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Barriers to Water Flow in Vasopressin-Treated Toad Urinary Bladder

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Summary. Unstirred layers of water complicate the measurement of water permeability across epithelia. In the toad urinary bladder, the hormone vasopressin increases the osmotic water permeability of the granular epithelial cell's luminal membrane, and also leads to the appearance of aggregates of particles within this membrane. The aggregates appear to be markers for luminal membrane osmotic water permeability. This report analyzes the relationship between transbladder osmotic water flow and aggregate frequency, and demonstrates that flow across the bladder is significantly attenuated by unstirred layers of water or by structural barriers other than the luminal membrane when the luminal membrane is made permeable by vasopressin. This analysis in addition yields unique values for the permeabilities of both the luminal membrane and the barriers to water flow which lie in series with it.

Key words: vasopressin, permeability, water flow, aggregates, series barriers

Calculations of the osmotic water permeability of an epithelium based on steady-state water flow measurements tend to underestimate true values because of the effects of unstirred layers of water in apposition to the structural permeability barriers. These unstirred layers lead to dissipation of the probe-solute's gradient due to diffusion in the unstirred layers, sweeping away of the probe-solute by the osmotic water flow, and the development of gradients in initially symmetrically distributed solutes which oppose the initial solute gradient (Diamond, 1979). While these effects are most prominent in "leaky" epithelia such as gallbladder and renal proximal tubule, they also appear to be significant in "tight" epithelia, including renal collecting tubule and toad urinary bladder (Hays, 1972; Schafer, Patlak & Andreoli, 1974). For these reasons, estimation of osmotic water permeability in most epithelia is a complex undertaking.

The urinary bladder of the Dominican toad *Bufo marinus* is a "tight" epithelium which has been extensively used in delineating the mechanism of action of the antidiuretic hormone vasopressin. In comparison to other epithelia, however, it offers two distinct advantages for the derivation of accurate values for osmotic water permeability. First, antidiuretic hormone causes a large increase in the osmotic water permeability of the luminal membrane of the granular cells of the bladder epithelium, and the permeability can be independently altered by changing the concentration of vasopressin. Second, a close and specific correlation has been demonstrated between vasopressin-stimulated osmotic water permeability and the occurrence of vasopressin-induced particle aggregates within the granular cell luminal membrane, as revealed by freeze-fracture electron microscopy both in the presence of varying concentrations of vasopressin (Chevalier, Bourguet & Hugon, 1974; Kachadorian, Wade & DiScala, 1975), and in studies using agents which specifically inhibit or enhance vasopressin-stimulation of water flow (Kachadorian et al., 1977; Levine, Kachadorian, Verna & Schlondorff, 1980). Indeed, we have suggested that these luminal membrane particle aggregates may be the sites for transluminal membrane water movement or, as a minimum, markers for regions of the luminal membrane which are highly water-permeable and carry the bulk of vasopressin-responsive osmotic water flow (Kachadorian, Muller, Rudich & DiScala, 1979). Moreover, similar particle aggregates have been reported in other vasopressin-responsive epithelia, including frog skin (Brown, Grosso & deSousa, 1980)

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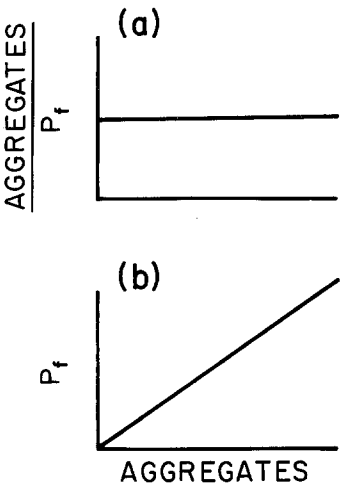

Fig. 1. R(series)=0. The graph of Eq. (4) is shown at the bottom (*b*), and is a straight line passing through the origin. The graph of Eq. (5) is at the top (*a*), and is a straight line with slope=0

