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Clarification of the Intercellular Space Phenomenon in Toad Urinary Bladder

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Summary. Vasopressin has been noted to increase the size of the intercellular spaces of toad bladder epithelium even in the absence of an osmotic gradient. The present studies demonstrate that the same phenomenon may be obtained in the presence or absence of a transepithelial gradient of glutaraldehyde indicating that the effect is not a fixation artifact. Morphologic evidence is presented demonstrating continuity between the epithelium and underlying smooth muscle. The data support the concept that not only net water flow, but also changes in smooth muscle tone can appreciably affect the epithelial geometry.

Net fluid transport across rabbit gallbladder [12, 15] and renal tubules [9] has been correlated with the extent of opening of the epithelial intercellular spaces. Similarly, vasopressin increases both osmotic water flow and the size of the intercellular spaces of toad bladder [1, 2, 8, 14], suggesting that the spaces increase as a direct consequence of the increased water flow [14]. This concept has been brought into question by recent observations demonstrating that vasopressin can increase the intercellular spaces in the absence of net fluid transport [5]. Since vasopressin is an effective relaxant of the smooth muscle of toad bladder, the effect of various smooth muscle relaxants and contractants was investigated. The results indicated that the enlargement of the intercellular spaces could be correlated with changes in smooth muscle tone of the bladder, even in the absence of changes in transepithelial fluid flow [6]. It was therefore concluded that while transepithelial water flow is an established cause of enlarged intercellular spaces, enlargement of the spaces may also arise from extraepithelial effects in epithelia containing a muscular submucosa.

This conclusion is subject to two substantial criticisms. First, from a technical point of view, tissues were fixed by addition of glutaraldehyde

to the serosal side of the tissue only. To the extent that the reflection coefficient for glutaraldehyde is significantly greater than zero, an osmotic gradient would be thereby introduced across the bladder. Then, addition of glutaraldehyde to the vasopressin-treated tissue might facilitate net water movement, increasing the intercellular spaces [10]. Second, from a conceptual viewpoint, it is difficult to appreciate how changes in the contractile state of the submucosal smooth muscle might significantly alter the geometry of the epithelium. The present report is addressed to these criticisms and reviews the established data.

Materials and Methods

Toads (*Bufo marinus*) were obtained both from National Reagents, Bridgeport, Connecticut, and the Pet Farm, Miami, Florida. Hemibladders excised from doubly-pithed animals were mounted in lucite double chambers, permitting examination of adjoining experimental and control quarter-bladders. Tissues were bathed with isotonic Ringer's solution on both mucosal and serosal surfaces (Na^+, 113; K^+, 3.5; Ca^{++}, 0.9; Cl^-, 116; HCO_3^-, 2.4 mM; pH, 7.5 to 8.0, and tonicity, 220 mOsm/kg H_2O).

The electrical potential was maintained at zero except for 9-sec intervals, each 30 sec, during which the potential was alternately changed to (+)10 and (−)10 mV; continuous recording of the current passed across the tissue thus provided a record both of short-circuit current and transepithelial electrical resistance [3].

After a baseline control period, vasopressin (Pitressin, Parke, Davis and Co., Detroit, Michigan) was added to a final serosal concentration of 200 mU/ml.

Fixation was performed 15 min after hormonal administration by addition of 50% glutaraldehyde to a final total concentration throughout the mucosal and serosal media of 1%. The glutaraldehyde was added either to the serosal medium alone, as in previous protocols [5, 6], or simultaneously to mucosal and serosal media to achieve the same final concentration of fixative.

Other hemibladders were simply immersed in beakers in the above Ringer's solution for a period of 3 hr with vigorous stirring and aeration of the bath to guarantee adequate oxygenation and to prevent the establishment of transient osmotic gradients. Some of these were incubated as above but loosely stretched by being pinned to an open wax frame. Fixation was performed by addition of glutaraldehyde to a final concentration of 1% and stirring was continued for 90 min at which time bladders were removed. Subsequent preparation of the tissue for phase and electron-microscopy did not differ from previous protocols [7].

Results

To determine the osmotic significance of the addition of glutaraldehyde to the serosal medium, a series of three experiments was performed, where vasopressin was added to the serosal medium of the experimental half, and glutaraldehyde to the mucosal and serosal media of both halves of the preparation, in similar concentrations. The phase micrographs obtained from these tissues were analyzed by the statistical technique previously

Table 1. Effect of vasopressin in the absence of a glutaraldehyde concentration gradient [a]

Experiment	Number open	Number closed	% Change [b]
407 A	922	347	64.06
B	288	916	
414 A	904	279	70.65
B	217	886	
518 A	897	261	70.75
B	304	1022	
			68 ± 2.2 % [c]

[a] Vasopressin was added to the serosal medium of each experimental side "A"; glutaraldehyde was subsequently added to the mucosal and serosal media of "A" and "B", in similar concentrations.

