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EVALUATION BY CAPACITANCE MEASUREMENTS OF ANTIDIURETIC HORMONE INDUCED MEMBRANE AREA CHANGES IN TOAD BLADDER

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A technique for estimating effective transepithelial capacitance in vitro was used to investigate changes in epithelial cell membrane area in response to antidiuretic hormone (ADH) exposure in toad bladder. The results indicate that transepithelial capacitance increases by about 30% within 30 min after serosal ADH addition and decreases with ADH removal. This capacitance change is not blocked by amiloride and occurs whether or not there is a transepithelial osmotic gradient. It is blocked by methohexital, a drug which specifically inhibits the hydro-osmotic response of toad bladder to ADH. We conclude that the hydro-osmotic response of toad bladder to ADH is accompanied by addition of membrane to the plasmalemma of epithelial cells. This new membrane may contain channels that are permeable to water. Stimulation of Na^+ transport by ADH is not related to membrane area changes, but appears to reflect activation of Na^+ channels already present in the cell membrane before ADH challenge.

Introduction

The toad bladder has been used extensively as a model system for the study of hormone action on epithelia since Leaf et al. [1] described an increase in sodium transport induced in this organ by antidiuretic hormone (ADH). It is well established that ADH also increases the permeability of the toad bladder to urea and water. However the effect of ADH on water permeability can be dissociated from the changes in sodium and urea transport [2–4]. A number of recent papers have suggested that the hydro-osmotic response to ADH involves aqueous channels in the apical membrane [5–7]. Freeze-fracture electron microscopic studies have shown that the ADH-induced increase in water permeability is associated with the appearance of distinctive intramembrane particle aggregates in the apical membrane [8,9] that may be the site of increased water permeability [10]. Similar aggregates are found in the membranes of certain cytoplasmic vacuoles before ADH exposure; therefore, it has been suggested that the aggregates move from cytoplasmic membrane elements to the apical membrane [11,12]. If aggregates are inserted into the apical membrane by fusion of the aggregate-containing vacuoles it follows that the area of the apical membrane may increase during the response to ADH. Indeed, morphometric measurements by Gronowicz et al. [13] suggest that membrane is added to the apical surface following ADH exposure.

A non-destructive method for examining the influence of ADH on epithelial sodium transport and hydraulic conductivity was devised by Cuthbert and Painter [14]. These authors speculated that an increase in the parallel arrangement of

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Abbreviation: ADH, antidiuretic hormone.

water filled Na^+ pathways might cause an increase in the capacitance of an epithelium (in this case frog skin) after ADH challenge. A small but significant increase in capacitance of about 7% over baseline was measured 60 min after ADH addition. A direct test of the hypothesis that conductive channels will cause a capacitance increase is to measure the capacitance before and after blocking the Na^+ channels with amiloride. Such an experiment was performed by Clausen et al. [15] with no significant change in capacitance being measured. An alternate explanation might then be an actual increase in the area of the lipid portion of the apical membrane.

In this paper we reexamine the phenomenon of epithelial membrane area increase mediated by ADH. The method we employ is based on the rapid and accurate determination of epithelial capacitance using voltage relaxation techniques. The data indicate that there is an increase of about 30% in area. In addition, we correlate the area increase with water flow but not Na^+ transport. The magnitude and site of the membrane area change was also evaluated by morphometric analysis.

Theoretical considerations

It has been well established that a biological membrane behaves as a parallel resistor-capacitor circuit, and that the measured capacitance is directly proportional to the area of the membrane in question. Additionally, the value of the capacitance is approximately described by the identity; 1 $cm^2 \cong 1$ μF. Lewis et al. [16] have shown that the electrical response of rabbit urinary bladder epithelium to a square current pulse can be used to calculate the transepithelial capacitance when the apical membrane resistance (and time constant) has been made large compared to the basolateral membrane resistance. This method will yield reasonable estimates of the epithelial capacitance as long as the junctional resistance is very large. A finite junctional resistance will yield incorrect estimates for apical capacitance. Because one cannot always be assured that the junctional resistance is either infinite or constant, we used the lumped model described by Lewis et al. [16] for estimating capacitance. In brief, this method involves passing a square transepithelial current pulse, recording the voltage response, and then fitting this voltage response to the sum of two exponentials. Such a procedure will yield two resistor values and two capacitor values. As pointed out by Lewis and Diamond [17] these resistor-capacitor values are complex functions of the actual apical and basolateral membrane resistances and capacitances and junctional resistance.

