Importance of Amino Acids on Vasopressin-stimulated Water Flow

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Abstract

The presence of several naturally occurring amino acids in the serosal bath of toad urinary bladder significantly alters the hydrosmotic response of this tissue to vasopressin. We found that histidine, glutamate, and lysine increase vasopressin-stimulated water flow by 75%, 60%, and 43%, respectively. In contrast, alanine did not alter vasopressin-stimulated water flow, whereas glutamine decreased it by 25%. The effect of each amino acid represents intracellular events because their effects on theophylline-stimulated water flow were similar to those found with vasopressin. However, the site of action of amino acids varied, with some operating at steps before and others at steps after cyclic AMP generation. The fact that the metabolically inactive D-histidine and D-glutamate are as effective as their metabolically active L-counterparts suggests that the action of amino acids depends upon some physicochemical properties of their molecules. The ability of amino acids to influence the hydrosmotic effects of vasopressin was shown to be independent of prostaglandin generation, ionic composition, and molecular charge.

In the case of histidine, we were able to obtain some understanding of the mechanism responsible for its action. We first showed that the effect of histidine does not depend upon its metabolism. In addition to D-histidine being as effective as the metabolically active L-histidine, we also showed that histidine is effective when its metabolism is abolished by low ambient temperature and also when its incorporation into proteins was prevented by cycloheximide. These findings suggest that histidine operates through some physicochemical property localized on its molecule. We were able to show that this property resides on the imidazole part of histidine. Imidazole, similar to histidine, increases vasopressin-stimulated water flow. Methylation of histidine on the imidazole ring completely abolished its effectiveness in increasing vasopressin-stimulated water flow. In contrast, methylation of histidine at the side chain increased vasopressin action similar to that found for histidine.

We provide evidence that the physicochemical property of the imidazole ring of histidine is that of chelating Zn^{++} intracellularly, and that the intracellular site of action of histidine is closely linked to microtubules formation and/or action.

Introduction

The importance of amino acids in biologic systems has been appreciated in the past, and new roles for these naturally occurring substances continue to be elucidated as research in several fields progresses. It has classically been accepted that as protein precursors they are essential for the structure and function of all cells, and that their catabolism to provide substrate for gluconeogenesis and lipogenesis is significant in the maintenance of cell energetics. More recently, it has become apparent that amino acids are involved in the control of a great variety of important cell functions, and that they represent significant substances in cell physiology as well as metabolism.

Amino acids have now been shown to be important determinants of intracellular ion composition and concentration. In the central nervous system, neutral amino acids such as γ-aminobutyric acid, glycine, and taurine have been shown to induce hyperpolarization of neurons by altering Cl^- permeability (1, 2). In the same tissue, both glutamate and aspartate are able to trigger depolarization by increasing Na^+ and Ca^{++} transport (1–4). In both muscle and kidney cells, a reciprocal relationship exists between intracellular concentrations of the cationic amino acids, arginine and lysine, and of K^+ (5–13). Noteworthy is evidence that cationic amino acids can also enhance H^+ exit from cells, resulting in an intracellular alkalosis (8). Heavy metals, such as copper and zinc, are known to have significant affinity for histidine and other amino acids (14–21), and at least copper uptake is influenced by the amino acid concentration gradient across the cell membrane (14). In addition to exerting effects on cell composition, amino acids have been shown to be of importance in regulating intracellular osmolality. Shifts in intracellular amino acid concentrations maintain cell osmoregulation in the face of changes in extracellular osmolality (22–25).

Evidence exists linking the amino acids to several hormone systems that depend upon cyclic AMP (cAMP) generation. Cationic amino acids operate upon both α- and β-cells of the islets of Langerhans in the pancreas (26), thereby stimulating secretion of insulin and glucagon (26). Glutamate has been shown to influence release of luteinizing hormone in the hypothalamic–hypophyseal tract (27). Amino acids have been shown to have a definite effect on cAMP generation in the nervous system (28, 29).

Ample evidence exists, therefore, for amino acids altering membrane permeability, affecting electrolyte composition of cells, binding heavy metals, controlling osmoregulation, and influencing cAMP production. In that it is well known that the response to vasopressin is dependent upon electrolyte composition (30–32), heavy metals (33), cell tonicity (34), and

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cAMP production (35), the current studies were designed to examine whether amino acids were able to influence vasopressin-stimulated water flow in the toad urinary bladder, a close analogue of the mammalian collecting duct.

In our present study, we found that the magnitude and direction of change in vasopressin-stimulated water flow was characteristic for each of the amino acids we examined. Some increased and some decreased vasopressin-stimulated water flow, whereas others did not alter water flow significantly. The site of action of various amino acids also seems to differ, with some operating at steps before and some operating at steps after cAMP generation. The effect of a given amino acid does not appear to be specifically related to its charge or metabolism but rather appears to reflect some physicochemical property of the molecule. The effect of certain amino acids can be abolished by changing the ionic composition of the serosal bath. However, we present evidence that this does not represent a direct relation of ion composition to the action of amino acids, but it simply reflects the dependence of amino acid uptake upon ionic composition.

We have attempted to identify the mechanism involved in the action of histidine. We first demonstrated that the effects of histidine are unrelated to its metabolism (both catabolism and protein synthesis). These findings suggest that histidine works through some physicochemical property. When we examined the effects of various analogues, it became apparent that the property responsible for the effects of histidine resides on its imidazole ring. Further experiments suggest that histidine operates by chelating zinc intracellularly. We present evidence that zinc interferes with the formation or function of microtubules. We propose that chelation of zinc intracellularly by histidine prevents its action at the microtubule level, thus enhancing the effectiveness of vasopressin.

