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Structural Responses to Voltage-Clamping in the Toad Urinary Bladder

I. The Principal Role of Granular Cells in the Active Transport of Sodium

V.A. Bobrycki, J.W. Mills, A.D.C. Macknight* and D.R. DiBona**

Laboratory of Renal Biophysics, Massachusetts General Hospital, Boston, Massachusetts, Department of Physiology, University of Otago, Dunedin, New Zealand, and Department of Physiology and Biophysics and Nephrology Research and Training Center, University of Alabama in Birmingham, Birmingham, Alabama 35294

Summary. The structural consequences of clamping the transepithelial potential difference across the toad's urinary bladder have been examined. Reducing the potential to zero (short-circuiting) produced no apparent changes in the morphology of any of the four cell types which comprise the epithelium. Computer assisted, morphometric analysis of quick frozen specimens revealed no measurable difference in granular cell volume between open- and short-circuited preparations. However, when the open-circuit potential was quantitatively reversed (serosa negative with respect to mucosa), some of the preparations showed a marked increase in granular cell volume. To examine this more systematically twelve preparations were voltage-clamped at 50 mV (serosa negative); eight of the twelve revealed prominent granular cell swelling relative to control, short-circuited preparations. Only in this group of eight had the external circuit current fallen substantially during the clamping interval. Mitochondria-rich cells were not affected detectably. Application of the diuretic amiloride prior to clamping at reversed potential prevented granular cell swelling in every case. Goblet cells which were often affected by the −50 mV clamp were not protected by the diuretic. Granular cell swelling thus appeared to be dependent on sodium entry at the mucosal surface. We also observed that, after voltage reversal, the apical "tight" junctions of the bladders were blistered as they are with hypertonic mucosal media. This blistering was associated with an increase in passive ionic permeability and was not prevented by application of amiloride. This finding is consistent with the evidence that the junction is a complex barrier with asymetric, and hence, rectifying properties for intrinsic ionic conductance as well as hydraulic permeability. These findings, together with others from the literature, lead to the conclusion that the granular cells constitute the principal, if not sole, elements for active sodium transport across toad urinary bladder and that they swell when sodium entry exceeds the transport capacity of the pump at the basal-lateral surface.

The study of sodium transport across epithelia has been greatly aided by the application of voltage-clamping techniques and there is broad agreement that these methods are generally nondestructive. To a large extent, this appears to be true; in particular, experience has shown that voltage-clamping the transmural potential at zero (short-circuiting) does not dramatically decrease the long term viability of isolated amphibian bladder or skin. With regard to these "tight" epithelia, the finding that short-circuit current is equivalent to net sodium transport (Ussing & Zerahn, 1951; Leaf, Anderson & Page, 1958) has facilitated an understanding of the mechanism of transepithelial active transport and its hormonal regulation. The presence of passive ionic pathways across epithelia complicates understanding of ion movement in response to finite applied potential differences, and it has been noted (Mandel & Curran, 1972; Bindslev, Tormey, Pietras & Wright, 1974) that the conductance of these electrical shunts is related in a nonlinear way to variations in transmural voltage difference. Indeed, the active pathway for sodium exhibits a complex responsiveness to potential as well (e.g. Finkelstein, 1964; Civan, 1970). It is thought that the mechanism for active transcellular sodium transport and its attendant metabolic machinery can be affected by

* *Permanent address:* Department of Physiology, University of Otago, Dunedin, New Zealand.

** *Mailing address and to whom reprint requests should be made:* Department of Physiology & Biophysics and Nephrology Research and Training Center, University of Alabama in Birmingham, University Station, Birmingham, Alabama 35294.

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externally applied electrical forces and that the fluid composition of the epithelial cell interior should be sensitive to imposed alterations in transcellular ion flow.

From this reasoning, a knowledge of the relationship between cell volume and transmural clamping voltage should yield important information with respect to the disposition of ionic barriers and to the capacity of the tissue to transport sodium actively. Voûte and Ussing (1968) reported several years ago that short-circuiting the isolated frog skin resulted in a swelling of the cells of the *stratum granulosum,* the layer of cells just beneath the outer cornified surface cells. When the skin was fixed after a brief period in which the spontaneous potential difference was quantitatively reversed (thus making the inside solution as negative with respect to the outside as it had previously been positive), this layer of cells was still more dramatically affected. They reasoned that the swelling occurred because this layer of cells was the primary site of active transport. It was later verified (Voûte & Hanni, 1973) that this "reactive cell layer" (RCL) was specifically affected even when the tissue was fixed by rapid freezing rather than by chemical means. The swelling was not due, therefore, to a fixation artifact.

The argument for concentration of transport activity in the RCL has been seriously challenged, however, by other findings. Smith (1971), from determinations of tissue capacitance, concluded that the sites for active extrusion of sodium had to be distributed over a very large fraction of the cell membranes within this multilayered epithelium and that, therefore, the frog skin must function as a syncytium. The widespread occurrence of gap junctions in this tissue (Farquhar & Palade, 1965) supports this possibility, which is consistent with the similarities in cellular ion content between the various cell layers revealed by electron microprobe analysis (Rick et al., 1978*b*). In addition, studies of $Na^+ - K^+$-ATPase distribution (Mills & DiBona, 1977; Mills, Ernst & DiBona, 1977) favor this view, emphasizing the presence of the sodium pump only on the membranes of living cells [in contrast to the earlier study of Farquhar & Palade (1966)]. The ouabain-binding sites are sparse within the *stratum granulosum* (the RCL) but heavily concentrated in the middle and deeper layers of the epithelium, the *strata spinosum* and *germinativum.*

Consideration of the morphological results obtained by Voûte and Ussing, together with the evidence that all of the epithelial cell layers in frog skin are involved in transepithelial sodium transport, prompted us to examine the consequences of voltage-clamping of the toad urinary bladder. In this tissue, a single layer of cells traverses the epithelium from the mucosal surface to the basement membrane (DiBona, Civan & Leaf, 1969*a*). Here cells of three types bridge the epithelium so that parallel elements – rather than series elements as in the frog skin – comprise the potential sites for transepithelial sodium transport, though there is still controversy as to the relative contributions of the different cell types to this process. The present experiments were designed to investigate the effects of transmural voltage-clamping on the volume of these epithelial cells and to assess the dependency of cellular volume on the level of sodium transport.

Materials and Methods

Female specimens of the toad *Bufo marinus* were obtained from the Dominican Republic (National Reagents, Inc., Bridgeport, Conn.) and were maintained without food for up to three weeks on moistened wood chips. Urinary hemibladders were dissected from doubly-pithed toads and mounted in Lucite double-chambers to provide experimental and control sheets of 2.5 cm^2 area from the same tissue. The serosal aspect of the tissue was supported on nylon mesh and a slight excess of mucosal to serosal bathing medium was applied to hold the tissue flat with a hydrostatic pressure of 0.5 to 1.5 cm.

The sodium Ringer's solution consisted of (mM): Na^+, 117; K^-, 3.5; Ca^{2+}, 1.0; Cl, 117; HPO_4^{2-}, 2; the pH was 7.8 and osmolality was 228–235 mOsm/kg H_2O. Amiloride was a generous gift of Merck, Sharpe and Dohme (Rahway, N.J.).

