Effect of Inhibitors on Sodium-Independent Lysine Transport in Intact Intestine (37159)

PAUL K. FREDERICK, SHELDON REISER, AND PHILIP A. CHRISTIANSEN

Gastrointestinal Research Laboratory and Departments of Medicine and Biochemistry, Indiana University School of Medicine and Veterans Administration Hospital, Indianapolis, Indiana 46202

Sodium is required for the active transport of most organic solutes (1). A sodium gradient across the cell membrane is believed to be necessary to provide the energy for active transport. Lysine transport clearly is an exception to the sodium gradient hypothesis as active lysine transport has been demonstrated in the epithelial cells of toad bladder (2), rat kidney cortex slices (3), and rabbit ileum (4) in the absence of external sodium. The possible mechanisms involved in this non–sodium-dependent transport have stimulated our interest. Munck and Schultz (5), and Reiser and Christiansen (6) have shown that intracellular leucine will stimulate the influx of lysine into rabbit and rat intestine in the absence of sodium. Reiser and Christiansen feel that this stimulation of influx is mediated by an exchange transport system which requires no energy and therefore no external sodium. We wonder if the sodium-independent portion of lysine transport in mammalian intestine is due to an exchange transport with an as yet unidentified substance. Support of this hypothesis can be obtained circumstantially by comparing the characteristics of the sodium-independent component of lysine transport with the characteristics of the sodium-independent lysine transport accelerated by intracellular leucine. This report describes the effect of various inhibitors on these two sodium-independent lysine transport systems in intact intestine with special emphasis on the data as they provide support for the above hypothesis.

Methods. Wistar strain, male rats weighing 150–250 g were used as a source of intestinal tissue. The animals were fed on a standard diet and watered *ad libitum* and were not deprived of food prior to sacrifice.

All animals were killed by decapitation, the small intestine quickly removed and 15 cm trimmed from both the proximal and distal ends. Everted sacs 40 cm in length were prepared and filled with the standard incubation media and incubated for 15 min at 37° in 100 ml of oxygen gassed incubation media with or without 1 m*M* leucine. After preincubation the sac was cut open at one end and the inside medium drained. The residual intestinal segment was washed in either saline (with sodium experiments) or isotonic choline chloride (without sodium experiments), divided into segments approximately 10 cm in length, randomized, filled with standard incubation medium lacking lysine and inulin, and tied into sacs according to the method of Wilson and Wiseman (7). The sacs were then incubated at 37° in 5 ml of oxygenated incubation media for 30 min in a water bath with shaker oscillating 50–75 times per minute. The standard incubation medium employed was an oxygenated Krebs–Ringer–Tris buffer, pH 7.4, containing 118 m*M* sodium chloride, 25 m*M* Tris chloride, 4.7 m*M* potassium chloride, 2.5 m*M* calcium chloride, 1.2 m*M* magnesium sulfate, 1.2 m*M* potassium phosphate (monobasic), 8,000–15,000 counts/min per ml tritiated inulin and ^{14}C radioactive (5,000–12,000 counts/min per ml) and nonradioactive L-lysine to a final concentration of 1 m*M*. On the basis of previous results 118 m*M* sodium gives optimum amino acid transport (8). In the studies using sodium-free incubation media, choline chloride was used as an isotonic replacement for the sodium chloride of the Krebs–Tris buffer. The sodium concentration of the incubation media was determined by direct analysis in a Coleman flame photometer.

Following the incubation the sacs were removed from the flasks, and excess incubation

medium was removed by touching the sacs on the side of a glass beaker. The sacs were opened, the inside medium was collected, and the empty sacs were washed, blotted and weighed. The residual tissue was then homogenized in 4 times its weight of 5% trichloroacetic acid to make a 20% homogenate. The homogenate was centrifuged, and aliquots of the supernatant, as well as the inside media, were counted in a Tri-Carb liquid-scintillation spectrometer (Packard) in a system containing xylene–dioxane–ethanol (5:5:3), naphthalene (40 g/liter), 2,5-diphenyloxazole (5 g/liter), and 1,4-bis-2-(5-phenyloxazolyl)-benzene (100 mg/liter). The spectrometer was adjusted to permit less than 0.01% ^{3}H efficiency on the ^{14}C channel and 10% efficiency of the ^{14}C on the ^{3}H channel. These settings allowed for approximately 60% ^{14}C counting efficiency and 22% ^{3}H counting efficiency. An extracellular fluid space was calculated from the distribution space of [^{3}H]inulin (corrected for 10% ^{14}C contribution) in the residual sac and expressed as percent of tissue wet weight (9).

