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THE EFFECT OF THE CONVULSANT ALLYLGLYCINE (2-AMINO-4-PENTENOIC ACID) ON THE ACTIVITY OF GLUTAMIC ACID DECARBOXYLASE AND THE CONCENTRATION OF GABA IN DIFFERENT REGIONS OF GUINEA PIG BRAIN*

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Abstract—Intraperitoneal administration of allylglycine to guinea pigs resulted in convulsions approximately 3 hr later. The concentration of GABA and the activity of GAD were significantly reduced in three brain areas, namely the cochlear nucleus, inferior colliculus and cerebral cortex, with the smallest changes being observed in the cortex. There were large *in vitro* regional variations in the extent of the allylglycine inhibition in brain areas from guinea pig, cat and rat, with those areas rich in GAD activity being least affected. Endogenous GAD activities in the brain regions were found to be inversely correlated with the percentage allylglycine inhibition ($P < 0.005$). Other inhibitors of GAD activity i.e. NaCl, Zn^{2+} and thiosemicarbazide showed no such regional variation of inhibition. The results suggest that the regional differences in allylglycine inhibition reflect anomalies of the metabolism of the drug *per se*, and probably do not indicate regional differences in GABA turnover and metabolism.

Little information is available concerning the rate of turnover of GABA in discrete brain regions, and the relative contributions made to the turnover by 'metabolic' and 'transmitter' compartments. In part, this is due to the absence of a specific inhibitor of glutamic acid decarboxylase (EC 4.1.1.15 L-glutamate 1-carboxylase, GAD) the rate limiting enzyme of GABA synthesis [1]. Potential candiates such as the convulsant hydrazides are known to inhibit a host of pyridoxal phosphate dependent enzymes in addition to GAD, and thus do not possess the required selectivity of action [2]. Antibodies to GAD are the most specific inactivators of the enzyme but seem unable to penetrate the blood–brain barrier [3]. Alberici *et al.* [4] and Rodriguez de Lores Arnaiz *et al.* [5] have suggested that the convulsant allylglycine may be a useful tool in the study of the distribution of GABA mediated systems. These authors found that the administration of allylglycine led to a reduction of GAD activity and GABA concentration in the brains of convulsed animals and that this was accompanied by ultrastructural changes in the nerve endings. In addition several studies have employed subconvulsive doses of allylglycine in model studies related to the involvement of GABA in epileptic-like seizures [6–8]. In a previous publication [9] we have shown that the interaction between allylglycine and GAD is likely to be more complex than first envisaged by Alberici *et al.* [4]. Allylglycine appears to be metabolized by brain tissue with the concomitant production of a metabolite or metabolites which in turn inactivate GAD. This type of "pseudosubstrate" transformation has recently been described by Fowler [10]. Such results questioned the suitability of allylglycine for *in vivo* studies and prompted further investigations. In the present paper we report some anomalous properties of allylglycine inhibition in different brain regions, both *in vivo* and *in vitro*. Since the long term objective of this work was the application of allylglycine to the study of GABAergic innervation within the auditory system, the cochlear nucleus and inferior colliculus were amongst those regions investigated.

MATERIALS AND METHODS

DL-[1-^{14}C]Glutamic acid (sp. act. = 25 mCi/m-mole), 4-amino-*n*-[U-^{14}C]butyric acid (sp. act. = 232 mCi/m-mole) were obtained from the Radiochemical Centre, Amersham. DL-Allylglycine, NADP, bovine serum albumin, α-ketoglutaric acid (sodium salt) and 2-mercaptoethanol were obtained from Sigma (London) Chemical Co. Pyridoxal-5′-phosphoric acid, dithiothreitol, thiosemicarbazide and Triton X-100 were all purchased from B.D.H., Atherstone, Warwicks. Gabase (cell free preparation from Pseudomonase spp.) was obtained from the Cambrian Chemical Co. Ltd., Croydon, Surrey.