Transmural potential difference was monitored with calomel electrodes; voltage-clamping was applied through chlorided silver electrodes; electrical continuity from electrode reservoirs to the bathing media was by means of 3M KCl agar bridges. Tips of the voltage sensing bridges were positioned in the chambers within 1 mm of the tissue surface; current-sending bridges were placed 2.5 cm away to assure uniform flux density under voltage clamping conditions. The electrophysiological apparatus used was essentially of the design used previously (DiBona & Civan, 1973) except that no feedback loop to correct for solution resistance was applied since all experiments employed identical mucosal and serosal media. Voltage offset for clamping tissue potential at levels other than zero was applied through the positive input of one of the clamping amplifiers.

At the end of the experimental protocol, tissue was preserved by one of two methods. The rapid-freezing technique employed was a minor modification of the technique used by Stirling (1972). Initial freezing of the tissue was in liquid propane cooled in liquid nitrogen. Tissue removed from the chamber was immersed in propane within five seconds and kept at this temperature for processing the next day in a Stumpf-Roth freeze-dry apparatus. Vapor fixation in OsO_4 and embedding in Spurr resin were conducted as we have previously described (Mills et al., 1977).

When chemical fixation was employed, suitable volumes of 25% glutaraldehyde were added simultaneously to serosal and mucosal baths to provide a 1% solution of fixative on either side of the tissue. The applied voltage clamp was maintained in the presence of the fixative for 15 to 30 min and in each case beyond the point when there was no further change in external circuit current. Subsequent processing of tissue was as before (DiBona & Civan, 1972) using a phosphate buffer at pH 7.4, post-fixation in OsO_4, alcohol dehydration and embedding in an Epon-Araldite resin.

Sections for light and electron microscopy were cut on either a Reichert OmU_2, LKB-Ultratome III or a Sorvall MT-5000 microtome. Thin sections were stained with uranyl acetate and lead citrate and examined in either a Philips EM-200 or EM-301 electron microscope.

Quantitative light microscopic analysis of epithelial cell profiles in toluidine blue-stained, 1-μ thick sections was performed in the comparative study of open- *vs.* short-circuited tissue. This analysis was done by spark-pen tracing of outlines, both on photographic prints and, through an illuminated cursor, on the screen of a Princeton 801 graphics terminal interfaced to a Digital PDP 11/10 computer. Programs for conversion of input coordinate positions and computation of enclosed area were written in BASIC. Objectivity in this data reduction was established by coding the material to be examined; consistency of chosen criteria for cell boundaries was assured as one of us (VAB) performed all of the profile tracing. For each sample, individual cells were traced as encountered on randomly selected views until the mean value of cross-sectional area varied by no more than 5% with additional measurements. Thirty-two to 73 granular cells and 18 to 55 basal cells were thus counted for each specimen. In other studies, where cell volume changes were very evident, documentation of swelling or its absence was achieved by blind survey. Six investigators were provided with a minimum of three coded micrographs from each control and each experimental sample where the only identified variable was pairing.

Results

Comparison of Open-Circuited vs. Short-Circuited Tissues

In a series of eight experiments, paired quarter-bladders were maintained for 65–95 min in Ussing chambers with one member of the pair (experimental) short-circuited ($V_B=0$) and the other (control) left open-circuited. (These preparations were selected as those which had initial spontaneous potential differences of at least 30 mV and with a match of quarter-bladders to within 15% in this respect.) Over the period of incubation, the control tissue PD fell from 45 ± 6 mV ($\bar{x}\pm$ SEM) to 38 ± 6 mV; after an initial brief period of instability, short-circuit current (I_o) in the experimental tissue was nearly constant; $I_o^{10'}=19\pm4\ \mu A\cdot cm^{-2}$, $I_o^{60'}=16\pm3\ \mu A\cdot cm^{-2}$. These tissues were fixed with glutaraldehyde at the end of incubation. Subsequent light and electron microscopy revealed no obvious differences in cellular architecture, intercellular space geometry or in the structure of the epithelial "tight" junctions between control and experimental members of any of the eight pairs. It was concluded that, if cellular volume had been altered by sustained clamping at $V_B=0$, these changes were not detectable with conventional chemical fixation.

Four additional experiments were run with the identical protocol but tissue was fixed by rapid freezing as described under Materials and Methods. These tissues are only suitable for light microscopic examination. However, the findings were qualitatively the same as with gluteraldehyde fixation. Fig. 1 presents comparative light microscopic views of paired open- *vs.* short-circuited preparations. While the degree of tissue stretch grossly affected the relative shape of the epithelium from one experiment to another, paired tissues within each of the experiments were reported as identical by each of six observers presented with coded samples.

Appreciating that minor differences in cellular volume might be indistinguishable in this manner, these frozen-dried samples were examined by the computer-assisted morphometric analysis described in Materials and Methods. Results of that analysis are presented in Table 1.[1]

Sampling limitations made it possible to comment on only the granular and basal cell populations. Comparison of granular cell, GC, (or basal cell, BC) volumes, *per se*, yielded no significant difference between open- and short-circuited preparations. The wide standard deviations in this form of analysis, which result largely from the different degrees of stretch from bladder to bladder, were substantially reduced when data were expressed as the fractional volume of the granular cells, GC/(GC+BC) (Table 2). It is clear that little or no *measurable* volume change is associated with short-circuiting of the tissue. It should be appreciated that in cells with volumes of the order of 2000 μ^3, a volume increase of as much as 20% would be reflected in less than a 1 μ increase in its effective radius; therefore, small changes in cell volume would not be detected with these methods.

Voltage-Clamping at Reversed Potential

Voûte and Ussing (1968) had noted that the effects of short-circuiting on RCL cell volume were increased dramatically when tissue was clamped at a level that reversed the open-circuit potential difference (i.e. holding the inside potential as negative with respect to the outside as it had previously been positive). We, therefore, applied this "voltage-reversal" procedure to a set of paired preparations for periods of 30 to 90 min, after which tissue was fixed either by freezing or with glutaraldehyde. Clamping tissue potential difference in this way resulted in a very obvious distension of granular cell profiles in 3 of 5 quick

[1] Absolute volumes of these cells can be derived in this way as well but resulting accuracy depends very heavily on proper estimation of cell shape. Assuming that sections are infinitely thin with respect to displayed cross-sectional areas, the measured areas are directly proportional to actual volume. For the purpose of this study, *changes* in volumes were our principal concern.

