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Specificity of intestinal brush-border proline transport: cyanine dye studies

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The ability of rabbit jejunal brush borders to transport inhibitors of the imino carrier was investigated in membrane vesicles by measuring their ability to depolarize the membrane potential. Membrane potentials were monitored using a voltage-sensitive cyanine dye. Piperidine and pyrrolidine carboxylic acids, which are potent inhibitors of Na^+-dependent proline transport ($K_i < 0.5$ mM) depolarize the potential in a Na^+-dependent, saturable manner indicating transport. On the other hand, *N*-methylated amino acids, which are fair inhibitors (K_i 2–10 mM), do not depolarize the membrane to any significant extent, but they competitively inhibit the L-proline transport signal. This indicates that these analogs are nontransported inhibitors of the imino carrier. The poor inhibitors niacin and pipolinic acid ($K_i > 60$ mM) depolarize the membrane about twice as much as proline and with low K_f values. This suggests separate carriers for these substrates.

In studies of amino acid transport, it is common practice to measure substrate specificity by competition experiments. Even when competitive inhibition is observed, it is unclear whether or not the inhibitor is actually transported. Normally it is impractical to measure transport owing to the expense or availability of radiolabeled amino acids and their analogs. So in this study we have used a rapid, inexpensive electrical method to examine the transport of inhibitors of the intestinal brush-border imino carrier.

The imino carrier is a fairly specific Na^+-dependent system in rabbit intestine which handles L-proline but not neutral amino acids [1–3]. In guinea pig the imino carrier also accepts α-aminomonocarboxylic acids [4]. The specificity of this rabbit carrier has been examined by competition experiments [3], and a fairly rigid set of structural requirements have been defined. Namely, all analogs with inhibitor constants (K_i) less than 0.5 mM are piperidine or pyrrolydine 2-carboxylic acids or esters. Although α-amino acids ($K_i = \infty$) interact poorly with this imino carrier, the *N*-methylated amino acids all have K_i values between 2 and 10 mM.

We have examined the transport of these two classes of inhibitors using as an assay their ability to depolarize the brush-border membrane. All substances known to be transported across intestinal and renal brush borders by Na^+-cotransport depolarize the membrane in a Na^+-dependent, saturable manner [5]. Evidence is obtained that the pyrrolidine and piperidine analogs of proline are transported by the imino carrier, but that the *N*-methyl acylic inhibitors are probably not.

Intestinal brush-border membrane vesicles were

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Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; diS-C_3-(5), 3,3'-dipropylthiadicarbocyanine iodide; MeAIB, α-methylaminoisobutyric acid.

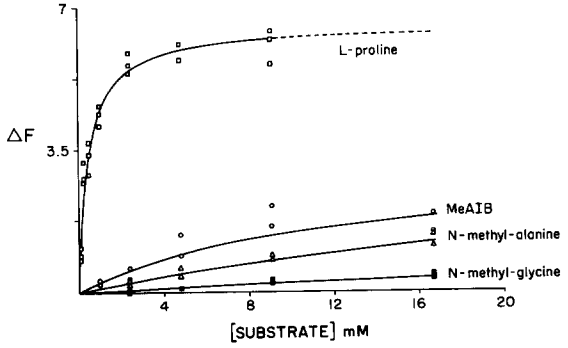

Fig. 1. Na^+-dependent ΔF values for L-proline and *N*-methylated amino acids. Experiments were conducted in duplicate or triplicate on one batch of membranes as described in Table I.