Lewis and DeMoura (unpublished) have demonstrated that the relationship between the apical and basolateral capacitors and the calculated capacitors ($C_1 + C_2$) is independent of membrane and junctional resistors.

$$\frac{C_1 C_2}{C_1 + C_2} = \frac{C_a C_{bl}}{C_a + C_{bl}} \tag{1}$$

This is the effective capacitance (C_T) of the epithelium and is numerically equal to the value of two series capacitors.

Materials and Methods

Large female *Bufo marinus* from the Dominican Republic were obtained from National Reagents, Inc., Bridgeport, CT, maintained on a moist peat moss at room temperature and fed *Tenebrio* larvae weekly. Hemibladders were removed from doubly-pithed toads and mounted on acrylic tubes in a manner similar to Bentley [18]. The bladders were rinsed inside and out with Ringer's (composition below) and held in air-bubbled Ringer's until needed for an experiment. This time varied from 15 min to approx. 2 h. For the experiment, the bladders were mounted in modified Ussing chambers (see Lewis and Diamond [17]). Electrical transepithelial voltage was measured using either Ag-AgCl wires or 3 M KCl agar bridges connected to Ag-AgCl KCl half cells. In both cases, the electrodes were placed approx. 5 mm from the epithelium. Current was passed from Ag-AgCl wires at the rear of each chamber. All electrodes were connected to a voltage-clamp. Current was passed using a WPI model 305 optical isolator as a source and a Horizon Northstar microcomputer as stimulus controller. The processor-generated pulse was displayed on a Tektronix storage oscilloscope and stored as digitized data on mini disk at a

sampling interval of 100 μs or 1 ms. Also, continuous open-circuit transepithelial potential (V_T), clamped V_T, and applied current (I_{app}) were recorded on a strip-chart recorder (Houston Inst.). In most cases, enough current was passed to yield a total voltage deflection (ΔV_T) of at least 20 mV. The length of the pulse varied as necessitated by the time required for the epithelium to reach a new steady-state V_T.

Analysis

The off-pulse of each data file (pulse) was displayed on a Tektronix storage oscilloscope as the linear ΔV_T vs. time and as ln ΔV_T vs. time to a resolution of 0.1 mV. One or two curves were fit to the data by computed least-squares regression. The extent of the linear portion on the log plot at long times was determined visually and the regression for that portion computed. Subsequently, the computed ΔV_T at early times based on the computed regression was subtracted from each data point and regression was performed on the differences if two time constants were apparent from the logarithmic plot. The results were presented as $y = \Delta V_x e^{-1/\tau_x}$ for each regression line. Given the I_{app}, resistance (R_x) for each regression was calculated as $\Delta V_x / I_{app}$, capacitance (C_x) as τ_x / R_x. Transepithelial resistance was taken to be the sum of the R_x values, and effective transepithelial capacitance (C_T) as in Eqn. 1. All results were expressed as the fractional change in transepithelial capacitance (ΔC_T), because this analysis does not allow us to assign either of the computed C_x values or τ_x values to either the apical or basolateral membranes.

Morphometry

Morphometric analysis was performed on epithelia whose capacitance and response to ADH had been determined. Hemibladders were mounted in a double Ussing-type chamber (see Ref. 19) so that control and experimental capacitance measurements could be performed on contiguous tissue areas. The area of the slot-shaped opening of each chamber was 2.0 cm^2. Capacitance measurements were made on both tissue areas prior to and for 20 min subsequent to the addition of arginine vasopressin (ADH) to one chamber. 20 min after ADH addition, the tissues in both chambers were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) and prepared for routine electron microscopy. The surface-to-volume ratios of the luminal and basolateral membranes of granular cells were determined using standard techniques [20], and the amplification factors for these membranes were determined by dividing the boundary lengths of each cell membrane by the linear length of epithelium examined.

