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Ionic and Metabolic Requirements for the Hydroosmotic Response to Antidiuretic Hormone in Toad Urinary Bladder

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Summary. A study has been conducted to determine the ionic and metabolic requirements for full expression of the hydroosmotic response to antidiuretic hormone in the toad urinary bladder. By appropriate manipulation of incubation conditions it can be shown that there is a pool of serosal sodium necessary for a full hormone response. This serosal sodium pool is not related to the transepithelial sodium transport pool. A full hydroosmotic response also requires serosal potassium; however, no specific anion requirement was demonstrated. Additionally, anaerobic or aerobic metabolism support a full hydroosmotic response equally well.

The toad urinary bladder is used extensively as a simple *in vitro* model for the mammalian distal nephron (Leaf, 1965). Antidiuretic hormone (ADH) increases water reabsorption across this tissue by increasing both the rate of ^3H_2O diffusion and bulk water flow down an osmotic gradient. In addition ADH simultaneously stimulates active transcellular sodium transport as measured by short-circuit current (SCC). In a previous study (Davis *et al.*, 1974) we observed that the macrolide antibiotic cytochalasin B inhibited the hydroosmotic response to ADH without altering basal bulk water permeability or ^3H_2O diffusion, or the increase in ^3H_2O diffusion induced by ADH. Additionally, although cytochalasin increased ^{22}Na, ^{36}Cl, and ^{14}[C]-urea fluxes and decreased transepithelial potential, it did not alter basal or ADH-induced increase in SCC. These results suggested that there may be a relation between sodium translocation, distinct from transepithelial sodium transport, and bulk fluid movement. In the present study we have further defined the role of ion metabolism in supporting bulk transepithelial fluid movement.

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Materials and Methods

Large female toads (*Bufo marinus*) were purchased from National Reagents, Bridgeport, Conn., and maintained on moist bedding before use. Paired hemibladders, removed from doubly pithed toads, were initially incubated in a phosphate buffered Ling-Ringer's solution of the following composition (mM): NaCl, 92.7; KCl, 2.5; $CaCl_2$, 1.0; $MgSO_4$, 1.2; $NaHCO_3$, 7.8; NaH_2PO_4, 2.0; NaH_2PO_4, 1.2; pH 7.4–7.6; osmolarity 200–220 mosmol/kg H_2O (Ling, 1962). In experiments where $[Na^+]$ was altered in the incubation medium, KCl was not added, 5.0 mM Tris-HCl was used as a buffer, and 2.5 mM $KHCO_3$ replaced the $NaHCO_3$. An appropriate amount of NaCl was added and the buffer adjusted to 210 mosmol/kg H_2O by addition of choline chloride. Potassium-free buffer was prepared by deletion of KCl from the Ling-Ringer's buffer described above. For experiments in which the anion composition of the buffer was altered, 10 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (Sigma, St. Louis, Mo., (HEPES)) was utilized as buffer, and chloride-, phosphate- and sulfate-free buffer was prepared utilizing 2.5 mM $KHCO_3$ and 1.0 mM $CaCO_3$. Buffer osmolarity was adjusted by the addition of sodium cyclamate (Sigma). Bicarbonate-free buffer was prepared by deletion of $NaHCO_3$ from the buffer and bubbling the air used to aerate the incubation media through 0.5 M NaOH rather than deionized water.

For bulk water permeability studies, hemibladders were mounted on hollow glass rods (Bentley, 1958). The mucosal surface was bathed in 3 ml of the appropriate dilute (1:5) buffer or deionized water. The rod and attached hemibladder were incubated in a vessel containing 15 ml of continuously aerated buffer. Water movement down the imposed osmotic gradient was determined gravimetrically. To provide for equilibration with buffers of varying ionic composition or with metabolic inhibitors, all tissue was preincubated for 1 hr with 3 buffer changes (both mucosal and serosal) during the preincubation period.

The ^3H_2O (New England Nuclear, Cambridge, Mass.) flux determinations were carried out on tissue mounted between the halves of double compartment Lucite chambers (Goodman, Allen & Rasmussen, 1969). Each chamber was filled with 6 ml of the appropriate full strength buffer. After a 1-hr preincubation period with 3 buffer changes, ^3H_2O (10^6 cpm) was added to the mucosal side of the tissue. Flux was determined by sampling the serosal chamber every 20 min. After each 20-min period both mucosal and serosal bathing solutions were changed and ^3H_2O readded. After base line flux rates were established, arginine vasopressin (AVP-Pitressin, Parke Davis) was added and further flux rates determined. Radioactivity in buffer samples (200 μl aliquots) was measured in a model 3385 Packard Liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.) in a toluene-methyl cellusolve counting solution. Ouabain and acetazolamide were obtained from Sigma Chemical Company, St. Louis, Mo.; 8-(*p*-chlorophenylthio)adenosine-3′,5′-cyclic-monophosphoric acid (8 CPT-cAMP) from ICN Pharmaceuticals, Cleveland, Ohio.