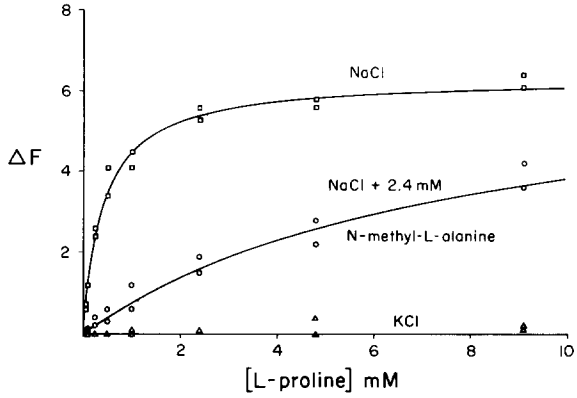

Fig. 2. Effect of *N*-methyl-L-alanine on the L-proline ΔF values. Experiments were conducted as described in Table I in the absence or presence of 2.4 mM *N*-methyl-L-alanine. The inhibitor was added before L-proline to ensure that the proline ΔF was recorded. Note that the 2.4 mM *N*-methyl-L-alanine ΔF is less than 0.5 units (see Fig. 1).

prepared from rabbit jejunum by a Ca^{2+}-differential centrifugation procedure [1] and membrane potentials were monitored [6] with a voltage-sensitive fluorescent dye (3,3′-dipropylthiadicarbocyanine iodide, diS-C_3-(5)). All amino acids and analogs were purchased from Sigma Chemical Co., St. Louis, MO, and Aldrich Chemical Co., Milwaukee, WI, except for *N*-methyl-L-alanine which was from Vega Biochemicals, Tucson, AZ.

Figs. 1 and 2 show the Na^+-dependent change in fluorescence produced by L-proline as a function of proline concentration under zero-*trans* conditions, i.e. intravesicular Na^+-free and proline-free conditions. The curves can be fitted by a function representing a single saturable process.

$$\Delta F = \frac{\Delta F_{max}[Pro]}{K_f + [Pro]}$$

where the maximum change in fluorescence (ΔF_{max}) is about 6 units (approx. 10 mV) and the

TABLE I

KINETICS OF Na^+-DEPENDENT AMINO ACID TRANSPORT

ΔF_{max} and K_f values obtained for substrates in intestinal brush-border membrane vesicles.For each substrate the kinetic parameters were measured using dis-C_3-(5) as described previously [6]. Vesicles contained 300 mM mannitol and 50 mM Tris-Hepes (pH 7.5) and were added to cuvettes containing 100 mM NaCl or KCl, 100 mM mannitol and 50 mM Tris-Hepes (pH 7.5). After 1 min, substrates (pH 7.5) were added to the cuvette to give eight different concentrations (0–18 mM) and the maximum change in fluorescence ΔF of dis-C_3-(5) was recorded (in triplicate) at λ_{ex} 620 nm and λ_{em} 699 nm with a band width of 2 nm. There was no significant change in ΔF in KCl, but in NaCl ΔF varied with substrate concentration as a single saturable function (see Figs. 1 and 2). ΔF_{max} and K_f were obtained by iterative non-linear regression. All values were obtained with a single batch of membranes in one experiment. Essentially identical results were obtained in two other membrane preparations (identical K_f and similar ΔF_{max} values). * Inhibitor constants (K_i values) obtained in a parallel series of experiments using radioactive tracer uptakes [3], in which Na^+-dependent 50 μM L-proline uptakes were measured in the presence and absence of inhibitors (see Ref. 1).

	ΔF_{max}	K_f (mM)	K_i (mM) *
L-Pipecolic acid	5.0±0.1	0.32±0.02	0.18
L-Proline	4.9±0.2	0.42±0.04	0.29
OH-L-proline	4.8±0.2	1.2 ±0.1	0.15
L-Proline methyl ester	5.1±0.3	1.9 ±0.2	0.38
Betaine	5.4±0.5	7.2 ±1.3	1.5
Picolinic acid	9.9±0.3	2.4 ±0.2	59.0
Niacin	9.5±0.6	6.5 ±0.7	375.0
L-Phenylalanine	17.8±1.6	7.4 ±0.9	16.0

L-proline concentration producing 0.5 $\Delta F_{max}(K_f)$ is approx. 0.5 mM.

