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Active and Passive Na^+ Fluxes Across the Basolateral Membrane of Rabbit Urinary Bladder

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Summary. The apical membrane of rabbit urinary bladder can be functionally removed by application of nystatin at high concentration if the mucosal surface of the tissue is bathed in a saline which mimics intracellular ion concentrations. Under these conditions, the tissue is as far as the movement of univalent ions no more than a sheet of basolateral membrane with some tight junctional membrane in parallel. In this manner the Na^+ concentration at the inner surface of the basolateral membrane can be varied by altering the concentration in the mucosal bulk solution. When this was done both mucosal-to-serosal ^{22}Na flux and net change in basolateral current were measured. The flux and the current could be further divided into the components of each that were either blocked by ouabain or insensitive to ouabain. Ouabain-insensitive mucosal-to-serosal Na^+ flux was a linear function of mucosal Na^+ concentration. Ouabain-sensitive Na^+ flux and ouabain-sensitive, Na^+-induced current both display a saturating relationship which cannot be accounted for by the presence of unstirred layers. If the interaction of Na^+ with the basolateral transport process is assumed to involve the interaction of some number of Na^+ ions, n, with a maximal flux, M_{MAX}, then the data can be fit by assuming 3.2 equivalent sites for interaction and a value for M_{MAX} of 287.8 pM cm^{-2} sec^{-1} with an intracellular Na concentration of 2.0 mM Na^+ at half-maximal saturation. By comparing these values with the ouabain-sensitive, Na^+-induced current, we calculate a Na^+ to K^+ coupling ratio of 1.40 ± 0.07 for the transport process.

Key words rabbit urinary bladder · Na^+ flux · Na–K-ATPase · Na^+ transport · basolateral membrane · electrogenic transport

Introduction

In tight epithelia, Na^+ transport is a two-step process. First Na^+ ions move passively down their electrochemical gradient at the apical membrane into the cell and then Na^+ is actively transported out of the cell across the basolateral membrane by a (Na + K)-ATPase. Information about both of these steps is important to an understanding of the regulation of Na^+ transport in epithelial tissues and to the more global relationship of Na^+ transport to fluid and electrolyte balance in the total organism.

Unfortunately, an examination of either of the two steps involved in epithelial Na^+ transport is complicated by the nature of the tissue. In particular, the apical and basolateral membranes in series with the poorly accessible intracellular compartment make investigation of the intact basolateral ATPase of epithelia particularly difficult. Consequently, most information about $Na^+ - K^+$ transport systems has come from the examination of the properties of other systems such as erythrocytes, nerve axons, and muscle. Although the properties of (Na + K)-ATPase in epithelial tissues is probably similar to that found in other cells, it is necessary to verify enough similar properties to convince ourselves that we can apply the complete quantitative description obtained in, say, erythrocytes to the ATPase of epithelial cells.

In a previous effort to obtain quantitative information about the (Na + K)-ATPase in rabbit urinary bladder, we examined short-circuit current as a function of intracellular Na^+ activity using Na^+-specific microelectrodes (Eaton, 1981). This work provided information about the activity of the basolateral (Na + K)-ATPase as a function of intracellular Na^+ activity. Nonetheless, the study could not give a direct measure of the Na^+ flux due to the ATPase. A way to more precisely define the dependence of Na^+ flux on intracellular Na^+ activity was suggested by the work of Lewis, Eaton, Clausen and Diamond (1977) and Lewis, Wills and Eaton (1978) in which the polyene antibiotic, nystatin, was used to increase the permeability of the apical membrane. The permeability was increased to the extent that the apical membrane no longer formed a rate-limiting barrier for the movements of univalent ions into the epithelial cells.

After the application of nystatin, they (Lewis et al., 1978) demonstrated that it was possible to activite a rheogenic Na^+ transport system at the basolateral membrane by addition of small amounts of Na^+ to the mucosal solution. The major complications of the work were (1) only the net current

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produced by the transport system could be measured, and (2) the symmetrical potassium solutions required to measure the transport current were so unlike the normal milieu of the (Na+K)-ATPase that there was some question of extending the results to the normal physiological state.

In this paper, we have tried to answer these problems. First, we have designed a special bathing solution which approximates the univalent cation concentration of the cell interior. This solution, in effect, allows us to "dialyze" the intracellular compartment of nystatin-treated epithelia. Secondly, we have measured the unidirectional, ouabain-sensitive Na^+ fluxes at various intracellular Na^+ concentrations in order to obtain quantitative information about the effect of intracellular Na^+ concentration on the basolateral (Na+K)-ATPase. We have then compared these fluxes to the rheogenic component of Na^+ transport to estimate the coupling between Na^+ and K^+ at the basolateral membrane.

Materials and Methods

Urinary bladders were obtained from adult New Zealand white rabbits. The tissue was dissected, mounted according to the procedures described in Lewis and Diamond (1976), and placed between modified Ussing chambers (8.5 ml/chamber) which were designed to reduce edge damage and facilitate the addition and removal of isotopic samples. The exposed area of tissue was 1 cm^2.

Electrical Measurements

Transepithelial voltage (V_T) was monitored with two 0.1 M KCl agar bridges 10 mm apart on opposite sides of the tissue. These are connected externally to Ag–AgCl wires. The serosal solution was the ground reference. In the serosal chamber there is a Ag–AgCl wire connected to a virtual ground current to voltage converter used to determine transepithelial current (I_T). In the mucosal chamber, a platinum-platinum black electrode was connected to a current source. The current source and voltage electrodes led to a voltage clamp. V_T and a signal proportional to transepithelial current were displayed on two digital voltmeters and a two-channel chart recorder. During flux experiments, the tissue was generally left in an open-circuit condition to avoid ion accumulation or depletion due to a maintained current gradient. Membrane resistance was monitored by applying a short constant current pulse and measuring the tissue voltage response. In experiments to measure the rheogenic component of transport, the tissue was voltage clamped at the potential of the tissue in the absence of mucosal Na^+ (zero current condition). The change in this current when Na^+ was added was defined as the Na^+-induced current.

