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ALLYLGLYCINE, AN INHIBITOR OF THE UPTAKE OF L-LEUCINE AND L-PROLINE IN RAT BRAIN SLICES

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Abstract—The convulsant allylglycine inhibited the uptake of L-leucine and L-proline in rat brain slices and was without influence on the uptake of acetylcholine, dopamine, L-glutamic acid, glycine and L-lysine under the conditions studied. L-Leucine uptake could also be inhibited by the "large neutral" amino acids L-methionine, L-phenylalanine and L-valine. No kinetic studies of L-leucine uptake were possible due to the inability to measure initial rates of uptake. The time course of L-leucine uptake was not linear under a variety of conditions and appeared to be complicated by a rapid efflux of radioactive label in the form of acidic metabolite(s). L-Proline uptake was mediated by a "high affinity" saturable system (K_m 50 μM), and was non-competitively inhibited by allylglycine (K_i *ca.* 50 μM). L-Leucine competitively inhibited (K_i 75 μM) L-proline uptake, although L-leucine uptake was unaffected by L-proline. L-Norleucine, DL-pipecolic acid and L-azetidine-2-carboxylic acid inhibited L-proline uptake.

ALLYLGLYCINE (DL-2-amino-4-pentenoic acid) is a convulsant[1,2] which produces ultrastructural changes in the cerebral cortex,[3] cerebellum[4] and hippocampus.[5] The convulsant action of this amino acid has been interpreted on the basis of a competitive inhibition of the activity of the enzyme L-glutamate 1-carboxylyase (GAD) which leads to a decrease in the levels of the inhibitory transmitter γ-aminobutyric acid (GABA).[4] A recent report that allylglycine inhibits the incorporation of L-leucine into protein by nerve endings isolated from rat brain, but is without influence on L-leucine incorporation into protein by a brain microsomal preparation, suggested that allylglycine might inhibit L-leucine uptake.[6] We have investigated the influence of allylglycine on the uptake of a number of substances in rat brain slices and have found it to inhibit the uptake of L-leucine and L-proline.

METHODS

Uptake was measured as described previously[7,8] using slices (0·1 × 0·1 × 2 mm) of rat cerebral grey matter incubated at 37° (unless stated otherwise) in freshly oxygenated Krebs–Ringer medium. For the preliminary testing of the possible effects of allylglycine on uptake, the slices were preincubated for 15 min with 0·1 mM allylglycine, and the uptake of radioactive substrate measured during 10 min incubation. Subsequent experiments on L-leucine and L-valine uptake were made using various incubation periods.

In studies of the inhibition by allylglycine of the uptake of L-proline, the concentration of both the substrate and inhibitor were varied in order to determine

values for the apparent kinetic constants, K_m, K_i and V_{max}. IC_{50} Values (concentration causing 50 per cent inhibition of uptake) were determined graphically as described previously.[8]

Materials

Radiochemicals. [Methyl-^{14}C]acetylcholine, sp. act. 10 mCi/m-mole, [side chain-1,2-^{3}H]dopamine, 390 mCi/m-mole, [U-^{14}C]L-glutamate, 10 mCi/m-mole, [4,5-^{3}H]L-leucine, 1 Ci/m-mole, [U-^{14}C]L-lysine, 8 mCi/m-mole, and [U-^{3}H]L-proline, 266 mCi/m-mole, were purchased from The Radiochemical Centre, Amersham. [2-^{3}H]Glycine, 10 Ci/m-mole, was purchased from New England Nuclear, Boston.

Drugs and amino acids. These were purchased from the suppliers indicated: allylglycine, D-allo-hydroxyproline, imidazole-4-acetic acid, L-methionine DL-sulphoximine, L-proline and sodium *p*-chloromercuriphenylsulphonate (Sigma); 2,4-dinitrophenol and ouabain (Fluka); sodium azide, L-glutamic acid, mercuric chloride and L-valine (BDH); L-norleucine (Mann); D- and L-azetidine-2-carboxylic acids, L-hydroxyproline, 5-hydroxytrytamine, L-leucine, noradrenaline, L-methionine and DL-pipecolic acid (Calbiochem); iodoacetic acid and L-phenylalanine (Koch-Light); cycloserine (Nutritional Biochemicals), γ-aminobutyric acid (Roche). Dr. J. C. Watkins prepared L-prolyl-L-proline, 2-pyrrolidone, and L-2-pyrrolidone-5-carboxylic acid.

