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L-PROLINE UPTAKE IN HUMAN FIBROBLASTS : EVIDENCE FOR A HIGH-AFFINITY SYSTEM IN ADDITION TO SYSTEM A

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Proline uptake was studied in human skin fibroblasts by simultaneous running of kinetic and inhibition experiments on the same cell lines. Two systems for proline uptake were shown : a high-affinity system not inhibited by α-(methylamino)isobutyric acid and a low affinity system inhibited by this amino acid (i.e. system A). These results appear to be of interest, firstly because up till now, system A was considered preferable for proline uptake in human fibroblasts, and secondly because they illustrate the need for combined inhibition and kinetic studies of amino acid uptake, especially when the substrate concentration range used and the respective Km of the systems do not allow their détection by kinetic analysis alone. Furthermore, this high-affinity system may have major physiological implications.

We previously studied the effect of cycloleucine on the kinetics of amino acid uptake in cultured human fibroblasts and defined one uptake system for proline only (1). Since L-proline may be considered as a tracer amino acid in evaluating the transport activity by system A in this cell type (2), we examined the inhibition of proline uptake by Me-AIB, and to our surprise obtained results suggesting the existence of more than one proline uptake system. We therefore conducted simultaneous kinetic and inhibition experiments on the same human skin fibroblast cell lines.

MATERIALS AND METHODS

Human fibroblasts were obtained from skin biopsies taken from children during abdominal surgery. Five different cell lines were grown under perfectly standardized conditions as previously described (3). We adopted the cluster tray method described by Gazzola et al. (4) with minor modifications.

Abbreviations : Me-AIB : α-(methylamino) isobutyric acid, P.B.S-G Phosphate-buffered saline solution, pH 7.45, supplemented with 5.56 mM glucose, System A, alanine-preferring system, System ASC, alanine, serine and cysteine-preferring system.

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For measurement of amino acid transport, about 3 X 10^4 cells between passages 3 and 6 were seeded in 2 cm^2 wells of disposable multiwell trays (Costar) and incubated for 72 or 96 h in 1 ml of growth medium, always replaced 24 H before each experiment. At this time the cells were just reaching confluency as estimated visually with an inverted phase microscope. The procedure for amino acid transport then began with 90 min of preincubation with PBS-G, after which excess liquid was removed and the experimental cover immediately placed over the drained monolayers. The 24 tubes in the experimental covers each contained 0.3 ml of PBS-G, comprising 0.5 µCi of L-[U-^{14}C] proline (260 mCi/mmol, CEA-Gif sur Yvette France) plus the desired concentration of the non radioactive L-proline (Sigma Chemical Co) and when necessary, of the inhibitor. The contents of the 24 tubes were simultaneously transferred to the monolayers in 24 wells by vigorous horizontal shaking of the whole tray and cover, and incubated for 1 minute at 37°C. Transport assays were terminated by dumping the uptake medium in one motion and immediately washing the 24 wells three times with 2 ml of ice-cold 0.154 M NaCl (total washing time : 10 sec). The cluster trays were then drained and the monolayers extracted with 250 µl of 1N NaOH by incubation and gentle shaking for at least 2 hours at room temperature. Next, 100 µl of dissolved cells was counted for radioactivity in 15 ml of counting liquid (Picofluor) and 100 µl was used for protein determination by the method of Lowry et al (5). The counting efficiency of the NaOH samples was 85 %. Quenching was assessed by external standardization with a Packard 460 c scintillation spectrometer. Average protein content was 10-15 µg per 100 µl NaOH solution, i.e 25-40 µg per well. For experiments in the absence of sodium, choline chloride replaced sodium chloride in the incubation mixture and washing medium.

