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INHIBITION OF SODIUM-DEPENDENT L-LEUCINE UPTAKE IN RAT BRAIN SYNAPTOSOMES

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Abstract—Synaptosomes isolated from adult rat cerebral cortices were used for studying the uptake of L-leucine by the Na^+-dependent route. Three non-metabolizable amino acid analogues, which had been used previously to discriminate the Na^+-dependent A-type uptake system of animal cells, were employed in this study. It was found that Na^+-dependent uptake of leucine was insensitive to inhibition by 2-aminoisobutyric acid (AIB) and *N*-methylaminoisobutyric acid (MeAIB) whereas *N*-methylalanine (NMA) was markedly inhibitory. Inhibition by NMA was stereospecific—only the L-isomer had a pronounced effect. Na^+-dependent uptake of leucine as well as its inhibition by L-NMA were rather insensitive to changes in pH from 6 to 9. Kinetic analysis of inhibition by L-NMA of Na^+-dependent uptake revealed a non-competitive type of inhibition with a K_i value of approximately 0.5 mM.

The uptake of L-leucine into rat brain synaptosomes takes place largely by a Na^+-independent process [1–3] although there is a significant Na^+-dependent uptake component which has been previously identified and partially characterized [4]. For Na^+-independent uptake studies, 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic adid (BCH) which has been found to be a specific inhibitor of the Na^+-independent L-type transport system in Ehrlich ascites tumor cells [5], is used to discriminate Na^+-independency of L-leucine uptake into synaptosomes in a Na^+-containing environment [3]. For the discrimination of Na^+-dependent uptake, several non-metabolizable amino acid analogues such as 2-aminoisobutyric acid (AIB), *N*-methylaminoisobutyric acid (MeAIB) and *N*-methylalanine (NMA) have been used to characterize the A-type uptake system for neutral amino acid transport in animal cells [6, 7]. The Na^+-dependent ASC-type uptake system is distinguished from the L-type and A-type systems by its insensitivity to inhibition by the above-mentioned analogues [6–8].

Although the physiological role of the Na^+-dependent component for leucine uptake in rat brain synaptosomes is unknown at present [4], it is the purpose of this investigation to explore further this uptake system with the use of several A-type transport system inhibitions (AIB, MeAIB and NMA) in order to provide a better insight into its uptake mechanism. In this communication, we report on the insensitivity of leucine uptake to inhibition by the first two inhibitors. In particular we also wish to highlight the specific effect that NMA has on synaptosomal Na^+-dependent uptake of L-leucine.

MATERIALS AND METHODS

Materials. Uniformly labeled L-[U-^{14}C]leucine (sp. act 342 mCi/mmol) was obtained from Amersham International (Amersham, U.K.). 2-Aminoisobutyric acid (AIB), *N*-methylaminoisobutyric acid (MeAIB), *N*-methyl-D-alanine (D-NMA) and *N*-methyl-L-alanine (L-NMA) were obtained from the Sigma Chemical Co. (St Louis, MO). Other chemicals of the finest grade available were purchased from either (Sigma) or from Merck (Darmstadt, F.R.G.).

Preparation of synaptosomes. Synaptosomes were prepared according to the method of Kurokawa *et al.* [9] as modified by Tan *et al.* [4]. Cerebral cortices obtained from adult male Wistar rats (200–250 g) were homogenized in 10 vol. of ice-cold 0.32 M sucrose in a motorized homogenizer, using a loose-fitting Teflon pestle (IKA-Werk, 200 rpm with 10 up and down strokes). All subsequent procedures were carried out at 0–4°. The homogenate was centrifuged at 1000 *g* for 10 min and the pellets were discarded. The supernatant was centrifuged at 10,000 *g* for 15 min and the resultant pellets were washed once with 20 vol. of 0.32 M sucrose, resuspended in 0.32 M sucrose (2 mL/g of wet weight of tissue) and then layered on 3–13% (w/v) discontinuous Ficoll density gradients in 0.32 M sucrose. The tubes were centrifuged for 20 min at 21,000 *g* using a SW41Ti rotor in a Beckman ultracentrifuge (model L8-7). Synaptosomes obtained from the 3–13% Ficoll interface were removed, and diluted with 4 vol. of 0.32 M sucrose and then centrifuged at 30,000 *g* with a JA-20 rotor in a Beckman high speed centrifuge (model J2-21) for 25 min. The snyaptosomal pellets were resuspended in a medium containing 10 mM Tris–HCl and 250 mM sucrose, pH 7.4.

