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ELECTRICALLY AND OSMOTICALLY INDUCED CHANGES IN PERMEABILITY AND STRUCTURE OF TOAD URINARY BLADDER

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SUMMARY

The purpose of this investigation was to explore the effects of electrical gradients on the permeability and structure of the toad urinary bladder and to compare these results with those produced by osmotic gradients. From this we hoped to gain new insights into permeation pathways across epithelia.

A marked increase in conductance begins within 20 ms, when voltage gradients of several hundred mV are applied in either direction across the bladder. This is accompanied by increased permeability of both mannitol and 1,7-heptanediol.

Electron microscopy shows that these gradients also produce "blisters" within the tight junctions of the bladder epithelium. However, this structural change occurs only after the conductance change is complete.

Osmotic gradients applied in either direction across the epithelium produce conductance and permeability changes virtually identical to those produced electrically. Blisters are formed in the tight junctions only when the mucosal solution is made hypertonic.

We conclude: (a) Blister formation in tight junctions does not cause the conductance and permeability changes. (b) Both polar and non-polar permeation pathways are altered. (c) Both types of gradient probably act by basically the same mechanism.

INTRODUCTION

While surveying current–voltage relations in epithelia, we observed that electrical currents could produce large increases in the conductance of the toad urinary bladder.

Similar increases in conductance are known to be produced in this bladder by osmotic gradients [1]. Accompanying this osmotically induced change, blisters develop in the tight junctions of the bladder epithelium [2–4]. This structural alteration has been interpreted as evidence for the role of tight junctions as permeation pathways in this and other high resistance epithelia.

Our paper reports the effects of electrical gradients on the conductance, nonelectrolyte permeability and ultrastructure of the toad urinary bladder. It compares these effects with those produced by osmotic gradients.

We find the two types of gradient produce remarkably similar changes, and from these we can arrive at several tentative conclusions: (1) Both gradients produce their effects by ultimately similar mechanisms. (2) Changes occur in both polar and non-polar permeation pathways. (3) Blister formation in tight junctions is not the cause of these effects. (4) No inferences may be made about the relative importance of cellular and junctional permeation routes across this tissue.

METHODS

Urinary bladders obtained from toads (*Bufo marinus*) were mounted between lucite chambers. The area of exposed tissue was 1.3 cm^2. Each half chamber contained 16 ml of saline, which was composed of 105.4 mM NaCl, 2 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$ buffered to pH 7.3 with 2.125 mM Na_2HPO_4–0.375 mM NaH_2PO_4. The saline in each half chamber was continuously gassed with 100% O_2 and vigorously agitated by means of magnetic stirring bars. Osmotic gradients were imposed across the tissue by the addition of mannitol, sucrose or urea to the saline bathing either the mucosal or the serosal surface of the bladder.

Transmural electrical potentials (P.D.) and conductances ($G=1/R$) were measured and recorded essentially as described previously for the rabbit gall bladder [5]. (The Devices stimulator was replaced by a Devices relay unit in order to pass current pulses longer than 1 s, and the P.D. and currents were recorded continuously on a two channel chart recorder.)

Bladders destined for electron microscopy were mounted between two plastic rings prior to assembly in the chambers, as described previously for studies on the gall bladder [6]. Like DiBona and Civan [3], fixation was started by adding 50% glutaraldehyde to the solution in each half chamber to produce a final concentration of 1%. During the initial fixation period, electrical or osmotic gradients continued to be maintained across the tissue, and no significant changes in conductance were observed. After 15 min the tissue rings were removed from the chambers, and fixation was continued for 2 h at room temperature in 1% glutaraldehyde in 0.11 M cacodylate buffer containing 2 mM $CaCl_2$ at pH 7.3. The tissues were subsequently washed overnight in cold buffer, post-fixed in 1% OsO_4, and embedded, sectioned and stained by conventional methods.

Permeabilities were measured by radioactive tracer techniques. Carbon-labelled mannitol and 1,7-heptanediol were obtained from ICN, Irvine, Calif., and the samples were assayed by liquid scintillation counting. Isotope fluxes were measured across the tissues in both directions; their magnitudes were independent of direction. The fluxes were corrected for unstirred layer effects as described previously for the gall bladder [7]. As judged from diffusion potential transients and from butanol fluxes, the thickness of the unstirred layers was about 200 μm (Pietras R. J. and Wright, E.M., unpublished); accordingly a small correction was applied to the 1,7-butanediol fluxes.

