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ENZYME-ACTIVATED IRREVERSIBLE INHIBITION OF RAT AND MOUSE BRAIN 4-AMINOBUTYRIC ACID-α-KETOGLUTARATE TRANSAMINASE BY 5-FLUORO-4-OXO-PENTANOIC ACID

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SUMMARY: γ-Aminobutyric acid-α-ketoglutarate aminotransferase from rat and mouse brain was irreversibly inhibited by 5-fluoro-4-oxo-pentanoic acid, an analogue of succinic semi-aldehyde. The inhibition was concentration and temperature-dependent, and was initiated solely with the pyridoxamine form of the enzyme. All of the results to date are consistent with a catalytic mechanism for the inhibition. Intraperitoneal administration of 5-fluoro-4-oxo-pentanoic acid to rats or mice produced a dose-dependent, irreversible inhibition of brain γ-aminobutyric acid-α-ketoglutarate aminotransferase and elevation of brain GABA levels. No inhibition of glutamate decarboxylase, aspartate aminotransferase or alanine aminotransferase was observed.

INTRODUCTION: GABA-T is a pyridoxal-phosphate dependent enzyme which catalyzes the reversible transamination of GABA to succinic semialdehyde. As an obligatory part of this reaction sequence, the pyridoxal form of the enzyme is converted to the pyridoxamine form. The pyridoxal form is then regenerated by transamination with α-ketoglutarate. There is a great interest in GABA-T because it plays a central role in the catabolism of GABA, a major inhibitory neurotransmitter in mammalian brain. Inhibition of this enzyme in mammalian brain leads to elevated brain GABA levels [for a review see (1) and references therein], therefore inhibition of this enzyme has been a target for attempts to potentiate GABA-ergic neurotransmission. Recently, the concept of enzyme-activated irreversible inhibition (2,3,4) of enzymes has been used to design selective, potent inhibitors of GABA-T [e.g. ethanolamine-O-sulphate (5);

Abbreviations: GABA, γ-aminobutyric acid; GABA-T, γ-aminobutyric acid-α-ketoglutaric acid aminotransferase (E.C. 2.6.1.19); SSADH, succinic semialdehyde-NAD; oxidoreductase (E.C. 1.2.1.16); NAD, β-nicotinamide adenine dinucleotide; αKG, α-ketoglutarate; MFL, 5-monofluorolevulinate, 5-fluoro-4-oxo-pentanoic acid.

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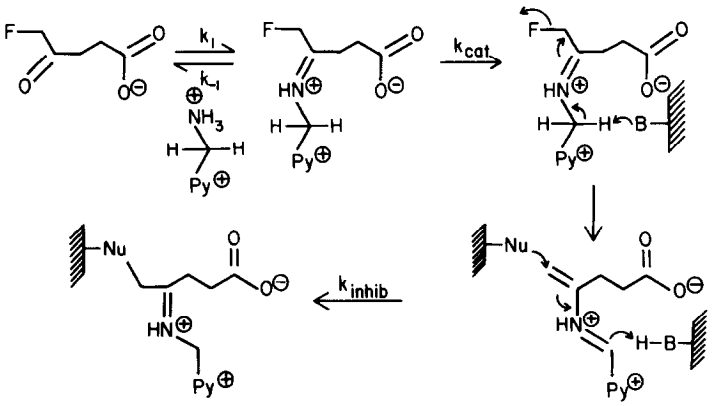
γ-acetylenic GABA (6); γ-vinyl GABA (7); isogabaculine (8) and ω-fluormethyl analogues of GABA and β-alanine (9,10)].

To date all "k_{cat} inhibitors" or "suicide substrates" of GABA-T have been amine analogs of GABA or β-alanine and therefore have had to interact with the pyridoxal form of the enzyme. In vivo, as GABA-T is inhibited and brain GABA levels rise, the equilibrium between the pyridoxal and pyridoxamine forms of the enzyme will be shifted towards the pyridoxamine form, which has previously been shown to be refractory to inhibition by existing k_{cat} inhibitors (6,7, 11). This is the probable reason why, to date, it has been difficult to achieve total inhibition of this enzyme in vivo using the k_{cat} approach.

