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INFLUENCE OF STEREOISOMERS OF 4-FLUOROGLUTAMATE ON RAT BRAIN GLUTAMATE DECARBOXYLASE

JAROSLAV DRŠATA[a,*], MILOSLAVA NETOPILOVÁ[a] and VLADIMÍR TOLMAN[b]

[a]*Faculty of Pharmacy in Hradec Králové, Department of Biochemical Sciences, Charles University in Prague, Heyrovského 1203, 500 05 Hradec Králové, Czech Republic;* [b]*Institute of Experimental Botany, Czech Academy of Sciences, Vídeňská 1083, 14220 Prague 4, Czech Republic*

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Inhibition of rat brain glutamate decarboxylase (GAD, EC 4.1.1.15) by individual stereoisomers of 4-fluoroglutamate (4-F-Glu) and 2-fluoro-4-aminobutyrate (2-F-GABA) was studied. All stereoisomers of 4-F-Glu inhibited decarboxylation of L-glutamate catalysed by the enzyme preparation. At 1×10^{-2} M concentration, the most potent inhibitor of GAD was D-*erythro*-4-F-Glu with about 70% inhibition in the presence of 1.23×10^{-2} M L-glutamate. The inhibition by all stereoisomers was of the competitive type. K_i values ranged from 2×10^{-3} M for the D-*erythro* isomer to 1.1×10^{-2} M for the D-*threo* and L-*erythro* isomers. The influence of all stereoisomers was reversible as shown by dialysis except for a small amount in the case of the D-*erythro* isomer. The inhibition was independent of external pyridoxal-5′-phosphate added. No inhibition of rat brain GAD was found with 2-fluoro-4-aminobutyrate stereoisomers.

Keywords: Rat brain glutamate decarboxylase; Stereoisomers of 4-fluoroglutamate; Competitive reversible inhibition

INTRODUCTION

L-Glutamate decarboxylase (GAD, EC 4.1.1.15) catalyses decarboxylation of L-glutamic acid to GABA, the major inhibitory neurotransmitter in the mammalian CNS.[1] The substrate of GAD, L-glutamic acid itself, beside its participation in general amino acid metabolism, acts as an excitatory

* Corresponding author.

neurotransmitter in the CNS. *In vivo* administration of potent GAD inhibitors in laboratory animals can cause convulsions and this is used to induce experimental epileptic states.[2] For these reasons, specific or less specific inhibitors of GAD have been prepared for research or potential pharmacological use.[3] These include compounds with potent pharmacological effects, which are based on structural analogies with natural substrates of pyridoxal-dependent enzymes engaged in amino acid metabolism.[4]

Among the inhibitors of amino acid metabolism, fluorinated amino acids represent an important group because substitution of a fluorine atom for hydrogen influences chemical reactivity of the amino acid without introducing a significant steric change.[5] One such compound is 4-fluoroglutamate (4-F-Glu), which was synthesised in 1961 as a mixture of four stereoisomers.[6] The influence of 4-fluoroglutamate on GAD was studied with the enzyme from *Escherichia coli*[7,8] and from the calf brain.[7]

As for the influence of 4-fluoroglutamate on other enzymes, Firsova *et al.*[9] found noncompetitive inhibition of glutamine synthetase by D,L-4-fluoroglutamate, while Unkeless and Goldman[10] found both diastereoisomers of 4-fluoroglutamate to be alternative substrates of glutamate dehydrogenase (EC 1.4.1.3), glutamine synthetase (EC 6.3.1.2), carboxypeptidase G (EC 3.4.2.6), and leucine aminopeptidase (EC 3.4.1.1). The same authors also described thermodynamic and kinetic aspects of the reaction of 4-fluoroglutamate with D-glutamate cyclase. Other authors[11] found D,L-*threo*-4-fluoro-glutamate to be a chain-terminating inhibitor of folylpolyglutamate synthesis. Vidal-Cros *et al.*[12] studied the interaction of GAD from *E. coli* with a similar compound – 3-fluoroglutamate.

