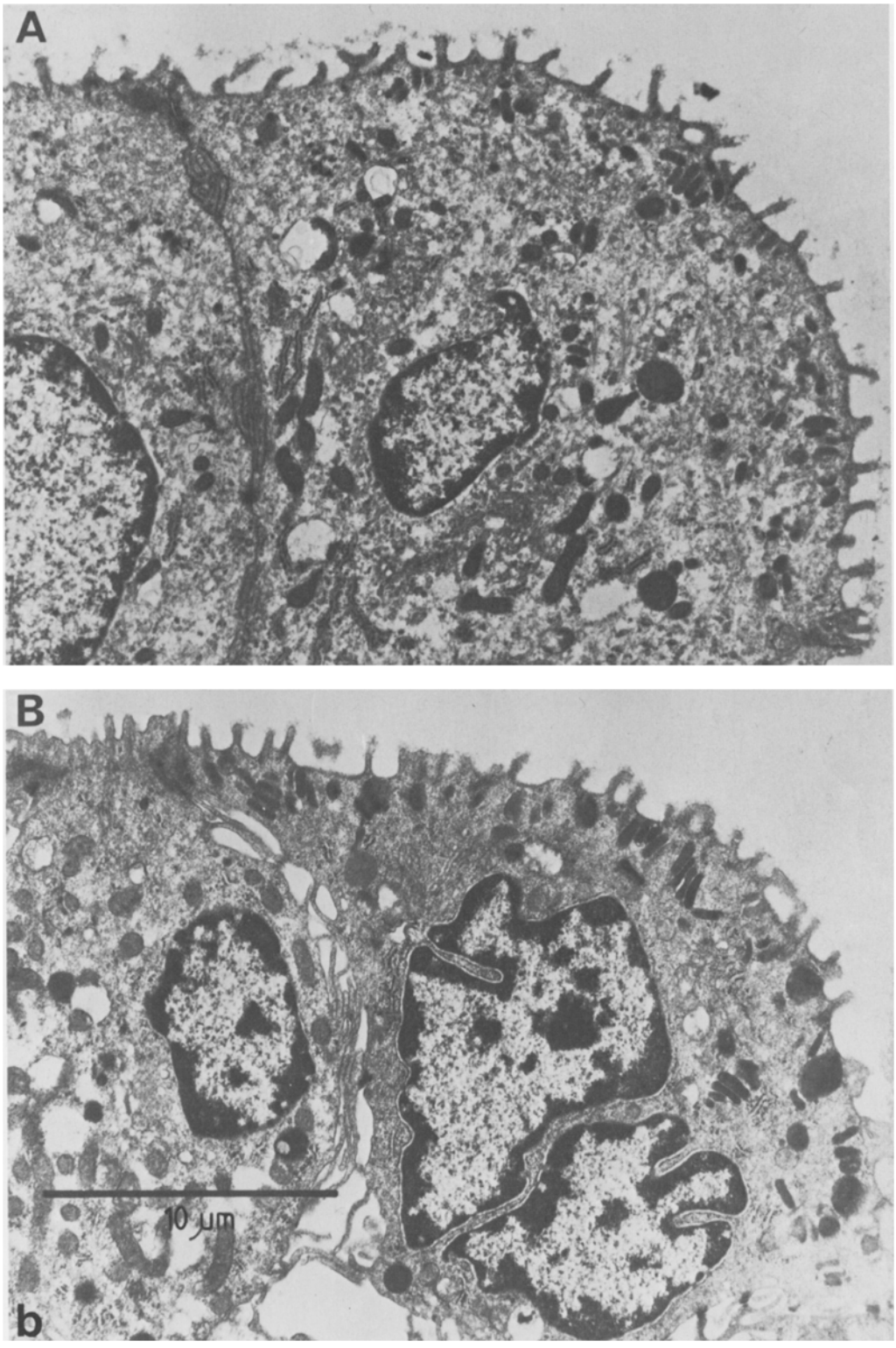


Fig. 6. (*a*) Light micrographs of toad bladder epithelial cells incubated in NaCl Ringer's mucosa and serosa (*A*) or in Na^+ gluconate Ringer's mucosa and serosa (*B*). (Magnification 500×). (*b facing page*) Electron micrographs of toad bladder epithelial cells incubated in NaCl Ringer's mucosa and serosa (*A*) or in Na^+ gluconate Ringer's mucosa and serosa (*B*). (Magnification 4750×)

conclude that for the majority of preparations nystatin was very ineffective in reducing the apical resistance and that this could account for the very small increase in transepithelial voltage and minor decrease in transepithelial resistance. However, using capacitance measurements, one can demonstrate that the apical membrane resistance has been reduced dramatically. Lewis et al. (1977) demonstrated that when the apical membrane resistance is large compared to the basolateral membrane resistance, the calculated capacitance (*see* Materials and Methods) will approximate the apical membrane capacitor. Conversely, when the basolateral resistance greatly exceeds the apical membrane resistance (as expected after full nystatin action) the calculated capacitance will approximate that of the basolateral membrane. Epithelial capacitance was measured before and up to approximately 4 min after the addition of nystatin. Table 3 shows (for the 14 experiments) that even though the transepithelial resistance changed only slightly, the capacitance increased from a mean of 2.0 $\mu F/cm^2$ to 12.9 $\mu F/cm^2$ and the resistance decreased from 7600 to 5100 Ω cm^2. Such a large increase in capacitance is indicative of a dramatic decrease in apical membrane resistance.

