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TRANSMITTER SYNTHESIS AND CONVULSANT DRUGS: EFFECTS OF PYRIDOXAL PHOSPHATE ANTAGONISTS AND ALLYLGLYCINE

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Abstract—Glutamic acid decarboxylase (GAD) activity in mouse brain homogenates was inhibited after intraperitoneal administration of convulsant doses of three pyridoxal phosphate antagonists, methyldithiocarbazinate (MDTC, 45 mg/kg) thiosemicarbazide (TSC, 100 mg/kg) or 4-deoxypyridoxine (4-DP, 250 mg/kg). Dopa-decarboxylase (DCA) activity in the same homogenates was inhibited to a similar or lesser extent than GAD activity. Addition of pyridoxal phosphate to the homogenates relieved or abolished the inhibition of both GAD and DCA. GAD activity in mouse brain homogenates was inhibited (21 per cent) after intraperitoneal administration of a convulsant dose of allylglycine (AG, 200 mg/kg) which is not a pyridoxal phosphate antagonist. Addition of AG to mouse brain homogenates caused an inhibition of GAD activity. The inhibition was increased by preincubation of the homogenate with AG. In contrast, cerebral DCA activity was unchanged after a convulsant dose of AG, or after addition of AG to brain homogenates with or without preincubation. Brain GABA concentration was decreased after 4DP (250 mg/kg) but dopamine and serotonin concentrations were unchanged. Homovanillic acid and 5-hydroxyindoleacetic acid were significantly increased (26 and 96 per cent respectively). Brain GABA concentration was decreased after AG, while brain monoamine and monoamine metabolites were unchanged (except for a 28 per cent decrease in 5HT at 60 min). The experiments demonstrate that inhibition of DCA activity is not the primary or critical mechanism in the convulsant action of hydrazides and allylglycine.

Hydrazides and other pyridoxal phosphate antagonists that induce convulsions inhibit the cerebral enzyme glutamic acid decarboxylase (GAD: L-glutamate 1-carboxy-lyase; EC 4.1.1.15) [1–3]. The relationship between changes in brain GABA content and the occurrence of convulsions after hydrazide administration is not a simple one [4–7]. However, a correlation between the degree of inhibition of cerebral GAD activity and the onset of seizures has been shown for a pyridoxal phosphate antagonist, 4-deoxypyridoxine, and two other compounds not related to hydrazides, 3-mercaptopropionic acid and allylglycine [8]. A recent study of three sulphur-containing hydrazides—methyldithiocarbazinate (MDTC), thiocarbohydrazide, and thiosemicarbazide (TSC)—failed to reveal a correlation between GAD inhibition and seizure onset [9].

Pyridoxal phosphate (PLP) is required as coenzyme by all cerebral decarboxylases and transaminases, including GABA-transaminase (GABA-T; 4-aminobutyrate: 2-oxoglutarate aminotransferase EC 2.6.1.19) the enzyme responsible for the further metabolism of GABA [10]. One enzyme, L-aromatic amino acid decarboxylase (DCA) is apparently responsible for both the conversion of L-DOPA to dopamine (3,4-dihydroxy-L-phenylalanine carboxy-lyase, EC 4.1.1.26) and of L-hydroxytryptophan (5HTP) to serotonin (5HT) (5-hydroxy-L-tryptophan carboxy-lyase, EC 4.1.1.28) [11]. Cerebral transmitter systems utilising dopamine, noradrenaline and serotonin play an important role in regulating the level of sleep and wakefulness and the level of motor activity [12, 13]. Changes in these systems influence seizure thresholds in several animal models of epilepsy [14, 15]. Gey and Georgi [16] have shown in a very diverse group of centrally active drugs that there is a tendency for drugs which lead to a reduction in DCA activity to increase motor activity or induce seizures, whereas anaesthetic or depressant drugs are associated with enhancement of DCA activity. Thus, convulsions induced in rats by thiosemicarbazide (15–25 mg/kg) were associated with 30–35 per cent inhibition of DCA. However, under normal circumstances, the rate limiting step in the synthesis of catecholamines is tyrosine hydroxylase [17]. The rate of 5HT synthesis is controlled by tryptophan hydroxylase activity for which substrate availability (i.e. intraneuronal L-tryptophan concentration) is the limiting factor [18]. Thus, it is not certain that a partial inhibition of DCA will lead to a critical reduction in the synthesis of cerebral monoamines.