RESULTS

Allylglycine, 0·1 mM, clearly inhibited the uptake of L-leucine and L-proline in rat brain slices (Table 1). The uptake of any of the other substances studied was not appreciably influenced by this concentration of allylglycine, but 1 mM allylglycine has been reported to inhibit the high affinity uptake of GABA (39 per cent inhibition) in slices of rat brain[9] and of glycine (18 per cent inhibition) in slices of rat spinal cord.[10]

L-Leucine uptake

Measurement of the time course of uptake of L-leucine at 0·01, 7·7 and 100 μM external concentration showed that uptake of radioactivity was not linear with respect to time. Higher tissue to medium ratios were obtained when the slices were

TABLE 1. EFFECT OF ALLYLGLYCINE ON THE UPTAKE OF VARIOUS SUBSTANCES IN RAT BRAIN SLICES

Substance	Concn (μM)	Probable uptake system	% Inhibition by 0·1 mM allylglycine
Glycine	0·01	Small neutral amino acids	N.S.
L-Leucine	0·01	Large neutral amino acids	52 ± 4
L-Proline	0·01	Imino acids	72 ± 3
GABA	0·01	Specific for GABA	N.S. (see Ref. 27)
L-Lysine	1	Basic amino acids	N.S.
L-Glutamate		Acidic amino acids	
	1000	low affinity	N.S.
	0·01	high affinity	N.S. (see Ref. 8)
Dopamine	0·01	Catecholamines	N.S.
Acetylcholine	1	Choline derivatives	N.S.

Slices were preincubated with allylglycine for 15 min, and uptake measured after incubation for a further 10 min. Results are means ± S.E. of quadruplicate experiments. N.S. = not significant.

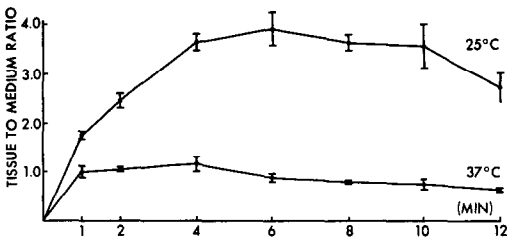

FIG. 1. Time course of the uptake of L-leucine (μM) in rat brain slices at 25 and 37°. Tissue to medium ratios were calculated on the basis of an 80% water content of the slices. Values are means ± S.E. of quadruplicate determinations.

incubated at 25° than at 37° (Fig. 1). Allylglycine (300 μM) inhibited the uptake of radioactivity at all times examined (Fig. 2).

Analysis of the radioactivity in the slices after 2 min incubation with 20 μM [^{3}H]L-leucine by thin layer chromatography (Avicel plates developed with *n*-butanol–acetic acid–water, 4:1:1) indicated that more than 95 per cent had the same R_f as that of L-leucine, and that no radioactivity was incorporated into material insoluble in 75% aqueous ethanol.

Analysis of the radioactivity in the medium after incubation with 0·01 μM [^{3}H]L-leucine at various times using cation and anion exchange resins indicated a time dependent formation of labelled acidic material (Table 2), which was not absorbed by Dowex 50(H^+) but was absorbed by Dowex 1 (acetate). This material may represent acidic metabolites of L-leucine, such as α-ketoisocaproic acid and isovaleric acid, released from the slices into the medium during the incubation.

In addition to allylglycine, a number of other amino acids—L-methionine, L-phenylalanine and L-valine—inhibited L-leucine uptake (Table 3). Work on mouse brain slices indicates that these amino acids may be taken up by the same "large neutral" amino acid transport system.[11,12] L-Methionine DL-sulphoximine and L-proline did not inhibit L-leucine uptake and thus cannot be substrates for the transport system.