Methods

Female Dominican toads (National Reagents, Bridgeport, CT) were double-pithed and glass bungs were tied into both hemibladders in situ. The bladders were excised and washed three times with amphibian phosphate-buffered Ringer's solution (120 meq Na^+, 4 meq K^+, 1 meq Ca^{++}, 116 meq Cl^-, 5 mM phosphate, osmolality 250 mosM/kg, pH 7.4). All hemibladders were filled with 8 ml of distilled water. Experimental hemibladders were suspended in a 35-ml serosal bath of the above normal phosphate Ringer's containing the appropriate amino acid at pH 7.4, while their paired hemibladder was placed in 35 ml of Ringer's alone. We allowed a 1-h incubation period for both hemibladders to ensure maximal amino acid uptake in the experimental tissue (36). After the incubation period, osmotic water flow was determined gravimetrically (37) at 15-min intervals. After obtaining a baseline water flow in the first 15-min period, the hemibladders were exposed to either maximal or submaximal concentrations of vasopressin (86 mU/ml or 2 mU/ml), cyclic adenosine 3'5'-monophosphate (10 mM cAMP), or theophylline (5 mM), and water flow was recorded for two subsequent 15-min intervals.

In the series of experiments where the effect of altering the serosal bath pH or Ca^{++} was examined, 5 mM Tris was used in place of 5 mM phosphate in our Ringer's solution for better buffering of alkaline pH and to avoid Ca^{++} precipitation. Unless stated otherwise, the pH of all solutions was carefully maintained at pH 7.4 throughout all experiments. In separate control studies, we determined that the amino acids did not alter [^{14}C]sucrose permeability when compared to untreated hemibladders thus excluding changes in the integrity of the epithelia.

L-Alanine and L-glutamate were purchased from Fischer Scientific Co., Fair Lawn, NJ. L-Glutamate, L-lysine, L-histidine, D-glutamate, D-histidine 1-methyl histidine, α-methyl histidine, imidazole, vasopressin, cAMP, and theophylline were purchased from Sigma Chemical Co., St. Louis, MO. Naproxen was kindly provided by Syntex Laboratories, Palo Alto, CA. Comparisons between experimental and control hemibladders were performed using the paired t test.

Results

Effects of amino acids

Vasopressin-stimulated water flow. The effects of certain amino acids on vasopressin-stimulated (2 mU/ml) water flow are shown in Table I. Lysine, histidine, glutamate, and guanidinosuccinate added to the serosal bath significantly increased vasopressin-stimulated water flow. Glutamine on the other hand significantly diminished vasopressin-stimulated water flow. Addition of the neutral amino acid alanine did not alter vasopressin-stimulated water flow. Basal water flows were not affected by any of these amino acids (not shown). The effectiveness of histidine was shown to extend to even maximally (vasopressin, 86 mU/ml) stimulated water flow (45.9±3.5 μl/min vs. 35.1±4.7 μl/min, $n = 8$, $P < 0.02$, histidine treated vs. control, respectively).

The length of the incubation period appeared to be of minor importance. In experiments where there was no incubation, histidine (5 mM) was quite effective in increasing the effect of vasopressin (2 mU/ml) (42.7±6.6 vs. 24.9±7.1 μl/min, $n = 5$, $P < 0.002$). This finding is consistent with the fact that the bulk of amino acid uptake in toad bladders occurs within the first 10 min (36).

The concentrations of amino acids used in these studies are similar to those routinely used in in vitro experiments. However, we did examine the effectiveness of lower concentrations approaching the normal levels of amino acids in plasma. We found that 1 mM histidine, glutamate, and lysine were as effective as the 5 mM used in most of the experiments cited so far. In the case of histidine, we extensively examined its dose response. In these studies, we found that concentrations as low as 0.1 mM were effective in increasing vasopressin-

Table I. Effects of Amino Acids on Vasopressin-stimulated Water Flow*

Amino acid	Control	Experimental	Effect	P value
	µl/min	*µl/min*	*% control*	
Histidine (5 mM, $n = 10$)	16.2±1.9	28.4±3.5	+75%	<0.01
Glutamate (5 mM, $n = 7$)	22.3±2.4	35.7±2.5	+60%	<0.005
Lysine (1 mM, $n = 4$)	38.4±9.2	54.9±10.5	+43%	<0.01
Guanidinosuccinate (5 mM, $n = 4$)	18.0±5.0	24.5±6.2	+36%	<0.05
Alanine (5 mM, $n = 4$)	28.9±8.5	28.9±7.8	NC	NS
Glutamine (1 mM, $n = 6$)	37.6±6.1	27.9±5.3	−25%	<0.05

The concentration of each amino acid in the serosal bath of Ringer's solution is shown in parentheses. Water flow after vasopressin stimulation is shown in microliters per minute for both control (Ringer's solution only) and experimental (Ringer's + amino acid) hemibladders. n, number of experiments; NC, no change; NS, no statistical significance. Results are shown as mean±SEM.
* 2 mU/ml.

Table II. Response of Vasopressin-stimulated Water Flow to Variable Concentrations of Serosal Histidine

	Histidine concentration in serosal bath		Water flow in response to vasopressin (2 mU/ml)			
	Control	Exp.	Control	Exp.	*n*	Significance
	mM	*mM*	*μl/min*	*μl/min*		*P*
A.	0	5	16.2±1.9	28.4±3.5	10	<0.01
	0	1	16.9±2.2	26.8±2.7	5	<0.05
	0	0.5	18.3±2.0	27.1±4.8	8	<0.05
	0	0.3	20.6±2.8	27.9±2.1	5	<0.05
	0	0.1	25.9±3.2	30.4±2.9	12	<0.05
B.	5	1	30.7±5.1	27.8±5.1	6	NS
	5	0.5	30.8±4.1	29.4±3.4	6	NS
	5	0.3	40.0±3.9	34.6±1.2	4	NS

Dose response for the effect of histidine on vasopressin-stimulated water flow. (*A*) The effects of various concentrations of histidine added to the serosal bath of the experimental hemibladder on vasopressin-stimulated water flow compared with its paired control hemibladder bathed in regular Ringer's solution devoid of histidine. (*B*) The effectiveness of variable histidine concentrations in the serosal bath of the experimental hemibladders compared to a control hemibladder bathed in Ringer's containing 5 mM histidine. *n*, number of paired experiments.

stimulated water flow (Table II). When we directly compared the effectiveness of low concentrations of histidine to 5 mM, we found no statistical difference in terms of their effects upon the hydrosmotic action of vasopressin (Table II).

