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Apical Membrane Area of Rabbit Urinary Bladder Increases by Fusion of Intracellular Vesicles: An Electrophysiological Study

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Summary. Mammalian urinary bladder undergoes, in a 24-hour period, a series of slow fillings and rapid emptying. In part the bladder epithelium accommodates volume increase by stretching the cells so as to eliminate microscopic folds. In this paper we present evidence that once the cells have achieved a smooth apical surface, further cell stretching causes an insertion of cytoplasmic vesicles resulting in an even greater apical surface area per cell and an enhanced storage capacity for the bladder. Vesicle insertion was stimulated by application of a hydrostatic pressure gradient which caused the epithelium to bow into the serosal solution. Using capacitance as a direct and nondestructive measure of area we found that stretching caused a 22% increase in area. Removal of the stretch caused area to return to within 8% of control. An alternate method for vesicle insertion was swelling the cells by reducing mucosal and serosal osmolarity. This perturbation resulted in a 74% increase in area over a 70-min period. Returning to control solutions caused area to decrease as a single exponential with an 11-min time constant. A microtubule blocking agent (colchicine) did not inhibit the capacitance increase induced by hypoosmotic solutions, but did cause an increase in capacitance in the absence of a decreased osmolarity. Microfilament disrupting agent (cytochalasin B, C.B.) inhibited any significant change in capacitance after osmotic challenge. Treatment of bladders during swelling with C.B. and subsequent return to control solutions increased the time constant of the recovery to control values (22 min). The Na^+-transporting ability of the vesicles was determined and found to be greater than that of the apical membrane. Aldosterone increased the transport ability of the vesicles. We conclude that some constituent of urine causes a loss of apical membrane permeability. Using electrophysiological methods we estimated that the area of cytoplasmic vesicles is some 3.3 times that of the apical membrane area. We discuss these results in a general model for vesicle translocation in mammalian urinary bladder.

Key Words vesicle fusion · Na^+ transport · aldosterone · mammalian urinary bladder · cytoskeleton · channel degradation

Introduction

During a contraction/filling cycle in the mammalian urinary bladder the epithelial cells dramatically change their shape. It is generally believed that in the contracted bladder the cells are goblet-shaped with an irregularly folded apical membrane, a smooth lateral membrane and convoluted basal membrane. As the bladder fills with urine the apical membrane is smoothed. The basal membrane becomes less convoluted as the lateral membrane becomes more convoluted. The cells now become cuboidal. Further stretching of the epithelium past this stage requires addition of new membrane to the apical surface. Evidence for such a scheme of cell shape change and membrane insertion comes from electrophysiological studies of Lewis and Diamond (1976), and morphometric analysis by Porter, Kenyon and Badenhausen (1967) and Minsky and Chlapowski (1978). The latter authors examined uptake of ferritin during bladder contraction (Porter et al., 1967), while Lewis and Diamond (1976) used measurements of membrane capacitance and tissue dry weight to establish that new membrane might be added to the apical surface.

Close inspection of electromicrographs indicates that the apical membrane of the mammalian urinary bladder has an unusually thickened membrane of ca. 12 nm. Upon freeze fracture (Staehelin, Chlapowski & Bonneville, 1972) this thick membrane was found to occupy 73% of the apical surface and was composed of polygonal plaques with a substructure of hexagonally arranged particles. The remaining 27% of the apical membrane is 7 nm in thickness and corresponds to the normal lipid bilayer.

Freeze fracture of the cytoplasm (Staehelin et al., 1972) revealed fusiform vesicles with identical plaques containing the same hexagonal particles.

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The cytoplasm of the more apical cell layer of mammalian urinary bladder contains in the contracted state a significant density of these vesicles in the supra nuclear zone (Leeson, 1962; Richter & Moize, 1963). In addition, these vesicles as well as the apical membrane plaques are all tethered by a dense network of filaments of 7-nm diameter (Minsky & Chlapowski, 1978) which coalesce in the region of desmosomes.

The exact role of these vesicles and attached filaments has been speculative. Some feel that the filaments act as a supportive structure to maintain cell integrity as well as oppose or counter the forces exerted during the contraction/filling cycle of the bladder (Staehelin et al., 1972). Others speculate a direct role of the filaments in the movement of cytoplasm vesicles into and out of the apical membrane (Minsky & Chlapowski, 1978).

In this paper, using electrophysiological methods, a physiological function for the vesicles and microfilaments is proposed. Using a series of perturbations the existance of vesicle movement into and out of the apical membrane as well as the necessity for an intact microfilament system, for this movement, is demonstrated. Last, the ionic permeability properties of the vesicle membrane and the relative area of cytoplasmic vesicles to that of the apical membrane is estimated.

Materials and Methods

Urinary bladders were obtained from male New Zealand rabbits. After removing the bladders and dissecting away the underlying muscle coats the epithelium was mounted between temperature-controlled modified Ussing chambers as previously described by Lewis (1977).

Ag-AgCl electrodes were placed close to and on opposite sides of the epithelium and were used to sense the spontaneous transepithelial potential (V_T, serosa as reference). Current (I) passing Ag-AgCl electrodes were placed in the rear of each half-chamber. Both voltage-sensing and current-passing electrodes were connected to an automatic voltage clamp. Voltage and current outputs from the clamp were connected to an A/D converter interfaced to a small laboratory computer. To measure transepithelial resistance (R_T) a computer-controlled current pulse (ΔI) was passed across the epithelium, the resulting voltage deflection (ΔV_T) was measured (300 msec after initiation of the current pulse) and the resistance then calculated using Ohm's law. (The amplitude and direction of the current pulse was set so that V_T depolarized and ΔV_T was greater than 10 mV but less than 30 mV. The resistance of the epithelium is near linear in this range.) In addition I_{sc} (short-circuit current) was calculated by dividing the measured V_T by the calculated R_T. All measurements (V_T, ΔV_T, ΔI, I_{sc} and R_T) were stored on magnetic disk and displayed on a printer along with the time of the measurement. Voltage and current channels were also continuously monitored on a storage oscilloscope (Tektronix; Beaverton, Oregon) and a dual-channel paper chart recorder (Houston Instruments).

Solutions

The standard solution used in the studies was a NaCl Ringer's with a composition in mM of: 111.2 NaCl, 25 $NaHCO_3$, 5.8 KCl, 2 $CaCl_2$, 1.2 $MgSO_4$, 1.2 K_2HPO_4 and 11.1 glucose. In experiments designed to swell the cells, the following solution (in mM) was used: 43.1 NaCl, 25 $NaHCO_3$, 2.9 KCl, 1 $CaCl_2$, 0.6 $MgSO_4$, 0.6 K_2HPO_4 and 5.5 glucose. Both solutions were aerated with 95% O_2 and 5% CO_2, buffered to pH 7.4 at 37°C.

Colchicine (Sigma) was added to the solutions at a final concentration of 0.1 mM. Cytochalasin B (Sigma) was dissolved in methanol and then added to both chambers to a final concentration of 35 μM. Amiloride (a gift from Merck, Sharp and Dohme) was added to only the mucosal chamber to a final concentration of 10^{-4} M, a dose known to cause 100% inhibition (Lewis & Diamond, 1976).

To irreversibly inhibit the amiloride-sensitive current, *p*-chloromercuribenzenesulfonic acid (PCMBS) was added to the mucosal chamber at a final concentration of 0.5 mM.

To increase Na^+ transport by the rabbit urinary bladder, animals were placed on a low Na^+ diet (Purina) for 2 weeks prior to sacrificing (Lewis & Diamond, 1976; Schwartz & Burg, 1978).