The Na^+/K^+-ATPase inhibitor ouabain, and the thiol reagent *p*-chloromercuriphenylsulphonate inhibited L-leucine uptake, indicating that this uptake is dependent on sodium ions and thiol groups, in common with the transport of other amino acids.

The metabolic inhibitors, 2,4-dinitrophenol and iodoacetic acid, also inhibited L-leucine uptake (24 ± 6 and 41 ± 1 per cent inhibition respectively) after preincubation with the slices at 1 mM for 30 min.

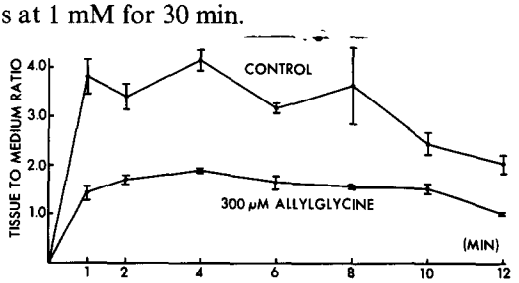

FIG. 2. Time course of the uptake of L-leucine (7·7 μM) in rat brain slices at 37° in the presence and absence of allylglycine (300 μM). Details as for Fig. 1.

TABLE 2. ANALYSIS OF RADIOACTIVITY IN THE MEDIUM DURING INCUBATION OF [^{3}H]L-LEUCINE WITH RAT BRAIN SLICES

Time (min)	Radioactivity (dis/min) after treatment with Dowex 50(H^+)	Dowex 1 (acetate)
2	456	23
6	1032	51
12	1169	11

Rat brain slices (100 mg instead of the usual 10 mg) were preincubated in medium (10 ml) at 37° for 15 min, [^{3}H]L-leucine (0·01 μM) added and incubation continued. Aliquots (1 ml) were taken at 0, 2, 6 and 12 min, filtered, the filters washed with saline (10 ml), the combined filtrates and washings treated with Dowex 50(H^+) resin (5 ml) to remove [^{3}H]L-leucine and centrifuged. Aliquots (1 ml) of the supernatants were assayed for radioactivity and the remainder treated with Dowex 1 (acetate) (0·5 ml/ml of supernatant) to remove acidic material, centrifuged and further aliquots (1 ml) assayed for radioactivity. Values are means of duplicate experiments from which the zero time blanks were subtracted.

L-*Proline uptake*

The time course (Fig. 3) of the uptake of L-proline (0·01 μM) differed from that of L-leucine in being linear for at least 4 min. The effect of various inhibitors of L-proline uptake was measured using a 4 min incubation period, whereas a 2 min period was used in the kinetic studies. Thin layer chromatography showed that, no metabolism of L-proline or incorporation into protein occurred during the 4 min incubation period.

TABLE 3. INHIBITORS OF L-LEUCINE UPTAKE IN RAT BRAIN SLICES

Inhibitor	% Inhibition
p-Chloromercuriphenylsulphonate	104 ± 1
L-Phenylalanine	86 ± 1
L-Valine	69 ± 6
L-Methionine	67 ± 3
Allylglycine	62 ± 3
Ouabain	57 ± 5
L-Methionine-DL-sulphoximine	N.S.
L-Proline	N.S.

Slices were preincubated with inhibitor (0·1 mM) for 15 min, and uptake measured after incubation for a further 2 min with L-leucine (0·01 μM). Results are means ± S.E. of quadruplicate experiments, N.S. = not significant.

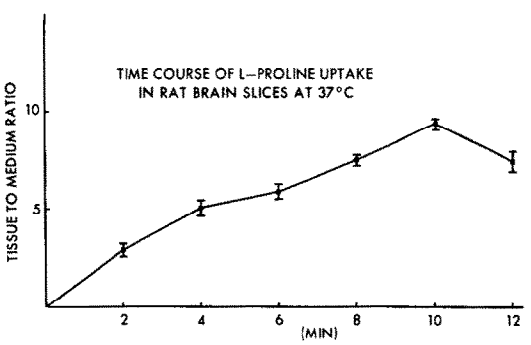

FIG. 3. Time course of the uptake of L-proline (0·01 μM) in rat brain slices at 37°. Details as for Fig. 1.

