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INHIBITION BY AMINOACETONITRILE AND PROPARGYLAMINE OF GLYCINE CLEAVAGE SYSTEM FROM RAT BRAIN AND LIVER MITOCHONDRIA

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Abstract—Glycine degradation in the CNS is catalyzed by the glycine cleavage system (GCS). We have tested several compounds with a structural analogy to glycine for their inhibition of the GCS. Compounds having an inhibitory activity, such as aminoacetonitrile and propargylamine, are characterized by having a primary amine group and a π-electron system present in the group substituting for the carboxylic function. The inhibition by these compounds of glycine degradation may be of pharmacological interest given the neurotransmitter role of glycine.

Glycine is a compound having an inhibitory neurotransmitter activity in the spinal cord [1]. A neurotransmitter role for this aminoacid has also been postulated in higher areas of the CNS [2]. Glycine degradation in vertebrates is mainly carried out by the enzyme complex known as the glycine cleavage system (GCS) [3, 4], which splits glycine to form CO_2, NH_3 and methylene tetrahydrofolate, which is derived from the α-carbon of glycine. This enzyme system is located in the inner mitochondrial membrane [5, 6] and is composed of four proteins: P-protein (pyridoxal phosphate dependent), H-protein (a lipoic acid containing protein), T-protein (a tetrahydrofolate-requiring enzyme) and L-protein (a lipoamide dehydrogenase, whose function is to regenerate the lipoic acid) [7, 8]. These proteins have been purified by the group of Prof. Kikuchi, who have also elucidated the reaction mechanism and the function of each protein on the overall cleavage reaction [9]. The reaction is fully reversible in *in vitro* conditions and thus the GCS can catalyze glycine synthesis from CO_2, NH_3 and methylene tetrahydrofolate [8, 9]. However, glycine synthesis by this system does not appear to have appreciable physiological significance [4] since, when the enzyme activity is deficient, an increase in glycine levels is observed [10, 11]. The GCS can also catalyze the $^{14}CO_2$–glycine exchange reaction [8, 9]. This exchange of the carboxyl group of glycine with $^{14}CO_2$ can be considered as an indication of P-protein activity.

The role of the GCS in glycine metabolism is now well established by the fact that genetic deficiency of GCS activity is the cause of large increases in glycine levels in the CNS [10]. Thus the *in vivo* role of the GCS is glycine degradation. Given the neurotransmitter role of glycine, an increase of its concentration in the CNS may be of pharmacological interest. Consequently we have studied the inhibition of the GCS by compounds structurally related to glycine, in order to obtain preliminary information on the requirements for a molecule to be an inhibitor of the GCS. There are very few reports on inhibitors of the GCS; cysteamine [12] has been shown to be an inhibitor of the liver GCS *in vitro* and di-*n*-propylacetate inhibits the GCS in a reversible manner *in vitro* and reduces the activity of the enzyme complex *in vivo*, probably by reduction of the level of a protein component (P-protein) [13].

MATERIALS AND METHODS

[1-^{14}C]Glycine (sp. act. 45–55 mCi/mmole) and $Na_2{}^{14}CO_3$ (sp. act. 30–50 mCi/mmole) were purchased from Commissariat à l'Energie Atomique (Saclay, France) and diluted to the sp. act. required. Biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO) and the inhibitors from Aldrich (Beerse, Belgium).

GCS activity was determined as described previously [14]. The reaction medium contained (in 250 μl): 0.125 μmoles of pyridoxal phosphate, 0.75 μmoles of tetrahydrofolate, 1.25 μmoles of dithiothreitol, 1.25 μmoles of NAD, 2.5 μmoles of [1-^{14}C]glycine (0.1 μCi/μmole) and 12.5 μmoles of Tris adjusted with HCl to pH 8. The reaction was started by the addition of 1 mg of mitochondrial protein and carried out at 37° for 1 hr, and stopped with 200 μl of 20% (v/v) $HClO_4$. The $^{14}CO_2$ produced was recovered in a well containing 0.2 ml of 1 M ethanolic solution of hyamine hydroxyde.

The $^{14}CO_2$–glycine exchange reaction was determined as described by Kochi *et al.* [13] with slight modifications. The reaction mixture contained (in a final volume of 500 μl): 25 μmoles of potassium phosphate buffer (pH 6.2), 5 μmoles of glycine, 10 μmoles of $K_2{}^{14}CO_3$ (0.2 μCi/μmole), 0.25 μmoles of pyridoxal phosphate and 2.5 μmoles of dithiothreitol. The reaction was started by adding 2 mg of mitochondrial protein, carried out at 37°C for 1 hr

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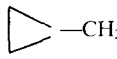
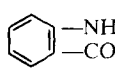
Table 1. Effects of several compounds on GCS activity from rat brain and liver mitochondria

