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THE EFFECTS OF ALLYLGLYCINE ON GABA SYNTHESIS *IN VIVO*

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Abstract—The relative incorporation of ^{14}C into GABA and glutamate following the intracerebroventricular injection of D-[U-^{14}C]glucose has been determined in mice pretreated with either 2 m-mole/kg allylglycine interperitoneally (i.p.) or saline as control. The proportion of total counts appearing in glutamate declined slightly between 1 hr and 3 hr post-injection, but the proportion of total counts in GABA decreased significantly ($P < 0.001$) from control over the same period. There was a strong positive correlation between the ratio of counts in GABA to glutamate and the time after injection of allylglycine. A significant fall ($P < 0.05$) in the specific activity of GABA was observed from 1 hr post-injection of allylglycine onwards. These effects preceded the convulsions induced by allylglycine by at least 1–1.5 hr, but the maximal effect was observed at the onset of convulsions at 2.5–3 hr. The results support the view that the convulsant actions of allylglycine or its deaminated metabolite are due to the inhibition of glutamate decarboxylase and consequent reduction in GABA synthesis.

The convulsant properties of DL-C-allylglycine (2-amino-4-aminopentenoic acid) were first reported by Schneider, Cassir and Chordikian [1], although a mechanism of action was not proposed until Alberici De Canal, Rodriguez De Lores Arnaiz and De Robertis [2] found that the brain GABA level was reduced by 40 per cent during the convulsions. Later workers [3–6] have found that although allylglycine inhibits glutamate decarboxylase (L-glutamate 1-carboxylase, EC 4.1.1.15, GAD) both *in vivo* and *in vitro*, the inhibition is of a complex mixed type, and may, in fact, be due to a metabolite of allylglycine [7]. This view is supported by the evidence that the maximum brain and blood levels of allylglycine do not correspond with the onset of the convulsions [6], and that there is a latency of 90 min following the i.p. injection of allylglycine before any inhibition of GAD can be detected [3, 6].

The type of convulsions induced by allylglycine more closely resembles those produced by pentylenetetrazol (i.e. intermittent episodes of clonic fore and hind limb movements [8]) rather than the characteristic running fits and full tonic extensions observed with the specific GAD inhibitors mercaptopropionic acid and thiomalic acid [9, 10].

It has been shown that mercaptopropionate, which produces convulsions within 5 min of an i.p. injection, causes a measurable inhibition of GAD within that time and a significant fall in the rate of GABA synthesis [3, 11, 12]. The present work therefore examines the rate of GABA synthesis in vivo following the administration of allylglycine in order to determine whether or not a reduced rate of synthesis can be correlated with the onset of convulsions. The method employed consisted of injecting mice intracerebroventricularly (i.c.v.) with D-[U-^{14}C]glucose and determining the relative incorporation of ^{14}C into glutamate and GABA [12].

MATERIALS AND METHODS

Animals. Adult female LACG mice weighing between 28–32 g were used in all experiments.

Sources of chemicals. All reagents were of analytical (A.R.) grade whenever possible and were obtained from the Sigma Chemical Co., Kingston-upon-Thames, U.K. Allylglycine was checked for purity by two-dimensional t.l.c. of its dansyl derivatives [6]. Radiochemicals were obtained from the Radiochemical Centre, Amersham, U.K.

Administration of drugs and metabolites. Allylglycine was made up in 0.9% (w/v) saline, buffered to pH 7.4 with 20 mM sodium phosphate buffer, and injected i.p. D-[U-^{14}C]glucose (0.5 mCi/m-mole) was injected i.c.v. in 5 μl of 0.9% saline by the method of Brittain and Handley [13] using a 3 mm 27 gauge needle and a Hamilton syringe.

Measurement of incorporation of ^{14}C into brain amino acids. Mice were i.p. injected with allylglycine or saline as control between 60–180 min prior to the i.c.v. injection of D-[U-^{14}C]glucose. The mice were sacrificed at 2, 5 or 8 min later by total immersion in liquid nitrogen. The cortex was exposed and 15–25 mg of grey matter removed, refrozen, weighed, then homogenized in 0.12 ml of ice-cold 80% (v/v) ethanol using a hand-operated micro-homogenizer. The homogenate was left to stand for 20 min at 0° then centrifuged at 900 *g* for 5 min. Aliquots of the supernatant were then subjected to high voltage paper electrophoresis as described previously to separate GABA and glutamate for subsequent scintillation counting [12]. The overall recoveries of the amino acids were calculated by use of standard samples of [U-^{14}C]GABA and L-[U-^{14}C]glutamate. Some samples were subjected to the dansylation procedure of Roberts and Keen [14] so that the absolute concentrations of endogenous glutamate and GABA could be calculated.