Solutions

The composition (in mM) of the original bathing solution (NaCl saline) was 111.2 NaCl, 25 $NaHCO_3$, 5.8 KCl, 2.0 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4 and 11.1 glucose. After the tissue had stabilized, the mucosal solution was replaced with nystatin bathing solution; the composition of which was 25 K_2SO_4, 25 $KHCO_3$, 3.2 $MgSO_4$, 160 sucrose, 1.2 KH_2PO_4 and 11.1 glucose.

All solutions were adjusted to pH 7.4 and bubbled with 95 –5% O_2—CO_2. Ouabain and nystatin were obtained from Sigma Chemical Company.

Nystatin Treatment

After the tissue was bathed on the serosal surface in NaCl saline and on the mucosa with nystatin bathing solution, nystatin stock solution (5 mg/ml in 95% ethanol) was added to the mucosal chamber to produce a nystatin concentration of 50 mg/liter (1% ethanol).

We originally chose nystatin as the ionophore of choice because of several important properties: (1) nystatin has an extremely steep dose response curve. (2) Pores formed by nystatin are highly permeable to univalent ions (with a slight preference for cations over anions) but very poorly permeable to divalent ions and especially divalent anions (Russell, Eaton & Brodwick, 1977). The pores formed are so permeable to univalent ions that the mobility of ions through the pore is almost the same as in water (Holz & Finkelstein, 1970). (3) Nystatin forms permeable pores when applied to only one side of a membrane (Marty & Finkelstein, 1975; Russell et al., 1977).

Because of these properties, we (Lewis et al., 1977) tested the effect of nystatin on the mucosal membrane. In the previous work, which we have again confirmed under the conditions used for the experiments in this paper, we found a reduction in the apical membrane resistance after application of nystatin to the extent that no measurable voltage drop could be detected across the apical membrane.

Since the cell interior and the mucosal solution become in effect continuous for the movement of univalent ions after treatment with nystatin, cell viability can only be ensured by bathing the mucosal surface of the epithelium with a solution that is similar to the normal intracellular contents. We have found that the four most important factors in the composition of this solution are (1) high K^+ concentration, (2) very low Ca^{++} concentration, (3) low concentration of permeable anions, and (4) higher than normal Mg^{++} concentration to maintain the integrity of the tight junctions in the absence of Ca^{++}. To meet these requirements we have developed the nystatin bathing solution outlined above. To the basic solution small amounts of Na_2SO_4 can be added to stimulate the basolateral transport system.

^{22}Na Flux Measurements

Carrier-free $^{22}NaCl$ was diluted approximately 10-fold with distilled water to produce a solution with a specific activity of 1 mCi/ml. Fifty µl of this stock was added to the mucosal chamber (to the serosal chamber for backflux experiments). Immediately a 10-µl sample was removed from the mucosal chamber as a measure of specific activity and a 100-µl sample was removed from the serosal chamber as a measure of background. The serosal sample was replaced with 100 µl of cold NaCl saline. Because fluxes across the bladder are very low, the counts in the mucosal chamber at the beginning and end of the experiment were virtually the same. At 10-min intervals 100-µl samples were removed from the serosal chamber and replaced with 100 µl of cold serosal solution. Twenty-five samples were taken prior to the addition of ouabain and 15 samples after the addition. The first 10 samples were generally discarded to assure stability of flux. All samples were added to 20 ml scintillation vials with 1 ml of water

and 10 ml of Toluene-Triton-X-omnifluor cocktail and counted for 10 min or to 1% accuracy. Since the specific activity of the serosal chamber was always very low no correction for isotopic backflux was made. Na^+ flux was calculated according to the method of Lewis and Diamond (1976).

Results

Normal Response to Nystatin and Mucosal Na^+ Ion

The typical response of the urinary bladder to treatment with the nystatin bathing saline (Na^+-free, K-sulfate saline; *see* Materials and Methods) and the subsequent addition of nystatin is shown in Fig. 1. The tissue is kept in an open-circuit condition for the duration of the experiment. When both mucosal and serosal surfaces are bathed in normal rabbit Ringer's, the initial transepithelial potential for the tissue in Fig. 1 is -9 mV (mucosal potential with respect to a serosal ground). As described in Materials and Methods, the mucosal surface is then bathed in a Na^+-free, K-sulfate Ringer's in which the concentrations of K^+, Cl^-, Ca^{++}, Mg^{++} and HCO_3^- are similar to those of the cell interior. This solution produces a small hyperpolarizing response which is apparently due to the anionic assymetry between the mucosal and serosal solutions since a potassium chloride saline applied to the mucosa depolarizes the transepithelial potential to close to 0 mV. When nystatin is subsequently added to the mucosal solution, the transepithelial potential rapidly hyperpolarizes reflecting the normal potential of the basolateral membrane (Lewis et al., 1978). The tissue then undergoes a short, transient change in potential, followed by stabilization at a transepithelial potential compatible with an extremely low apical membrane resistance and a basolateral membrane potential which reflects the potassium concentration of the mucosal solution (Lewis et al., 1978). In the absence of further manipulation, the tissue is stable at this potential for 3–5 hr. Under these conditions, we view the tissue as functionally consisting of a sheet of basolateral membrane in parallel with some high resistance tight junctional membrane. As far as the movement of univalent ions, the apical membrane has been functionally removed (Lewis et al., 1977, 1978; Russell et al., 1977).

After the potential has stabilized, the Na^+ concentration of the mucosal solution can be altered by addition of small amounts of concentrated Na_2SO_4. Addition of Na^+ ion activates the basolateral Na $-$K transport system (addition of a similar quantity of K_2SO_4 has no effect). Since the system is electrogenic, activation leads to a small hyperpolarization of transepithelial potential. Addition of 10^{-4} M ouabain abolishes the potential.

