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Inhibition of Urea-Linked Water Flux and [^{14}C]Urea Transport across the Toad Bladder by Amiloride[1] (41125)

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Abstract. Urea movement across the toad bladder was measured with [^{14}C]urea and by determining the amount of water flow obligated to urea movement along a urea concentration gradient from mucosa to serosa. This "urea-linked volume flux" was measured gravimetrically. Both the urea-linked volume flux and the [^{14}C]urea transport were stimulated by vasopressin and reversibly inhibited by 0.1 m*M* amiloride by about 50%. Amiloride had no effect on vasopressin-stimulated osmotic water flux across the bladder wall. Calculations on the amount of water obligated to urea movement indicate that the mucosal urea solution becomes concentrated during its passage through the bladder wall in the presence of vasopressin. This concentrating effect of the permeability barrier is less pronounced in the presence of amiloride. It is postulated that amiloride blocks specific urea channels in the rate-limiting barrier, channels which are capable of selectively enhancing urea movement and retarding water flow. Since amiloride is also a well-known inhibitor of sodium transport in this tissue, the present study raises the question as to whether amiloride acts on two different sets of channels or blocks only one which is shared by both sodium and urea.

The antidiuretic hormone, vasopressin, increases the permeability of the isolated urinary bladder of the toad to urea (1) and to water (2) by mechanisms which are thought to be related to those involved in altering the permeability of the mammalian collecting duct to these substances (3, 4). Although the hormone exerts its characteristic effect on membrane permeability only when applied to the serosal surface of the bladder, it has been suggested that the ultimate effector site for vasopressin is at or near the mucosal cell face of the bladder epithelium (5–9). There has been mounting evidence in recent years to suggest that the effect of vasopressin on water permeability may involve a different process than the hormonal effect on urea permeability. Thus, Handler and Orloff (10) showed that the urea permeability response of the bladder to vasopressin could be eliminated by prolonged incubation in cycloheximide without inhibiting the osmotic water permeability response. Levine *et al.* reported that phloretin (11) and acetamide (12) specifically inhibited urea movement across the bladder, and we observed a similar antagonism between thiourea and urea (13). These observations are consistent with the view that urea movement across the bladder wall does not occur by simple diffusion, but requires a special transport process. We have suggested that a "urea channel" may be involved in the translocation of urea rather than a classical mobile carrier, because the urea transport process is preserved following glutaraldehyde fixation (13).

In the present study we have investigated the effect of the diuretic drug, amiloride, following a preliminary report by Hays (14) that this compound inhibits urea transport. We found that amiloride is, indeed, a very effective inhibitor of [^{14}C]urea transport in the toad bladder, while having no inhibitory action on vasopressin-stimulated osmotic water flux. In the medullary collecting duct of the mammalian nephron water and urea are reabsorbed simultaneously in the presence of vasopressin (3, 4) and one would expect the movement of urea to influence the movement of water. In order to measure this influence of bulk urea transport on net osmotic water flow, toad bladders were filled with a 205 m*M* urea solution and sus-

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pended in an equiosmotic NaCl solution. As urea and water move from mucosa to serosa across the bladder wall, the bladder loses weight, which can be readily quantitated with a semimicrobalance. Vasopressin markedly increases this "urea-linked volume flux" (13) presumably by a separate action on rate-limiting urea and water permeability barriers. In the present study we have tested the effects of amiloride on this urea-linked volume flux and have found it to be markedly inhibited. Amiloride has been used extensively as a blocker of sodium transport in anuran epithelial membranes, and radiolabeled compounds have been employed as markers for estimating the number of sodium entry sites at the mucosal cell face of the vasopressin target cell (15–17). Our results indicate that amiloride is not a specific blocker of sodium channels, unless one postulates that sodium and urea penetrate the membrane by the same pathway, and there is no evidence at present that they do.