RESULTS

Current induced conductance changes

The conductance of the urinary bladders was determined from the voltage response upon switching d.c. current pulses on or off. The rise in voltage after the

onset of a constant current pulse approximated a single exponential which corresponded to a tissue capacitance of about 0.5 μF/cm^2. At low current densities (10 μA/cm^2) the instantaneous conductance was independent of voltage and varied in different preparations between 0.15 and 0.7 m$\Omega^{-1}\cdot$cm^{-2}. At these current densities the voltage remained stable after the capacitive surge and no transients such as conductance changes or polarization effects were observed. The instantaneous current–voltage curves were approximately linear up to about $\pm$350 mV, but thereafter became markedly non-linear (see Fig. 1). (As explained below, we think that the non-linear component is simply due to the fact that the time constant for the current induced conductance changes approaches the time constant for the capacitative element.)

However, at higher current densities (>20 μA/cm^2) voltage transients were observed. An example of this is shown in Fig. 2 where the voltage decreased with time after passing current in either direction across the tissue. In this bladder, initial resistance 4800 $\Omega\cdot$cm^2, passing a current of 78 μA/cm^2 reduced the resistance to 4200 $\Omega\cdot$cm^2 within 150 ms of switching the pulse on in one direction, and to 3300 $\Omega\cdot$cm^2 upon passing the same current in the opposite direction. Evidence that the voltage transient was in fact due to a drop in resistance (increase in conductance), was provided by (i) the magnitude of the voltage step upon switching the current on or off, and (ii) the relative magnitude of the voltage steps produced by short current pulses immediately before and after the passage of the long pulse. Currents of longer duration than those shown in Fig. 2 demonstrated that the conductance reached a stable value (the same value for currents passage in either direction) within 20 s.

The greater the current density the larger the increase in the conductance. This

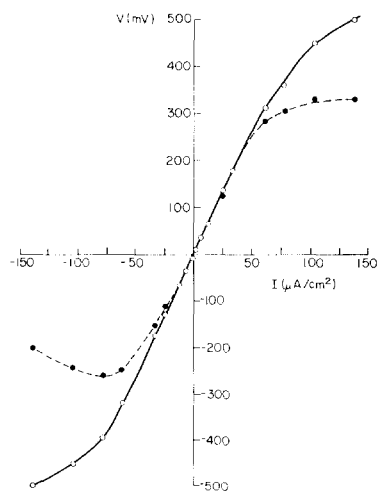

Fig. 1. Current–voltage relations for the toad urinary bladder incubated in Ringer's solution. The voltage drop across the bladder on passing constant current pulses was recorded 20 ms (○—○) and 400 ms (●---●) after the onset of the current pulse. The spontaneous transmural P.D., which ranged from 20 to 60 mV in these experiments, was subtracted from all voltages, and the curves have been corrected for the voltage drop between the salt bridges used to monitor the P.D. values. The voltage (mV), serosa with respect to mucosa, is plotted on the ordinate against the current (μA/cm^2) on the abscissa. This result is from a single bladder, but is typical of all those studied.

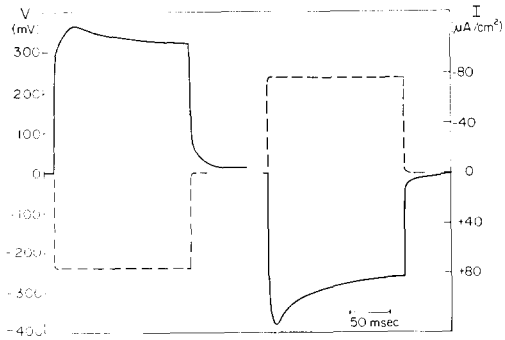

Fig. 2. Records, redrawn oscilloscope traces, showing voltage transients across the toad urinary bladder. Current pulses (78 μA/cm^2) were passed in both directions across the tissue for 150 ms, and both the voltage and current traces on the oscilloscope were recorded on film. These records were obtained from the same bladder as the experiment shown in Fig. 1. Voltages (———) and currents (– – –) are plotted on the ordinate with respect to time on the abscissa. The voltages are given as the serosa with respect to the mucosa.