Guinea pigs of either sex were killed by stunning and exsanguination, and whole brains (including brain stem and cerebellum) removed prior to the dissection of the following selected regions. (1) Cochlear nucleus: this was readily identified as a compact almost separate nucleus, situated where the eighth

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Abbreviations used: GAD; glutamic acid decarboxylase. GABA; 4-aminobutyric acid. GABA-T; GABA-transaminase.

nerve enters the medulla. (2) Inferior colliculus: excised 1–2 mm above the surface of the tectum. (3) Cerebral cortex: gray matter was sampled to a depth of approx 3 mm from a region in the vicinity of the auditory cortex. (4) Medulla: a segment of the medulla was taken at the level of the olivary nuclei. (5) Superior colliculus: excised 1–2 mm above the surface of the tectum. (6) Cerebellum: whole cerebellum was used.

The range of wet weights for the areas dissected in guinea pig brain were as follows: whole brain, 2–3 g; cochlear nucleus, 10–18 mg; inferior colliculus, 25–35 mg; cerebral cortex, 30–45 mg; superior colliculus, 30–35 mg; medulla, 54–70 mg; whole cerebellum, 220–300 mg.

The same procedure was adopted for the dissection of cat and rat brain regions except for the method of sacrifice. Cats were killed by an overdose of Nembutal and rats by chloroform anaesthesia. Dissections were usually complete within 3–4 min after death. For GAD determinations, tissues were homogenized in hypotonic 10 mM sodium phosphate (pH 6.8) buffer prior to assay. For GABA determinations, tissue homogenates were made 75% with respect to alcohol by the addition of absolute ethanol. Alternatively, tissues were homogenized directly in 75% ethanol. Insoluble material was removed by centrifugation, the pellet rehomogenized in 75% ethanol and sedimented once again by centrifugation. The original and washing supernatants were combined and evaporated to dryness. The samples were resuspended in a fixed vol. of 10 mM sodium phosphate buffer (pH 7.4) and centrifuged at 100,000 *g* for 60 min to remove any cloudiness. Suitable aliquots of the supernatant (up to 25 μl) were taken to dryness in microtubes in a vacuum desiccator prior to GABA assay. For sample volumes greater than 25 μl, this process was repeated in multiples of 25 μl. In this way the assay was linear with volume up to 100 μl. To check recovery, a suitable aliquot of [^{14}C]GABA was added prior to the initial homogenization in ethanol. The percentage recovery in this extraction procedure was 102 ± 2 (S.E.M. for 10 experiments).

GAD assay. Glutamate decarboxylase was assayed radiometrically as previously described [9]. The reaction mixture consisted of the following components in a total volume of 1 ml (final concentrations in m-mole^{-1} or as stated): L-sodium 1-[^{14}C]glutamate, 4.0 (final sp. act. 0.03 μCi/μmole); dithiothreitol 1.0; pyridoxal-5′-phosphate, 0.2; sodium phosphate buffer (pH 6.8), 50.0; bovine serum albumin, 1.0 mg; Triton X-100, 0.25%. Unless stated otherwise, assay tubes were incubated for 2–3 hr and the reaction terminated by tipping the acid from the sidearm into the main reaction vessel. GAD reaction rates have been calculated in terms of μmole glutamic acid decarboxylated g^{-1} hr^{-1}.