Capacitance Measurements

The apical membrane area was determined by measuring the epithelial capacitance where 1 $\mu F \simeq 1$ cm^2 of actual membrane area (Cole, 1972). In brief, a transepithelial constant current pulse was passed across the epithelium. The resulting voltage response was digitized every 100 μsec or 1 msec, depending upon whether the current pulse was shorter or longer than 254 msec, respectively. The voltage response was fitted by the sum of two exponentials, yielding two resistor values and two capacitor values (R_1, R_2 and C_1, C_2, respectively). As noted by Lewis, Clausen and Diamond (1976) and Clausen, Lewis and Diamond (1979), these resistor and capacitor values are complex functions of the apical and basolateral resistances and capacitances (R_a, R_{bl} and C_a, C_{bl}, respectively) and junction resistance (R_j). However, a meaningful value can be derived from the following relationship:

$$\frac{C_1C_2}{C_1 + C_2} = \frac{C_aC_{bl}}{C_a + C_{bl}} = \frac{C_a}{\left(\frac{C_a}{C_{bl}}\right) + 1} = C_T \qquad (1)$$

This C_T (effective capacitance) is then equal to the value for two series capacitors (Eq. 1). However, C_T is dependent on the ratio of apical-to-basolateral capacitance. If C_a is much less than C_{bl} then $C_T \cong C_a$. Clausen, Lewis and Diamond (1979), estimated a C_a/C_{bl} of 1 : 5, thus C_T will underestimate C_a by 20%. If indeed we measure a change in C_T this then will represent the minimal change in apical capacitance. Appendix A gives a full explanation and verification of the method for rabbit urinary bladder. All data is given as mean ± SEM. Where appropriate either a paired or unpaired *t*-test was performed to determine significance.

Results

In this section we first show that there is a change in effective capacitance of the epithelium caused by

Table. Change in capacitance and resistance of the urinary bladder induced by bowing the epithelium into the serosal chamber[a]

	$C_T(\mu F)$	ΔC_T	$R_T(\Omega\ \mu F)$	ΔR_T
Control	2.25 ± 0.21	0	18,700 ± 1,500	0
Stretch	2.69 ± 0.23	0.22 ± 0.04	11,100 ± 4,900	−0.30 ± 0.18
Return	2.43 ± 0.21	0.08 ± 0.02	22,600 ± 9,300	0.13 ± 0.12

[a] The fractional change in capacitance and resistance (ΔC_T and ΔR_T) during stretch and return are normalized to the control value, $n = 4$.

stretch or cell swelling. Next we investigate the control of vesicle insertion and withdrawal. Lastly, we determine whether these vesicles contain amiloride-sensitive transport pathways and the relative area of cytoplasmic vesicles to the apical membrane.

Stretch and Cell Swelling Cause a Capacitance Increase

The urinary bladder can accommodate a volume increase by first unfolding of an initially folded apical membrane and second by insertion of cytoplasmic vesicles into (at least) the apical membrane. Evidence for the later possibility is found in the morphological analysis of Minsky and Chlapowski (1978) and suggested by the data of Lewis and Diamond (1976).

Two methods were used to investigate the possibility of insertion of cytoplasmic vesicles into the apical membrane. The first is to mount the epithelium in the chamber without the normally present nylon mesh which supports the preparation from the serosal side. The epithelium is stretched by applying a hydrostatic pressure oriented so as to bow it into the serosal compartment. If no new membrane is added then the capacitance will remain constant since the epithelium will bow out until all membrane folds are eliminated. If the capacitance increases this might indicate that apical membrane area is being increased by insertion of vesicles into (at least) the apical cell membrane. The Table demonstrates that for four preparations capacitance increased by a mean of 22%, and after removal of the gradient the capacitance returned to within 8% of the initial value in less than 5 min. The resistance of these preparations, during stretch, did decrease but once resistance is normalized to area (i.e. capacitance) there was no significant change (*see* Table). This indicates that stretch increases apical membrane capacitance, perhaps by the fusion, into the apical membrane, of cytoplasmic vesicles.

An alternative approach to stimulation of vesicle translocation might be by cell swelling. Such swelling might cause a mobilization of vesicles to accommodate the increase in cell volume without a concomitant stress on the cell membranes.

Exposure of the epithelium to $\frac{1}{2}$ osmotic strength Ringer's (*see* Materials and Methods for composition) on both serosal and mucosal surfaces causes no change in epithelial capacitance for the first 15 min. After this time capacitance rapidly increases over the next 45 min and reaches a stable value some 74% greater than that of control (Fig. 1). The change in resistance during such an osmotic perturbation is also shown in Fig. 1. There was no significant change in resistance during the period of reduced osmolarity ($P > 0.2$). Upon returning both solutions to control Ringer's, the increased capacitance is rapidly returned to near control values (Fig. 1). This rapid return can be fit to an equation of the form:

$$C_T(t) = C_T(o)e^{-tK} \tag{2}$$

where $C_T(o)$ is the capacitance immediately before returning to isosmotic solution, $C_T(t)$ is the capacitance at any time "t" after establishing isosmotic solutions and K is the proportionality constant which in electrical terminology is the inverse time constant, i.e. the time required to reach 37% of the control value. This time constant is 11.3 ± 1.1 (n = 4) min and reflects the withdrawal of vesicles for a random model.[1]

The magnitude of the capacitance change cannot be accounted for by an increase in only the basolateral capacitance since an initial C_a/C_{bl} ratio of 1:5 (Clausen et al., 1979) would result in a maximum change of 20% as compared to the mean change of 74% (the smallest change observed was 56%).

[1] *See* Appendix for proof that the time course of C_T closely follows that of C_a.

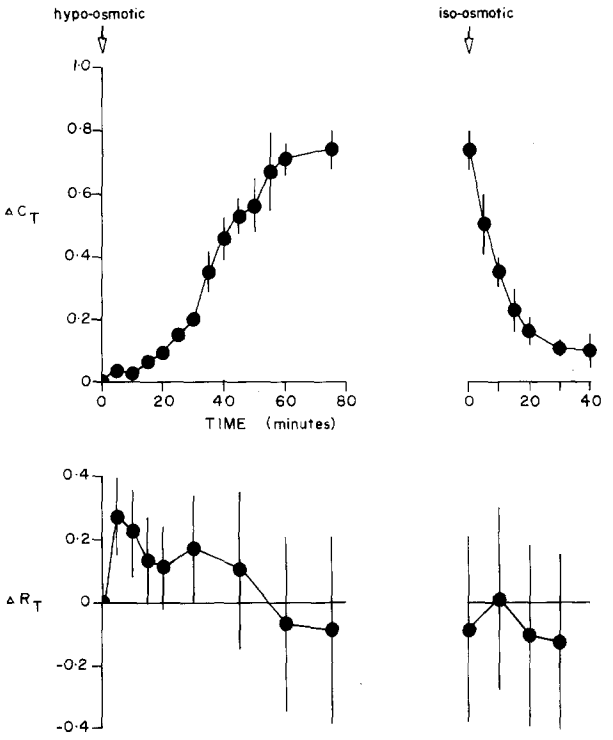

Fig. 1. The effect of ½ osmotic strength Ringer's on ΔC_T (fractional increase in effective capacitance) and R_T (normalized to capacitance as defined in Eq. 1). Note that ΔC_T starts to increase after 15 min. The magnitude of this increase (74%) requires an increase in the apical membrane capacitance of at least 44% (*see* Discussion). The change in resistance shows no consistent trend, indicating a variability by individual bladders to accommodate the osmotic challenge. The initial $C_T = 2.26 \pm 0.16$ μF (for a 2 cm^2 chamber opening), $n = 8$. Returning the bladder to control Ringer's results in a rapid exponential decrease in the capacitance with a time constant of 11 min. R_T was substantially lower than before swelling and I_{sc} (*not shown*) was greater. Vertical bars are SEM for $n = 4$

Control of Vesicle Fusion

In response to epithelial stretch or swelling, capacitance was noted to change. Electron micrographs indicate a dense filamentous network binding the cytoplasmic vesicles with the apical membrane, tight junctions and cellular desmosomes. This section addresses the question of whether microtubules and/or microfilaments are involved in vesicle insertion and withdrawal at the apical membrane.

If microtubules control the movement of cytoplasmic vesicles, then the use of colchicine should inhibit the translocation of cytoplasmic membrane constituents. A 3-hr incubation of the epithelium in colchicine caused a 17% increase in capacitance (Fig. 2*a*). Figure 2*b* shows that the amount of increase in capacitance during colchicine treatment is dependent on the initial capacitance value, i.e. the degree of stretch. A highly stretched preparation gives the largest absolute change in capacitance (from 2 to 2.6 μF), while a less stretched preparation gives a small change in capacitance (from 3.05 to 3.2 μF). Subsequent exposure to hypoosmotic solutions caused a rapid increase in capacitance as compared to control conditions (Fig. 2*a*). Return to control Ringer's caused a decrease of capacitance (*not shown*) with a time course similar to control preparations. This indicates that microtubules are not essential for membrane translocation. The other possibility for control of vesicle translocation are microfilaments. A well-known agent for microfilament disruption is cytochalasin B (C.B.). Pretreatment of the bladder with C.B. caused no alteration in epithelial capacitance but did result in a nonspecific decrease in transepithelial resistance (Fig. 3), as has been reported for toad urinary bladder (Taylor et al., 1973). Challenging the C.B. pretreated urinary bladder with the hypoosmotic solutions does not elicit any change in the epithelial capacitance (Fig. 3), suggesting that an intact microfilament system is important for vesicle translocation.