Materials and Methods. Experiments were carried out during the summer and winter months on toads of the species *Bufo marinus* from the Dominican Republic (National Reagents Inc., Bridgeport, Conn.). Animals weighed between 190 and 260 g; they were kept at room temperature and allowed free access to water. Toads were double pithed and their urinary bladders were isolated, split, and mounted as half-bladders on the ends of glass cannulae as detailed earlier (18). The mucosa was on the inner and the serosa on the outer surface of a bladder sac which held approximately 7 ml of fluid. An osmotic pressure gradient was established across the bladder wall by filling the bladder with a solution that had all the components of Ringer's fluid, except that the concentration of NaCl was reduced below the usual value of 110 m*M* as indicated in the figures. A urea concentration gradient was established across the bladder wall by filling the bladder with a solution that had all the components of Ringer's fluid, except that all the sodium chloride, i.e., 110 m*M*, was replaced by equiosmotic concentrations of urea. This solution is referred to as "urea–Ringer's." The bladders were suspended in an outside (serosal) bath of Ringer's fluid, which had the following composition, in millimoles per liter: NaCl, 110; KCl, 3.5; $CaCl_2$, 1; $MgCl_2$, 1; dextrose, 5.5; Trizma (Sigma Chemical Co., St. Louis, Mo.), 10; pH, 7.4. The osmolality of this solution was 235 mosm/kg H_2O as measured cryoscopically with a Fiske osmometer.

The permeability coefficients of the bladder wall for urea and for inulin were measured with [^{14}C]urea (ICN) and [^{3}H]inulin (New England Nuclear). Pitressin (Parke–Davis, Detroit, Mich.) was used as a vasopressin source. Amiloride was kindly supplied by Merck, Sharp, & Dohme. Net volume fluxes across the bladder wall were measured gravimetrically according to the general method of Bentley (2). Bladders were wiped on a glass dish and weighed rapidly on a semimicrobalance. With practice, bladders can be wiped and weighed with a consistancy of ±5 mg.

Results. In the experiment shown in Table I the effect of mucosal amiloride on tracer urea flux in the mucosal-to-serosal direction was tested in bladders bathed on both surfaces with Ringer's fluid. Amiloride at a concentration of 0.1 m*M* inhibited [^{14}C]urea flux by 60% in the absence of vasopressin. Vasopressin increased urea flux

TABLE I. INHIBITION OF TRACER UREA FLUX BY AMILORIDE

	K_{trans} [^{14}C]urea ($\times 10^{-7}$ cm/sec)	
	Period I 0–60 min no hormone	Period II 60–120 min with pitressin
Hemibladder A no amiloride	11.8 ± 9.7 (100%) $P < 0.001$	135 ± 26 (SD) (n = 6) (100%) $P < 0.001$
Hemibladder B with amiloride	3.7 ± 1.9 (40 ± 17%)	75 ± 23 (55 ± 10%)

Note. Matched hemibladders from six toads were filled with regular Ringer's fluid (i.e., 110 m*M* NaCl–Ringer's) and suspended into regular Ringer's fluid. Amiloride at a final concentration of 0.1 m*M* was added to the mucosal bath of B hemibladders. After 60 min pitressin (20 mU/ml) was added to the serosal baths. Values are expressed as K_{trans} [^{14}C]-urea ± SD as well as percentage of the urea flux across hemibladder B compared to A. The P value in the table was calculated from the percentage values.

more than 10-fold, and this vasopressin-stimulated tracer urea flux was inhibited by 45% in the presence of amiloride.

In the experiment shown in Fig. 1 the effects of amiloride on the urea-linked water flux across the bladder wall were tested. Hemibladders were filled with urea–Ringer's containing 0.1 m*M* of amiloride and suspended into an equiosmotic solution of Ringer's fluid. Control, matched hemibladders from the same animals were treated identically, except that amiloride was omitted from the mucosal solution. As is shown in the figure vasopressin markedly increased the mucosal-to-serosal movement of water along the urea concentration gradient, i.e., the urea-linked volume flux. This urea-linked volume flux was markedly reduced in bladders containing amiloride, i.e., by 65% during the first 30-min interval of exposure to hormone and by 33% during the second 30-min interval.

In order to learn whether the effect of amiloride on urea-linked volume flux is due to inhibition of urea or of water movement across the bladder wall, the experiment in Fig. 2 was carried out. The protocol for this experiment was identical to the one in Fig. 1, except that hemibladders were filled with Ringer's fluid diluted to 190 mOsm/kg H_2O. This relatively small osmotic pressure gradient across the bladder wall (45 mOsm/kg H_2O) was chosen so that the net water flux in this experiment approximated the net urea-linked water flux in the experiment in Fig. 1. As is apparent from the results in Fig. 2, amiloride did not inhibit the vasopressin-stimulated water flux along an osmotic pressure gradient at all.