As previously mentioned 4 of the 18 experiments demonstrated a biphasic response of voltage and resistance to nystatin action: a fast change, similar in time course to the majority of experiments, followed by a slower decrease in resistance and increase in voltage (taking up to an hour to reach a new steady-state value). If the rate of nystatin action was slower for these four preparations then the capacitance would increase significantly after 4 min. Figure 10 demonstrates that this does not happen. In the first 4 min capacitance changes from 2.5 to 15.3 $\mu F/cm^2$ and cell resistance from 60 to 6.7 $k\Omega$ cm^2, after 1 hr resistance decreased to 4 $k\Omega$ cm^2 but the capacitance increased only slightly from 15.3 to 15.7 $\mu F/cm^2$. The large decrease in resistance but small increase in capacitance indicates that the slow phase is due to a decrease in basolateral resistance. Two lines of evidence suggest that the



slow decrease in cell resistance is due to the appearance of K^+-selective pathways in the basolateral membrane. First, the addition of 3 mM Ba^{2+} to the serosal solution of nystatin-treated bladder always reduced the cell emf to a value of *ca.* 20 mV, as shown in Fig. 11. Thus bladders with a low cell emf showed little response to Ba^{2+}, while bladders which demonstrated the slow hyperpolarization

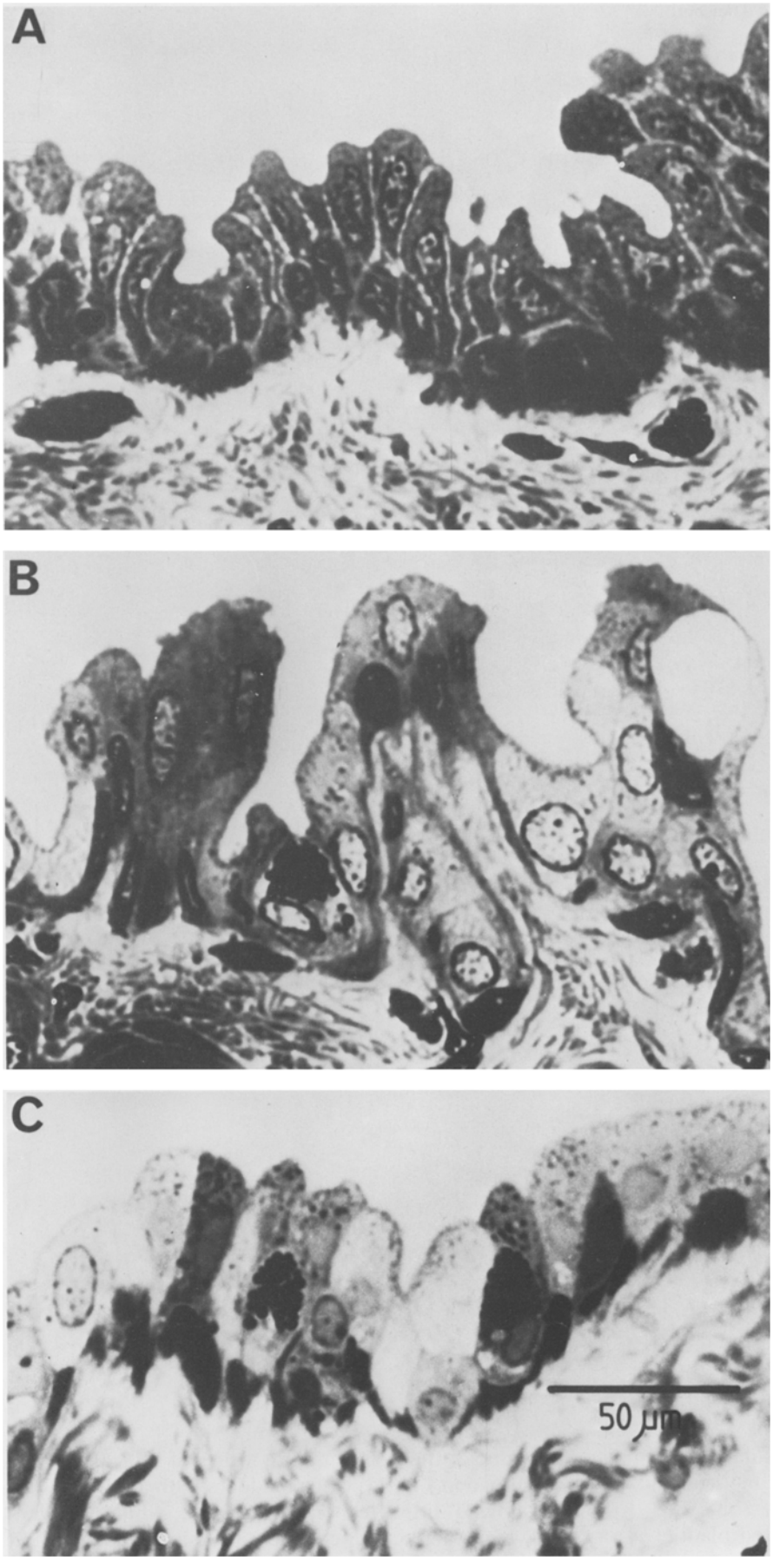


Fig. 7. Light micrographs of toad bladder epithelial cells incubated in gluconate medium (*A*), acetate medium (*B*) or hypo-osmotic gluconate medium (*C*). (Magnification 500×)