We have, therefore, re-examined the action of convulsant doses of MDTC, TSC, 4DP and allylglycine on cerebral GAD activity and compared this with the concurrent inhibition of DCA (assessed by the decarboxylation of L-DOPA). We have then compared the actions of 4-deoxypyridoxine (which shows the most marked inhibition of DCA) and of allylglycine (which produces a marked inhibition of GAD and no inhibition of DCA activity, on cerebral concentrations of GABA, dopamine, noradrenaline and serotonin and of, as potential indicators of dopamine

and serotonin turnover, their acid metabolites, homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5HIAA).

MATERIALS AND METHODS

Adult DBA/2 mice, 20–30 g, were used for the preparation of brain homogenates and for *in vivo* studies (except for the experiment in which cerebral monoamines were measured).

GAD and DCA activity in vitro. Mice were decapitated, the whole brains removed, weighed and a 10 per cent (w/v) homogenate prepared in ice-cold 50 mM phosphate buffer, pH 6.0, containing 0.2% (v/v) Triton X-100 and 2.5 mM AET (2-amino ethyl-isothiouronium bromide hydrobromide). The homogenate was allowed to stand for 10 min in ice and was centrifuged at 3,000 *g* at 4° for 15 min. The pellet was discarded and the supernatant used as the source of enzymes. GAD activity was estimated by the method of Roberts and Simonsen [19] as modified by Tapia and Awapara [20]. Final concentrations in the reaction mixture (1.0 ml) were: L-glutamate (5.6 μmoles) L-[I-^{14}C]glutamate (0.05 μCi) adjusted to pH 6.3 with KOH, phosphate buffer, pH 6.3 (50 μmoles) AET (2.5 μmoles) and brain homogenate (equivalent to 20 mg wet wt. tissue). Pyridoxal 5'-phosphate (PLP, 0.05 μmole) and drug were added where indicated. Samples were incubated at 37° for the stated times, together with appropriate blanks. DCA activity was estimated by the method of Lamprecht and Coyle [21]. Final concentrations in the reaction mixture (1.0 ml) were: L-3,4-dihydroxyphenylalanine (0.34 μmole) dissolved in water containing 1 mg/ml. ascorbic acid), L-3,4-dihydroxyphenyl [1-^{14}C]alanine (0.1 μCi), phosphate buffer pH 6.8 (75 μmoles), EDTA (0.2 μmole) AET (2.5 μmoles) and brain homogenate (equivalent to 20 mg wet wt tissue). PLP and drug were added where indicated. Samples were incubated for the stated times at 37°, together with appropriate tissue blanks. GAD and DCA activities were estimated using substrate concentrations which were twice the K_m under these assay conditions. In some experiments, enzyme and drug were pre-incubated at 37° in the assay medium (without substrate) for 30 min (GAD) or 20 min (DCA).

GAD and DCA activity (in vivo). Mice were injected with DL-allylglycine (AG, 200 mg/kg) 4-deoxypyridoxine hydrochloride (4DP, 250 mg/kg), methyldithiocarbazinate (MDTC, $H_2NNHC{:}\ SSCH_3$, 45 mg/kg) thiosemicarbazide (TSC, 100 mg/kg) or saline and killed at the stated times by decapitation. Brain homogenates were prepared and assayed as above. Enzyme activity in control homogenates or from saline injected animals was assayed concurrently with homogenates to which drugs had been added or from drug injected animals. Body temperature was recorded by a rectal probe and controlled by external heating.