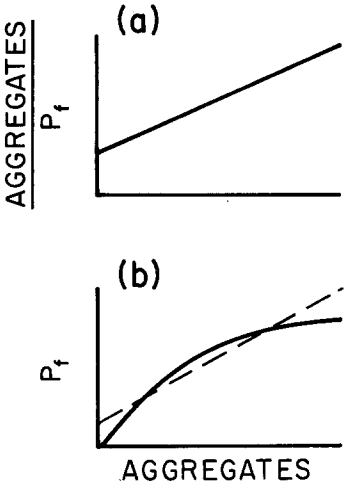

Fig. 2. R(series)>0. The curved line which describes Eq. (4) is shown at the bottom (*b*). The broken line in (*b*) is a straight line which fits the data points well, but has a non-zero y intercept, overestimates both end points, and underestimates central points. The graph of Eq. (5) is at the top (*a*), and is a straight line with positive slope. Note that for both Fig. 1*a* and Fig. 2*a* the y intercept=$1/P$(aggregate), while the slope=$1/P$(series)

and rat renal papillary collecting duct (Harmanci, Kachadorian, Valtin & DiScala, 1978).

We examined the following hypothesis: If the concentration of luminal membrane aggregates is in fact a true marker for the osmotic water permeability of the luminal membrane [that is, P(luminal membrane) is proportional to aggregate frequency N(agg)], then analyzing the relationship between aggregate frequency and whole tissue water permeability [P(tissue)] as aggregate frequency is increased by vasopressin should yield true values for P(luminal membrane).

These relations may be formalized in the following manner: Permeability barriers in series:

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

where R(series)=the effective resistance of nonluminal membrane tissues and unstirred layers of water to osmotic flow. This resistance is assumed to be constant. Thus,

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

If P(luminal membrane) is proportional to aggregate frequency, then:

$$P(\text{luminal membrane}) = P(\text{aggregate}) \times N(\text{agg}) \quad (\textit{see footnote 1}) \quad (3)$$

where P(aggregate) is the luminal membrane water permeability‹associated with a single aggregate, and N(agg)=the number of aggregates/cm^2 of luminal membrane.

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

$$P(\text{tissue}) = \frac{P(\text{aggregate}) \times N(\text{agg}) \times P(\text{series})}{[P(\text{aggregate}) \times N(\text{agg})] + P(\text{series})} \quad (4)$$

and

$$N(\text{agg})/P(\text{tissue}) = [1/P(\text{series})] \times N(\text{agg}) + 1/P(\text{aggregate}). \quad (5)$$

These equations are somewhat similar in form to those which describe saturation kinetics of enzymatic reactions. In particular, Eq. (4) demonstrates that P(tissue) approaches P(series) as the frequency of aggregates N(agg) increases, while Eq. (5) implies that a graph of N(agg)/P(tissue) *vs.* N(agg) should be linear if the model accurately describes conditions in the bladder. It is a particularly useful formulation because it also yields values for P(series) [=1/slope] and P(aggregate) [=$1/y$ intercept].

Figs. 1*b* and 2*b* show the results expected for the graphs of Eq. (4) in each of two situations: 1) R(series)=0 (i.e. no series barrier), and 2) R(series)>0 (i.e. series barrier present). In the first instance, Eq. (4) reduces to P(tissue)=P(aggregate)×N(agg) and thus describes a straight line which passes

[1] More precisely, Eq. (3) should be

$$P(\text{luminal membrane}) = P(\text{aggregate}) \times N(\text{agg}) + P(\text{basal})$$

where P(basal) is the permeability of the luminal membrane in the absence of aggregates. We correct for this difference by subtracting the basal water permeability from all stimulated permeability measurements. While this leads to an overestimation of luminal membrane permeability in the stimulated state, the error involved is very small, since basal water permeability is only about 1% of fully stimulated permeability. (For the tissues shown in Figs. 5 and 6 basal permeability was $2.8 \pm 0.3 \times 10^{-4}$ cm/sec while stimulated tissue permeability was on the order of 250×10^{-4} cm/sec.) The calculated overestimation of aggregate permeability from this approximation is only about 2–3%.

through the origin, while in the second, it is a curve passing through the origin. Note that while one might fit a straight line to the experimental data points even when R(series) > 0, such a line would not pass through the origin, and would overestimate the points at the ends of the curve, and underestimate those in the center.

Similarly, Figs. 1*a* and 2*a* show the results for the graphs of Eq. (5) in the two situations outlined above. Here, the discrimination between situations 1 and 2 is more clearly seen than in Eq. (4). In particular, the straight line fitting Eq. (5) is horizontal in the "no series barrier" situation, while it has a positive slope in the "series barrier" case.