In the present paper, the authors studied all four stereoisomers of 4-fluoroglutamate and their decarboxylation products (*R*)-(+)-2-fluoro-GABA and (*S*)-(−)-2-fluoro-GABA, prepared by one of them,[13] with respect to their influence on GAD from the rat brain.

MATERIALS AND METHODS

Materials

The rat brain was used as the source of GAD and the enzyme preparation was obtained as described previously.[14,15] The protein content was determined according to the method of Lowry.[16] Specific GAD activity of the preparations was about 65 nkat/g prot. Individual stereoisomers of 4-fluoroglutamate and 2-fluoro-GABA were prepared according to the literature.[13]

D,L-4-Fluoroglutamic acid (approx. 1 : 1 *erythro/threo* mixture)[6] was separated into racemic diastereomers, which were further resolved into the antipodes through their diastereomeric salts with chiral bases.[17] D-antipodes were also prepared, together with the enantiomers of 2-fluoro-4-aminobutyric acid, by the action of glutamate decarboxylase (*E. coli* (Sigma)) on either D,L-*erythro*- or D,L-*threo*-4-fluoroglutamic acid.[18] Chemicals used in GAD assay: L-1-^{14}C-glutamate (Radiochemical Centre Amersham, specific activity 1.65 MBq · mmol^{-1}), pyridoxal-5′-phosphate (Koch-Light Labs.), 1,4-dithiothreitol (DTT, Merck). Bray's scintillation cocktail (Spolana, Neratovice).[19] All chemicals used in the GAD assay were at least of analytical grade.

GAD Assay and Evaluation of Inhibition

Enzyme activity was determined radiometrically.[15] The incubation mixture contained 60 µl of the enzyme preparation, labelled L-glutamate in a final concentration of $2.5–12.3 \times 10^{-3}$ and 1×10^{-4} M pyridoxal-5′-phosphate in 0.1 M sodium phosphate buffer, pH 6.7. In the experiments with 4-fluoroglutamate, samples contained the compound or its individual stereoisomers in concentrations ranging mostly between 1 and 4×10^{-2} M. The mixture was incubated at 37°C for 30 min and the liberated $^{14}CO_2$ was trapped in 0.1 ml of 30% KOH. The radioactivity was measured in dioxane scintillation cocktail using 1219 Rackbeta scintillation counter LKB Wallac (Radioisotope Laboratory, Charles University Faculty of Pharmacy, Hradec Králové). GAD activities were calculated on the basis of the known amount and specific radioactivity of $^{14}CO_2$ produced in the catalysed reaction and expressed in nkat per g of protein. The influence of fluorinated glutamate derivatives was expressed as a % of control activity. All data are the mean ± SD of four to six measurements. Significance of differences between means was determined using Student's *t*-test.

K_i Values and Inhibition Kinetics

Racemic 4-fluoroglutamate ($0–1 \times 10^{-2}$ M) was used in order to screen any inhibitory effect of the compound on rat brain GAD. In the case of 2-fluoro-GABA, only 1×10^{-2} M concentration was used to check the effect. Because of the limited amount of individual stereoisomers of 4-fluoroglutamate that could be prepared for the inhibition experiments in comparison with the relatively high concentrations that were required to reach a marked GAD

inhibition, inhibition kinetics was studied using a variable concentration of the substrate and a constant concentration of 4-fluoroglutamate only. The K_i values, and type of inhibition, were then obtained graphically using the Lineweaver and Burk (1/v versus 1/s) plot.

Influence of the Coenzyme on GAD Inhibition

GAD activity was measured in the presence of 4-fluoroglutamate and in control samples after addition of different concentrations of pyridoxal-5′-phosphate, representing sub-saturation (no external pyridoxal-5′-phosphate added) and saturation (1×10^{-4} and 1×10^{-3} M) of the enzyme with the coenzyme.[14]

Influence of Pre-Incubation of 4-F-Glu with GAD on Enzyme Inhibition

Individual stereoisomers of 4-fluoroglutamate (2×10^{-2} M) were pre-incubated with the enzyme preparation for 24 h, the assay mixture was then completed and GAD activity measured. The results were compared with those of control samples without 4-fluoroglutamate, which were processed in the same way.