Estimation of GABA and cerebral monamines. Mice (Swiss S) were injected with AG(200 mg/kg, i.p.) 4DP (250 mg/kg) or saline, and were observed for the onset of seizure activity. Animals were killed at the stated times by decapitation, the brains removed and divided midsagitally through the superior longitudinal fissure and frozen in liquid nitrogen (within 1 min of killing) and kept at −76° until homogenised. One half of the brain was used to estimate cerebral monoamines, dopamine (DA), noradrenaline (NA) serotonin (5HT) and 5-hydroxyindoleacetic acid (5HIAA) and the other half to estimate GABA. Homovanillic acid (HVA) was estimated on whole brain. Cerebral monoamine, monoamine metabolites and GABA were estimated as previously described [22]. Differences between control and drug-treated enzyme activities and cerebral metabolites were compared by Student's *t*-test.

Radioactivity counting. Samples were prepared and counted as previously described [9].

Materials. Isotopes were purchased from the Radiochemical Centre, Amersham. Drugs, coenzymes, enzymes and other chemicals were purchased from Sigma Chemical Co. Methyldithiocarbazinate was a gift from Phillips Duphar Laboratories.

RESULTS

In vitro *experiments.* The addition of allylglycine (AG) to mouse brain homogenates caused a significant inhibition of GAD activity (Table 1). Control activity decreased with increasing time of incubation; the percentage inhibition produced by AG increased as the duration of the assay was extended. Inhibition was not markedly changed by increasing the concentration of AG (within the range used). Addition of PLP did not change the percentage inhibition of GAD by AG.

Preincubation of brain homogenate with AG (50 mM) increased the GAD inhibition markedly (Table 2) compared to no preincubation (cf. Tables 1 and 2). Unlike the experiments without preincubation (Table 1), addition of PLP (either at the start of the preincubation or at the start of the assay) reduced the inhibitory effect of AG on GAD.

Control DCA decreased with duration of incubation but, in contrast to GAD DCA activity was not consistently modified by AG (50 or 100 mM) (Table 1). The one out of 20 results showing a significant change ($P < 0.05$) is probably the result of random factors as there is no evidence for a trend with time. Preincubation of mouse brain homogenates with AG (50 mM) in the presence or absence of PLP did not significantly alter DCA activity (Table 2).

In vivo *experiments.* AG (200 mg/kg, i.p.) induced seizures after a mean latency of 95 min. A significant inhibition of GAD activity was seen in brain homogenates subsequent to this (Table 3). Addition of PLP to the homogenates did not alter this inhibition. DCA activity in the same homogenates was not decreased compared to control (Table 3).

GAD activity was inhibited in brain homogenates from mice killed 30 min after MDTC (45 mg/kg i.p.; mean time to seizure onset 15 min), 30 min after TSC (100 mg/kg i.p.; no seizures observed up to 30 min) and 60 min after 4DP (250 mg/kg i.p.; mean time to seizure 45 min) (Table 4). Addition of PLP to the homogenates greatly reduced or abolished the GAD inhibition following i.p. administration of MDTC, TSC or 4DP. DCA activity was inhibited in the same brain extracts to a similar or a lesser extent than GAD activity (Table 4). As with GAD activity, addi-

Table 1. Effect of AG on GAD and DCA activity in mouse brain homogenates *in vitro*

Duration of incubation (min)	GAD Activity								DCA Activity					
	Control		AG Concentration 50 mM		70 mM		100 mM		Control		AG Concentration 50 mM		100 mM	
	−PLP	+PLP	−PLP	+PLP	−PLP	+PLP	−PLP	+PLP	−PLP	+PLP	−PLP	+PLP	−PLP	+PLP
10	13.82 ±0.25	22.52 ±0.36	90 ± 1†	92 ± 1†	90 ± 5	85 ± 2†	84 ± 1†	80 ± 6†	3.31 ±0.10	4.39 ±0.26	96 ± 2	97 ± 2	98 ± 6	102 ± 4
20									2.90 ±0.09	4.48 ±0.08	98 ± 2	98 ± 1	90 ± 5	97 ± 1
30	12.53 ±0.16	20.02 ±0.09	83 ± 1†	81 ± 2†	84 ± 1†	77 ± 3†	78 ± 3†	75 ± 1†	2.35 ±0.05	4.05 ±0.09	101 ± 2	99 ± 1	100 ± 2	101 ± 2
40									1.94	3.89	103 ± 11	108 ± 8	111 ± 2	114 ± 1
60	10.17 ±0.13	17.69 ±0.25	76 ± 1†	74 ± 2†	84 ± 2†	71 ± 1†	79 ± 1†	70 ± 1†	±0.25 1.81	±0.34 3.45	94 ± 1	101 ± 4	87 ± 1*	101 ± 2
90	9.77 ±0.11	15.94 ±0.09	73 ± 1†	63 ± 1†	78 ± 1†	56 ± 2†	74 ± 2†	53 ± 2†	±0.09	±0.08				