RESULTS AND DISCUSSION

. Inhibition of proline uptake

We chose an inhibitor-to-substrate ratio of 10 (6,7). These experiments were performed on cell lines 1 and 2. As shown in Table I, six proline concentrations were incubated for 1 min, in the presence or absence of inhibitor. The proline concen-

Table I

Effect of Me-AIB, serine (Ser) or glycine (Gly) on L-proline uptake in human fibroblasts

	L-proline concentration (mM)	INHIBITOR									
		None		Me-AIB			Ser** or Gly***			Me-AIB and Ser		
		Protein*	Uptake	Protein*	Uptake	% Inhib	Protein*	Uptake	% Inhib	Protein*	Uptake	% Inhib
Cell line 1	0.5	24	71.3	22	21.5	69.8	20	14.8	79.2	29	9.6	86.9
	0.2	24	47.3	25	19.5	58.8	21	18.1	61.7	31	11.9	74.8
	0.1	29	60.5	24	25.2	53.3	22	26.1	56.9	31	17.1	71.7
	0.05	25	67.4	23	32.6	51.6	22	35.5	47.3	31	26.8	60.2
	0.025	42	52.3	40	37.4	29.5	32	48.0	8.0	44	34.0	35.0
	0.010	40	71.1	34	52.5	26.2	28	65.0	8.5	44	46.4	34.0
Cell line 2	0.5	36	20.4	34	12.5	38.7	35	16.7	18.1			
	0.2	41	39.0	42	24.1	38.2	40	29.5	24.0			
	0.1	38	41.2	36	26.3	36.2	34	31.3	24.0			
	0.05	38	43.8	35	34.4	21.5	34	37.5	14.4			
	0.025	39	50.1	42	36.6	26.9	36	49.3	1.6			
	0.010	42	57.8	38	51.2	11.4	34	57.8	0			

Uptake (dpm/µg protein) was measured at 1 min incubation time in two different cell lines for proline concentrations ([S]) ranging from 0.010 to 0.5 mM in the presence of 10 [S] inhibitor, as described under Materials and Methods. Each result is the average of 6 experimental values.

* µg per well
** Effect of Ser on cell line 1
*** Effect of Gly on cell line 2

tration range of 0.010 to 0.50 mM allowed preferential investigation of high-affinity systems. Three amino acids, methylamino isobutyric acid (Me-AIB), serine and glycine were used to test the contribution to proline uptake of the A, ASC and imino-glycine transport systems respectively. None of the three amino acids inhibited proline uptake by more than 30 % for the two lowest proline concentrations of 0.010 and 0.025 mM. Even when 10 [S] of Me-AIB and 10 [S] of serine were simultaneously added, the inhibition did not exceed 35 % (Table I). This finding indicates that a system other than the A, ASC or iminoglycine systems intervenes in proline uptake.

The role of sodium-ion in proline uptake was also tested (results not shown) and inhibition of this uptake was found to be stronger in the absence of sodium than in the presence of Me-AIB.

We noticed that the percentage of inhibition by Me-AIB was much larger for cell line 1 than for cell line 2. Despite the standardization of our experimental conditions, we also noticed that, probably owing to the different growth rates of these lines, the protein content of the wells (i.e cell density) was smaller in cell line 1 than in cell line 2. These results are in agreement with the well-known sensitivity to cell density of the Me-AIB-inhibited system A, whose activity decreases as cell density increases. Consequently, the greater inhibition of proline uptake by Me-AIB in cell line 1 may be due to the fact that the density of this cell line is lower than that of cell line 2.

One factor limiting the study of proline uptake in human fibroblasts is that, at the present time, no specific substrate for the ASC system has been characterized in these cells. Serine may be considered the best substrate for the ASC system, even though it is known to be transported by both the ASC and A systems (6). In Chinese hamster ovary cells, the use of Me-AIB, serine and glutamine allowed Moffett et al (8) to define a new system contributing to proline uptake which they called system P. In human fibroblasts, the inhibition studies reported in the literature indicate that at a concentration of 1 mM, proline is a preferential substrate for system A (9, 10). Russell et al (11) only observed 20 % inhibition by Me-AIB for a proline concentration of 0.2 mM after 15 min incubation, and attributed this non-inhibitable uptake to system ASC. These results may be explained by the fact that, as shown here, the participation of system A in proline uptake decreases with the substrate concentration. However, at a concentration of 0.1 mM Gazzola et al (12) found 80 % inhibition by Me-AIB, but their experimental conditions (preincubation in the presence of serum) were known to enhance the uptake by system A.