In the course of these and similar experiments we have noted marked variability between different toads in the sensitivity of the urea transport to vasopressin as well as in the degree to which amiloride inhibits this transport. It was, therefore, necessary to demonstrate in the same hemibladder that amiloride blocks urea movement while not affecting water flow. This study is shown in Table II. Again, amiloride was found to have no inhibitory action on osmotic water flux (see Period II) while depressing urea-linked volume flux by 53% (see Period IV). Moreover, the inhibitory effect of 0.1 m*M* amiloride was completely

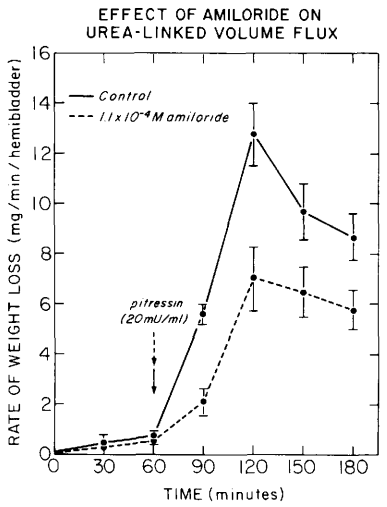

FIG. 1. Six contralateral hemibladders were filled with 235 mosm/kg H_2O urea–Ringer's without (●——●) or with 0.1 m*M* amiloride (●– – –●) and suspended in equiosmotic Na–Ringer's fluid to which 20 mU/ml pitressin was added at t = 60 min. Values are given as mean responses ± SEM.

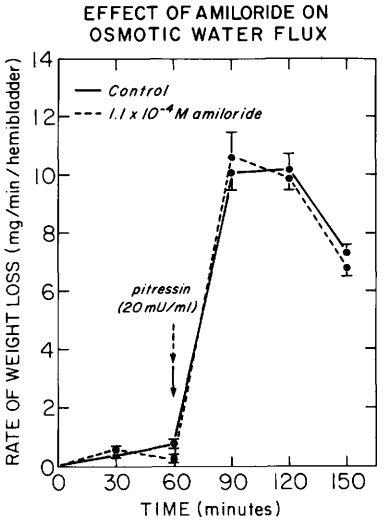

FIG. 2. Six contralateral hemibladders filled with Na–Ringer's fluid which had been diluted to 190 mosm/kg H_2O without (●——●) or with (●– – –●) 0.1 m*M* amiloride and suspended in 235 mosm/kg H_2O Na–Ringer's fluid to which 20 mU/ml pitressin was added at t = 60 min. Values are given as mean responses ± SEM.

TABLE II. EFFECT OF AMILORIDE ON OSMOTIC WATER FLUX AND UREA-LINKED WATER FLUX

	Rate of weight loss (mg/min/hemibladder)			
	Period I 0–30 min (Osm. gradient basal)	Period II 30–60 min (Osm. gradient pitressin)	Period III 120–150 min (Urea gradient basal)	Period IV 150–180 min (urea gradient pitressin)
		No amiloride		
Control hemibladder	0.9 ± 0.4	12.0 ± 1.9	0.4 ± 0.3	5.9 ± 1.9 $P < 0.01$
		0.1 m*M* Amiloride		
Experimental hemibladder	0.8 ± 0.5	12.1 ± 1.9	0.4 ± 0.3	2.8 ± 0.8

Note. Control and experimental hemibladders taken from the same toad were filled with 75 m*M* NaCl–Ringer's fluid (173 mOsm/kg H_2O) and suspended in 110 m*M* NaCl–Ringer's (235 mOsm/kg H_2O). Amiloride at a final concentration of 0.1 m*M* was added to the mucosal bath of the experimental hemibladders. At t = 30 min pitressin (20 mU/ml) was added to the serosal baths. At t = 60 min bladders were removed to hormone-free Ringer's for an hour and then refilled with urea–Ringer's fluid (235 mOsm/kg H_2O). Amiloride again was added to the mucosal solution of the experimental hemibladders. At t = 150 min hemibladders were again challenged with 20 mU/ml pitressin. Values given are the mean responses with the standard deviation of experiments carried out on six toads.