is shown in Fig. 1 where we have plotted the instantaneous I/V curve and the I/V curve constructed from the voltages recorded 400 ms after switching on the pulse. It can also be seen from this figure that there is a definite threshold for the current (or voltage) dependent conductance change. Comparison of I/V curves from bladder to bladder suggests that it is the voltage gradient rather than the current per se which produces the effect. For example, in one tissue with an initial resistance of 5650 $\Omega \cdot$cm^2 the threshold was reached at a current of 22 μA/cm^2, whereas in another tissue with an initial resistance of only 1040 $\Omega \cdot$cm^2 a current of 120 μA/cm^2 was required. In other words, the threshold was reached when the voltage across the tissue was 100–200 mV. Not shown in the figure is our finding that the time course of the conductance increase gets shorter the higher the current density. For example, in one experiment currents of 190 and 420 μA/cm^2 increased the conductance with half times of 2 and 0.1 s, respectively. As noted above this probably accounts for the non-linear region in the instantaneous I/V curves.

The final point to be noted about these current-induced conductance changes is that they are completely reversible. For example, in one tissue where the conductance was increased from 0.7 to 2.1 m$\Omega^{-1} \cdot$cm^{-2} by passing a current of 400 μA/cm^2 for 44 min the conductance returned to the original value within 2.5 min of switching off the current. In general the conductance changes produced by currents up to 500 μA/cm^2 were all fully reversible as long as the currents were applied for less than 10–15 min.

Similar current induced conductance increases were observed in another high resistance epithelium, the frog skin (Bindslev, N. and Wright E.M., unpublished), but in a low resistance epithelium, the gall bladder, we [8] found these effects were generally obscured by resistance changes associated with changes in dimensions of the lateral intercellular spaces.

Civan [9] has also studied current–voltage relationships in the toad bladder. Most of his observations were made with shorter current pulses at lower voltage gradients than we find necessary to produce sizeable conductance changes. Nevertheless he noted small conductance increases with the larger gradients.

Osmotically induced conductance changes

In general we confirm the reports by others [1–4] that osmotic gradients increase the conductance of the toad urinary bladder. Briefly, we find addition of non-electrolytes to the mucosal fluid increases the conductance, e.g. mannitol (300 mM) added to the mucosal solution increased the conductance in one preparation from 0.3 to 0.8 $m\Omega^{-1} \cdot cm^{-2}$ in about 20 min. The potency of non-electrolytes in producing the effect is in the order, urea > mannitol > sucrose.

Furthermore we find that addition of non-electrolytes to the serosal fluid also lowers the resistance, but in general higher concentrations are required in the serosal solution to trigger the increase in conductance, e.g. whereas addition of 300 mM mannitol to the serosal fluid had no effect, 500 mM mannitol lowers the resistance by 80–90% (cf. Table I and Fig. 7). The large concentrations required to produce this effect from the serosal side probably explains why it has not been reported by others.

Morphological changes associated with osmotic and electrical gradients

Since it was suggested that osmotically induced conductance increases in the tissue are due to the formation of blisters in the tight junctions we undertook electron microscopic studies in the hope of finding a morphological basis for the current induced conductance increase. Control bladders were compared with those whose conductance was increased by passage of current, and to confirm the work of others we also included bladders whose conductance was modified by osmotic gradients. In all cases we directed most of our attention to the structure of the tight junctions.

In the control bladders (Fig 3) the plasma membranes in the apical region of adjacent epithelial cells came together to form typical pentalaminar tight junctions. These were approximately 0.3–0.5 μm in depth, and appeared uniformly close throughout this distance.

Passage of current for several minutes altered the structure of these junctions regardless of the direction in which the current was passed. The uniform closeness of the junctional membranes (Fig. 4) was interrupted by focal dilations or blisters. We found blisters within 89% of the junctions examined, sometimes near the top, sometimes near the bottom of the junctional complex. In either case they appeared to be sealed off above and below by normally fused membranes. The blisters usually occurred singly, but sometimes in groups of two to three. They were nearly circular in cross section, with diameters ranging from 10 to 240 nm. The average diameter was 110 nm.