. Kinetic studies

The present inhibition studies show that a system other than system A is involved in proline uptake. It is probably a very high affinity system as it was particularly effective at very low proline concentrations (0.010 and 0.025 mM). To check this probability we conducted further kinetic studies on three other cell lines, 3, 4

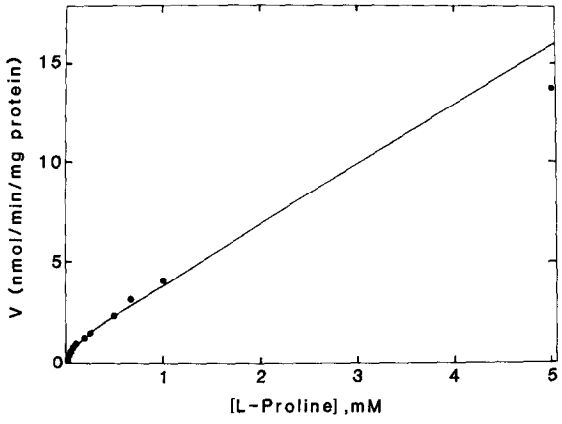

Figure 1.

Kinetics of L-proline uptake, in the presence of Me-AIB, in a human skin fibroblast cell line.

Initial rates of uptake (1 min) were measured for proline concentrations ([S]) ranging from 0.010 to 5 mM in the presence of 10 [S] Me-AIB as described under Materials and Methods. Each point is the average of 6 experimental results. Fitting is obtained with the model described under table II.

and 5. We used a wide range of 13 concentrations from 0.010 to 5 mM, in the absence and presence of 10 [S] Me-AIB and chose an incubation time of 1 min after ensuring that at the end of this period, proline uptake was still linear with time. Kinetic transport parameters were determined by non-linear regression analysis (13) as previously described (1). Different mathematical models were systematically tested.

- In the presence of Me-AIB

The existence of a saturable process was particularly striking (e.g. in cell line 4, Fig.1).

The appropriate model is : $v = Vmax \frac{[S]}{Km + [S]} + K_D [S]$, and this Me-AIB non-inhibitable system has a high affinity : Km = 0.0505 to 0.124 mmol/l (Table II)

- Therefore, in the absence of Me-AIB, the correct model is :

$$v = Vmax_1 \frac{[S]}{Km_1 + [S]} + Vmax_2 \frac{[S]}{Km_2 + [S]} + K_D [S]$$

This model includes five unknown constants, which could not be calculated in the absence of fair initial estimates. Therefore, for the high-affinity system, we took account of the constants obtained for the system not inhibitable by Me-AIB. Owing to the high affinity of this system and to our concentration range, we postulated that the low affinity system (i.e the Me-AIB inhibitable system) was preponderant. Therefore the initial estimates for the latter system were based on the constants defined assuming the existence of only one saturable system. Accordingly, the mathematical procedure was modified in order to take simultaneous account of the experiments with and without inhi-

Table II

Kinetic parameters of the Me-AIB non-inhibitable system for L-proline uptake, in human fibroblasts

	Cell line 3	Cell line 4	Cell line 5
V_{max}	1.61 ± 0.24	0.866 ± 0.126	1.19 ± 0.20
K_m	0.124 ± 0.026	0.0560 ± 0.0115	0.0505 ± 0.0172
K_D	1.75 ± 0.23	3.01 ± 0.29	1.75 ± 0.38

Approximate initial rates of uptake (1 min) were measured in three different cell lines for proline concentrations ([S]) ranging from 0.010 to 5 mM in the presence of 10[S] Me-AIB, as described under Materials and Methods. As shown in figure 1 the model fitting the experimental results is :

$$v = V_{max} \frac{[S]}{K_m + [S]} + K_D [S]$$

kinetic parameters are expressed ± S.D. (K_m as mmol/l ; V_{max} as nmol/min/mg protein)