Lysine transport is expressed in the two following forms:

(a) Intracellular accumulation is the millimolar concentration of lysine in the cellular water after 30 min, assuming a water content of approximately 80% of the tissue wet weight and a tissue density of 1 g/ml (10). This parameter was calculated on the basis of a modification of a formula used by Crane and Mandelstam (11) which now takes the following form: m*M* lysine in cellular water= (m*M* lysine in homogenate supernatant $\times$ homogenate volume) — (extracellular space $\times$ tissue wet weight $\times$ 0.8 $\times$ initial m*M* lysine in medium)/(1 — extracellular space) $\times$ (tissue wet weight $\times$ 0.8).

(b) Serosal appearance is expressed as the amount (μmoles) of lysine appearing in the serosal medium per 500 mg tissue wet weight after 30 min.

The counting rate of each individual experiment was mathematically treated so that 10,000 counts/min per ml were equivalent to the initial concentration of the lysine. This permitted the conversion of counts to μmoles lysine from identical experiments having somewhat different initial counting rates. A paired-difference *t* test was used to determine if a significant difference existed between lysine transport in the absence or presence of the inhibitors. References to statistical significance pertain to the 5% probability level or below.

Ethacrynic acid and probenecid were used as the pure powders and were kindly supplied by Merck, Sharp and Dohme Research Laboratories, West Point, Pennsylvania.

Results. The purpose of the experiments, Table I, was to determine the effects of the metabolic inhibitors on sodium-dependent and sodium-independent lysine transport and to serve as a basis of comparison of the effect of the same inhibitors on the stimulation of lysine transport by intracellular leucine. In the absence of extracellular sodium, the intracellular accumulation of lysine was reduced by about 30% but still attained levels significantly greater ($p < 0.001$) than unity. The maintenance of an essentially sodium-free environment during these and similar studies was confirmed by the finding that only 4.5 $\pm$ 0.2 m*M* sodium (mean $\pm$ 1 SEM; $n = 156$) was present in the mucosal medium after incubation. In contrast to lysine, the intracellular accumulation of leucine was reduced 66% and did not reach levels greater than that of the initial medium in the absence of extracellular sodium. These results show the presence of a sodium-independent active transport process in rat intestine that appears to be relatively specific for basic amino acids as typified by lysine. Dinitrophenol (DNP) eliminated active lysine transport in the presence and absence of sodium with intracellular accumulation values not attaining values greater than unity. These results represent strong evidence for the view that the maintenance of non–sodium-dependent lysine transport requires energy. Like DNP, the sulfhydryl inhibitor *N*-ethylmaleimide (NEM) produced a decrease in lysine intracellular accumulation regardless of the presence or absence of sodium. Five millimolar ethacrynic acid was used as an inhibitor of the sodium and potassium dependent ATPase since 1 m*M* ouabain added to the incubation medium produced little if any inhibition of lysine transport. Isolated epithelial cell preparations

TABLE I. Effect of Metabolic Inhibitors on the Sodium-Dependent and Sodium-Independent 30-min Intracellular Accumulation of Lysine.[a]

	Intracellular lysine accumulation (m*M*/30 min)					
	With sodium			Without sodium		
Inhibitor	Inhibitor absent	Inhibitor present	% Decrease	Inhibitor present	Inhibitor absent	% Decrease
0.4 m*M* Dinitrophenol	1.97 ± 0.14	0.85 ± 0.07	56.9	1.56 ± 0.10	0.90 ± 0.06	42.3
	$P < 0.001$			$P < 0.001$		
0.4 m*M* *N*-ethylmaleimide	1.91 ± 0.08	1.35 ± 0.10	29.3	1.62 ± 0.07	1.36 ± 0.10	16.0
	$P < 0.001$			$P < 0.05$		
5 m*M* Ethacrynic acid	2.20 ± 0.16	1.00 ± 0.08	56.1	1.39 ± 0.08	1.26 ± 0.10	9.3
	$P < 0.001$					
1 m*M* Sodium fluoride	2.12 ± 0.15	1.76 ± 0.08	17.0	1.54 ± 0.08	1.45 ± 0.08	5.8
	$P < 0.05$					
5 m*M* Probenecid	2.05 ± 0.13	1.43 ± 0.14	30.2	1.58 ± 0.12	1.58 ± 0.14	
	$P < 0.001$					
5 m*M* Adenosine triphosphate	2.20 ± 0.13	1.53 ± 0.10	30.5	1.45 ± 0.10	1.03 ± 0.08	29.0
	$P < 0.001$			$P < 0.01$		