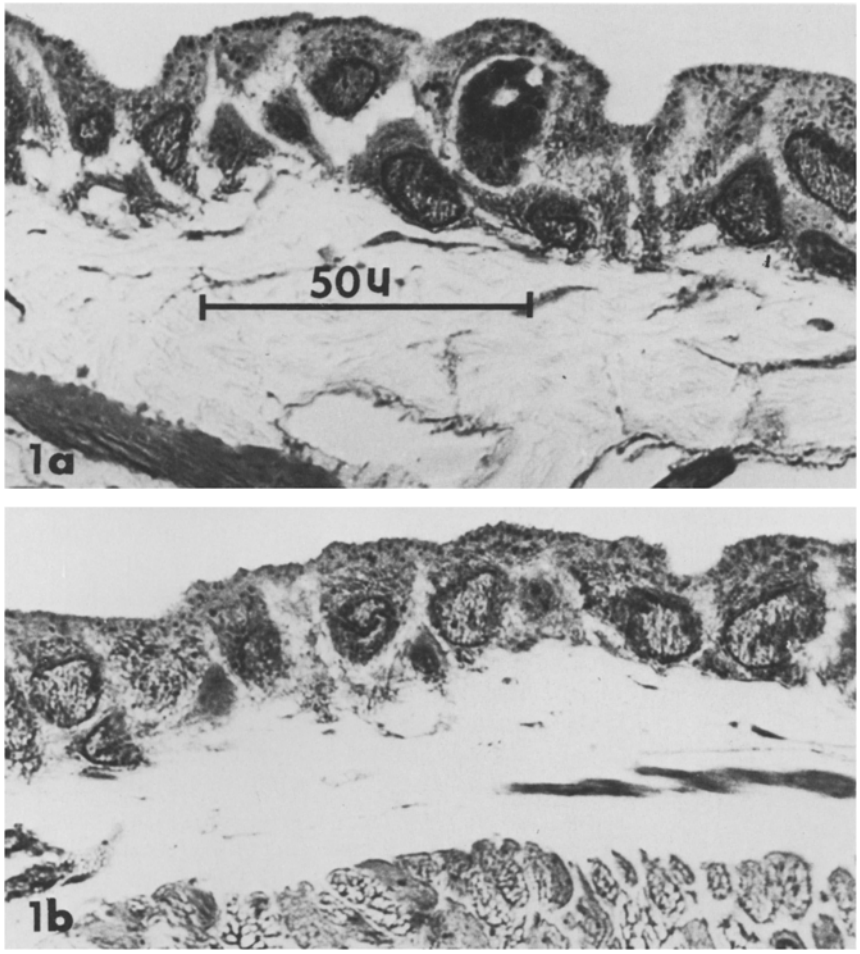

Fig. 1. Light microscopic views from paired quarter-bladders to compare epithelial cell volumes in open- (*a*) *vs.* short-circuited (*b*) conditions. As suggested in this illustration, no differences were apparent nor were any found after morphometric analysis. Samples were quick frozen; sections stained with toluidine blue. Magnification of each figure: 800×

Table 1. Cross-sectional areas of epithelial cells in open- *vs.* short-circuited bladders

Pair	Open-circuit (μ^2)	Short-circuit (μ^2)	Difference (SC-OC)
	(Granular cells)		
A	199.6	229.2	29.6
B	51.2	47.7	−3.5
C	164.4	156.9	−7.5
D	108.8	62.93	−45.87
Mean diff. = $-6.8\ \mu^2$; SD = $30.9\ \mu^2$; SEM = $15.5\ \mu^2$			
	(Basal cells)		
A	92.75	144.43	51.68
B	36.23	30.55	−5.68
C	83.88	89.95	6.07
D	53.01	41.09	−11.92
Mean diff. = $10.0\ \mu^2$; SD = $28.8\ \mu^2$; SEM = $14.4\ \mu^2$			

Table 2. Relative volume of cell types:[a] Open- *vs.* short-circuited bladders

Pair	Open-circuit	Short-circuit	Difference (SC-OC)
A	0.685	0.615	−0.07
B	0.59	0.61	0.02
C	0.66	0.64	−0.02
D	0.67	0.60	−0.07
Mean diff. = 0.035; SD = 0.044; SEM = 0.022			

[a] Granular cells/(granular plus basal cells).

frozen samples and in 4 of 6 where chemical fixation was used. A pair of quarter-bladders in which voltage-reversal produced a most uniform pattern is illustrated in Fig. 2*a* and *b*. In this case, the protocol involved clamping the serosa at −65 mV relative to the mucosa for a period of 60 min before glutaraldehyde fixation. While we felt it necessary to perform electron microscopy (*below*) in order to examine the volume response in detail, the presence or absence of swelling was readily seen with light microscopy of toluidine blue, 1-μ thick sections. It was also observed that swelling, when present, was confined to granular cells and, occasionally, goblet cells. Mitochrondria-rich and basal cells were not obviously affected. (Because swelling was evident when present and because we thought it unlikely that absolute volumes could be

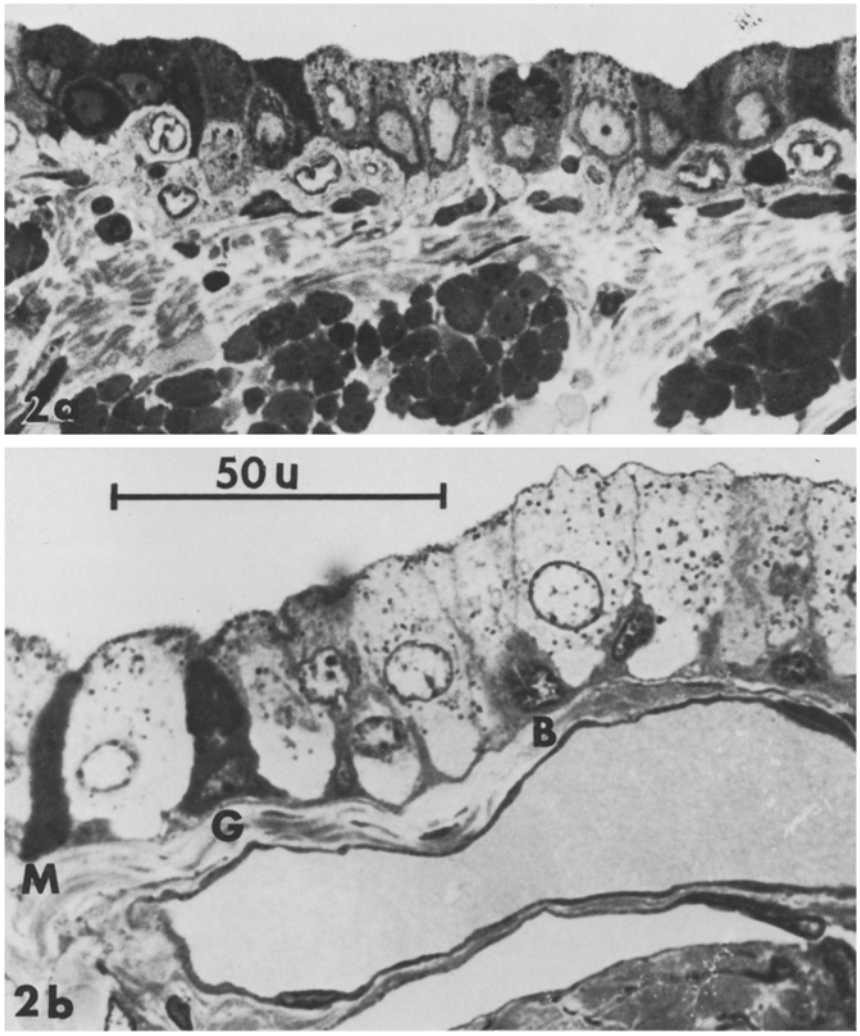

Fig. 2. Comparative light microscopic views from paired short-circuited (*a*) and voltage-reversed (*b*) preparations. In (*b*), the mitochondria-rich (*M*), goblet (*G*) and basal cells (*B*) appear dense and compact as in Fig. 1*a* while there is marked distension of the bulk of the epithelial cells – the granular cells. Many of the granular cells show a prominent bulge at their basal margin; this was more evident by electron microscopy (*see text* and Fig. 6*b*). Glutaraldehyde fixation; sections stained with toluidine blue. Magnification of each: 880 ×

preserved throughout the procedures for tissue preservation, we discontinued the computer-assisted profile analysis in favor of larger sample populations and blind examination of coded samples.)

