

THE EFFECT OF OSMOTIC FLOW ON THE DISTRIBUTION OF HORSERADISH PEROXIDASE WITHIN THE INTERCELLULAR SPACES OF TOAD BLADDER EPITHELIUM

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INTRODUCTION

Numerous studies have suggested that the lateral intercellular spaces can serve as part of the pathway for water movement across epithelia (2, 3, 5, 6, 11, 14, 15, 20, 22, 23, 24). The primary evidence for this view has been the frequent observation in several tissues that increases in net fluid transport is accompanied by marked widening of intercellular spaces. However, recent work has demonstrated that widened intercellular spaces can occur without net water transfer, possibly due to contractile activity of smooth muscle (8, 9). It has also been observed that the degree of stretch imposed on tissue can markedly affect the width of intercellular spaces (9). Thus, it would be useful to follow water movement through intercellular spaces by a technique other than size changes.

Horseradish peroxidase has been used extensively as a tracer to assess the permeability of cell junctions (4, 7, 12, 16). Although the zonula occludens which occurs at the luminal border of the toad bladder epithelium is impermeable to peroxidase (19), the intercellular space is easily penetrated by peroxidase from the basal (serosal) side of the epithelium (19). This observation was utilized in the present studies to detect the flow of water through the intercellular spaces by correlating movement of the tracer with osmotic flow.

MATERIALS AND METHODS

Female toads, *Bufo marinus*, (National Reagents, Inc., Bridgeport, Conn.) were pithed and the bladders were mounted as sacs tied to plastic tubes with the mucosal surface inside. The bladders were placed in

an aerated Ringer's solution of 111.4 mM NaCl, 3.5 mM KCl, 2.4 mM Na HCO₃, 0.88 mM CaCl₂, with a pH of 7.6-8.2 and tonicity of 220 milliosmols/kg H₂O. As previously noted (9) the ultrastructure of the toad bladder epithelium and the intercellular spaces can be dramatically altered by stretch. A relatively constant preparation was achieved by having the mucosal solution inside the tube rise about a centimeter above the level of the serosal bathing medium.

After a basic equilibration period of 20 min with Ringer's solution on both sides of the bladder, all bladders (except peroxidase controls) had an additional 30 min equilibration period with the same Ringer's containing 1.5 mg/ml horseradish peroxidase (Worthington Biochemical Corp., Freehold, N. J.), RZ = 1.2, and 1.5 mg/ml bovine albumin fraction V (Fisher Scientific Company, Pittsburgh, Pa.) in the serosal medium. Although the results obtained were essentially the same in the absence of albumin, we found, as have others (12), that the albumin is useful in fixing the peroxidase. Preliminary experiments also demonstrated that it made no difference if peroxidase was present in the mucosal solution as well as the serosal medium during the equilibration period. In experiments with vasopressin (Pitressin, Parke, Davis & Co., Detroit, Mich.), this compound was present during this 30 min equilibration period at a concentration of 200 mU/ml. After the incubation period the mucosal solution was removed and replaced with either full strength Ringer's solution or 1/5 Ringer's solution for periods of 1, 4, or 10 min. The serosal medium remained the same and thus the bladder was constantly exposed to peroxidase on its serosal surface. Tissue was fixed in 2.5% glutaraldehyde (Polysciences, Inc., Warrington, Pa.) in 0.09 M cacodylate buffer at pH 7.4 for 1-3 hr. Freezing and sectioning was unnecessary since the bladders are so thin. Although, in some of the experiments reported in this paper, the staining reaction was carried out on the following day as recommended by Graham and Karnovsky (13), the amount of reaction product was found to be greater if the incubation is carried out immediately after glutaraldehyde fixation for 1 hr and brief washing. All cases reported as lacking peroxidase were treated in this fashion. The incubation medium contained 5 mg of 3,3',4,4'-biphenyltetramine tetrahydrochloride (Matheson, Coleman, and Bell, Cincinnati, Ohio) in 10 ml of 0.05 M Tris buffer, pH 7.6, and 0.1 ml of 1% H₂O₂. Tissue was incubated for 15 min at room temperature, washed, postfixed for 1 hr in 1.3% osmium tetroxide buffered with *s*-collidine, dehydrated in alcohol, and flat-embedded in Epon. Sections were cut perpendicular to the surface of the bladder on an LKB Ultratome with a diamond knife, picked up on uncoated 75 × 300 mesh copper grids, stained with uranyl acetate and lead citrate, and examined in an RCA EMU-4B electron microscope.