Site of action. To evaluate the site of action of amino acids, we examined their effect on cAMP- and theophylline-stimulated water flow. Histidine increased both cAMP- and theophylline-stimulated water flow (Table III). These findings suggest that this amino acid acts predominantly by increasing a step subsequent to cAMP generation. Glutamate increased theophylline-stimulated water flow but had no effect on cAMP-stimulated water flow (Table IV), suggesting its site of action is after vasopressin binding to its receptor site but before cAMP generation. Lysine and guanidinosuccinate were similar to glutamate in that they did not alter cAMP-stimulated water flow, but enhanced theophylline-stimulated water flow (not shown). These studies suggest that amino acids operate on several steps occurring in response to vasopressin stimulation. The similarities of the effects of the acidic glutamate to the basic amino acid lysine demonstrate that the effects are probably independent of charge.

The independent action of each individual amino acid on vasopressin-stimulated water flow was further demonstrated in another series of experiments. In these studies, we compared the effect of a combination of two amino acids influencing vasopressin-stimulated water flow in the same direction (each 2.5 mM) to either amino acids alone (5 mM) as shown in Table V. Histidine's effect to increase vasopressin-stimulated water flow was additive to either that of glutamate or guanidinosuccinate. These findings are consistent with histidine's enhancement of an intracellular step which is different from that of the other two amino acids. Glutamate was not additive to guanidinosuccinate, suggesting that these two amino acids alter water flow by operating upon the same step.

Dependence upon metabolism. To evaluate whether the effects of the amino acids depend upon their metabolism, we

Table III. Effects of Serosal Histidine on Theophylline and cAMP-stimulated Water Flow*

		Osmotic water flow			
Serosal solution		Baseline		Theophylline (5 mM)	
		μl/min		*μl/min*	
NPR	$n = 6$	0.6±0.1	NS	7.5±1.5	$P < 0.05$
NPR + histidine		0.5±0.1		13.6±2.6	
				cAMP (10 mM)	
NPR	$n = 6$	1.2±0.2	NS	16.0±1.9	$P < 0.02$
NPR + histidine		1.2±0.1		21.4±2.1	

The effects of 5 mM serosal histidine on 5 mM theophylline- or 10 mM cAMP-stimulated water flow are shown. Symbols as per Table I. NPR, normal phosphate Ringer's solution.
* 5 mM.

Table IV. Effects of Serosal Glutamate on Theophylline- and cAMP-stimulated Water Flow*

		Osmotic water flow			
Serosal solution		Baseline		Theophylline (5 mM)	
		μl/min		*μl/min*	
NPR	$n = 9$	1.5±0.2	NS	10.5±2.1	$P < 0.05$
NPR + glutamate		1.9±0.2		17.6±2.7	
				cAMP (10 mM)	
NPR	$n = 9$	1.6±0.2	NS	19.0±2.5	NS
NPR + glutamate		1.3±0.3		18.7±3.2	

The effects of 5 mM sucrose glutamate on 5 mM theophylline- or 10 mM cAMP-stimulated water flow are shown. Symbols as per Table I. NPR, normal phosphate Ringer's solution.
* 5 mM.

Table V. Effects of Combination of Serosal Amino Acids on Vasopressin-stimulated Water Flow

Amino acids added to serosal bath		Vasopressin-stimulated water flow			
Control (mM)	Experimental (mM)	Control	Experimental	*n*	*P*
		μl/min	*μl/min*		
GSA (5)	His (2.5) + GSA (2.5)	28.1±4.1	45.4±1.8	4	<0.01
His (5)	His (2.5) + GSA (2.5)	34.0±8.1	40.0±8.0	4	<0.01
Glu (5)	His (2.5) + Glu (2.5)	30.8±4.8	47.6±4.0	4	<0.05
His (5)	His (2.5) + Glu (2.5)	41.5±4.7	46.6±4.2	4	<0.02
GSA (5)	GSA (2.5) + Glu (2.5)	40.6±4.1	37.6±4.5	4	NS
Glu (5)	GSA (2.5) + Glu (2.5)	35.1±9.5	32.4±8.2	4	NS

In these studies we examined whether the effects of several amino acids, each shown to increase vasopressin stimulated water flow, is additive. The effects on vasopressin-stimulated water flow of a combination of two amino acids (each in a concentration of 2.5 mM—experimental) added to the serosal bath is compared to serosal bath containing Ringer's solution plus only one of the two amino acids (concentration 5 mM—control). *n*, number of experiments; NS, no statistical significance; His, histidine; GSA, guanidine succinate; Glu, glutamate.

examined the effect of D-amino acids on vasopressin-stimulated water flow. Similar to L-amino acids, D-amino acids significantly increased vasopressin-stimulated water flow. In the presence of 5 mM D-histidine, vasopressin-stimulated water flow increased significantly compared to control (32.6±3.0 vs. 18.5±2.2 μl/min, $n = 5$, $P < 0.025$). Vasopressin-stimulated water flow was also increased by the addition of D-glutamate, 5 mM/liter in the serosal bath (34.3±3.1 vs. 22.8±2.9 μl/min, $n = 5$, $P < 0.02$). In separate experiments with paired hemibladders, we directly compared the effect of a D-amino acid to the effect of the naturally occurring L-amino acid. The results of these studies showed that D- and L-amino acids have identical effects on vasopressin-stimulated water flow (29.7±2.3 vs. 29.0±2.1 μl/min, $n = 4$, D- vs. L-histidine, and 19.8±3.2 vs. 19.3±3.0 μl/min, $n = 6$, D- vs. L-glutamate). In that D-amino acids are not metabolized in most biologic systems (26), except for a few bacteria, it is unlikely that their observed effects are related to their metabolism. The observed similarities of D- and L-amino acids suggest that their effects on vasopressin-stimulated water flow do not reflect their metabolic fate.