GABA assay. With some minor modifications, GABA was assayed by the method of Graham and Aprison [11], based on the enzymic conversion of GABA to succinic acid with the concomitant formation of NADPH. To each tube (750 × 100 mm) was added 50 μl incubation mixture containing the following components (final concentrations in m-mole^{-1}): tetra-sodium pyrophosphate buffer (pH 8.4), 71; α-ketoglutarate, 7.4 (previously neutralized with Na_2CO_3); NADP, 0.18; 2-mercaptoethanol, 0.18 μl; Gabase, 70 μg. Blank tubes and GABA standards also received 50 μl incubation mixture. Tissue samples, tissue and reagent blanks, and GABA standards were treated identically throughout the procedure. All tubes were incubated at 38° in a Dubnoff shaker for 45 min. After the incubation period, 100 μl of phosphate buffer was added containing 400 mM Na_3PO_4 and 200 mM $Na_2HPO_4 \cdot 12H_2O$ in order to destroy the excess NADP and the tubes were heated for 15 min at 60°. Finally, the NADPH produced in the reaction was converted to a fluorescent NADP product by adding 100 μl of 10 N NaOH containing 0.075% H_2O_2 and heating at 60° for 10 min. Seven hundred μl of distilled water was added and the fluorescence read in an Aminco Bowman spectrophotofluorometer (λ excitation = 355 nm, λ emission = 460 nm). The lower limit of the assay's sensitivity was 0.25 nmole and a linear relationship existed between fluorescence and the amount of GABA up to 5nmole. Reagent blanks but not tissue blanks were routinely assayed.

To retain uniformity, quantitative data have been expressed as mean ± S.E.M. wherever possible.

RESULTS

The convulsive properties of allylglycine as reported by Alberici *et al.* [4] were confirmed. Intraperitoneal administration of allylglycine (300 mg kg^{-1} body wt ≡ 2.61 m-mole kg^{-1}) produced convulsions in eight out of nine animals with a mean latency of 174 ± 10 min (range 135–210 min). Onset of convulsions was preceded by a period of hyperexcitability with running and jumping which lasted for several minutes. Table 1 shows that the three regions tested in convulsed animals showed significant reductions in both GAD activity (24–35 per cent) and GABA concentration (33–59 per cent) over controls. Reductions in GAD activity and GABA concentration were greatest in the inferior colliculus and cochlear nucleus, whilst cerebral cortex was least affected. Although previous *in vivo* studies with allylglycine have been confined to whole brain [7] [12], cerebral cortex [4] and cerebellum [5], the observed percentage loss of GAD and GABA in cerebral cortex is in good agreement with the findings of Alberici *et al.* [4].

Possible regional variation in allylglycine inhibition was also investigated in *in vitro* systems. Of the three regions tested there was a large variation in the extent of inhibition at different allylglycine concentrations, the order of inhibition being cochlear nucleus > inferior colliculus > cerebral cortex in order of decreasing severity (Fig. 1a). In some experiments cochlear nucleus was inhibited completely at 5 mM allylglycine whilst under the same conditions the inferior colliculus and cerebral cortex were inhibited 70% and 30% respectively. Within experimental error no regional variation of inhibition was noted for three other categories of GAD inhibitors [9], i.e. thiosemicarbazide (Fig. 1b); NaCl (Fig. 1c); and Zn^{2+} (Fig. 1d), although the shapes of the dose–inhibition curves were dissimilar. It thus appears that the degree of allylglycine inhibition varies considerably according to the brain region from which the homogenate is prepared (Table

Table 1. The effect of convulsive doses of allylglycine on GABA concentrations (μmole g^{-1} wet wt) and GAD activities (μmole g^{-1} wet wt hr^{-1}) in cochlear nucleus, inferior colliculus and cerebral cortex *in vivo*. Guinea pigs were injected i.p. with 300 mg kg^{-1} body wt of allylglycine in 0.9% sterile saline ($\equiv$ 2.61 m-mole kg^{-1}). Control animals received either no injection or injection of saline. At the onset of convulsions (mean latency = 174 $\pm$ 10 min), animals were sacrificed and individual cochlear nuclei, inferior colliculi and cerebral cortices dissected out and homogenized in 1.2 ml 10 mM sodium phosphate buffer (pH 6.8). Suitable aliquots were then taken for GAD assay or processed for GABA assay

	Control (n)	Convulsed (n)	Δ%*	P-value†
Cochlear Nucleus				
GAD	3.08 ± 0.20 (14)	2.00 ± 0.22 (10)	35	<0.005
GABA	1.36 ± 0.09 (12)	0.82 ± 0.10 (14)	40	<0.001
Inferior Colliculus				
GAD	11.70 ± 0.23 (14)	7.68 ± 0.25 (11)	34	<0.001
GABA	3.82 ± 0.16 (22)	1.56 ± 0.08 (16)	59	<0.001
Cerebral Cortex				
GAD	6.75 ± 0.25 (11)	5.15 ± 0.26 (6)	24	<0.001
GABA	2.54 ± 0.09 (11)	1.71 ± 0.15 (8)	33	<0.001

* Mean percentage reduction of GAD activity or GABA concentration in convulsive animals.

