CONCISE PUBLICATIONS

Relationship of Aggregated Intramembranous Particles to Water Permeability in Vasopressin-Treated Toad Urinary Bladder

WILLIAM A. KACHADORIAN, SHERMAN D. LEVINE, JAMES B. WADE, VINCENT A. DISCALA, and RICHARD M. HAYS

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

ABSTRACT It has been previously demonstrated with freeze-fracture electron microscopy that vasopressin induces specific structural alterations of the luminal membrane of granular cells from toad urinary bladder in a dose-dependent fashion. These alterations consist of aggregated intramembranous particles and are observed both in the presence and absence of an osmotic gradient. We examined the effect of methohexital, a selective inhibitor of vasopressin-stimulated water flow, and the effect of phloretin, a selective inhibitor of urea permeability, on the structure of the granular cell luminal membrane. Methohexital treatment of the vasopressin-stimulated toad bladder reduced both the osmotic water flow and vasopressin-induced alterations of membrane structure to the same extent. Phloretin reduced urea permeability but not water flow or particle aggregation. Since neither agent affects vasopressin-stimulated sodium movement, these findings indicate that the phenomenon of particle aggregation is specifically related to vasopressin-induced water permeability and not to changes in urea or sodium permeability.

INTRODUCTION

The freeze-fracture technique is a method by which biologic membranes are internally fractured and prepared for examination with transmission electron microscopy (1). By convention, the inner (protoplasmic) membrane fracture face resulting from application of this technique has been designated fracture face P and the apposed outer (exoplasmic) face, fracture face E (2). On either of these membrane fracture faces, particle-like structures (intramembranous particles) are found in characteristic distributions according to membrane type; and considerable evidence suggests that these structures represent proteins intercalated within the membrane (3–6).

Recently, freeze-fracture electron microscopy was used to demonstrate that oxytocin stimulation of isolated frog bladder induces a structural alteration in epithelial cell luminal membranes (7). This alteration consists of the aggregation of intramembranous particles at multiple sites and is seen on the inner membrane fracture face. A similar observation in isolated toad urinary bladder after vasopressin stimulation has also been made and the specific localization of aggregates to luminal membranes of granular cells and the organization of aggregated particles in

This work was presented in part at the 8th Annual Meeting of the American Society of Nephrology, Washington D. C., November 1975.

Dr. Levine is the Molly Berns Senior Investigator of the New York Heart Association. Dr. Wade's present address is the Department of Physiology, Yale University School of Medicine, New Haven, Conn. 06510.

Received for publication 17 May 1976 and in revised form 8 November 1976.

linear arrays have been established (8). Since for both preparations intramembranous particle aggregation occurs in the absence, as well as in the presence, of an osmotic gradient (9, 10), the aggregation response can not be considered a consequence of net water flow per se, but rather appears to represent a primary hormonal effect. In addition, since the frequency of particle aggregation sites and the cumulative area of membrane occupied by them are linearly related to measured rates of vasopressin-induced osmotic water flow (8, 10), it appears that these sites of aggregation may be of functional significance in the mechanism of action of vasopressin.

The present investigation concerns the specific role of these vasopressin-induced alterations in membrane structure. Since vasopressin stimulates transepithelial movement of water and many solutes, notably sodium and urea, sites of intramembranous particle aggregation could be related to any or all of these vasopressin-induced functional changes. To deal with this problem we have (*a*) selectively inhibited vasopressin-stimulated osmotic water transfer across toad bladder with methohexital, which does not alter vasopressin-induced changes in sodium or urea movement (11); (*b*) selectively inhibited urea permeability with phloretin, which does not alter induced changes in osmotic water or sodium movement (12); and (*c*) determined in each case the extent to which the aggregation response was modified. Our data support the hypothesis that vasopressin-induced structural alterations of granular cell luminal membranes from toad urinary bladder relate specifically to induced changes in water permeability.