Are microfilaments also necessary for movement of the vesicles back into the cytoplasm? To study this, the bladder was exposed first to hypoosmotic solutions for 60 min, then C.B. was added to both chambers. After 60-min exposure to C.B both solutions were returned to normal osmolarity and the effective capacitance was monitored. Again the capacitance decreased in an exponential fashion; however, the time constant was increased to 22.5 ± 1.7 ($n = 3$) min as compared to 11.3 min for the control.

Na^+ Permeability Properties

Lewis and Diamond (1976) reported that the urinary bladder was capable of actively transporting Na^+ from urine to plasma and that aldosterone increased this transport rate. Lewis and Wills (1983) have also noted that the selective permeability of the amiloride pathways is increased to Na^+ after aldosterone treatment. This section investigates the permeability properties of the vesicles. A third method, utilizing a mechanical perturbation, was used to maximize the insertion of vesicles into the apical membrane. In brief, a rapid series of hydrostatic pressure pulses (called punching[2]) are applied to the epithelium from the apical side. Figure 4 illustrates the effect of such a procedure on V_T, R_T and I_{sc}. Immediately after a punch V_T and R_T are greatly decreased (R_T is usually about 200 Ω μF). Over the next 40 min both V_T and R_T increase to new stable values. V_T and I_{sc} are greater and R_T is less than

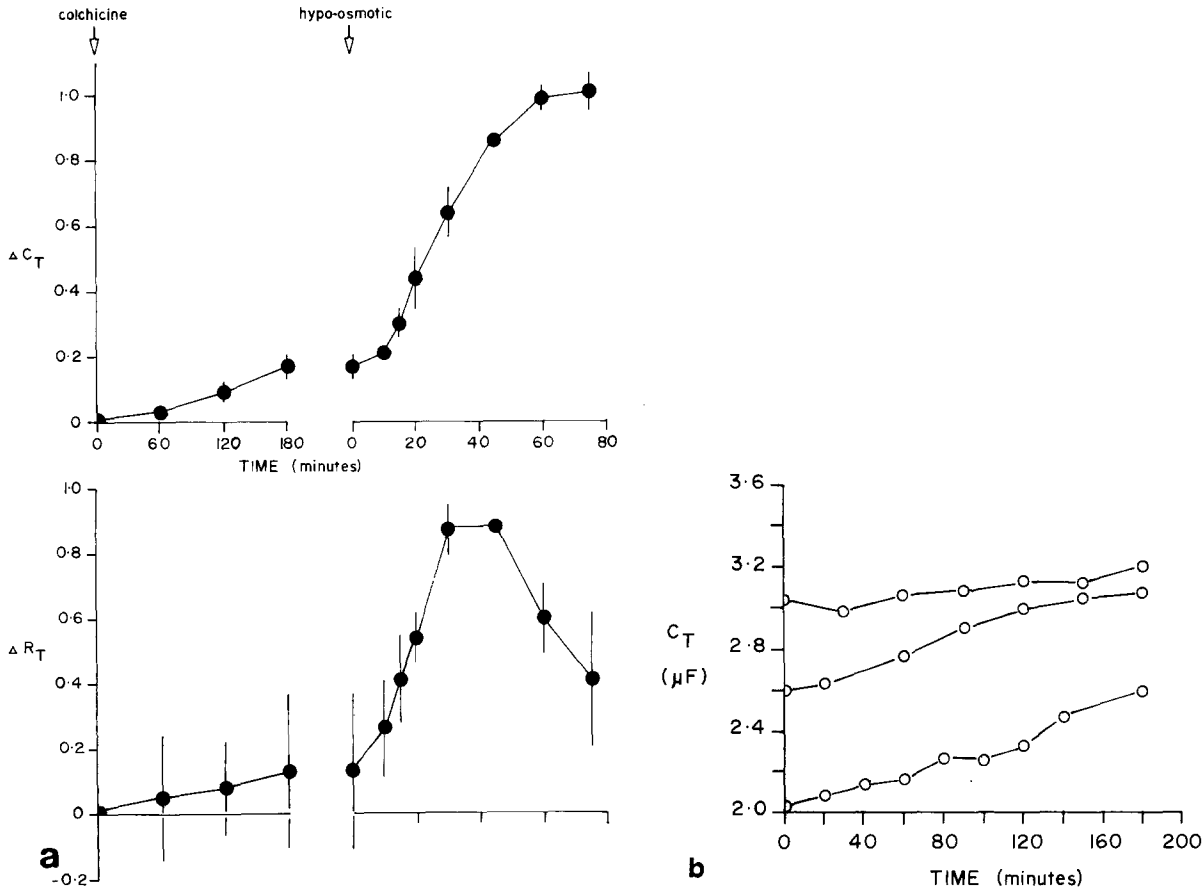

Fig. 2. (*a*) Effect of colchicine on ΔC_T and R_T before and during osmotic challenge. Note that colchicine elicits a 17% increase in ΔC_T. The rate of capacitance change with osmotic challenge is more rapid than in control conditions. In contrast to Fig. 3, R_T always increased. Vertical bars are SEM for $n = 3$. The initial $C_T = 2.56 \pm 0.27$ μF (for 2 cm^2 chamber opening). (*b*) Change in capacitance as a function of time after colchicine. Nominal chamber area is 2 cm^2. Note that the more stretched a preparation is (lower C_T) the larger is the capacitance increase during cholchicine inhibition of microtubular assembly

before punching. Capacitance is not changed by this procedure but there is a significant enhancement of apical Na^+conductance, i.e., amiloride-sensitive Na^+ transport (from 0.4 ± 0.1 to 4.7 ± 0.7 μA/μF, $n = 10$, SEM).

Pretreating the bladder with C.B. and then punching greatly reduces the increase in amiloride-sensitive Na^+ transport, implying that microfilaments are required for vesicle insertion during punching. To reach a constant amiloride response 2.2 ± 0.2 ($n = 5$) punches were required (Fig. 5).[2] The possibility that aldosterone might increase the Na^+ permeability of the vesicles was investigated by measuring the maximum amiloride-sensitive current (achieved by punching) of bladders from rabbits placed on a low Na^+ diet for 2 weeks, compared to a control group of animals which were fed a normal diet for 2 weeks. Bladders from diet animals had twice the amiloride-sensitive current (5.7 ± 0.65 μA/μF, $n = 4$ and 2.4 ± 0.7 μA/μF, $n = 4$, respectively). This indicates that aldosterone increases the permeability of not only the apical membrane but also the cytoplasmic vesicles.

Estimating the Area of Cytoplasmic Vesicles

Morphological evidence indicates that there is a great reserve of cytoplasmic vesicles even in the stretched or distended bladder. In this section we will attempt to calculate the area offered by the vesicles and compare it to the apical area.

The method is as follows: First we "punch" the preparation to a constant amiloride-sensitive I_{sc}.

[2] Each punch is composed of rapidly raising and lowering the mucosal solution 6 to 8 times in the absence of a serosal solution.

