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AMINO ACID ACCUMULATION IN THE TOAD BLADDER RELATIONSHIP TO TRANSEPITHELIAL SODIUM TRANSPORT

SAMUEL O. THIER

Massachusetts General Hospital (Renal Unit) and Department of Medicine, Harvard Medical School, Boston, Mass. (U.S.A.)

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SUMMARY

Amino acid accumulation has been studied in the toad bladder. The mucosal surface of the epithelial cells is impermeable to amino acid movement in either direction. Concentrative uptake of amino acid occurs through the serosal surface. The uptake of the neutral amino acid α-aminoisobutyric acid is sodium dependent, ouabain sensitive and unaffected by anaerobiosis. The concentrative uptake of amino acids may be independent of processes for transepithelial sodium transport, though the energy-requiring steps for both processes are thought to be in the serosal surface of the mucosal epithelial cells. The last observation makes unlikely the postulate that sodium serves a non-specific role in the production of energy for transport.

INTRODUCTION

The active transport of amino acids by a variety of tissue preparations *in vitro* has been shown to be dependent upon Na^+ (refs. 1–5). Though the mechanism by which amino acid transport is linked to Na^+ remains unknown, two major possibilities have been proposed. CSAKY[6], noting that several seemingly unrelated non-electrolytes required sodium for concentrative transfer, suggested that sodium functioned intracellularly in the production of energy generally available for active transport. CRANE[7], on the other hand, has suggested a coupled influx of sodium and non-electrolyte across the cell membrane. Extrusion of sodium by an energy-dependent step and a diminished affinity for the non-electrolyte at the inner membrane surface would result in concentrative uptake of the non-electrolyte.

The isolated toad bladder, a tissue of polar nature, actively transports sodium from mucosal to serosal surface by a process which can be measured as the equivalent short-circuit current[8]. This tissue, therefore, provides a system in which sodium and amino acid movements can be studied simultaneously and in which the concentrations of these species can be varied independently across a directed membrane.

The purpose of the present study is to describe the accumulation of neutral (α-aminoisobutyric acid) and cationic (L-lysine) amino acids by the toad bladder and the relation of these processes to transepithelial sodium transport.

It is concluded that neutral and cationic amino acids are accumulated by separate, saturable uptake mechanisms and that these mechanisms may be independent of those for active transepithelial sodium transport.

METHODS

Isolated urinary hemibladders of the toad, *Bufo marinus*, were studied in the double chamber designed by SHARP AND LEAF[9]. The double chamber consists of two parallel identical compartments for control and experimental conditions. The hemibladder is mounted so that half of the tissue (one quarter bladder) covers each compartment of the chamber; one quarter bladder and its compartment serve as the control. Independent electrical circuits for each quarter bladder allow monitoring of transepithelial potentials and short-circuit currents. All bathing media are continuously aerated unless otherwise stated. The composition of the solution bathing either the serosal or mucosal surface of either quarter bladder may be varied independently. This use of a single half bladder for control and experimental conditions corrects for the variation among the toads. In each table experimental data are presented with values from a given animal on the same horizontal line. Each horizontal line, therefore, represents experimental data from a different animal. The significance of results obtained in paired double chamber experiments was determined by partial analysis of variance.

The bathing solutions were frog Ringer's (Na^+, 114; K^+, 3.5; Cl^-, 117; HCO_3^-, 2.4; Ca^{2+}, 0.89 mM; pH 7.8, 220 mosM/kg water) except when Na^+ was replaced isoionically with choline$^+$ or K^+ or was replaced with Mg^{2+} (final osmolality, 187 mosM per kg water). Unless otherwise stated, bathing media on both surfaces were frog Ringer's solution. When sodium was removed from either mucosal or serosal surface the opposite surface was always bathed with frog Ringer's solution.

