J. Membrane Biol. 52, 181–184 (1980)

The Journal of
Membrane Biology

Effect of Osmotic Gradient on ADH-Induced Intramembranous Particle Aggregates in Toad Bladder

Stephen J. Ellis, William A. Kachadorian, and Vincent A. DiScala

Renal Service, U.S. Public Health Service Hospital, Staten Island, New York 10304

Summary. Paired toad urinary bladders were prepared without or with an osmotic gradient (175 mosM) across them, stimulated for 2.5 ($n=6$), 5 ($n=6$), 30 ($n=6$) or 60 ($n=6$) min with ADH (20 mU/ml), and studied by freeze-fracture electron microscopy. Water permeability at these times was assessed in additional bladders ($n=6$ for each case) after tissue fixation according to the technique of Eggena. After both 60 and 30 min of ADH stimulation, the presence of a gradient compared with the absence of one was associated with fewer aggregates (242 ± 35 *vs.* $382 \pm 14 \times 235\,\mu m^{-2}$ at 60 min, $P<0.01$; 279 ± 36 *vs.* $470 \pm 51 \times 235\,\mu m^{-2}$ at 30 min, $P<0.01$) and lower water permeability (8.4 ± 1.1 *vs.* $18.8 \pm 1.8\,\mu g \times min^{-1} \times cm^{-1} \times mosM^{-1}$ at 60 min, $P<0.005$; 9.2 ± 1.0 *vs.* $22.0 \pm 2.1\,\mu g \times min^{-1} \times cm^{-2} \times mosM^{-1}$ at 30 min, $P<0.001$). In addition, with a gradient both maximum water permeability and maximum aggregate frequency were reached nearly together; a similar correspondence occurred without a gradient. We conclude that in the presence of an osmotic gradient both the ADH-associated aggregates and the water permeability response to ADH are prevented from reaching the higher levels observed in bladders not exposed to a gradient.

Key words: Antidiuretic hormone; water permeability; toad urinary bladder; *Bufo marinus*; membrane structure and function.

Freeze-fracture electron microscopy has been used to demonstrate that vasopressin (ADH) stimulation of toad bladder is accompanied by the appearance of intramembranous particle aggregates on the protoplasmic (*P*) fracture face of granular cell luminal membrane [2, 3, 10, 11]. Selective inhibition or enhancement of ADH-induced water flow results in a decrease or increase, respectively, in aggregate frequency and/or cumulative aggregate area [9, 11, 12]; therefore, it has been suggested that this morphologic change is specifically related to the ADH-induced increase in transbladder water permeability. Moreover, it has been further postulated that the aggregation response may be a marker for luminal membrane permeability [8]. In light of the postulated specificity of particle aggregation as a marker for transbladder water permeability, the following observations would appear to require additional clarification: (i) transbladder osmotic water flow with a constant osmotic gradient has been reported to reach a maximum 5 min after the maximum aggregation response has been reached [4]; and (ii) as compared to the absence of an osmotic gradient, the presence of an osmotic gradient is known to inhibit the ADH-induced increase in water permeability [6]; however, an osmotic gradient has been reported to have no effect on the aggregation response [4]. The purpose of the present experiments is to re-examine these two observations. In contrast to previous studies [1, 4, 5, 8–11], we used the fixed-sac method of Eggena [6] to assess ADH-associated increases in osmotic water permeability because this approach allows for the measurement of both fixed water permeability, and nonosmotic gradient-associated water permeability.

Materials and Methods

Urinary hemibladders from double pithed Dominican toads (*Bufo marinus*) were prepared as sacs on the end of glass tubes. After they were washed inside (mucosal surface) and out (serosal surface) with Ringer solution (111 mM NaCl, 3.5 mM KCl, 2.5 mM $NaHCO_3$, and 1.0 mM $CaCl_2$; pH 7.6–8.2; 220 mosM/kg H_2O), they were suspended in an aerated Ringer bath and their mucosal volumes were replaced with an amount of Ringer solution sufficient to fill the bladders to capacity. During a 30-min equilibration period transbladder electrical potential was measured with calomel electrodes and a Keithley electrometer (610 C). If the potential