METHODS

Paired hemibladders from double-pithed female Dominican toads (*Bufo marinus*) were mounted as sacs on the end of glass tubes; one from each pair served as an experimental bladder, the other as a control. After each bladder was washed three times on both mucosal and serosal surfaces with phosphate-buffered Ringer's solution (111 mM NaCl, 4 mM KCl, 0.5 mM $CaCl_2$, 1 mM $NaHPO_4$, 4 mM Na_2PO_4; pH 7.4; and 230 mosmol/kg H_2O), each was filled with 8 ml Ringer's solution diluted 1:2 with distilled water which contained tracer amounts of [^{14}C]urea (New England Nuclear, Boston, Mass.), and then suspended in a 35-ml Ringer bath. For studies with methohexital ($n = 6$), the serosal baths of the experimental bladders contained 0.3 mM methohexital (Brevital, Eli Lilly and Company, Indianapolis, Ind.). In the phloretin studies ($n = 4$), the mucosal baths of the experimental bladders contained 0.1 mM phloretin (K and K Laboratories, Inc., Plainview, N. Y.) in 0.5% ethanol and those of the controls contained ethanol alone. In all cases, stirring of both mucosal and serosal baths was provided by rotating magnets.

Isotope permeability (K_{trans}[^{14}C]urea) for both control and experimental bladders was determined for a single 15-min period before and for two consecutive 15-min periods after the addition of vasopressin (Pitressin, Parke, Davis & Company, Detroit, Mich.) to the serosal baths at a final concentration of 86 mU/ml. These two latter periods were averaged into a single 30-min period for ease of data presentation. Osmotic water flow was measured gravimetrically for both experimental and control bladders for 15 min before and 30 min after the addition of vasopressin (13). At the end of the experiments in which methohexital was used, transepithelial electrical potential was evaluated.

After final measurements of transport function were made, bladders were immersed in 2.5% glutaraldehyde containing 0.1 M cacodylate buffer (pH 7.4) for 1 min to keep them from shrinking when emptied, removed and rapidly drained, cut from their glass tubes, and then replaced in the same fixative for 15 min. Thereafter, they were placed in 0.1 M cacodylate buffer, coded, and stored under refrigeration until processed for freeze fracture.

Freeze fracture was done with a Balzers freeze-etch unit (BAF 301) (Balzers High Vacuum Corp., Santa Anna, Calif.) on tissue samples after cryoprotectant treatment with glycerol, as previously reported (8, 10). Tissue replicas after freeze fracture were studied with an RCA (EMU 4B) (RCA Corp., Camden, N. J.) or Zeiss (EM 10) (Carl Zeiss, Inc., New York) electron microscope without knowledge of their status. Since vasopressin-induced intramembranous particle aggregation is observed on the inner fracture face P of granular cell luminal membranes, a single random micrograph, representing 22 μm^2, was taken of this membrane fracture face from each of approximately 10 different granular cells for every bladder studied. Thus, for each bladder, the total area of granular cell luminal membrane used for structural quantitation approximated 220 μm^2. At a final magnification of 45,000 ×, the number of aggregates was counted and, for bladders treated with methohexital and their controls, the area of membrane which each aggregate occupied was measured by planimetry, using an Elograph graphical digitizer (E241) (Elographics Inc., Oak Ridge, Tenn.) and a Wang programmable calculator (720C) (Wang Laboratories, Inc., Lowell, Mass.).

In separate experiments with methohexital which paralleled those already described, active sodium transport, as measured by short-circuit current, was evaluated with five pairs of bladders prepared as sacs, in accord with the procedure of Walser (14).

In all experiments, data for paired hemibladders were compared for statistical differences ($P < 0.05$) by Student's t test (15) unless otherwise specified.

RESULTS

Figure 1 illustrates vasopressin-induced intramembranous particle aggregation on fracture face P of granular cell luminal membrane from a control bladder studied in this investigation, and is in accord with previous observations (8, 10). On the apposed fracture face E of the same membrane type, distinctive areas were observed in which depressions (impressions) made by the aggregated particles were organized in parallel arrays.