Table 1. Incorporation of ^{14}C into GABA and glutamate

Time after injection of D-(U-^{14}C) glucose, min	No. of observations	Tissue	Total counts d.p.m./mg wet wt	% Total counts in GABA	% Total counts in glutamate
2	5	Whole brain	2673 ± 214	1.96 ± 0.68	23.8 ± 0.7
5	4	Whole brain	1629 ± 247	4.72 ± 0.40	30.1 ± 2.8
2	6	Cerebral Cortical grey matter	*817.6 ± 231.5	3.97 ± 1.44	28.2 ± 3.4
5	6	Cerebral Cortical grey matter	878.5 ± 101.8	8.38 ± 0.56	49.7 ± 3.6
8	6	Cerebral Cortical grey matter	*381.7 ± 114.8	4.75 ± 0.84	57.3 ± 2.9

Mice were i.c.v. injected with 2.5 μCi of D-(U-^{14}C)glucose at zero time and killed at the time intervals shown. Each value is the mean ± S. E. M.

* Mean < mean at 5 min, $P < 0.02$.

The results have been expressed as the means ± S.E.M. of at least five observations. Statistical analysis of the data was performed using the Student's "*t*"-test for measuring the significance of the difference between the means of independent groups.

RESULTS

The incorporation of ^{14}C into glutamate and GABA was determined at intervals of 2, 5 and 8 min in otherwise untreated mice (Table 1). The overall recovery of radioactivity in the ethanol supernatant fraction was about three times higher in a whole brain extract at 2 min than in the cerebral cortical grey matter. This was undoubtedly due to the gradual penetration of the glucose from the site of injection (into the third and fourth ventricles) to the cerebral cortex. A certain amount of glucose is probably lost by leakage from the site of injection into the sagittal sinus since the total recovery of activity in the brain was never greater than 78 per cent of the total injected. Over longer periods there will be gradual loss of activity due to the complete metabolism of glucose to CO_2. This loss of activity was apparent at the 8 min time interval (Table 1). For these reasons the time interval of 5 min between the injection of the glucose and the sacrifice of the mouse was chosen for the subsequent experiments. Also, the percentage incorporation into GABA of ^{14}C was at its largest and most consistent value at this time.

The effect of allylglycine on the incorporation of label into GABA and glutamate is shown in Table 2. There was some variation in the total counts recovered in the ethanol supernatant fraction (657–1000 d.p.m./mg wet wt) although none of the mean values of any group was significantly different ($P > 0.05$) from any of the other group means. The percentage counts in glutamate tended to decline from the control value at zero time although only at 60 and 180 min post-injection was the fall in activity statistically significant ($P < 0.05$). In contrast, the activity in GABA showed a highly significant fall ($P < 0.001$) of 50 per cent from control at 60 min post-injection. The counts in GABA continued to decline until, at 180 min post-injection, they were only 7.7 per cent of control. These values therefore indicate a very marked reduction in the rate of GABA synthesis.

The correlation of this reduction in GABA synthesis with time was determined by plotting the log of the ratio of the percent counts in GABA to the percent counts in glutamate against time. The resultant straight line (Fig. 1) had a highly significant

Table 2. Effects of allylglycine on the incorporation of ^{14}C into GABA and glutamate

Time after injection of allylglycine, min	No. of observations	Total counts in brain tissue (d.p.m./mg wet wt)	% Total counts in glutamate	% Total counts in GABA
Control (T = 0)	6	878.5 ± 101.8	49.7 ± 3.6	8.38 ± 0.56
60	6	656.7 ± 132.2	*36.2 ± 2.8	†4.13 ± 0.65
90	7	1000 ± 183.2	36.8 ± 4.6	†1.96 ± 0.23
120	6	702.4 ± 209.7	40.4 ± 6.9	†1.94 ± 0.40
180	5	707.9 ± 178.8	*31.4 ± 3.9	†0.649 ± 0.161

Mice were i.p. injected with 2 m-mole/kg of allylglycine at zero time and then injected i.c.v. with D-[U-^{14}C]glucose (2.5 μCi) at the time intervals shown and killed 5 min later. Each value is the mean ± S. E. M.

* Mean < control mean, $P < 0.05$.

† Mean < control mean, $P < 0.001$.

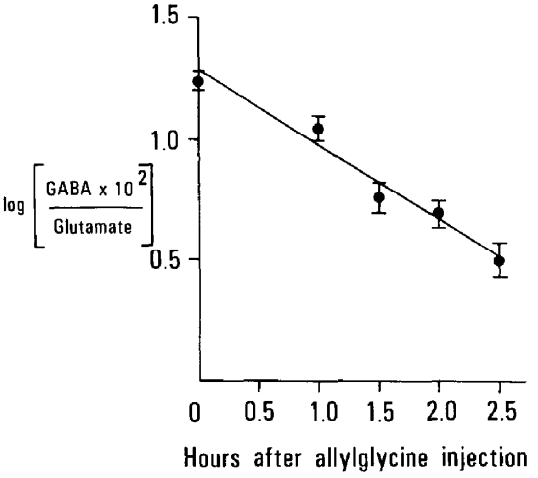

Fig. 1. The relative incorporation of ^{14}C into GABA following allylglycine. Mice were injected with 2 m-mole/kg allylglycine i.p. then i.c.v. injected with 2.5 μCi of D-[U-^{14}C]glucose at the time intervals shown. After a further 5 min the relative incorporation of ^{14}C into GABA and glutamate was determined. The graph expresses the regression of

$$\text{Log}\frac{\%\text{ total counts in GABA} \times 10^2}{\%\text{ total counts in glutamate}}$$

on time. Each point represents the mean ± S.E.M. of at least five determinations. The correlation coefficient was calculated by the method of least squares and found to be − 0.986; the regression coefficient of x on y was highly significant, $P < 0.001$.

