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Effect of Distension on ADH-Induced Osmotic Water Flow in Toad Urinary Bladder

William A. Kachadorian and Sherman D. Levine

Membrane Research Laboratory, Renal Service, U.S. Public Health Service Hospital, Staten Island, New York 10304, and Department of Medicine, Albert Einstein College of Medicine, Bronx, New York 10461

Summary. We recently described a method by which the resistance to water flow of the luminal membrane of ADH-stimulated toad bladder can be quantitatively distinguished from that of barriers lying in series with it. This method requires estimates of both total bladder water permeability (assessed by transbladder osmotic water flow at constant gradient) and luminal membrane water permeability (assessed by quantitation of the frequency of ADH-induced luminal membrane particle aggregates). In the present study we examined the effect of bladder distension on transepithelial osmotic water flow before and during maximal ADH stimulation. Base-line water flow was unaffected by bladder distension, but hormonally stimulated flow increased systematically as bladders became more distended. Distension had no effect on the frequency of ADH-induced intramembranous particle aggregates. By comparing the relationships between aggregate frequency and hormonally induced water permeability in distended and undistended bladders, we found that distension appeared to enhance ADH-stimulated water flow by decreasing the resistance of the series permeability barrier while the apparent water permeability associated with each single luminal membrane aggregate was unaffected. In that bladder distension causes tissue thinning, the series resistance limiting ADH-stimulated water flow appears to be accounted for by deformable barriers within the bladder tissue itself, probably unstirred layers of water.

Key words antidiuretic hormone · toad urinary bladder · tissue distension · osmotic water permeability

Introduction

The isolated toad urinary bladder has been extensively used to study the mechanism of action of antidiuretic hormone (ADH). ADH acts upon the epithelial cells which line the bladder, increasing their luminal membrane permeability to water and small solutes, most notably sodium and urea. It seems clear that ADH-stimulated water movement occurs through pore-like pathways [10, 12, 17, 26] which are selective for water alone [21, 23, 24, 29] and restricted to granular-type cells only [6, 7, 28]. Freeze-fracture electron microscopy reveals that ADH also causes the occurrence of intramembranous particle aggregates in granular cell luminal membrane [3, 19]. These aggregates are derived preformed [5, 14] from membranes of long, cytoplasmic tubular structures [13, 30] which fuse with the luminal membrane consequent to ADH treatment [27, 31]. The hypothesis that aggregates may be or may contain actual sites for ADH-stimulated water flow across the luminal membrane has not yet been directly proved. Nonetheless, there is considerable evidence which indicates that, as a minimum, aggregates are accurate markers of hormonally stimulated luminal membrane water permeability [2, 4, 8, 9, 14–20, 23].

Because of the profound effect of ADH on luminal membrane water permeability, there has been a tendency to underestimate the importance of barriers to transbladder flow other than the luminal membrane. In fact, in our original studies in which 17 fully distended bladders were stimulated with various concentrations of ADH, the relationship between hormonally enhanced osmotic water permeability and luminal membrane aggregates appeared to be linear [19]. We recently re-examined this question in a larger group of fully distended, ADH-stimulated bladders [22]. We found that the relationship between aggregates and osmotic water permeability was generally similar to that which we had observed earlier; however, it became apparent that the linear regression line relating aggregates and induced water permeability consistently overestimated water permeability at both low aggregate frequency (i.e., it did not pass through the origin) and high aggregate frequency, while it tended to underestimate water permeability at intermediate aggregate frequency. A more detailed analysis of these data revealed that the data closely approximated the "saturation curve" relationship expected if aggregate frequency were proportional to luminal membrane water permeability while a (constant) flow barrier in series with the luminal membrane became increasingly limiting for transbladder water movement as luminal permeability approached a maximum.

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Based upon this formulation for barriers in series, we calculated that in fully distended bladders, maximally stimulated with ADH, this series barrier contributes between one-half and two-thirds of the total transbladder resistance to water flow [22]. Our data did not, however, permit us to localize the series barrier within the bladder tissue. The present report includes an approach to this issue, based upon alterations in series barrier resistance that accompany different states of bladder distension.