Table I summarizes the results obtained in similar experiments with seven cyclic analogs of L-proline and also shows their inhibitor constants obtained in parallel experiments [3]. The results with L-proline, L-hydroxyl proline and L-phenylalanine are similar to those obtained previously [6]. Examination of Table I shows that the pyrrolidine and piperidine carboxylic acids and derivatives all depolarize the membrane to the same extent, i.e. ΔF_{max} values are not different, and the ranking of the K_f values agree with the K_i values. However, the pyridine carboxylic acids (picolinic acid and niacin) and L-phenylalanine depolarize the membrane about 2–3-times greater than L-proline, ΔF_{max} 10–18 vs. 6 units, and there are marked differences between the K_f and K_i values. Whereas the niacin and L-phenylalanine K_f values are similar (approx. 8 mM) there is a 200-fold difference in the K_i values, and the relative rankings of picolinic acid and L-phenylalanine do not agree. These results suggest that a system (or systems) separate from the imino carrier handles the transport of these three substrates in intestinal brush borders.

Fig. 1 also shows the effect of three *N*-methyl amino acids on the fluoresence of diS-C_3-(5). All three gave rather small and variable ΔFs up to 18 mM. This is reflected in the estimates of ΔF_{max} and K_f: MeAIB ΔF_{max} 4 ± 1 and K_f 12 ± 2 mM; *N*-methyl-L-alanine ΔF_{max} 3 ± 1 and $K_f > 17$ mM; *N*-methylglycine (sarcosine) $K_f > 30$ mM. In contrast, *N,N,N*-trimethylglycine (betaine) gave the same ΔF_{max} as L-proline (5.4 ± 0.5) and a K_f of 7.2 ± 1.3 mM. These K_f values are 3–6-times higher than the K_i values obtained in tracer experiments [3].

The effect of one of the *N*-methylated amino acids on the L-proline depolarization was investigated. At a fixed, saturable L-proline concentration (9 mM), the concentration of *N*-methyl-L-alanine was varied up to 10 mM. The Na^+-dependent L-proline signal was completely blocked with 50% inhibition at 2.4 mM *N*-methyl-L-alanine (not shown). We next measured the effect of 2.4 mM *N*-methyl-L-alanine on the concentration dependence of the L-proline signal (Fig. 2). There was marked inhibition of the proline ΔF at low, but not high, proline concentrations. ΔF_{max} and K_f were 6.4 ± 0.1 units and 0.42 ± 0.02 mM in the absence of *N*-methyl-L-alanine and 6.9 ± 1.4 units and 8.0 ± 2.4 mM in its presence. The apparent change in the affinity of L-proline yields an inhibitor constant of 0.12 mM (see Ref. 7). On the other hand, there was no effect of this analog on the L-phenylalanine signal: the ΔF_{max} and K_f values were 14 ± 1 units and 8 ± 1 mM in the absence and 12 ± 1 units or 6 ± 1 mM in the presence of 2.4 mM *M*-methyl-L-alanine. These experiments indicate that *N*-methyl-L-alanine is a competitive inhibitor of Na^+-dependent L-proline, but not L-phenylalanine, transport.

On the basis of these and parallel radioactive tracer experiments [3,4], we conclude that the potent inhibitors ($K_i < 0.5$ mM) of proline transport (piperidine and pyrrolidine carboxylic acids) are also transported. A similar conclusion applies to *N,N,N*-trimethylglycine (betaine). However, the *N*-methylated amino acids which are fairly good inhibitors (K_i 2–10 mM) of proline transport (measured by tracer uptakes and membrane potentials) appear to be poorly transported themselves. In fact, the initial rate of Na^+-dependent 50 μM MeAIB uptake is only 13% of the L-proline uptake (Ref. 1; Fig. 1e, and Table I) despite the similarity between the MeAIB K_i (0.78 mM) and the proline K_m (0.55 mM) (Ref. 1, Table IV). Thus inhibitors such as these may prove useful in measuring binding kinetics to the imino carrier in both brush borders and solubilized proteins. Finally, the results with phenylalanine, niacin, and picolinic acid suggest the presence of carriers separate from the imino carrier. There is strong independent evidence for a separate phenylalanine carrier [1,2]. In kidney, niacin may be handled by a monocarboxylic carrier [8], but there is no evidence for this carrier in rabbit intestinal brush borders (see Ref. 6).

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