Dependence upon ionic composition. We next examined the effect of amino acids under conditions of altered serosal bath composition. These studies were designed to explore the previously mentioned relationship between intracellular concentrations of certain ions and amino acids, as well as the possible dependence of their uptake mechanisms on a given electrolyte.

In the presence of histidine (Table VI), vasopressin-stimulated water flow was increased even when the serosal bath had no K^+, low Ca^{++}, high Ca^{++}, high pH, low pH, or no Na^+ (replaced by either choline or K^+). Hence, the effect of histidine appears to be independent of electrolytic composition.

Similar to the observations with histidine, the effect of glutamate was also independent of serosal pH, K^+, and Ca^{++} (Table VII). However, elimination of serosal Na^+, with replacement by either choline or K^+, abolished the enhancement of vasopressin-stimulated water flow noted in the presence of this amino acid. The dependence of glutamate on serosal Na^+ may reflect the effect of Na^+ on glutamate uptake rather than any special role of serosal Na^+ in glutamate's action. To assess this, we repeated the previous experiment but ensured glutamate uptake by preincubation of experimental hemibladders with glutamate in Na^+-containing Ringer's solution before the experiment. Under these conditions, we were able to show that glutamate-pretreated bladders increased vasopressin-stimulated water flow (28.3±5.4 vs. 17.3±4.8 μl/min, $n = 6$, $P < 0.05$) even though Na^+ was absent from the serosal bath for 1 h before, as well as during, vasopressin stimulation.

The effectiveness of glutamine in decreasing vasopressin-stimulated water flow was also dependent upon serosal bath Na^+, and could not be shown in its absence. In one set of experiments where both hemibladders were incubated in Ringer's solution plus 1 mM glutamine, addition of ouabain to the

Table VI. Effects of Variations of Serosal Bath Composition on Histidine Increase of Vasopressin-stimulated‡ Water Flow*

Serosal bath	Control	Histidine (5 mM)	*P* value
	μl/min	*μl/min*	
High Ca^{++} (10 mM) ($n = 6$)	3.3±0.4	8.8±1.9	<0.025
Low Ca^{++} (0.1 mM) ($n = 6$)	6.5±0.4	20.0±4.0	<0.02
Low pH (7.1) ($n = 6$)	6.0±0.7	11.9±2.1	<0.02
Low pH (8.5) ($n = 6$)	26.0±3.6	58.4±5.1	<0.05
Low K^+ (0 mM) ($n = 6$)	9.9±2.4	28.0±5.2	<0.01
Choline Ringer's ($n = 7$)	15.4±1.8	27.9±3.3	<0.005
K^+ Ringer's ($n = 8$)	8.2±1.0	13.8±1.9	<0.005
Naproxen (10^{-5} M) ($n = 6$)	36.1±2.6	50.2±3.6	<0.02

Changes in the composition of Ringer's solution are shown under "Serosal bath" where the solute existing in a concentration different from normal Ringer's solution is identified and its concentration is shown in parentheses. The paired hemibladders used for a given experimental set differs from each other only in terms of the absence of histidine (control) or presence of histidine 5 mM in the serosal bath. *n*, number of paired experiments.

* 5 mM.

‡ 2 mU/ml.

Table VII. Effects of Variations of Serosal Bath Composition on Glutamate Increase of Vasopressin-stimulated‡ Water Flow*

Serosal bath	Control	Glutamate	*P* value
	μl/min	*μl/min*	
High Ca^{++} (10 mM) (n = 6)	9.4±2.2	14.1±2.0	<0.02
Low Ca^{++} (0.1 mM) (n = 6)	17.6±2.5	20.0±3.1	<0.02
Low pH (7.1) (n = 6)	15.5±4.8	26.6±5.8	<0.02
Low pH (8.5) (n = 6)	33.3±3.3	41.9±0.8	<0.05
Low K^+ (0 mM) (n = 6)	15.7±2.5	18.6±2.9	<0.05
Naproxen (10^{-5} M) (n = 6)	45.6±4.9	58.0±3.3	<0.05
Choline Ringer's (n = 8)	19.2±2.9	20.8±2.3	NS
K^+ Ringer's (n = 4)	8.3±2.4	8.2±1.6	NS

Changes in the composition of Ringer's solution are shown under "Serosal bath" where the solute existing in a concentration different from normal Ringer's solution is identified and its concentration is shown in parentheses. The paired hemibladders used for a given experimental protocol differ from each other only in terms of absence of glutamate (control) or presence of glutamate 5 mM in the serosal bath. n, number of paired experiments.
* 5 mM.
‡ 2 mU/ml.

experimental hemibladder (thus eliminating Na^+ transport across the basolateral membrane) resulted in much higher water flow in response to vasopressin compared to its paired control (20.9±4.5 vs. 13.1±3.0 μl/min, n = 8, $P < 0.005$). Because glutamine is known to decrease vasopressin-stimulated water flow, the prevention of its effect by ouabain supports the notion that its uptake is Na^+ dependent.