After exposure to aerated bathing media containing ^{14}C-labeled amino acids added to either mucosal or serosal medium for 15–120 min, tissues were removed from the chambers, rinsed in frog Ringer's solution, blotted, weighed and placed in 2.0 ml of distilled water. The tissue amino acid pool was equilibrated with the distilled water by boiling for 6 min. Aliquots (0.2 ml) of aqueous tissue extract and bathing media were counted in a liquid scintillation spectrometer. The counting solution was water (0.2 ml), ethanol (2.8 ml), and 0.010 % POPOP (*p*-bis-[2-(5-phenyloxazolyl)]-benzene), 0.413 % PPO (2,5-diphenyloxazole), in toluene (7.0 ml). One-dimensional ascending paper chromatography (butanol–acetic acid–water (4:1:2, by vol.)) of the tissue supernatant indicated that over 95 % of the radioactivity for α-aminoisobutyric acid and 75 % of the radioactivity for lysine were recovered at the appropriate R_F values for the amino acid studied.

Total tissue water was determined to be the difference in weight before and after drying overnight in a tared vessel at 105° in a vacuum oven. The distribution of [^{14}C]-inulin was determined and defined as the extracellular fluid space. The distribution ratio, (counts/min per ml intracellular fluid)/(counts/min per ml medium), was calculated as previously described[10]. The percentage of wet tissue weight present as water and the tissue inulin space were essentially unchanged by changes in the composition of the bathing media. Though there was a suggestion of a decreased inulin space in potassium Ringer's solution, the difference from control values was not

significant. Therefore, previously determined values for total tissue water (80 % of wet weight) and inulin space (40 % of wet weight) were used in calculating distribution ratios[11].

That 90 % of tissue radioactivity was in the mucosal epithelial cell layer, which accounted for 33 % of the tissue weight, was demonstrated by separating mucosal epithelial cells from serosal connective tissue by scraping with a glass slide and determining the radioactivity in each. The separated epithelium and serosal connective tissue were each handled in the manner described for the intact tissue. Monitoring of transepithelial voltage and short-circuit current was performed as previously described[8].

Uniformly ^{14}C-labeled L-lysine, 222 mC/mmole, and [*carboxy*-^{14}C]inulin, 0.10 mC/38 mg (mol. wt., 3000–4000), were purchased from the New England Nuclear Corp. [^{14}C]α-Aminoisobutyric acid, 9.6 or 19 mC/mole, was purchased from Volk Radiochemical Corporation. All unlabeled amino acids were purchased from Mann Research Laboratories and Nutritional Biochemical Corporation.

RESULTS

Amino acid movement from mucosal and serosal bathing solutions

In initial studies no significant transepithelial movement of amino acid occurred from either mucosal or serosal bathing medium. Furthermore, there was no significant penetration of mucosal cells from mucosal medium. Therefore, in all subsequent studies amino acids were added only to the serosal bathing medium.

Effect of the short-circuited state

In the open-circuited state a potential difference between the serosal medium and the more negatively charged cell interior might favor the intracellular accumulation of cationic amino acids. This possibility was examined by placing either α-aminoisobutyric acid (0.065 mM) or lysine (0.065 mM) in a double chamber, one half of which was open and the other half short-circuited. There was no difference in amino acid accumulation in the open as compared to the short-circuited state despite initial transbladder potentials of up to 55 mV. All subsequent studies were carried out with the bladder short-circuited allowing continuous monitoring of sodium transport.

There was no effect of amino acids on transepithelial sodium transport as measured by the short-circuit current under the conditions of the present study.

Intracellular accumulation of amino acids from serosal medium

Lysine and α-aminoisobutyric acid are accumulated against a concentration gradient by independent saturable processes which approach a steady state (influx equal to efflux) by 90 min (Fig. 1). Studies of the saturability of the transport processes were performed during the linear phase of accumulation (30 min for lysine and 45 min for α-aminoisobutyric acid) (Fig. 2). Since progressively higher concentrations of lysine in the medium produced distribution ratios approaching 1.0, a correction for simple physical diffusion was made according to the theoretical considerations discussed by ROSENBERG, BLAIR AND SEGAL[10] and ROSENBERG, DOWNING AND SEGAL[12]. However, after 45 min α-aminoisobutyric acid did not appear intracellularly in any significant quantity when concentrative uptake was inhibited by removing serosal

sodium. Since simple diffusion of α-aminoisobutyric acid could not be demonstrated, no correction was made in calculating the uptake of this amino acid.