Materials and Methods

In order to evaluate these possibilities, we examined the relationship between aggregate frequency and water flow in two groups of tissues. The first group of tissues provided the initial impetus for formal examination of the series barrier hypothesis, and consisted of a set of 64 bladders, some of which we have reported previously as part of evaluations of various selective stimulators and inhibitors of water flow (Kachadorian et al., 1977; Levine et al., 1980). These tissues were all prepared for transport measurements and fixed for electron microscopy in a single laboratory, but were exposed to varying osmotic gradients, as well as a variety of chemical stimulators and inhibitors, in addition to varying concentrations of vasopressin. Furthermore, tissue distension was not standardized, and tissue weights were not determined.

On the basis of the data from these tissues, we performed a second independent series of experiments, which were directed specifically toward evaluation and quantitation of the series barrier. In this series of studies, we measured water flow and aggregate frequency in bladders which were uniformly distended, of known weight, and with a single osmotic gradient, but which received varying concentrations of vasopressin. Thus the only variable among the second group of tissues was the vasopressin concentration used.

A total of 34 fully-distended hemibladders obtained from female Dominican toads were studied in the presence of vasopressin concentrations from 0.6 to 20 mU/ml, the latter concentration being one which yields maximal trans-bladder water flow. All tissues were prepared as sacs and were placed in an aerated Ringer's solution (in mM: 111 NaCl, 3.5 KCl, 2.5 $NaHCO_3$, 1.0 $CaCl_2$; pH 7.9; 220 mOsm) and filled to capacity with Ringer's diluted 1:5 with water. Bladders were filled until a stable hydrostatic pressure of about 2–4 cm of water was present on the mucosal side. Bladder surface area was calculated from measurement of mucosal volume capacity (generally about 12 ml), on the assumption that the fully-distended bladder is a smooth sphere. All bladders had spontaneous open-circuit potentials greater than 20 mV.

Water flow was measured gravimetrically (Bentley, 1958) before addition of vasopressin and at 5-min intervals beginning immediately upon addition of vasopressin, for a total of 30 min. The peak 5-min water flow rate, corrected for the basal flow rate and normalized for bladder surface area, was used for all permeability calculations. At the end of the 30-min flow measurement, the bladders were fixed in glutaraldehyde, coded, and granular cell luminal aggregate frequency was assessed by freeze-fracture electron microscopy without knowledge of tissue status as in our previous work (Kachadorian et al., 1975).

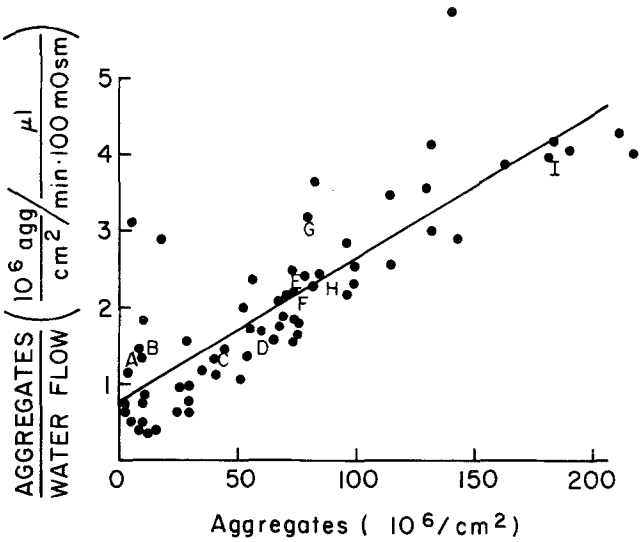

Fig. 3. Relationship of aggregate frequency and aggregate frequency/water flow in 64 hemibladders. Letters *A–I* refer to mean values from experimental protocols shown in Table 1. • are individual data points. Line is calculated from Eq. (5)

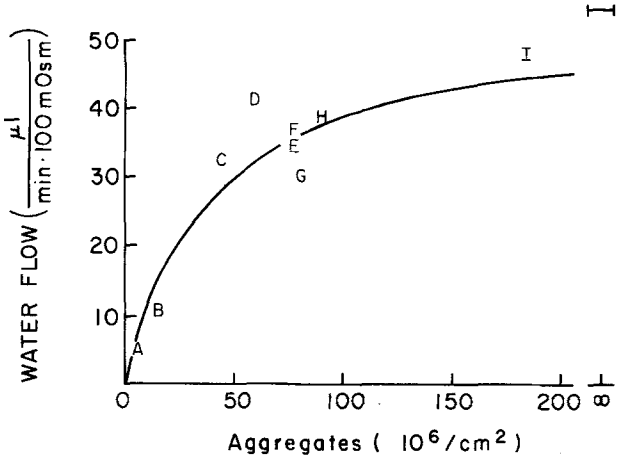

Fig. 4. Relationship between aggregate frequency and water flow in same tissues as those shown in Fig. 3. Line is calculated from Eq. (4) using values obtained from Eq. (5)