reversed by bathing the bladder mucosa for a period of 1 hr in Ringer's fluid. This is shown in Table III. Thus, during Period IV hemibladders "A" exhibited a urea-linked volume flux of 5.9 mg/min/hemibladder in the presence of vasopressin. Subsequently, during Period V the urea-linked flux was depressed to 2.6 mg/min/hemibladder, when 0.1 m*M* amiloride was added to the mucosal solution. Following a 1-hr washout period in hormone and amiloride free Ringer's fluid the urea-linked volume flux was reestablished during Period VI to 6.0 mg/min/hemibladder in the presence of vasopressin, but in the absence of amiloride. Similar results were obtained in matched hemibladders ("B") which had been exposed to amiloride and vasopressin first, then washed and exposed to vasopressin alone.

If one assumes that the urea-linked flux is an isoomotic flux, i.e., that the 205 m*M* urea solution presented to the mucosal surface moves across the bladder wall as a 205 m*M* urea solution, then it is possible to calculate from the rate of weight loss of the bladder a permeability coefficient of the bladder wall for urea. These calculated permeability coefficients for urea are shown in Table III where they are compared to permeability coefficients for urea measured directly by including trace amounts of [^{14}C]urea in the mucosal solutions. The permeability coefficients for urea determined by these two independent methods were very similar, but only in the presence of amiloride. In the absence of amiloride (and in the presence of vasopressin) the permeability coefficients for [^{14}C]-urea were considerably greater than for unlabeled urea. This observation implies that the assumption that urea moves across the bladder wall as an isoosmotic solution is not valid, especially in the absence of amiloride. Thus, for example in Period IV, the permeability coefficients were 87.4 × 10^{-7} cm/sec for [^{14}C]urea and 57.8 × 10^{-7} cm/sec for unlabeled urea flux. The latter value was probably underestimated, because the 5.9 mg of mucosal solution which passed through the bladder wall did not have a concentration of 205 m*M* urea, but rather 87.4/57.8 × 205 m*M* = 310 m*M* urea. The same bladder treated with amiloride in Period V had permeability coefficients for [^{14}C] urea of 26.0 × 10^{-7} cm/sec and for unlabeled urea of 25.5 × 10^{-7} cm/sec, so that the urea solution calculated to move across the bladder wall under these conditions is 209 m*M*, which is virtually identical to the 205 m*M* urea solution presented to the mucosal bladder surface. On averaging the results in Periods IV, V, and VI it is estimated that the 205 m*M* urea solution penetrates the bladder wall in the presence of vasopressin and in the presence of amiloride as a 246 mmole/liter urea solution, whereas in the presence of vasopres-

TABLE III. REVERSIBILITY OF AMILORIDE INHIBITION OF UREA-LINKED WATER FLUX AND [^{14}C]UREA FLUX IN THE PRESENCE OF VASOPRESSIN

	Period IV 150–180 min	Period V 240–270 min	Period VI 330–360 min
	Urea gradient pitressin	Urea gradient pitressin *amiloride*	Urea gradient pitressin
Hemibladder A			
Rate of weight loss (mg/min/hemibladder)	5.9 ± 1.9	2.6 ± 0.5	6.0 ± 1.7
K_{trans} urea[a] ($\times 10^{-7}$ cm/sec)	57.8	25.5	58.8
K_{trans} [^{14}C]urea[b] ($\times 10^{-7}$ cm/sec)	87.4 ± 50	26.0 ± 11.7	130.4 ± 75.9
	Urea gradient pitressin *amiloride*	Urea gradient pitressin	Urea gradient pitressin *amiloride*
Hemibladder B			
Rate of weight loss (mg/min/hemibladder)	2.8 ± 0.8 $P < 0.01$[c]	8.7 ± 3.7 $P < 0.01$	3.4 ± 1.2 $P < 0.02$
K_{trans} urea[a] ($\times 10^{-7}$ cm/sec)	27.5	85.3	33.3
K_{trans} [^{14}C]urea[b] ($\times 10^{-7}$ cm/sec)	33.6 ± 26.5 $P < 0.1$	143.8 ± 6.9 $P < 0.01$	46.2 ± 33.8 $P < 0.05$