The toad bladder is an easily distensible organ. Gfeller and Walser have studied toad bladder morphology in relation to different filling volumes [11]. They demonstrated that the bladder's luminal surface area remains virtually constant with changes in filling volume. At maximal filling volume, the luminal membrane is practically a flat sheet with some microvilli. As filling volume decreases, the luminal membrane becomes undulating and microvilli become both more numerous and more prominent. In addition, as bladder filling approaches maximum, epithelial cells and supporting tissues are distended so that the luminal and serosal bathing media are separated by a smaller thickness of tissue.

Walser has previously demonstrated that bladder distension increases transbladder sodium movement [32]. This effect is reversible and appears to involve only the conductance of the active sodium transport pathway [25, 32]. In contrast, transepithelial urea movement both in the absence and presence of ADH has been shown by Lief et al. to be unaffected by bladder distension [25].

In the present investigation we measured the effect of bladder distension on transbladder water movement before and during bladder stimulation with both maximally and submaximally stimulating concentrations of ADH. We found that while the occurrence of aggregates in granular cell luminal membrane was unchanged, "maximal" ADH-stimulated transbladder osmotic water flow was systematically enhanced as bladder distension increased. Comparison of the relationships between aggregate frequency and induced osmotic water permeability in undistended and distended bladders at various concentrations of ADH suggests that distension increased osmotic water flow by decreasing the resistance of the series permeability barrier while the luminal membrane water permeability associated with each aggregate was not appreciably altered. In view of the morphologic effect of bladder filling, which we confirmed, our data are consistent with the view that the series barrier resistance limiting ADH-stimulated water movement in toad bladder is largely, if not totally, accounted for by tissue unstirred layers of water.

Materials and Methods

In the first set of experiments in this study the effect of various degrees of bladder filling on ADH-stimulated osmotic water flow was examined. Paired bladders from 16 female Dominican toads (*Bufo marinus*) were mounted as sacs on the ends of glass tubes, suspended in vigorously aerated Ringer solution (111 mM NaCl, 3.5 mM KCl, 2.5 mM $NaHCO_3$, 1.0 mM $CaCl_2$; 220 mosmol/Kg H_2O), and filled either to total capacity[1] or to a lesser extent with Ringer's diluted 1:5 with distilled water. For all bladders, filling was always sufficient to bathe the entire mucosal surface. In an additional series of experiments, paired bladders from six other toads were prepared in the same manner, except that the mucosal bathing medium of the submaximally distended tissues was stirred by a suspended, rotating magnet.

After a 30-min equilibration period, transbladder open-circuit potential was measured, and if less than 20 mV, the experiment was terminated. Otherwise, water movement was determined gravimetrically [1] first for a 30-min base-line period and then for a 20-min period in the presence of ADH (Pitressin, Parke-Davis, Detroit, Mich.) at a concentration (20 mU/ml serosal solution) sufficient to induce a maximal hydro-osmotic response. After the final water flow measurement, each submaximally filled bladder was maximally filled with a measured volume of fluid and bladder volume capacity was calculated. All bladders were then emptied, blotted gently to remove adherent fluid, and weighed. The total luminal surface area of every bladder was calculated from its maximal volume capacity on the assumption that the lumen of a fully distended bladder sac is a smooth sphere [11]. This surface area estimate and wet tissue weight were both used to normalize water flow for the various bladders studied here.

In all sac preparations studied a mucosal hydrostatic pressure of approximately 2–4 cm H_2O was always present. Bladders that were maximally filled, tended to have higher mucosal hydrostatic pressures than those which were submaximally filled. In validation experiments with fully distended, paired bladders ($n=6$), we found no measurable effect of a difference in hydrostatic pressure between 2.4 ± 0.2 and 5.0 ± 0.2 cm H_2O on either base-line or ADH-stimulated transbladder osmotic water movement.