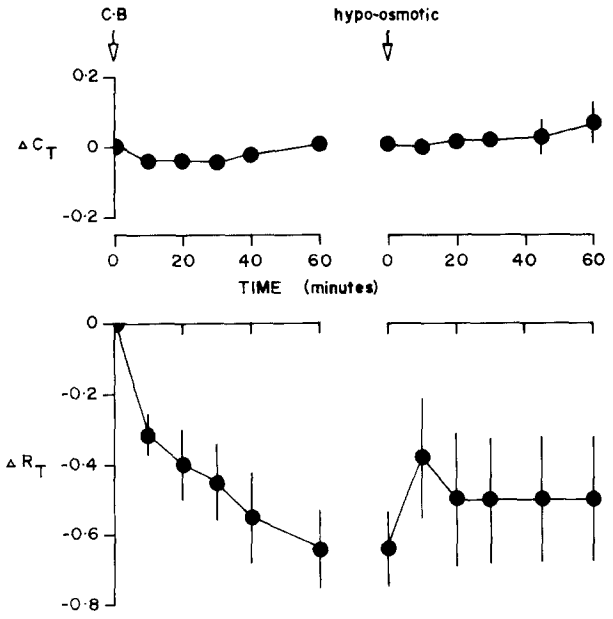

Fig. 3. Effect of cytochalasin B on capacitance and R_T before and during osmotic challenge. Two points are noteworthy. First C.B. inhibits any change in capacitance during osmotic challenge. Second C.B. induces a conductive increase in the absence of osmotic challenge. Similar conductance changes have been measured in the toad bladder, indicating an ionophore-like action of C.B. Vertical bars are SEM for $n = 3$. The initial $C_T = 2.14 \pm 0.11$ μF

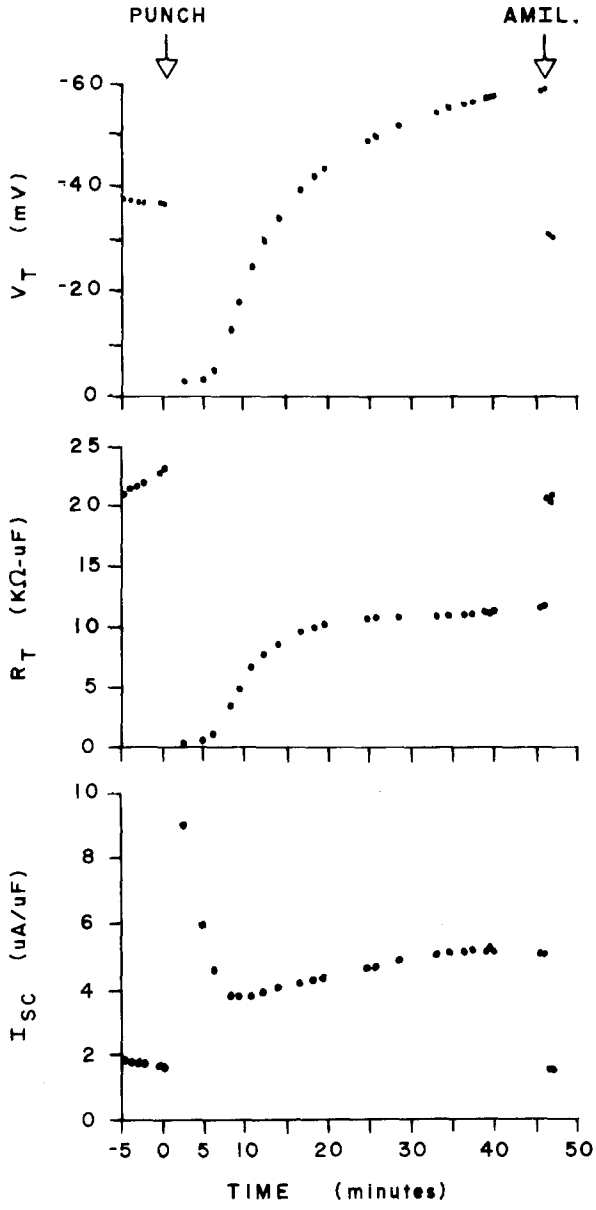

Fig. 4. The time-dependent recovery of V_T, R_T and I_{sc} after "punching" a preparation (*see text*)

Thus, the permeability properties of the apical membrane is equal to the permeability properties of the vesicles. Next we irreversibly inhibit all amiloride-sensitive pathways (in the apical membrane) using the sulfhydryl reactive agent PCMBS (*see* Fig. 6). We have now made the apical membrane permeability properties totally different than the vesicles and have thus tagged the vesicles that make up the apical membrane. The last step is to punch the epithelium to a new constant amiloride-sensitive current. In this last state we have mixed apical tagged vesicles with the cytoplasmic vesicles. Now the apical membrane has two populations of vesicles, those of apical origin (i.e., PCMBS-treated) and those of cytoplasmic origin. If one can measure the number of vesicles treated with PCMBS and divide that by the number of vesicles *not* treated with PCMBS, this will yield a ratio of apical area to cytoplasmic vesicle area. The number of remaining PCMBS-treated vesicles is equal to the difference in amiloride-sensitive I_{sc} immediately before PCMBS treatment to that after PCMBS treatment plus punching. The number of vesicles from the cytoplasm is equal to the amiloride-sensitive I_{sc} measured after PCMBS plus punching. The value for pool to apical area is 3.3 ± 1.2 ($n = 6$). Thus there is a greater surface area of cytoplasmic vesicles than for the apical membrane.

Discussion

Evidence for Vesicle Fusion

The first report of the presence of cytoplasmic vesicles in the mammalian urinary bladder epithelium (mouse in this case) was by Walker (1960). Walker noted that this epithelium consisted of three histo-

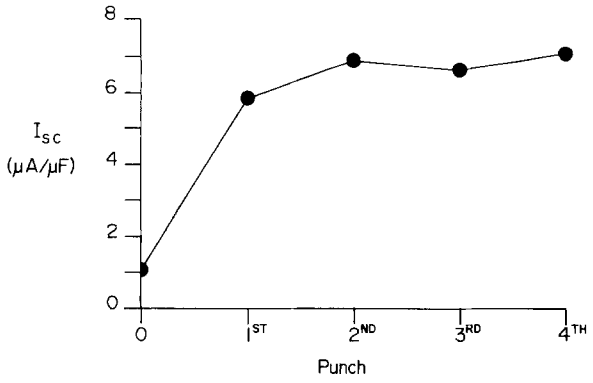

Fig. 5. An example of the number of "punches" required to bring I_{sc} to a constant elevated value (for description *see text*)

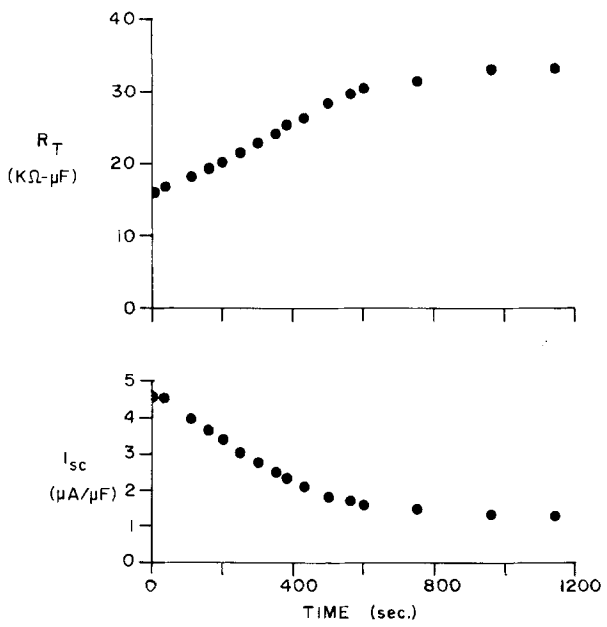

Fig. 6. These data indicate that the sulfhydryl reactive agent PCMBS irreversibly inhibits amiloride-sensitive current. At time zero PCMBS (0.5 mM) was added to only the mucosal solution. Over the next 5 min V_T decreased (*not shown*), R_T increased and I_{sc} decreased. Washing the PCMBS out of the mucosal solution caused no significant increase in I_{sc}

logically distinct cell layers, with the largest cells being in direct contact with the urine. Close inspection of the most apical layer showed that the cells were packed with discoidal vesicles and a dense filamentous network. Although Walker speculated that the function of the cytoplasmic vesicles was secretory, subsequent work by Porter, Kenyon and Badenhausen (1965, 1967) disproved this function and proposed instead that the vesicles are added to or removed from the apical membrane of the bladder during distension or contraction, respectively. Minsky and Chlapowski (1978) using morphological techniques, demonstrated that the predominant mechanism for reducing apical area was by vesicle formation (as suggested by Hicks, 1965, and Porter et al., 1967) and not membrane infolding (as suggested by Staehelin et al., 1972; Koss, 1969). This former mechanism has the advantage of reducing apical area thus offering a smaller surface area for loss of ions and water during subsequent bladder filling. The mechanism of this transfer of vesicles into and out of the apical membrane was not known, but Minsky and Chlapowski (1978) speculated that the filaments might play a role in the translocation (this is discussed below). Electrophysiological evidence for vesicle insertion is found in the data of Lewis and Diamond (1976) when they correlated epithelial capacitance with tissue dry weight for a number of different preparations. If only membrane is being unfolded then a plot of capacitance against dry weight will have a single slope with an extrapolated intercept of zero weight for zero capacitance. This however was not found. Inspection of their Figure (Fig. 3, p. 10, Lewis & Diamond, 1976) shows two slopes, one which at high capacitance and weight will extrapolate to the above intercept, the other slope intersecting the capacitance axis at ca. 1 μF per cm^2 of chamber opening. Dry weight decreased by 245% but capacitance decreased only 14%. The simplest interpretation of their data is as follows: the initial decrease in dry weight and capacitance is a result of the elimination of first macroscopic and then microscopic folds; at this point the apical membrane is flat. A further decrease in dry weight (caused by stretch) *can only* occur if vesicles are added to the apical membrane.