[a] Calculated on the basis of the measured rate of weight loss above on the assumption that the 205 m*M* urea solution on the mucosa moves across 17 cm^2 bladder surface area as an isoosmotic solution.
[b] Calculated from direct measurements of [^{14}C]urea fluxes across the bladder wall.
[c] *P* values refer to the significance of difference between hemibladders A and B with the paired *t* test.

sin but in the absence of amiloride a 370 m*M* urea solution crosses the bladder wall. These calculations, of course, do not take into account any urea added or subtracted from the transport pool by bladder metabolism.

High concentrations of urea (205 m*M*) were employed in these experiments and could have resulted in disruption of epithelial integrity and shunting of [^{14}C]urea across the membrane by nonspecific pathways. In order to estimate this shunt pathway for urea, hemibladders used in the experiment depicted in Table III were subsequently exposed to [^{3}H]inulin. These data are shown in Table IV. The permeability coefficient of these hemibladders for inulin was found to be about 10% of the permeability coefficient of these hemibladders for urea. This finding implies, that after having exposed the bladder mucosa intermittently for 420 min to 205 m*M* urea, the bladder was "leaky" to the extent that roughly 10% of net [^{14}C]urea transport could have occurred via pathways large enough to permit inulin movement across the bladder wall. In other words, the specific urea transport in the presence of vasopressin and in the absence of amiloride was estimated to be 90% of the total urea transport under the conditions of this experiment. When corrections are made for the nonspecific shunting (assuming that amiloride does not inhibit this pathway), urea transport is inhibited by 72% by amiloride. Without these corrections for the shunt pathway inhibition would have been 65% in this experiment (see Period VI). In Table V the extent of nonspecific shunting of the tracer urea experiments discussed earlier (Table I) is examined. These bladders had not been exposed to high mucosal urea solutions, but

TABLE IV. SHUNT PATHWAY FOR UREA IN TOAD BLADDER AS MEASURED BY [^{3}H]INULIN

	K_{trans} ($\times 10^{-7}$ cm/sec), pitressin		Specific urea transport VI–VII ($\times 10^{-7}$ cm/sec)	
	Period VI 330–360 min [^{14}C]Urea	Period VIII 420–450 min [^{3}H]Inulin		
Hemibladder A	No amiloride 130.4 ± 75.9	No amiloride 12.8 ± 5.2	117 ± 74 $P < 0.05$	(100%) ($P < 0.001$)
Hemibladder B	0.1 m*M* Amiloride 46.2 ± 33.8	No amiloride 14.8 ± 9.7	31 ± 25	(28%)

Note. This study is a continuation of the study shown in Table III. Hemibladders A and B were filled with urea–Ringer's fluid to which trace amounts of [^{14}C]urea were added in Period VI and trace amounts of [^{3}H]-inulin in Period VII. There was a 1-hr rest period with regular Ringer's bathing both bladder surfaces in the absence of hormone at t = 360 min. Specific urea transport was calculated by subtracting the K_{trans} [^{3}H]inulin from the K_{trans} [^{14}C]urea. Note, that when the specific urea transport in hemibladder B is expressed as a percentage of the specific urea transport in the matched hemibladder A from the same toad, the difference becomes statistically more significant. Values given are the mean responses with the standard deviation of experiments carried out on six toads.

they had been removed from toads for a total of 180 min and had been exposed for 60 min to vasopressin during this interval. As is shown in Table V the [^{3}H]inulin flux across these bladders in the presence of vasopressin was 8 × 10^{-7} cm/sec. This value is 6% of the [^{14}C]urea flux measured in the same hemibladders during an earlier period (compare hemibladder "A", Period III, Table V, with hemibladder A, Period II, Table I). When corrections are made for this nonspecific shunting of [^{14}C]urea, amiloride inhibits tracer urea flux in the experiment of Table I by 47 instead of by 45% without corrections. Thus, nonspecific shunting of urea in the present experiments did not alter the degree of amiloride inhibition of urea transport substantially. It is of interest, that the permeability of the bladder wall to inulin was not increased during a 60-min period of exposure to 205 m*M* urea, as is shown for the A hemibladders during Period IV in Table V. Thus factors other than mucosal urea are primarily responsible for the shunt pathway observed in the present study.