Since GABA-T catalyzes a reversible reaction, and recognizes aldehyde (succinic semialdehyde) and keto (α-ketoglutarate) as well as amino acid (GABA and L-glutamate) substrates, it should be possible to employ the k_{cat} approach to irreversibly inhibit this enzyme using a suitable keto analogue (Scheme I).



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In this communication we report the selective irreversible inhibition of GABA-T by 5-fluorolevulinate, an analogue of succinic semialdehyde. This inactivator initiates the inhibitory process by interacting with the pyridoxamine form of the enzyme.

MATERIALS AND METHODS: 5-Fluorolevulinate was prepared as described by Rando (12). All other reagents were of the highest grade commercially available.

GABA-T was partially purified from rat brain by following the initial steps of the procedure of Fowler & John (13) with the addition of an $(NH_4)_2SO_4$ fractionation (45-65%). The resulting preparation had a specific activity of 27 μmoles/h/mg protein. SSADH was partially purified by following the method of Pitts et al. (14). Protein was determined by the method of Bradford (15) using bovine serum albumin as the standard.

GABA-T activity was determined by coupling the formation of succinic semialdehyde to the SSADH-dependent reduction of NAD and following the formation of NADH at 340 nm as previously described by Lippert (7). Time-dependent inhibition of GABA-T *in vitro* was determined as previously described (7) except that all volumes were halved.

The elimination of fluoride ion from 5-fluorolevulinate was determined using a fluoride ion electrode (Orion Cat. #96-09) calibrated with solutions of NaF (10^{-5} M - 10^{-2} M).

For the *in vivo* determinations, mice were killed by decapitation and the whole brain was removed, divided in half by sectioning sagitally before freezing in liquid N_2 within 2 minutes after death. One half was used for enzyme determinations as previously described (16); the other half, while still frozen, was homogenized in 4 volumes 0.3 M $HClO_4$ (4:1 v/w) and was used for the determination of GABA, by employing a modification of the method of Scott & Jacoby (17) using a preparation of GABA-T/SSADH from *Pseudomonas* (Sigma Cat. #G-7509). Since the Cl_3COO^- anion inhibits this bacterial GABA-T and HCl produced turbid extracts, neither Cl_3CCOOH nor HCl were suitable alternatives to $HClO_4$.

RESULTS AND DISCUSSION: Incubation *in vitro* of GABA-T with 5-fluorolevulinate in the presence of GABA or L-glutamate produced time-dependent inhibition of GABA-T activity which went to completion (Fig. 1). GABA or L-glutamate can transaminate with GABA-T and form the pyridoxamine form of the enzyme. We propose that 5-fluorolevulinate can transaminate with the pyridoxamine form of the enzyme and eliminate fluoride ion, thereby producing a reactive α,β-unsaturated imine (see Scheme 1). This is the same intermediate that has been proposed to cause the inhibition of GABA-T upon incubation with 4-amino-5-fluoropentanoic acid (9,10).

Incubation of GABA-T with 5-fluorolevulinate in the presence of 0.1 mM α-KG did not lead to inhibition (Fig. 1). α-KG at 1/10 its Km value was used to ensure that the enzyme was exclusively in the pyridoxal form without saturating the active site with this substrate. When both α-KG and GABA were present with the inhibitor in the preincubation medium, thereby allowing the enzyme to shuttle back and forth between the pyridoxal and pyridoxamine forms, 5-fluorolevulinate inhibited GABA-T at a rate intermediate between that observed in the presence of either GABA or α-KG alone.