[a] Each value represents the mean value from 12 rats ± SEM. A paired-difference *t* test was used to obtain the probability values. A *P* of 0.05 or less was considered significant and only these probability values are shown.

obtained from rat intestine have been found to be very sensitive to ouabain (12), therefore one can postulate that the serosal membrane of intact rat intestine may be relatively impermeable to ouabain. Ethacrynic acid has been shown to inhibit sodium absorption in the loop of Henle (13) and in hamster intestine (14) and inhibit the extrusion of sodium from guinea-pig kidney cortex slices (15). Ethacrynic acid significantly inhibited lysine intracellular accumulation by 56% in the sodium containing system. The comparatively very small 9% inhibition of the sodium-independent lysine intracellular accumulation confirms that the major action of ethacrynic acid is limited to sodium-dependent processes. One millimolar sodium fluoride inhibited the sodium-dependent lysine accumulation by 17% while not significantly influencing the magnitude of the sodium-independent lysine accumulation. These results indicate that the sodium-dependent component of lysine transport derives a portion of its energy from glycolysis while the sodium-independent component does not. The small increase in the mucosal sodium concentration due to addition of 1 m*M* sodium fluoride would not be expected to influence these results. Probenecid is an established inhibitor of renal transport that can block either reabsorption or secretion of several organic compounds (16). Binder *et al.* (14) demonstrated that 5 m*M* probenecid inhibited methionine and lysine transport in hamster small intestine, while having no effect on glucose, sodium or water transport. Probenecid was found to specifically inhibit the sodium-dependent component of lysine intracellular accumulation. ATP was used as in inhibitor in these studies on the basis of previous results which showed that ATP inhibited amino acid uptake by isolated intestinal epithelial cells (17). Five millimolar ATP significantly inhibited lysine accumulation in intact intestine by about 30% both in the presence or absence of extracellular sodium.

In isolated intestinal epithelial cells intracellular leucine produced a stimulation of both the influx and steady-state levels of lysine (6). Work with intact intestine (5, 6) showed an increase in transmural transport but no consistent increase in intracellular accumulation. In the present study, Table II, we were similarly unable to show consistent increases in lysine intracellular

TABLE II. Effect of Inhibitors on the Stimulation of the Serosal Appearance of 1 m*M* Lysine by Intracellular Leucine in a Na^+-Free System.[a]

Inhibitor	Serosal appearance, μmoles lysine/500 mg tissue/30 min: Krebs–Tris preincubated	Krebs–Tris + 1 m*M* leucine preincubated	% Change
None (72)	0.21 ± 0.01	0.30 ± 0.01	+43
	$P < 0.001$		
0.4 m*M* Dinitrophenol (12)	0.14 ± 0.01	0.16 ± 0.01	+14
0.4 m*M* *N*-ethylmaleimide (12)	0.22 ± 0.03	0.31 ± 0.02	+41
	$P < 0.05$		
5 m*M* Ethacrynic acid (12)	0.16 ± 0.01	0.27 ± 0.04	+69
	$P < 0.05$		
1 m*M* Sodium fluoride (12)	0.24 ± 0.02	0.35 ± 0.04	+46
	$P < 0.05$		
5 m*M* Probenecid (12)	0.16 ± 0.02	0.27 ± 0.02	+69
	$P < 0.01$		
5 m*M* Adenosine triphosphate (12)	0.20 ± 0.02	0.23 ± 0.02	+15

[a] Values expressed as mean ± SEM from the number of individual experiments found in the parentheses. Probability values were obtained and expressed as in Table I.

accumulation due to intracellular leucine. Munck and Schultz (5) showed an increase in mucosal-to-cell flux and cell-to-serosal flux of lysine in the presence of intracellular leucine. The increase in cell-to-serosal flux may explain why the intracellular accumulation of lysine is not raised by intracellular leucine. On the basis of these considerations, we interpret the increase in lysine serosal appearance as a reflection of the increase in the mucosal-to-cell lysine flux which occurs in the presence of intracellular leucine (6). In agreement with previous findings (5, 6) the relative magnitude of the leucine stimulation of lysine transport was found to be about the same in the presence or absence of sodium. Of the inhibitors tested, Table II, only DNP and ATP decreased the relative magnitude of the leucine stimulation of lysine serosal appearance.