Aggregation sites which were seen in vasopressin-stimulated bladders exposed to methohexital appeared structurally indistinguishable from those associated with vasopressin alone. In quantitative terms, however, striking differences were observed. In Table I are listed these quantitative structural data as well as corresponding functional data. Both the number of sites of intramembranous particle aggregation and the cumula-

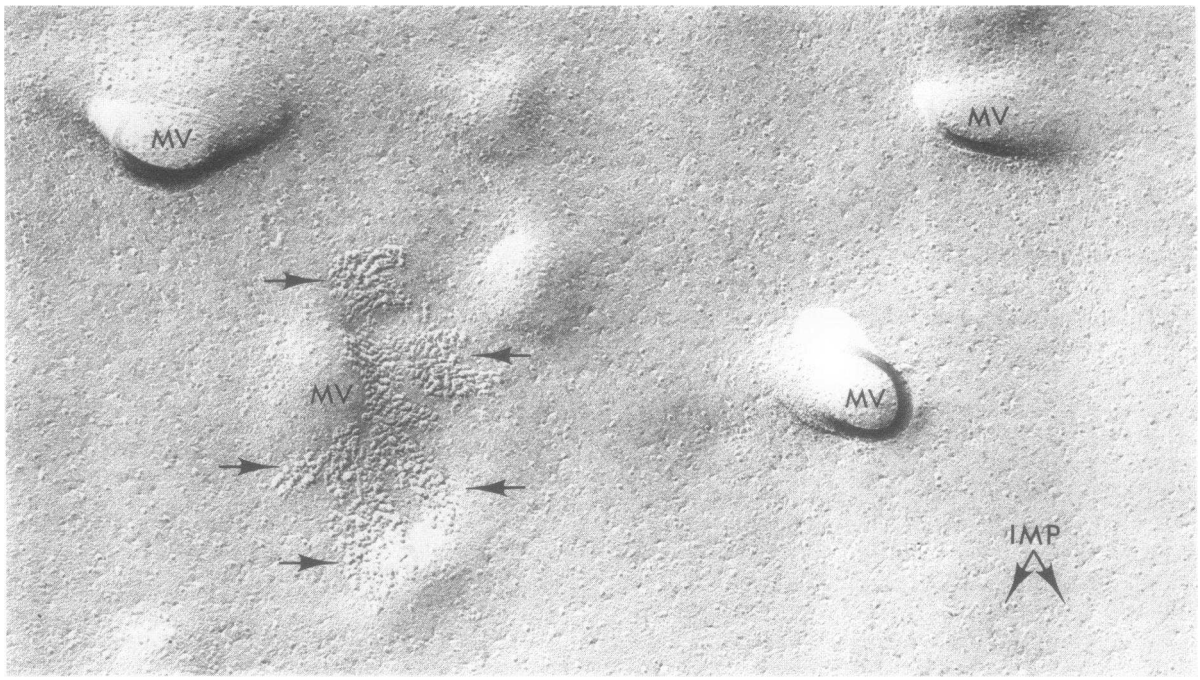

FIGURE 1 Vasopressin-induced intramembranous particle aggregation on fracture face P of control granular cell luminal membrane. Aggregates (arrows) are seen between and near the bases of microvilli (MV). Unaggregated intramembranous particles (IMP) appear as small projections which are randomly distributed. × 75,000

tive area of membrane which these sites occupied were less ($P < 0.005$) in methohexital-treated bladders than in the controls. In addition, vasopressin-stimulated osmotic water flow was diminished by methohexital ($P < 0.001$) whereas neither urea permeability nor electrical potential was significantly altered. In relative terms, the 69% inhibitory effect of methohexital on water flow followed closely the 74% reduction in aggregate frequency and the 79% reduction in the cumulative area of membrane occupied by the aggregates.

The increase in short-circuit current after vasopressin in methohexital-treated bladders was the same as that seen in paired controls. This has been shown

TABLE I

Effects of Methohexital and Phloretin on Functional and Structural Characteristics of Vasopressin-Treated Bladders

	K_{trans} Urea	Osmotic water flow	Potential difference	Frequency of aggregation sites	Cumulative area of aggregation sites
	$\times 10^{-5}$ cm/s	mg/min	mV	no./220 μm^2 membrane	$\times 10^{-3}$ μm^2/220 μm^2 membrane
Methohexital studies (n = 6)					
Control	358±49	30.7±2.0	56±18	111±17	1,171±209
Methohexital	309±70	9.5±2.1	48+14	29±7	247± 63
Δ (Control-methohexital)	50±28	21.1±1.6	7±13	82±13	924±174
$P <$	0.2	0.001	0.6	0.005	0.005
Phloretin studies (n = 4)					
Control	384±17	27.3±1.5	—	261±34	—
Phloretin	65±3	27.6±1.2	—	250±39	—
Δ (Control-phloretin)	319±16	−0.3±0.8	—	11±17	—
$P <$	0.001	0.8	—	0.6	—

previously in six paired experiments for bladders studied in Lucite chambers (11), where the increase in short-circuit current was not significantly different, and is presented here for five pairs of bladders mounted as sacs (Fig. 2).