While it was clear that cellular volume could be affected by significant alterations in transmural potential difference, reversal of the spontaneous potential did not appear to provide a reliable protocol for a detailed examination of this phenomenon. Spontaneous potential difference is a nonphysiological index of tissue activity in a quantitative sense to the extent that its *in vitro* value may be affected by "edge damage" (Dobson & Kidder, 1968; Walser, 1970; Helman & Miller, 1971). It seemed more rigorous, then, to clamp systematically each of the tissues at a given value, $V_B = -50$ mV, rather than at a value determined by spontaneous potentials which were highly variable. A group of twelve experiments yielded the electrical results displayed in Fig. 3. The plot shows the time-dependent fall in the current through the quarter-bladder clamped at -50 mV compared to that in a paired, short-circuited, control preparation. Data are plotted for 12 experiments as the ratios of final and initial external current in the voltage-reversed preparations (I^t_{-50} and I^o_{-50}, respectively) normalized by division by the short-circuit current of the paired control tissue (I^t_{sc} and I^o_{sc}). While the absolute data are not shown in this representation, it should be noted that external current in the short-circuited control preparations ($V_B = 0$) was fairly constant, varying by no more than 16% up to the time when the experiment was concluded by chemical fixation of the tissue. Subsequent identification of cell swelling by six observers provided with coded micrographs resulted in a unanimous conclusion. Eight of the twelve pairs showed an obvious difference in that voltage-reversed preparations displayed swollen granular cells. Examination of the external current traces recorded during the voltage-clamping revealed that, for these eight experiments, the current required

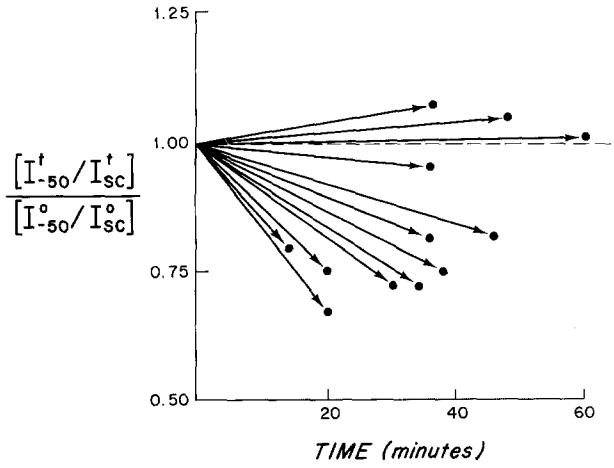

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Fig. 3. Plot of time-dependent change in external circuit current in twelve tissues clamped at −50 mV (serosa negative). The ordinate expresses the ratio of final to initial external current in a voltage-reversed preparation (I^t_{-50} and I^o_{-50}) normalized by these values for the short-circuited control tissues (I^t_{sc} and I^o_{sc}). A value of 1.00 indicates no change. Current fell relative to that in the control tissue in eight of the twelve experiments; these eight tissues were readily identified by light microscopy where each showed prominent granular cell swelling

to maintain the clamp had fallen by 20 to 40% in the −50 mV preparations relative to that in the control short-circuited preparations. In the four cases where swelling was absent, external circuit current dropped by less than 5%, or actually increased relative to the control tissue value. This result suggested that a change in cellular volume reflected a failure of the tissue to maintain a high level of transmural ion flow in the presence of voltage-reversal. When the tissue is bathed in a standard Ringer's solution, the major components of this flow are sodium and chloride with only sodium utilizing a transcellular pathway (Macknight & Leaf, 1978*b*). Logically, therefore, the swelling observed might be dependent on sodium flux through the cells. This proposition was tested by application of the diuretic, amiloride.

Dependence of Volume Change on Sodium Flux

Six experiments were conducted using the protocol detailed in Fig. 4. After a suitable baseline period during which each of the paired quarter-bladders was short-circuited (V_B=0), one had 10^{-4} M amiloride added to its mucosal medium. When the external current in this tissue had fallen to a minimum (0.5 to 2.5 μA), both quarter-bladders were clamped at V_B= −50 mV. The difference in the values of the initial external currents upon voltage-reversal reflects inhibition by amiloride of the flux of sodium through the active transport pathway. External current was followed for an additional 60 min before the tissues were fixed. The untreated quarter-bladders displayed a time-dependent fall in external current as in prior experiments of this type, while the amiloride-treated tissues exhibited a slight but significant increase ($p<0.01$) which began only after the first 30 min of clamping at −50 mV. Examination of coded samples revealed no evidence of swelling in the presence of the diuretic while the untreated preparations showed marked granular cell distension in 4 of the 6 cases. This finding is illustrated in Fig. 5.

Cellular Specificity of the Response

In those instances where substantial changes in cellular volume were produced by tissue depolarization, the responsiveness of the various cell types was different. The granular cells, the majority population lining 98% of the mucosal surface (DiBona, 1978) were the obvious index by which each of the test epithelia were classified, and they appeared to be far more sensitive to applied potential than the mitochondria-rich or basal cells which did not appear other than normal with the different procedures. Goblet cells varied in their responsiveness to voltage-reversal – often not affected when surrounding granular cells were grossly swollen but, on other occassions, appearing severely distended and having discharged their content of mucous droplets. Consistent with their not being involved in transmural sodium transport, voltage-dependent volume changes in the goblet cells were not prevented by the addition of amiloride (*cf.* Fig. 5). Fig. 6*a* and *b* are electronmicroscopic views of bladder epithelium fixed with glutaraldehyde after one hour of voltage-reversal. In this instance, the tissue potential was held at −62 mV. The features shown here are comparable to those seen in those tissues held at −50 mV where external circuit current had fallen appreciably. In each of the Figures it is apparent that mitochondria-rich cells were not markedly affected while adjacent granular cells showed clear signs of volume expansion. The granular cell (GC) in Fig. 6*a* is an example of the extent to which some of these cells appeared damaged; it shows dispersed cytoplasmic constituents, distended profiles of rough endoplasmic reticulum and a spherical, euchromatic nucleus. However, mitochondria of the granular cells are not rounded-up or evidently disrupted as has been seen often with swelling caused by dilution of the serosal medium (e.g. DiBona, Civan & Leaf, 1969*b*; DiBona, 1979). A feature of the swelling in this instance is that, in many granular cells, the apical (or urinary) pole of the cell seems less affected than the basal portion where one often sees a prominent bulge (Fig. 6*b*). Consistently, these regions were noted to