The method of Lowry *et al.* [10] was employed to determine the protein content of the synaptosomes using bovine serum albumin as the standard. The yield of synaptosomes was 6–10 mg/g weight weight of brain tissues.

Synaptosomal uptake assays. Uptake studies were carried out immediately after the preparation of synaptosomes. All incubations were carried out in

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Table 1. Effect of AIB, MeAIB and L-NMA on synaptosomal L-[^{14}C]leucine uptake*

Inhibitor	Uptake of L-[^{14}C]leucine (pmol/mg protein/min)			% Inhibition
	TS	TNaS	TNaS–TS	
No inhibitor	322 ± 21	474 ± 17	152	0
AIB (0.5 mM)	320 ± 17	467 ± 17	147	3.3
AIB (5 mM)	331 ± 17	476 ± 14	145	4.6
MeAIB (0.5 mM)	317 ± 13	484 ± 22	167	−9.9
MeAIB (5 mM)	326 ± 15	479 ± 10	153	−0.7
L-NMA (0.5 mM)	330 ± 6	399 ± 12	69	54.6
L-NMA (5 mM)	334 ± 8	329 ± 14	−5	103.3

* Synaptosomes were incubated at 25° for 2 min at a leucine concentration of 30 μM in either Medium TS or Medium TNaS. Uptake rates were mean ± SE of six experiments as measured by analysis of variance.

triplicate at 25° for 2 min and contained 0.25 mg synaptosomes, 0.1 μCi L-[^{14}C]leucine and other selected substances in a final volume of 1 mL. The incubation medium used was either Medium TS (10 mM Tris–HCl, 10 mM choline chloride and 250 mM sucrose, pH 7.4) or Medium TNaS (10 mM Tris–HCl, 10 mM NaCl and 250 mM sucrose, pH 7.4). At the end of the incubation period, leucine uptake was terminated by adding 3 × 5 mL of ice-cold Medium TS without choline chlorice and with gentle filtration on 25 mm glass fibre filters (Whatman GF/F). The filters were prepared for liquid scintillation counting as described previously [4].

Non-specific binding of L-[^{14}C]leucine to the filters were determined in parallel incubations at the end of which the filters were washed with 3 × 5 mL of ice-cold water instead of Medium TS. The radioactivity that remained on the water-washed filters were then subtracted from that found in the corresponding filters washed with medium TS, and the resultant values were taken to represent uptake of ^{14}C-labeled leucine into synaptosomes. The uptake rate of leucine was linear over a 3 min period at 25° at a synaptosomal protein concentration of 0.25 mg/mL. No significant incorporation of L-[^{14}C]leucine into trichloroacetic acid-insoluble proteins was observed under the conditions for leucine uptake [4].

Definitions of uptake systems. The abbreviations and definitions of the A-type (alanine-preferring), L-type (leucine-preferring) and ASC-type (alanine-serine-cysteine-preferring) uptake systems are essentially the same as those previously defined for the Ehrlich cell's amino acid transport systems [8, 11].

RESULTS

Inhibition of synaptosomal uptake of L-leucine by AIB, MeAIB and NMA

The effect of selected A-type uptake inhibitors on L-[^{14}C]leucine uptake into rat brain synaptosomes was studied in a medium without Na^+ (Medium TS) as well as in a medium containing Na^+ (Medium TNaS) which measured Na^+-independent uptake and total uptake of leucine respectively. Na^+-dependent uptake, the uptake due to Na^+, is the difference in uptakes assayed in Medium TNaS and Medium TS. It was found that AIB, MeAIB and L-NMA had no effect on Na^+-independent uptake (Table 1). In the presence of a Na^+ concentration of 10 mM, there was an increase in uptake of leucine to about 47% at an external leucine concentration of 30 μM. L-NMA showed marked inhibitory effects on this increase in the presence of Na^+ and complete inhibition of this component was observed at an inhibitor concentration of 5 mM. In contrast, both AIB and MeAIB showed no significant effect on Na^+-dependent uptake of L-leucine under similar conditions.