[b] $\% \text{ Change} \equiv \frac{(\%\text{ Open})_A - (\%\text{ Open})_B}{(\%\text{ Closed})_B}$ (*cf.* Ref. [6]).

[c] Mean ± SEM.

used [6]. A population of approximately 1,000 cells from each side was examined. Cells were considered in the "open" configuration if intercellular spaces were visible on both sides, or if a space was present between the lateral-basal margin and the underlying basement membrane observed in the tissue section. At an instrumental magnification of 2,000 X, the intercellular spaces of the experimental half, treated with vasopressin, were markedly greater than the spaces of the control half of the preparation (Table 1). The average increase in fractional cell population in the open configuration, divided by the control fractional cell population in the closed configuration, was 68 ± 2.2 (SEM) %. Thus, vasopressin increased the intercellular spaces, even in the absence of transepithelial gradients of salt and glutaraldehyde, a conclusion valid at the 0.005 probability level.

To further investigate the problem, a second series of three experiments was performed where vasopressin was added to the serosal media of both experimental and control quarter-bladders. After 15 min, glutaraldehyde was added to the serosal bath of the control, and to both serosal and mucosal baths of the experimental side. Examination of these tissues by phase microscopy using the same technique indicated that the simultaneous addition of glutaraldehyde to mucosal and serosal media did not affect the enlargement of the intercellular spaces induced by vasopressin (−1 ± 2.5 %) (Table 2).

It was of interest to study the tissue morphology under conditions where "free shortening" of the bladder wall through smooth muscular contraction was permitted, contrary to the conditions of the chamber experiments. Hemibladders immersed for prolonged periods of time in

Table 2. Addition of vasopressin to serosal media of both "A" and "B" with subsequent addition of glutaraldehyde to serosal bath of "B" and to serosal and mucosal baths of "A"

Experiment	Number open	Number closed	% Change
444 A	698	394	−4.6
B	743	391	
446 A	905	430	−2.2
B	810	372	
468 A	686	321	+3.9
B	772	383	
			−1 ±2.5% [a]

[a] Mean ± SEM.

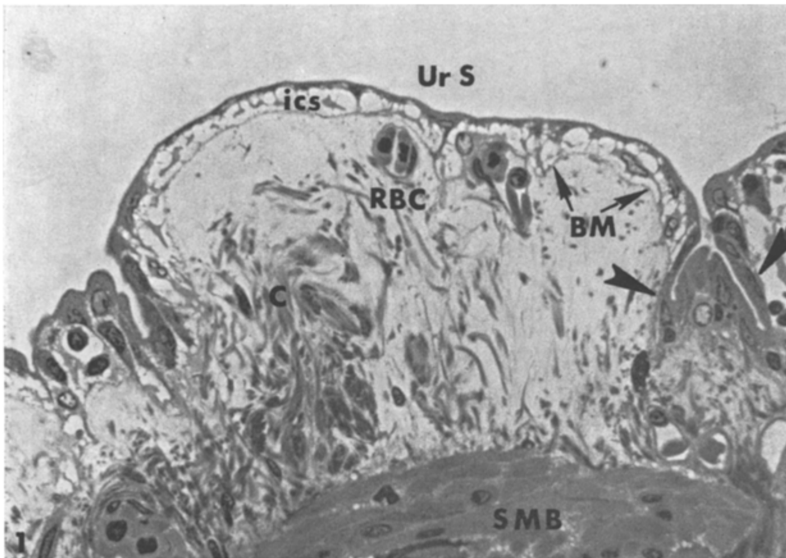

Fig. 1. Phase micrograph of loosely mounted hemibladder. The intercellular spaces (ICS) of the epithelium are markedly open except in the invaginated region at the right (between heavy arrows). The crest region (center) is noticeably poor in submucosal elements; a similar appearance was noted previously following addition of muscle relaxants or following loose mounting of the preparations (*cf.* [6]). This illustration also serves to demonstrate the phase microscopic appearance of quarter-bladders treated with vasopressin, regardless of the fixation method. Smooth muscle bundle, SMB; red blood cells, RBC; basement membrane, BM; collagen bundles, C; urinary surface, UrS. ×250

beakers of well-aerated Ringer's solution characteristically contract. The tissue was loosely stretched across a wax frame to simplify orientation and prevent markedly oblique sectioning. Under these experimental conditions, it is easily appreciated (Fig. 1) that the musculature tends to pull the tissue