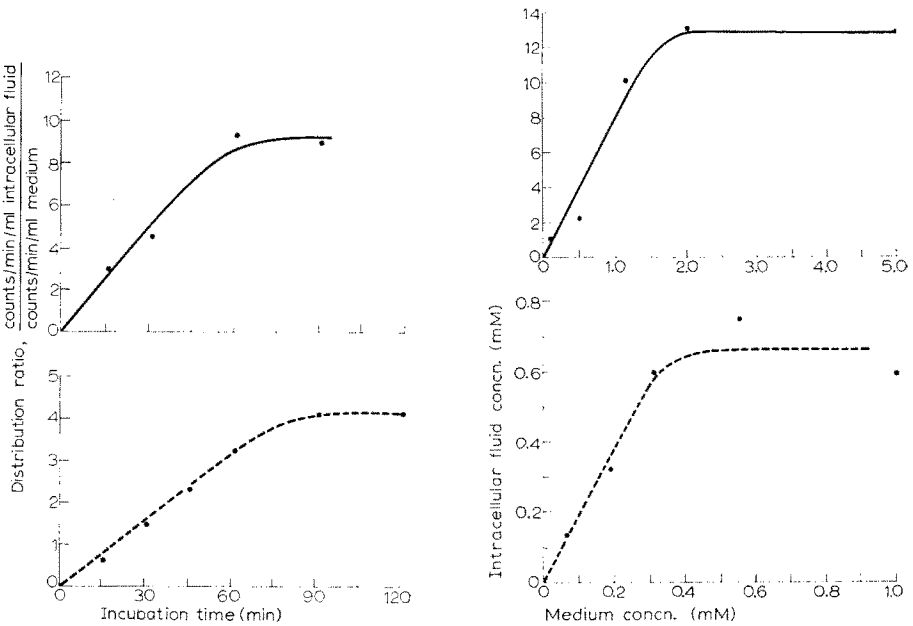

Fig. 1. (Left.) The distribution ratios of α-aminoisobutyric acid (– – –) and L-lysine (——) as a function of time. Frog Ringer's solution was the bathing medium for mucosal and serosal surfaces. Amino acids were added to the serosal medium to a concentration of 0.065 mM.

Fig. 2. (Right.) The effect of increasing external α-aminoisobutyric acid (– – –) and L-lysine (·····) concentrations on the intracellular concentration of these amino acids. Experimental conditions were comparable to those in Fig. 1 except that the tissues were incubated for 30 min with lysine and for 45 min with α-aminoisobutyric acid.

Specificity of the uptake mechanisms for neutral and cationic amino acids was demonstrated by incubating α-aminoisobutyric acid and lysine with related and unrelated amino acid for 90 min (Table I). α-Aminoisobutyric acid uptake was inhibited by neutral glycine but not by cationic arginine, while lysine uptake was inhibited by arginine but not by glycine.

Effect of removing serosal sodium

Replacement of serosal sodium with choline, magnesium, or potassium while the mucosal surface is bathed with frog Ringer's solution abolishes concentrative uptake of α-aminoisobutyric acid (Table II), but not energy-dependent transepithelial sodium transport which is maintained at 50 % of control levels[13]. The failure to concentrate α-aminoisobutyric acid intracellularly in the absence of serosal sodium could be explained by assuming either an absolute requirement for sodium in order to penetrate the cell or a role for sodium in determining the rate of amino acid entry. To differentiate between these possibilities α-aminoisobutyric acid uptake was studied in the absence of serosal medium sodium for up to 3 h. A gradual increase in the concentration of intracellular α-aminoisobutyric acid to distribution ratios of greater than 1.0 indicated that the requirement for sodium was not absolute, but rather that the

TABLE I

AMINO ACID ACCUMULATION BY TOAD BLADDER: EFFECT OF OTHER AMINO ACIDS

Amino acids were added to the serosal bathing medium. All bathing media were frog Ringer's solution. Incubation time was 90 min and the media were oxygenated by continuous bubbling with air.