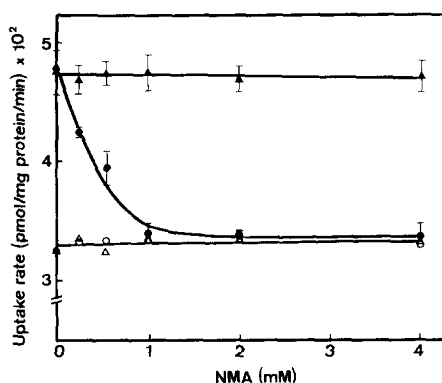

Fig. 1. Effect of NMA on uptake of L-[^{14}C]leucine by rat brain synaptosomes. Uptake rates were measured at 25° for 2 min at a leucine concentration of 30 μM in the presence of L-NMA in Medium TS (○——○), L-NMA in Medium TNaS (●——●), D-NMA in Medium TS (△——△) and D-NMA in Medium TNaS (▲——▲).

Stereospecific effect of NMA on Na^+-dependent uptake

The effect of D-NMA and L-NMA on Na^+-dependent uptake of L-leucine can be seen on Fig. 1. The D-isomer had no effect on leucine uptake whereas the L-isomer inhibited uptake in a concentration-dependent manner. The Na^+-dependent uptake component was totally abolished at L-NMA concentrations of 2 mM and higher. Using a Dixon plot where the reciprocals of the uptake velocities of leucine were plotted against the concentration of L-NMA, it was found that L-NMA inhibited Na^+-dependent leucine uptake of rat brain synaptosomes with a K_i of 0.53 mM (Fig. 2).

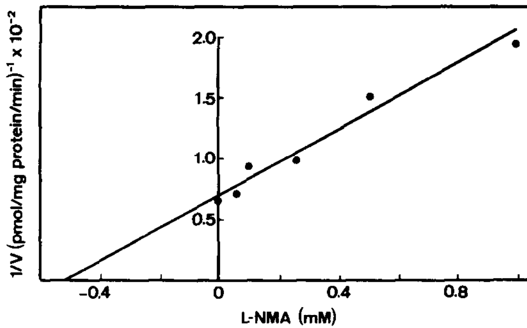

Fig. 2. Dixon plot of L-NMA inhibition of Na^+-dependent L-[^{14}C]leucine uptake. Uptake rates were measured at 25° for 2 min at a leucine concentration of 30 μM in the presence of different concentrations of L-NMA. Na^+-dependent uptake rates were obtained from the difference in uptake rates assayed in Medium TS and Medium TNaS.

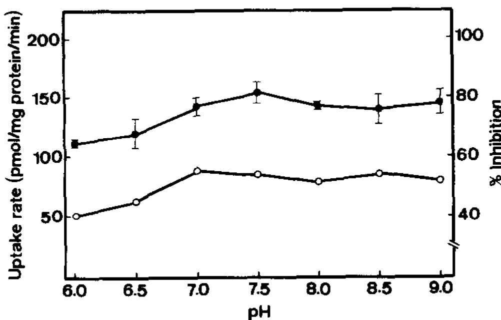

Fig. 3. Effect of pH on Na^+-dependent L[^{14}C]leucine uptake. Uptakes were measured at 25° for 2 min at a leucine concentration of 30 μM over a pH range from 6 to 9. Results were expressed as Na^+-dependent uptake in absence of inhibitor (●——●) and % inhibition of Na^+-dependent uptake in presence of L-NMA (○——○).

Effect of pH on L-NMA inhibition of Na^+*-dependent uptake*

The uptake of L-leucine in the presence and absence of L-NMA was investigated over a pH range from 6 to 9. The results are shown on Fig. 3. Na^+-dependent uptake of leucine as well as its inhibition by L-NMA appeared to be insensitive to pH changes over the pH range studied.

Effect of substrate concentration on leucine uptake

The effect of L-NMA on leucine uptake was studied as a function of leucine concentration from 0 to 100 μM. The results are shown on Fig. 4. A concentration of 0.5 mM L-NMA was used in the experiment and inhibition was measured over the range of leucine concentrations indicated above. Velocities of uptake due to the Na^+-dependent component were obtained in the presence and absence of L-NMA. Lineweaver–Burk plots of the data (Fig. 5) showed two linear lines meeting at a point on the x-axis (K_m = 90 μM), demonstrating that L-NMA inhibited Na^+-dependent uptake of L-leucine in a non-competitive manner. The V_{max} was reduced from