Blisters were found in 87% of the junctions exposed to hypertonic mucosal solutions, confirming earlier reports [2–4]. The smaller of these blisters were identical to those produced by current. However, the blisters on the whole averaged 3 times larger, with a mean diameter of 305 nm. 40% of the junctions had dilations between 250 and 1000 nm in diameter (Fig. 5), whereas none of the blisters produced by current were in this size range.

The question of whether these obvious changes in structure of the tight junctions provide the explanation for the conductance increases seems to be ruled out by two additional observations: (1) In bladders which were fixed only 20 s after applying the current, we could find no junctions with clearly defined blisters of any size, even though the increase in conductance was virtually complete. A few images do suggest, however, that blisters may be beginning to form (e.g. Fig. 6). (2) When the conductance was increased by the addition of mannitol (500 mM) to the serosal solution

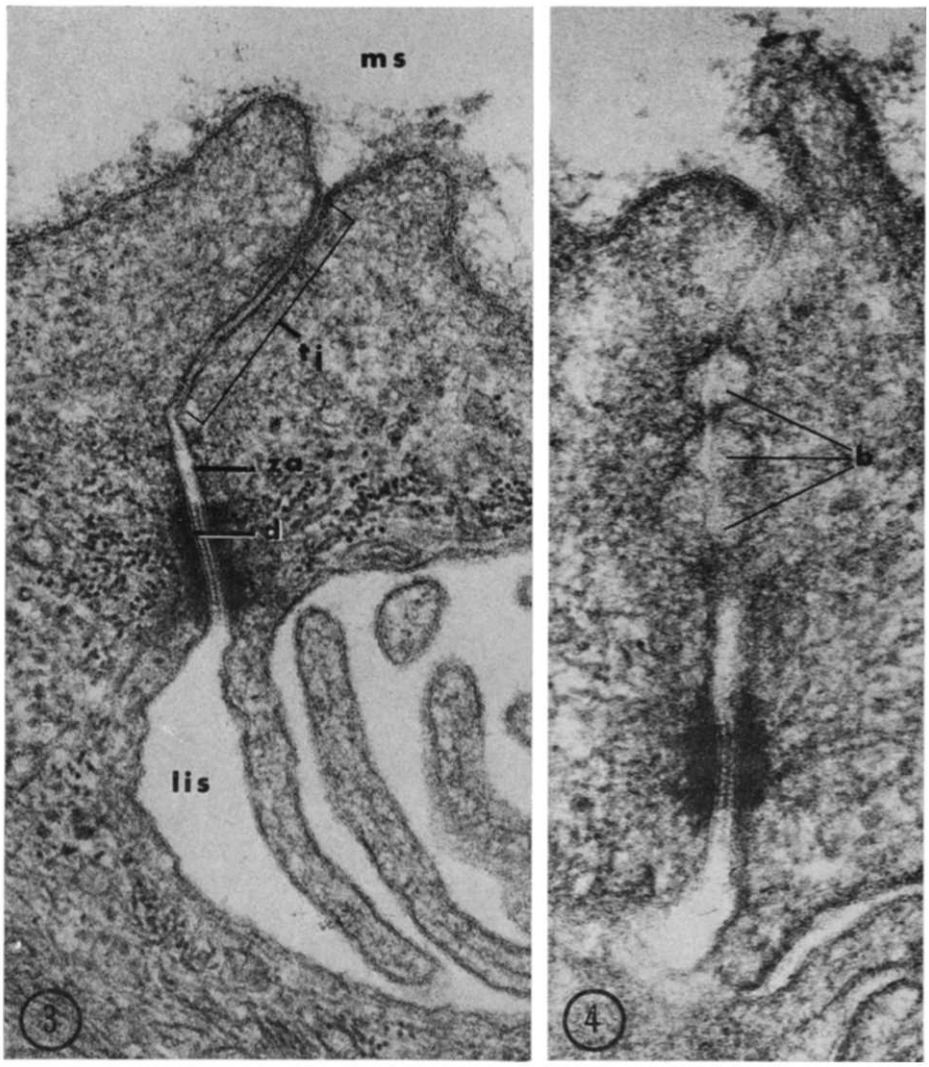

Fig. 3. Control. Resistance 1570 $\Omega \cdot cm^2$. This, as all subsequent pictures, is an electron micrograph showing the apical region of two adjacent toad bladder epithelial cells. ms is the mucosal bathing solution. A typical "junctional complex" is present, consisting of a tight junction (tj), zonula adhaerens (za) and a desmosome (d). Below the complex, the lateral intercellular space (lis) becomes quite broad. Magnification, 100000 ×.