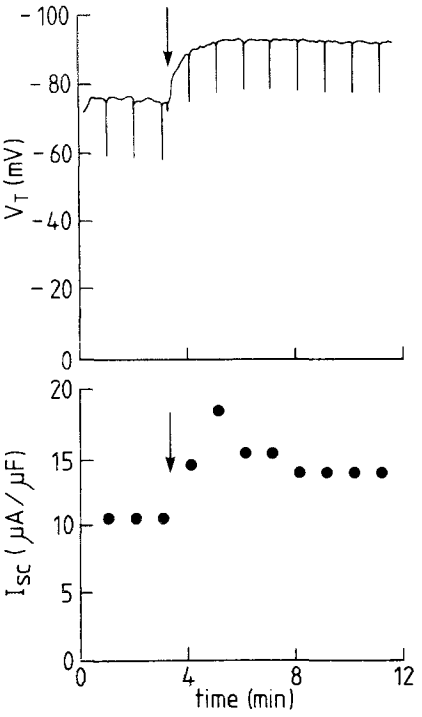


Fig. 8. Effect of substitution of acetate for serosal Cl^- on V_T and I_{sc}. Initially the hemibladder was bathed with NaCl Ringer's on both the serosal and mucosal surfaces. Replacement of serosal Cl^- by acetate is indicated by the arrow

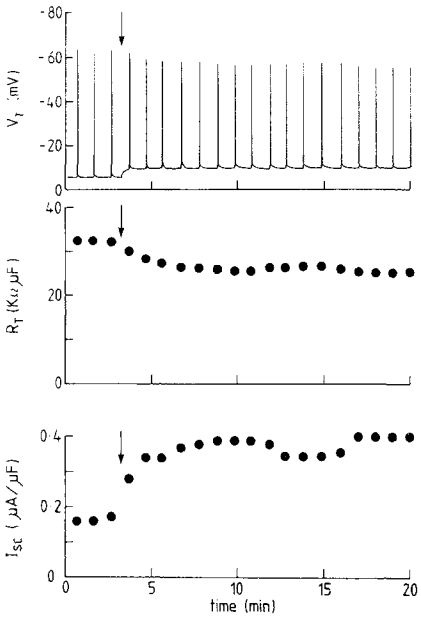


Fig. 9. A typical response of a hemibladder, bathed by gluconate Ringer's, following the addition of nystatin to the mucosal chamber. The bladder was bathed by a K^+ gluconate mucosal solution and a Na^+ gluconate serosal solution. The addition of nystatin (730 U · ml^{-1}) to the mucosal chamber is indicated by the arrow. Note that, although the bladder responded rapidly to the addition of the nystatin, there was little change in either V_T or R_T

demonstrated, after reaching a new steady state, a marked response to serosal Ba^{2+} addition. Second, the calculation of the selective permeability of the basolateral membrane (by equimolar replacement of Na^+ by K^+) demonstrated a Na^+ to K^+ permeability ratio of *ca.* 0.05 for bladders with biphasic response (Fig. 12) and *ca.* 0.3 for bladders with a monophasic response (*see* Table 3).

In summary, even though large decreases in tissue resistance or increases in transepithelial voltage were not generally found, capacitance measurements reveal that nystatin has altered apical membrane resistance profoundly.

ii) Response to Nystatin as a Function of the Serosal Anion

Figure 9 illustrates the response of the epithelium to the addition of nystatin with K^+-gluconate mucosa and Na^+-gluconate serosa. If the epithelium is preincubated instead in mucosal K^+-acetate and serosal Na^+-acetate solutions, and then nystatin is added to the mucosal solution, the resistance and voltage responses differ significantly as illustrated in Fig. 13. The voltage rapidly increases and plateaus within 4 min while, with a similar time course, the resistance rapidly decreases to a plateau value

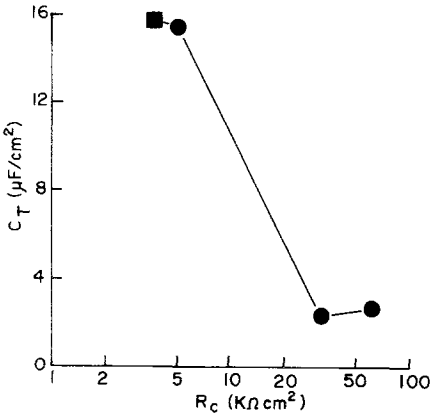


Fig. 10. The value of transepithelial capacitance (C_T) over a 4-min interval (●) and 1 hr (■) following the addition of nystatin (730 U · ml^{-1}) to the mucosal chamber. Points represent values for a single hemibladder bathed by mucosal K^+ gluconate solution and serosal Na^+ gluconate solutions. *See* text for details

which is significantly lower than before nystatin. Table 3 summarizes the transepithelial voltage, resistance, and capacitance, the cell emf (E_c), cellular resistance, and the Na to K permeability ratios of the paracellular pathway (P^i_{Na}/P^i_K) and of the cellular pathway (P^c_{Na}/P^c_K), before and after the addition of

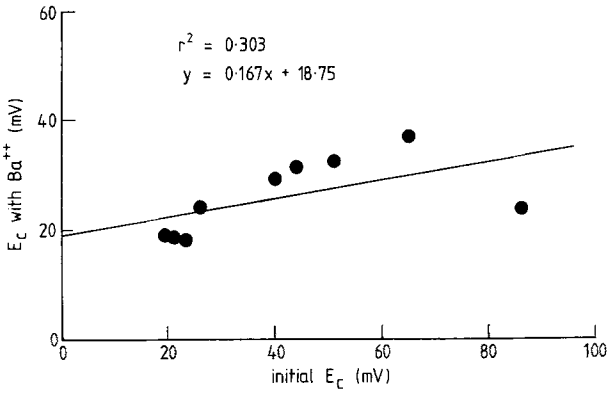


Fig. 11. The relation in gluconate-Ringer's between the steady-state cell emf (E_c) of nystatin-treated hemibladders, and the cell emf following the addition of 3 mM Ba^{2+} to the serosal solution. Each point represents a value for an individual hemibladder. Solid line represents line of best fit for the points