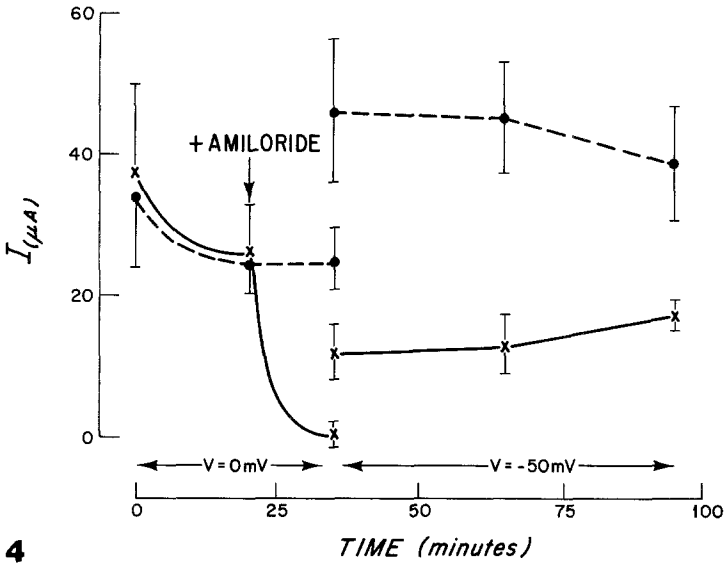

Fig. 4. Protocol and results for six experiments to test the dependence of cell swelling on sodium entry. Solid line: amiloride-treated quarter-bladders; dashed line: control paired quarter-bladders. Tissues were held at 0 mV until the amiloride addition (10^{-4} M) to the mucosa of the experimental preparation had dropped short-circuit current to zero. Then both preparations were clamped at −50 mV and held for one hour. The untreated preparation showed a fall in external current over this interval (*as noted above*); amiloride-treated tissues showed a rise in external current and, therefore, in passive conductance

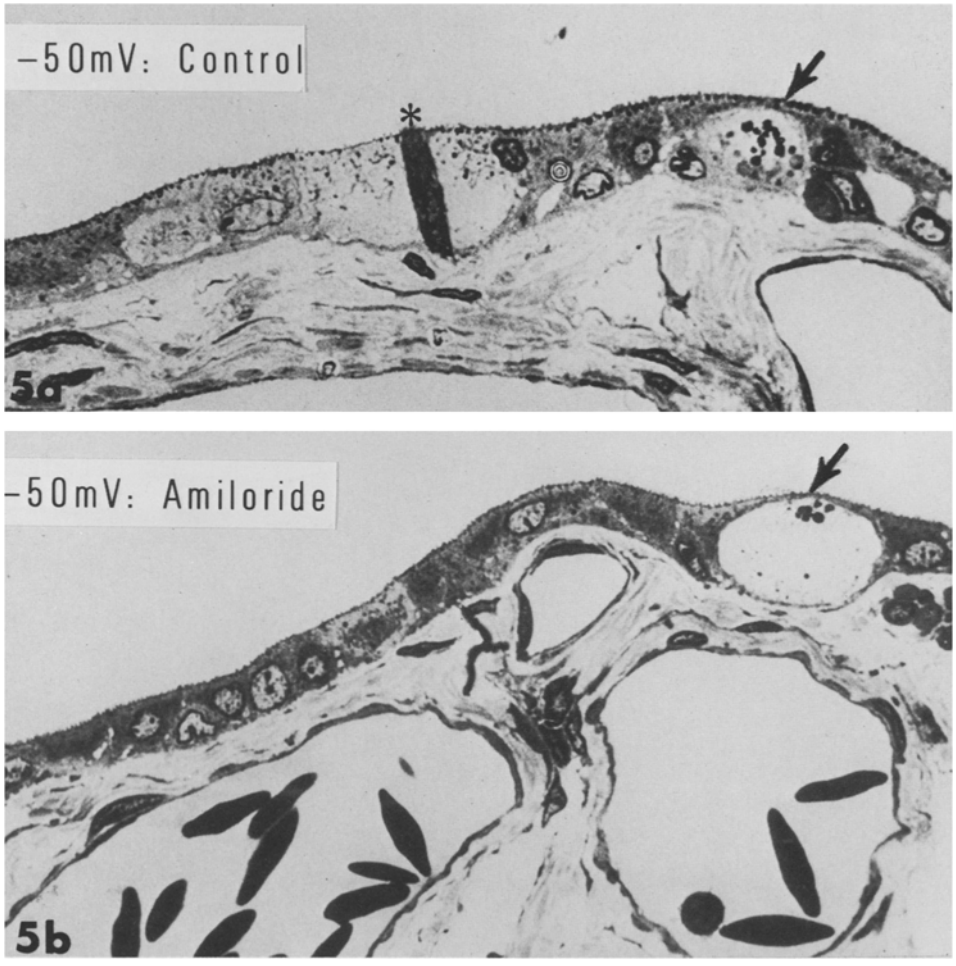

Fig. 5. Comparative views of a pair of quarter-bladders from the experiments described in Fig. 4. The degree of swelling as evidenced by dispersion of cytoplasmic contents is variable in the control preparation (*a*) but the epithelium is everywhere thicker than in the amiloride-treated tissue (*b*). Observations of this sort provided ready identification of these samples in four of the six cases where the control tissue was swollen. Note that the mitochondria-rich cell (*) in (*a*) is compact in appearance although bounded on either side by grossly swollen granular cells. Goblet cells (unlabeled arrows) were often swollen as well and, unlike granular cells, not protected by amiloride application. Glutaraldehyde fixation; toluidine blue-stained sections. Magnification: 300×