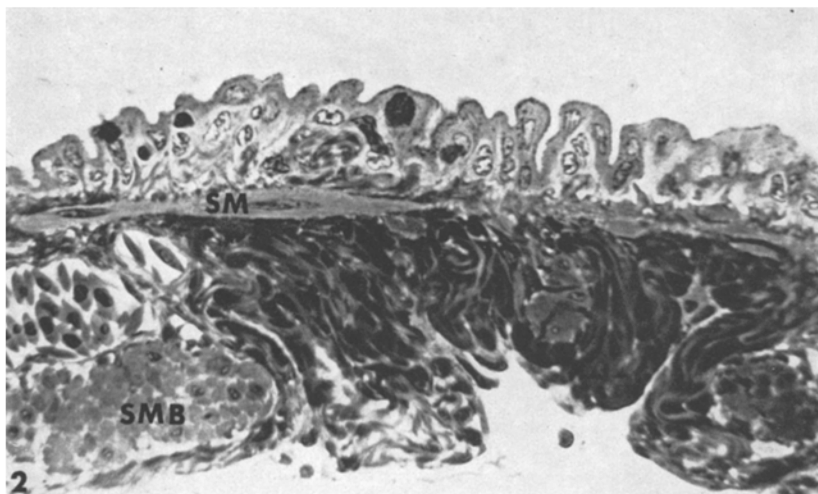

Fig. 2. Phase micrograph of tightly stretched quarter-bladder without vasopressin treatment. Note that intercellular spaces cannot be discerned and that smooth muscle cells (SM), in characteristic spindle shape, lie close to the basal surface of the epithelium. Large bundles of muscles are not characteristically seen in such a position but rather, as the one in this section (SMB), at some distance from the epithelium. $\times 250$

into a discrete pattern of folds. At the crests of these folds are cells surrounded by enlarged spaces, while the valleys of these folds seem more heavily invested with basal cells and with surface cells in a much more closed configuration.

This finding suggests that the action of the smooth muscle on the epithelial geometry is not completely diffuse, but rather that there may be specific points of attachment between the epithelium and the musculature. Sections from twelve samples were examined by electron-microscopy in an attempt to verify this. In eleven of the twelve samples examples have been found where, as in Fig. 2, the basement membrane of the epithelium and the membrane of a smooth muscle cell come into close proximity.

In the eleven instances thus far noted, the smooth muscle cell in question approached to within 150 Å of the basement membrane (as in Fig. 3), but in no case was it in apparent association with a smooth muscle bundle; such bundles are characteristically found several microns deeper in the submucosa.

The occasional focal densities of the smooth muscle plasma membrane have been previously noted, and it was suggested that they function as intercellular connections within a bundle of smooth muscle cells [4]. In this case, they are reminiscent of the hemidesmosomes marking the basal margin of the basal cells. However, the local densities of the basement membrane