In a second set of experiments, morphologic effects of distension were examined by thin-section electron microscopy in bladder pairs unexposed ($n=2$) and exposed to ADH ($n=6$). In addition, for the bladders exposed to hormone, the frequency of induced intramembranous particle aggregates was assessed in freeze-fracture replicas. The procedure in these experiments was generally similar to that described above. Since the intent of these experiments was to examine morphologic aspects of bladder distension, however, the submaximally distended bladders (which were filled approximately between 40 and 60% of capacity) were not fully distended at the end of an experiment. Instead, both they and the maximally distended paired tissues, while still secured to glass tubes, were fixed with an isotonic solution of buffered glutaraldehyde (1.3% in 0.05 M cacodylate buffer at pH 7.4) from the serosal side for 1 min to preserve microscopic shape, then drained and cut from their tubes, and replaced in the same fixative for 30 additional min. The morphologic methods which followed thereafter are described elsewhere [15, 19].

In the final experimental set, 24 bladders from 14 toads were filled to $68 \pm 1\%$ of volume capacity (as determined at the end

[1] Bladders were considered to be filled to capacity and fully distended when the continued addition of solution to the preparation resulted only in increasing the volume contained in the cannula with no further increase in the volume of the bladder. Bladder volume capacity was calculated by subtracting the volume of solution in the cannula from the total volume added to the preparation.

of an experiment) and exposed to ADH concentrations between 0.1 and 20 mU/ml. Induced osmotic water flow was assessed for each 5-min interval during 30 min of stimulation. Peak values occurred regularly by about the 15th min of treatment and were used to calculate hormonally induced osmotic water permeability. These same bladders, after being filled to capacity, were then fixed in glutaraldehyde and intramembranous particle aggregate frequency for them was assessed in freeze-fracture replicas. Total luminal surface area was estimated in these bladders as in the first set of experiments, from measurements of bladder volume capacity. In the presence of a comparable transbladder osmotic gradient, aggregate frequency is both maximal and constant from 5–30 min after ADH treatment begins [8, 9]. The data derived from these experiments were used to estimate the water permeability associated with a single luminal membrane aggregate and the water permeability of the series resistance barrier by the method which we recently reported [22]. In brief, for permeability barriers in series:

$$R_{\text{tissue}} = R_{\text{luminal membrane}} + R_{\text{series}} \quad (1)$$

where R_{tissue} and $R_{\text{luminal membrane}}$ are the resistances to osmotic flow of the entire tissue and of the luminal membrane, and R_{series} is the resistance of the nonluminal membrane portion of the tissue and unstirred layers of water. R_{series} is assumed to be constant. With permeability (P) as the reciprocal of resistance:

$$1/P_{\text{tissue}} = 1/P_{\text{luminal membrane}} + 1/P_{\text{series}}. \quad (2)$$

If $P_{\text{luminal membrane}}$ is proportional to aggregate frequency:

$$P_{\text{luminal membrane}} = P_{\text{agg}} \times N_{\text{agg}} \quad (3)$$

where P_{agg} is the luminal membrane water permeability associated with a single aggregate, and N_{agg} is the number of aggregates per cm^2 of luminal membrane.

From Eqs. (2) and (3) we may derive:

$$P_{\text{tissue}} = \frac{P_{\text{agg}} \times N_{\text{agg}} \times P_{\text{series}}}{(P_{\text{agg}} \times N_{\text{agg}}) + P_{\text{series}}} \quad (4)$$

$$N_{\text{agg}}/P_{\text{tissue}} = (1/P_{\text{series}}) \times N_{\text{agg}} + 1/P_{\text{agg}} \quad (5)$$

with P_{tissue} calculated from water flow per unit area of fully distended tissue. These equations are similar in form to those which describe saturation kinetics of enzymatic reactions. Equation (4) demonstrates that P_{tissue} approaches P_{series} as N_{agg} increases, while Eq. (5) implies that a graph of $N_{\text{agg}}/P_{\text{tissue}}$ *vs.* N_{agg} should be linear if the model accurately describes conditions in the bladder. From Eq. (5), P_{series} = (1/slope) and P_{agg} = (1/y-intercept).