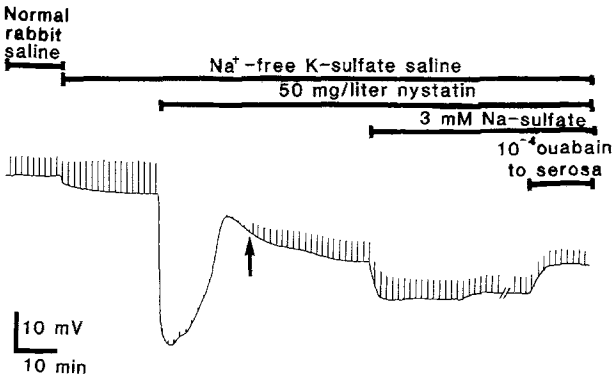

Fig. 1. Transepithelial potential in response to mucosal application of nystatin. The serosal solution contains normal rabbit saline throughout. The contents of the mucosal chamber are given as bars at the top of the Figure. The initial transepithelial potential is -9 mV (serosal ground). The upward deflections represent the transepithelial voltage response to a fixed-size current step. As such the magnitude of the deflection is a measure of transepithelial resistance. Initially the current step is 0.5 μA. At the heavy arrow, the step was increased to 5 μA because of the decreased tissue resistance after application of nystatin. The maximum hyperpolarization produced by nystatin is -53 mV while the steady-state level prior to addition of Na^+ was -33 mV

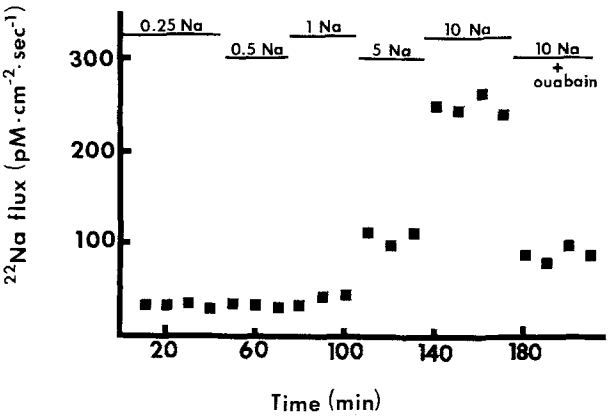

Fig. 2. Mucosal-to-serosal Na^+ flux associated with various mucosal concentrations of Na^+. The Na^+ concentration in mM of the mucosal solution and time of application is indicated by the numbers and bars at the top of the Figure. As the Na^+ concentration is increased, the Na^+ flux increases. At the end of the experiment, ouabain is added to the serosal solution to demonstrate that a substantial portion of the Na^+ flux is inhibitable by ouabain

In Fig. 2, the unidirectional flux of Na^+ ion from mucosal to serosal solution is examined under the same conditions as those in Fig. 1. After replacing the mucosal solution with nystatin bathing solution and adding nystatin to the mucosal solution, ^{22}Na plus varying concentrations of Na_2SO_4 are added to the mucosal solution and the mucosal-to-serosal Na^+ flux is measured. For low concentrations of Na^+ ion the mucosal-to-serosal flux is extremely

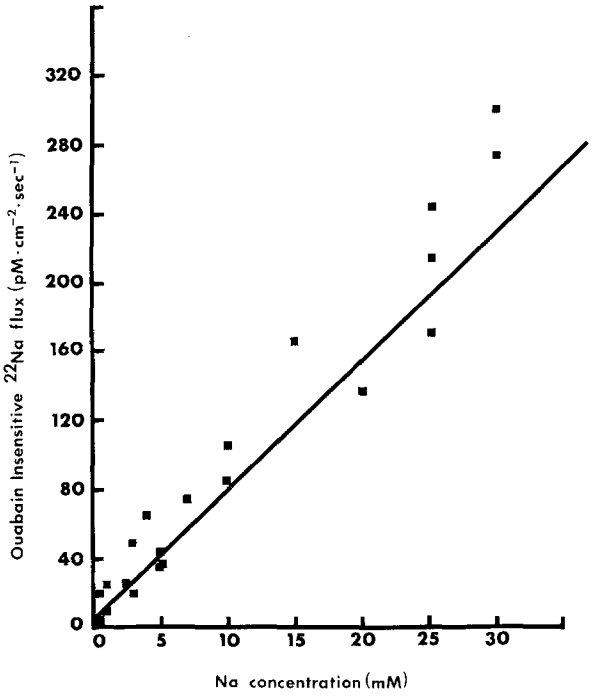

Fig. 3. Ouabain-insensitive Na^+ flux versus mucosal (intracellular) Na^+ concentration. The data from all experiments was pooled to show that there was a linear relationship between ouabain-insensitive mucosal-to-serosal Na^+ flux and the concentration of Na^+ at the intracellular surface of the basolateral membrane

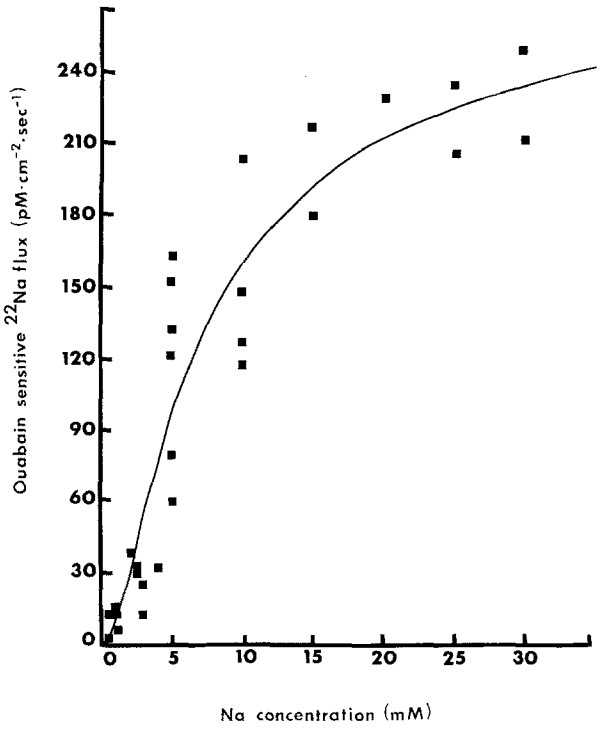

Fig. 4. Ouabain-sensitive Na^+ fluxes versus mucosal (intracellular) Na^+ concentration. The data from all experiments were pooled to show that there was a sigmoidal relationship between ouabain-sensitive mucosal-to-serosal Na^+ flux and the concentration of Na^+ at the intracellular surface of the basolateral membrane. The solid line through the points is the best-fit to the equation:

$$\frac{M_{Na}}{M_{MAX}} = \frac{1}{\left(1 + \frac{K_{Na}}{[Na]_i}\right)^n}$$

with the values for M_{MAX} of 287.8 pM cm^{-2} sec^{-1}, $n = 3$, and $K_{Na} = 2.0$ mM

low. At higher concentrations (associated with the appearance of the electrogenic basolateral hyperpolarization) the unidirectional flux increases dramatically. Ouabain blocks a substantial component of the Na^+-induced flux.