Results

Deletion of serosal Na^+ and choline substitution has no effect on baseline water movement. However, after the addition of AVP (65 mU/ml) there is a reversible reduction in the hydroosmotic response (Fig. 1). This effect of choline substitution is observed when either 1:5 strength Ling-Ringer's buffer or deionized water is present in the mucosal bath. Additionally, substitution of deionized water for dilute Ling-Ringer's buffer in the mucosal bath had no effect on the hydroosmotic response

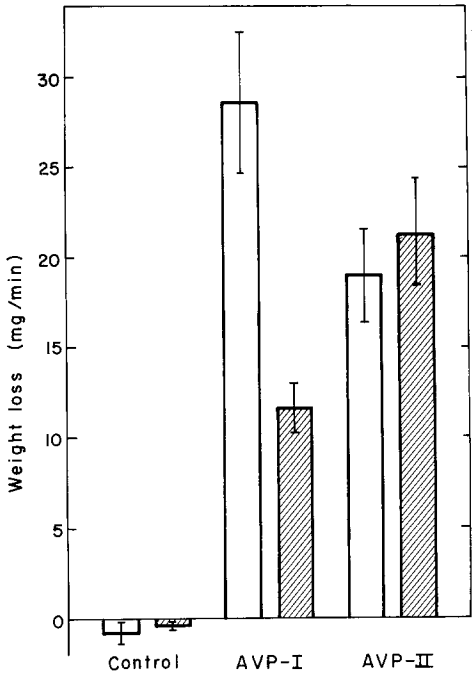

Fig. 1. The effect of replacement of serosal sodium by choline on the hydroosmotic response to AVP. Matched hemibladders were preincubated in a serosal bathing solution containing either sodium (□) or choline (▨) and a mucosal bathing solution consisting of deionized water. During the preincubation both the mucosal and serosal buffers were changed every 20 min. At the end of the preincubation a 20-min basal weight loss was determined. AVP (65 mU/ml) was then added to the serosal bathing solution of all hemibladders, and weight loss was determined over the next 40 min. At the end of this period the bathing solutions were again changed. Sodium-containing buffer was used in all serosal and deionized water in all mucosal bathing solutions. After three 20-min washes, water permeability had returned to basal rates and AVP (65 mU/ml) was again added and weight loss determined. As shown during the first exposure to AVP the hydroosmotic response in choline-exposed hemibladders was reduced. This reduction was reversible since a comparable AVP response was observed when both sets of hemibladders were incubated in sodium-containing serosal bathing solution ($n=8$; data are expressed as mean ± SEM)

when either choline or sodium was present in the serosal bath. When the serosal [NaCl] is varied from 0 to 90 mM, there is a striking increase in the hydroosmotic response as the [NaCl] is increased from 20 to 25 mM. Between 0 and 20 mM NaCl there is no apparent effect of increasing [NaCl], and there is no further effect of increasing [NaCl] above 25 mM (Fig. 2). Deletion of serosal K^+ also had a profound inhibitory effect upon the hydroosmotic response to ADH (Fig. 3). In the absence of K^+ the AVP-induced increase in bulk water flow was reduced by approximately 50%. The inhibitory effect of either serosal Na^+ or K^+

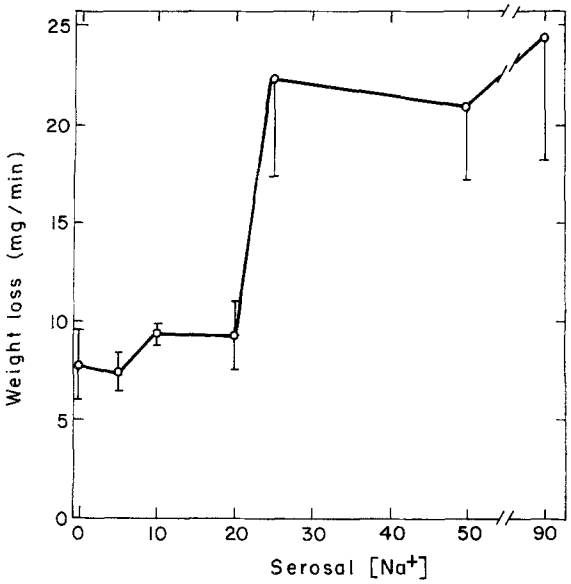

Fig. 2. The effect of increasing serosal sodium concentration on the hydroosmotic response to AVP. Hemibladders were then preincubated for 1 hr with three buffer changes in serosal bathing solutions containing increasing [Na^+]. AVP (65 mU/ml) was then added and weight loss determined over the next 40 min. A minimum of 5 hemibladders were used for each experimental point. (Data are expressed as the mean ± SEM)