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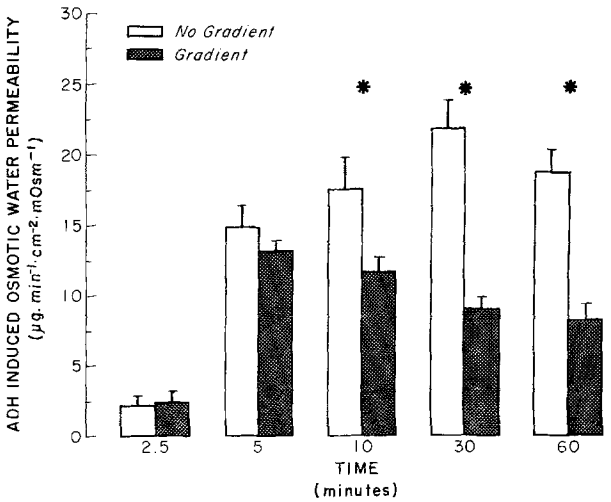

Fig. 1. Osmotic water permeability in glutaraldehyde-fixed, paired bladders after various periods of ADH exposure with and without a gradient. In each group $n=6$. Means ±SEM are shown. Asterisk indicates that difference between paired means is statistically significant at a probability level <0.05

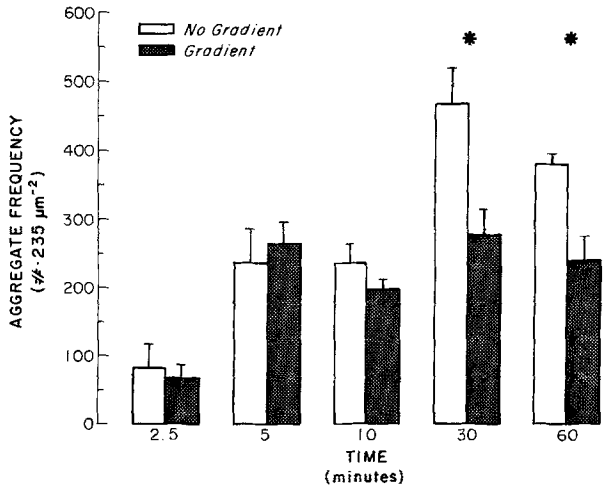

Fig. 2. Aggregate frequency in paired bladders after various periods of ADH exposure with and without a gradient. In each group $n=6$. Means ±SEM shown. Asterisk indicates that difference between paired means is statistically significant at a probability level <0.05

Table 1. Time course study of the effect of osmotic gradient on the ADH-induced aggregation response, cumulative aggregate area

Time (min)	Cumulative aggregate area ($\mu m^2 \times 235\ \mu m^{-2}$ cell membrane)		
	Gradient (175 mosM)	No gradient	P value for Δ (gradient-no gradient)
2.5	0.5±0.1	0.6±0.2	NS
5	2.1±0.3	1.8±0.4	NS
10	1.7±0.2	1.8±0.2	NS
30	1.7±0.3	3.2±0.4	$P<0.02$
60	1.2±0.2	2.3±0.2	$P<0.01$

Values are means ±SEM ($n=6$).

across either hemibladder of a pair was less than 20 mV, the experiment was terminated; otherwise an experimental period began. Bladder pairs were exposed to a serosal ADH (Pitressin, Parke-Davis) concentration of 20 mU/ml for either 2.5 ($n=12$), 5 ($n=12$), 10 ($n=12$), 30 ($n=12$) or 60 ($n=12$) min. Five min prior to stimulation with ADH, the mucosal contents of bladders comprising a pair were replaced either with Ringer solution diluted 1:5 with distilled water or with full strength Ringer. For morphologic studies half of the bladder pairs for each time period ($n=6$) were simultaneously fixed from mucosal and serosal surfaces with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 15 min, and then stored at 4 °C for two to four weeks in vials containing 0.1 M cacodylate buffer. Freeze fracture was performed on these tissues in a Balzers freeze-etch unit (BAE 301) after treatment for at least 60 min with 25% glycerol in 0.1 M cacodylate buffer as previously described [10], and the resulting platinum-carbon replicas were examined with either an RCA (EMU 4B) or Zeiss (EM 10A) electron microscope. A single micrograph of a standard area ($\sim 23.5\ \mu m^2$) of fracture face P from the luminal membrane of 10 to 14 separate and randomly selected granular cells was taken. The number of aggregates present was expressed per reference area of membrane ($235\ \mu m^2$). The surface area of each aggregate was evaluated by planimetry with a Elographics graphical digitizer (E 241) on-line to a Wang programmable calculator (720 C).