Fig. 4. Current passage, 4 min. Resistance was dropped from 1510 to 490 $\Omega \cdot cm^2$ by passing 460 $\mu A/cm^2$ for 4 min. The continuity of the tight junction is interrupted by a series of circular blisters (b) about 90 nm in diameter. Their appearance is somewhat blurred because their dimensions are of the same order as the thickness of the section. These blisters are identical in appearance to the smaller of those produced by osmotic gradients. Magnification 100000 ×.

(Fig. 7), we did not observe any signs of even incipient blistering in any junction.

The lateral intercellular spaces of the epithelium were also examined in these experiments. Previous studies [6] had shown that variations in the widths of these spaces

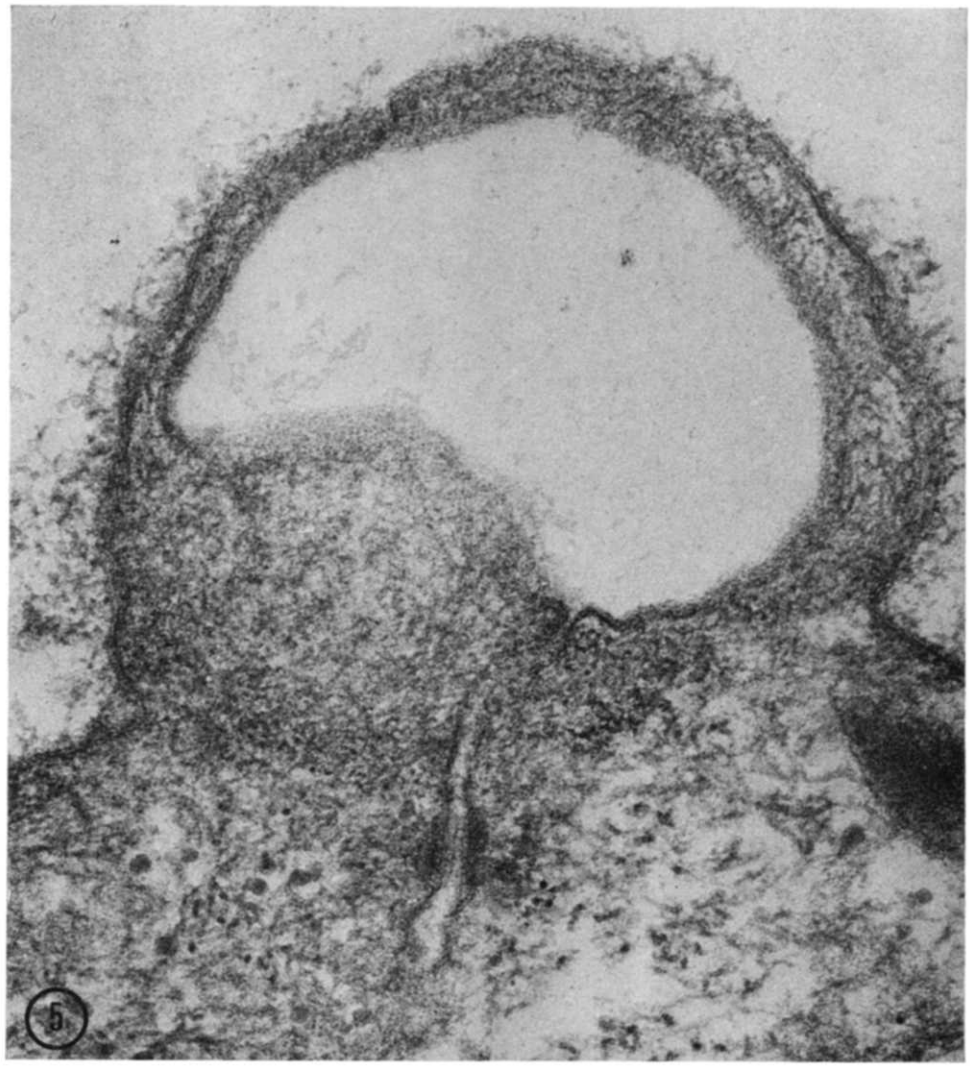

Fig. 5. Hypertonic mucosa. Resistance was dropped from 2010 $\Omega \cdot cm^2$ to 920 $\Omega \cdot cm^2$ 20 min after 300 mM mannitol was added to the mucosal bathing solution. The resulting blister within the tight junction is 840 nm in maximum diameter, and is typical of the larger junctional disruptions produced only by osmotic gradients. Magnification, 100000 ×.