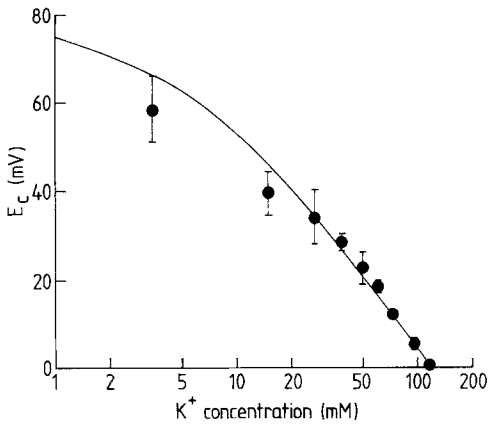


Fig. 12. Changes in the cell emf (E_c) of nystatin-treated hemibladders as a function of serosal K^+ concentration. The curve was generated from the constant field equation assuming a Na^+ to K^+ permeability ratio of 0.05 and that gluconate behaved as an impermeant anion

nystatin to bladders in experiments in which either gluconate or acetate was the dominant medium anion. The most notable features are that, after nystatin, V_T and E_c (now the basolateral emf) are larger, and R_c and P^c_{Na}/P^c_K are smaller when the bladder is bathed in an acetate Ringer's compared to a gluconate Ringer's.

As with acetate, transepithelial voltage increased, and transepithelial resistance decreased rapidly in response to mucosal nystatin when the serosal solution was $\frac{1}{2}$ osmotic strength Na^+-gluconate Ringer's. As illustrated in Fig. 7, the hypo-osmotic medium prevented the cellular shrinkage associated with isosmotic gluconate. Under these conditions basolateral emf approximated 60 mV. This result adds further support to the argument that the changes in plasma membrane permeability are dependent not on the anionic species bathing the membrane but rather on the state of the cell volume. This hypothesis predicts that, in nystatin-treated tissue, substitution of hypo-osmotic gluconate for serosal gluconate should increase the basolateral membrane emf. Conversely, changing from a hypo-osmotic to an isosmotic serosal gluconate medium should decrease it. In two experiments changing from isosmotic to hypo-osmotic media increased the cell emf (on average) from 31 to 59 mV, with a concommitant decrease in resistance, a steady-state value being reached in less than 10 min. Conversely, changing from a hypo-osmotic to an isosmotic Ringer's decreased the cell emf from 64 to 33 mV (with a corresponding increase in resistance). Thus, as predicted, nystatin did not appear to blunt the responses of cell emf to osmotic perturbations.

iii) Sodium Activation of the Na^+ Pump

Since the apical membrane has been effectively removed as a barrier to the movement of monovalent anions and cations, it is possible to determine whether the basolateral Na^+ pump is rheogenic, and, if it is, to examine the current generated by the pump as a function of cell Na^+ concentration. The protocol followed here is the same as that developed by Lewis et al. (1978) and Lewis and Wills (1983). In brief, after full expression of the action of nystatin on the apical membrane with a mucosal K^+-gluconate Ringer's, the Na^+-gluconate serosal solution was replaced by a K^+-gluconate Ringer's. Next, aliquots of NaCl were added first to the serosal solution and then to the mucosal solution. Only after mucosal addition was a hyperpolarization measured. The polarity indicated that the Na^+ pump was generating a net current. Addition of 10^{-4} M ouabain reduced this hyperpolarization to zero in approximately 5 min. Measurement of the hyperpolarization and of the membrane resistance allows calculation of the current generated at each mucosal Na^+ concentration. Figure 14 is a plot of mucosal Na^+ concentration versus current generated by the pump. The curve is obviously sigmoidal and is best described by the model for three identical independent sites:

$$I_p = \frac{I_{max}}{\left(1 + \frac{K_m}{Na^+}\right)^3}$$

where I_p is the current generated by the pump, I_{max}

Table 3. Effects of nystatin on bladders incubated in gluconate or acetate media[a]

Serosal medium	V_T (mV)	R_T (kΩ · μF)	C_T (μF · cm $^{-2}$)	E_c (mV)	R_c (kΩ · μF)	P^i_{Na}/P^i_K	P^c_{Na}/P^c_K
Na^+ gluconate	7 ± 1	18.3 ± 2.0	[b]2.0 ± 0.3			0.75 ± 0.02	
+ nystatin (n = 18)	17 ± 2	11.3 ± 1.1	[b]12.9 ± 1.9	32 ± 3	38.8 ± 5.5		0.28 ± 0.03
Na^+ acetate	7 ± 2	35.0 ± 6.5	3.3 ± 0.3			0.71 ± 0.04	
+ nystatin (n = 8)	60 ± 4	4.4 ± 0.9		73 ± 2	5.6 ± 1.2		0.03 ± 0.004

[a] (1) In all cases, the mucosal medium contained the K^+ salt of the anion; (2) Technical difficulties prevented the measurement of tissue capacitance following nystatin in bladders incubated with acetate; (3) The junctional permeability ratio (P^i_{Na}/P^i_K) was calculated (before nystatin addition) using the assumption that $R_c \cong \infty$, i.e., $V_T = E_j$ (*see* Materials and Methods).
[b] n = 14.

is the maximum current generated by the pump, K_m is the rate constant for association (it is *not* the concentration for half-maximal stimulation). The best fit parameters are I_{max} = 22 μA/cm² and K_m = 8.25. The implications of such a kinetic scheme will be addressed in the Discussion.