Solutions

Two Ringer's solutions were used in these experiments. The first was a NaCl Ringer's of the following composition: NaCl, 111 mM; KCl, 3.5 mM; $NaHCO_3$, 2.5 mM; and $CaCl_2$, 1.0 mM. The pH of this solution was 8.0 when bubbled with air. The second Ringer's was that used by Handler [21]. It contained NaCl, 90 mM; $NaHCO_3$, 25 mM; KCl, 3 mM; $CaCl_2$, 1 mM; $MgSO_4$, 0.5 mM; and KH_2PO_4, 0.5 mM; and had a pH of approx. 7.8 when bubbled with 97% O_2/3% CO_2. For experiments in which an osmotic gradient was applied, the stock Ringer's was diluted to half-strength. Amiloride (a gift from Merck, Sharp and Dohme) was added to the mucosal bath at a concentration of 10^{-4} M. Methohexital (as Brevital R, Eli Lilly and Co.) and arginine vasopressin (from Sigma Chemical Co.) were added to the serosal bath at concentrations of $3 \cdot 10^{-4}$ M and 20 mU/ml, respectively.

Results

ADH induced increases in capacitance and current

Epithelial capacitance and current were monitored for 60 min prior to serosal ADH addition and then for 30 min following addition of serosal ADH when both mucosal and serosal solution were of identical composition (i.e. no osmotic gradient). There were no significant spontaneous changes in capacitance prior to ADH treatment. Upon addition of ADH, effective capacitance increased by 36% after 25 min (see Fig. 1). Similarly, both short circuit current (I_{sc}; N.B, calculated as V_T/R_T) and transepithelial conductance increased. I_{sc} doubled in the first 10 min and then decayed, a response which is quite typical for this preparation. Transepithelial conductance (data not shown) also increased by ≅ 70% reaching a peak

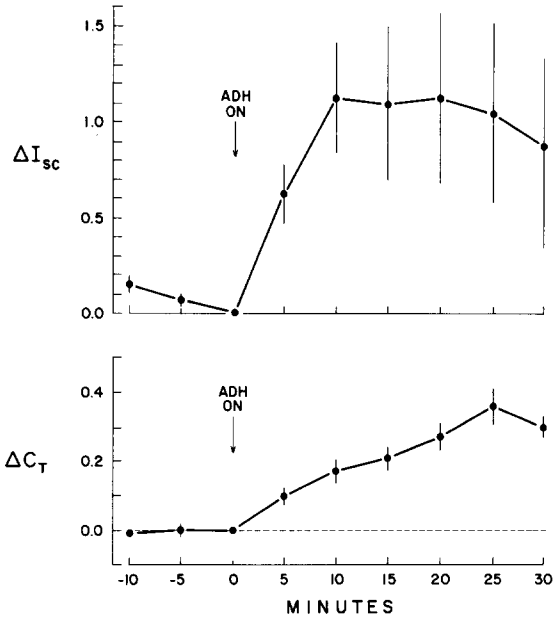

Fig. 1. ADH-induced changes in capacitance (ΔC_T) and short circuit current (ΔI_{sc}) under control conditions, i.e. no drug addition, no osmotic gradient ($N = 8$).

value at 25 min. Results very similar to these were recently found by Warncke and Lindemann [22] using impedance analysis.

Area changes or artifact?

An increase in capacitance might represent an increase in the actual area of the epithelium brought about by a fusion of intracellular vesicles with the apical membrane. However there are other possibilities which can result in an increase in capacitance without an area change. These are: (i) Cell swelling which might alter membrane thickness, dielectric constant, and/or the dimensions of the lateral intercellular, spaces. (ii) Alteration of membrane resistance of a single cell type in an epithelium composed of a heterogeneous cell population.

We have investigated the possibility that apparent capacitance changes occur in the absence of actual area changes in the following experiments.