Discussion. We have shown previously (13) that when the isolated toad bladder is filled with a urea solution and suspended in a solution containing equiosmotic amounts of sodium chloride (as measured by freezing point depression) the bladder loses volume, and this volume loss can be readily measured by weighing the bladder on a semimicrobalance. We have referred to this weight loss as the urea-linked volume flux. This term is not meant to imply that diffusion of urea down its concentration gradient drags water with it by a solute–solvent interaction. In fact, water flow across the bladder is diminished by urea compared to the situation in which urea is absent. Nevertheless, the term urea-linked volume flux is useful to indicate the association and dependency of water movement on urea movement and to distinguish this water flux

TABLE V. EFFECT OF 205 m*M* MUCOSAL UREA ON [^{3}H]INULIN PERMEABILITY

	K_{trans} [^{3}H]inulin ($\times$ 10^{-7} cm/sec)	
	Period III 180–240 min NaCl–Ringer's mucosa	Period IV 240–300 min Urea–Ringer's mucosa
Hemibladder A	7.7 ± 0.8	8.0 ± 0.6 (SD) (n = 6)
Hemibladder B	7.5 ± 0.6	

Note. This is a continuation of the experiment in Table I. Matched hemibladders from six toads were equilibrated in hormone-free, amiloride-free Ringer's fluid for 60 min. At t = 180 min. bladders were filled with 110 m*M* NaCl–Ringer's containing [^{3}H]inulin and suspended into 110 m*M* NaCl–Ringer's containing Pitressin at a final concentration of 20 mU/ml. After 60 min the mucosal solution in the A bladders was replaced by 205 m*M* urea–Ringer's containing [^{3}H]inulin. Net inulin fluxes are expressed as the mean ± SD.

from the water movement along an osmotic gradient (and against a sodium chloride concentration gradient).

We have shown in the present study that amiloride inhibits the vasopressin-induced, urea-linked volume flux as well as the [^{14}C]urea flux, while having no inhibitory effect on osmotic water flux across the bladder. The inhibitory effect of amiloride is readily reversed. Under the particular circumstances of these experiments, i.e., prolonged exposure to high mucosal urea solutions, 6 to 10% of the total urea transport was found to occur via a shunt pathway large enough to transport [^{3}H]inulin. However, corrections for this nonspecific urea movement did not substantially alter values obtained for amiloride inhibition. It is possible that the shunt pathway for urea was overestimated here to the extent that some ^{3}H could have penetrated the bladder wall without inulin. It is also conceivable that the nonspecific urea pathway was underestimated here, because under our experimental conditions such a pathway had to be large enough to admit an inulin molecule.

The vasopressin-sensitive permeability barriers for water and for urea are thought to be located at or near the mucosal cell face of the epithelium. It is of interest in this regard, that no structural correlates of urea permeability have been described to date, although it has been possible to correlate changes in osmotic water permeability with changes in vasopressin-induced luminal intramembranous particle aggregates seen by freeze-fracture studies (19, 20). We have shown previously (13) that the vasopressin-induced, urea-carrier system can be stabilized by glutaraldehyde fixation without losing its characteristic function of transporting urea and being inhibited by thiourea. Thus, the urea carrier behaves as a urea-specific channel. Amiloride presumably blocks urea from gaining access to this channel. In this regard it is of interest that the mucosal urea solution appears to be moving through the rate-limiting membrane as a hypertonic solution, especially in the absence of amiloride. Thus, when the bladder was rendered permeable to urea by vasopressin and exposed on the mucosa to a 205 m*M* urea solution, we have calculated in the present study that the solution moving across the bladder wall consisted of 370 m*M* urea. This finding confirms our earlier observation based on chemical measurements of urea fluxes with the urease reaction method (13) which suggested that the mucosal urea solution becomes concentrated during its passage through the membrane. The present study indicates that amiloride diminishes the concentration of the urea solution moving through the membrane from a concentration of 370 to 246 m*M*. This observation suggests that amiloride might be blocking specific urea channels and that these specific urea channels are somehow endowed with the capacity of enhancing urea movement and restricting water movement.

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