In this study we found that the epithelial capacitance could be increased by two methods. The first was by applying a hydrostatic pressure gradient oriented to bow the epithelium into the serosal chamber. This resulted in a 22% increase in area. Removal of the pressure gradient caused area to return to within 8% of control. The second approach was to induce cell swelling, which resulted, after one hour, in a 74% increase in capacitance (i.e. apical area). Again, the simplest interpretation (which is supported by morphological evidence, *see below*) is the addition of membrane to the apical surface from a cytoplasmic store and not an unfolding of a highly convoluted apical membrane. An alternate method was to apply a series of hydrostatic pressure pulses (punching). Figure 7 shows a possible mechanism for vesicle insertion and removal. We hypothesize that the hydrostatic pressure compresses the cells accelerating both the apical membrane and vesicles towards each other. Upon contact some vesicles "kiss" into the membrane. After the pressure pulse

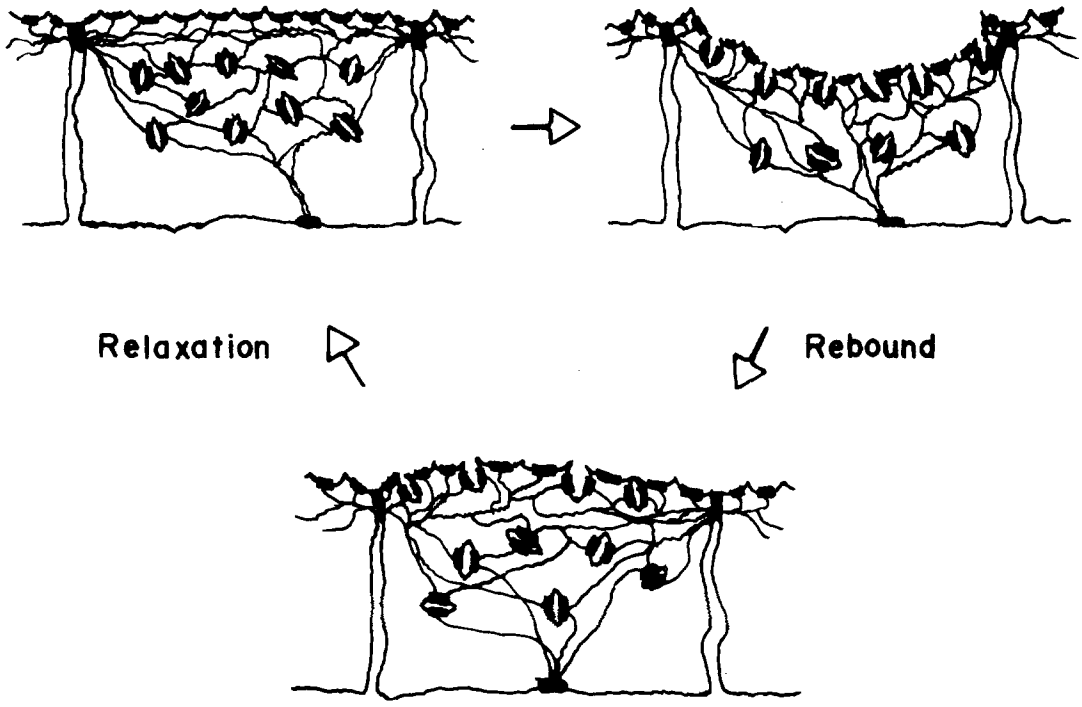

Fig. 7. A cartoon illustration of the possible mechanism for vesicle insertion caused by "punching." *See text* for details

the apical membrane would rebound, and since the vesicles are tethered by microfilaments, some will be pulled out of the apical membrane into the cytoplasm.

In the remainder of this discussion we will consider in turn the role of the cytoskeleton in vesicle insertion and withdrawal, the response of the cells to stretch and swelling and lastly the influence of urine composition on the vesicle properties.

Role of the Cytoskeleton

The mechanism of vesicle fusion is, at the simplest, a three-step process. First one must maneuver the vesicle close to the apical membrane, the lipid portions (interplaque regions) must fuse, and finally the vesicle opens up forming a tight seal with the interplaque regions of the apical membrane. The role of microfilaments in the first step is strongly suggested by the inhibitory effect of cytochalasin B on capacitance change by osmotic challenge (Fig. 3) and also current changes by punching. Such capacitance measurements or current changes preclude the possibility that increases in the apical membrane area occur by simply unfolding of apical membrane. Thus filaments are necessary for incorporation. The role of microtubules in the regulation of vesicle insertion/withdrawal is less clear. Colchicine, a drug known to inhibit microtubules did not alter the response to osmotic challenge but by itself caused an increase in area. The absolute increase in area was inversely proportional to the starting capacitance value (*see* Fig. 2*b*). Thus at a starting value of 3 μF the capacitance increase was minimal while at a starting value of 2 μF the change was maximal. A possible interpretation of these results is that microtubules control the general shape of the cells and thereby decrease the stress on the apical and basolateral membranes during stretch. Inhibition of this shape-retaining role by inhibition of microtubule polymerization might transfer the stresses implicit during stretch to the apical membrane, which then reduces this stress by addition of vesicles.

The processes mediating vesicle withdrawal are less clear. After returning the preparation to isosmotic conditions either without or with colchicine treatment the area changes return to near baseline values within 40 min, suggesting that microtubules are not involved in vesicle removal. If the preparation is treated with C.B. during an osmotic challenge the time course for return to baseline is retarded; however, the apical area does ultimately return to near control values. This indicates that microfilaments are important for insertion but not necessarily for removal. Possible interpretations for this is either an incomplete action of C.B. on microfilament disruption, or that the close apposition of vesicles, during cell shrinkage, leads to a natural contact of dimers resulting in a retraction of mem-

brane. At this time, we cannot distinguish between these possibilities or others.

The mammalian urinary bladder is not the only preparation which increases or regulates its transport ability by insertion of membrane that contains transport proteins. Others include, the gastric mucosa (H^+ secretion; Diamond & Machen, 1983 for review), turtle urinary bladder (H^+ secretion; Gluck, Cannon & Al-Awqati, 1982), and the toad urinary bladder (H_2O absorption; Wade, Stetson & Lewis, 1981; Warncke & Lindemann, 1981; Stetson, Lewis, Alles & Wade, 1982). Colchicine has been found to inhibit the stimulation of transport for these three epithelia (Kasbekar, Obrink & Flemstrom, 1978; Arruda, Sabatini, Mola & Dytko, 1980; Taylor et al., 1973, respectively). Cytochalasin B, however, does not alter gastric mucosal H^+ secretion (Kasbekar et al., 1978), has not been tested on the turtle bladder, and inhibits the hydroosmotic response of the toad bladder (Taylor et al., 1973).

Thus, as opposed to mammalian urinary bladder, the above epithelia rely on assembled microtubules for vesicle translocation. This difference might reflect the active contraction of microtubules for movement of vesicles into a membrane as opposed to a more passive movement of vesicles in response to cell shape change as is the case for the mammalian bladder.

Cell Shape and Vesicle Movement

Upon distension the epithelial cells change from a cube (of ca. 20 μm in height) to a disc (of ca. 5 μm in height). In the collapsed state the microfilaments are oriented perpendicular to the apical membrane with attachment points to vesicles, apical membrane, tight junctions and desmosomes in both lateral and basal membrane (Minsky & Chlapowski, 1978). As the cell is stretched the cell diameter increases and the filaments start to orient themselves more parallel to the apical membrane. This results in a lifting of vesicles toward and in contact with the apical membrane. Since microfilaments connect the lateral and basal membranes, stretching causes the basal membrane to move toward the apical membrane. The above cycle is reversed when the bladder collapses.