Brain homogenates were prepared and assayed for GAD and DCA activity with and without the stated concentrations of AG, in the absence (−PLP) or presence (+PLP) of exogenous PLP, as described in Methods. The reaction was terminated at the times stated. Results are presented as mean control activity (μmoles/g/hr) ±S.E.M. together with the mean per cent of control activity (±S.E.M.) for drug treated homogenates (n = 4–6). Differences between activity in control and drug-treated homogenates were compared by Student's t-test and are denoted by *$P < 0.05$, †$P < 0.01$.

Table 2. Effect of preincubation with AG on GAD and DCA activities in mouse brain homogenates

Duration of preincubation	Duration of assay	GAD Activity Control	GAD Activity 50 mM AG	GAD % Cont.	DCA Activity Control	DCA Activity 50 mM AG	DCA % Cont.
20 min − PLP	20 min − PLP				2.92 ± 0.45	2.51 ± 0.37	86%
20 min + PLP	20 min − PLP				4.31 ± 0.70	4.39 ± 0.65	102%
20 min − PLP	20 min − PLP				3.19 ± 0.41	2.72 ± 0.39	85%
20 min − PLP	20 min + PLP				4.80 ± 0.80	4.02 ± 0.83	84%
30 min − PLP	30 min − PLP	8.47 ± 0.55	3.59 ± 0.27	42%†			
30 min + PLP	30 min − PLP	19.05 ± 0.54	10.00 ± 0.45	52%†			
30 min − PLP	30 min − PLP	9.75 ± 1.08	2.49 ± 0.28	26%†			
30 min − PLP	30 min + PLP	17.71 ± 0.62	7.47 ± 0.67	42%†			
60 min − PLP	30 min − PLP	10.72 ± 2.45	3.31 ± 0.63	31%†			
60 min + PLP	30 min − PLP	27.75 ± 4.95	13.59 ± 2.67	49%†			
60 min − PLP	30 min − PLP	9.59 ± 2.16	2.03 ± 0.47	21%†			
60 min − PLP	30 min + PLP	25.14 ± 4.78	9.68 ± 1.72	39%†			

Mouse brain homogenates were preincubated with the assay mixture (less substrate) in the presence and absence of AG and PLP for the stated times. The assays were initiated by the addition of substrate without (− PLP) or with (+ PLP) added PLP, and were terminated at the stated times. Enzyme activity (μmoles/g/hr) is expressed as mean ± S.E.M., together with the per cent of control activity for 4 estimations. Differences between control and drug treated homogenates were compared by Student's *t*-test and denoted by *P < 0.05, †P < 0.01.

Table 3. Effect of AG (200 mg/kg) on mouse brain GAD and DCA activities *in vivo*

Treatment and duration	GAD − PLP	GAD + PLP	DCA − PLP	DCA + PLP
Saline	10.96 ± 0.57	23.08 ± 0.67	2.84 ± 0.06	4.19 ± 0.15
AG 30 min	10.87 ± 0.43	22.51 ± 0.62	3.18 ± 0.16*	4.36 ± 0.20
	99%	98%	112%	104%
AG 60 min	9.86 ± 0.38	20.69 ± 0.25	2.92 ± 0.03	4.36 ± 0.14
	90%	90%	103%	104%
AG 120 min	8.66 ± 0.38†	18.72 ± 0.50	2.80 ± 0.08	4.02 ± 0.20
	79%	81%	99%	96%

Mice were injected i.p. with 200 mg/kg AG or saline and killed at the stated times. GAD and DCA activities were measured as described in Methods, without (− PLP) or with the addition (+ PLP) of exogenous PLP.