In Fig. 3, individual aggregate surface area is histographically illustrated for all aggregates observed in vasopressin-stimulated bladders with and without methohexital treatment. In each case, aggregate size is asymmetrically distributed. Application of an appropriate nonparametric statistical test (Wilcoxan test for unequal observations [15]) indicates that the slight decrease in the mean area of aggregates in methohexital-treated bladders (8.7×10^{-3} μm^2) compared with the mean area of those in controls (10.7×10^{-3} μm^2) is statistically significant ($P < 0.01$).

As shown in the lower half of Table I, phloretin reduced vasopressin-stimulated urea movement by 82% but did not exert any effect on either hormonally-induced osmotic water flow or on the number of aggregates per area of granular cell luminal membrane.

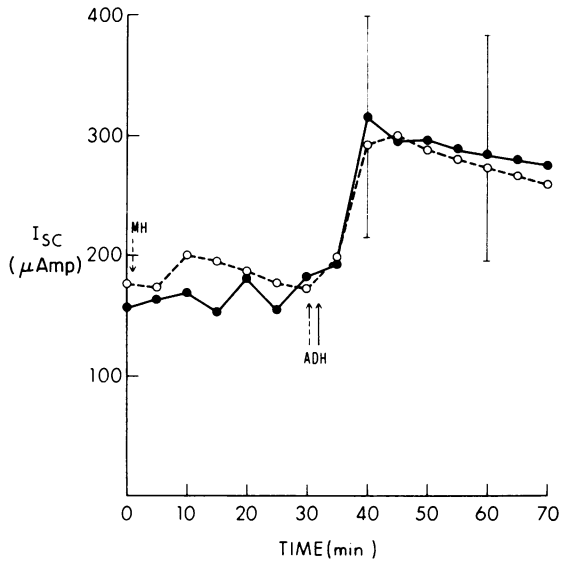

FIGURE 2 Short-circuit current (mean±SEM) before and after vasopressin in paired control (●——●) and methohexital-treated (O - - - O) bladders prepared as sacs ($n = 5$).

DISCUSSION

The results of this investigation indicate that the vasopressin-induced structural alterations found in granular cell luminal membranes from toad bladder correlate specifically with alterations in osmotic water permeability. Both the present and an earlier study (11) indicate that methohexital inhibits the effect of vasopressin on osmotic water movement across the toad bladder, but does not affect vasopressin-induced changes in urea permeability or active sodium transport. Phloretin, on the other hand, inhibits urea permeability, but it does not alter the aggregation response to vasopressin. In view of these results, and because methohexital treatment affected vasopressin-stimulated osmotic water flow quantitatively to about the same extent as vasopressin-induced particle aggre-

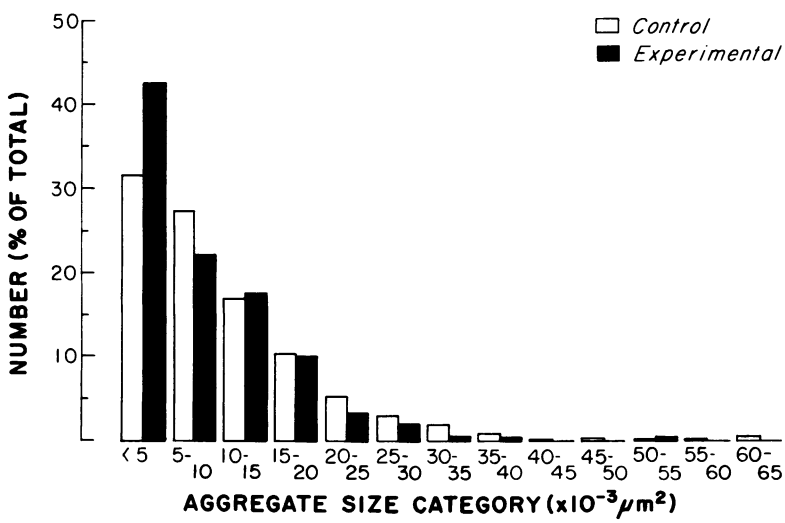

FIGURE 3 Frequency histogram of aggregate size for control bladders treated with vasopressin and for paired experimental bladders treated with vasopressin plus methohexital. Relative to control, methohexital exposure is associated with an increase in the proportion of smaller aggregates and a decrease of larger aggregates such that mean aggregate size is reduced significantly ($P < 0.01$) from 10.7×10^{-3} to 8.7×10^{-3} μm^2.