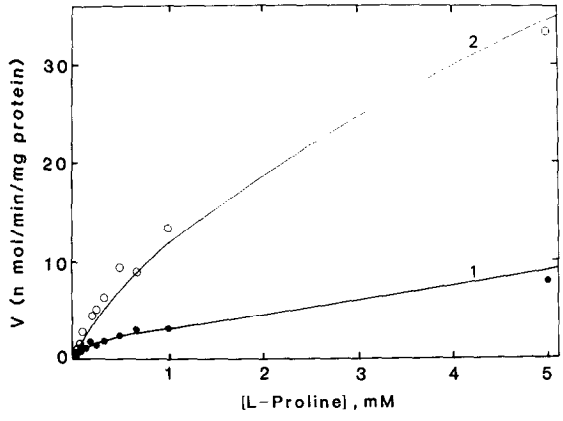

Figure 2.

Kinetics of L-proline uptake in human skin fibroblasts in the presence and absence of Me-AIB.

Initial rates of uptake (1 min) were measured for proline concentrations ([S]) ranging from 0.010 to 5 mM, in the presence or absence of 10 [S] Me-AIB, as described under Materials and Methods.

- ● experimental points in the presence of Me-AIB ; each point in the average of 6 results.
- ○ experimental points in the absence of Me-AIB ; each point is the average of 6 results.

Curves 1 and 2 respectively represent the process not inhibitable by Me-AIB and the inhibitable one (i.e system A uptake) plus a diffusion process, fitted as described under Table III.

Table III

Kinetic parameters for L-proline uptake in human fibroblasts

		Cell line 3	Cell line 4	Cell line 5
Me-AIB non inhibitable system	$Vmax_1$	2.04 ± 0.46	1.00 ± 0.16	1.47 ± 0.26
	Km_1	0.191 ± 0.053	0.0697 ± 0.0134	0.0889 ± 0.0261
Me-AIB inhibitable system	$Vmax_2$	44.66 ± 13.69	7.51 ± 3.38	49.7 ± 23.5
	Km_2	3.74 ± 1.36	1.41 ± 0.75	4.70 ± 2.78
	K_D	1.49 ± 0.38	2.78 ± 0.35	1.51 ± 0.40

Approximate initial rates (1 min) of uptake were measured in three different cell lines for proline concentrations ([S]) ranging from 0.010 to 5 mM. For each cell line, experiments were simultaneously conducted in the presence and absence of 10 [S] Me-AIB. Both results were taken into account by the model .

$$v = Vmax_1 \frac{[S]}{Km_1 + [S]} + Vmax_2 \frac{[S]}{Km_2 + [S]} + K_D [S]$$

kinetic parameters are expressed ± S.D. (*Km* as mmol/l ; *Vmax* as nmol/min/mg protein).

bitor. This enabled us to obtain correct fitting of the experimental points for each cell line (e.g : cell line 3, Fig 2) and well-defined kinetic constants for the inhibitable and non inhibitable systems and for the diffusion process (Table III).

The variability of the kinetic parameters observed from one cell line to the other may be due to differences of cell density in addition to the variability inherent to this live material. Nevertheless, the existence of the two systems is obvious for each cell line.

The results of the kinetic studies reported in the literature are more homogeneous than those for inhibition, as none of the investigators showed the presence of more than one uptake system when performing kinetic studies alone (1,2,9,10,11,14).

One of the most striking conclusions which may be drawn from these results for proline uptake in human fibroblasts is that system A is not the only system accounting for such uptake, neither is it the system exhibiting the highest affinity for proline. Nevertheless, it may account for much of the uptake, and thus mask the existence

of another system. Our search for a second system proved fruitful, inasmuch as we combined careful kinetic studies over a large concentration range with inhibition studies, we were able to demonstrate the presence of an Na^+-dependent, high-affinity system (K_m = 0.07 to 0.19 mmol/l) not inhibitable by Me-AIB. This system was shown to differ from both the A and ASC systems. It is not used by glycine, but we have not yet been able to characterize it further. The existence of such a system seems important, as up till now, proline has been considered the best substrate for uptake by system A in human fibroblasts. Furthermore, in view of the usual proline levels in plasma (0.060 to 0.260 mmol/l) it may have major physiological implications.

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