Incubation times: GAD 60 min, DCA 20 min.

Activities (μmoles/g/hr) are expressed as the mean ± S.E.M., together with per cent activity compared to controls for 8 determinations. Differences between control and drug treated animals were compared by Student's *t*-test, and denoted by *P < 0.05, †P < 0.01.

Table 4. The effect of MDTC, TSC and 4DP on mouse brain GAD and DCA activities *in vivo*

Treatment	GAD − PLP	GAD + PLP	DCA − PLP	DCA + PLP
Saline	14.05 ± 0.93	29.17 ± 0.98	3.43 ± 0.13	5.27 ± 0.20
MDTC 45 mg/kg	8.44 ± 0.79†	25.46 ± 1.57*	2.87 ± 0.13*	4.71 ± 0.14
30 min	60%	87%	84%	89%
TSC 100 mg/kg	10.50 ± 0.96*	26.50 ± 1.46	2.39 ± 0.11†	4.77 ± 0.08*
30 min	75%	91%	70%	91%
4DP 250 mg/kg	4.64 ± 0.48†	25.57 ± 1.35*	1.71 ± 0.06	5.09 ± 0.11
60 min	33%	88%	50%	97%

Mice were injected i.p. with the stated dose of the drug or saline and killed at the stated times. GAD and DCA activities were measured in brain homogenates as described in Methods, with (− PLP) or with (+ PLP) the addition of exogenous PLP. Incubation times: GAD 60 min, DCA 20 min. Activities (μmoles/g/hr) are expressed as the mean ± S.E.M., together with the per cent activity compared to controls for 6–8 determinations. Differences between control and drug treated animals were compared by Student's *t*-test, and are denoted by *P < 0.05, †P < 0.01.

Table 5. The effect of 4DP and AG on cerebral GABA, monoamine and monoamine metabolites in mouse brain *in vivo*

Treatment dose and time	No. of animals	GABA	DA	HVA	5HT	5HIAA	NA
Control	6	1.28 ± 0.03	525.7 ± 29.3	221.7 ± 16.1	579.5 ± 60.9	265.8 ± 25.9	553.3 ± 43.0
4DP (250 mg/kg)							
20 min	6	92 ± 6	102 ± 8	102 ± 7	97 ± 4	134 ± 29	110 ± 6
60 min	4	51 ± 2†	115 ± 10	126 ± 9*	79 ± 4	196 ± 23†	76 ± 7*
AG (200 mg/kg)							
60 min	6	86 ± 4†	107 ± 13	101 ± 9	72 ± 3*	102 ± 13	117 ± 9
120 min	6	72 ± 3†	100 ± 10	105 ± 3	98 ± 9	89 ± 11	92 ± 7

Mice were injected i.p. with the stated doses of the drugs or saline and killed at the stated times. GABA (μmoles/g) and monoamine and monoamine metabolites (ng/g) were estimated as described in Methods. Results are expressed as means ±S.E.M. or per cent of control estimation (±S.E.M.) for the number of determinations shown. Differences between control and drug treated were compared by Student's *t*-test, and are denoted by *$P < 0.01$, †$P < 0.05$.

tion of PLP to the homogenates reduced or abolished this inhibition.

Since 4DP was the most effective of the four drugs at inhibiting GAD and DCA activities, the concentrations of cerebral monoamines, monoamine metabolites and GABA were determined in mouse brain after treatment with 4DP, and compared to treatment with AG. Animals were sacrificed before (20 min 4DP; 60 min AG) or after (60 min 4DP; 120 min AG) the onset of convulsions. GABA concentration was significantly reduced at 60 min and 120 min after AG (Table 5). The concentrations of DA and its principal metabolite HVA were unchanged at both times. A reduction in 5HT concentration (28 per cent) was seen at 60 min after AG but not at 120 min. 5HIAA and NA concentrations were unchanged after AG. The concentration of GABA was reduced 60 min after 4DP (Table 5). DA and HVA concentrations were unchanged at 20 min after 4DP but HVA concentration was significantly elevated (26 per cent) 60 min after 4DP. 5HT and 5HIAA concentrations were unchanged 20 min after 4DP, but 5HIAA was significantly elevated (96 per cent) 60 min after 4DP. NA concentration was unchanged 20 min after 4DP but was significantly reduced (24 per cent) 60 min after 4DP (Table 5).