(a) Effect of changes in cell volume on capacitance. One reported morphological effect of ADH stimulation is dilation of the lateral intercellular spaces of the epithelium [23]. Because the opening of these intercellular spaces might be responsible for the increases in capacitance, a series of experiments was performed in which the osmotic gradient across the epithelium was altered by varying the mucosal and serosal bath osmolalities. The following bath combinations were examined: 90 mM NaCl (220 mosM) Ringer's on mucosa, 60 mM NaCl (165 mosM) Ringer's on serosa; 90 mM NaCl Ringer's on mucosa, 30 mM NaCl (110 mosM) Ringer's on serosa; half strength Ringer's on mucosa, full strength Ringer's + 220 mosM mannitol (440 mosM) on serosa. These maneuvers were designed to either swell or shrink the epithelial cells, thus changing the intercellular space geometry in as many ways as possible. In no case did any of these maneuvers cause an appreciable increase in the total epithelial capacitance, although dramatic changes in I_{sc} and conductance were observed (data not shown). These experiments then indicate that changes in tissue geometry are not responsible for the capacitance change associated with ADH action.

(b) Effect of transport inhibition on capacitance. Because the natriuretic drug amiloride appears to block sodium conductance in granular cells but not in mitochondria-rich cells [24,25], we applied amiloride to examine the influence of changes in granular cell membrane resistance on effective capacitance. Using four separate bladders, the current, conductance and capacitance were followed for at least 75 min without the addition of ADH. In this control experiment there was no significant increase in any of these parameters. The subsequent addition of amiloride did not change effective capacitance, but did cause a decrease in transepithelial conductance and I_{sc}. The later descreased to near zero. This experiment then indicates that the change in capacitance is not associated with selective resistance changes of a sub-population of cells.

Osmotic gradient with ADH

The initial experiments showing an ADH-induced increase in capacitance were performed without an osmotic gradient. It was important to determine whether a capacitance increase also occurs in the presence of an osmotic gradient because that is the in vivo condition. Therefore, a series of four hemibladders was stimulated in the presence of an osmotic gradient; the mucosal bath

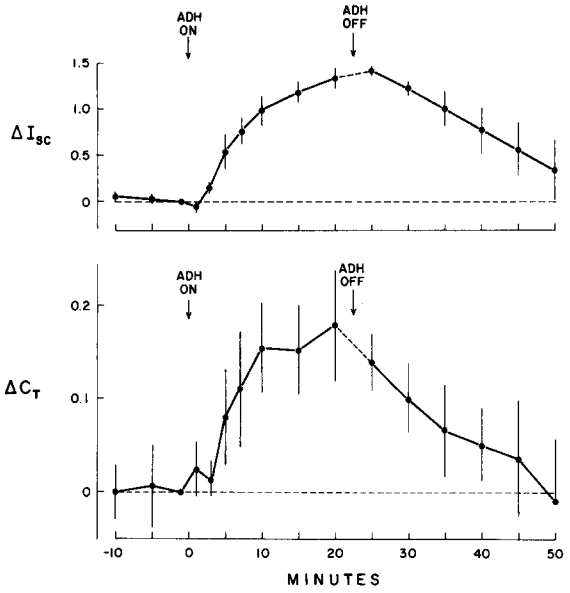

Fig. 2. ΔI_{sc} and ΔC_T with ADH addition and removal in the presence of an osmotic gradient ($N=4$).

was half-strength Ringer's, the serosal bath was full-strength Ringer's. It was also important to show that the increase in capacitance is reversible after ADH removal if it reflects a true physiologic phenomenon. Therefore, ADH was removed from the serosal bath 20 min after its addition. Fig. 2 shows the results of this experiment. Total capacitance, I_{sc} and transepithelial conductance all increased promptly after the addition of ADH. By 20 min they all appear to be nearing a peak. In the case of capacitance, this peak is approx. 18% greater than control values. After ADH removal, all parameters decreased in a linear manner. Capacitance reversed rather quickly, reaching initial values 25 min after ADH removal. I_{sc} and transepithelial conductance lagged behind capacitance, with conductance requiring the greatest time to reach initial levels.