could explain osmotically induced conductance changes in the rabbit gall bladder. However, because of the high resistance of toad bladder epithelium, the width of the spaces in this tissue are too large to explain even a small fraction of the observed conductance changes.

Osmotic and current induced changes in non-electrolyte permeability

In an attempt to characterize further these conductance changes in the toad urinary bladder, we used two non-electrolytes to probe for changes in membrane

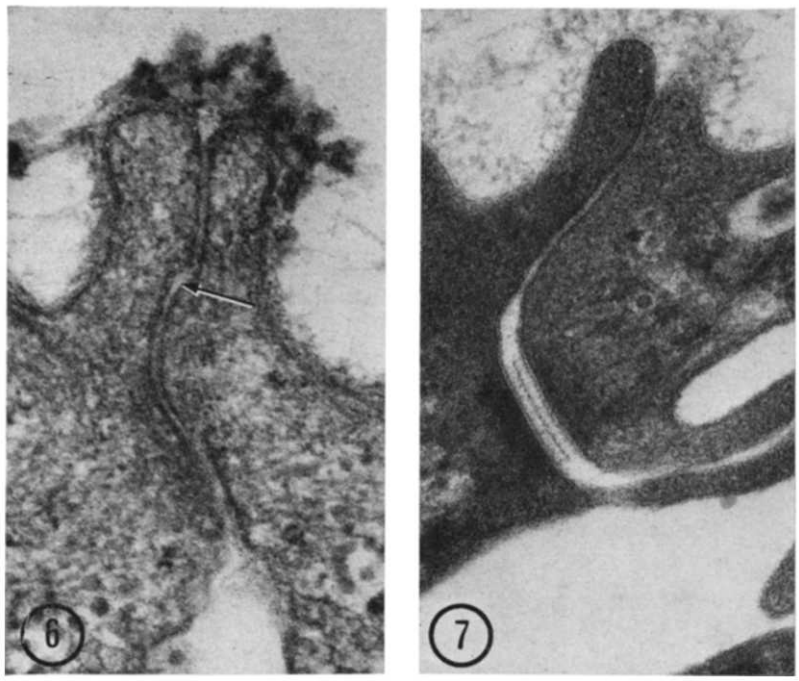

Fig. 6. Current passage, 20 s. Fixation was initiated after 20 s, by which time 390 $\mu A/cm^2$ of current had lowered the resistance from 2150 $\Omega \cdot cm^2$ to a steady 430 $\Omega \cdot cm^2$. No blisters are present, although the arrow suggests an area where one might be beginning to form. Magnification 100000 ×.

Fig. 7. Hypertonic serosa. Resistance was dropped from 4040 $\Omega \cdot cm^2$ to 400 $\Omega \cdot cm^2$ after addition of 500 mM mannitol to the serosal bathing solution. Fixed after 75 min. The density of the cytoplasm is the result of cell shrinkage. The junction appears perfectly intact. Its narrower appearance is an illusion due to the fact that the dense lines of the unit cell membranes have about the same opacity as the surrounding cytoplasm. Magnification, 100000 ×.

structure. Mannitol, (oil–water partition coefficient $1 \cdot 10^{-6}$) was chosen as an example of a solute with a low permeability that we expect to permeate across the tissue via a polar route, and 1,7-heptanediol (oil–water partition coefficient $3 \cdot 10^{-2}$) was chosen as a solute with a high permeability that we expect to permeate via a lipid route (Pietras, R. J., Bindslev, N. and Wright. E. M., unpublished). The protocol in these experiments was first to measure the fluxes of these solutes in the unmodified bladders for 2–3 h, and then to apply either osmotic or electrical gradients to produce substantial increases in conductance. In the case of the electrical experiments current pulses lasting 14–20 s were passed at 1 min intervals for 1.5 h. (This procedure was to avoid a further slow decline in the tissue resistance.) At least two flux experiments were carried out for each compound under each condition.