Relationship of Mucosal Na^+ Concentration to Na^+ Flux

With the exception of a few experiments similar to the one depicted in Fig. 2, the majority of the experiments consisted of treating the tissue with nystatin and then applying one concentration of Na^+, determining the unidirectional flux and then applying 10^{-4} M ouabain to block the component of Na^+ transport associated with the basolateral ATPase. This allowed us to have, for each Na^+ concentration, a specific measure of the Na^+ flux generated by ouabain-sensitive active transport and the flux associated with other Na^+ permeation mechanisms. In Fig. 3, ouabain-insensitive Na^+ fluxes are plotted as a function of mucosal Na^+ concentration. The relationship is linear for even the highest Na^+ concentrations examined.

The linearity of the relationship supports our original assumption that the apical membrane of the cell can no longer be a rate-limiting step in determining the mucosal-to-serosal flux as it is in the normal tissue prior to treatment with nystatin (Lewis & Diamond, 1976).

In a similar fashion we can calculate the ouabain-sensitive Na^+ flux from the difference in the flux before and after treatment with ouabain. From Fig. 4, it is apparent that the relationship between ouabain-sensitive Na^+ flux and Na^+ concentration is quite different from the same relationship for ouabain-insensitive Na^+ flux. The ouabain-sensitive flux displays a distinct "foot" or threshold at low Na^+ concentrations and saturates at high Na^+ concentrations.

Possible Contributions of Unstirred Layers

Our primary concern after finding the saturating relationship between ouabain-sensitive flux and mu-

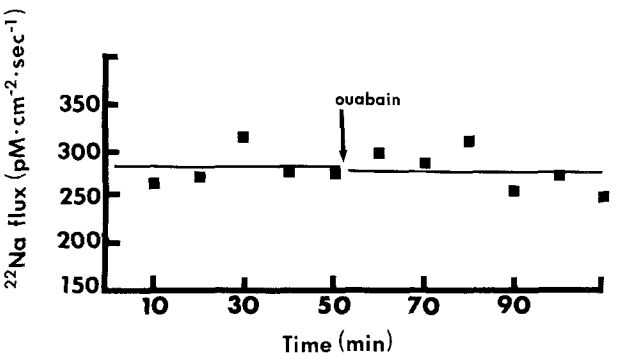

Fig. 5. Serosal-to-mucosal Na^+ flux. This Figure demonstrates that there is no significant component of ouabain-sensitive serosal-to-mucosal Na^+ flux. The lack of any transient change in the flux after application of ouabain also implies little contribution to the flux of an unstirred layer inside the cell

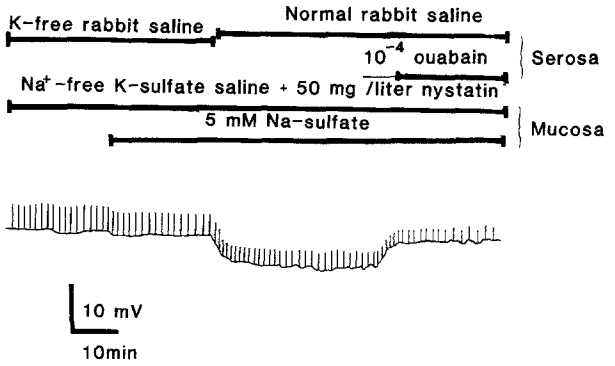

Fig. 6. Transepithelial potential in response to K^+-free serosal solution. The composition of the serosal and mucosal solutions and the duration of action is given by the captions and bars at the top of the Figure. Initially the tissue has Na^+-free nystatin bathing solution on the mucosal surface plus 50 mg/l nystatin and K-free rabbit saline on the serosal surface. The original transepithelial potential was -1 mV (serosal ground). The upward voltage deflections represent the transepithelial voltage response to a current step of 5 μA. The lack of hyperpolarizing electrogenic response to the mucosal application of Na^+ suggests that K^+-free solution on the serosa is a fairly effective transport blocker. Subsequent return to normal serosal K^+ produces a substantial electrogenic response. The lack of any transient changes on application of normal serosal K^+ suggests that Na^+ transport is not limited by the rate of diffusion of K^+ through an unstirred layer

cosal Na^+ was that the shape of the relationship might be strongly affected by the presence of unstirred layers at either the mucosal or serosal surface of the tissue. To investigate this possibility, we performed a number of experiments.

First, we felt that there might be an unstirred layer at the intracellular surface of the basolateral membrane. At very high rates of active Na^+ transport, this layer might be depleted of Na^+ to the extent that the depletion caused Na^+ diffusion rather than Na^+ transport to be rate limiting.

If this were true, we reasoned that there would be substantial recycling of any Na^+ that entered the cell interior from the serosal solution. That is, the unstirred layer coupled with the active transport process would reduce the serosal-to-mucosal Na^+ flux by an amount similar to any possible reduction of the mucosal-to-serosal flux. Also, if the serosal-to-mucosal flux was substantially reduced by the activity of the Na^+ transport mechanism removing Na^+ from an unstirred layer, then blocking the system with ouabain should increase the serosal-to-mucosal flux. In Fig. 5, a typical experiment in which serosal-to-mucosal Na^+ flux was measured shows that ouabain has little or no effect on the flux. In 5 additional experiments there was also no ouabain sensitive serosal-to-mucosal Na^+ flux. For all 6 of the experiments in which serosal-to-mucosal Na^+ flux was measured the ratio of Na^+ flux before the application of ouabain to the flux after application was 1.01 ± 0.031. We, therefore, concluded that an unstirred layer on the intracellular surface of the basolateral membrane was not the rate-limiting step in the flux of Na^+ across the tissue.

The results of the previous experiment did not preclude the presence of a rate-limiting unstirred layer at the serosal surface of the basolateral membrane. To investigate this possibility, we measured the mucosal-to-serosal Na^+ flux first in a K^+-free serosal solution, followed by a normal K^+ containing serosal saline, and finally after serosal addition of ouabain. We reasoned that with the pump blocked in K^+-free solution, Na^+ gradients in any serosal unstirred layer that was present would be reduced. Upon application of K^+ containing saline to the serosa which reactivates the transport system, we would expect a transient in Na^+ flux larger than the steady-state flux when the Na^+ gradients in the unstirred layer were present. We could also ascertain the effectiveness of K^+-free solution as a transport blocker by comparison with the effects of ouabain on Na^+ flux.