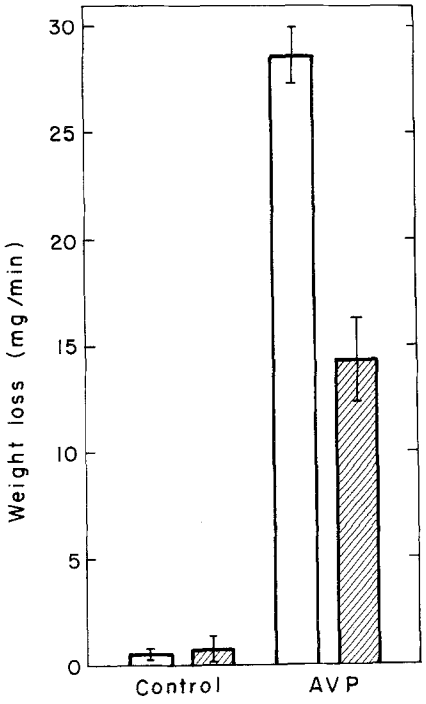

Fig. 3. The effect of serosal potassium deletion on the hydroosmotic response to AVP. Matched hemibladders were preincubated for 1 hr in a serosal bathing solution containing 2.5 mM KCl (□) or one without added potassium (▨). A basal 20-min weight loss period was recorded, and then AVP (65 mU/ml) was added to all hemibladders. Weight loss was then determined over the next 40 min. ($n=8$; data are expressed as the mean ± SEM)

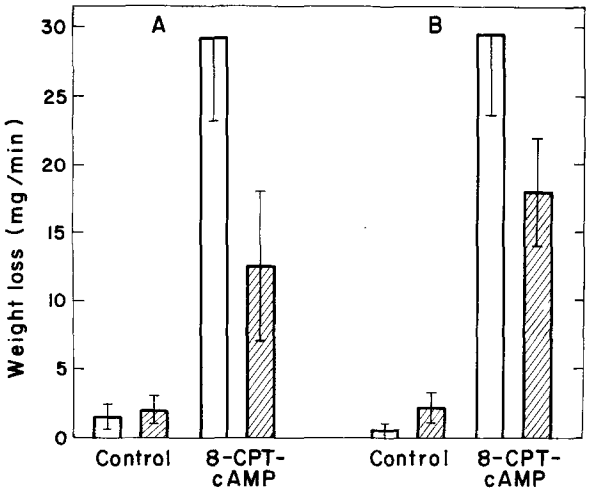

Fig. 4. The effect of replacement of serosal sodium by choline (*A*) or deletion of serosal potassium (*B*) on the hydroosmotic response to 8-CPT-cAMP. Matched hemibladders were preincubated as described in Figs. 1 and 3, respectively. [(*A*): □, serosal sodium; ▨, serosal choline; (*B*): □, 2.5 mM serosal KCl; ▨, no K^+]. At the end of the preincubation, a 20-min basal weight loss was determined and 8-CPT-cAMP (5×10^{-5} M) then added to all hemibladders. Weight loss was then determined over the next 40 min. [(*A*): $n=8$; (*B*): $n=6$; data are expressed as the mean ± SEM]

deletion on the hydroosmotic response is also observed when 8 CPT-cAMP, rather than AVP, is used to induce the hydroosmotic response. In both cases the degree of inhibition of the hydroosmotic response is comparable to the inhibition observed when AVP is employed (Fig. 4).

To determine if the inhibition of the hydroosmotic response in the absence of serosal Na^+ or K^+ were due to a failure of the hormone to induce its effect upon mucosa permeability, we investigated the effect of choline substitution for Na^+ or K^+ deletion on ^3H_2O diffusion. As shown in Fig. 5, deletion of Na^+ had no effect on ^3H_2O permeability in the absence of AVP or on the time course or magnitude of the AVP-induced increase in this parameter. Similarly, deletion of K^+ from the bathing solution did not alter baseline ^3H_2O water permeability or the increase induced by AVP (data not shown).