Results

Effect of Bladder Distension on Transbladder Osmotic Water Flow Before and During ADH Stimulation

In the absence of ADH, osmotic water flow across the undistended bladders was not different from flow across the fully distended bladders over the range of filling volumes used (30–95% of maximal volume). With ADH treatment, however, a direct linear relationship between hormonally induced water flow and bladder filling became evident. Figure 1 demonstrates the close relationship between the degree of bladder filling (expressed as percent of bladder volume capacity) and ADH-induced osmotic water flow in the submaximally filled bladders (expressed as percent of flow in the paired, maximally filled tissues)[2]. Although the water flow data in Fig. 1 are normalized for tissue weight, a similar relationship holds for the same type of data plot when water flow is normalized for total luminal surface area instead (r=0.94; P< 0.01). In addition, considering only the submaximally filled bladders studied in these experiments, the linear relationship between percent bladder filling and ADH-induced water flow was still evident when absolute flow rates were normalized for either tissue weight (r=0.90; P<0.01) or total luminal surface area (r=0.89, P<0.01). Finally, the relationship shown in Fig. 1 was not altered by stirring of the mucosal bath in the six submaximally filled bladders that were additionally studied.

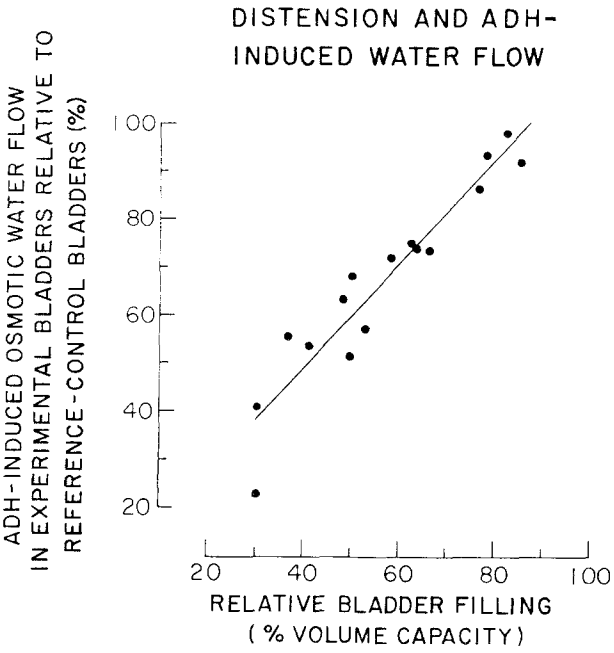

Fig. 1. Relationship between ADH-induced water flow in experimental bladders and bladder filling. For this data plot water flow was first factored by tissue weight and then expressed as a percentage of flow in paired reference-control bladders. The least squares regression line is $\tilde{Y}=4.6+1.08X$; (r=0.95; P<0.01). For a similar data plot with water flow normalized for total luminal surface area instead, a similar relationship ($\tilde{Y}=15.4+0.88X$; r=0.94; P<0.01) was also observed

In other experiments the effect of bladder distension was found to be reversible: paired, maximally filled bladders were exposed to a maximal concentra-

[2] The fully distended and submaximally distended bladders used to generate the data in Fig. 1 were approximately the same size (91.8±7.5 *vs.* 97.7±4.4 mg). The calculated transbladder osmotic potential (gradient) for water flow in the bladders comprising these two groups at the end of these experiments tended to be slightly less for bladders that were submaximally distended (166.1±2.0 *vs.* 169.2±1.8 mosmolal). This difference, for the purposes of this report, is not substantive.