The results of these experiments are summarized in Table I, where it can be seen that both the mannitol and 1,7-heptanediol permeabilities increased whenever the conductance was increased by, (a) exposing the mucosal surface to hypertonic solutions, (b) exposing the serosal surface to hypertonic solutions, and (c) passing current

TABLE I

OSMOTIC AND CURRENT INDUCED PERMEABILITY CHANGES

Results of six individual experiments in which the conductance of the bladder was modified by either osmotic gradients or current. In the osmotic experiments sucrose (500 mM) was added to the serosal solution (serosa hypertonic) or urea (300 mM) was added to the mucosal solution (mucosa hypertonic). The initial values refer to the conductance and permeability of the bladder prior to placing a gradient across it. Values cited are means obtained from multiple determinations in each experiment. Standard errors for each value are less than 10%. Additional experiments for each condition gave similar results.

	Conductance ($m\Omega^{-1} \cdot cm^{-2}$)		Permeability (10^{-7} cm/s)	
	Initial	Final	Initial	Final
Mannitol fluxes				
(i) mucosa hypertonic	0.5	3.8	2	23
(ii) serosa hypertonic	0.5	4.3	2	26
(iii) applied current	0.8	3.1	6	22
Heptanediol fluxes				
(i) mucosa hypertonic	0.5	3.8	260	490
(ii) serosa hypertonic	0.5	1.8	170	320
(iii) applied current	0.3	1.9	101	206

across the tissue. (Urakabe, et al. [1] also found that the addition of 240 mM urea to the mucosal fluid increased the permeability of the toad urinary bladder to sucrose and tritiated water, but no such increase was observed when they added this concentration to the serosal fluid.) The mannitol permeability increased in approximately the same proportion as the conductance, but the increase in the 1,7-heptanediol permeability was proportionately much less. It should be noted however, that the absolute increase in the heptanediol permeability was much greater than the increase in mannitol permeability for a given change in the conductance. Finally, it is also apparent from this table that hypertonic solutions on the serosal face of the tissue produce very similar changes in permeability to those produced by hypertonic mucosal solutions.

DISCUSSION

Structural evidence for site of permeability change

These experiments demonstrate that the permeability and ultrastucture of the toad urinary bladder can be modified by the passage of direct current across the tissue. Currents greater than 20 $\mu A/cm^2$ increased the electrical conductance, non-electrolyte permeability, and, at least after passing the current for a few min, these effects were accompanied by blistering of the tight junctions. Similar effects were observed when the mucosal solution was made hypertonic by the addition of non-electrolytes. This latter observation has led others to the conclusion that blistering of the junctions is the cause of the conductance increase [2–4]. Our results are inconsistent with this hypothesis for two reasons: (i) Addition of sucrose (500 mM) to the serosal solution causes increases in conductance and permeability indistinguishable from those produced by other treatments, yet does not produce blisters in the junctions. (ii) There is no temporal correlation between the increase in conductance and the appearance of blisters when current is passed across the tissue.

An increase in the leakiness of the tight junctions might nevertheless go a long way towards explaining both the current and osmotic effects. There is evidence that changes in the penetrability of tight junctions need not be accompanied by obvious changes in their structure. First, in the frog skin, where hypertonic urea solutions also increase the conductance and permeability [10], ionic lanthanum gains access to normal looking junctions when the skin is exposed to hypertonic solutions [11]. Secondly, a preliminary report [12] on the toad bladder also shows that ionic lanthanum, but not colloidal lanthanum or ruthenium red, gains access into the tight junctions upon addition of urea to the mucosal solution. These authors also conclude that there is no unequivocal relationship between the appearance of blisters within the junction and the penetration of ionic lanthanum. Furthermore Wade et al. [4] have found $BaSO_4$ precipitates within junctions where no blisters are apparent. Though such observations demonstrate alterations in the submicroscopic structure of junctions, they do not necessarily prove that these altered junctions are the principal pathway for the increased permeation of other substances.