Reversibility of the Effect

The mixture of the GAD preparation with individual stereoisomers of 4-fluoroglutamate was pre-incubated for 30 min and then dialyzed against 0.1 M phosphate buffer, pH 6.7, for 2 h, with repeated exchange of the buffer. GAD activity was then measured and compared with the control experiment without 4-fluoroglutamate carried out in the same way, and with control results without dialysis.

RESULTS AND DISCUSSION

Preliminary screening of the effect of racemic 4-fluoroglutamate on rat brain GAD suggested that concentrations in the range 10^{-2} M should be used in further studies (Table I).

At a concentration of 1×10^{-2} M, the individual stereoisomers showed relative differences in their influence on GAD activity, the D-*erythro* isomer being the most potent inhibitor (69.8% inhibition of decarboxylation of L-glutamate, see Figure 1). The second most potent inhibitor was the L-*threo*

TABLE I Inhibition of rat brain glutamate decarboxylation by racemic 4-fluoroglutamate in the presence of 1.23×10^{-2} M L-glutamate

Concentraion of 4-*F-Glu* (mM)	*GAD activity* (nkat/g prot.)	*% of inhibition*
0	30.6 ± 2.9	0
0.1	28.5 ± 0.6	6.9
1.0	26.6 ± 3.4	13.0
10	16.3 ± 3.0	46.7

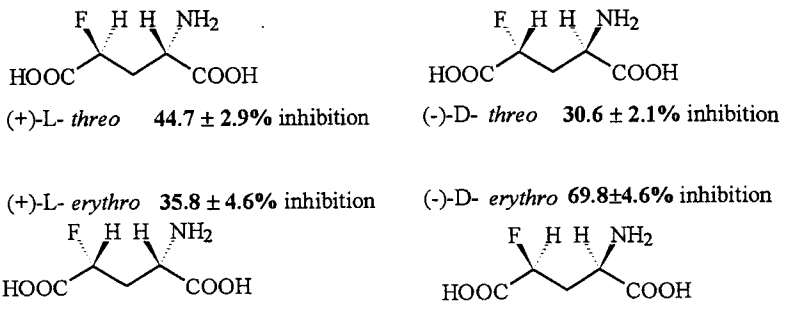

FIGURE 1 Formulas of 4-fluoroglutamate stereoisomers and their relative influence in 1×10^{-2} M concentration on GAD activity in the presence of 1.23×10^{-2} M L-glutamate.

isomer (44.7% inhibition), which has the same space arrangement at C4 i.e. (4*S*) configuration. L-*erythro* and D-*threo* isomers with the (4*R*) arrangement were less effective (35.8% and 30.6% inhibition, respectively). This suggests that the (4*S*) arrangement at C4 is more effective than the (4*R*) one (see Figure 1). However, the same configuration at C2 is present in both the most and the least effective isomers (D-*erythro* and D-*threo*) and suggests that the configuration at C2 is less decisive for the inhibitory effect.

The kinetics of GAD inhibition were estimated first with racemic 4-fluoroglutamate (results not presented here) and with individual stereoisomers (Figure 2). In all cases, the effect was of a competitive character, as it might be expected on the basis of the similarity in structure of 4-fluoroglutamate with that of L-glutamate as the natural substrate of GAD, and from the results with GAD from other sources.[7,10] Unkeless and Goldman[7,10] found that only the D-isomers of 4-fluoroglutamate are true inhibitors of GAD from *E. coli*, while the L-isomers are alternative substrates. In the light of this view the present authors considered it important to examine if there was any effect on GAD of the possible decarboxylation products of 4-fluoroglutamate, i.e. (*R*)-(+)- and (*S*)-(−)-2-fluoro-GABA. At 1×10^{-2} M