In Fig. 6, the electrical responses of the tissue to the treatments described above are depicted. Prior to the responses shown in the Figure, the mucosal surface of the tissue was perfused with the nystatin-bathing solution plus nystatin and the serosal surface with rabbit saline from which all the K^+ had been removed (*see* Materials and Methods). When 15 mM Na^+ is added to the mucosal solution, we would ordinarily expect a hyperpolarizing response (*see* Fig. 1); however, in the absence of serosal K^+, little if any hyperpolarization occurs.

Addition of K^+ to the serosal solution with a mixing time of approximately 3 sec leads to a prompt hyperpolarization with no transients. Serosal

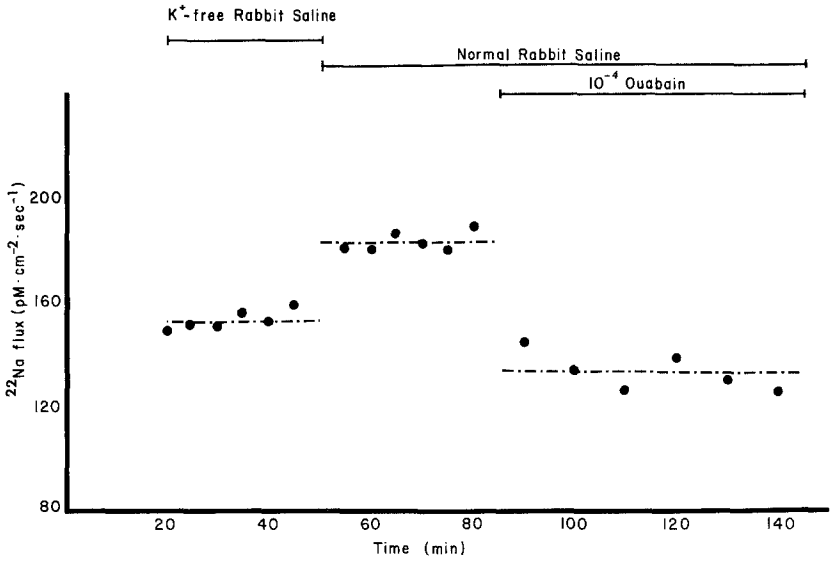

Fig. 7. The effect of K^+-free and ouabain on mucosal-to-serosal Na^+ flux. In an experiment similar to Fig. 6, K^+-free solution is shown capable of blocking the mucosal-to-serosal flux of 10 mM Na^+ to almost the same extent as 10^{-4} M ouabain. The absence of transients in the flux suggests that the rate-limiting step in mucosal-to-serosal Na^+ flux is the transport process and not any unstirred layers associated with the tissue

application of ouabain completely blocks the hyperpolarization. When the Na^+ flux associated with these treatments is examined (Fig. 7), there is a substantial reduction of Na^+ flux by the K^+-free saline on the serosa. Nonetheless, there is no evidence for any transient increase in Na^+ flux after addition of K^+ to the serosa. The same results were obtained in three additional experiments. These results suggest that if there is a serosal unstirred layer, its effect on mucosal-to-serosal Na^+ flux is smaller than the sample-to-sample variation in the flux.

Rheogenic Component of the Response

We were interested in comparing the current generated by the Na^+ transport process to the ouabain-sensitive Na^+ flux. From these two values, the $Na^+ - K^+$ coupling ratio for the transport system can be calculated. In making this measurement, we were essentially repeating the similar observation of Lewis et al. (1978) except that we were making the measurement under circumstances which more nearly corresponded to the normal physiologic state of the cell (and, of course, to our measurements of Na^+ flux). To perform the experiment, we applied the nystatin-bathing solution and nystatin just as in Fig. 1. When the cell potential had stabilized, the cell was voltage-clamped at that potential (the zero current potential). Various concentrations of Na^+ were then added to the mucosal solution and the deviation from zero current for each Na^+ concentration was measured. To be sure all of this current was associated with rheogenic transport, ouabain was added to the serosal solution and any ouabain-insensitive Na^+ current was determined for the various Na^+ concentrations. Except for the very highest

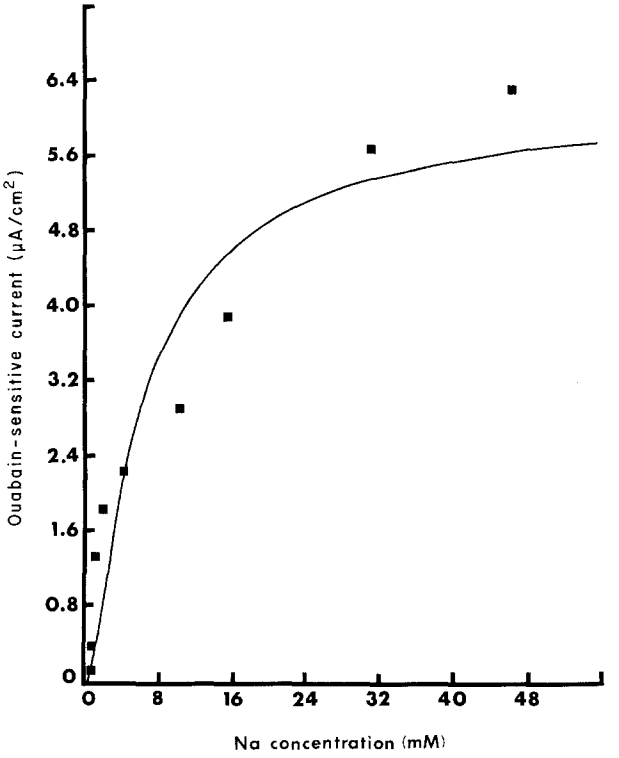

Fig. 8. Ouabain-sensitive Na^+ current. The relationship between the ouabain-sensitive component of Na^+-induced current and the Na^+ concentration at the intracellular surface of the basolateral membrane is a saturating function. The Na^+-to-K^+ coupling ratio for the basolateral Na^+ transport process can be determined from this data and the data from Fig. 4 (*see text*)

Na^+ concentrations used (>25 mM) there was no significant ouabain-insensitive Na^+ current. Figure 8 shows the ouabain-sensitive Na^+ currents obtained in the above manner on a single tissue for various mucosal Na^+ concentrations.