gation, it is reasonable to conclude that of the effects of vasopressin stimulation on water, urea, and sodium movement across toad bladder, only osmotic water movement relates to these induced structural alterations. Further support for this conclusion is the finding that colchicine, which disrupts microtubules and inhibits the stimulation of water flow, but not sodium transport, by vasopressin (16), also reduces the number of aggregates and the fractional area of membrane occupied by them (17).

It seems clear that the aggregation response to vasopressin is not a consequence of water flow per se since it occurs in the absence of an osmotic gradient when net water flow is negligible (9, 10). On the basis of two paired experiments which we performed, aggregates seen in the absence of a gradient were at least as numerous as those seen in the presence of one.

The mechanism by which vasopressin modifies the permeability of toad bladder involves mediation by intracellular cyclic AMP (cAMP) (18, 19). When cAMP is used to stimulate toad bladder, alterations in granular cell luminal membrane occur which are identical to those induced with vasopressin stimulation (9, 10). Since methohexital appears to inhibit vasopressin-stimulated osmotic water flow by inhibiting cAMP synthesis (20), the finding in this study of fewer membrane alterations in methohexital-treated bladders is probably best explained by a reduction of intracellular cAMP concentration.

In this connection, it is interesting that the mean size of aggregation sites associated with vasopressin plus methohexital was significantly less than that associated with vasopressin alone. With levels of vasopressin between 0.1 and 200 mU/ml, a change in mean aggregate size was not previously noted (10), and thus the finding of smaller aggregates in methohexital-treated bladders may represent a second, direct effect of methohexital per se on the mobility of membrane proteins. The significance of smaller aggregates in methohexital-treated bladders is open to question since the reduction in size seems slight, but nevertheless this effect is consistent with the small discrepancy observed between relative reductions of aggregate frequency (74% decrease) and cumulative area of membrane affected by the aggregation phenomenon (79% decrease) in methohexital-treated bladders.

The results of this investigation suggest more firmly than before (8, 10) that the vasopressin-induced alterations in toad bladder granular cell luminal membrane relate specifically to changes in membrane water permeability. It should not be concluded, however, that the intramembranous particles which are aggregated by vasopressin are necessarily actual sites for water transfer. Nevertheless this possibility should be seriously considered, especially since the water permeability barrier altered by vasopressin is specifically located in granular cell luminal membranes (21, 22) and, in the case of red cell membranes, intramembranous particles apparently can represent sites for water passage (23).

ACKNOWLEDGMENTS

We thank Ms. C. Uiterwyk and Mr. R. Worthington for providing technical assistance and Mrs. P. Collins for typing this manuscript.

This research was supported by grant SI-74-26 from the Central Clinical Investigations Committee of the U. S. Public Health Service, Bureau of Medical Services, Division of Hospitals and Clinics, grants AM-18710 and HL-13979 from the National Institutes of Health, and a grant-in-aid from the New York Heart Association.