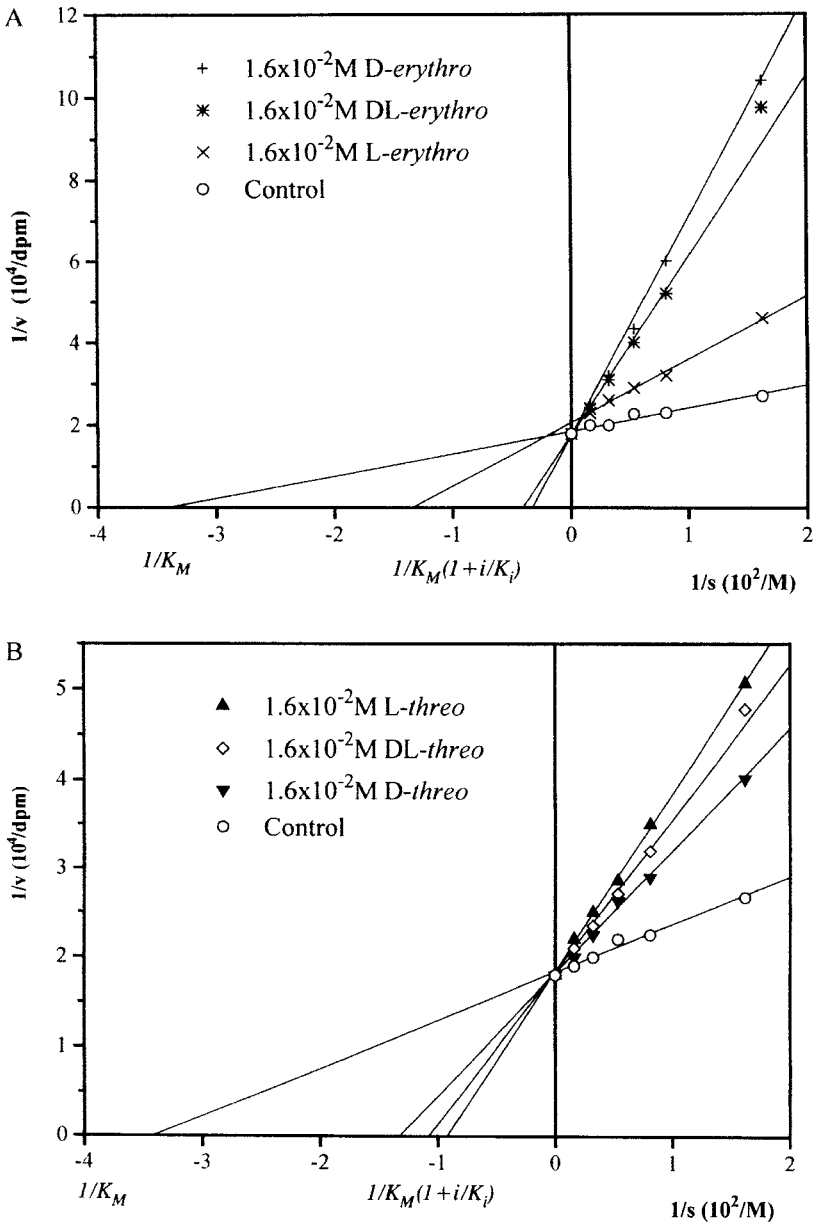

FIGURE 2 Kinetics of glutamate decarboxylase inhibition by stereoisomers of 4-fluoroglutamate depicted in the Lineweaver–Burk plot. Concentration of individual stereoisomers = 2×10^{-2} M. (A) *Erythro*- isomers; (B) *threo*- isomers.

concentration, the individual stereoisomers of 2-fluoro-GABA showed no influence on GAD activity (Table II). The present authors attempted to determine whether the process of decarboxylation of L-fluoroglutamate stereoisomers was rapid enough to-decrease the inhibitor concentrations and, thus, inhibitory effect on L-glutamate decarboxylation. The experiment with pre-incubation of the GAD preparation with individual stereoisomers of 4-fluoroglutamate surprisingly suggested that after 24 h pre-incubation the relative inhibition of GAD was paradoxically more pronounced with L-isomers than with D-isomers in comparison with the starting point (Figure 3). These results suggest that decarboxylation of L-isomers is very slow. A decrease in GAD inhibition by D-isomers might be ascribed to a

