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Metabolic Dependence of the Offset of Antidiuretic Hormone-Induced Osmotic Flow of Water Across the Toad Urinary Bladder

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Summary. The elevated osmotic permeability to water induced by antidiuretic hormone (ADH) in the isolated urinary bladder of the toad is rapidly reversed by removal or washout of the ADH. This return to normal water permeability is delayed by the suppression of production of metabolic energy by any of three maneuvers: (i) low temperature (2 °C); (ii) inhibition of oxidative phosphorylation (10 mM azide or 0.5 mM 2,4 dinitrophenol); or (iii) inhibition of glycolysis (10 mM iodoacetate or 10 mM 2-deoxyglucose). Moreover, exposure to cytochalasin B, 2.1×10^{-5} M, either before or after initiation of the hormonal effect also delays the return of water permeability to normal following removal of ADH. When considered within constraints imposed by models which predict ADH's action on water permeability to be either via modulation of the fluidity of lipids in the membrane or via the figuration of proteins ("pores") in the lipid membrane, these observations on the inhibition of the reversal of ADH stimulation of water flow are more consistent with the protein (pore) theory and place limitations on the mechanisms by which proteins in such pores can return to the resting or impermeable state.

The isolated urinary bladder of the toad responds to the application of antidiuretic hormone (ADH) with an increase in water permeability (Hays & Leaf, 1962), amide permeability (Leaf & Hays, 1962), and active transport of sodium (Leaf, Anderson & Page, 1958). Although impressive evidence indicates that 3′–5′-cyclic adenosine monophosphate is the intracelluar messenger for the ADH response (Handler & Orloff, 1973), the sequence of biochemical and molecular steps subsequent to the generation of the cyclic-AMP remains to be elucidated fully.

The regulation by ADH of osmotic water permeability is believed to occur at the apical or urinary plasma membrane (DiBona, Civan & Leaf, 1969; Civan & Dibona, 1974) of the granular rich cells, the predominant cell type in this tissue. The locus for osmotic regulation within

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this apical membrane might reside in either the lipids composing the membrane or in the proteins in the lipid membrane. Accordingly, two major hypotheses have been advanced to account for the effect of ADH on water permeability (reviewed by Andreoli & Schafer, 1976): the fluidity modulation hypothesis, which holds that water permeability is regulated by the packing, orientation, structure, or motion of phospholipids in the apical membrane (Petras & Wright, 1975); and the pore hypothesis, which holds that water traverses the apical membrane via protein-lined pores or via aqueous channels (Finkelstein, 1976), possibly in protein.

We recently tested the fluidity modulation hypothesis by estimating the microviscosity of toad urinary bladder membranes; we found that the change in microviscosity produced by cyclic AMP was unlikely to be large enough to account for the large change in water permeability known to be produced in this tissue (Masters, Yguerabide & Fanestil, 1978). This finding and concurrent studies by others (Finkelstein, 1976) suggest that attention should be focused on the proteins in the apical plasma membrane as the more likely molecular locus for the formation and dissolution of aqueous channels involved in regulation of water permeability. Indeed, impressive structural studies, accomplished with freeze-fracture techniques, provide evidence that aggregates or clusters of intramembranous particles (protein) appear to be specifically related to the effect of ADH upon water permeability (Wade, DiScala & Karnovsky, 1975; Chevalier, Bourquet & Hugon, 1974). Available circumstantial evidence incriminates these aggregates in the apical plasma membrane of granular rich cells as the most likely locus of "pores" involved in osmotic flow of water (Wade, Kachadorian & DiScala, 1977).