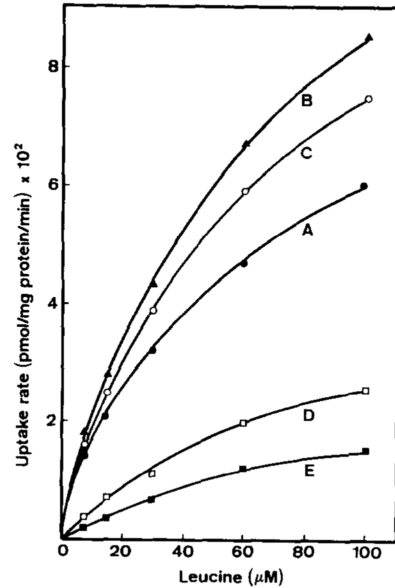

Fig. 4. Effect of leucine concentration of L-[^{14}C]leucine uptake. Uptake rates were measured at 25° for 2 min at various concentrations of leucine. Medium TS (●——●, curve A), Medium TNaS (▲——▲, curve B), Medium TNaS + 0.5 mM L-NMA (○——○, curve C). Curve D (□——□) representing Na^+-dependent uptake rates, was obtained from the difference in uptake rates between curve B and curve A. Curve E (■——■) representing Na^+-dependent uptake rates in the presence of 0.5 mM L-NMA, was obtained from the difference in uptake rates between curve C and curve A.

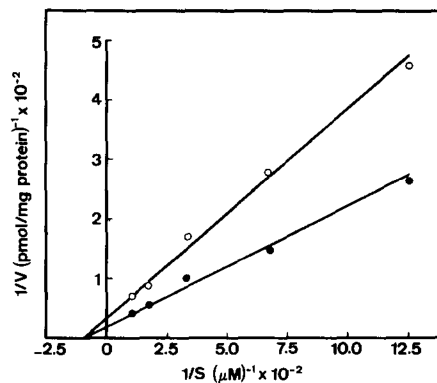

Fig. 5. Lineweaver–Burk plots of Na^+-dependent uptake of leucine in the absence of L-NMA (●——●) and in the presence of 0.5 mM L-NMA (○——○). The data were obtained and replotted from curves D and E of Fig. 4, respectively. The lines were fitted by a least square regression method.

464 pmol/mg protein/min in the uninhibited state to 268 pmol/mg protein/min in the presence of 0.5 mM L-NMA.

DISCUSSION

Several transport systems with overlapping specificities have been described for the uptake of neutral

amino acids by animal tissues [12, 13]. These transport systems have been characterized with the aid of a number of non-metabolizable amino acid analogues. For uptake of the branched-chain amino acid leucine, the Na^+-independent L-type transport system appears to be predominant [1, 2] and this uptake is efficiently blocked by BCH (a non-metabolizable analogue) in various cell types [5] as well as in synaptosomes [3]. Besides the observation of Na^+-dependent uptake, L-leucine is also transported into synaptosomes by a prominent Na^+-dependent process [4]. Under appropriate conditions of uptake, this component could contribute up to one-third of the total rate of leucine uptake (Table 1). Investigation of this Na^+-dependent uptake component using A-type system uptake inhibitors give rather unexpected results. These inhibitors (AIB, MeAIB and NMA) have been previously shown to inhibit specifically the A-type Na^+-dependent component [6, 7, 12] and any residual uptake which is insensitive to inhibition by these inhibitors is often viewed to be a result of uptake due to the ASC-type uptake system [7, 13, 14]. Our findings show that all the three analogues lack the ability to inhibit leucine uptake in the absence of Na^+ and these results are consistent with previous observations that these inhibitors do not inhibit Na^+-independent uptake. In addition, AIB and MeAIB are also non-inhibitory towards Na^+-dependent uptake which could mean that Na^+-dependent uptake is unlikely to be that of the A-type. It is of interest that the principal Na^+-dependent system for neutral amino acid transport in rabbit ileum is quite unlike the A-type uptake system as it is not inhibited by either AIB or MeAIB [15]. This is often extrapolated to mean that the Na^+-dependent component of amino acid uptake is that of the ASC-type since there is a lack of model substrate restricted to the ASC-type uptake system [14, 16]. In contrast, however, NMA shows marked inhibition towards the Na^+-dependent uptake component of leucine uptake. A small NMA-inhibitable component of branched-chain amino acid transport was also observed in human placental tissues [6]. Hence caution must be exercised here in claiming ASC-type involvement since NMA is a known A-type uptake system inhibitor [6]. Thus it appears that the Na^+-dependent component of leucine uptake in synaptosomes is neither truly A-type or truly ASC-type as defined earlier by the use of such analogues.