Must the cell volume and basolateral membrane area also change during bladder distension? A simple calculation to determine the maximum apical area increase at constant cell volume and basolateral area is as follows. We start with a cube of unitary dimensions. We then increase by equal amounts the length and depth of both the top (i.e. apical area) and bottom of the cube. At constant basolateral area we calculate the new cell height, and from this height and the new length and depth we calculate the new volume. Performing such a simple calculation we find that apical area could be increased by a factor of 240%. This value is almost identical to the maximum decrease in dry weight (245%) caused by stretching for a constant capacitance as determined by Lewis and Diamond (1976). In addition, from our measured value of apical area to vesicle pool area there is a more than adequate amount of vesicles to accommodate such an area change.

Some unresolved questions are: how do the lower cell layers accommodate stretch, is the length of the apical cell perimeter constant and, if not, do the tight junctions reform during distension and bladder collapse?

Urine Modifies Apical Membrane Properties

There are two observations which suggest that the properties of the apical membrane are altered during exposure to urine. The first is that stimulation of vesicle fusion into the apical membrane by stretch, cell swelling or punching results in an enhancement of the net Na^+ transport across the epithelium. Recent experiments by Loo, Lewis, Ifshin and Diamond (1983), using fluctuation analysis, demonstrated that the increase in Na^+ transport ability of the apical membrane was a result of an increase in apical membrane channel density and not single-channel currents. Inhibition of vesicle translocation into the apical membrane (by pretreatment with C.B.) inhibits the increase in Na^+ transport normally produced by punching. These observations suggest that first the vesicles transfer Na^+ channels from the cytoplasm to the apical membrane, and second that once in the apical membrane the channels are modified by some constituent of the urine resulting in a decrease in channel density. The second observation deals with the aldosterone stimulation of Na^+ transport. In this regard placing rabbits on low Na^+ diets for two weeks resulted in an increase in net Na^+ transport both before and after stimulation of vesicle fusion. This raises two interesting mechanisms for aldosterone action. The first is a stimulation of channel production during vesicle synthesis by the golgi. Thus during each expansion/contraction cycle of the bladder, vesicles with a higher channel density are inserted, resulting in an increase in the magnitude of Na^+ transport. An alternate explanation is the activation of quiescent channels that pre-exist in both the apical membrane and cytoplasmic vesicles. Recent experiments (*unpublished observation*) indicate that the latter pos-

sibility exists in the rabbit bladder, i.e., activation of pre-existing quiescent channels.

Effect of PCMBS

There have been a number of studies on the effect of the sulfhydryl reactive agent (PCMBS) on the amiloride-sensitive Na^+ transport system present in many tight epithelia. Dick and Lindemann (1975) reported that PCMBS concentrations up to 10 mM (apical solution) resulted in a rapid and reversible increase in Na^+ transport across frog skin. Benos, Mandel and Simon (1980) also demonstrated such a stimulation of Na^+ transport by 1 mM PCMBS in bullfrog skin. In addition they found that continued exposure to PCMBS resulted in an irreversible decrease in Na^+ transport, with a steady-state value 10% lower than the control I_{sc}. In both of the above studies, amiloride efficiency for inhibiting Na^+ transport was unaltered.

Gottlieb, Turnheim, Frizzell and Schultz (1978), found that exposure of the apical membrane of rabbit descending colon to 1 mM PCMBS did not result in an increase in Na^+ transport but irreversibly abolished the amiloride-sensitivity of the preparation, whether amiloride was present during PCMBS treatment or not.

The response of the amiloride-sensitive Na^+ current to PCMBS, in the rabbit urinary bladder, is similar to the long-term effects of PCMBS on frog skin, i.e., there was a decrease of amiloride-sensitive current. The rate of decline was in the range of 0.4 to 0.8 $\mu A/\mu F$ min, which is similar to the rate measured by Benos et al. (1980) estimated from their Fig. 1. Preliminary calculations on the rate of decline as a function of I_{sc} indicated a Michaelis-Menten kinetic scheme for inhibition. Unlike frog skin and rabbit descending colon, PCMBS caused a decrease in I_{sc} equal to that caused by 10^{-4} M amiloride, i.e., PCMBS acts like an irreversible amiloride although the two sites of action are most likely quite different.

Conclusion

In this paper we have attempted to demonstrate that the mammalian urinary bladder epithelium accommodates area changes by the insertion and withdrawal of cytoplasmic vesicles. This method of area change is in keeping with the primary function of the urinary bladder, which is to maintain the urine composition as close to that of the kidney filtrate as possible. Thus upon micturition the bladder must minimize the available surface area so as to reduce the movement of urine into the plasma. Such a mechanism is performed by reducing apical area by an orderly withdrawal of apical membrane into the cytoplasm. The structure which accomplishes this function is a dense network of cytoplasmic microfilaments. During Na^+ depletion these vesicles have an enhanced Na^+ permeability to counter the natural diffusion of Na^+ from plasma to urine.

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Appendix A

In this Appendix we first describe the circuit and equations used to calculate C_T, next the method for measurement of C_T, and finally confirm the validity of the method using resistor-capacitor networks of known values.

As previously shown by Lewis et al. (1976) the equation which describes the voltage response ($V_T(t)$) to a current step (ΔI) is:

$$V_T(t) = \Delta I(R_T - R_1 e^{-t/R_1C_1} - R_2 e^{-t/R_2C_2}) \tag{A1}$$

where t is time. It is important to note that even though an equivalent circuit of an epithelium has 5 circuit parameters (Fig. A1*a*) (3 resistors and 2 capacitors) the voltage response is described by 4 parameters, i.e. R_1, R_2, C_1 and C_2 (Fig. A1*b*) or as demonstrated by Suzuki et al. (1982) a completely different equivalent circuit. These 4 parameters are functions of all circuit elements.

The relationship of the apical and basolateral membrane resistors and capacitors and junctional resistor to the four-parameter model (R_1, R_2, C_1 and C_2) is:

$$\frac{C_1C_2}{C_1 + C_2} = \frac{C_aC_{bl}}{C_a + C_{bl}} = C_T \tag{A2}$$

$$\frac{R_1 + R_2}{R_1C_1R_2C_2} = \frac{R_a + R_{bl}}{R_aC_aR_{bl}C_{bl}} \tag{A3}$$

$$\frac{1}{R_1C_1} + \frac{1}{R_2C_2} = \frac{R_a + R_j}{R_aR_jC_a} + \frac{R_{bl} + R_j}{R_{bl}R_jC_{bl}} \tag{A4}$$

$$\frac{1}{R_1C_1R_2C_2} = \frac{R_a + R_{bl} + R_j}{R_aR_{bl}R_jC_aC_{bl}}. \tag{A5}$$

It must be emphasized that R_1, R_2, C_1 and C_2 bear no direct resemblance to the true membrane parameters (R_a, R_{bl}, C_a and C_{bl}) unless $R_j \gg R_a$ and R_{bl}.

In this section we will demonstrate that by recording the complete time response of the voltage to a square current step (as opposed to a single time point) the effective capacitance defined by Eq. (2) as

$$C_T = \frac{C_1C_2}{C_1 + C_2}$$

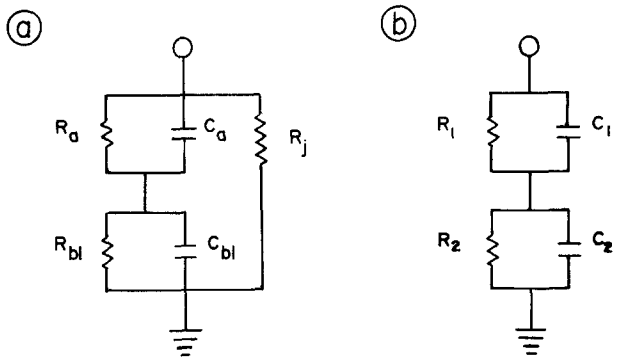

Fig. A1. Electrical equivalent circuit for the rabbit urinary bladder. (*a*) is the representation for an epithelium in which the individual morphological parameters are represented as discrete elements. Because of the nonuniqueness of the model it can be represented by a four-element model, shown in (*b*) and described by Eqs. (A2), (A3) and (A4)