† Statistical significance of difference in mean values for control and convulsive animals.

2). This effect is not species specific since both rat and cat brain regions varied in allylglycine susceptibility, although they were similarly inhibited by additions of NaCl. When the percentage inhibition (of GAD by allylglycine) was plotted against endogenous regional GAD activity, it was found that a significant inverse correlation ($r = -0.687$, $P < 0.005$) existed, although cerebral cortex from any source appeared to be little affected by allylglycine (Fig. 2).

Previously we have shown that the extent of allylglycine inhibition in a partially purified preparation of GAD from guinea pig whole brain is dependent

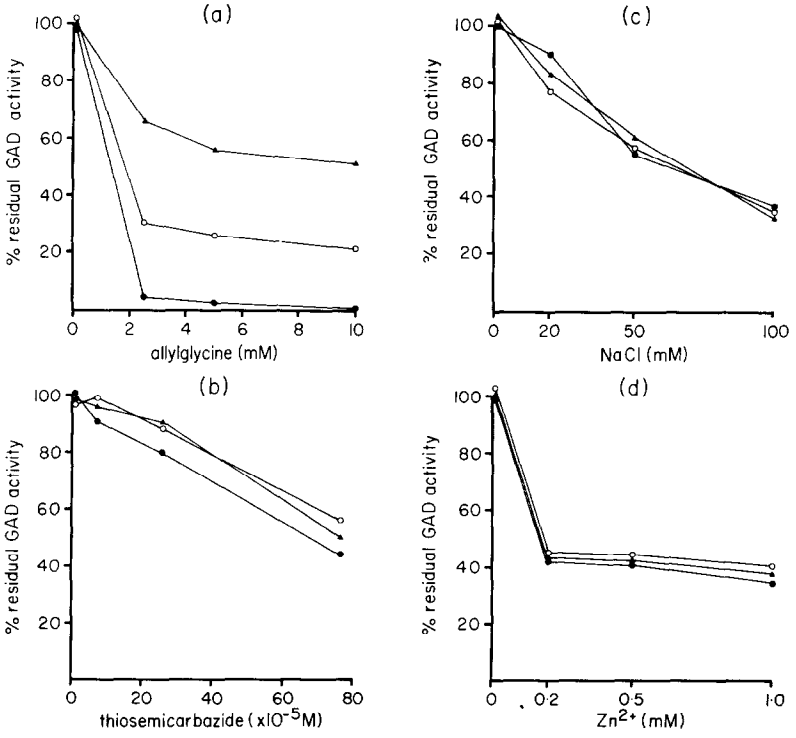

Fig. 1. Inhibition of GAD in crude homogenates from cochlear nucleus (●), inferior colliculus (○) and cerebral cortex (▲) by (a) allylglycine (b) thiosemicarbazide (c) NaCl and (d) Zn^{2+} *in vitro*. Five per cent (w/v) homogenates were prepared in 10 mM sodium phosphate buffer (pH 6.8) and incubated with each inhibitor at the concentration indicated for 1.5 hr (5 mg tissue homogenate/assay). Controls were run for each tissue (water replaced inhibitor) and percentage residual GAD activity calculated at each inhibitor concentration.