Does cell Na^+ influence the capacitance change?

Although it is well documented that ADH increases cell cAMP levels, this does not necessarily mean that cAMP directly influences capacitance. To test the possibility that the capacitance change might be secondary to the ADH-induced increase in sodium transport, bladders were pretreated with 10^{-4} M amiloride. After conductance and I_{sc} had decreased to new steady-state levels the bladders were challenged with ADH (Fig. 3). 30 min after the addition of ADH, total capacitance had increased by approx. 48% when compared to the value 1 min before the addition of ADH. I_{sc} increased from an average of 2.1 ± 1.0 (S.E.) to 9.0 ± 3.2 during the same interval. Both capacitance and I_{sc} had not reached a plateau by 30 min. Because the capacitance increase occurs even when Na^+ transport is inhibited by amiloride, it is unlikely that cell Na^+ is a trigger for capacitance changes.

Inhibition of capacitance change by methohexital

Although the evidence provided in the previous sections indicates that changes in cell volume and Na^+ transport are not responsible for the capacitance changes, is the capacitance increase associated specifically with the increase in water permeabililty?

Levine et al. [26] demonstrated that methohexital added to the serosal solution at a concentration of $3 \cdot 10^{-4}$ M inhibits the ADH-induced hydro-osmotic response of the toad urinary bladder,

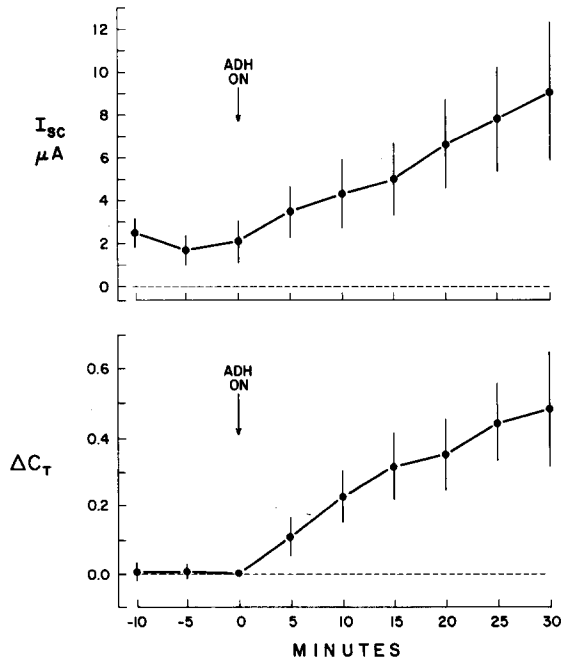

Fig. 3. ADH-induced changes in absolute I_{sc} and ΔC_T in the presence of mucosal amiloride ($N=6$).

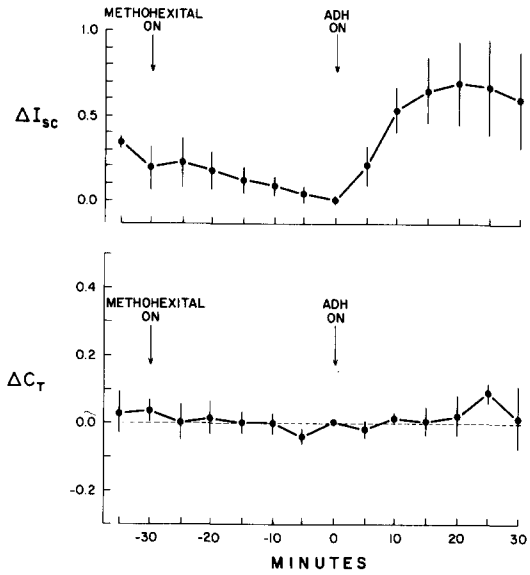

Fig. 4. ADH-induced changes in ΔI_{sc} and ΔC_T in the presence of serosal methohexital ($N=6$).