In order to measure osmotic water permeability, the remaining bladder pairs ($n=6$ for each time period) were fixed after ADH exposure according to the technique of Eggena [6] by replacing the mucosal solution with 1% glutaraldehyde in 0.05 M cacodylate buffer and fixing for 5 min. Following removal of this glutaraldehyde solution and after two mucosal washes with 1/5 strength Ringer and final replacement of the mucosal volumes with 1/5 strength Ringer, bladders were placed in a full strength Ringer bath. Water flow was then measured gravimetrically [1] for 15 min and expressed per unit bladder surface area which was estimated by measurements of mucosal volume (assuming sacs to be spheres).

Student's t test for paired or unpaired data (as required) was used to evaluate differences in the water flow and aggregation responses. Regression analysis was performed by the method of least squares. A level of $P<0.05$ was regarded as significant.

Results and Discussion

After either 2.5 or 5 min of ADH stimulation, osmotic water permeability was not affected by an osmotic gradient (175 mosM) (Fig. 1). However, after 10, 30, or 60 min of ADH stimulation, the presence of a gradient inhibited osmotic water permeability as compared to the absence of a gradient (Fig. 1). In connection with this observation, aggregate frequency, as well as total membrane area occupied by aggregates, was less in the presence than in the absence of an osmotic gradient after 30 or 60 min of ADH stimulation (Fig. 2, Table 1). Moreover, the gradient-associated inhibition of water flow and the gradient-associated decrease in aggregate frequency, when expressed as percentages, were linearly related such that the line of best fit for the relationship approximately intercepted the origin and had a slope close to 1 (Fig. 3).

With an osmotic gradient present, osmotic water

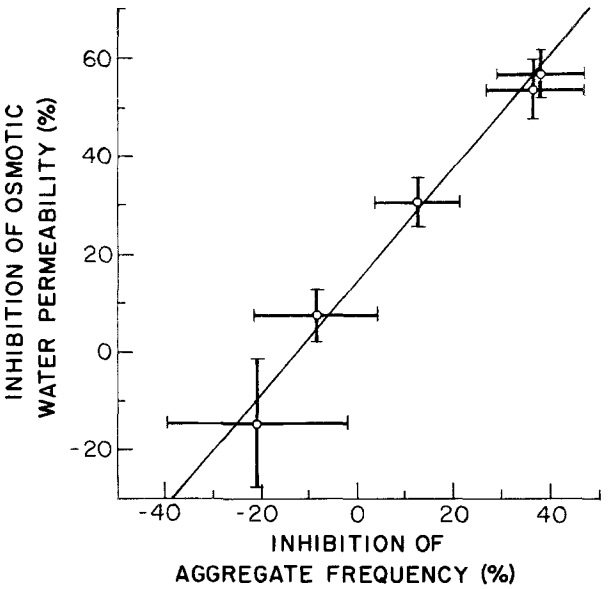

Fig. 3. Percent inhibition ((no gradient − gradient)/(no gradient)) × 100 of osmotic water permeability *vs.* percent inhibition of aggregate frequency. Means ± SEM are shown. For each observation $n=6$. $r=0.99$; $P<0.005$. Least squares regression line: $y=0.15+1.17x$

permeability reached a maximum and aggregate frequency plateaued at some time after 2.5 min but by 5 min of ADH stimulation. Without an osmotic gradient, osmotic water permeability reached a maximum and aggregate frequency plateaued at some time after 10 min but by 30 min of ADH stimulation (Figs. 1 and 2).