In contrast, the effect of lysine on vasopressin-stimulated water flow appears to be Na^+ independent, in that elimination of Na^+ by replacement with choline did not abolish the observed increase in water flow (10.2±1.5 vs. 6.8±1.4 μl/min, n = 5, $P < 0.01$). However, K^+ appears to be very significant in the effectiveness of this amino acid, inasmuch as replacement of serosal Na^+ by K^+ completely abolished any increase in vasopressin-stimulated water flow (6.8±1.4 vs. 7.8±2.0 μl/min, n = 5, NS). These findings are consistent with the uptake characteristics of lysine in this tissue. Lysine uptake has been shown to be independent of Na^+, but inversely dependent upon K^+ (36, 38).

In the same series of experiments, we also evaluated the importance of endogenous prostaglandins in modulating the effects of amino acids. This was felt necessary in view of the close linkage of electrolyte changes to prostaglandin synthesis (35), the effects of amino acids upon cell phospholipid metabolism (39), and also the fact that prostaglandins represent a well-known intracellular modulator of vasopressin action (40–42). To evaluate the significance of endogenous prostaglandins on the action of a given amino acid, we added naproxen 10^{-5} M to the serosal bath of both hemibladders for 1 h before to vasopressin stimulation. This maneuver has been shown to reduce cyclooxygenase activity to undetectable levels (41, 42), thus eliminating endogenous prostaglandin production (42) in the toad bladder. We found that both histidine and glutamate increase vasopressin-stimulated water flow independent of endogenous prostaglandins (Tables VI and VII).

Effects of histidine

Dependence on metabolism. The similarity of the effects of the metabolically inactive D-histidine to the naturally occurring L-histidine (Table VIII), suggests that histidine increases vasopressin action not through its metabolism but probably through some physicochemical property. To test this further, we examined the effects of histidine on vasopressin-stimulated water flow under conditions that influence its intermediate metabolism and protein synthesis.

We first examined the effects of histidine with intermediate metabolism abolished for all practical purposes by lowering

Table VIII. Independence of Effect of Histidine on Vasopressin-stimulated Water Flow from Its Metabolism

Serosal bath	n	Osmotic water flow: Baseline		Osmotic water flow: Vasopressin (2 mU/ml)	
		μl/min		*μl/min*	
NPR	5	0.9±0.2	NS	18.5±2.2	$P < 0.025$
NPR + D-histidine (5 mM)		0.6±0.2		32.6±3.0	
NPR T = 7°C	4	2.2±0.4	NS	3.6±0.3	$P < 0.05$
NPR + histidine (5 mM), T = 7°C		1.2±0.4		7.9±1.2	
NPR + cycloheximide (2 μg/ml)	5	1.2±0.3	NS	31.3±3.2	$P < 0.01$
NPR + histidine (5 mM) + cycloheximide (2 μg/ml)		1.9±0.4		48.0±3.7	

This table shows the results of three experiments. In the first, the metabolically inactive D-histidine is shown to increase vasopressin-stimulated water flow similarly to its metabolically active counterpart L-histidine. In the second experiment, histidine increased vasopressin-stimulated water flow even in the absence of metabolism (7°C). In the third experiment, inhibition of protein synthesis by 3 h of pretreatment with cycloheximidine did not prevent histidine from increasing vasopressin-stimulated water flow.

the environmental temperature to 7°C. In these experiments paired hemibladders were incubated for 30 min in the presence (experimental) or absence (control) of 5 mM histidine in the serosal bath at 22°C to ensure histidine uptake. The bladders were placed then in fresh serosal baths of identical composition (with or without histidine) precooled for ~2 h at 7°C. Both hemibladders were transferred to the new cold solution, allowing 30 min for equilibration. After the equilibration period, osmotic water flow was determined during a 15-min baseline period and two 15-min periods after vasopressin stimulation. As shown in Table VIII, histidine quite effectively increased vasopressin-stimulated water flow even in the cold. These findings strongly support the notion that the effects of histidine are unrelated to intermediary metabolism.

We then examined the effects of histidine when protein synthesis is inhibited, thus precluding the incorporation of histidine into proteins. Both hemibladders were incubated for 3 h in Ringer's solution containing cycloheximide 2 mg/ml. These experiments were performed with toads received in the winter (January and February) because cycloheximide has been shown to abolish protein synthesis in toad bladders completely during winter (43). We then placed the hemibladders in fresh Ringer's solution containing no histidine (control) or 5 mM histidine (experimental). Both solutions contained cycloheximide to ensure continuous inhibition of protein synthesis. As shown in Table VIII, under conditions of protein synthesis inhibition, serosal histidine resulted in significantly increased vasopressin-stimulated water flow. These findings suggest that histidine increased the hydrosmotic effects of vasopressin through a mechanism independent of incorporation into and/or production of new proteins.

Taken together, all the experiments presented thus far strongly argue that the action of histidine is independent of its metabolism.

Localization of the molecular structure responsible for the effects of histidine. Histidine has an unusual structure compared to other amino acids, and this structure allows for research at the molecular level using rather simple techniques. Histidine is composed of an imidazole ring and a side chain. To identify which part of the molecule of histidine is responsible for its effects, we first examined the effects of imidazole on vasopressin action.

Imidazole has been previously shown to alter vasopressin-stimulated water flow via its effects on prostaglandin and thromboxane synthesis (44). In our studies we preincubated both hemibladders in Ringer's solution containing naproxen 10^{-5} M, an agent that inhibits cyclooxygenase activity to undetectable levels (40–42), thus eliminating prostaglandin and thromboxane synthesis. We then moved both hemibladders to new serosal baths also containing naproxen 10^{-5} M. This maneuver was felt necessary to eliminate preexisting prostaglandins and thromboxanes. The serosal bath of the experimental hemibladder differed from that of the control hemibladder in that it also contained 5 mM imidazole. We found that in the absence of endogenous prostaglandin and thromboxane synthesis, imidazole acted much like histidine, in that it significantly increased vasopressin-stimulated water flow (Table IX).