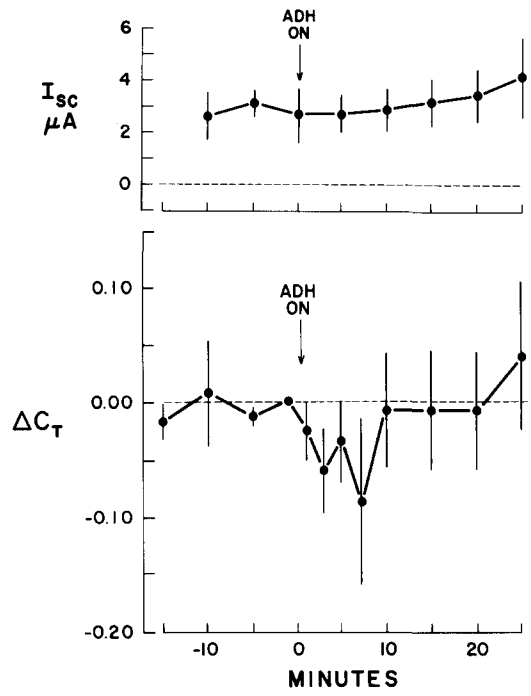

Fig. 5. ADH-induced changes in absolute I_{sc} and ΔC_T in the presence of mucosal amiloride and serosal methohexital ($N=4$).

but leaves essentially unaltered the increase in urea and Na^+ permeability. Fig. 4 shows the effect of methohexital on the effective capacitance and I_{sc}. Note that the capacitance change was completely inhibited while I_{sc} increased in the usual manner.

Similar experiments with amiloride in the mucosal solution show that serosal methohexital also blocks the capacitance change in response to ADH when current changes are inhibited (Fig. 5). However, it was noted that the small ADH-induced increase in I_{sc} that occurs in the presence of amiloride (Fig. 3) was further reduced ($P<0.002$ by analysis of variance) by methohexital (Fig. 5). In experiments with methohexital the transepithelial conductance increased by 20 to 90% with addition of ADH. Thus ADH-induced changes in capacitance appear to be correlated specifically with the increase in water permeability, not with amiloride-sensitive changes in Na^+ transport or conductance.

TABLE I

EFFECT OF ADH ON ΔC_T, CELL SURFACE DENSITY (S_v) AND MEMBRANE AMPLIFICATION

Values are means ± S.E. $N=5$.

	Control	ADH	ADH − control [a]
ΔC_T	0.01 ± 0.02	0.25 ± 0.05 [b]	0.24 ± 0.04 [b]
S_v ($\mu m^2/\mu m^3$)			
Apical membrane	0.98 ± 0.16	0.89 ± 0.07	−0.09 ± 0.14
Basolateral membrane	2.04 ± 0.26	1.72 ± 0.16	−0.33 ± 0.13
Membrane amplification			
Apical membrane	1.64 ± 0.12	2.12 ± 0.21	+0.49 ± 0.27
Basolateral membrane	3.50 ± 0.37	4.24 ± 0.74	+0.72 ± 0.84

[a] Mean ± S.E. of ADH − control for each paired bladder.

[b] $P<0.01$ by paired Student's t-test.

Morphometry

The morphometric data derived from five double-chamber experiments are displayed in Table I. Both the apical and basolateral membrane surface density (S_v) appeared to decrease in response to ADH, probably as a result of an ADH-induced cell volume increase [27]. While mean values for apical and basolateral membrane amplification were increased (by 29% and 21%, respectively) in response to ADH, these changes were not significant on a paired basis (Student's t-test, $P > 0.05$) due to large variability in the ADH-induced change among bladders.

Discussion

It was the purpose of this paper to investigate the influence of ADH on the capacitance of the toad urinary bladder for a series of conditions, The major findings are as follows: (i) The epithelial capacitance of the bladder in the presence or absence of an osmotic gradient increases by $\sim 20\%$ to $\sim 36\%$ over a time of 30 min following ADH addition to the serosal solution.

(ii) This capacitance change is not associated with cell swelling, shrinkage, or Na^+ transport, nor is it a function of a heterogeneous cell population. Thus it probably represents a real increase in epithelial surface area.