Compound (4 mM)	Structure	% inhibition	
		Brain	Liver
1 Aminoacetonitrile	$H_2N—CH_2—C\equiv N$	76 ± 3	71 ± 2
2 Aminopropionitrile	$H_2N—CH_2—CH_2—C\equiv N$	0	
3 Methylaminoacetonitrile	$H_3C—NH—CH_2—C\equiv N$	15 ± 2	
4 Propargylamine	$H_2N—CH_2—C\equiv CH$	88 ± 1	84 ± 1
5 Cyclopropylmethylamine		21 ± 1	18 ± 1
6 *o*-Aminobenzoic acid		28 ± 1	23 ± 2

Each value is the mean ± S.E. of six experiments. Control activities were 0.193 ± 0.007 nmoles/min × mg protein (brain) and 0.842 ± 0.053 nmoles/min × mg protein (liver).

Table 2. Reversibility of the inhibitory effect of aminoacetonitrile (AAN) and propargylamine (PPA) on the GCS activity

Preincubation	Activity (nmoles/min × mg protein)	
	Liver mitochondria	Brain mitochondria
0°	0.918 ± 0.092	0.224 ± 0.027
0°, 10 mM AAN	0.876 ± 0.075	0.212 ± 0.018
0°, 10 mM PPA	0.921 ± 0.110	0.196 ± 0.012
37°	0.177 ± 0.012	0.108 ± 0.007
37°, 10 mM AAN	0.341 ± 0.015	0.101 ± 0.004
37°, 10 mM PPA	0.316 ± 0.022	0.099 ± 0.010

Mitochondria (5 mg/ml) were preincubated in the presence of the inhibitors. After 1 hr, the mitochondria were washed 3 times by centrifugation and the GCS activity determined. Each value is the mean ± S.E. of six experiments.

and stopped by the addition of 0.5 ml of 25% acetic acid. An aliquot of 0.8 ml was introduced into a scintillation counting vial and dried by heating under vacuum to remove the remaining $^{14}CO_2$. The radioactivity of [^{14}C]-glycine was then determined.

Sprague–Dawley (Charles River, CD®, St Aubin les Elbeuf, France) male rats weighing about 200 g and fasted for 24 hr were used. Liver mitochondria were prepared as described by Schneider [15]. Brain mitochondria were prepared from the P_2 fraction [16] by centrifugation in a sucrose gradient [12 ml of sucrose (1.2 M), 12 ml of sucrose (0.8 M) and the P_2 fraction suspended in 12 ml of sucrose (0.32 M)] at 54,000 *g* for 90 min in a Beckman SW28 rotor. Mitochondria were recovered at the bottom of the gradient. Brain and liver mitochondria were resuspended in 50 mM Tris adjusted with HCl to pH 8 containing 1 mM dithiothreitol at 20 mg mitochondrial protein/ml. Protein content was measured spectrofluorimetrically as indicated by Resch *et al.* [17]. Kinetic constants (K_m and V_{max}) have been calculated by a statistical program in a HP-97 as described by Zivin and Waud [18].

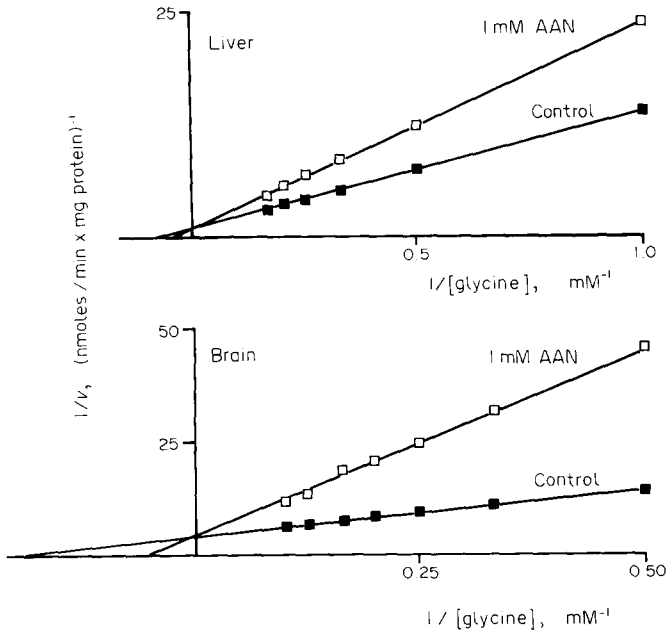
Table 3. Inhibition of $^{14}CO_2$–glycine exchange by aminoacetonitrile (AAN) and propargylamine (PPA)

Compound (4 mM)	% inhibition	
	Brain	Liver
AAN	12 ± 2	13 ± 2
PPA	50 ± 3	53 ± 3

Each value is the mean ± S.E. of six experiments. Control activities were 0.167 nmoles/min × mg protein (brain) and 0.182 nmoles/min × mg protein (liver).