The inhibition reaction followed pseudo-first-order kinetics for at least 3 half-lives, and was concentration (Fig. 2), temperature and pH-dependent. The pH profiles of the rate of transamination of GABA and the rate of inhibition by 5-fluorolevulinate were similar; the maximal rate for each was at pH 8.5. The rate of transamination and inhibition when expressed as the

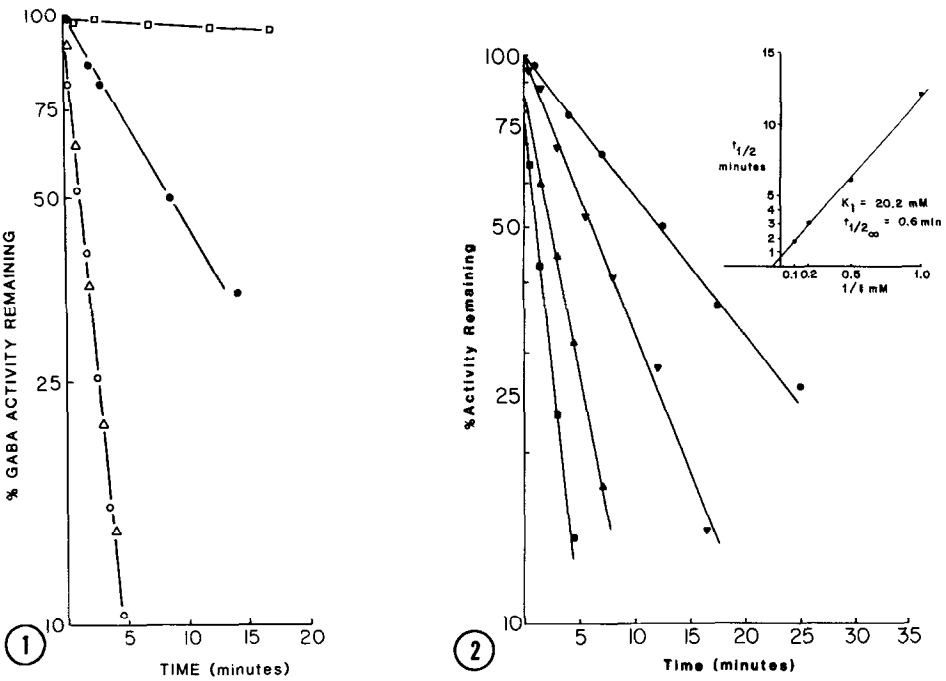

Fig. I. The effect of GABA, L-glutamate and α-KG on the inactivation of GABA-T in vitro. GABA-T (0.02 I.U.) was incubated with 5-fluorolevulinate 1 mM in pyrophosphate buffer 100 mM pH 8.6, at 25° containing mercaptoethanol 5 mM and either GABA 1 mM (△), L-glutamate 1 mM (○) or α-KG 0.1 mM (⊡) or both substrates (●) in a total volume of 0.1 ml. A 10 µl aliquot was withdrawn at each indicated time and assayed for residual GABA-T activity.

Fig. II. Time and concentration-dependent inhibition of GABA-T by 5-fluorolevulinate in vitro. GABA-T (0.02 I.U.) was incubated at 0° with pyrophosphate buffer 100 mM pH 8.6, mercaptoethanol 5 mM, GABA 1 mM and 5-fluorolevulinate at 1 mM (●); 2 mM (▼), 5 mM (▲), or 10 mM (■). At the indicated times a 10 µl aliquot was withdrawn and assayed for residual GABA-T activity. In the insert the $t_{\frac{1}{2}}$'s for the inhibition were plotted against the reciprocal of the inhibitor concentration in order to determine the K_i and the first order rate constant for the inhibition at 0°.

fraction of the rate observed at pH 8.5 decreased to 88% and 65% respectively at pH 9.5, to 78% and 43% at pH 7.5 and to 35% and 30% at pH 6.5. At 25°, the reaction rate increased linearly with inhibitor concentration up to 1 mM ($t_{\frac{1}{2}}$=1.5 min). To detect Michaelis complex formation, the enzyme was incubated with 5-fluorolevulinate (1-10 mM) in the presence of 1 mM GABA at 0°. At this temperature, the inhibition reaction rate was approximately 1/10 that found at 25°, so that concentrations of inhibitor as high as 10 mM could be employed conveniently (Fig. 2). Using the procedure of Kitz and Wilson (18) as modified by Jung & Metcalf (6), the K_I was found to be 20.2 mM and the

calculated first-order rate constant for the reaction at 0^{o} was 1.2 min^{-1} (corresponding to a half-life of the enzyme of 0.6 min. in the presence of saturating concentrations of inhibitor). 5-Fluorolevulinate even at 10 mM did not inhibit aspartate aminotransferase (E.C. 2.6.1.1) or alanine aminotransferase (E.C. 2.6.1.2).