Since the onset and the offset of the action of ADH on water permeability occurs within minutes of the addition (Edelman, Petersen & Gulyassy, 1964) or removal (Bourquet *et al.*, 1975) of the hormone, the mechanisms involved in the rapid formation and dissolution of these aggregates will be required for an understanding of the sequence of events involved in the action of the hormone. Prior studies with agents which alter microtubule function or attack microfilaments (Taylor *et al.*, 1973, 1975; Davis *et al.*, 1974; DeSousa, Grosso & Rufener, 1974) suggest that these intracellular filamentous structures might be involved in the formation of the aggregates. Earlier studies with inhibitors of energy production had also suggested that metabolic energy was necessary for the onset of action of ADH on water flow (Handler, Petersen & Orloff, 1966). In addition, the direct relationship between temperature and water flow has been studied in tissues where water permeability was "fixed"

with glutaraldehyde (Eggena, 1972). In contrast, events involved in the offset of action of ADH have not been evaluated. Therefore, we report our initial studies designed to evaluate possible mechanisms involved in the offset of action of ADH on water permeability and, by inference, upon the disappearance of the intramembranous aggregates postulated to be involved in the pores. We base the interpretation of our studies on the fate of intramembranous particles in the plasma membrane of lymphocytes, where membrane proteins may undergo lateral migration, cluster, or be endocytosed (Schreiner & Unanue, 1976; Silverstein, Steinman & Cohn, 1977).

Materials and Methods

Toads, *Bufo marinus*, from the United States were purchased from College Biological Supply, Escondido, California, and were stored at room temperature on moist peat moss. Animals were doubly pithed and the urinary bladders were excised into a standard Ringer's solution which contained (in mM/liter): NaCl, 111; KCl, 3.35; $NaHCO_3$, 2.4; $CaCl_2$, 2.7. Osmotic flow of water was determined gravimetrically (the reproducibility of weighing was ± 5 mg) according to Bentley (1958) in periods of 30 min. Paired hemibladders were mounted as bags (mucosa inside) on the ends of hollow glass cannulae, washed three times with Ringer's solution, and filled with 5 ml of Ringer's solution which had been diluted 1:5 with distilled water (1/5 Ringer's). The bags were suspended in 40 ml of aerated Ringer's solution. Unless indicated otherwise, the following protocol was employed. Basal water flow in the absence of experimental intervention was measured for one or two consecutive 30-min periods. Basal water flow averaged 0.063 g per 30 min. Where indicated, ADH (20 mU/ml; arginine vasopressin from Sigma Chemical Co., St. Louis, Missouri) was added to the serosal side of hemibladders and water flow was measured for one subsequent 30-min period. In two sham experiments, to determine if prolonged exposure to inhibitors alone altered osmotic water permeability, ADH was omitted and 2-deoxyglucose or 2,4-dinitrophenol was added in the concentrations and at the times as though ADH had actually been present. Following the 30-min exposure to ADH (or sham exposure to ADH), the solution inside the bags was replaced with 5 ml 1/5 Ringer's and the ADH was washed out by replacing the serosal solution twice with 40 ml of Ringer's solution. During the washout of ADH one of each pair of hemibladders served as the control and the other hemibladder was exposed to low temperature, azide, 2,4-dinitrophenol, 2-deoxyglucose, iodoacetate or cytochalasin B. In the experiments with low temperature, the effect of ADH was generated at room temperature but only the experimental hemibladders were washed with Ringer's solution at 2 °C and maintained at 2 °C by submerging the incubation beakers in an ice bath. In the experiments with azide, 10 mM sodium azide (Sigma Chemical Co.) and 5 mM glucose were included in the 1/5 Ringer's solution on the mucosal side and in the standard Ringer's solution on the serosal side of the experimental hemibladders during and subsequent to the second of two 30-min periods of basal water flow. The control tissues received only 5 mM glucose. In the experiments with dinitrophenol, 0.5 mM 2,4-dinitrophenol (Sigma Chemical Co.) was added to the serosal solution during the last 5 min of the ADH exposure and was present in both mucosal and serosal solutions during the washout. In the experiments with 2-deoxyglucose, 10 mM 2-deoxyglucose (Sigma Chemical Co.) was added to both mucosal and serosal solution beginning 5 min after addition of ADH. In the experiments with iodoacetate, 10 mM sodium iodoacetate (Calbio-

chem Corp., La Jolla, Calif.) was added to all mucosal and serosal solutions to which the experimental hemibladders were exposed, beginning 5 min after the addition of ADH. In the experiments with cytochalasin B, 2.1×10^{-5} M cytochalasin B (Sigma Chemical Co.) was added to the serosal solution beginning 15 min before or 5 min after the addition of ADH.