RESULTS

We have tested the effect of some compounds structurally related to glycine on the GCS activity. When the glycine carboxylic function was substituted by a —C≡N the resulting compound [aminoacetonitrile (AAN)] inhibited the GCS activity of the liver and brain. This compound has been previously described as an inhibitor of glycine decarboxylation in wheat leaves [19]. At a concentration of 4 mM the higher homologue of AAN is not an inhibitor whereas its *N*-monomethyl derivative is a weak inhibitor of GCS [compounds **2** and **3** (Table 1)]. Substitution of the glycine carboxylic function by other functions also having a π- or pseudo π-electron system gives a compound with high inhibitory activity [propargylamine (PPA)] and a compound with weaker inhibitory activity, cyclopropylmethylamine [compounds **4** and **5** (Table 1)]. Ortho-aminobenzoic acid, which has the two functions present in the



AAN (mM)	V_{max} (nmoles/min x mg)	K_m (mM)
0	0.975 ± 0.089	12.83 ± 1.47
1	0.909 ± 0.235	20.34 ± 6.14

K_i = 1.70 mM

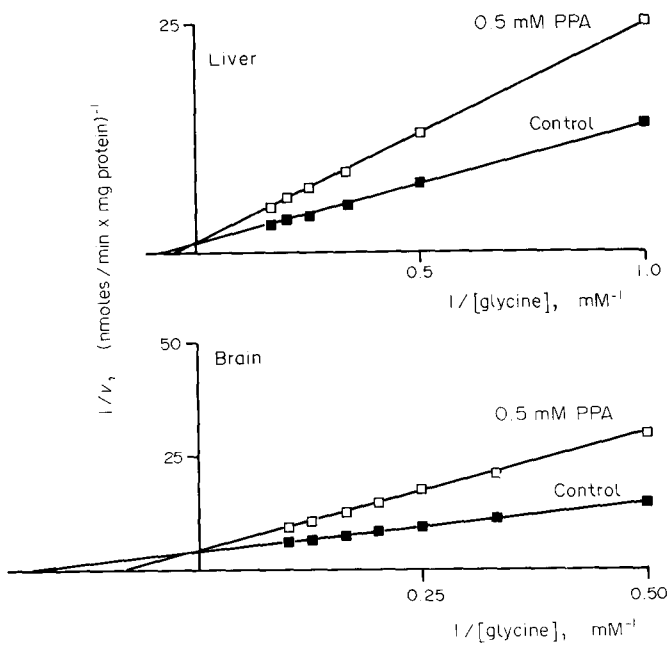
AAN (mM)	V_{max} (nmoles/min x mg)	K_m (mM)
0	0.247 ± 0.007	5.29 ± 0.31
1	0.236 ± 0.042	19.19 ± 4.33

K_i = 0.38 mM

Fig. 1. Inhibition by aminoacetonitrile (AAN) of the GCS from liver and brain mitochondria. Kinetic parameters were calculated from sextuplicate determinations of the GCS activity at each glycine concentration.

glycine molecule linked by a double bond included in a phenyl ring also shows a weak inhibitory activity.

We have studied the characteristics of GCS inhibition by AAN and PPA. As shown in Table 2, both are reversible inhibitors, since incubation for 1 hr of the mitochondrial suspension in the presence of each inhibitor, followed by a washing, does not produce a significant effect on the GCS activity. It is to be noted that incubation of the enzyme at 37° for 1 hr causes partial inactivation. Temperature effects are higher on the liver (81% inactivation) than on the brain enzyme (52% inactivation). However, AAN and PPA partially protect the liver but not the brain enzyme from heat inactivation.



PPA (mM)	V_{max} (nmoles/min x mg)	K_m (mM)
0	0.975 ± 0.089	12.83 ± 1.47
0.5	0.920 ± 0.098	22.03 ± 2.71

K_i = 0.69 mM

PPA (mM)	V_{max} (nmoles/min x mg)	K_m (mM)
0	0.247 ± 0.007	5.28 ± 0.31
0.5	0.237 ± 0.033	12.55 ± 2.11

K_i = 0.36 mM

Fig. 2. Inhibition by propargylamine (PPA) of the GCS from liver and brain mitochondria. Kinetic parameters were calculated from sextuplicate determinations of the GCS activity at each glycine concentration.