Amino acid	*Accumulation ratio* $\frac{counts/min\ per\ ml\ intracellular\ fluid}{counts/min\ per\ ml\ media}$			
	Control	+ *Glycine (2.4 mM)*	*Control*	+ L-*Arginine (2.4 mM)*
[^{14}C]α-Aminoisobutyric acid (0.065 mM)	2.82	1.29	2.85	3.76
	2.33	1.29	5.07	2.82
	3.87	1.32	3.79	5.44
	2.48	1.16	4.27	3.79
	4.41	1.69	5.54	4.35
	4.31	2.29	6.59	7.23
Mean	3.37	1.50	4.69	4.57
$\Delta \pm$ S.E.		−1.87 ± 0.27		−0.12 ± 0.26
P		< 0.01		< 0.50
L-[^{14}C]Lysine (0.065 mM)			4.53	3.10
			7.64	2.71
	14.09	17.07	10.53	2.67
	18.01	18.15	8.86	3.30
	8.40	8.26	8.54	6.86
	10.44	9.37	9.40	3.39
	14.11	11.40	6.31	3.68
	10.04	9.96	4.03	1.82
Mean	12.52	12.37	7.48	3.44
$\Delta \pm$ S.E.		−0.15 ± 0.65		−4.04 ± 0.81
P		> 0.50		< 0.01

TABLE II

AMINO ACID ACCUMULATION BY TOAD BLADDER: EFFECT OF REMOVING SEROSAL SODIUM

Experimental conditions were the same as in Table I with the exception that the serosal bathing medium was varied as indicated. Frog Ringer's solution bathed the mucosal surface.

Amino acid	*Accumulation ratio* $\frac{\text{counts/min per ml intracellular fluid}}{\text{counts/min per ml media}}$					
	Control	K^+ *Ringer*		*Control*	Mg^{2+} *Ringer*	
						Control	*Choline Ringer*
[^{14}C]α-Aminoisobutyric acid (0.065 mM)	4.24	0.01		2.47	0.30		3.21	0.06
	3.90	0.14		4.39	0.49		4.30	0.06
	1.89	0.09		3.69	0.37		5.35	0.44
	7.40	0.31		3.70	0.42		3.72	0.47
Mean	4.36	0.16		3.56	0.40		4.15	0.26
$\Delta \pm$ S.E.		−4.20 ± 1.08			−3.16 ± 0.36			−3.89 ± 0.42
P		< 0.05			< 0.01			< 0.01
L-[^{14}C]Lysine (0.065 mM)	5.56	4.84		11.54	8.81		9.15	9.05
	12.01	7.55		3.50	6.26		14.78	13.23
	10.90	5.52		7.15	6.05		9.06	7.26
	9.17	4.70		7.31	7.54		7.50	7.85
	8.26	4.82		6.55	6.42		8.52	8.51
	8.04	4.74		8.24	6.38		12.34	7.76
Mean	8.82	5.36		7.38	6.91		10.23	8.94
$\Delta \pm$ S.E.		−3.46 ± 0.34			−0.47 ± 0.48			−1.29 ± 0.71
P		< 0.001			> 0.30			> 0.10

absence of sodium altered the rate of amino acid accumulation. There was no significant difference in serosal sodium concentration as measured by flame photometer at 90 min *versus* 3 h (range 3 to 5 mM). The presence of finite amounts of sodium in the serosal medium at 90 and 180 min is thought to result from transepithelial leakage and efflux from cells. It is of note that there was no significant increase in serosal sodium between 90 and 180 min while the accumulation of α-aminoisobutyric acid increased to distribution ratios above 1.0 during the same period. The uptake of lysine is unaffected by removal of serosal sodium except when potassium is substituted for sodium. With potassium Ringer's solution as the serosal bathing medium lysine uptake was inhibited but not abolished (Table II).

Effect of anaerobiosis and ouabain

To study amino acid accumulation during diminished transepithelial sodium transport, bladders were maintained under anaerobic conditions (less than 0.1 vol. % oxygen[8]) or in the presence of 10^{-4} M ouabain. Transepithelial sodium transport and α-aminoisobutyric acid accumulation were depressed by both experimental conditions. Lysine uptake, however, was unaffected by either condition (Table III). Transepithelial sodium transport is 20–50 % of control levels with anaerobiosis and less than 30 % of control levels with 10^{-4} M ouabain.