TABLE II Glutamate decarboxylase activity in the presence of 2-fluoroglutamate stereoisomers. Concentration of L-glutamate = 1.23×10^{-2} M, concentration of 2-F-GABA = 1×10^{-2} M

	Activity (nkat/g prot.)	*% of control*
Control	56.8 ± 2.8	100
(+)2-F-GABA	57.5 ± 2.7	101.1
(−)2-F-GABA	58.2 ± 2.3	102.5

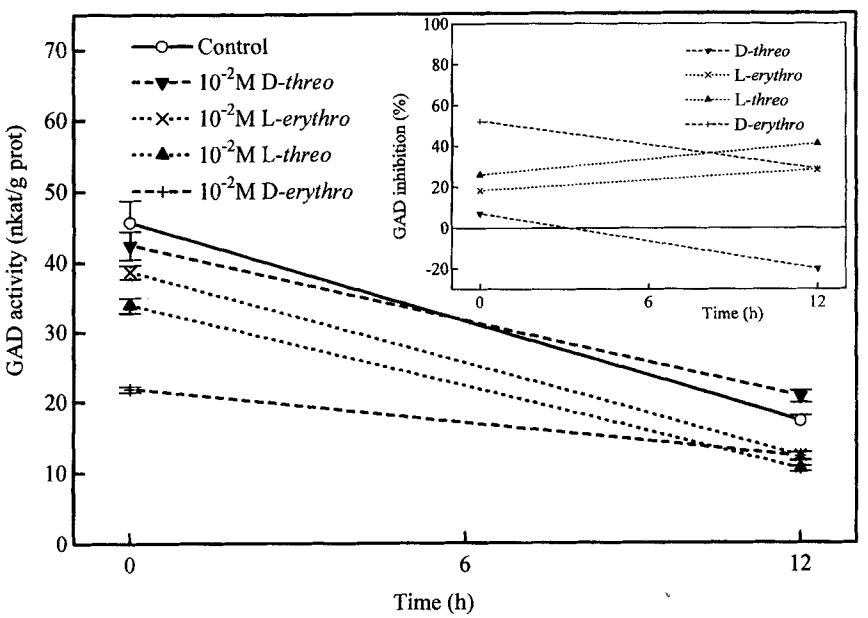

FIGURE 3 Time profile of the influence of stereoisomers of 4-fluoroglutamate in 1×10^{-2} M concentration on GAD activity in the presence of 1.23×10^{-2} M L-glutamate.