In one set of experiments, the flux of ^{14}C-*n*-butyramide (American Radiochemical Corporation, Sanford, Fla.) from mucosa to serosa was examined in tissues mounted as a sheet (1 cm^2) between Lucite chambers containing 10 ml Ringer's solution bubbled with air and vigorously stirred with magnetic stirrers. (The magnetic stirring "bars" and stirrer were those supplied for use with an oxygen electrode system by Yellow Springs Instruments, Yellow Springs, Ohio.) From 30–45 min after 1.31×10^{-6} M ^{14}C-*n*-butyramide was added to the mucosal solution, the first of several 30 min flux periods was initiated. At the beginning and end of each flux period aliquots were removed from the mucosal (100 µl sample) and serosal (1 ml sample) solutions and the volume on both sides was restored to the original 10 ml with fresh Ringers. Radioactivity was quantitated by liquid scintillation spectrometry with an efficiency of $85 \pm 2\%$. The flux of *n*-butyramide was determined in paired tissues at room temperature for two 30-min periods in the absence of ADH. Since there was no difference in the flux rate in these 2 periods, the values were averaged and taken as the basal flux in the absence of ADH. ADH (20 mU/ml) was added to the serosal solutions of both hemibladders at the end of the second 30-min basal flux period. Thirty minutes later one of each pair of hemibladders (experimental) was rapidly chilled by placing the entire flux chamber in a -30 °C freezer until the temperature was < 5 °C, when the chamber was transferred to a cold room (~ 5 °C). The aeration and stirring of solutions was interrupted only while the chamber was in the freezer. The flux of ^{14}C-*n*-butyramide was again determined for two 30-min periods, the results of which were again averaged because the values were not different in the two periods. The paired (control) hemibladder was treated in a manner identical to the experimental tissue except that all maneuvers were conducted at room temperature (~ 23 °C). Permeability to *n*-butyramide was calculated and expressed as 10^{-5} cm/sec. Since unstirred layer corrections were not necessary unless the permeability was about 10-fold greater than that of *n*-butyramide (Pietras & Wright, 1975), corrections for unstirred layer effects were not made.

Results were calculated in absolute terms (grams of water flow per hemibladder per 30 min). All values are means $\pm$ SEM. Statistical significance was calculated using Student's *t*-test for paired observations with a *P* value of 0.05 or less as the criterion of significance.

Results

The Effect of Low Temperature on the Offset of ADH-Induced Osmotic Flow of Water

Exposure of tissues stimulated at room temperature by ADH to solutions lacking ADH at 2 °C significantly retarded the offset of hormone-induced osmotic water flow. The absolute rate (Table 1, expt. *A*) of water flow was significantly greater ($P = 0.001$) in the cold-exposed tissues during the first and second 30-min periods after initiation of washout of ADH.

Table 1. Effects of inhibitors of energy production on absolute rate of osmotic water flow during offset of ADH stimulation[a]