The serosal anions were replaced with cyclamate, an organic anion. Elimination of chloride, phosphate, and sulfate had no effect on the hydroosmotic response if Na^+ and K^+ were present in the serosal bathing solution (Table 1). Additionally, both deletion of serosal bicarbonate and incubation in the presence of 0.5 mM acetazolamide, a carbonic anhydrase inhibitor, to inhibit endogenous bicarbonate generation

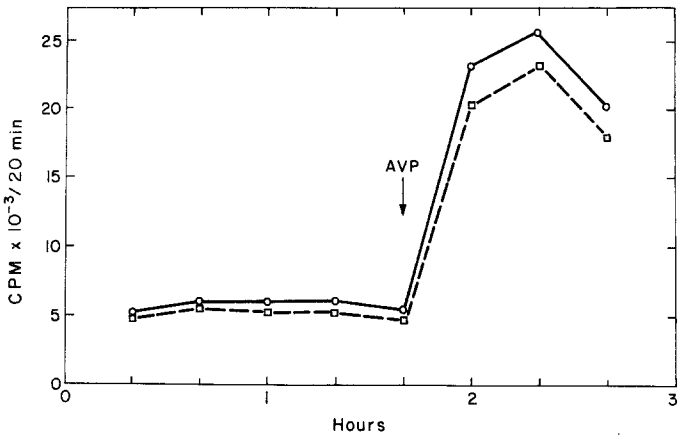

Fig. 5. The effect of replacement of serosal sodium by choline on ^3H_2O diffusion across the toad urinary bladder. After mounting a hemibladder in a double compartment Lucite chamber, one-half was bathed on both the serosal and mucosal surfaces with full strength sodium-containing buffer (□---□) and the other half with full strength choline-containing buffer (○–○–○). After a 1-hr preincubation, ^3H_2O was added to the mucosal bathing solutions, and ^3H_2O appearance was monitored in the serosal chambers. At the indicated time AVP (65 mU/ml) was added. This experiment represents 1 of 4 replicate experiments performed. No significant difference in the response in any experiment was observed when sodium- and choline-containing buffers were compared. Similarly, in 4 experiments no difference was observed when the response of tissue incubated in regular buffer was compared to the response of tissue incubated in the absence of KCl

Table 1. Effect of anion replacement on the hydroosmotic response to AVP[a]

Mucosa	Serosa	Weight loss (mg/min)	
Deionized H_2O	Ling-Ringer's	22.4 ± 1.2	$n=5$
	Na cyclamate	19.0 ± 2.6	
1:5 Ling-Ringer's	Ling-Ringer's	19.8 ± 0.9	$n=5$
	Na cyclamate	20.7 ± 2.8	
Deionized H_2O	Ling-Ringer's	30.5 ± 4.4	
	Ling-Ringer's		$n=6$
	HCO_3-free	34.4 ± 3.0	
	Diamox 5×10^{-4} M		

[a] Hemibladders were preincubated for 1 hr in the appropriate buffers. Basal weight loss was recorded for 20 min. AVP was then added and weight loss determined over the next 40 min. Basal weight loss in all hemibladders was < 1 mg/min. (Data are expressed as the mean $\pm$ SEM).

Table 2. Effect of ouabain and metabolic inhibitors of the hydroosmotic response to AVP[a]

	Weight loss (mg/min)	
Control	25.5±3.2	$n=6$
Ouabain (10^{-4} M)	26.4±2.0	
Control	24.8±3.7	$n=6$
NaCN (1 mM)	27.3±2.3	
Control	21.1±3.2	$n=6$
Iodoacetic acid (0.1 mM)	24.4±2.7	
Control	28.3±2.2	$n=6$
NaCN (1 mM)+ iodoacetic acid (0.1 mM)	6.6±2.2	

[a] A protocol similar to the one employed in Table 1 was used for these experiments.

(Maren, 1967) had no effect on the hydroosmotic response. Finally, incubation of the toad urinary bladder in the presence of the ($Na^+ + K^+$). ATPase inhibitor, ouabain (Skou, 1965), or the metabolic inhibitors, CN^- (Slater, 1967) or iodoacetic acid (Harris, Meriwether & Park, 1963) alone had no effect on the AVP-induced hydroosmotic response (Table 2). However, in the presence of both CN^- and iodoacetate a marked inhibition of the hydroosmotic response was observed.