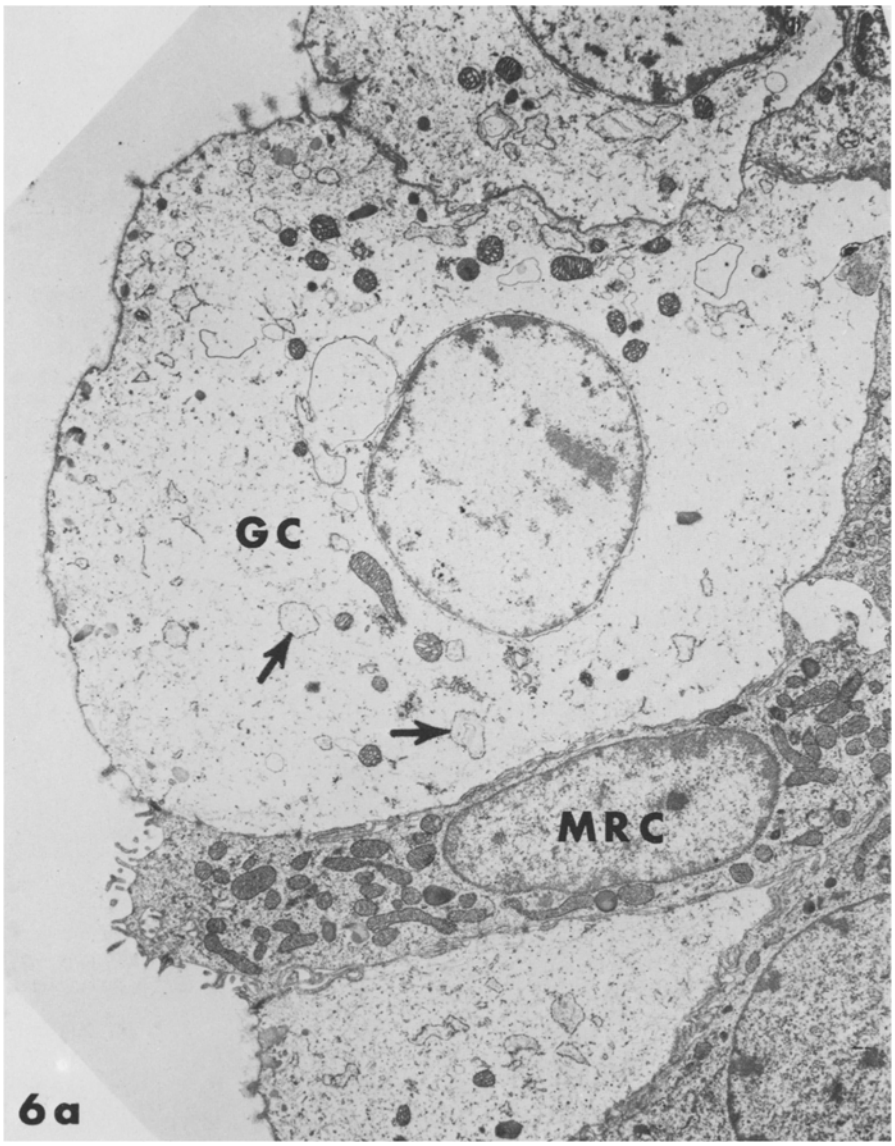

Fig. 6. (*a*) Electron micrograph of toad bladder epithelium fixed after 60 min of being clamped at −62 mV. The granular cell (*GC*) has expanded to fill all of the intercellular space and shows a near spherical nuclear profile with little remaining heterochromatin, distended profiles of rough endoplasmic reticulum (unlabeled arrows) and sparse cytoplasmic ground substance particularly in the basal portion of the cell (urinary surface is to the left). The mitochondria-rich cell (*MRC*) appears normal but compressed between adjacent granular cells as in the view in Fig. 5*a*. Uranyl acetate and lead citrate stain; Magnification: 4800×

contain electron dense particles 10–20 nm in diameter but no formed organelles. Retrospectively, one can appreciate this swelling of the basal cytoplasm in the light micrograph of Fig. 2*b* as well.

Electronmicroscopic examination of several pieces of tissue from preparations in which external current had not fallen significantly with $V_B = -50$ mV and where no swelling was apparent by light microscopy, yielded no remarkable features by which to distinguish them from open-circuited preparations.

Shunt Pathway Sensitivity

Bindslev et al. (1974) have previously noted that application of a potential reversing voltage clamp to the toad bladder produces bullous distensions or blisters of the "tight" junctions, comparable to those elicited by application of hypertonic mucosal media (DiBona, 1972; Wade, Revel & DiScala, 1973). Consistent with the observations in a series of papers (DiBona & Civan, 1973; Civan & DiBona, 1974, 1978), it might be expected that these junctions – polarized, anisotropic structures – would exhibit a change in form and in ionic conductivity with a reversal of the naturally occurring transmural potential difference. The observed change in passive ionic permeability which might be attributed to the paracellular pathway was small and detectable only in the presence of amiloride where transcellular conductance was presumably nullified. In Fig. 4, the results of 6 experiments show a 60% increase in passive conductance (0.22 to 0.36 mmhos/2.5 cm^2 of tissue) over 60 min for which we sought a structural correlate. We assumed that "edge-damaged" tissue conductance did not increase with time and that the change in some physiologic passive pathway across the tissue

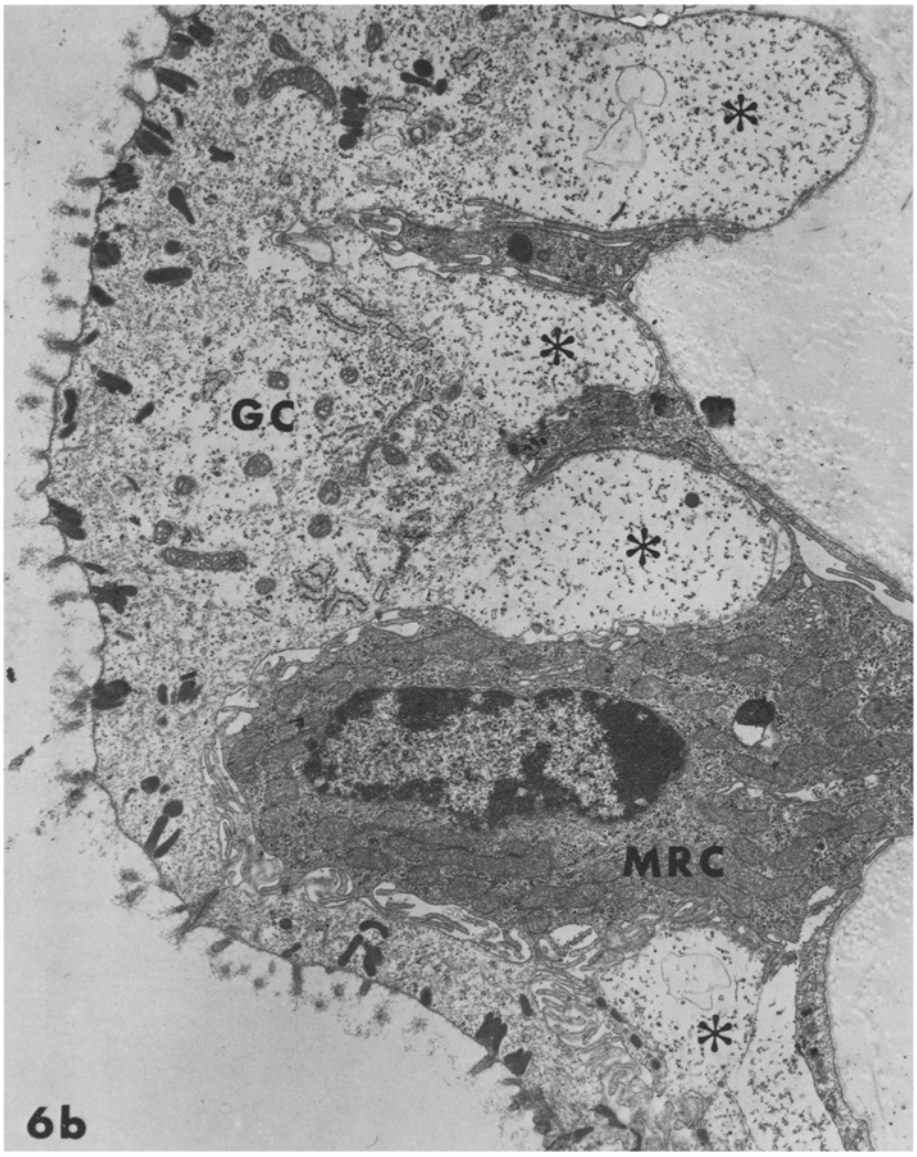

Fig. 6. (*b*) Electron micrograph of a less prominently swollen region of the tissue illustrated in (*a*). Here the granular cells (*GC*) appear normal in terms of the apical portion of the cell with compact terminal web and flattened saccules of endoplasmic reticulum; it is clear that the basal portion of the cell is distended in pockets or bulges (*) which contain electron-dense particles about the size of the free ribosomes. The mitochondria-rich cell (*MRC*) which does not reach the surface in this view shows no sign of cellular volume expansion. Preparations like this one where external current fell by 44% over 60 min of voltage reversal typically displayed the heterogeneity exemplified by these Figures. Uranyl acetate and lead citrate stained. Magnification: 8100×

was substantially greater than the 60% observed in the preparation overall. Three of these amiloride-inhibited, voltage-reversed preparations were selected at random and examined by electron microscopy where it was found that 7–10% of observed junction profiles (14 of 186, 9 of 106, and 12 of 147) showed at least one clear distension at a magnification of 5000×. This observation is in fair agreement with our earlier findings (DiBona & Civan, 1973) where there was a positive correlation between the increase in paracellular conductance and the degree of junction deformation.