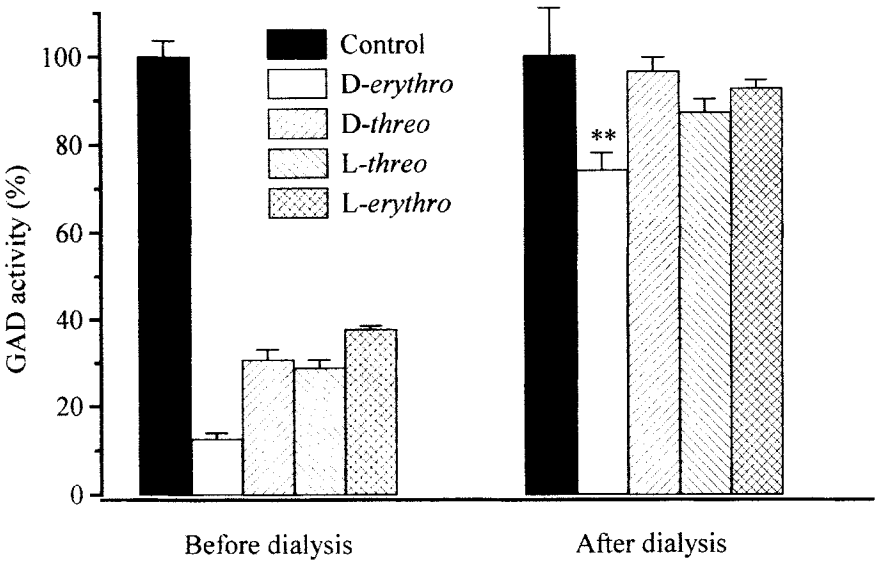

FIGURE 4 An attempt to restore glutamate decarboxylase activity by dialysis of the mixture of GAD preparation with individual stereoisomers of 4-fluoroglutamate. Concentration of F-Glu stereoisomers before dialysis: 3.6×10^{-2} M; $^{**}p = 0.01$.

stabilising effect of the compound bound on the enzyme molecule but the reason for an increase in the effect of L-isomers on GAD is not clear.

According to results from dialysis experiments the interactions of L-isomers and of the D-*threo* isomer with GAD seem to be reversible. In the case of the D-*erythro* isomer, partial irreversible binding or more pronounced denaturation of the enzyme molecule in the presence of the compound could not be excluded (Figure 4).

Addition of an external coenzyme, pyridoxal-5′-phosphate confirmed the common experience that the enzyme from the rat brain is not saturated with the coenzyme.[13]. Pyridoxal-5′-phosphate did not affect GAD inhibition by racemic 4-fluoroglutamate, which suggests that the inhibitor cannot be removed from the system by a direct reaction with the coenzyme and that there is no difference in the affinity of the compound to the holo- and apoenzyme of GAD (Figure 5).

From the results presented in this paper it may be concluded that stereoisomers of 4-fluoroglutamate inhibit decarboxylation of L-glutamate by GAD in a reversible and competitive manner. Since L-glutamate concentration in the rat brain tissue is of the order of 10^{-3} M only, even lower concentrations of 4-fluoroglutamate than those used in the *in vitro* experiments presented here might be effective as GAD inhibitors *in vivo*.

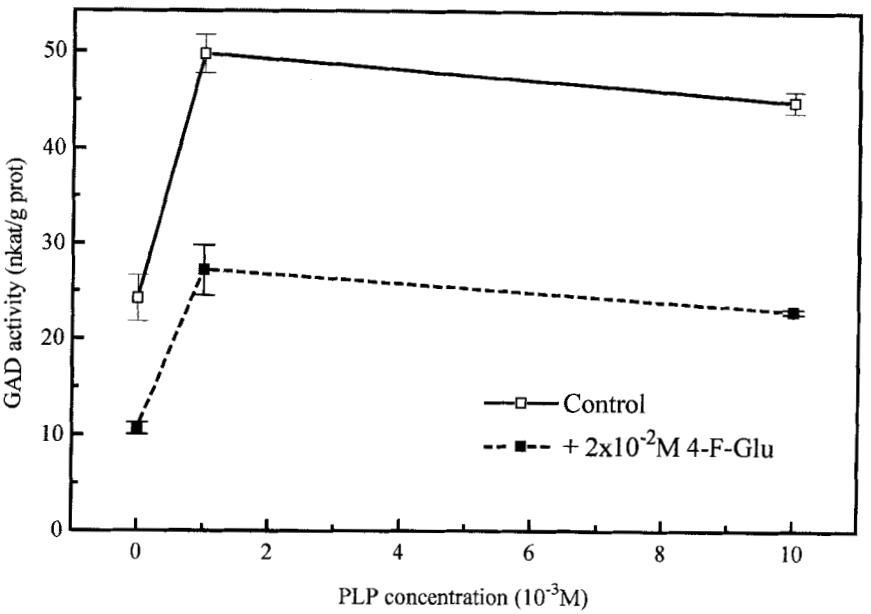

FIGURE 5 Influence of addition of pyridoxal-5′-phosphate on glutamate decarboxylase inhibition by racemic 4-fluoroglutamate.

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