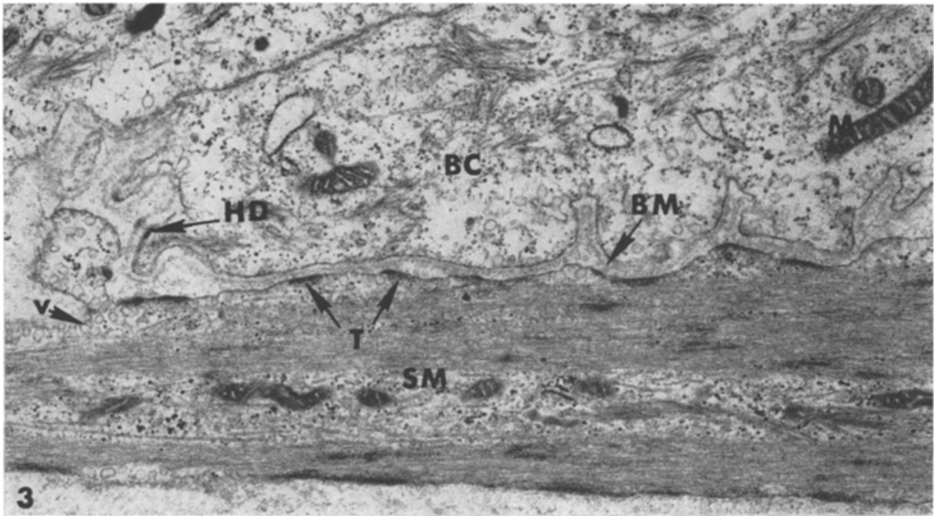

Fig. 3. Electron micrograph of an untreated quarter-bladder. In this view, the basal surface of a basal cell (BC) is shown to follow the contours of the basement membrane. Hemidesmosomes (HD) on the basal cell membrane are occasionally apposed by a focal density on the basement membrane. Thickenings (T) of the smooth muscle (SM) cell membrane are not apposed by such densities but are at least as close (ca. 150 Å) to the basement membrane as the basal cell hemidesmosomes. Vesicles, v; mitochondria, M. $\times$ 15,000

often seen in apposition to the basal cell hemidesmosomes were not found in apposition to the thickenings of the muscle membranes observed in the present study.

Discussion

Vasopressin has been noted to increase the size of the intercellular spaces in the absence of an osmotic gradient across toad bladder [5, 6], when fixation is performed by addition of glutaraldehyde to the serosal medium alone. Since a similar compound, formaldehyde, crosses toad bladder rapidly [13], it seemed likely that addition of glutaraldehyde to the serosal bath would provide a transient insubstantial osmotic gradient. However, Grantham, Cuppage and Fanestil [10] have recently observed a small net water flow under these conditions, and have suggested that the increased flow *per se* is responsible for the reported dilation of intercellular spaces. Furthermore, using light microscopy of unfixed tissue, they were unable to observe dilation of the spaces unless an osmotic gradient was present.

The current experiments, however, confirm and extend the initial observation [5, 6], since: (a) vasopressin increased the size of the intercellular

spaces in the absence of transepithelial gradients of glutaraldehyde and salt, and (b) the size of the intercellular spaces was increased to a similar extent in the presence and absence of a transepithelial gradient of glutaraldehyde. The data have thus confirmed that vasopressin can increase the size of the intercellular spaces of toad bladder by a mechanism independent of net transepithelial water flow. The failure of Grantham *et al.* to observe such an increase suggests that their technique provided insufficient resolution; in fact, their photomicrographs demonstrated cellular outlines only under limited conditions – in the presence of vasopressin and a dilute mucosal medium.

The data of the present study must be considered in the context of the previous observations [6] that: (a) the morphologic and transport effects of vasopressin may be dissociated by using very low concentrations of hormones, (b) under certain conditions, the intercellular spaces may be reduced despite a concurrent increase in net water flow, and (c) smooth muscle contractants may decrease, and relaxants may increase, the intercellular spaces without observable changes in net transepithelial flow. The conclusion seems inescapable, therefore, that at least part of the vasopressin-induced increase in intercellular spaces must be mediated by a relaxation of the submucosal smooth muscle.

If changes in smooth muscle tone can indeed significantly affect epithelial geometry, stretching of the tissue or an increase of the mucosal hydrostatic pressure should decrease the intercellular spaces, a phenomenon which has, in fact, been observed [6]. In this regard, the results of Carasso *et al.* [1] which, at first appear contradictory, are, in fact, supportive. They observed that the intercellular spaces in frog urinary bladder were open only when both an osmotic gradient and antidiuretic hormone were present. Their experimental situation, involved a horizontal mounting of the preparation and the application of a mucosal hydrostatic pressure of 20 cm of water, which circumstances should lead to space closure according to the present theory. Indeed, initial fixation in the experiments of Carasso, Favard, Bourguet, and Jard [1] was from the serosal side only, documenting further that establishment of a gradient in this fashion in the presence of antidiuretic hormone is inconsequential with regard to intercellular space geometry.

The current study further supports this concept, that smooth muscle activity can alter the size of the intercellular spaces, by providing a morphologic basis for the continuity between the epithelium and the underlying contractile elements.

It is, then, well established for amphibian urinary bladder that, in the presence of an osmotic gradient, antidiuretic hormones will permit water flow from mucosa to serosa, and that this flow of water enlarges the epithelial intercellular spaces. However, any attempt to correlate the degree of space enlargment with osmotic water flow must take into account the consequences of the experimental procedure. The degree of stretch in mounting the preparation, the presence of a hydrostatic pressure differential, and the tone of the submucosal smooth muscle all influence the intercellular space geometry and must be rigorously controlled if investigation in this field is to be fruitful.

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