TABLE III

AMINO ACID ACCUMULATION BY TOAD BLADDER: EFFECT OF OUABAIN AND ANAEROBIOSIS

Experimental conditions were the same as in Table I except that N_2 was substituted for air in one set of experiments and that ouabain (10^{-4} M) was added to the serosal medium in the second set of experiments.

Amino acid	*Accumulation ratio* $\frac{counts/min\ per\ ml\ intracellular\ fluid}{counts/min\ per\ ml\ media}$			
	Control	N_2	*Control*	*Ouabain* (10^{-4} *M*)
[^{14}C]α-Aminoisobutyric acid (0.065 mM)	2.62	1.70	16.64	10.65
	4.89	1.29	20.10	7.13
	6.18	2.94	7.10	4.39
	2.22	1.84	4.11	2.97
	3.73	1.76	4.49	2.30
	2.57	2.21	4.17	1.74
			2.35	0.98
			1.83	1.15
Mean	3.70	1.96	7.60	3.91
$\Delta \pm$ S.E.		-1.74 ± 0.58		-3.69 ± 1.45
P		< 0.05		< 0.05
L-[^{14}C]Lysine (0.065 mM)	7.26	9.06	13.13	16.40
	7.21	7.48	15.38	12.63
	8.27	7.66	6.55	8.31
	8.06	7.83	5.21	4.39
	8.41	9.61	8.67	8.12
	9.62	9.98	8.15	12.39
Mean	8.14	8.60	9.52	10.37
$\Delta \pm$ S.E.		$+0.46 \pm 0.26$		$+0.85 \pm 0.59$
P		> 0.30		> 0.20

These results did not indicate whether a reduction in transepithelial sodium transport inhibited neutral amino acid uptake or whether the interference with energy production produced a less specific effect.

Effect of removing mucosal sodium

When transepithelial sodium transport is abolished by removing mucosal sodium while the serosal surface is bathed with frog Ringer's solution, α-aminoiso-

TABLE IV

AMINO ACID ACCUMULATION BY TOAD BLADDER: EFFECT OF REMOVING MUCOSAL SODIUM

Experimental conditions were the same as in Table I except that choline Ringer's solution was used as the mucosal bathing medium. Frog Ringer's solution bathed the serosal surface.

	Accumulation ratio $\frac{counts/min\ per\ ml\ intracellular\ fluid}{counts/min\ per\ ml\ media}$			
	[^{14}C]α-*Aminoisobutyric acid (0.065 mM)*		L-[^{14}C]*Lysine (0.065 mM)*	
	Control	*Choline Ringer*	*Control*	*Choline Ringer*
	7.00	9.37	8.50	12.63
	7.52	5.90	8.27	14.08
	3.19	2.53	10.85	14.44
	1.52	2.43	10.74	14.63
	2.65	4.95	10.50	7.63
	4.56	3.45	8.23	14.81
	4.01	3.52	11.37	10.77
	5.42	9.04	7.09	10.78
			5.88	12.13
			10.05	11.14
Mean	4.48	5.15	9.15	12.30
$\Delta \pm$ S.E.		$+0.67 \pm 0.38$		$+3.15 \pm 0.68$
P		> 0.10		< 0.01

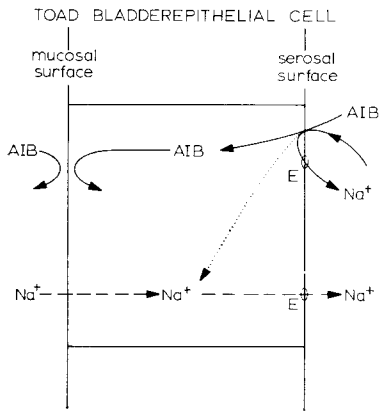

Fig. 3. Schema of α-aminoisobutyric acid (AIB) and sodium interaction in the toad bladder epithelial cell. Net movement of α-aminoisobutyric acid into the cell by a sodium-dependent process is indicated at the serosal surface. (α-Aminoisobutyric acid may cross the serosal border in either direction, but cannot cross the mucosal surface.) The energy-dependent (E) active transepithelial transport process for sodium extrusion is represented by the dashed line (– – –). The sodium which enters the cell with alpha-aminoisobutyric acid may enter the active transport pool (. . . .), or may be extruded by a separate energy-dependent process (E).

butyric acid accumulation is unaffected indicating that transepithelial sodium transport and α-aminoisobutyric acid accumulation are independent processes.