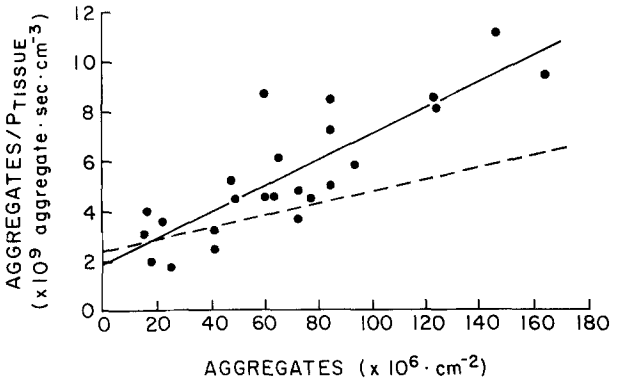

Fig. 2. Relationship between aggregate frequency and aggregate frequency divided by hormonally induced osmotic water permeability (P_{tissue}) in submaximally distended bladders (solid line). $r=0.84$; $P<0.01$. Dashed line represents the relationship between these variables in fully distended bladders [22]

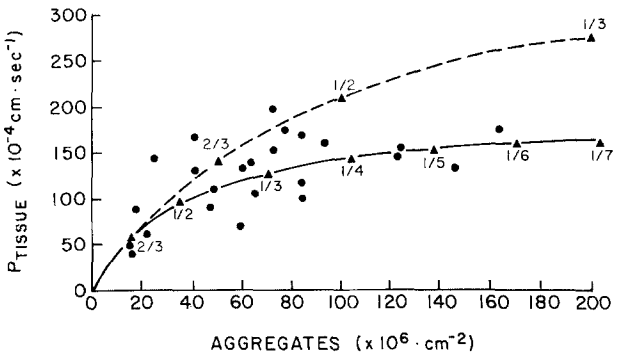

Fig. 3. Relationship between aggregate frequency and hormonally induced osmotic water permeability (P_{tissue}) in submaximally-distended bladders (solid line). Dashed curve represents the relationship between these variables in fully distended bladders [22]. Fractions of tissue flow resistance of the luminal membrane are indicated (▲)

tion of ADH, and water flows were measured to ensure pairing. Removal of 30–50% of the mucosal bathing medium from one bladder of each of six pairs led to a prompt decrease in water flow which was of the same magnitude as that shown in Fig. 1.

Effect of Bladder Filling on Toad Bladder Morphology

Thin-section electron microscopy revealed that in both the absence and presence of ADH, submaximally distended bladders were thicker than paired bladders which were maximally filled. In addition, the mucosal surface of the submaximally filled bladders appeared more undulating than that of the bladders maximally filled, but deep invaginations were not seen in either group. Intercellular space dilation associated with ADH treatment appeared to be about the same in both groups of bladders. In general, both in the absence and presence of ADH, microvilli in the submaximally filled bladders appeared to be more prominent and more numerous than in bladders that were fully distended; however, for the bladder groups studied these differences were neither consistent nor striking.

In the ADH-treated bladders studied by freeze-fracture electron microscopy, luminal membrane intramembranous particle aggregates were never observed on microvilli, irrespective of bladder filling. This is consistent with our previous experience. Aggregate frequency in the submaximally filled bladders ($220 \pm 38 \times 235\ \mu m^{-2}$) was not statistically different (paired t test) from that in the bladders which were maximally filled ($189 \pm 18 \times 235\ \mu m^{-2}$). In fact, the observation that aggregate frequency tended ($0.2 < P < 0.3$) to be greater in the submaximally filled bladders, may be accounted for by the slight tendency for nonmicrovillar membrane area in these bladders to be reduced with a lesser level of distension.

The Relationship between ADH-Induced Osmotic Water Permeability and Luminal Membrane Aggregates in Submaximally Distended Bladders

Figure 2 illustrates the relationship ($r=0.84$; $P<0.01$) between aggregate frequency (N_{agg}) and aggregate frequency normalized for induced osmotic water permeability (N_{agg}/P_{tissue}) in the submaximally distended bladders receiving various levels of ADH. From the equation for the regression line that is shown in Fig. 2, we calculate (*see* Materials and Methods, Eq. (5)) that the water permeability of the series barrier in these tissues is 191×10^{-4} cm/sec and that the water permeability associated with a single luminal membrane aggregate is 5.5×10^{-10} cm^3/sec. The regression line relating aggregate frequency and N_{agg}/P_{tissue} which we derived previously for fully distended bladders [22] is included in Fig. 2 for comparison.