Expt.	Condition	Time (min)[b]			
		0–30	30–60	60–90	90–120
A	Control $n=8$	0.99 ± 0.057	0.56 ± 0.043	0.23 ± 0.038	0.17 ± 0.026
	2 °C	1.12 ± 0.10	0.89 ± 0.010[e]	0.40 ± 0.020[e]	0.24 ± 0.029
B	Control $n=9$	1.42 ± 0.078	0.53 ± 0.054	0.19 ± 0.030	0.10 ± 0.018
	Azide	1.08 ± 0.088[e]	0.79 ± 0.095[c]	0.51 ± 0.070[e]	0.30 ± 0.040[e]
C	Control $n=11$	1.16 ± 0.11	0.43 ± 0.049	0.18 ± 0.047	0.15 ± 0.032
	Dinitrophenol	1.03 ± 0.10	0.64 ± 0.113	0.43 ± 0.074[d]	0.30 ± 0.043[d]
D	Control $n=9$	1.09 ± 0.041	0.30 ± 0.113	0.14 ± 0.020	0.08 ± 0.013
	Deoxyglucose	0.87 ± 0.10[c]	0.47 ± 0.056[d]	0.28 ± 0.068[c]	0.22 ± 0.053[c]
E	Control $n=8$	1.44 ± 0.056	0.56 ± 0.032	0.20 ± 0.022	0.10 ± 0.017
	Iodoacetate	1.40 ± 0.067	0.91 ± 0.07[e]	0.39 ± 0.0031[e]	0.19 ± 0.013[c]

[a] Values are grams per 30 min per hemibladder, expressed as mean ± SEM.
[b] Antidiuretic hormone (20 mU/ml) present during 0–30 min interval only. Other experimental details in text.
[c] $P<0.05$.
[d] $P<0.025$.
[e] $P<0.01$.

The Effect of Inhibitors of Oxidative Metabolism on the Offset of ADH-Induced Osmotic Flow of Water

The presence of 10 mM sodium azide produced no effect on basal water flow (data not shown), but significantly ($P<0.01$) reduced the ADH-induced water flow by about one-fourth (Table 1, expt. *B*). Despite inhibition of water flow in the presence of ADH, azide significantly retarded the offset of the action of ADH. The absolute water flow was significantly greater in the azide-treated tissues in all three 30-min periods after initiation of washout of ADH (Table 1, expt. *B*).

Addition of 0.5 mM 2,4-dinitrophenol, beginning 25 min after addition of ADH, failed to significantly increase ($P=0.10$) water flow during the first 30 min after removal of hormone (Table 1, expt. *C*). However, the osmotic water flows in the second and third 30-min periods after

washout of ADH were about two-fold greater in the inhibitor-treated tissues than in the controls ($P=0.01$). In a sham experiment where all maneuvers were the same, except ADH was omitted, 0.5 mM 2,4-dinitrophenol did not alter osmotic water flow. The water flow during the third washout period (90–120 min after sham addition of the hormone) was 0.033 ± 0.0057 g in the controls and 0.053 ± 0.013 g in the tissues exposed to dinitrophenol (mean difference was 0.020 ± 0.013, $n=6$).

The Effect of Inhibitors of Glycolysis on the Offset of ADH-Induced Osmotic Flow of Water

2-Deoxyglucose (10 mM), added 5 min after ADH, reduced the osmotic flow during the period of exposure to ADH by about 20% ($P=0.05$). Despite inhibition of water flow in the presence of ADH, deoxyglucose significantly retarded the return of water flow to normal during all three periods (Table 1, expt. *D*). In a sham experiment, where all maneuvers were the same, except ADH was omitted, 10 mM deoxyglucose did not alter osmotic water flow. The water flow during the third washout period (90–120 min after sham addition of the hormone) was 0.034 ± 0.0099 g in the controls and 0.047 ± 0.011 g in the tissues exposed to 2-deoxyglucose ($P=0.4$, $n=6$).

Since exposure of tissues to 10 mM iodacetate simultaneously with the addition of ADH produced nearly complete inhibition of hormone-stimulated water flow (data not shown), the iodoacetate was added 5 min after addition of ADH. The absolute rate of water flow was greater in the iodoacetate treated tissues during all three 30-min periods after initiation of washout (Table 1, expt. *E*).