Discussion

The results of this study may be summarized as follows: *a*) epithelial cell volume was not affected detectably when the potential difference across the toad bladder was nullified by short-circuiting; but *b*) voltage-clamping the tissue at a reversed potential (serosa negative) resulted in a selective and pronounced swelling of granular cells; *c*) granular cell swelling was completely prevented by prior exposure of the mucosal surface of the tissue to a concentration of amiloride which was sufficient to abolish completely the active transport of sodium; and *d*) reversal of the transmural potential increased the conductance of a passive shunt pathway concurrent with structural alteration of the tight junctions. Interpretation of these results requires consideration of the toad bladder epithelium in terms of correlations between its structure and its permeability characteristics.

Transmural Potential and Cellular Volume

Cellular swelling will occur by osmosis if total cellular solute is increased relative to bathing medium concen-

trations. Consequently, the effect of transmural potential difference on cellular ionic balance is of primary interest. Since the major ions in the bathing media are sodium and chloride, it is presumably these ions which are gained by the swollen cells when tissue transmural potential difference is reversed. Sodium enters the cells predominantly from the mucosal medium across the apical cellular membrane which is permeable to this ion almost exclusively (Macknight & Leaf, 1978*a*). Efflux from cells to serosa is through the basal-lateral cellular membrane by a system involving a ouabain-sensitive ($Na^+ + K^+$)-ATPase – the sodium pump. There seems to be little passive diffusion of sodium across the basal-lateral membranes (Beauwens & Al-Awqati, 1976; Canessa, Labarca & Leaf, 1976; Macknight & McLaughlin, 1977; Rick et al. 1978*a*). Influx and efflux of sodium appear to be balanced under both open- and short-circuited conditions since Na^+-content does not seem to vary under these conditions where a constancy of cellular volume has also been measured (Macknight, Civan & Leaf, 1975).

Cellular swelling in voltage-reversed tissues might be derived from either an increased influx of sodium, a decreased efflux, or both.[2] Since macroscopic electrical neutrality must be preserved, increased cellular sodium would necessitate either an equivalent loss of cellular potassium or a gain of chloride; each of these ions exchanges predominantly with the serosal medium (Robinson & Macknight, 1976*b*; Macknight, 1977). Therefore, when the tissue is clamped with the serosa negative, charge will be carried across the tissue in part by sodium moving through the cells from mucosa to serosa and in part by sodium entering cells from the mucosal medium and either potassium being lost to, or chloride being gained from, the serosal medium. Since cells actually swell, an uptake of chloride rather than any loss of potassium would appear to be the predominant ion movement accompanying the gain in sodium.

The entry of sodium to the cells across the apical membrane is thought to be a passive process with its rate determined by the electrochemical potential gradient, $\Delta\tilde{\mu}$, and by the permeability of the membrane to sodium. Each of these parameters may be affected by an applied voltage-clamp. Reversing the spontaneous polarity of the tissue will enhance $\Delta\tilde{\mu}$ through an increase in the electrical driving force. Any externally applied electromotive force that makes the serosa more negative with respect to the mucosa will be fractionally distributed across transcellular ionic pathways as increased electronegativity of the potential difference from mucosa to cell and that from cell to serosa. The magnitude of the change will be determined by the relative electrical resistances of the apical (R_M) and basal-lateral membranes (R_B). The greater $R_M : R_B$, the greater the decrease in apical membrane potential, and therefore, the greater the absolute increase in electrical driving force and, consequently, in $\Delta\tilde{\mu}$. There is considerable evidence that with mucosal medium sodium concentrations greater than 20 to 30 mM, the sodium entry mechanism is saturated under both open- and short-circuited conditions (Frazier, Dempsey & Leaf, 1962; Leb, Hoshiko & Lindley, 1965). In the present experiments, with mucosal medium sodium concentration of 120 mM, the fact that amiloride-inhibitable cellular swelling occurred in voltage-reversed tissues provides further evidence for voltage-sensitivity of the entry mechanism (Biber & Sanders, 1973; Cuthbert & Shum, 1976) and, therefore, of apical membrane resistance. Consequently, both the driving force for sodium entry and the permeability of the apical membrane to sodium seem to have been enhanced by the potential-reversing voltage-clamp.

The possibility remains, for cells which are swollen, that the active transport step for sodium at the basal-lateral cellular membrane may have been affected as well. Reversing potential across the tissue must result in some decrease in the basal-lateral membrane potential (in this case, inversely proportional to the ratio, $R_M : R_B$). Any resulting decrease in the electrical gradient across the basal-lateral membrane should facilitate transport from cell to serosa to the extent that the work required to transport sodium across this membrane will be reduced. Similarly, any increase in cellular sodium activity as a consequence of increased rate of entry to the cells from the mucosal medium should facilitate sodium transport from cell to serosa, again reflecting the decreased electrochemical gradient for sodium across this membrane. Consequently, decreased pump activity would not be predicted from consideration of an effect on basal-lateral membrane potential or on cellular sodium activity. However, as discussed below, it is possible that the active transport mechanism, *per se*, may be affected during voltage-reversal since current flowing across the tissue does decrease with time.

Among preparations where voltage was clamped at serosa-negative levels, swelling was restricted to tissues in which the current in the external circuit fell (Fig. 3). Since the clamping voltage was held constant, transmural conductance must have decreased in these samples. Three possibilities may have contributed to this decrease. First, as pump capacity is initial-

[2] The limited potassium conductance of the mucosal membrane makes its increased entry during voltage-reversal an unlikely factor in cellular ion accumulation. If it were important, however, one would not expect swelling to be prevented by amiloride as has been observed.

ly exceeded, current can be carried across the tissue by sodium entering the cells from the mucosal medium and by chloride gained from the serosal medium. As sodium accumulates in the cell the electrochemical gradient driving sodium into the cell will decrease, reducing the rate of sodium and chloride entry, so that transepithelial current will decrease. Second, as cellular volume and sodium concentration increase, and cellular potassium concentration decreases, the conductance of the apical membrane to sodium may decrease (Lewis, Eaton & Diamond, 1976; Robinson & Macknight, 1976*a*; Turnheim, Frizzell & Schultz, 1978). Third, it is possible that the sodium pump, forced to work at its maximum capacity, may not be able to sustain the transport rate indefinitely; current and conductance through this sodium-selective pathway would then decrease. The first and second possibilities would tend to limit cellular swelling, but the third would enhance it and therefore, might be favored since swelling was tightly correlated with a fall in external circuit current.

It is concluded, therefore, that the primary cause of cellular swelling in voltage-reversed tissues is an increased rate of entry of sodium to the cells from the mucosal medium that results in saturation of the sodium pump and cellular accumulation of sodium, chloride and water. The observation that amiloride completely prevents this swelling offers strong support for this interpretation.