Results

The results of the preliminary evaluation for the presence of a series barrier to osmotic water flow are shown in Figs. 3 and 4. The letters *A–I* are the mean values for the nine experimental protocols shown in Table 1. Because of the difficulty in accurately assessing bladder surface area, the data are shown in terms of osmotic water flow, rather than P(tissue). Despite the large variation in experimental protocols, the similarity of the data in Figs. 3 and 4 to Fig. 2*a* and 2*b* is striking, and the correlation coefficients for the figures are highly significant ($r=0.84$, $p<0.001$ for Fig. 3). Nonetheless, interpretation of this heterogeneous group of tissues is not unambiguous, so an additional series of bladders was examined.

Table 1. Conditions used for relating osmotic water flow and luminal membrane aggregate frequency

Set	*n*	Aggregate frequency	Water	Gradient	Serosal additions	
		(10^6 agg/cm^2)	(μl/min)	(mOsm)	Vasopressin (mU/ml)	Other (mM)
A	6	5± 1	9.4±2.5	205	2	NaCl 10
B	6	14± 3	12.8±2.8	115	85	methohexital 0.3
C	6	44±13	61.8±6.1	185	1	MIX 0.1[a]
D	12	59± 9	77.9±2.7	185	5	MIX 0.1
E	6	76±18	74.2±3.7	205	2	hydrazine 20
F	10	76±11	42.5±2.4	115	85	–
G	6	80±13	60.3±9.0	195	90	NaCl 5
H	6	89±21	72.9±5.0	185	100	MIX 0.1
I	6	183±11	95.7±3.5	195	90	hydrazine 10

[a] MIX = methylisobutylxanthine.

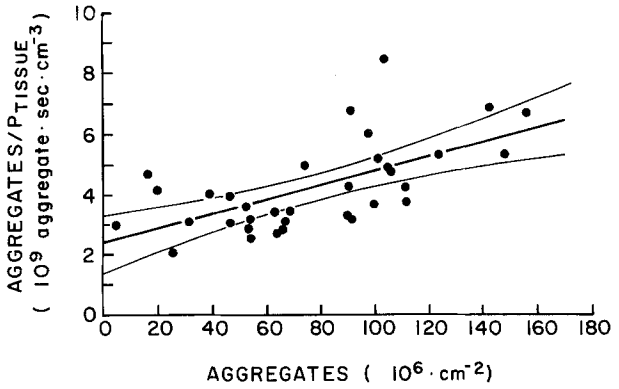

Fig. 5. Relationship between aggregate frequency and [aggregates/P(tissue)] in 34 hemibladders stimulated by various concentrations of vasopressin. The 95% confidence intervals for the least-squares regression line (——) are indicated by ——. Y intercept = 2.38×10^9 aggregates × sec/cm^3. Slope = 23.9 sec/cm

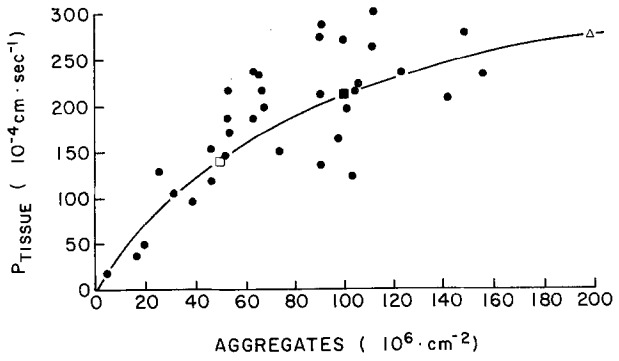

Fig. 6. Relationship between aggregate frequency and P(tissue) in the same hemibladders as in Fig. 5. The line is calculated from Eq. (4) using values obtained from Eq. (5). □, ■ and △ are points at which the luminal membrane provide two-thirds, one-half, and one-third of the total tissue flow resistance, respectively

Evaluation of the second group of tissues, all of which were treated identically except for the vasopressin concentration used, revealed similar results. Fig. 5 shows the relationship between N(agg) and N(agg)/P(tissue) in these bladders. As in the first group, the relationship between the two parameters appears linear, the slope is clearly positive, and the correlation is highly statistically significant ($r = 0.63$, $p < 0.01$). The least-squares regression line using Eq. (5) yielded values of: P(series) = 418×10^{-4} cm/sec, P(aggregate) = 4.2×10^{-10} cm^3/aggregate/sec.