This report is based on the examination of 47 sac preparations from 12 toads. Each treatment discussed was performed a minimum of five times.

RESULTS AND DISCUSSION

After an exposure of 30 min to horseradish peroxidase in the serosal medium, the tracer was consistently localized within the intercellular spaces of the epithelium. If such a preparation was subsequently exposed to an osmotic gradient by substituting diluted (1:5) Ringer's solution for the mucosal medium without treatment with vasopressin, there was no change in the position of the peroxidase (Fig. 1); nor was there any substantial change if the bladder was treated with vasopressin but not exposed to an osmotic gradient (Fig. 2). However, if the epithelium was both treated with vasopressin and exposed to an osmotic gradient for 4 min or longer, the tracer was apparently dislodged from the intercellular spaces (Fig. 3). Evidence which suggested that peroxidase had previously been in contact with the epithelium could still be found. For example, tracer was observed in vacuoles at the basal and lateral surfaces (Fig. 3, *V*), and occasionally there was staining of desmosomes and basement membrane. In four out of the five bladders examined after they were exposed to an osmotic gradient for only 1 min, there was a significant amount of peroxidase remaining within the intercellular spaces (Fig. 4). Although there was considerable variability in these intermediate cases, their intercellular spaces appeared to have less peroxidase activity than those of bladders not exposed to an osmotic gradient. In several cases these bladders exposed to an osmotic gradient for only 1 min were found to have some intercellular spaces containing tracer and others virtually empty.

The bladders exposed to both vasopressin and an osmotic gradient tended to have wider intercellular spaces than those exposed to the alternative treatments. Thus, the possibility that the absence of peroxidase might be secondary to a morphological change and subsequent leaching out of the tracer during processing had to be eliminated. When tissue exposed to vasopressin and an osmotic gradient but no peroxidase was fixed for 30 min in a glutaraldehyde solution containing horseradish peroxidase and then processed normally, the wide intercellular spaces were clearly packed with tracer (Fig. 5). This demonstrates that the basic result could not be easily