To confirm that the physicochemical property responsible for the action of histidine resides on its imidazole ring, we then examined the effects of two different methyl histidines on vasopressin-stimulated water flow. α-Methyl histidine has the methyl group in the α position of the side chain, thus leaving the imidazole ring intact. In contrast, 1-methyl histidine has its methyl group on the imidazole ring. As shown in Table IX, α-methyl histidine is quite as effective as the naturally occurring L-histidine in increasing vasopressin-stimulated water flow. In contrast, 1-methyl histidine did not alter the effect of vasopressin (Table IX). To ensure further that the latter finding does not represent any seasonal variability, we directly compared α-methyl histidine to 1-methyl histidine in paired hemibladders (Table IX). α-Methyl histidine resulted in a much greater increase of water flow in response to vasopressin.

On the basis of these findings, we conclude that histidine increases vasopressin-stimulated water flow through some physicochemical property that resides on its imidazole ring.

Physicochemical properties of the imidazole ring. Our findings that histidine increases vasopressin-stimulated water

Table IX. Importance of the Imidazole Ring with Regard to Vasopressin-stimulated Water Flow

		Osmotic water flow			
Serosal bath	*n*	Baseline		Vasopressin (2 mU/ml)	
		ml/min		*ml/min*	
NPR + Naproxen 10^{-5} M	6	1.8±0.2	NS	39.3±1.8	$P < 0.01$
NPR + Naproxen 10^{-5} M + Imidazole (5 mM)		1.7±0.3		51.0±1.5	
NPR	4	2.3±0.3	NS	9.2±2.3	$P < 0.005$
NPR + α-methylhistidine (5 mM)		1.8±0.3		24.0±3.2	
NPR	5	2.5±0.2	NS	14.6±2.5	NS
NPR + 1-methylhistidine (5 mM)		2.0±0.2		14.8±2.6	
NPR + α-methylhistidine (5 mM)	4	1.2±0.4	NS	18.3±3.0	$P < 0.01$
NPR + 1-methylhistidine (5 mM)		1.2±0.4		40.5±4.0	

This table underlines the importance of the imidazole ring to increase vasopressin-stimulated water flow. In the first experiment, the effects of imidazole on the action of vasopressin is seen independent of prostaglandins and thromboxanes whose synthesis is inhibited by preincubation with Naproxen 10^{-5} M. Methylation of histidine on the imidazole ring (1-methyl histidine) completely abolishes the action of this amino acid, whereas methylation away from the imidazole ring results in a compound ring (α-methyl histidine) equally potent to the mother compound.

flow independent of pH, Ca^+, Na^+, and K^+ substantially narrow the search for possible mechanisms of action. Indeed, the only remaining property of the imidazole ring that has been found to occur regularly in nature is that of chelating heavy metals (45). We thus examined whether the action of histidine is the result of intracellular binding of metals that are known otherwise to decrease the action of vasopressin such as Zn^{++} (46) and Cu^{++} (47). Of the two, only binding of Zn^{++} may explain all the findings of histidine (see discussion). We, therefore, concentrated on the possibility that histidine chelates Zn^{++} intracellularly. As a first step in that direction, we attempted first to show that histidine binds Zn^{++} intracellularly and reverses its inhibitory effects on vasopressin. We were able to confirm results previously reported by Bentley (46) by noting that, after exposure to Zn^{++} 10^{-4} M, the inhibitory effect on vasopressin-stimulated water flow is maintained even when Zn^{++} was removed from the Ringer's bathing for 30 min preceding vasopressin stimulation (35.0±3.5 vs. 50.1±5.0 μl/min, $n = 4$, $P < 0.05$). These effects seen in the absence of serosal Zn^{++} presumably reflect intracellular actions of Zn^{++}. When we repeated the above experiments but replaced the serosal bath of both the Zn^{++} exposed hemibladder and its paired control with Ringer's solution containing histidine for 30 min prior to vasopressin stimulation, the effects of intracellular Zn^{++} were completely reversed (61.0±4.6 vs. 60.5±4.0 μl/min, $n = 4$, NS). These findings strongly argue that histidine enters the cell and reverses the effects of zinc, consistent with histidine that chelates zinc intracellularly.

The fact that Zn^{++} modifies microtubule formation and results in kinked microtubules in various tissues (see Discussion), coupled with the known importance of microtubules in supporting vasopressin action (48, 49), represented the basis for our considering that the effects of zinc, and indirectly of histidine, may be due to alteration of microtubule formation and/or function. To test this hypothesis, we examined the effects of Zn^{++} and histidine in bladders in which we inhibited microtubule formation by incubating both hemibladders for 4 h before vasopressin stimulation in Ringer's solution containing colchicine 4×10^{-4} M. We found that neither Zn^{++} (15.6±1.4 vs. 15.8±0.9 μl/min, $n = 6$, NS) nor histidine (18.8±2.3 vs. 18.0±2.0 μl/min, $n = 5$, NS) influenced vasopressin-stimulated water flow when microtubule formation was inhibited.

Discussion

Relatively little is known about how plasma amino acids affect functions of the kidney. Glutamine has an established role as an ammonia precursor, contributing both to acid-base homeostasis and preservation of cations (50). In addition, under certain conditions, glutamine serves as a significant source of energy (51). Amino acid have been shown to have variable importance in Na^+ reabsorption (52), as well as tubular regeneration after tubular injury (39). The role of amino acids in relation to the concentrating mechanism has only been minimally examined. Epstein and his co-workers (53) have presented evidence that perfusion of the isolated rat kidney with amino acid restores concentrating ability. These authors showed that the amino acids preserve the integrity of epithelia of the loop of Henle. However, their data do not allow for assessment of any direct effect the amino acids may have on vasopressin action. Handler and Orloff (54) found that cysteine decreased vasopressin-stimulated water flow in the toad urinary bladder, and suggested that the observed effect was related to the action of cysteine on adenyl cyclase.