Discussion

In this paper, we have tried to directly measure the Na^+ transport characteristics of the basolateral (Na+K)-ATPase in rabbit urinary bladder. There are several possible difficulties with such measurements. We were at first worried about any possible effects that basolateral membrane potential might have on the activity of the ATPase. Several investigators have suggested that large potential gradients can affect the activity of the transport system (Helman, Nagel & Fisher, 1979; Macchia & Helman, 1979). Fortunately, in our work the potential changes at the basolateral membrane were never greater than 10 mV while ouabain-sensitive Na^+ fluxes were being measured. Potential changes of this magnitude should produce little if any effect on the activity of the ATPase (Brinley & Mullins, 1974; Chapman & Johnson, 1978).

Of course the greatest concern in this work was possible problems with unstirred layers. To exclude these problems, our primary rationale was that, if unstirred layers were a rate-limiting step in the movement of Na^+ across the tissue, then under conditions when the transport system is stopped or started abruptly there should be transient events associated with establishing new steady-state concentration values in the unstirred layers. We saw no evidence for any transients in the potential associated with the transport process or in the ouabain-sensitive Na^+ flux.

Another method of estimating the contribution of unstirred layers is to calculate the concentration gradient necessary to drive a diffusive flux across the unstirred layer of the same magnitude as the measured ouabain-sensitive flux. If we assume an unstirred layer at the mucosal membrane of 100 μm (Barry & Diamons, 1970) with an average cell height of 30 μm (Lewis, Eaton & Diamond, 1976), the longest diffusive path from mucosal bulk solution to the basolateral membrane would be 130 μ. Under steady-state flux conditions with 30 mM Na^+ in the mucosal bulk solution maximum Na^+ flux is about 290 pM cm^{-2} sec^{-1}. If we assume free solution mobility for Na^+ ion, a gradient of only 0.1 mM across the 130 μ unstirred layer will produce a diffusive flux of the measured magnitude. At lower flux rates associated with lower mucosal Na^+ concentrations, the necessary gradient is even lower. Even if the diffusion constant in the unstirred layer were 10 times smaller or the unstirred layer 10 times thicker, there would still only be about a 3% difference between the bulk solution Na^+ concentration and that at the intracellular surface of the basolateral membrane. The situation at the serosal surface is somewhat worse since the unstirred layer is probably about 300 μ with a reduction of the Na^+ mobility to about 77% of its free solution mobility (Barry & Diamond, 1970). Nonetheless, the gradient necessary to produce diffusive flux equal to the measured flux is only 0.29 mM. Considering the normally high concentration of Na^+ in the serosal solution (137 mM) this is a negligible gradient from the basolateral membrane to the serosal bulk solution. The previous analysis underscores the fact that although the low transport rate of the rabbit urinary bladder produces very small short-circuit currents, without such low transport rates the experiments described in this paper might not be possible.

Na^+ Permeability of the Basolateral Membrane

Although the primary purpose of the experiments described in this paper was to describe the characteristics of the basolateral Na^+ transport process, examination of the ouabain-insensitive Na^+ flux also gives information about the passive Na^+ permeability of the basolateral membrane.

By determining the net ouabain-insensitive Na^+ flux from the difference between the serosal-to-mucosal and the mucosal-to-serosal fluxes, we can calculate the permeability from the relationship (Hodgkin & Horowicz, 1959)

$$P_{Na} = M_{Na} \cdot \frac{RT}{V_{bl}F} \cdot \frac{1-\exp(V_{bl}F/RT)}{[Na]_s - [Na]_m \exp(V_{bl}F/RT)} \quad (1)$$

where P_{Na} is the Na^+ permeability, M_{Na} is the net Na^+ flux, V_{bl} is the basolateral membrane potential, $[Na]_s$ and $[Na]_m$ are the serosal and mucosal Na^+ concentrations, respectively, and R, T and F have their usual meanings. For the three experiments in which the serosal-to-mucosal flux was measured with 10 mM mucosal Na^+, the mucosal-to-serosal flux was assumed to be the mean value of all experiments with 10 mM mucosal Na^+ (92 pM cm^{-2} sec^{-1}).

Using Eq. (1), we calculate a Na^+ permeability of $(8.5 \pm 6.2) \times 10^{-7}$ cm sec^{-1}. Considering the substantial difference in methods, this value corresponds well with the value of 1×10^{-7} cm sec^{-1} obtained in rabbit urinary bladder by electrical measurements (Lewis et al., 1978). The relatively close agreement with the previous report supports our view that we have a relatively intact basolateral membrane.

Characteristics of the Basolateral Transport Mechanism

Since unstirred layers do not appear to be a serious problem, we used our results to calculate some of the properties of the basolateral transport system.

For a situation in which a specific number of Na^+ ions, n, interact with surface sites of a membrane-bound ATPase and the number of these surface sites is finite, the ratio of measured Na^+ flux to maximum Na^+ flux is given by the Langmuir equation (Hagiwara & Takahashi, 1967; Garay & Garrahan, 1973):

$$\frac{M_{Na}}{M_{MAX}} = \frac{1}{\left(\frac{K_{Na}}{[Na]_i} + 1\right)^n} \quad (2)$$

where $[Na]_i$ is the internal Na^+ ion concentration, K_{Na} is the internal activity of Na^+ ion that leads to half-maximal flux, while M_{Na} and M_{MAX} are Na^+ flux at a given Na^+ concentration and the maximal Na^+ flux at very high Na^+ concentrations, respectively. Although this equation is often associated with specific types of enzyme reactions, it is more generally useful in describing many saturating processes (Ainsworth, 1977).

Although the actual reaction sequence associated with the (Na+K)-ATPase may be more complicated than the several successive single-step reactions implied by Eq. (2), various investigators have had considerable success in describing the kinetics of (Na+K)-ATPase in terms of Eq. (2) if the reaction is examined under normal conditions of intra- and extracellular ion concentrations (for a review, *see* Glynn & Karlish, 1975).