($P < 0.001$) regression coefficient, indicating a close correlation.

By direct assay of the amino acid levels in the cerebral cortical extracts it was possible to calculate the specific activities of GABA and glutamate (Table 3). A significant ($P < 0.05$) and increasing fall in the specific gravity of GABA was detectable from 60 min post-injection. A slight, but not significant ($P > 0.05$), fall was observed in the specific activity of glutamate (Table 3). The product of the total counts in GABA and the specific activity of glutamate was calculated for each time interval (Table 3) in order to provide a relative estimate of the GABA synthesis. By correcting for any change in the specific activity of the glutamate in this way, a highly significant ($P < 0.01$) reduction in GABA synthesis became apparent from 1 hr post-injection.

DISCUSSION

Various methods have been used to estimate the turnover of GABA in the brain, and it has been recognized that GABA, or at least the rate of GABA synthesis, is more critical for the maintenance of the inhibitory processes in the brain than is the overall GABA concentration [11, 12, 18, 19]. Estimations of the rate of GABA synthesis are therefore essential in studies where the biochemical effects of a drug interfering with the GABA system are being correlated with the behavioural sequelae of that drug.

Previous studies have shown that, following allylglycine, time dependent decreases in brain GABA level and GAD activity can be observed prior to the onset of convulsions [3, 6]. The fall in GABA level, however, only became apparent after 2 hr post-injection, whereas, in the present work, a significant decrease in the specific activity of GABA was observed from 1 hr post-injection [6]. Thus, there is as might be expected, a fall in GABA synthesis occurring well before any reduction in overall GABA level can be detected. This reduction in GABA synthesis should correlate with the inhibition of GAD; however, any estimate of GAD activity, unless measured under suboptimal conditions, invariably underestimates the actual inhibition produced by a reversible inhibitor due to the dilution of the inhibitor concentration during the extraction and assay of the enzyme activity [12]. This problem has been overcome in studies of the reversible GAD inhibitor 3-mercaptopropionic acid by using either high concentrations of tissue extract or low concentrations of glutamate as substrate [12, 15]. The latter technique has been used previously to demonstrate that allylglycine, at the same dose level used in the present work, causes a detectable, though insignificant, fall in GAD activity from 90 min post-injection [6]. The delay in observing a fall in brain GABA level and GAD activity beyond the time

Table 3. Time course of the effect of allylglycine on the specific activities of GABA and glutamate

Time after injection (min)	Cerebral cortical GABA concentration (μmoles/g wet wt)	Specific activity (μCi/mmol) GABA	Glutamate	Relative rate of GABA synthesis, calculated as the product of total counts in GABA (nCi/g wet wt) and the specific activity of glutamate
0	1.32 ± 0.10 (6)	27.9 ± 5.10 (6)	24.0 ± 4.26 (6)	802.9 ± 172.8 (6)
60	1.21 ± 0.09 (6)	*10.8 ± 3.09 (6)	11.8 ± 4.07 (6)	†145.4 ± 43.4 (6)
90	1.30 ± 0.11 (6)	†8.57 ± 1.90 (7)	23.0 ± 5.39 (7)	†204.9 ± 32.4 (7)
120	1.00 ± 0.09 (6)	†7.29 ± 2.69 (6)	15.2 ± 8.13 (6)	†93.9 ± 23.2 (6)
180	†0.71 ± 0.07 (6)	†2.75 ± 0.946 (5)	12.8 ± 4.80 (5)	†26.7 ± 8.0 (5)

Mice were i.p. injected with 2 m-mole/kg of allylglycine at zero time and then i.c.v. injected with D-[U-^{14}C]glucose (2.5 μCi) at the time intervals shown and killed 5 min later. Each value is the mean ± S.E.M. of the number of observations indicated in parenthesis.

* Mean different from mean at zero time, $P < 0.05$.

† Mean different from mean at zero time, $P < 0.01$

when GABA synthesis is significantly reduced may not merely be due to the limitations of the experimental techniques used, but may reflect a selective inhibition of a small rapidly turned over transmitter pool of GABA in the nerve terminals; the larger metabolic pool of GABA with its slower turnover, in effect, masking the change in effective GABA level.

If, as has been suggested, a metabolite of allylglycine, possibly 2-keto-4-pentenoic acid is responsible for the inhibition of GAD, then the gradually increasing effects observed here on the specific activity of GABA may be due to gradually increasing concentration of this active metabolite [7, 20]. The peak brain concentration of allylglycine occurs within 30 min of an i.p. injection and does not correlate with the maximal reduction in specific activity of GABA observed at 3 hr post-injection [6].

The present results therefore provide further support for the view that a metabolite of allylglycine, possibly 2-keto-4-pentenoic acid, is responsible for the inhibition of GAD and the resultant reduction in GABA synthesis, and suggests that this metabolite may well be quite rapidly acting [6, 7].

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