Physiological evidence for site of permeability change

The fact that both current and osmotically induced conductance changes are associated with proportional increases in mannitol permeability shows that both procedures open up a non-specific polar pathway across the epithelium. This pathway could well be through the tight junctions, although a route through altered plasma membranes cannot be completely ruled out.

The opening up of a polar pathway is probably not the sole explanation for the permeability changes we have observed. As noted above, the absolute increase in 1,7-heptanediol permeability is much greater than with mannitol. If the effects on conductance are due simply to the opening of the tight junctions or some other free solution shunt pathway, we would expect similar absolute increases in permeability for the two molecules, owing to the fact that in size, shape and diffusion coefficients they are so alike. The absolute increase in 1,7-heptanediol permeability is almost an order of magnitude greater than that with mannitol. 1,7-heptanediol is highly lipid soluble and thus is thought to cross the epithelium via a lipid pathway. This suggests that both electrical and osmotic gradients alter the composition and/or organization of membrane lipids. Although it is reasonable to suppose that this effect is on the cell plasma membranes, the possibility cannot be excluded that lipoid elements in the tight junction are involved.

It should be noted that Mandel and Curran [13] observed that voltage clamping increased the permeability of the frog skin. They found that when the skin was depolarized to -100 mV (inside negative) the permeability to urea, mannitol, Cl^-, K^+ and Na^+ increased markedly. It is possible that this effect is related to those reported here, particularly since we also have observed voltage dependent conductance changes in frog skin.

Mechanism of permeability change

The close similarity between the changes produced by electrical and osmotic gradients at once suggests that these effects have a common origin. Namely, both gradients produce water flows which might disturb the submicroscopic structure of the tight junctions and/or the cell membranes. In fact, the formation of blisters within

the tight junctions lends strong support to the view that water is flowing through the junctions to produce pockets of elevated hydrostatic pressure.

Electrical currents can in principle produce water flows by at least two mechanisms. One is the classical electrokinetic phenomenon of electroosmosis. The other is the so called transport number effect (see Barry and Hope [14,15]).

Osmotic gradients indubitably produce water flows in this tissue, but unfortunately there is no simple correlation between the ability of solutes to generate water flows and their ability to elicit conductance changes. For example, urea is more effective than mannitol in lowering the resistance of the bladder, and yet urea would be expected to produce smaller water flows because it is more permeable than mannitol [16]. DiBona and Civan [3] and Wade et al. [4] have suggested that a critical factor might be the size of the solute molecules and their ability to penetrate the tight junctions. However, inspection of the data of Urakabe et al. [1] shows that the ability of solutes to increase permeability is not in the sequence of either their permeability coefficients or their size.

Clear evidence to support the hypothesis that water flow causes the permeability changes is thus lacking. However, this is not surprising in view of the anatomical complexity of the bladder wall where there are at least two parallel pathways across the epithelium, one through the cells, and one through the tight junctions. Furthermore, the formation of blisters in the tight junction suggests that this is a complex structure consisting of several regions with different permeability and mechanical properties. In the absence of detailed knowledge of the relative significance of the two pathways and of the properties of each of their subcomponents, it is hazardous to predict the precise relationship between permeability changes and net water flow, since there may be little relationship between overall properties and the properties of any one subcomponent. Thus, permeabilities and reflection coefficients have little bearing on the response of a small region, such as part of the tight junction.

Other mechanisms must also be considered. For instance, the electrical gradients employed might cause a reversible dielectric breakdown of the cell membranes and/or the tight junctions; such an effect, "punch through", has been described in *Chara* cells [17, 18]. The Wein effects [19] might also contribute to the effects of current on conductance, but we believe this is unlikely to explain the concomitant permeability changes.

In conclusion, we have demonstrated that both electrical and osmotic gradients increase the permeability and conductance of the toad bladder. Contrary to the opinion of others [2–4], such changes are probably not due to blisters in the tight junctions. Although the origin of these effects is not clear, both types of gradient appear to increase the flux of solutes through both polar and non-polar pathways. These pathways are likely to involve changes in both the tight junctions and the plasma membranes of the epithelium. Stresses caused by water flow are the most likely common explanation. Further elucidation of these phenomena will require a fuller knowledge of the properties of the various alternate pathways across the epithelium.

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