TABLE 4. INHIBITORS OF L-PROLINE UPTAKE IN RAT BRAIN SLICES

Inhibitor	% Inhibition	IC_{50} (μM)
Mercuric chloride	106 ± 2	
L-Norleucine	94 ± 5	16
DL-Pipecolic acid	91 ± 2	45
Ouabain	81 + 8	
Allylglycine	52 ± 3	60
L-Leucine	41 ± 4	105
L-Azetidine-2-carboxylic acid	38 ± 5	200
L-Hydroxyproline	14 ± 4	1000

Slices were preincubated with inhibitor (0·1 mM) for 15 min, and uptake measured after incubation for a further 4 min with L-proline (0·01 μM). Results for per cent inhibition are means ± S.E. of quadruplicate experiments. Results for the IC_{50}'s were determined graphically as described previously.[8]

A number of drugs and amino acids inhibited L-proline uptake (Table 4). Allylglycine was amongst the most potent inhibitors affording 50 per cent inhibition of uptake at a concentration of 60 μM. Kinetic analysis (Fig. 4) of this inhibition by allylglycine (75 and 150 μM) indicated that allylglycine was a non-linear non-competitive inhibitor of L-proline uptake with a K_i of 50 μM, based on the data obtained with 75 μM allylglycine. The apparent kinetic constants for L-proline uptake measured over the range 5–50 μM were K_m 50 μM, V_{max} 0·10 μmole/g/min.

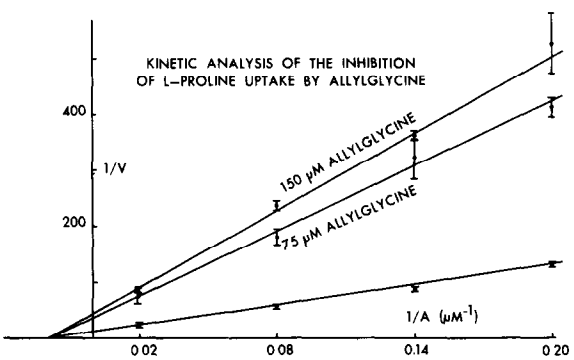

FIG. 4. Kinetic analysis of the inhibition of the uptake of L-proline by allyglycine. Ordinate: reciprocal of the initial velocity of uptake (V) measured over 2 min incubation at 37° and expressed as μmoles/g/min. Abscissa: reciprocal of the substrate concentration (A) expressed as μM. Allylglycine was preincubated with the slices for 15 min at 37° before addition of substrate.

The inhibition of L-proline uptake by L-leucine (Table 4) is of particular interest since L-proline did not inhibit L-leucine uptake (Table 3). Kinetic analysis of the inhibition of L-proline uptake by L-leucine (75 and 150 μM) indicated that L-leucine was a linear competitive inhibitor with a K_i of 75 μM.

Of the drugs and amino acids examined, mercuric chloride and ouabain inhibited L-proline uptake (Table 4), and the following were without significant effect: D-azetidine-2-carboxylic acid, cycloserine, GABA, L-glutamic acid, glycine, D-allo-hydroxyproline, 5-hydroxytryptamine, imidazole-4-acetic acid, noradrenaline, L-prolyl-L-proline, 2-pyrrolidone, L-pyrrolidone-2-carboxylic acid. The metabolic inhibitors, 2,4-dinitrophenol and iodoacetic acid at 1 mM, inhibited L-proline uptake (48 ± 2 and 30 ± 2 per cent inhibition respectively).