Cessation of transepithelial sodium transport is accompanied by a significant increase in lysine accumulation. This increase is noted regardless of whether mucosal sodium is replaced with choline or potassium and is not explained by changes in fluid spaces or in the percentage of lysine metabolized (as determined by chromatography of cell water). This increased accumulation remains unexplained (Table IV).

DISCUSSION

Amino acids penetrate the toad bladder only from the serosal surface. The failure of intracellularly concentrated amino acids to enter the mucosal bathing medium indicates that the mucosal surface membrane is essentially impermeable to amino acid movement in either direction (Fig. 3).

The separation of transport mechanisms with neutral α-aminoisobutyric acid accumulated by a sodium-dependent, ouabain-sensitive process and cationic lysine accumulated by a sodium-independent, ouabain-insensitive process is similar to the separation previously noted in kidney cortex slices[1].

Previous investigators have demonstrated that the toad bladder transports sodium by energy-dependent processes from mucosa to serosa[8]. The evidence suggests that sodium enters passively at the mucosal border and is actively extruded from the serosal border of the mucosal cell[13]. Removal of serosal sodium only partially decreases sodium transport, while removal of mucosal sodium effectively abolishes it[14]. Sodium entering the toad bladder epithelial cell from the serosal surface has not been demonstrated to enter directly into the active transport pool for transepithelial sodium transport. Therefore, it may not be assumed that sodium entering from the serosal surface is extruded by the transepithelial transport mechanism, and the possibility of a separate energy-dependent pathway for extrusion of serosal sodium must be considered. Neutral amino acid transport is inhibited in the absence of serosal sodium, and persists in the absence of mucosal sodium; this suggests that the processes by which amino acids are accumulated may be independent of those for transepithelial sodium transport, despite the fact that the energy-dependent steps for each process are in the same cell border (Fig. 3).

The dependence of α-aminoisobutyric acid uptake upon serosal sodium may be explained by assuming a role for sodium in (a) the alteration of membrane permeability, or (b) the coupled movement of α-aminoisobutyric acid and sodium across the membrane, or (c) the production of energy non-specifically available for transport. The persistence of other energy-dependent processes at the serosal surface such as transepithelial sodium transport in the absence of serosal sodium indicates that the requirement for serosal sodium cannot simply be the requirement of a non-specific energy-producing process as suggested by CSAKY[6]. The persistence of α-aminoisobutyric acid uptake in the absence of mucosal sodium and its inhibition by ouabain suggests that a serosal sodium extrusion step is involved in the accumulation of α-aminoisobutyric acid and that more than a simple change in membrane permeability is involved. These data are most consistent with a role for sodium as outlined by CRANE[7]. This interpretation of the data assumes the presence of a serosal entry and energy-dependent extrusion of sodium which may be independent of the transepithelial sodium transport pathway.

The accumulation of lysine by the bladder is less well defined. The uptake mechanism is not inhibited by lack of sodium, lack of O_2 or the presence of ouabain. It is inhibited by the structurally similar amino acid arginine and by potassium. Though a poorly defined exchange of potassium and lysine has been described in muscle[15], no such substitution of lysine for intracellular potassium was demonstrated in kidney cortex slices[16]. In the present study the concentration changes in intracellular potassium which would be anticipated would be too small to measure. It is possible that intracellular or surface binding of lysine accounts for the apparent concentrative uptake of this amino acid independent of usual metabolic energy sources. N-Acetylation of lysine to produce a neutral molecule may provide some insight into the handling of lysine. Finally, the small but significant increase in lysine accumulation when mucosal sodium is removed remains unexplained. Cessation of transepithelial sodium transport may allow greater accumulation by increased influx, decreased efflux or a combination of the two. Future investigation of the steady-state kinetics of this system may help answer this problem.

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