When the data (presented in Fig. 4) is interpreted in terms of Eq. (2), several of the parameters in the equation can be interpreted physically. The parameter n represents the number of equivalent binding sites for Na^+ ion and consequently the number of Na^+ translocated per ATP split. M_{MAX} is a measure of the total number of available ATPase sites and their rate of turnover, while K_{Na} is simply a measure of the affinity of the ATPase sites for Na^+ ion.

To determine the values for these parameters, we have used a nonlinear, least-squares fitting algorithm (Brown & Dennis, 1972) to determine the solid line in Fig. 4. Such a nonlinear curve-fit does not introduce any of the biases associated with the various linearization methods described in the literature (Colquahoun, 1971; Ainsworth, 1977). The solid line in Fig. 4 is described by Eq. (2) with values for M_{MAX} of 287.8 pM cm^{-2} sec^{-1}, a Na^+ concentration at half saturation K_{Na} of 2 mM, and a value for the number of equivalent sites n of 3.2. (The regression coefficient for the fit was 0.938.)

Although the method of nonlinear fitting does not introduce biases into the parameter values obtained, we can represent the data in two alternative linearized formulations which have become common

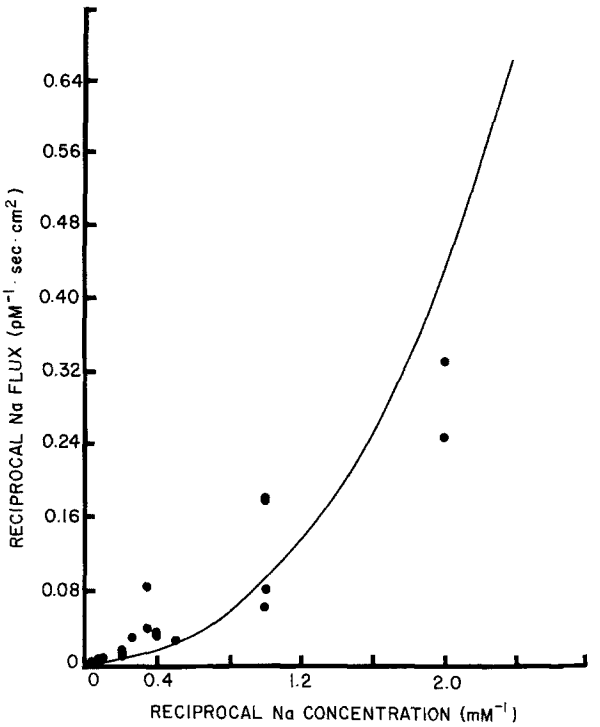

Fig. 9. Double reciprocal plot of Na^+ flux *vs.* Na^+ concentration. A traditional representation of reciprocal Na^+ flux versus reciprocal Na^+ concentration implies a Na^+ interaction leading to the flux that only involves one site. The calculated expectations for a model which involves 3 equivalent sites is represented by the solid line which appears to fit the data as well as a straight line (for more details, please *see text*)

for representing multiple site interactions (the Hill plot) and saturating functions (the Lineweaver-Burke plot). In Fig. 9, a traditional double-reciprocal plot of the data from Fig. 4 is presented. Such a plot implies a belief that the data can be described by an equation similar to Eq. (2) but with only one Na^+ interaction site ($n=1$). Although it is possible to draw a least-squares linear regression line through the data with a regression coefficient of 0.887, the slope (0.143 cm^2 sec) and intercept (-1.03×10^{-2} cm^2 sec pM^{-1}) of the line cannot be interpreted in a meaningful way. In particular, a negative intercept of a double-reciprocal plot of the sort in Fig. 9 has no physical meaning. Even if there were an error in the intercept large enough to produce a value for the maximal flux near the observed value (approximately 290 pM cm^{-2} sec), the plot would still predict a value for K_{Na} of greater than 40 mM, a value clearly above the half-maximal value of the data in Fig. 9. These observations imply that a value of $n=1$ for the model described by Eq. (2) is inappropriate. In fact, if Eq. (2) is replotted on the double-reciprocal plot with the model values obtained from the nonlinear fit, we obtain the solid

line in Fig. 9 which appears to fit the data as well as a straight line. If it is desirable to linearize Eq. (2) for any value of n, the method of Garay and Garrahan (1973) may be used. This method plots the nth root of the dependent variable (reciprocal flux in this case) *vs.* the reciprocal substrate concentration (sodium ion here) where n is the number of equivalent sites in Eq. (2). If we prepare such a plot for the data of Fig. 4 with $n=3$, it can be fit by a least-squares regression line with a regression coefficient of 0.937, an intercept of 0.150 and slope of 0.421. These values imply a maximal flux of 296.3 pM cm^{-2} sec^{-1} and a K_{Na} of 2.8 mM. Both values correspond well with the values obtained from the nonlinear curve fit.

Having established that a multiple site model appeared to explain the experimental data best (with 3 sites appearing to be the optimum number), we examined the possibility that the sites might not be strictly equivalent. In particular, the interaction of Na^+ with the first site might affect the binding of Na^+ to the second and third sites; that is, the sites might display strong cooperativity. If this were the case then Eq. (2) reduces to the following expression (Ainsworth, 1977; Benos, Mandel & Balaban, 1979):

$$\frac{M_{Na}}{M_{MAX}} = \frac{1}{1+\left(\frac{K_{Na}}{[Na]_i}\right)^n}. \tag{3}$$

Traditionally, a model of this form has been tested by plotting log $\{M_{Na}/(M_{MAX}-M_{Na})\}$ (sometimes known as LOGIT M_{Na}) *vs.* the log of the Na^+ concentration. In such a plot the slope is the number of cooperative sites and the intercept is equal to n times pK_{Na}.

Figure 10 shows such a plot. Again a straight line can be fit to the points (regression coefficient $=0.93$); however the points are better fit by the solid line which represents the replot of Eq. (2) with the best nonlinear-fit parameters (regression coefficient $=0.99$). Nonetheless, the Figure does serve to emphasize the multiple site nature of the Na^+ transporter. At high Na^+ concentrations, the slope of the best-fit line is close to one, implying, as expected, that the ATPase generally has 2 sites occupied a large proportion of the time and only requires 1 additional Na^+ to produce measurable flux. On the other hand at low Na^+ concentrations the slope is much greater (between 2 and 3) implying that the usual state of the ATPase is with either no sites or only one site occupied.