DISCUSSION

Gey and Georgi [16] concluded, from their study of thiosemicarbazide, aminooxyacetate, pentylenetetrazol, and amphetamine, that central excitant actions of these compounds were related to a reduction in DCA activity and not to observed increases or decreases in GAD activity. The group of drugs that they studied is heterogenous, with varied biochemical, pharmacological and behavioural effects. We have compared thiosemicarbazide with other pyridoxal phosphate antagonists (MDTC, 4DP) and the convulsant allylglycine. In confirmation of their observations, systemically administered TSC inhibits cerebral DCA activity and this inhibition can precede seizure onset. MDTC and 4DP in convulsant dosage also inhibit DCA activity; this is associated with a marked inhibition of GAD activity. Allylglycine, which is not a pyridoxal phosphate antagonist, does not inhibit DCA activity, either *in vitro* or following its systemic administration in convulsant doses.

Drug induced decreases in the cerebral content of monoamines are associated with a decreased threshold for seizure induction in several rodent test systems [14, 23]. However, our measurements of cerebral monoamines and their metabolites indicate that the reduction in cerebral DCA activity is probably not inducing seizures in this way. A 50 per cent inhibition of DCA activity after 4DP was accompanied by no changes in brain DA content and an increase in HVA content suggesting that the turnover of DA was increased [24]. The marked increase in 5HIAA is probably also indicative of an increased release of 5HT, although an effect of 4DP on reuptake or storage of 5HT is also possible. Impairment of transport of acidic metabolites may also contribute to the observed increases in HVA and in 5HIAA. Thus an impairment of 5HT synthesis after 4DP is not excluded by our data. However, larger decreases in 5HT concentration (e.g. 60 per cent after reserpine [25] or 61 per cent after *p*-chlorophenyl-alanine [22], do not lead to 'spontaneous' seizures, although they may be associated with a decrease in seizure threshold.

Allylglycine in a convulsant dose did not modify cerebral monoamine content, except for a decrease in 5HT content at 60 min. This could indicate a change in synthesis or storage of 5HT. The absence of any change in cerebral DA content, or in DCA activity, suggests that a change in decarboxylation rate is unlikely to be involved.

It is possible that transport of L-tryptophan into the brain could be reduced after 4DP or AG.

The 67 per cent inhibition of GAD activity following 4DP, 250 mg/kg, is in accordance with our previous observations of 58 per cent inhibition after 4DP, 225 mg/kg [8]. The 49 per cent reduction in brain GABA concentration is consistent with the rate limiting role of GAD in GABA synthesis.

There is massive evidence that GABA functions as an inhibitory transmitter in many sites in the CNS [26]. Drugs known to block its post-synaptic action (such as picrotoxin, bicuculine and tetramethylenedisulphotetramine) are powerful convulsant agents [3, 27]. In contrast, drugs blocking the postsynaptic actions of DA (e.g. haloperidol and pimozide) or of serotonin (e.g. methysergide) do not normally provoke seizures [28, 29]. There thus appears to be no reason to alter our previous conclusion that seizures after 4DP are most probably due to impaired synthesis of GABA [8].

Our earlier description [8] of the contrast between the weak inhibition of GAD when AG is added to brain homogenates and more marked inhibition observed when the drug is administered systemically, has been confirmed [30, 31]. Our suggestion that the GAD inhibition is due to formation of a metabolite of allylglycine has been supported by Orlowski *et al.* [31], who have produced evidence that transamination or oxidative deamination may be involved. It is clear from the *in vitro* (Tables 1 and 2) and *in vivo* (Table 3) experiments reported here that neither AG, nor an active metabolite responsible for GAD inhibition, inhibit DCA activity.

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