Discussion

Deletion of serosal sodium or potassium from the toad urinary bladder has been shown to decrease the hydroosmotic response to ADH (Bentley, 1959; Rasmussen *et al.*, 1960;, Finn, Handler & Orloff, 1966). In the present study this result has again been observed (Fig. 1). Additionally, it has been demonstrated that between 20 to 25 mM sodium is required for a full hydroosmotic response (Fig. 2). Schwartz, Rasmussen, Schoessler, Silver and Fong (1960) interpreted the decrease in the hydroosmotic response in the absence of serosal sodium as evidence that proper functioning of the sodium pump was necessary for maximal bulk water flow. However, evidence from the present study, discussed below, does not support that notion. The failure to observe a full hydroosmotic response in the absence of serosal sodium or potassium is not, however, due to failure of AVP to generate the necessary signal to alter mucosal

water permeability. In both the absence of Na^+ or K^+ the increase in ^3H_2O diffusion induced by AVP is equal to that induced by AVP in matched tissue incubated in a regular buffer (Fig. 5). Since the mucosal membrane is the diffusional barrier for water in the absence of AVP and the hormone increases the ^3H_2O permeability of this barrier (Parisi & Piccinni, 1973), the present results indicate that the serosal monovalent cations are involved in AVP action at a site distal to the mucosal face of the tissue. Additionally, since the inhibitory effect of monovalent cation deletion is also observed when an exogenous cyclic AMP derivative is used to induce the hydroosmotic response (Fig. 4), it is unlikely that the effect of serosal monovalent cation deletion on the AVP-induced hydroosmotic response is due dimply to an alteration in cyclic AMP generation or compartmentation. Finally there appears to be no anion selectivity for a full hydroosmotic response since replacement of chloride, phosphate, and sulfate with cyclamate or removal of bicarbonate had no effect on the hormone response.

To determine the importance of transepithelial ion transport in the hydroosmotic response, we employed the $(Na^+ + K^+)$-ATPase inhibitor ouabain, a potent inhibitor of transepithelial Na^+ transport in this tissue (Goodman *et al.*, 1969). Under our incubation conditions there was no effect of this agent the response to AVP (Table 2). This result contrasts with a previous report in which incubation with ouabain for 90 min produced an inhibition of the hydroosmotic response (Finn *et al.*, 1960). In our laboratory a similar inhibition was observed after a 90-min incubation with ouabain. When, however, the bathing solution is changed every 20 min no inhibition of the hydroosmotic response to AVP in the presence of ouabain is observed.

To determine the relationship between cellular [ATP] and the hydroosmotic response, AVP was added to the toad bladder in the presence of iodoacetate, an inhibitor of glycolysis (Harris *et al.*, 1963), and CN^-, an inhibitor of oxidative phosphorylation (Slater, 1967). At the concentrations employed, [ATP] is reduced to 10–25% of control levels. Under these conditions transcellular sodium transport, as measured by SCC, is inhibited (Goodman *et al.*, 1969; Handler, Preston & Orloff, 1969). By contrast, we observe no decrease in the AVP-induced hydroosmotic response in the presence of either inhibitor alone (Table 2). In the presence of both iodoacezate and CN^-, however, a decrease in the hydroosmotic response is observed. The present results agree with the previous observations of Rasmussen *et al.* (1960), i.e., that anaerobiosis or inhibition of glycolysis does not alter the hydroosmotic response to AVP.

However, Handler, Petersen and Orloff (1966) reported that iodoacetate or anaerobiosis caused an inhibition of the hormone-induced increase in water flow and that addition of pyruvate to the incubation buffer prevented the iodoacetate-induced inhibition of the hydroosmotic response. As discussed above, Handler *et al.* (1966) did not change their bathing solutions during incubation with inhibitor. In the present study and in the previous work by Rasmussen *et al.* (1960), however, the buffers were changed every 20 min during the course of the incubation. It thus appears that during prolonged incubation either metabolic product(s) accumulate which inhibit the hydroosmotic response or tissue viability is lost. Provision of pyruvate in the presence of iodoacetate during prolonged incubation may prolong tissue viability after endogenous substrate is utilized. When both glycolysis and oxidative phosphorylation were inhibited by addition of both iodoacetate and CN^- we observed an inhibition of the hydroosmotic response. Under these conditions tissue viability is almost certainly lost. Consequently, interpretation of this particular experimental result is most difficult.

The present study indicates that mucosal Na^+ does not influence the hydroosmotic response and that the serosal Na^+ pool which influences the hydroosmotic response is separate from the transepithelial transport Na^+ pool. In our previous study utilizing cytochalasin B (Davis *et al.*, 1974) we concluded that there was a pool of sodium, separate from the transepithelial transport Na pool, which was important for maintaining a full hydroosmotic response to AVP. The results of the present study support that conclusion and provide evidence that the pool is fully saturated with regard to the hydroosmotic response when serosal [Na^+] exceeds 25 mM. How serosal Na^+ functions to support the sustained movement of water across the toad bladder and what role serosal K^+ plays in this process must now be delineated.

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