REFERENCES

1. Branton, D. 1966. Fracture faces of frozen membranes. *Proc. Natl. Acad. Sci. U. S. A.* **55:** 1048–1056.
2. Branton, D., S. Bullivant, N. B. Gilula, M. J. Karnovsky, H. Moor, K. Mühlethaler, D. H. Northcote, L. Packer, B. Satir, P. Satir, V. Speth, L. A. Staehelin, R. L. Steere, and R. S. Weinstein. 1975. Freeze-etching nomenclature. *Science (Wash. D. C.).* **190:** 54–56.
3. MacLennan, D. H., P. Seeman, G. H. Iles, and C. C. Yip. 1971. Membrane formation by adenosine triphosphatase of sarcoplasmic reticulum. *J. Biol. Chem.* **246:** 2702–2710.
4. Pinto da Silva, P., S. D. Douglas, and D. Branton. 1971. Localization of A antigen sites on human erythrocyte ghosts. *Nature (Lond.).* **232:** 194–196.
5. Tillack, T. W., R. E. Scott, and V. T. Marchesi. 1972. The structure of erythrocyte membranes studied by freeze-etching. II. Localization of receptors for phytohemagglutinin and influenza virus to the intramembranous particles. *J. Exp. Med.* **135:** 1209–1227.
6. Tourtellotte, M. E., and J. S. Zupnik. 1973. Freeze-fractured *Acholeplasma laidlawii* membranes: nature of particles observed. *Science (Wash. D. C.).* **179:** 84–86.
7. Chevalier, J., J. Bourguet, and J. S. Hugon. 1974. Membrane associated particles: distribution in frog urinary bladder epithelium at rest and after oxytocin treatment. *Cell Tissue Res.* **152:** 129–140.
8. Kachadorian, W. A., J. B. Wade, and V. A. DiScala. 1975. Vasopressin: induced structural change in toad bladder luminal membrane. *Science (Wash. D. C.).* **190:** 67–69.
9. Bourguet, J., J. Chevalier, and J. S. Hugon. 1976. Alterations in membrane-associated particle distribution during antidiuretic challenge in frog urinary bladder epithelium. *Biophys. J.* **16:** 627–639.
10. Kachadorian, W. A., J. B. Wade, C. C. Uiterwyk, and V. A. DiScala. 1977. Membrane structural and functional responses to vasopressin in toad bladder. *J. Membr. Biol.* **30:** 381–401.
11. Levine, S. D., R. D. Levine, R. E. Worthington, and R. M. Hays. 1976. Selective inhibition of osmotic water flow by general anesthetics in toad urinary bladder. *J. Clin. Invest.* **58:** 980–988.
12. Levine, S., N. Franki, and R. M. Hays. 1973. Effect of phloretin on water and solute movement in the toad bladder. *J. Clin. Invest.* **52:** 1435–1442.
13. Bentley, P. J. 1958. The effects of neurohypophysial

extracts on water transfer across the wall of the isolated urinary bladder of the toad *Bufo marinus*. *J. Endocrinol.* **17:** 201–209.

14. Walser, M. 1969. Reversible stimulation of sodium transport in the toad bladder by stretch. *J. Clin. Invest.* **48:** 1714–1723.
15. Snedecor, G. W., and W. G. Cochran. 1967. Statistical Methods. Iowa State University Press, Ames, Iowa. 6th edition. 593.
16. Taylor, A., R. Maffly, L. Wilson, and E. Reaven. 1975. Evidence for involvement of microtubules in the action of vasopressin. *Ann. N. Y. Acad. Sci.* **253:** 723–737.
17. Kachadorian, W. A. 1976. The effect of colchicine on vasopressin (ADH)-induced intramembranous particle aggregation and water flow in toad bladder. *Kidney Int.* **10:** 587. (Abstr.)
18. Orloff, J., and J. S. Handler. 1962. The similarity of effects of vasopressin, adenosine 3′,5′-phosphate (cyclic AMP), and theophylline on the toad bladder. *J. Clin. Invest.* **41:** 702–709.
19. Handler, J. S., R. W. Butcher, E. W. Sutherland, and J. Orloff. 1965. The effect of vasopressin and of theophylline on the concentration of adenosine 3′,5′-phosphate in the urinary bladder of the toad. *J. Biol. Chem.* **240:** 4524–4526.
20. Levine, S. D., D. Schlondorff, R. E. Worthington, H. Weber, and R. M. Hays. 1976. Inhibition of vasopressin-stimulated adenylate cyclase by general anesthetics: its role in control of water permeability in toad urinary bladder. *Clin. Res.* **24:** 469A. (Abstr.)
21. DiBona, D. R., M. M. Civan, and A. Leaf. 1969. The cellular specificity of the effect of vasopressin on toad urinary bladder. *J. Membr. Biol.* **1:** 79–91.
22. Civan, M. M., and D. R. DiBona. 1974. Pathways for movement of ions and water across toad urinary bladder. II. Site and mode of action of vasopressin. *J. Membr. Biol.* **19:** 195–220.
23. Pinto da Silva, P. 1973. Membrane intercalated particles in human erythrocyte ghosts: sites of preferred passage of water molecules at low temperature. *Proc. Natl. Acad. Sci. U. S. A.* **70:** 1339–1343.