Our present studies attempted to define further whether amino acids may play a role in the physiology of the renal concentrating mechanism. We found that amino acids significantly influence the response of vasopressin in the toad bladder, a close analogue of the mammalian renal collecting duct. We showed that several amino acids act differently from each other. For example, histidine and glutamate are able to enhance vasopressin-stimulated water flow, whereas cysteine (54) and glutamine diminish it. We were also able to demonstrate that the amino acids operate through several mechanisms. Histidine increased the response to vasopressin due to enhancement of steps distal to cAMP generation, whereas glutamate enhanced vasopressin-stimulated water flow by altering steps to cAMP generation.

Although our present studies do not enable us to identify the precise mechanism by which each of the amino acids has its effect, certain conclusions may be drawn from the data presented. The observed effect of the amino acids appears unrelated to their charge, inasmuch as their classification as acidic, neutral, or basic does not correlate with their ability to modulate vasopressin-stimulated water flow. For example, both the acidic glutamate and the basic lysine increase vasopressin water flow by enhancing a step before cAMP generation.

Another way that amino acids may influence the response to vasopressin is by altering cellular ion concentration. Our experiments that examine the effect of changes in the ionic composition of the serosal bath indirectly address this possibility. Our data argue against the amino acids influencing vasopressin action by altering pH, Na^+, K^+, Ca^{++}, or membrane potential. The possibility that they act through altering prostaglandins, known modulators of vasopressin-stimulated water flow, seems untenable based upon our data that showed no difference in the effect of amino acid when prostaglandins are eliminated by the cyclooxygenase inhibitor naproxen.

We also believe that neither the metabolism of amino acids nor their incorporation into proteins accounts for their observed effects, in view of the similarities found among D- and L-compounds on vasopressin action. It should be emphasized that effects of amino acids unrelated to their metabolism have been previously observed. For example, direct stimulation of pancreatic insulin secretion is produced by both D- and L-amino acids (26).

The dependence of the action of amino acids on the composition of the serosal bath in toad bladders has been shown to correlate with their uptake characteristics. Similar to our functional studies, where the effectiveness of glutamate or glutamine was shown to be dependent upon extracellular Na^+, the uptake mechanisms of both of these amino acids are Na^+ dependent, and can be completely abolished by elimination of extracellular Na^+ or by addition of ouabain to the serosal bath (38). Lysine uptake has been shown to be inhibited by increased extracellular K^+, but it is not affected by eliminating Na^+ (36, 38), which is in agreement with our results for water flow. Histidine uptake is apparently unrelated to electrolyte composition (38) as was its functional characteristics. The close correlation of uptake of a given amino acid to its functional effectiveness suggests the importance of intracellular levels in regulating their action.

Our findings with histidine clearly illustrate the importance of the physicochemical properties of amino acids in modulating

the intracellular steps that follow vasopressin stimulation. The lack of importance of metabolism for this amino acid in relation to its action on the hydrosmotic effect of vasopressin was shown in two different ways. First, as we already mentioned, the metabolically inactive D-histidine alters the action of vasopressin in a way similar to the metabolically active L-histidine. Second, histidine is effective in increasing vasopressin-stimulated water flow even when intermediate metabolism is abolished by low ambient temperature, and also when incorporation of amino acids into protein is prevented by lengthy incubation with cycloheximide. Because the effects of histidine on vasopressin-stimulated water flow are unrelated to its metabolism, it follows that the actions of this amino acid reflect some physicochemical property of its molecule. Pursuing this further, we were able to show that it is the physicochemical properties of the imidazole ring of histidine that account for the action of this amino acid on vasopressin stimulation. We showed that imidazole, as well as a histidine analogue with its imidazole ring intact, shared with histidine the ability to increase vasopressin-stimulated water flow. In contrast, histidine analogues with a methyl group substituted for hydrogen in the imidazole ring failed to influence the hydrosmotic action of vasopressin.

The major physicochemical property that histidine shares with other imidazole derivatives is that of chelating heavy metals (45). It is well known that histidine contributes more than any other amino acid to plasma binding of Zn^{++} and Cu^{++} (14–20). In fact, it is the histidine content of protein that is responsible for their binding of heavy metals. For example, this is clearly the case in the binding of Zn^{++} and Cu^{++} by albumin (45), the binding of Fe^{++} by hemoglobin and cytochrome (45), and binding of cobalt by vitamin B_{12} (45). Consequently, this represents a major, if not the major, contribution of histidine to biologic phenomena, and underlies its unique position in physiology (45).

We reasoned, therefore, that histidine may affect vasopressin action by heavy metal chelation. For this to be correct it requires that the specific metal chelated should have effects on vasopressin-, cAMP-, and theophylline-stimulated water flow exactly the opposite of that of histidine. The only metal we found with such properties is Zn^{++} (46). Cu^{++} similar to Zn^{++} decreases the action of vasopressin (47). However, Cu^{++} operates at steps before cAMP generation inasmuch as it does not alter cAMP-stimulated water flow (47). This finding makes it unlikely that histidine increases the effects of vasopressin by binding Cu^{++}, because histidine also increases cAMP-stimulated water flow as well. Our findings that histidine reverses the effects of intracellular zinc clearly fit with the thesis that histidine increases water flow by chelating Zn^{++}, thus ameliorating the inhibitory effects of Zn^{++} on the action of vasopressin.