Discussion. The exchange transport system mediating the active uptake of lysine in intestinal cells has been shown to be independent of sodium (6). We proposed that the sodium-independent stimulation of lysine serosal appearance by intracellular leucine could be taken as a model of exchange transport for comparison with the properties of sodium-independent lysine transport. Supporting this concept are the data gathered by Reiser and Christiansen (18) regarding the kinetics of intracellular leucine-stimulated serosal appearance. The stimulation of lysine transport was found to be due to an increase in V_{max}. These findings are consistent with the kinetics describing the increase in lysine uptake due to the presence of intracellular leucine or alanine in isolated intestinal cells (unpublished results). Ethacrynic acid, sodium fluoride and probenecid did not inhibit either sodium-independent lysine intracellular accumulation or the stimulation of lysine serosal appearance by intracellular leucine in the absence of sodium. Conversely, DNP and ATP inhibited both of these sodium-independent transport processes. The only finding at variance with this correlation was the failure of NEM to produce a decrease of leucine-stimulated lysine transport comparable to the 16% inhibition of lysine intracellular accumulation. These results indicate that the action of the inhibitors on

these two sodium-independent lysine transport systems are generally similar and are therefore consistent with the hypothesis that sodium-independent lysine transport may be mediated by an exchange transport.

Summary. The effect of various inhibitors on the sodium-independent intracellular accumulation of 1 m*M* lysine and the sodium-independent stimulation of 1 m*M* lysine transmural transport by intracellular leucine was compared in intact rat intestine. Five millimolar ethacrynic acid, 1 m*M* sodium fluoride, and 5 m*M* probenecid did not inhibit either of these sodium-independent lysine transport systems while 0.4 m*M* DNP and 5 m*M* ATP inhibited both. NEM (0.4 m*M*) decreased lysine intracellular accumulation but not leucine-stimulated lysine transport. These results show that the action of the inhibitors on these two sodium-independent lysine transport systems are generally similar.

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1. Schultz, S. G., and Curran, P. F., Physiol. Rev. **50**, 637 (1970).

2. Thier, S. O., Biochim. Biophys. Acta **150**, 253 (1968).

3. Fox, M., Thier, S. O., Rosenberg, L., and Segal, S., Biochim. Biophys. Acta **79**, 167 (1964).

4. Munck, B. G., and Schultz, S. G., J. Gen. Physiol. **53**, 157 (1969).

5. Munck, B. G., and Schultz, S. G., Biochim. Biophys. Acta **183**, 182 (1969).

6. Reiser, S., and Christiansen, P. A., Biochim. Biophys. Acta **241**, 102 (1971).

7. Wilson, T. H., and Wiseman, G., J. Physiol. London **123**, 116 (1954).

8. Reiser, S., and Christiansen, P. A., Amer. J. Physiol. **212**, 1297 (1967).

9. Rosenberg, L. E., Downing, S. J., and Segal, S., Amer. J. Physiol. **202**, 800 (1962).

10. Spencer, R. P., and Samiy, A. H., Amer. J. Physiol. **200**, 501 (1961).

11. Crane, R. K., and Mandelstam, P., Biochim. Biophys. Acta **45**, 460 (1960).

12. Reiser, S., and Christiansen, P. A., Biochim. Biophys. Acta **225**, 123 (1971).

13. Goldberg, M., McCurdy, D. K., Foltz, E. L., and Bluemle, L. W., Jr., J. Clin. Invest. **43**, 201 (1964).

14. Binder, H. J., Katz, L. A., Spencer, R. P., and Spiro, H. M., J. Clin. Invest. **45**, 1854 (1966).

15. Proverbio, F., Robinson, J. W. L., and Whittembury, G., Biochim. Biophys. Acta **211**, 327 (1970).

16. Brazeav, P., *in* "Pharmacological Basis of Therapeutics" (L. S. Goodman and A. Gilman, eds.), 3rd Edition, p. 871. Macmillan, New York (1965).

17. Reiser, S., and Christiansen, P. A., Biochim. Biophys. Acta **233**, 480 (1971).

18. Reiser, S., and Christiansen, P. A., Clin. Res. **XVII**, 528 (1969).

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