(iii) After removal of ADH, the capacitance decreases in a linear manner to control values, thus supporting the reversible nature of the ADH effect.

(iv) The change in capacitance is not well correlated with I_{sc} changes.

(v) Methohexital, a drug known to block the hydro-osmotic response, was found to eliminate the capacitance change while leaving unaltered the change in I_{sc}.

(vi) There is an amiloride-insensitive current increase associated with the capacitance increase.

(vii) Due to variability in the data, morphometric techniques were unable to corroborate convincingly the changes in membrane area suggested by capacitance measurements.

Apical or basolateral membrane

Effective capacitance does not allow one to pinpoint at which of the two series membranes the change in capacitance is occurring. The largest changes will be observed if both membranes increase their respective capacitance. Although not statistically different from control values, the magnitude of the changes in membrane amplification observed by electron microscopy (25% for the apical membrane and of 21% for the basolateral membrane) is consistent with the measured capacitance changes. The recent work of Warncke and Lindemann [22] indicates that both membranes may be involved in this ADH response.

Capacitance and the hydro-osmotic responses

Using methohexital, we found that inhibition of capacitance is directly correlated with inhibition of the hydro-osmotic response. Moreover, Kachadorian et al. [28] demonstrated that the frequency of aggregates in the apical membrane and osmotic water flow are reduced 5 min after ADH removal, a time by which capacitance has begun to return to control levels. Therefore, changes in capacitance appear to be correlated with changes in aggregates and epithelial water permeability. However, the area of the apical membrane occupied by aggregates at the height of the ADH response is only some 2%. If the area changes by $\sim 20\%$ that leaves 18% of the change unaccounted for. This may be the result of at least two possibilities. (1) It is clear from freeze-fracture that aggregates occupy only a part of the membrane of the cytoplasmic vesicles which presumably fuse to the apical membrane in response to ADH. Perhaps, the additional bare membrane of these vesicles accounts for some larger fraction of the membrane that is added to the apical membrane. (2) Membrane from some other cellular compartment (e.g. granules) may also fuse by some specific or non-specific mechanism at the same time that aggregate-containing vesicles fuse. This addition may occur either at the apical or basolateral surface.

Capacitance and Na^+ transport

One of the interesting features of this series of experiments is that there is no correlation between capacitance and the amiloride-sensitive Na^+ transport. Indeed, the ADH-induced capacitance increase can be inhibited using methohexital without blocking the I_{sc} and conductance responses.

Using capacitance, we cannot differentiate be-

tween a variety of possible explanations for ADH-induced increases in Na^+ transport. We can say, however, that capacitance is not related causally to a change in Na^+ transport (refer to methohexital and amiloride experiments). Recently Cuthbert and Shum [29] found, using radiolabeled amiloride, that the number of amiloride-binding sites did not change during ADH stimulation. Also, Scott and Slatin [30] found that ADH increased in the apical membrane the number of proteins available for lactoperoxidase iodination, a response that could be blocked when the hydro-osmotic response was blocked selectively by colchicine. Moreover, when Na^+ transport was stimulated by aldosterone, a maneuver that does not change epithelial water permeability, no increase in membrane protein iodination was seen. These data and our observations are consistent with the possibility that the Na^+ channels already exist in the apical membrane in an inactivated state. Increases in I_{sc} and conductance by ADH may largely reflect activation of these channels.

Capacitance and residual current

While we found that amiloride inhibition of Na^+ transport does not influence the capacitance increase in response to ADH, pre-treatment of the bladder with amiloride and subsequent ADH stimulation does result in a measureable increase in I_{sc}. Thus, there appears to be a current increase that is amiloride insensitive. Is it associated with the capacitance change? This question is readily answered by comparing the amiloride-methohexital experiment (Fig. 5) with the amiloride experiment (Fig. 3). Pre-treatment with amiloride plus methohexital (Fig. 5) resulted in a small increase in current which is significantly less than the current response of bladders pre-treated with only amiloride (Fig. 3). This indicates that there is an amiloride-insensitive current associated with the capacitance change.

Acknowledgements

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