Table 2. *In vitro* variation in the allylglycine inhibition of GAD from different brain areas of guinea pig. Five per cent (w/v) homogenates were prepared in 10 mM sodium phosphate buffer (pH 6.8) and GAD activity measured in the presence of 2.5 mM allylglycine. The assay time was 1.5 hr and 5 mg tissue homogenate/assay was used. Results show normal GAD activities and percentage inhibition by allylglycine

Region	GAD activity (μmole g^{-1} hr^{-1})	Percentage inhibition
Cochlear Nucleus	3.89 ± 0.16 (7)	76 ± 5 (6)
Medulla	5.44 ± 0.66 (4)	68 ± 3 (4)
Cerebellum	6.10 ± 0.34 (4)	66 ± 3 (4)
Inferior Colliculus	12.06 ± 0.50 (7)	53 ± 5 (5)
Whole Brain	9.28 ± 0.49 (6)	44 ± 5 (4)
Superior Colliculus	18.44 ± 0.76 (4)	35 ± 1 (4)
Cerebral Cortex	8.55 ± 0.57 (4)	30 ± 2 (6)

on both the duration of assay and amount of enzyme preparation present [9]. It was therefore of interest to investigate these dependencies in the different brain areas in light of the varied susceptibility of the GAD to allylglycine inhibition. Table 3 shows that these dependencies are relevant for GAD obtained from all areas studied.

DISCUSSION

Intraperitoneal administration of allylglycine induced convulsions which were evident only after a latent period of 3 hr, even though the convulsive dose was twice that employed by Alberici *et al.* [4]. This requirement for relatively large doses of allylglycine which result in the onset of convulsions some considerable time later, has been observed by other workers [4] [7]. The latency of action is also seen in *in vitro* studies where inhibition is only evident after some 20 min of incubation [9]. Allylglycine induced convulsions were associated with reductions in both GAD activity and GABA concentration, which suggests that the drug may be similar in its effect to 3-mercaptopropionic acid [13], but different from other convulsants such as glutamyl hydrazine, aminooxyacetic acid and isonicotinic acid hydrazide which inhibit GAD activity *in vivo*, but elevate GABA concentrations [14, 15]. This is presumably due to the fact that these latter drugs effectively inhibit GABA-transaminase (EC 2.6.1.19 4-aminobutyrate: 2-oxoglutarate aminotransferase, GABA-T) as well as GAD, resulting in an increase in total brain GABA. However, both 3-mercaptopropionic acid [16] and allylglycine [4] do not inhibit GABA-T activity and thus a reduction in GAD activity is reflected directly in a decrease in GABA concentration.

The possibility that the reduction in GABA concentration is due to convulsions *per se* is unlikely, since administration of subconvulsive doses of allylglycine (200 mg kg^{-1} body wt) led to a 28 per cent reduction in GABA concentration in the cochlear nucleus 2 hr later. This conclusion is in agreement with the findings of Horton and Meldrum [7] who have shown

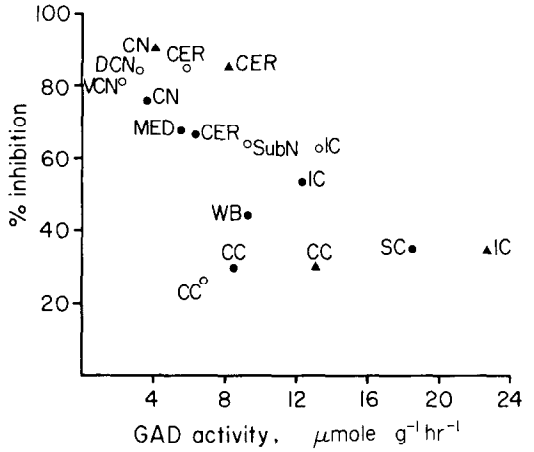

Fig. 2. Graph showing percentage inhibition of GAD by allylglycine under the conditions indicated in the legend to Table 2 in various brain regions from guinea pig (●), rat (▲) and cat (○). CN, cochlear nucleus; MED, medulla; CER, cerebellum; IC, inferior colliculus; WB, whole brain; SC, superior colliculus; SubN, substantia nigra; CC, cerebral cortex. The percentage inhibition was significantly correlated with endogenous GAD activity ($r = 0.687$, $P < 0.005$).