Besides the results presented here, several literature reports also support the presence of multiple Na^+ sites on the ATPase at the inner surface of the membrane. Three sites have been demonstrated in red blood cells (Garay & Garrahan, 1973) and squid axons (Brinley & Mullins, 1968). Also, Lewis et al. (1978) in rabbit urinary bladder and Garcia-Diaz and Armstrong (1980) in *Necturus* gallbladder have demonstrated a sigmoidal relationship between intracellular Na^+ and short-circuit current when the apical membrane was treated with nystatin. The relationship in rabbit urinary bladder could be fitted fairly well by a model similar to Eq. (1) with between two and four equivalent sites. Wills and Lewis (1980) and Eaton (1981) subsequently demonstrated a relationship between measured intracellular Na^+ and short-circuit current that could also best be described by multiple site models.

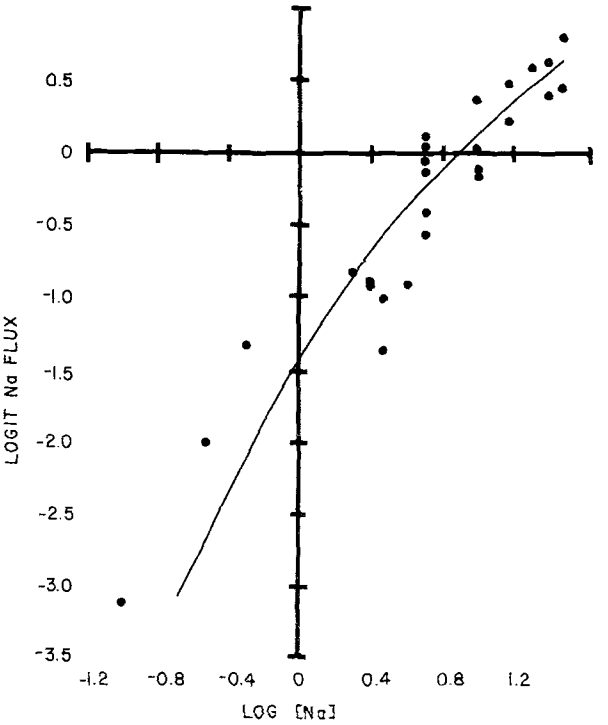

Fig. 10. Hill plot of Na^+ flux *vs.* Na^+ concentration. The log of the Na^+ concentration at the intracellular surface of the basolateral membrane is plotted *vs.* the logit of the Na^+ flux or log $\{M_{Na}/(M_{MAX}-M_{Na})\}$ where M_{Na} and M_{MAX} are the Na^+ flux at a specific Na^+ concentration and the Na^+ flux at very high Na^+ concentrations, respectively. The solid line is the calculated expectations for a three-site noncooperative model of Na^+ binding to the Na^+ transporter on the inner surface of the basolateral membrane (for more details, please *see text*)

Na–K Coupling Ratio

In their original formulation of a model for the tight epithelia, Koefoed-Johnson and Ussing (1958) proposed a neutral basolateral transport process with

exactly one Na^+ being exchanged for one K^+. They postulated this mechanism, in the absence of hard experimental evidence, mostly for its simplicity. However, subsequent to that time experimental results in preparations as diverse as snail neuron (Thomas, 1969) and red blood cells (Garay & Garrahan, 1973) as well as various epithelial tissues (Lewis et al., 1978; Rose & Nahrwold, 1976; Graf & Giebisch, 1979) have supported the possibility of nonunitary values for the ratio of Na^+ to K^+ transported per ATP. Under these conditions the transport system produces electrically measurable current which may also manifest itself as a potential drop across the basolateral membrane resistance. In our work, by comparing the ouabain-sensitive, Na^+-induced current across the basolateral membrane with the measured Na^+ flux, we can get a direct estimate of the Na^+-to-K^+ coupling ratio. Figure 7 shows the ouabain-sensitive Na^+ current for various mucosal Na^+ concentrations. The solid line drawn through the points is the best fit of the data to the expression similar to Eq. (2) (Eaton, 1981)

$$\frac{I_{Na}}{I_{MAX}} = \frac{1}{\left(1+\frac{K_{Na}}{[Na]_i}\right)^n} \tag{4}$$

where I_{Na} and I_{MAX} are the ouabain-sensitive, Na-induced current at a particular Na^+ concentration and the maximal current at high Na^+ concentrations, respectively. K_{Na}, n, and $[Na]_i$ are the same as in Eq. (2). For the best fit, the values are: for $I_{MAX} = 6.28\ \mu A/cm^2$, $K_{Na} = 1.8$ mM and $n = 3.0$. By converting the ouabain-sensitive current to flux, we obtain the rheogenic Na^+ flux for the transport system. When this is compared to the total mucosal-to-serosal Na^+ flux in the same preparations, we calculate a Na^+-to-K^+ coupling ratio of 1.40 ± 0.07 ($n = 9$). This is not significantly different from a Na^+-to-K^+ ratio of 3 to 2. A ratio of 1.58 Na^+-to-K^+ has been previously reported in rabbit urinary bladder (Wills & Lewis, 1980) while a ratio of 1.42 has been reported in turtle colon (Kirk, Halm & Dawson, 1980). The only objection to these reports was the suggestion that the transport system was tested under a situation in which the concentratons of intracellular Na^+ and K^+ might not have been normal, thus, possibly producing an abnormal operation of the transport system. Our experiments address this issue and confirm that the ratio is still 3:2 Na/K under normal intracellular and extracellular ion concentrations.

We are not truly surprised by our results since we have always felt that the (Na+K)-ATPase of virtually all cells would have similar properties. Indeed, the measured values for urinary bladder (number of sites = 3.2, $K_{Na} = 2.0$ mM) and red blood cell (number of sites = 3.0, $K_{Na} = 3.2$ mM) are almost identical. Nonetheless, we feel that it was important to verify the similarity since in subsequent work, investigators can apply the large amount of literature on the ATPase of red cells and excitable tissue in guiding their investigation of epithelial transport.

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