Studies on the effect of pH on Na^+-dependent uptake of L-leucine and its inhibition by L-NMA show little change in uptake rates when uptake was assayed at pH values of between 6 and 9, an observation which is more characteristic of an ASC-type system than that of an A-type system [8, 17]. Thus this system appears to be unique and distinct from the variety of transport systems encountered by Sershen and Lajtha [13] in mouse brain slices.

The distinctiveness of this Na^+-dependent uptake component for L-leucine is further shown by the stereospecific nature of its inhibition by the L-isomer of NMA and the non-competitive nature of its inhibition as demonstrated by the double-reciprocal plot. It is noteworthy that a similar non-competitive type of inhibition by vincristine on leucine uptake in rat cortical synaptosomes was previously inferred from Hill-type plots [18]. Research is now underway to further characterize the nature of this Na^+-dependent component for leucine uptake in rat brain synaptosomes.

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REFERENCES

1. Appel SH, Autilio LA, Festoff BW and Escueta AV, Biochemical studies of the synapses *in vitro* III. Ionic activation of protein synthesis. *J Biol Chem* **12**: 3166–3172, 1969.
2. Peterson NA and Raghupathy E, Characteristics of amino acid accumulation by synaptosomal particles isolated from rat brain. *J Neurochem* **19**: 1423–1438, 1972.
3. Tan CH, Leong MK, Ng FH and Thiyagarajah P, Sodium-independent synaptosomal uptake leucine. *Biochem Int* **14**: 161–166, 1987.
4. Tan CH, Leong MK and Ng FH, A novel sodium-dependent uptake system for L-leucine in rat brain synaptosomes. *Neurochem Int* **12**: 91–95, 1988.
5. Christensen NH, Handlogten ME, Lam I, Tager HS and Zand R, A bicyclic amino acid to improve discriminations among transport systems. *J Biol Chem* **244**: 1510–1520, 1969.
6. Enders RH, Judd RM, Donohue TM and Smith CH, Placental amino acid uptake. III. Transport system for neutral amino acids. *Am J Physiol* **230**: 706–710, 1976.
7. Shotwell MA, Kilberg MS and Oxender DL, The regulation of neutral amino acid transport in mammalian cells. *Biochim Biophys Acta* **737**: 267–284, 1983.
8. Christensen HN, Liang M and Archer EG, A distinct Na^+-requiring transport system for alanine, serine, cysteine, and similar amino acids. *J Biochem* **242**: 5237–5246, 1967.
9. Kurokawa M, Sakamoto T and Kato M, Distribution of Na^+, K^+ stimulated adenosine triphosphatase activity in isolated nerve ending particles. *Biochem J* **97**: 833–844, 1965.
10. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
11. Oxender DL and Christensen HN, Distinct mediating systems for the transport of neutral amino acids by the Ehrlich cell. *J Biol Chem* **238**: 3686–3699, 1963.
12. Christensen HN, Exploiting amino acid structure to learn about membrane transport. *Adv Enzymol* **49**: 41–101, 1979.
13. Shershen H and Lajtha A, Inhibition pattern by analogs indicates the presence of ten or more transport systems for amino acids in brain cells. *J Neurochem* **32**: 719–726, 1979.
14. Kilberg MS, Handlogten ME and Christensen HN, Characteristics of system ASC for transport of neutral amino acids in isolated rat hepatocyte. *J Biol Chem* **256**: 3304–3312, 1981.
15. Sepulveda FV and Smith MW, Discrimination between different entry mechanisms for neutral amino acids in rabbit ileal mucosa. *J Physiol* **282**: 73–90, 1978.
16. Franchi-Gazzola R, Gazzola GC, Dall'Asta V and Guidotti GG, The transport of alanine, serine and cysteine in cultured human fibroblasts. *J Biol Chem* **257**: 9582–9587, 1982.
17. Kilberg MS, Handlogten ME and Christensen MN, Characteristics of an amino acid transport system in rat liver for glutamine, asparagine, histidine, and closely related analogs. *J Biol Chem* **255**: 4011–4019, 1980.
18. Peterson NA, Raghupathy E and Estey SJ, Effects of vinca alkaloids on uptakes of amino acids by rat brain synaptosomes. *Biochem Pharmacol* **25**: 1389–1395, 1976.