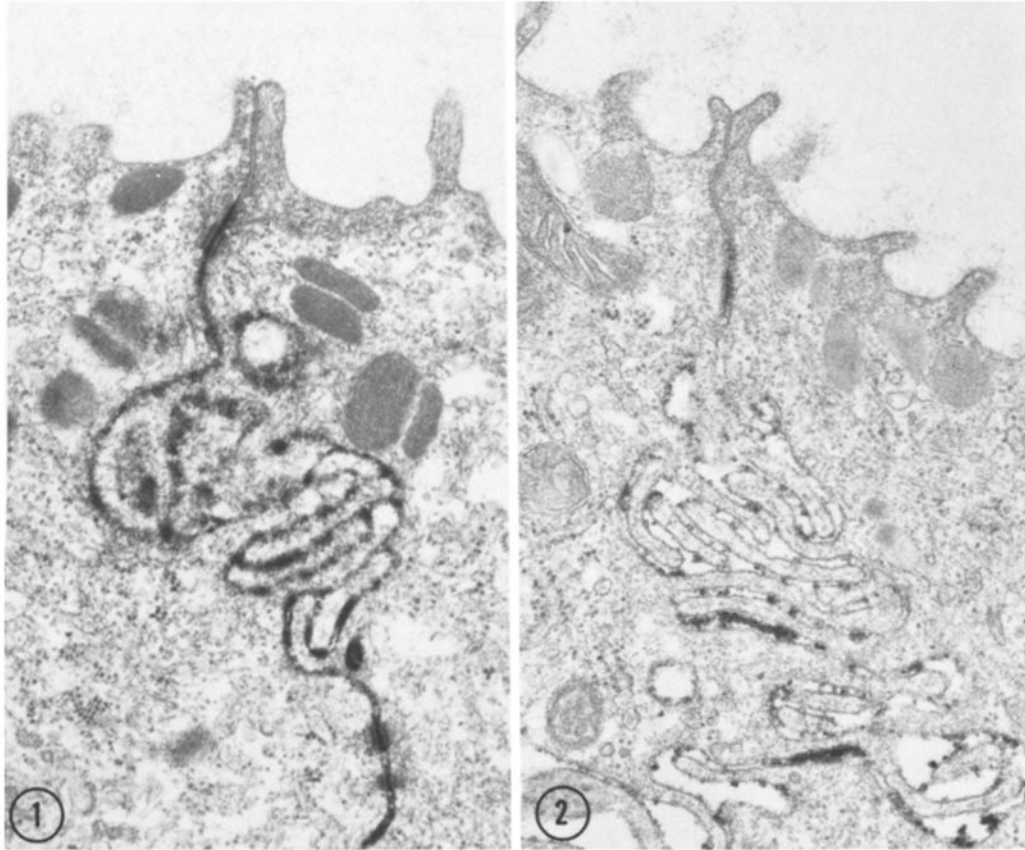


FIGURE 1 Region of apposition between two epithelial cells of toad bladder. Incubated with horseradish peroxidase on serosal surface for 30 min and then exposed to an osmotic gradient by substitution of 1:5 diluted Ringer's solution for the mucosal solution. No vasopressin present. Considerable peroxidase activity is localized within the intercellular space but stops at the level of the zonula occludens. $\times 29,000$.

FIGURE 2 Region similar to Fig. 1. Incubated with peroxidase on serosal surface in an isotonic medium containing vasopressin (200 mU/ml). In the absence of an osmotic gradient, peroxidase activity is still present in the intercellular space although perhaps reduced from that demonstrated in Fig. 1. $\times 29,000$.

attributed to washout of the tracer during processing. Peroxidase was not well retained in the extracellular spaces of the lamina propria even when peroxidase was added to the fixative. This parallels the observations made by others (7). It would seem, therefore, that at least a minimal barrier to diffusion and/or binding to cell membranes is necessary for the localization of peroxidase. It is not possible at the present to exclude the possibility that changes in these parameters affected the results. This, however, appears unlikely to be a critical factor in interpretation since bladders

exposed to an osmotic gradient for only 1 min (Fig. 4) demonstrate that significant peroxidase can be localized within widely dilated intercellular spaces and that the tracer is not necessarily bound to membranes.

Significant osmotic flow of water across the toad bladder occurs only when there is both an osmotic gradient and vasopressin present (1). Such water movement is thought to occur as bulk water flow through aqueous channels (17). This flow has been shown to affect the movement of solutes such as urea by solvent drag (18). The