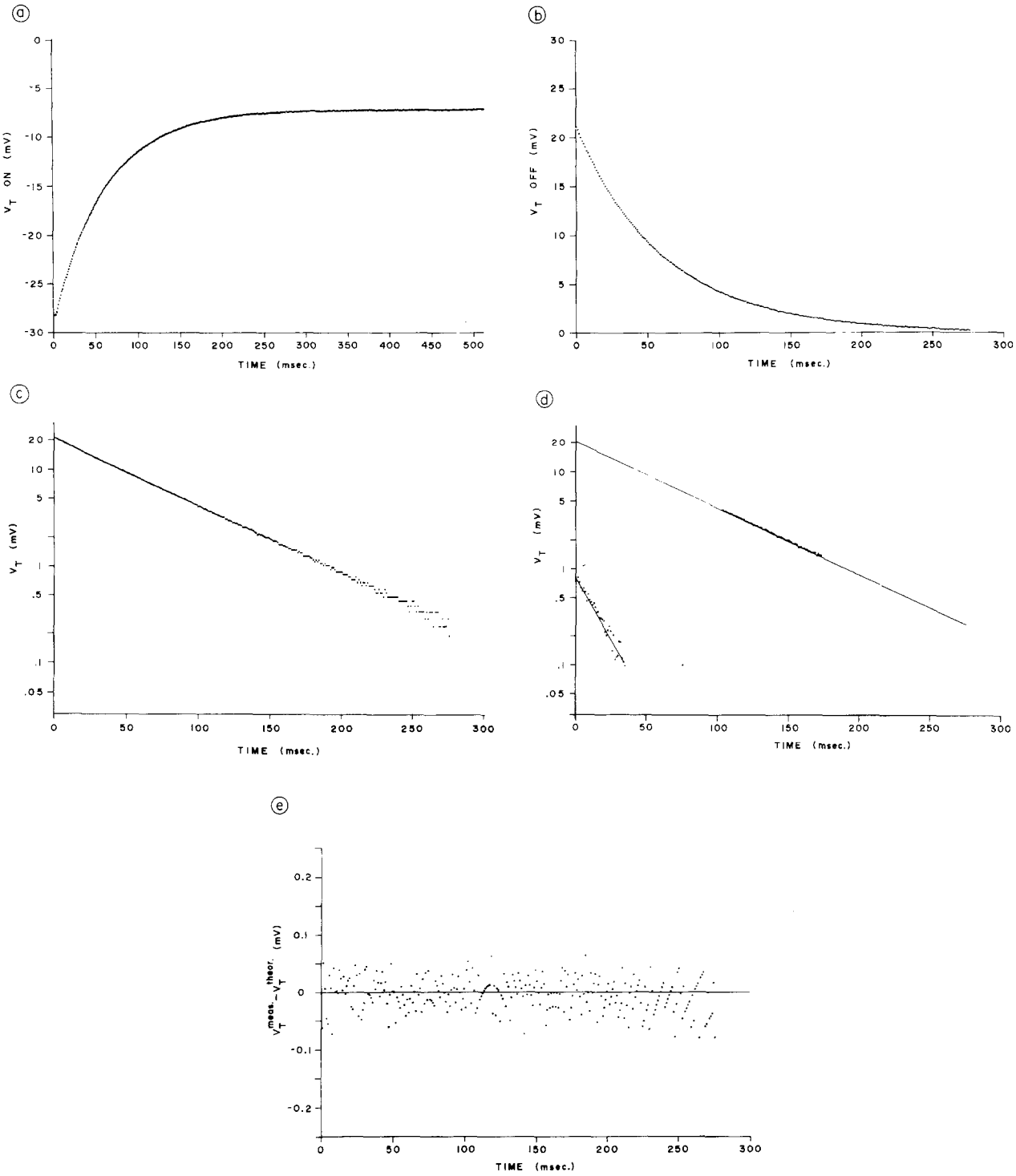

Fig. A2. Step-wise description for the determination of effective capacitance of an epithelium. Shown is the voltage response of the rabbit bladder epithelium to a square current pulse of 0.9 μA. Best fit values are R_1 = 22,550 Ω, C_1 = 2.82 μF, R_2 = 900 Ω, and C_2 = 18.56 μF

can be easily calculated and is independent of prior knowledge of membrane or junctional resistances. The determination of C_1 and C_2 is as follows: First, the voltage response to a square current pulse (ΔI) is digitized every msec (Fig. A2*a*) from a resting potential of ca. −28 mV (serosa ground) to ca. −7 mV. This on response is converted to an off response (Fig. A2*b*) by subtracting the steady-state voltage response from the prestimulus voltage. The absolute value of this voltage change is then displayed as logarithm of voltage against time (Fig. A2*c*). A linear regression is performed on data between two time points in the linear region of this curve (in this case between t = 100 and 170 msec), giving an intercept equal to V_1 and a slope equal to $1/R_1C_1$. The difference between this regression (linear form) and the measured data is then the second exponential (Fig. A2*d*). Again a

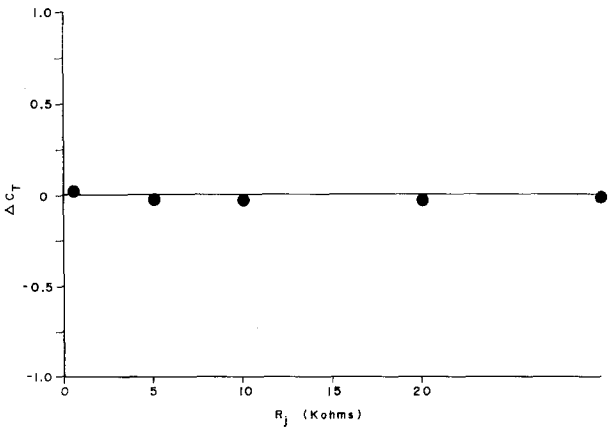

Fig. A3. An artificial membrane was constructed of known resistors and capacitors and the transient voltage response recorded and analyzed for the effective capacitance (C_T). The fractional change in effective capacitance ΔC_T (calculated by dividing the theoretical value into the difference between measured and theoretical values) is plotted as a function of changes in junctional resistance. Junctional resistance was varied from ∞ to 500 Ω, apical and basolateral resistors and capacitors were 20,000, 1000 Ω and 1 and 3 μF, respectively

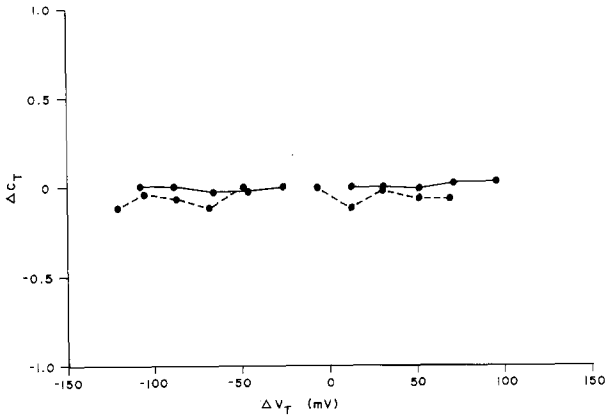

Fig. A4. The fractional change in effective capacitance as a function of the magnitude of the total voltage response. Solid line is a preparation that had significant amount of edge damage (R_T = 3000 Ω μF), the broken line was a preparation with minimal edge damage (R_T = 65,000 Ω μF). Capacitance was normalized to the value calculated for the smallest voltage deflection

linear regression analysis yields an intercept equal to V_2 and a slope equal to $1/R_2C_2$. R_1 and R_2 are calculated as $V_1/\Delta I$ and $V_2/\Delta I$, respectively, and C_1 and C_2 from the slope and corresponding resistor. To check goodness of fit, the difference between actual data and the "best" fit data is plotted to check for systematic over- or underestimates (Fig. A2*e*). Since the accuracy of the A/D converters is 50 μV, this example is an excellent fit.

The use of "exponential peeling" techniques like this have been criticized by several authors (Hegel, 1977; Clausen et al., 1979). The major criticism states that it is difficult to obtain unique estimates of the circuit parameters, i.e., widely different values of R_1, R_2, C_1 and C_2 are capable of fitting the response equally well. Fortunately, however, if the time constants are widely separated (say, by greater than a factor of 5), as is the case in rabbit urinary bladder (Clausen et al., 1979), then one can obtain good estimates from this analysis.