The Effect of Cytochalasin B on the Offset of ADH-Induced Osmotic Flow of Water

The addition of cytochalasin B (2.1×10^{-5} M) to the serosal solutions 5 min after ADH failed to diminish the osmotic flow of water during the 30-min exposure to ADH; however, addition of the cytochalasin B 15 min prior to ADH diminished the subsequent hormone-induced water flow by 28% (Table 2). Regardless of the time of addition, the return of water flow to lower levels was significantly retarded during all three washout periods (Table 2).

Table 2. Effect of cytochalasin B on absolute rate of osmotic water flow during offset of ADH stimulation[a]

Expt.[c]	Condition	Time (min)[b]			
		0–30	30–60	60–90	90–120
A	Control $n=6$	1.03 ± 0.11	0.31 ± 0.042	0.16 ± 0.027	0.11 ± 0.020
	Cytochalasin B	0.91 ± 0.74	0.57 ± 0.071[e]	0.30 ± 0.037[d]	0.20 ± 0.024[e]
B	Control $n=8$	1.66 ± 0.051	0.32 ± 0.041	0.12 ± 0.010	0.074 ± 0.020
	Cytochalasin B	1.19 ± 0.074[b]	0.49 ± 0.051[e]	0.25 ± 0.025[f]	0.15 ± 0.015[d]

[a] Values are grams per 30 min per hemibladder, expressed as mean ± SEM.
[b] Antidiuretic hormone (20 mU/ml) present during 0–30 min interval only.
[c] In expt. to *A*, cytochalasin B was added 5 min after ADH, while the addition was 15 min before ADH in expt. *B*. Other experimental details in text.
[d] $P<0.05$.
[e] $P<0.025$.
[f] $P=<0.01$.

Table 3. Effect of antidiuretic hormone and temperature on permeability to *n*-butyramide

Temperature[a]	Before ADH[b]	After ADH[b]	After / Before
~23 °C	2.30 ± 0.150	3.14 ± 0.113	1.38 ± 0.101[c]
~5 °C	2.48 ± 0.361	0.720 ± 0.106[d]	0.294 ± 0.0356[d]

[a] The temperature was ~23 °C before ADH for all tissues.
[b] Values are 10^{-5} cm/sec.
[c] Significantly different from 1.00; $P=0.05$.
[d] Significantly different from tissues at ~23 °C; $P<0.001$.

The Effect of Low Temperature on ADH-Induced Increase in Permeability to n-Butyramide

At room temperature, ADH significantly increased the permeability to *n*-butyramide by $38 \pm 10\%$ (Table 3), a finding previously reported (Pietras & Wright, 1975).[1] When tissues which had been exposed to ADH for 30 min at room temperature were cooled to ~5 °C (still in

[1] Although we confirm the finding that ADH increases permeability to *n*-butyramide, the basal permeability we measured was about fivefold greater than that reported (Pietras & Wright, 1974). We cannot explain this discrepancy.

the presence of ADH), the permeability to *n*-butyramide decreased to 30% of the value before ADH and to only 21% of the value in paired ADH-treated tissues at room temperature (Table 3).

Discussion

Suppression of the production of metabolic energy by (i) low temperature (Hays, Franki & Soberman, 1971), (ii) inhibitors of oxidative metabolism (Handler et al., 1966), or (iii) glycolysis (Handler et al., 1966) is known to suppress the onset or stimulation of osmotic flow of water produced by ADH. Since both the onset and, as shown in the present study, offset of water flow are inhibited by these maneuvers, it is not likely that the agents act directly on water permeability. Therefore, we infer that inhibition of metabolic energy production alters the action of ADH via some effect(s) indirectly related to water permeability. Although it is possible to postulate some new action of inhibitors of metabolism which indirectly affects water flow, it is more reasonable to deduce that the agents act by their known common mechanism – namely, inhibition of the production of energy. Therefore, we tentatively conclude that the offset of ADH-induced osmotic flow of water is dependent upon metabolic energy.