Zn^{++} has been found to influence microtubule formation in other tissues. It appears to influence both the rate of polymerization of tubulin as well as the morphology of microtubules (55, 56). Its action is very potent, to the extent that in Zn^{++}-treated tubulins, colchicine fails to have an effect (55, 56). The action of microtubules has been shown to be of particular importance for the hydrosmotic action of vasopressin.

Several lines of evidence suggest that Zn^{++} probably inhibits vasopressin-stimulated water flow by interfering with microtubule formation and/or function. Zn^{++} decreases vasopressin-theophylline-, and cAMP-stimulated water flow (46), in a way similar to that described for the microtubule inhibitor colchicine (48, 49). The similarities of actions of Zn^{++} and colchicine extend even further. Serosal hypertonicity also increases transepithelial water flow. This is achieved by activating most of the steps that follow cAMP generation during vasopressin stimulation, except for the steps involved in microtubule formation and action. This is demonstrated by the lack of effectiveness of colchicine on hypertonicity induced water flow (49). Similar to colchicine, we found that Zn^{++} is also ineffective to modify the hydrosmotic effect of serosal hypertonicity (16.6 ± 1.8 vs. 16.2 ± 1.0 μl/min, $n = 6$, NS, Zn^{++} 10^{-4} M-treated vs. control hemibladders, respectively). It is important to mention that histidine, which we suggest operates through Zn^{++} binding, is also ineffective on serosal hypertonicity-induced water flow (23.3 ± 0.9 vs. 23.6 ± 0.8 μl/min, $n = 12$, NS, histidine 5 mM-treated vs. control hemibladders). The finding that Zn^{++} and histidine are ineffective to influence the water flow induced by serosal hypertonicity, argues strongly that their action interferes with the formation and/or action of microtubules. It should be mentioned that Cu^{++} inhibits hypertonicity-induced water flow (42). This once more argues against the possibility that histidine may operate by binding Cu^{++}. It also suggests that Cu^{++} acts at a step different from the one used by Zn^{++} and histidine.

Probably the strongest evidence that Zn^{++} operates through an action at the microtubule level stems from the lack of additive effects to colchicine to inhibit vasopressin-stimulated water flow. This suggests that both operate at the same step, that is to say, a microtubule dependent one. The argument that colchicine may completely abolish an important step in the cellular cascade and represent a rate limiting step thus masking the effect of agents that operate at other steps is essentially untenable for two reasons. Colchicine is known to decrease vasopressin-stimulated water flow by about 40% only (48, 49). In addition, agents that decrease vasopressin action by operating at steps other than microtubules are additive to colchicine. For instance, Cu^{++}, a metal known to inhibit vasopressin-stimulated water flow at a step before cAMP generation (47), further decreased vasopressin-stimulated water flow when combined with colchicine (4.0 ± 0.6 vs. 9.1 ± 2.0 μl/min, $n = 4$, $P < 0.05$, Cu^{++} + colchicine- vs. colchicine-treated hemibladders). It is interesting to mention that the increase in vasopressin-stimulated water flow seen in the presence of histidine is also abolished when microtubule formation is prevented by colchicine. This suggests that the effects noted with histidine necessitate an intact microtubular system, and further strengthen our thesis that histidine binds Zn^{++} intracellularly thus permitting an uninterrupted microtubule formation and action.

The lack of effectiveness of both Zn^{++} and histidine when microtubular synthesis is inhibited could also be consistent with their operating at a step important for microtubular action rather than microtubular formation. For instance, Zn^{++} may bind with the histidine molecules of proteins so as to change their structure. Such reactions are quite often in nature. One example is found when Zn-peptide binding transforms insulin to the Zn hexamer as opposed to a single chain or dimer (57). One could, therefore, alternatively consider that histidine increases vasopressin-stimulated water flow by chelating Zn^{++} and thus inducing conformational changes in proteins involved in the generation and/or action of microtubules.

Let us now turn to the physiologic relevance of our

experiments and their potential applications. Amino acids regularly exist in plasma, the equivalent of the serosal bath, in concentrations of 0.1–1.0 mM (58, 59). The use of a 5 mM concentration of amino acids in most of our experiments does not subtract from the potential physiologic importance of our findings, in that in vitro systems often require higher concentrations to elicit physiologic effects seen in vivo (5). In addition, our finding that concentrations of amino acid similar to those in plasma are effective further supports the physiologic importance of our experiments. We should also mention that amino acid concentrations deep in the medulla of the kidney are several times higher than those observed in the peripheral plasma (60). Taken together, these observations support our argument that amino acids may represent physiologically important modulators of the response of sensitive epithelia to vasopressin.

The role of amino acids in vasopressin-stimulated water flow appears to be more complex than their merely influencing the effect of the hormone. In preliminary studies, we have found that vasopressin and cAMP alter amino acid uptake by the toad bladder epithelia (38). These findings suggest that feedback loops may exist in which the intracellular concentration of certain amino acids is altered in response to vasopressin, which in turn determines the end effect of the hormone.

The dependence of vasopressin-sensitive epithelia on amino acids is physiologically intriguing. Appropriate research is needed to further evaluate their effects in vivo and expand these observations to other species. The role of amino acids in preventing or treating conditions of vasopressin resistance or in sustaining function in perfused kidneys could potentially have important clinical applications.

The finding that normally occurring amino acids influence the action of vasopressin may have significant physiologic implications beyond identifying a new modulating system and providing a better understanding of the physiologic factors involved in the response of this hormone. The effects of intracellular amino acids to alter steps before and after cAMP generation may also apply to other peptide hormones that, similar to vasopressin, achieve their effects through cAMP generation and action. The possibility, therefore, that amino acid may have a more global role in modulating the action of peptide hormones remains to be ascertained.

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