We verified our ability to determine C_T by analyzing transients from an artificial epithelium composed of resistors and capacitors. Notably, we determined C_T for different values of junctional resistance. Figure A3 shows that the normalized value for C_T does not change as a function of junctional resistance even though R_j was varied from ∞ to 500 Ω (*see* Figure legend for details). Similarly, effective capacitance was independent of variations of apical or basolateral resistance changes over wide ranges of values (*not shown*).

As previously stated because of the finite junctional resistance we are unable to calculate individual membrane capacitances or resistances. If, however, an independent estimate of one of the five parameters is possible then the other four parameters can be calculated from the two exponentials, using Eqs. (A2) through (A5) (*see also* Suzuki et al., 1982). Although beyond the emphasis of this Appendix, it is interesting to note that one can solve for all 5 circuit parameters if one experimentally perturbs one of the circuit elements (e.g., alter R_a with amiloride) and analyzes two transients, one before and the other after the experimental perturbation.

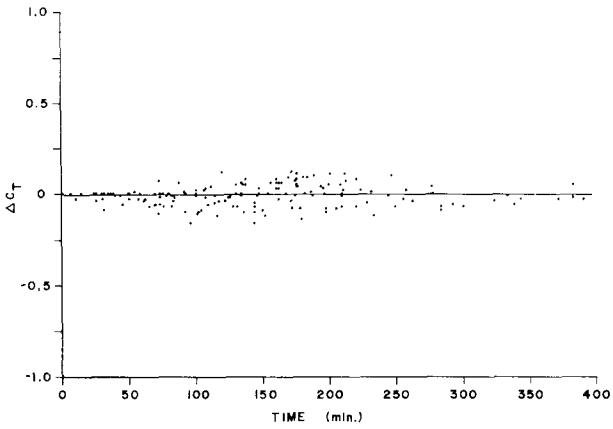

Fig. A5. A plot of the fractional change in effective capacitance as a function of time after mounting the tissue in the Ussing chambers. This Figure represents 167 determinations from 17 preparations. Note the relative independence of capacitance as a function of time. Capacitance was normalized to the value calculated at time zero

The constant value for C_T measured on an artificial epithelium validates both the theory and experimental system. A similar validation for the rabbit urinary bladder is shown below.

Voltage and Time Independence

Over a reasonable voltage range (Fig. A4) the fractional change in capacitance is minimal. This indicates that the circuit elements act in a linear voltage-independent manner.

To verify time independence of C_T, we monitored C_T as a function of time after initial mounting. This is seen in Fig. A5 which shows that C_T (normalized to the time zero value) is independent of the time after initiation of the experiment.

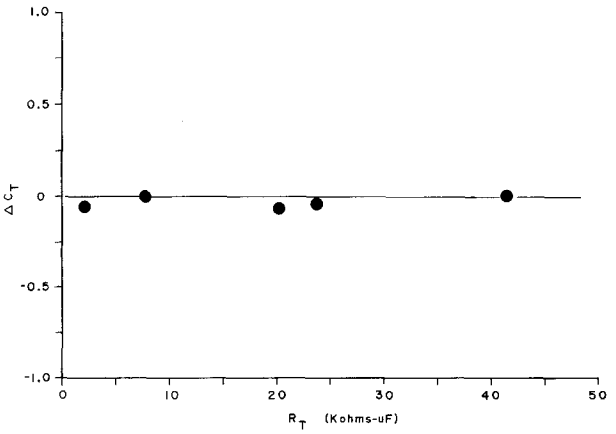

Fig. A6. Independence of effective capacitance on alteration of transepithelial resistance. The transepithelial resistance was decreased by removing both serosal and mucosal solutions, and then rapidly raising and lowering the mucosal solution. In this example the R_T decreased from 41,000 to 2,140 Ω μF. Over a period of 20 min the resistance increased to 24,300 Ω μF. C_T, however, did not measurably change. Capacitance was normalized to the value calculated for R_T of 41,000 Ω μF

Circuit Resistance (Independence)

This set of experiments demonstrates that C_T is independent of a variable cellular and junctional resistance. To perform this experiment, the transepithelial resistance was decreased by applying a hydrostatic pressure from mucosa to serosa. This pressure causes a decrease in junctional resistance and also the cell resistance pathway. Figure A6 shows that C_T remained constant during these alterations. A similar finding (*not shown*) also results when only the apical resistance was decreased using the pore-forming antibiotic gramicidin D.

Appendix B

In Results we demonstrated that upon replacement of the hypoosmotic solution with an isosmotic solution the effective capacitance (C_T) decreased as a single exponential. Since C_T contains values for not only C_a but also C_{bl}, would one expect C_T to decrease in such a manner if C_{bl} is constant and C_a changes as a single exponential? To investigate this we rewrite Eq. (2) to include both C_a and C_{bl} terms.

$$\Delta C_T(t) = C_T(t) - C_T(\infty) = \frac{[C_a(\infty) + \Delta C_a e^{-tk}]C_{bl}}{C_a(\infty) + \Delta C_a e^{-tk} + C_{bl}} - \frac{C_a(\infty)C_{bl}}{C_a(\infty) + C_{bl}} \quad \text{(B1)}$$

where $C_T(t)$ is the effective capacitance at any time t after changing into isosmotic solution, $C_T(\infty)$ and $C_a(\infty)$ are the effective and apical capacitance at infinite time after returing to isosmotic solutions, respectively, ΔC_a is the absolute change in apical capacitance ($\Delta C_a = C_a(t) - C_a(\infty)$ with $C_a(t)$ being the apical capacitance at any time t after changing from a hypoosmotic to an isosmotic solution).

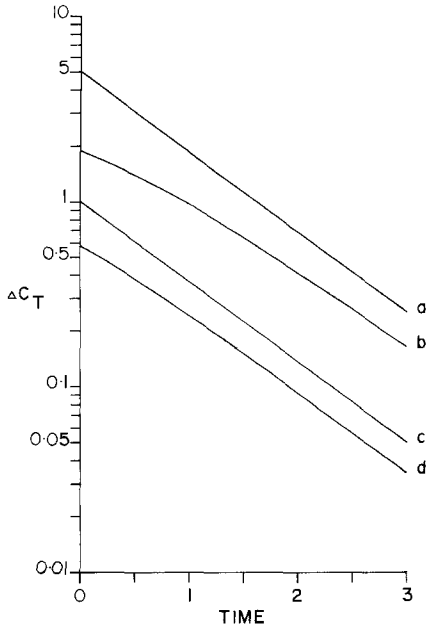

Fig. B1. The extent of the deviation from a single exponential of ΔC_T caused by a finite basolateral capacitance (C_{bl}). Curves *a*, *b*, *c* and *d* were generated using Eq. (B2). For all curves $C_a(\infty)$ and K were 1. ΔC_a was 5 for curves *a* and *b* and 1 for curves *c* and *d*. C_{bl} was 5 for curves *b* and *d* and infinite for *a* and *c*, i.e., for *a*, *c*, $\Delta C_T = \Delta C_a$. Fitting a single exponential to the calculated curves (*b* and *d*) yields for *b* an $r^2 = 0.9947$, an intercept of 2.11 and a slope of 0.85, while for *d* an $r^2 = 0.9997$ an intercept of 0.618 and a slope of 0.964. Although a finite C_{bl} results in a significant underestimate of the true ΔC_a, the proportionality constant K is not greatly altered

Re-arranging Eq. (B1) we have:

$$\Delta C_T = C_a e^{-tk} \left[\frac{C_{bl}}{C_a(\infty)\left(\frac{C_a(\infty)}{C_{bl}} + 2\right) + C_{bl} + \left(\frac{C_a(\infty)}{C_{bl}} + 1\right)\Delta C_a e^{-tk}} \right]. \quad \text{(B2)}$$

From this Equation we observe that the change in effective capacitance is a fraction of the change in apical capacitance. More importantly, it is not a constant fraction because of the exponential term in the denominator. The percent change of this term is dependent on the values of the apical and basolateral capacitance and the induced change in apical capacitance. The smaller the change in C_a and/or the larger the value for C_{bl} the smaller the error.

Figure B1 illustrates the effect of different values of ΔC_a on ΔC_T as a function of time. Even though the maximal change is greatly attenuated (at the largest C_a value of 5 μF) the plot is nearly exponential. Most importantly is that the proportionality factor ($k = 1/\tau$) is not dramatically altered (from a value of $\tau = 1$ to 1.18).

We conclude that although the maximal change in effective capacitance is an underestimate of the actual change, the time course of the change is well determined by a single exponential.