Conclusions concerning the role of metabolic energy in the molecular events leading to the onset and offset of action of ADH are less certain. Nevertheless, the probable rquirement for metabolic energy imposes constraints upon both the fluidity modulation hypothesis and the pore hypothesis concerning water flow. (*See* the introduction for a fuller definition of these hypotheses.) Low temperature produces a decrease in the molecular motion of phospholipids in artificial bilayers and in biological membranes (Chapman, 1975). Using polarization fluorescence of a lipophilic probe (Masters *et al.*, 1978), we have unpublished evidence that temperature alters the microviscosity of toad bladder cells in the expected fashion: when the temperature was changed from 35 to 25 °C, the microviscosity increased from 2.6 to 3.8 poise. Thus, lowering the temperature of solutions bathing the isolated urinary bladder of the toad from room temperature (20–25° C) to 2 °C should have produced an increase in the microviscosity (decrease in fluidity) of the cellular plasma membranes. This inference is confirmed by our finding that cold decreases the permeability of ADH-stimulated toad bladder to *n*-butyramide by 70–80% (Table 3). In addition, these results also exclude the possibility that an ADH-mediated

re-arrangement of lipids into a more permeable configuration could be "frozen" by lowering the temperature of the tissues. Thus, there is strong evidence to support the inference that cold temperature decreases the fluidity of the toad bladder. Despite this inference and despite the fact that lowering the temperature decreases the rate of osmotic flow of water in toad bladders with "fixed" water permeability (Eggena, 1972), in our studies the flow of water decreased more slowly at 2° C than at room temperature (Table 1, expt. *A*). Such findings are contrary to those predicted by a fluidity modulation hypothesis in which the hormonal action on flow of water is regulated by packing, orientation or mobility of membrane lipids.

Therefore, the energy dependence and inhibition by low temperature of the offset of ADH-induced osmotic water permeability is more consistent with a "pore" hypothesis. If water does traverse the membrane in protein pores such as might exist in the aggregates of intramembranous particles (AIMP) seen on freeze-fracture electron microscopy (Kachadorian, Wade & DiScala, 1975), one can postulate four possible fates for AIMP during the offset of action of ADH: (i) AIMP could be proteolytically destroyed, (ii) AIMP could diffuse apart laterally in the plane of the membrane in a random fashion, (iii) AIMP could be anchored to sub-plasma membrane component(s), such as microfilaments, which disperse the particles in the plane of the membrane, or (iv) AIMP could be endocytosed or internalized by the cell. Our finding that the offset of ADH-induced water flow depends upon metabolic energy enables tentative elimination of the first two possibilities, for they are processes which do not depend upon the proximate production of metabolic energy. These considerations led us to perform the experiments with cytochalasin B, for this agent impairs migration of particles in the plane of the plasma membrane (Schreiner & Unanue, 1976) and inhibits endocytosis (Axline & Reaven, 1974; Silverstein *et al.*, 1977). Thus, possibilities *iii* and *iv*, mentioned above, are supported by the finding that cytochalasin B inhibits the offset of ADH-induced water flow (Table 2). Unfortunately, our findings do not enable differentiation of an energy-dependent, cytochalasin B-sensitive redistribution or disperion of particles within the plane of the membrane, analogous to the aggregation or "caps" of antigen-antibody complexes in lymphocytes (Schreiner & Unanue, 1976), from an energy-dependent, cytochalasin B-sensitive endocytosis or internalization of AIMP, analogous to the endocytosis or membrane internalization in lymphocytes (Schreiner & Unanue, 1976). To conclude, our findings that metabolic energy is required

for and that cytochalasin B inhibits the offset of ADH-induced osmotic flow of water are consistent with recent biophysical (Masters *et al.*, 1978; Finkelstein, 1976) and structural (Chevalier, Bourquet & Hugon, 1974; Wade *et al.*, 1977) studies suggesting that ADH increases water permeability via a protein-pore mechanism. Moreover, these findings place constraints upon the potential pathways for reversal of the protein-pores to the basal, water-impermeable condition.

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