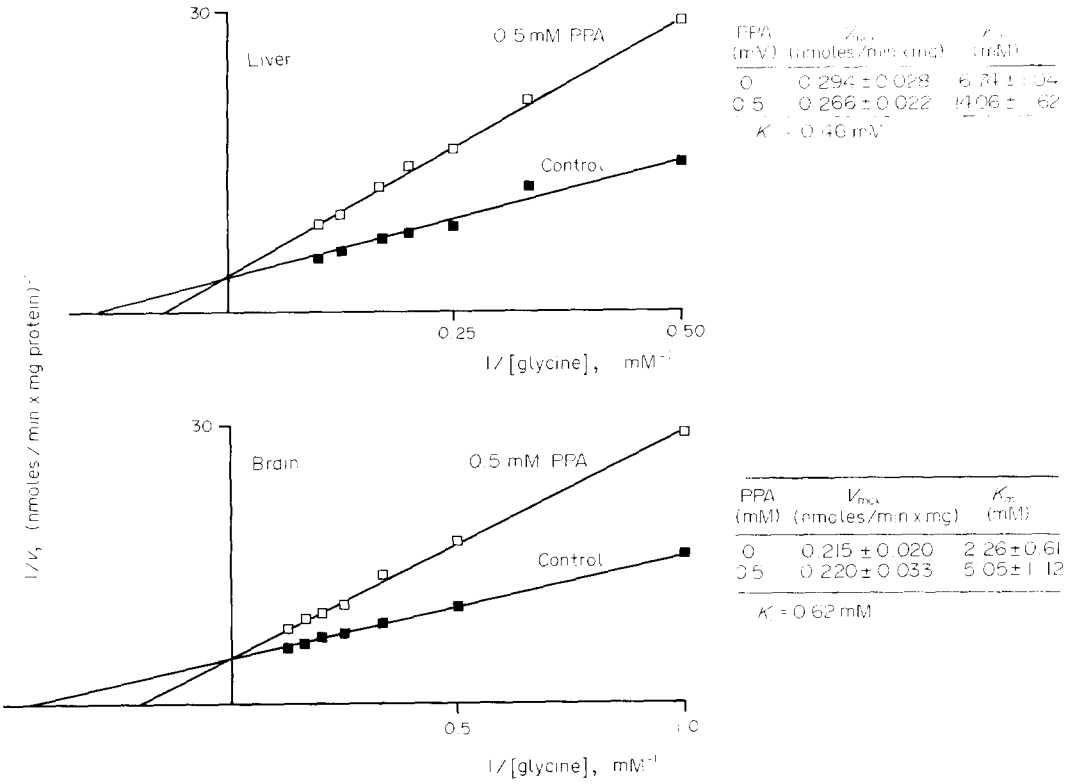


Fig. 3. Inhibition by propargylamine (PPA) of the $^{14}CO_2$–glycine exchange reaction. Kinetic parameters were calculated from sextuplicate determinations of the exchange activity at each glycine concentration.

The GCS can also catalyze the $^{14}CO_2$–glycine exchange reaction due to the labilization of the glycine carboxylic group, when this aminoacid forms an imine bond with the pyridoxal phosphate of P-protein. This exchange can be considered as an indication of P-protein activity. The effect of AAN and PPA on the exchange reaction has been tested. At the same concentration (4 mM) both compounds are weaker inhibitors of the $^{14}CO_2$–glycine exchange reaction than of the GCS activity (Table 3.). Kinetic characteristics for the inhibition of GCS activity by AAN and PPA have been studied. Both compounds are competitive inhibitors of the liver and brain mitochondrial enzyme systems (Figs. 1 and 2). Kinetics of the inhibition by PPA of the $^{14}CO_2$–glycine exchange reaction have also been studied; PPA has been found to be a competitive inhibitor of the exchange reaction in liver and brain mitochondria (Fig. 3).

DISCUSSION

Though the importance of the GCS on glycine degradation in the brain has been clearly established, there have been few reports on inhibitors of this enzyme. These inhibitors could be of pharmacological interest given the neurotransmitter role of glycine. Di-*n*-propylacetate has been reported to be an inhibitor of the GCS. However, di-*n*-propylacetate has other biochemical effects [20–22] and thus cannot be considered as a specific agent. In the present study we demonstrate that two compounds having an unsubstituted amino group, a function bearing π-electrons and a chain length similar to glycine are competitive inhibitors of the GCS reaction. Another compound having an unsubstituted amino group but no π-electron system (cysteamine) has also been described as a strong inhibitor of liver GCS activity *in vitro* [12]. To be cleaved glycine must react with the pyridoxal phosphate moiety of P-protein [2]; thus it is to be expected that AAN and PPA are inhibitors of the $^{14}CO_2$–glycine exchange reaction, catalyzed by P-protein alone. However, these compounds are weaker inhibitors of the $^{14}CO_2$–glycine exchange reaction than of the cleavage reaction, when tested with 10 mM glycine. This could be explained by the different experimental conditions of the two reactions (pH 6.2 for exchange, pH 8 for cleavage) and/or the higher affinity for glycine of the exchange than of the GCS.

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