The line calculated from Eq. (4) with these values is redrawn in Fig. 6, which relates N(agg) and P(tissue), together with the values for the individual bladder tissues. The correlation coefficient between the predicted and actual values for P(tissue) is 0.81 ($p < 0.01$). The points □, ■ and △ on this line correspond to loci at which the luminal membrane contributes two-thirds, one half, and one-third of the total resistance to transepithelial water flow, respectively. The data points which correspond to the bladders receiving high concentrations of vasopressin all fell within this range.

Discussion

We believe that the technique described provides a method for direct estimation of luminal membrane water permeability, together with a correction for the presence of barriers which lie in series with the luminal membrane, including both the effects of unstirred layers of water, and those of structural barriers within the bladder tissue, though we cannot on the basis of these data distinguish among the contributions of these series barriers. In the absence of vasopressin, essentially all of the resistance to water flow lies at the level of the luminal membrane (DiBona, Civan & Leaf, 1969). When maximally stimulating concentrations of vasopressin are present, we calculate that barriers other than the luminal membrane constitute one-half to two-thirds of the total resistance to flow (though the total resistance of the tissue is of course low compared to the unstimulated state). Thus estimates of luminal membrane osmotic water permeabil-

ity in the vasopressin-stimulated bladder which are derived from whole tissue water flows appear to underestimate true values by a factor of 2 to 3. The underestimation would be less severe when the bladder is submaximally stimulated.

While the most compelling evidence for the presence of a flow barrier in series with the luminal membrane is based upon the results from the tissues in which the only variable was the concentration of vasopressin, we were able to see a similar pattern of response in the tissues which were treated with a spectrum of inhibitors and stimulators of transport. These data are consistent with the hypothesis that these inhibitors and stimulators alter water permeability primarily by altering aggregate frequency, and not by altering the resistance to any other flow barriers which lie in series with the luminal membrane. Nonetheless, interpretation of this particular series of studies must be made with caution because of the many variables involved in its generation.

Our estimates of the flow resistance of the luminal membrane compared to that of the entire tissue correspond closely to those extrapolated from previous studies by others using several different techniques. Hays, for example, calculated a zero-time extrapolation of water flow in vasopressin-stimulated bladders upon sudden imposition of an osmotic gradient, and estimated that the zero-time flow rate was three times the steady-state value (Hays, 1972). Similarly, Hays and Leaf (1962) estimated epithelial cell volume by measurements of inulin space in the presence of an osmotic gradient with and without high levels of vasopressin. Recalculation of their data with the simplifying assumption of constant tissue dry weight and epithelial cell solute content suggests that cell swelling in the tissues receiving vasopressin decreased cell osmolality to a level about midway between those of the mucosal and serosal baths. Thus the osmotic gradient across the mucosal surface of the bladder was one-half that across the whole tissue, and consequently the luminal membrane resistance to water flow was about one-half the total tissue resistance. Finally, estimates of epithelial cell swelling in bladders exposed to vasopressin and an osmotic gradient can be derived from photomicrographs published by Peachey and Rasmussen (1961) and according to our calculations yield similar results.

While the data presented here suggest that the series barrier is constant, there is evidence that under some conditions it may vary in permeability. It is possible, for example, that time-dependent "intrinsic inhibition" of vasopressin-stimulated water flow, which leads to a decrease in water flow after prolonged exposure to vasopressin without any alteration of aggregate frequency (Kachadorian, Casey & DiScala, 1978), is the result of a modulated decrease in P(series). Nonetheless, in most situations the model of a fixed resistance barrier to osmotic flow (due to either structural elements or unstirred layers or both) in series with a luminal membrane whose permeability to water increases in proportion to aggregate frequency describes well the acute alterations in P(tissue) observed in the bladder as aggregate frequency is increased by vasopressin.

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