Discussion

We consider in turn five topics: *i*) the effect of mucosal anion substitution on Na^+ transport, *ii*) the site of action of nystatin, *iii*) passive and active properties of the basolateral membrane, *iv*) possible mechanisms by which basolateral membrane permeability could be modified by anions, and, *v*) the implications for Na^+ transport and cell volume of changing basolateral membrane permeability and resistance.

i) Effects of Mucosal Anions

Replacement of mucosal Cl^- with gluconate resulted in a rapid and reversible decrease in Na^+ transport with a concomitant increase in transepithelial resistance (Fig. 2). In an extensive study Singer and Civan (1971) demonstrated that Na^+ transport across toad urinary bladder was influenced markedly by the anion which replaced mucosal Cl^-. The anion most effective in stimulating Na^+ transport was SCN^-, while azide caused the most inhibition. These authors concluded that anions interact with fixed positive charges of weak field strength on the plasma membrane thereby affecting Na^+ conductance. Our results extend this interpretation and suggest that the interaction is on or near the external surface of the apical Na^+ channel, since gluconate modifies the competitive interaction between Na^+ and amiloride. Whether the effect is

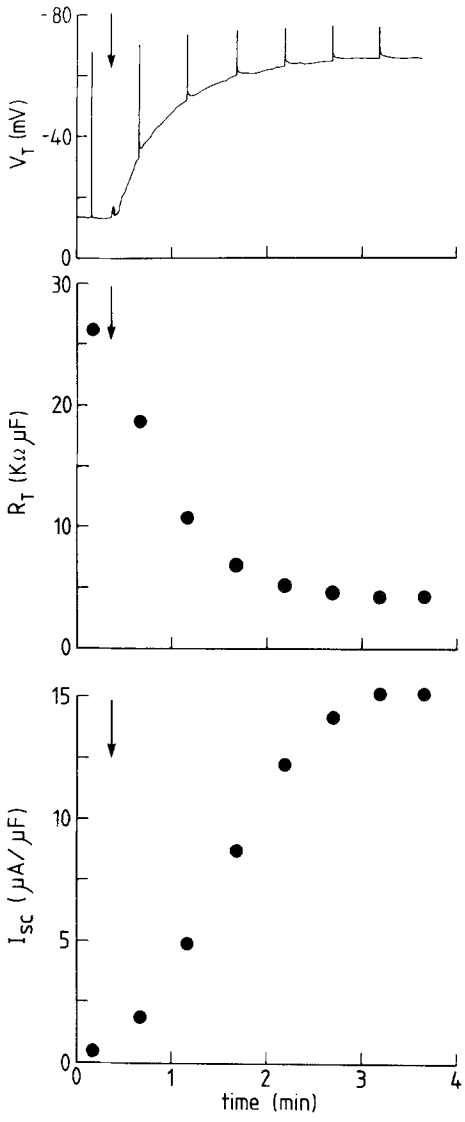


Fig. 13. Typical changes in V_T, R_T and calculated I_{sc} following the addition of nystatin to the mucosal chamber of a hemibladder bathed in K^+-acetate Ringer's mucosa, and Na^+-acetate Ringer's serosa. Nystatin was added at the arrow

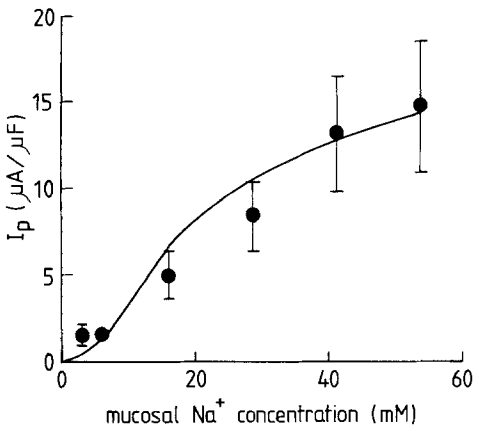


Fig. 14. Relation between mucosal Na^+ concentration and the current generated by the pump (I_p) in nystatin-treated hemibladders. Solid line represents best fit to points (*see* text). Vertical lines represent SEM when greater than symbol (n = 7)

by screening of surface charge, by increasing the rate constant for Na^+ binding to an external self-inhibition site, by decreasing the dissociation rate constant from this site, or by altering the field strength of the selectivity filter (which might result in a decrease in single-channel permeability), cannot be resolved using macroscopic methods. It might, however, be resolved using fluctuation analysis (*see* Lindemann & Van Driessche, 1977; Palmer et al., 1982).

In a microelectrode study Narvarte and Finn (1980) concluded that in toad urinary bladder apical membrane Na^+ conductance was decreased when gluconate replaced mucosal medium Cl^-. In addition these authors reported that E_{bl} was also depressed after mucosal gluconate and attributed this to a decreased rheogenic Na^+ transport consequent upon the decreased cell Na^+ with decreased apical Na^+ entry. It appears that basolateral K^+ permeability is sensitive to changes in cell volume (*see below*) and decreased Na^+ entry could result in a decreased cell volume with decreased basolateral K^+ permeability and increased basolateral membrane resistance. Since $E_{Na} = E_a^{Na} + E_b$, a prediction is that mucosal gluconate might decrease E_{Na} through a decrease of E_b. Although there was a small (2 mV) decrease in E_{Na} when replacing mucosal Cl^- with gluconate, it was statistically significant. This most probably reflects the inability of our method to detect small changes (~5%) in the driving force.