Cellular Specificity of the Response

The response of the granular cell to the imposed electrical gradients indicates that it is this cell type which is responsible for active transepithelial sodium transport. The relative constancy in cell volume in the transition from the open- to the short-circuited state suggests sufficient sodium pump capacity to accommodate the accelerated influx from mucosa to cell caused by the increased electronegativity of the cell interior. The cellular swelling which occurs with reversal of tissue potential suggests that, as the rate of sodium influx to the cell increases, the pump "capacity" is exceeded. Saturation of pump "capacity" as indicated by cell swelling, seems to occur at about -50 mV.

The hypothesis that the volume changes in the granular cells result from an increased rate of sodium entry with saturation of the transport process, was directly tested by abolishing sodium entry with amiloride. This prevented, specifically, the swelling of the granular cells as predicted. This result provides compelling evidence that granular cells are involved in, and perhaps fully responsible for, the amiloride-sensitive transepithelial transport of sodium across toad urinary bladder.

The mitochondria-rich cells appeared to be insensitive to reversal of transmural potential difference. One might argue that the constancy of volume in these cells reflects a greater sodium pump capacity. However, this reasoning is inconsistent with results from studies of $(Na^+ + K^+)$-ATPase localization (Mills & Ernst, 1975), and data obtained on cellular ions in electron microprobe experiments (Rick et al., 1978*a*). Rather, the lack of response by these cells would seem to indicate that their apical membranes have a significantly lower sodium conductance than those of the granular cells. While the abundance of mitochondria in these cells argues for their being involved in an energetic process, it may be that their primary function is in acidification of the urine as has been suggested (Rosen, Oliver & Steinmetz, 1974; DiBona, 1978). In any event, these studies do not unequivocally deny these cells a role in active sodium transport; positive identification of their physiological role remains to be established.

In contrast, one might presume that the greater sensitivity of the goblet cells to depolarizing voltages is due to their possessing high mucosal permeability to sodium with limited sodium conductance at their basal-lateral boundaries. However, their volume response was insensitive to amiloride application and, as argued earlier (DiBona, 1978) their absence in the bullfrog bladder, a competent absorber of sodium, renders their involvement in this process very unlikely. There was no detectable basal cell swelling .There is evidence for gap junctions between the granular and basal cells (Wade, 1978) and this, together with data obtained in electron microprobe experiments (Rick et al., 1978*a*). suggests a syncytium of these cell types. Some swelling might, therefore, have been predicted. But, since swelling reflects the balance between rate of entry and extrusion, its absence may simply indicate that gain of sodium through the gap junctions from the granular cells has not been accelerated sufficiently to exceed the maximum pump capacity of the basal cells or that cell-cell communication has been interrupted by tissue depolarization. Certainly, the absence of swelling in these experiments provides no substantive evidence against the hypothesis that granular and basal cells function as a syncytium.

Effects on the Paracellular Pathway

In addition to the effects on cellular morphology, reversed potentials induced amiloride-insensitive structural alterations (blisters) in the "tight" junc-

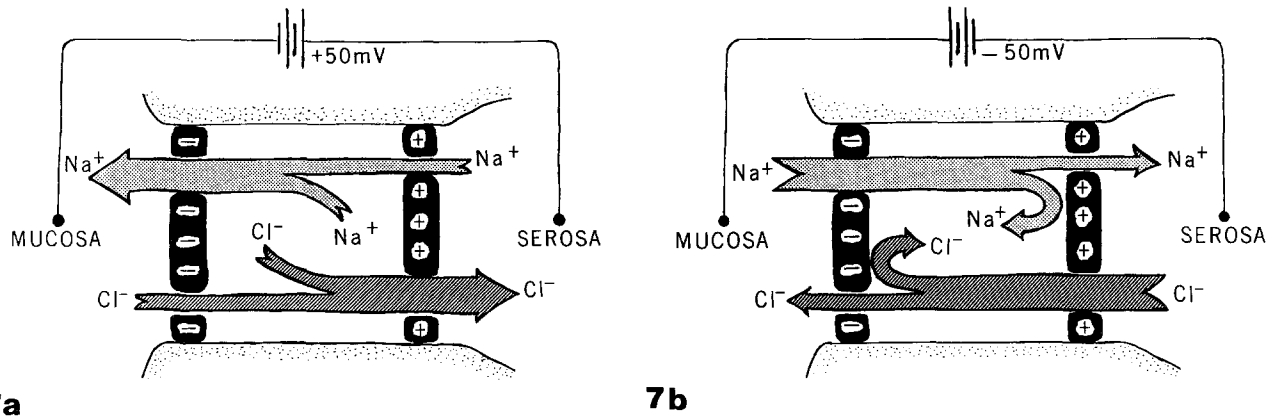

Fig. 7. Schematic views of the postulated effects of transmural potential on the ionic conductance of the "tight" junctions. In the open-circuit condition (*a*) where the serosa is positive with respect to mucosa, low conductance of the shunt pathway is attributed in part to the presence of series barriers charged negatively at the mucosal edge and positively in the portion of the junction facing the lateral intercellular space (serosal aspect). Ion flow to allow a 50-mV potential drop in this orientation would tend to deplete the Na^+ and Cl^- of the junctional space between barriers. Conversely, when the potential is reversed (*b*) as was done in this study, an increase in junction ionic conductance would be brought about by an obligatory accumulation of salt in the junctional space. Given reflection coefficients greater than zero and finite hydraulic permeability, it would also be expected that ion accumulation in the voltage-reversed state would result in an osmotic swelling of the junction leading to the occasional formation of a distinguishable "blister" as caused by mucosal hypertonicity

tions. The quantitative extent of these alterations seems consistent with the observed modest increase in passive conductance of the tissue; the larger increases in passive conductance associated with application of hypertonic mucosal media have been correlated with far more extensive alterations in junctional morphology (DiBona, 1972; DiBona & Civan, 1973). Since passive conductance has increased and junctions are blistered with voltage reversal, it is evident that the reduction in transepithelial conductance (in the absence of amiloride) is due to an increase in transcellular resistance.

The electrically induced blistering, similar to that reported by others (Bindslev et al., 1974) is best explained by a distribution of fixed charges within this complex structure such that the apical boundary of the junction possesses greater electronegativity than the underlying components. The rationale for this is elaborated upon in Fig. 7, where the junction is described as a composite barrier with an asymmetric distribution of transference numbers such that its internal ion concentration (and hence its overall conductance) varies with the direction of applied potential. Because these junctions are distensible, osmotic considerations are necessary as well. In the case of epithelial depolarization, where the salt concentration within a junction of this design would be increased, it is reasonable that osmotic swelling would occur causing focal blistering in the more compliant regions. Any separation of opposing cell membranes in this narrow zone (whether or not it results in a demonstrable deformation of the space) should contribute further to enhanced conductance. A formal analysis of this general arrangement has been provided by Katchalsky and Curran (1965). Further study of this aspect of junction structure is required to explain fully the role of this rate-limiting barrier in the transport activity of epithelia of this type.

In conclusion, these findings establish that a substantial reversal of the spontaneous potential difference across the toad urinary bladder may result in selective cellular swelling and junctional blistering. A major conclusion is that the granular cells have an important role in transepithelial active sodium transport in the toad bladder. The possibility of modulating cellular swelling in this way provides a potentially important tool for the morphological analysis of transport processes in epithelia. In the following paper (DiBona, Sherman, Bobrycki, Mills & Macknight, 1981), this procedure is used in an analysis of the natriferic action of vasopressin.

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