Figure 3 shows the actual values for aggregate frequency and ADH-stimulated osmotic water permeability, together with the curve that is the solution to Eq. (4). The corresponding curve which we previously calculated for fully distended bladders [22] is included as well.

Discussion

We recently described a method by which the resistance of the bladder's luminal membrane can be distinguished from that of the tissue barriers which lie in series with it [22]. We were not then, however, able to localize the series resistance within the bladder tissue and the apposing layers of water. The present study demonstrates that ADH-stimulated water flow

across the bladder can be systematically altered by tissue distension. Furthermore, in that luminal membrane surface area is constant with different degrees of tissue distension [11], this study also provides insight into the location of the series barriers.

Comparison of the regression lines for N_{agg} *vs.* (N_{agg}/P_{tissue}) in distended and undistended tissues (Fig. 2) suggests that the increase in permeability which accompanies distension is the result of an increase in the permeability of the series barrier (1/slope), while luminal membrane aggregate permeability (1/*y*-intercept) appears to remain virtually unchanged. Although the data shown in Fig. 3 for the relationship between aggregates and hormonally induced osmotic water permeability can be fit to a straight line rather than the curve shown, the straight line of best fit is not a theoretically reasonable approximation of the underlying relationship because its *y*-intercept (84.0×10^{-4} cm/sec) is far removed from the origin. Furthermore, the interpretation that the decreased resistance to water flow across fully distended bladders is localized within the tissue beyond the luminal membrane is independently suggested by the observation that tissue distension had no effect on the occurrence of luminal membrane aggregates in response to a standard maximal dose of ADH.

We believe that it is unlikely that bulk-phase unstirred layers play a significant role in this distension effect because vigorous stirring did not alter osmotic water flow. Similarly, we did not observe any deep invaginations of the luminal membrane which would effectively decrease the amount of membrane available for transport. The possibility of unstirred layers within microvilli deserves consideration here. If aggregates were found on the portion of the membrane which was folded into microvilli in the submaximally distended tissues, these aggregates might contribute little to overall permeability because of local unstirred layer effects within microvilli. However, aggregates were not seen on microvilli in any of the bladders examined, distended or not.

It is not altogether clear to us where in the bladder tissue the series barrier is localized. If discrete water conducting pathways exist as structures in membranes at post-luminal membrane sites (assuming lipid bilayers to be inelastic), we think that it is unlikely that they would be altered appreciably by bladder distension; they could, however, comprise constant, distension-independent barriers to flow. According to our observations, the dilation of intercellular spaces associated with ADH treatment appeared not to be affected by bladder distension. Therefore, we think that it is also unlikely that distension-related differences in ADH-stimulated water flow would be explained by changes in the resistance of pathways through nonmembranous structures (such as the basal lamina) within the tissue. It seems more reasonable to postulate that alterations in bladder geometry which accompany distension lead to changes in the thickness of unstirred layers of water within the tissue, decreasing series barrier resistance when the tissue is distended, and increasing resistance when the tissue is not.

In the absence of ADH, we found that distension had no effect on base-line osmotic water movement. The relatively low water permeability of the bladder's luminal membrane in the absence of hormone would explain this: clearly a low luminal membrane permeability would mask any absolute change caused by a series barrier. This may also explain why distension does not alter urea permeability (expressed as transport rate/bladder) [25], which is, even in the presence of ADH, considerably less than base-line water permeability.

Finally, for practical purposes, our results suggest that the effect of distension should be seriously considered in experimental design when rates of ADH-stimulated water flow across toad bladder are studied. In many of the fully distended tissues examined previously [22], the series barrier resistance was approximately two times greater than the resistance of the luminal membrane during maximal ADH stimulation, so that the luminal membrane provided about one-third of the total resistance to flow. In the submaximally distended tissues described here, the effect of the series barrier was even more significant. In these tissues, only about one-fifth of the total resistance could be localized to the luminal membrane during maximal hormone stimulation, and "maximum" water permeability was one-half of that in the fully distended tissues. Clearly, without standardization of bladder distension, quantitative interpretations of data related to osmotic water permeability are severely compromised.

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