ii) Site of Action of Nystatin

The polyene antibiotics nystatin and amphotericin B enhance net Na^+ transport in NaCl Ringer's when added to the mucosal medium. Lichtenstein and Leaf (1965), who first demonstrated this in toad urinary bladder, attributed the enhancement to an increased rate of entry of Na^+ to the cells across the apical plasma membrane whose ionic conductance had been increased nonspecifically. More recently, Reuss, Gatzy and Finn (1978), using microelectrode techniques, concluded that mucosal addition of amphotericin B resulted in a small decrease in apical resistance, but that the predominant decrease in resistance occurred at the tight junctions, or in a cell-type not impaled by the microelectrode. The following points, however, argue against a predominant effect of polyene antibiotics at a shunt site: a) in mammalian urinary bladder (Lewis et al., 1977) and in descending colon (Wills et al., 1979), the predominant effect of nystatin is at the apical membrane with the drug added to the mucosal solution; b) the large change in transepithelial capacitance found in the present study (Fig. 10) can only be accounted for by a reduction in apical resistance and not by a reduction in junctional or shunt resistance. A decrease in shunt resistance would attenuate any change in epithelial capacitance; c) in the present study basolateral membrane resistance seems to be a function of cell volume such that, as the cell swells, the basolateral membrane resistance decreases. Since the experiments of Reuss et al. (1978) were performed in Cl^--containing solutions, significant cell swelling might have occurred leading to a reduction of basolaterial resistance as a function of time. Membrane and junctional (or shunt) resistances were calculated by these workers (as well as others, e.g., Lewis et al., 1977) on the assumption of a constant basolateral resistance. Given this assumption, the equations used to calculate resistance demand that when tissue resistance falls, the calculated junctional resistance decreases dramatically.

Our results agree with the conclusion of Lichtenstein and Leaf (1965) and indicate that the action of nystatin is localized to only the apical plasma membrane.

iii) Passive and Active Properties of the Basolateral Membrane

In brief, the evidence that alterations in basolateral membrane permeability and resistance are linked to changes in cell volume is as follows: a) the cellular emf (sum of apical emf and basolateral emf) decreased from 130 to 68 mV (Table 2), when a permeant serosal anion (Cl^-) was replaced with an impermeant anion (gluconate). That cell volume also decreased was confirmed from measurements of

cellular composition as well as histologically (Fig. 6); b) replacement of Cl^- with an anion, acetate, the undissociated weak acid of which can enter cells, maintained the cellular emf. That cell volume had not decreased was confirmed histologically (Fig. 7); c) the site (apical or basolateral) of the change in cellular emf after serosal gluconate was localized by selectively shunting one membrane (the apical) with nystatin. This then allowed direct measurement of the basolateral membrane emf. In the presence of gluconate (shrunken cells) the basolateral emf was some 30 mV, while, in the presence of acetate or of $\frac{1}{2}$ osmotic strength serosal gluconate (where cell shrinkage was also prevented), the basolateral emf's were calculated as 79 and 60 mV, respectively; d) addition of 3 mM serosal Ba^{2+} decreased Na^+ transport as well as decreasing cell emf to 79 mV with Cl^- media while no further decrease in cell emf was measured after serosal Ba^{2+} when gluconate was the major serosal anion. Similarly, basolateral emf decreased after Ba^{2+} in cells whose volumes had been relatively well maintained (hypo-osmotic Ringer's) but not in cells shrunken in gluconate Ringer's.

MacRobbie and Ussing (1961) and more recently Ussing (1982) have proposed that cell swelling induces (in the basolateral membrane of frog skin) the appearance of Cl^- channels as well as the activation of a neutral NaCl entry mechanism. The purpose of this system being to regulate cell volume. Whether a similar situation occurs in the toad urinary bladder during volume perturbation is not known.

These arguments support the contention that the basolateral membrane emf is either directly or indirectly controlled by the state of cell volume. The basolateral emf (which under control conditions is predominantly a K^+ diffusion potential) could be reduced by two mechanisms; either addition or activation of parallel shunt channels, e.g. for Na^+, or removal (inactivation) of K^+ channels which are normally in parallel with a population of less selective (more leaky) channels. Figure 15 is a plot of cellular resistance (predominantly basolateral resistance) as a function of the cellular emf (now basolateral emf) after nystatin treatment. Since cell resistance increases as cell emf decreases, this suggests that highly selective K^+ channels are either removed or inactivated as the cells lose volume. These possibilities will be discussed below. The zero resistance intercept of this plot represents the emf resulting from ion diffusion through these highly selective channels, and is 78 mV. Since the Nernst potential (given that cell K^+ concentration equals that in the mucosal solution, 119.5 mM) is 90 mV, this indicates that the added channels are highly K^+ selective (*ca.* a P_{Na}/P_K of 0.007).

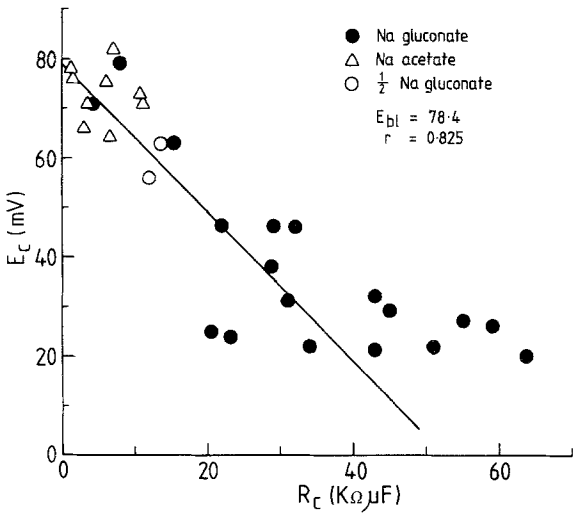


Fig. 15. Relation between E_{cell} and R_{cell} of nystatin-treated hemibladders in various serosal Ringer's solutions. (●), Na^+ gluconate; (△), Na^+ acetate; (○), $\frac{1}{2}$ Na^+ gluconate. In all cases the mucosal surface of the hemibladders was bathed with an equivalent K^+ Ringer's