DISCUSSION

The observed inhibition of L-leucine incorporation into protein in nerve endings by allylglycine[6] could be the result of inhibition of L-leucine uptake. Allylglycine may be a substrate for a "large neutral" amino acid transport system analogous to that described in mouse brain slices[11] which can transport L-leucine, L-methionine, L-phenylalanine and L-valine. There is evidence from *in vivo* studies that allylglycine and L-valine are transported from the blood into the brain by a common system and that L-valine can protect against the convulsant action of allylglycine.[13]

More detailed analysis of the mechanism of the observed inhibition of L-leucine uptake in brain slices by allylglycine is complicated by the inability to measure initial rates of uptake. The observed time course of L-leucine uptake might be interpreted on the basis of rapid metabolism of L-leucine in the slices and subsequent efflux of the metabolite(s), as has been observed for the uptake of GABA in rat brain slices.[14] The metabolism of L-leucine appears to be more temperature dependent than the uptake as higher tissue to medium ratios were observed at 25° than at 37°. Further investigation of the uptake of L-leucine in brain slices is clearly warranted.

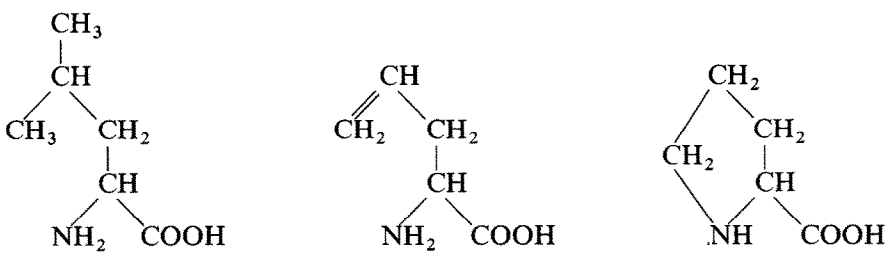

FIG. 5. Structures of leucine, allylglycine and proline drawn to emphasize similarities.

Allylglycine also inhibited L-proline uptake. This amino acid appears to be taken up by a different system (the "imino acid" system[12]) to that taking up the large neutral amino acids. There are structural similarities between leucine, allylglycine and proline as illustrated in Fig. 5. All three substances appear to be able to interact with the imino acid system (proline as a substrate, leucine and allyglycine as competitive and noncompetitive inhibitors respectively) but proline does not interact with the large neutral amino acid system.

It has been proposed[4] that allylglycine might be a useful tool for the study of GABA mediated systems in the brain, as it is a competitive inhibitor of GAD and causes ultrastructural changes in known GABA releasing neurones in the cerebellum. It can be calculated that the K_i for this inhibition is approximately 20 mM, i.e. some two orders of magnitude higher than that observed for inhibition of L-proline uptake.

It is interesting that the apparent K_m for L-proline uptake (50 μM) is comparable with that observed for the "high affinity" uptake of the likely amino acid transmitters, GABA (22 μM),[14] glycine (40 μM),[10] L-glutamic acid (20 μM)[7] and taurine (50 μM).[15] Other workers have reported on the existence of a "high affinity" uptake of L-proline into nerve endings.[16] As transport systems for amino acids with K_m's of this order have to date been described only for the amino acids likely to be synaptic transmitters, such a finding suggests that L-proline could also be a transmitter.

There is some circumstantial evidence to suggest that L-proline could be important in brain function. When administered microelectrophoretically, L-proline[17] and allylglycine[18,19] reduce the firing rate of neurones, but are relatively weak in comparison to glycine and GABA. L-Leucine and L-valine have little influence on neuronal firing.[20] The concentration of free L-proline in autopsied rat brain is 0·12–0·15 μmole/g[21] and similar concentrations are found in various regions of biopsied cat brain;[22] higher concentrations and some regional variations are found in autopsied human brain.[23] L-Proline antagonizes the change in transparency of the isolated chick retina induced by L-glutamate[24] and may influence short term memory.[25] Two types of hyperprolinemia have been described and both appear to be associated with mental retardation and seizures.[26]

Further investigations are warranted of the possibility that the convulsions induced by allylglycine are in part the result of interference with the uptake of L-proline.

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