Eggena's fixed-sac method, which is used in this study, has two advantages over the conventional "unfixed"-sac method for studying the correlation of ADH-induced morphologic and physiologic changes occurring in the presence and absence of an osmotic gradient. First, the effect of no osmotic gradient on the ADH-induced transbladder water permeability can be fixed with glutaraldehyde [6] and thereafter assessed by measuring water flow in the presence of a constant osmotic gradient. Second, the physiologic and morphologic events with which this study is concerned should be precisely coincident because with the fixed-sac method preserved momentary morphologic changes are being correlated with preserved momentary water permeabilities.

With Eggena's method we observed a close time correspondence between the maximal ADH-induced water flow and ADH-associated intramembranous aggregates. This contrasts with a previous study [4] in which the maximum water permeability response to ADH, as measured by the conventional "unfixed"-sac method, was reported to be delayed until 5 min after the maximum aggregation response. Since the conventional "unfixed" sac method failed to demonstrate that the aggregation response and water permeability reach a maximum at about the same time, we hypothesized that perhaps some inherent inaccuracy in the method itself might be responsible, and/or that the method was not selectively measuring changes in luminal membrane water permeability. An inherent inaccuracy may result from the inability of the conventional method to measure momentary water flow. If osmotic water permeability is measured over an interval of time, as with the conventional method, then the observed value would actually be an integrated, rather than a momentary reflection of water permeability. The ability to measure momentary water permeability would be especially important during the first 5 min following ADH stimulation when water permeability is changing rapidly.

The inability to selectively measure changes in luminal membrane water permeability could also account for the separation of water permeability and aggregation response maxima seen with the "unfixed"-sac method. This hypothesis would require (i) a post-luminal membrane site (i.e., granular cell cytoplasm, basolateral membrane, interstitum, basal cells and/or serosal cells) which undergoes a gradual increase in water permeability that reaches a maximum after 10 min of ADH stimulation, and (ii) in contrast to the permeability changes in the luminal membrane, a post-luminal membrane site permeability change which is not preserved by glutaraldehyde.

In examining the effect of an osmotic gradient as compared to its absence, we found that the osmotic gradient-induced inhibition of water permeability was linearly related to the osmotic gradient-induced inhibition of the aggregation response to ADH (Fig. 3). This finding differs from that of Dratwa et al. [4] who reported that the aggregation response was not influenced by an osmotic gradient. Possibly, these investigators did not note the effect which we observed because in their studies the influence of an osmotic gradient on the aggregation response was assessed with unpaired bladders.

With regard to a possible mechanism whereby an osmotic gradient might interfere with the ADH-induced aggregation response, Eggena has postulated that granular cell swelling, which occurs in response to incubation in a hypotonic medium or in response to bulk water flow, blunts the effect of ADH possibly by diluting intracellular cyclic adenosine monophosphate (cAMP) [7]. Since the aggregation response to ADH is known to be mediated via cAMP [2], lower intracellular levels of cAMP could account for a lower aggregate frequency; however, other mechanisms are possible.

In summary: (i) the presence as compared to the

absence of an osmotic gradient appears to reduce toad bladder responsiveness to ADH-induced increases in water permeability in concert with proportional reductions in the aggregation response, thus further confirming the close relationship between these two phenomena; and (ii) the fixed-sac method of measuring water permeability demonstrates that a chronological correlation between water permeability and aggregation response maxima actually occurs, thus suggesting that the previously observed chronological dissociation of the two maxima may be due to either an artifact inherent to the conventional "unfixed"-sac method for measuring water flow or to unfixable changes in the water permeability of a post-luminal membrane site, or to both.

We thank Mr. Stelio Fantoli and Mrs. Kristine Olsen for providing technical assistance, Ms. Alice Barry for typing this manuscript, and Drs. Jacqueline Muller and Stephen W. Rudich for their helpful suggestions.

This investigation was supported by NIH Grant AM 18710 from the National Institutes of Health.

This study was published as an abstract by the American Society of Nephrology for the 11th Annual Meeting in Washington, D.C., November, 1978.

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Received 11 July 1979; revised 19 October 1979