In that it transports net charge, as measured electrically and estimated using radioactive isotopes in a variety of epithelia, the Na, K-ATPase, i.e., the Na^+ pump, is rheogenic. By applying graded doses of Na^+ to a nystatin-treated preparation, the activation of the pump current, as a function of mucosal Na^+ can be described qualitatively (qualitatively since cell Na^+ concentration must be lower than the mucosal concentration). The kinetic equation which describes this relationship is not a simple first-order rate reaction but rather yields a sigmoidal curve indicating a more complex binding and activation than one Na^+ ion per cycle of the pump. The exact stoichiometry cannot be specified but, following the lead of Post and Jolly (1957) and in line with recent evidence from epithelia (Hviid Larsen, Fuchs, & Lindemann, 1979; Nielsen, 1979; Kirk, Halm & Dawson, 1980; Zeuthen & Wright, 1981; Eaton, Frace & Silverthorn, 1982; Lewis & Wills, 1983), it is most probably 3 Na^+ ions to 2 K^+ ions. It is beyond the scope of this paper to describe the complete ion kinetics of the pump which must await further research.

If indeed our analysis of cell emf and of basolateral emf is correct, we can calculate the intracellular Na^+ activity for the acetate and gluconate experiments using the equation:

$$E_c = E_a^{Na} + E_b.$$

Since we have measured E_c before the E_b after nystatin for each of the two media we can solve for E_a^{Na} (which is the Nernst potential for Na^+ of the

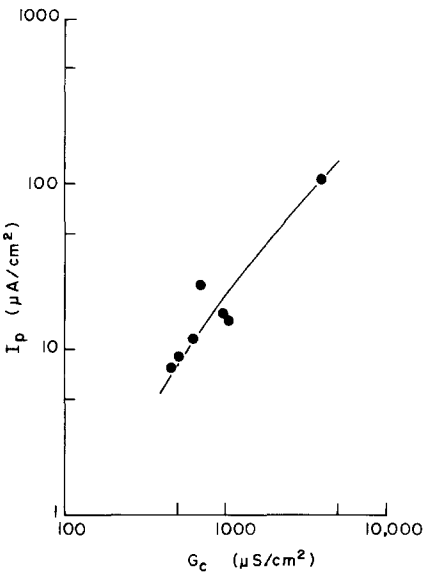


Fig. 16. A plot of the current generated by the pump (using 56 mM NaCl) versus the basolateral membrane conductance. The linear relationship between conductance and pump current indicates a constant ratio between pump density and K^+ channel density

apical membrane assuming perfectly Na^+-selective channels). For acetate, E_a^{Na} approximates 50 mV and for gluconate it approximates 41 mV, yielding calculated intracellular Na^+ activities of some 12 and 17 mM, respectively. These values are in excellent agreement with estimates for the sodium transport pool in toad urinary bladder epithelial cells obtained from chemical and isotopic analyses (Macknight, Civan & Leaf, 1975) and from electron microprobe analysis (Rick et al., 1978).

iv) Possible Mechanisms for Changes in Basolateral Membrane Permeability

There are at least three possible mechanisms to account for the appearance or disappearance of the basolateral membrane K^+ channels in response to volume changes. First, there could be activation of quiescent channels in the basolateral membrane, as has been postulated for the apical membrane Na^+ channel when aldosterone or ADH stimulate Na^+ entry (Li et al., 1982; Palmer et al., 1982). Secondly, there could be insertion of channels from an intracellular store, a mechanism normally mediated by cytoskeletal elements such as microtubules and/or microfilaments. Examples of channel insertion into the target membrane via cytoplasmic vesicles include water channels into the apical membrane of toad urinary bladder (Kachadorian, Wade & Di Scala, 1975), H^+-ATPase into the apical membrane of gastric mucosa (Forte, Machen & Forte, 1977) and turtle urinary bladder (Gluck, Cannon & Al-Awqati, 1982) and Na^+ channels into the apical membrane of rabbit urinary bladder, a phenomenon which occurs during bladder filling and which is solely mechanical in nature (Lewis & de Moura, 1982). There is some evidence for vesicle insertion in to the basolateral membrane of toad urinary bladder following ADH. Warncke and Lindemann (1981), using impedance analysis, reported that not only did the apical membrane surface area increase by 30% as a function of time after serosal ADH administration, but a similar increase occurred at the basolateral membrane. Although this increase in basolateral membrane area might be a direct response to ADH, it is also possible that it is a secondary response mediated by an increase in cell volume occurring during the stimulation of water and ion flows.

Thirdly, there could be modification of pre-existing channels. For example, an increase in single-channel permeability or an alteration of the duration of open time or closed time of a spontaneously fluctuating channel. Although there is no example of this latter mechanism, it has been reported that basolateral K^+ channels do fluctuate spontaneously (i.e. open and closed; Van Driessche et al., 1982) and a chemical messenger might alter either the opening or closing rate constants for a channel. It is not possible from our data to differentiate among these possibilities. Indeed, more than just one mechanism might be involved.

In addition to alterations of basolateral membrane permeability, is there also a modification of pump density? Again one can invoke the same mechanisms for an increase or alteration of pump properties. These are activation, incorporation and modification. Two findings suggest that pump density decreased when cellular volume decreased. First, the calculated intracellular Na^+ activity was a little higher in preparations treated with gluconate (which had lower rates of transport) than in control preparations (which had higher rates of transport). One might predict that when Na^+ transport decreases as a consequence of decreased apical entry, cell Na^+ should decrease, as it does after amiloride (Macknight et al., 1975), however, Na^+ increased.