All of the above incubations routinely contained 5 mM mercaptoethanol thereby excluding the possibility that 5-fluorolevulinate was being released from the active site and then inhibiting GABA-T via an affinity labelling mode. In addition, no F^{-} ion release was detected from a solution of 5 x 10^{-4} M of the compound in the presence of 5 x 10^{-3} M mercaptoethanol and 0.1 M potassium pyrophosphate buffer pH 8.6 over a period of 30 minutes which was the time corresponding to the longest incubation of the inhibitor with the enzyme. However, there was detectable F^{-} ion release (3.8 x 10^{-6} M/hr) corresponding to a second order rate constant of 0.024 M^{-1} min^{-1} upon prolonged incubation of 5-fluorolevulinate with mercaptoethanol. We therefore cannot at this time rule out the possibility that at least part of the observed inhibition of GABA-T is due to S_N2 attack on the Schiff base between 5-fluorolevulinate and enzyme-bound pyridoxamine phosphate by an essential thiol group in the enzyme's active site without prior activation of the inhibitor by the enzyme.

Administration of 5-fluorolevulinate to rats or mice produced a dose-dependent, rapid decrease in GABA-T activity with little effect on glutamate decarboxylase activity, resulting in a marked increase in brain GABA levels 4 hours after an intraperitoneal injection (Fig. 3). GABA-T inhibition increased linearly from 20% to 90% inhibition with the log of the dose from 25 mg/kg to 200 mg/kg (Fig. 3). At least 99% inhibition was achieved at 400 mg/kg, at which time GABA levels were 5 times control values. It is noteworthy that 5-fluorolevulinate caused almost complete inhibition of GABA-T at doses much lower than one would predict from its K_i and k_{cat} compared to inhibitors which interact with the pyridoxal form of the enzyme (7,10,19). It is notable also that the dose-inhibition curve is shallower at low doses and

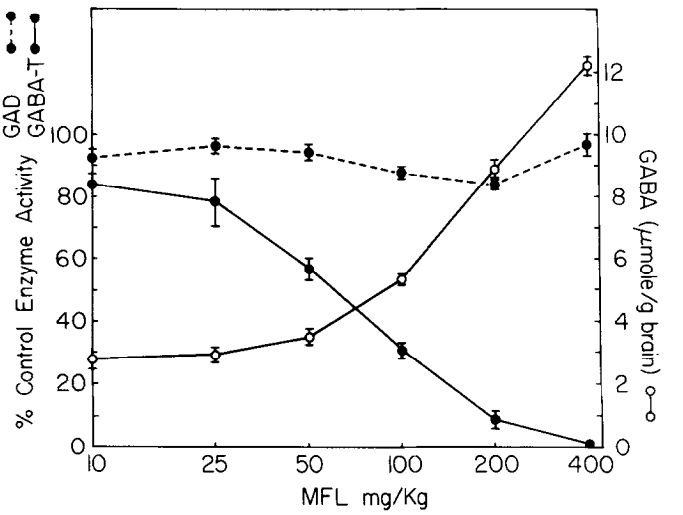

Fig. III. Dose effect relationship between mouse brain GABA-T and GAD activities and brain GABA levels 4 hours after an intraperitoneal dose of 5-fluorolevulinate. Control activities were 28.7 ± 0.7 μmoles/g/hr for GAD and 50.1 ± 2.7 μmoles/g/hr for GABA-T. Control GABA levels were 2.67 ± 0.04 μmoles/g. Control values and the values after 100 mg/kg and 200 mg/kg doses are the means ± S.E.M. of 10 animals. All other points are the means ± S.E.M. of 5 animals.

steeper at high doses of inhibitor than it would be if it were to follow a simple logistic function. This probably occurs at least in part because partial inhibition causes brain GABA levels to rise and push the pyridoxal-pyridoxamine equilibrium to the pyridoxamine form, the form of GABA-T with which 5-fluorolevulinate interacts before it can transaminate with and inhibit GABA-T.

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