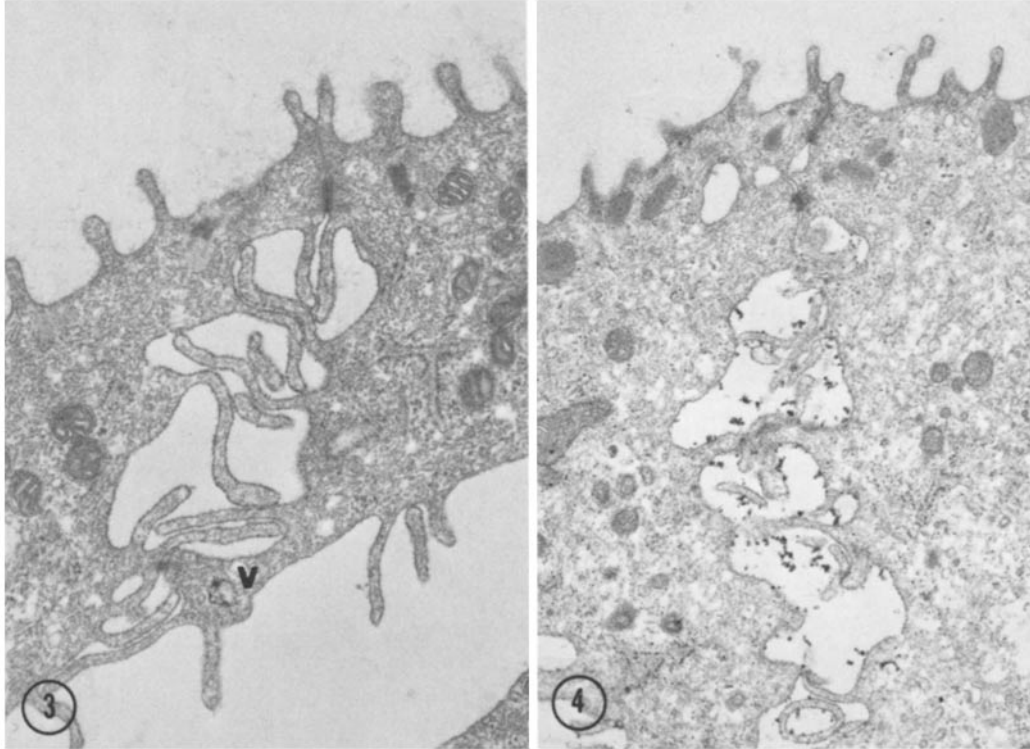


FIGURE 3 Epithelium incubated with peroxidase as in Figs. 1 and 2, but subsequently exposed to both vasopressin and an osmotic gradient (1:5) for 4 min. Peroxidase activity is almost completely absent from the intercellular space. Tracer is still found within some vacuoles (*V*). $\times 23,000$.

FIGURE 4 Tissue treated the same as that in Fig. 3, but exposed to an osmotic gradient for only 1 min. A significant amount of peroxidase remains within the intercellular space. $\times 14,000$.

present results would suggest that larger molecules such as proteins are also subject to solvent drag and that at least some bulk water flow occurs within the intercellular spaces of the toad bladder.

The present technique leaves unresolved the question of how water initially enters the intercellular space. It is not known if water must pass first into the cell across the apical cell membrane or if it is possible to bypass the cytoplasm completely and move directly across the zonula occludens into the intercellular space. Considerable evidence suggests that vasopressin increases the permeability of the apical cell membrane (17), and it has frequently been observed that the epithelial cells tend to swell under conditions of osmotic water flow (10, 21). Thus, since vasopressin has not been observed to affect the zonula occludens, the presently available evidence would

suggest that water probably enters the intercellular space from the cells.

The present technique does not allow an assessment of the fraction of the total water flow which moves through the intercellular spaces. It may be that a relatively small fraction of the total water moves through the intercellular spaces, but the studies presented here suggest that these spaces form a pathway through which at least some movement of water can occur.

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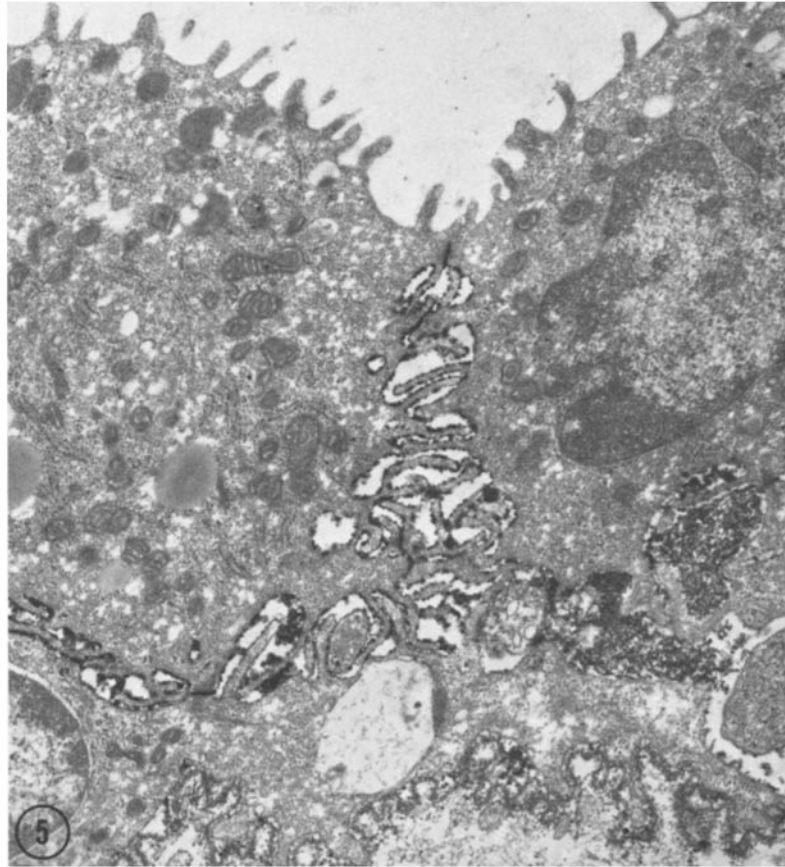


FIGURE 5 Epithelium exposed to an osmotic gradient in the presence of vasopressin for 10 min without peroxidase and then incubated in glutaraldehyde solution containing peroxidase for 30 min. The intercellular space is packed with peroxidase, demonstrating that peroxidase is not removed by the processing procedure. $\times 15,000$.

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