Secondly, a plot of the current generated by the pump (at an external Na^+ concentration of 56 mM) against basolateral membrane conductance (Fig. 16) resulted in a linear relationship. Since the variability in cell conductance is a consequence of activation/insertion of Ba^{2+}-sensitive K^+ channels, and since conductance and pump current are linearly related, this suggests that cell volume also regulates

pump activity by either activation/inactivation or insertion/withdrawal of pre-packaged vesicles containing both K^+ channels and pumps. An interesting calculation can be performed to determine how many pumps there are per K^+ channel. Assuming a single K^+ channel conductance of 30 pS, a net electrochemical gradient of 20 mV, and that the channel is always open, then approximately 4×10^6 ions will flow through the channel per second. The maximal turnover rate of a Na^+ pump is 150 cycles/second. If 2 K^+ are extruded every cycle then 300 K^+ ions are transported per pump per second. Since cell K^+ is constant this indicates that there are at least 13,000 pumps per K^+ channel.

It is tempting to speculate, therefore, that basolateral K^+ permeability and the capacity for Na^+ transport change in parallel. This would allow cell K^+ concentration to remain relatively constant in the face of variations in transepithelial Na^+ transport as has been observed experimentally in toad bladder epithelial cells (Macknight et al., 1980) and would thus minimize changes in cellular volume with changing rates of transport.

v) Implications for Na^+ Transport and Cell Volume

When the serosal solution bathing toad urinary bladder is made hyperosmotic, Na^+ transport decreases (Lipton, 1972; Bentley et al., 1973) while a hypo-osmotic serosal solution stimulates transport (Lipton, 1972). Can a model of variable basolateral K^+ permeability account for these shifts in transport?

With a hyperosmotic challenge the cells would shrink. From the present results we would predict that this would result in a decrease in basolateral K^+ permeability, a depolarization of the basolateral membrane potential and an increase in basolateral membrane resistance. These two latter changes would result in a decrease in the net electrochemical gradient for Na^+ entry across the apical membrane (under both open- and short-circuit conditions). Conversely, a hypo-osmotic serosal solution would increase basolateral membrane K^+ permeability, decreasing membrane resistance and hyperpolarizing the membrane potential. Consequently, the driving force for Na^+ entry across the apical membrane, would be increased (again for both open- and short-circuit conditions). Finn and Reuss (1975), in microelectrode studies, have demonstrated that a hypo-osmotic serosal solution causes a decrease in the resistance of the basolateral membrane whereas a hyperosmotic serosal solution causes an increase in this resistance. More recently, Davis and Finn (1982*a*,*b*) have reported that basolateral membrane resistance increases after amiloride as judged both by electrophysiological techniques and by the inhibition of cellular swelling which would otherwise occur rapidly with a high K^+ concentration in the serosal solution. Our observations and hypothesis are consistent with these experimental findings. They are also consistent with the findings of others in a variety of cell types which indicate that changes in cell volume under anisosmotic conditions can be minimized by changes in cell ionic contents. For example, in hypo-osmotic media the initial swelling is often counteracted by a loss of cellular solute, often K^+ with Cl^- (for a tight epithelium, e.g. Ussing, 1982). In some tissues this may reflect the activation of a cotransport process which, if electrically neutral, can have no effect directly on membrane resistance, but in others it appears that the pathways followed are independent, the linkage between the two ions being simply the need to preserve bulk electroneutrality (*see* Hoffmann, 1983, for a review). Thus a tendency for membrane K^+ permeability to increase with swelling may make an essential contribution to the limitation of cellular swelling under such conditions. Conversely, in hyperosmotic media, the initial cellular shrinkage is often partially corrected for by solute retention; again, a net uptake of K^+ and Cl^- is often associated with this. Here, a combination of increased rate of Na^+ and Cl^- entry, with the Na^+ subsequently being extruded from the cells by the Na^+, K^+-ATPase could be responsible for this and the increase in cellular K^+ would be facilitated by a decrease in the K^+ conductance of the plasma membrane.

Changes in basolateral membrane K^+ permeability may play an important role in epithelia in allowing changes in the rate of transepithelial transport of Na^+ to occur without concommitant changes in cellular K^+ content and in cellular volume. Schultz (1981) has discussed this issue in some detail. In addition, they may also be important in helping to maintain cellular integrity during transepithelial transport of water. Under physiological conditions the toad urinary bladder reabsorbs water and Na^+ from a dilute urine into a somewhat dehydrated plasma under the control of ADH. This hormone increases the water permeability of the apical membrane and water moves into the cell causing it to swell (DiBona, Civan & Leaf, 1969). This swelling could then trigger (either directly or indirectly) an increase in basolateral membrane K^+ permeability with resultant hyperpolarization of the basolateral membrane. Because current flows through the paracellular pathway, this increased cellular negativity and decreased basolateral membrane resis-

tance cause the apical membrane potential to become more cell interior-negative with respect to the urine. Thus the net electrochemical driving force for Na^+ entry is increased and this will help to maintain the cell osmolality which contributes to the driving force for net water movement from urine to plasma.

In conclusion, changes in cell volume may be more important as a signal for the regulation of ion channels in transporting epithelia than has been appreciated previously. This report offers evidence that a decrease in cell volume decreases basolateral K^+ permeability, probably by reducing the number of specific, Ba^{2+}-sensitive K^+ channels. This decrease in turn reduces Na^+ entry which would otherwise only exacerbate the problem of cellular and extracellular hyperosmolarity. Hypo-osmotic serosal solutions increase basolateral membrane permeability, enhance Na^+ entry and transepithelial Na^+ transport and thus reduce the possibility of dilution of the cell milieu and contribute to the correction of the hypo-osmolality of the body fluids. The mechanism which regulates this change in basolateral membrane permeability and associated parallel change in Na^+ pump density or kinetics is still unknown, though it is tempting to postulate a role for intracellular Ca^{2+} in such control. This particular system offers one more example of the intricate and inter-related homeostatic mechanism that cells possess to ensure their survival and normal operation.

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