Table 3. Time and tissue concentration dependence of allylglycine inhibition in cochlear nucleus, inferior colliculus and cerebral cortex *in vitro*. Tissues were homogenized in 10 mM sodium phosphate buffer (pH 6.8) and GAD activity determined in the presence of 2.5 mM allylglycine under the conditions indicated. Controls were run on each occasion with allylglycine omitted and results expressed as percentage inhibition

Conditions	Cochlear nucleus	Inferior colliculus	Cerebral cortex
	Percentage inhibition		
(a) 5 mg tissue homogenate; 25 min assay	43	19	3
(b) 5 mg tissue homogenate; 90 min assay	72	51	32
(c) 2 mg tissue homogenate; 90 min assay	71	52	31
(d) 5 mg tissue homogenate; 45 min assay	48	30	12
(e) 1 mg tissue homogenate; 45 min assay	35	9	0

reduced GAD activity in the brains of mice prior to convulsions. These results strongly suggest that the convulsions are primarily the result of a reduction of GABA in the brain, an explanation which is consistent with the findings of Wood and Peesker [12], who showed that aminooxyacetic acid, an inhibitor of GABA-transaminase, protects animals against allylglycine induced convulsions. The decrease in GABA concentration is therefore a more plausible explanation for the convulsant properties of allylglycine rather than its inhibition of the uptake of some amino acids into brain [7] or its blocking of synaptosomal protein synthesis [17].

The present *in vivo* results suggest that GAD in the inferior colliculus and cochlear nucleus is more strongly inhibited by allylglycine than that in the cerebral cortex. Quantitative assessment of such *in vivo* data should however be made with some caution for two main reasons.

(1) For practical convenience, tissues were homogenized in a standard volume of buffer which resulted in the formation of homogenates of different tissue concentration, e.g. cochlear nucleus 1%, inferior colliculus 2%, and cerebral cortex 3%. As the inhibition remains in the most dilute homogenates, where the endogenous level of allylglycine is extremely low, an inactivation mechanism rather than a competitive one is indicated *in vivo*. This is in agreement with the *in vitro* data of Table 3, where an increase in the tissue concentration leads to an increased inhibition. In the former context it is significant that Karlsson *et al.* [13] found that the *in vivo* inhibition of GAD by 3-mercaptopropionic acid, a fully competitive inhibitor of GAD [19], was not detectable under the assay conditions employed in the present study, due to reactivation of the enzyme.

(2) Regional rates of post-mortem GABA increase may be variable, according to the distribution of GAD [20].

As the allylglycine inhibition of GAD is pyridoxal phosphate dependent [9], the question arises as to whether the GAD mediates its own inactivation by converting the allylglycine to an inhibitory product or whether the allylglycine is metabolised by one or more other B_6-dependent enzymes. If GAD itself were solely responsible, then those areas which contain high endogenous GAD activity would be expected to show greatest *in vivo* and *in vitro* inhibition. However, *in vitro* experimental results show that the converse is true (Fig. 2). This finding strongly suggests that an enzyme with a uniform regional distribution, other than GAD, is primarily responsible for the metabolism of allylglycine. Brain regions relatively low in GAD activity would thus tend to show a greater percentage loss of activity than GAD rich areas such as the colliculi. Nevertheless, GAD may still be involved, in part at least, since Wu and Roberts [21] have recently shown that allylglycine is a weak inhibitor of purified mouse brain GAD.

Clearly the mechanism of allylglycine inhibition is most complex and is certaintly not of the simple competitive type as suggested by Rodriguez de Lores Arnaiz *et al.* [5]. Such uncertainty of action calls into question the suitability of use of allylglycine in some recent studies of GABA mediated systems [6–8]. Regional studies of changes in GABA concentration during 4-methoxypyridoxine [22] and 3-mercaptopropionic acid [13] induced convulsions are rightly accompanied by a thorough knowledge of the *in vitro* mode of action of the convulsant, a vital pre-requisite for such an approach. The possibility remains that regional effects of allylglycine in brain do not reflect different degrees of GABA transmitter function, but anomalies in the metabolism of the drug itself.

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