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GLUTAMIC ACID DECARBOXYLASE INHIBITION AND ULTRASTRUCTURAL CHANGES BY THE CONVULSANT DRUG ALLYLGLYCINE

MARTHA ALBERICI, GEORGINA RODRIGUEZ DE LORES ARNAIZ and EDUARDO DE ROBERTIS

Instituto de Anatomía General y Embriología, Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina

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Abstract—The effects *in vivo* and *in vitro* of the convulsant drug allylglycine on the activity of glutamic acid decarboxylase (GAD), aminobutyrate aminotransferase, glutamine synthetase and aspartate- and alanine-aminotransferases of the rat cerebral cortex were studied. The most significant result was the finding of an inhibition of GAD, during the period of convulsion, which was even greater by addition *in vitro* of the drug. This inhibition is not by way of the cofactor, pyridoxal phosphate. Preincubation of the homogenate with allylglycine enhanced the inhibition, while preincubation in buffer–substrate produced a protective effect.

The inhibition of GAD was correlated with a decrease of 40 per cent in the concentration of γ-aminobutyric acid in the cerebral cortex. In the convulsant rat, ultrastructural alterations of some nerve endings of the cerebral cortex were observed. After cell fractionation, such altered nerve endings were preferentially found in the GAD-rich (nonaminergic) fraction of isolated nerve endings. The possible mechanism of the convulsions induced by allylglycine is discussed and a specific effect on GAD-rich inhibitory nerve endings is postulated.

THE STUDY of the mechanism of action of convulsant drugs is of considerable interest in explaining certain pathological or therapeutically induced states in the human. In a previous paper from this laboratory, the effect of the convulsant, methionine sulfoximine (MSO)* was analyzed with neurochemical methods and electron microscopy. Although practically no changes were observed in the content of biogenic amines and related enzymes, the ultrastructural changes found were mainly related to the nonaminergic population of nerve endings and to some enzymatic systems involved in the metabolism of glutamate, glutamine and GABA. In addition to the known inhibition of glutamine synthetase,[1–3], a strong inhibition of alanine-aminotransferase and a lesser inhibition of glutamic acid decarboxylase (GAD) were found in the MSO-convulsant rats.[4]

In 1960, Schneider *et al.*[5] described the convulsant properties of the 2-amine-4-pentenoic acid [CH_2-$CHCH_2CH(NH_2)COOH$], allylglycine. More recently, McFarland and Wainer[6] studied in mice the dose–response relationship of this drug and

* Abbreviations used in this work are: MSO, methionine sulfoximine; GABA, γ-aminobutyric acid: GAD, L-glutamate 1-carboxyylase (EC 4.1.1.15); GABA-AT, aminobutyrate aminotransaminase (EC 2.6.1.19); GS, L-glutamate:ammonia ligase (ADP) (EC 6.3.1.2); Asp-AT, L-aspartate: 2-oxoglutarate aminotransferase (EC 2.6.1.1); Alan-AT, L-alanine: 2-oxoglutarate aminotransferase (EC 2.6.1.2).

the protection by barbiturates. So far, nothing has been reported about the possible mechanism of action of this convulsant drug. To attempt a study of this mechanism, the cerebral cortex of rats convulsing after administration of allylglycine was studied for the activity of the principal enzymes associated with the metabolism of glutamine, glutamate and GABA, whose subcellular localization in brain was previously studied in this laboratory.[7] In addition, the level of free amino acids in convulsant rats was determined and the effect *in vitro* of the drug on the enzymes was studied.

The main findings obtained consisted of an inhibition of GAD and a decrease in the concentration of GABA. These neurochemical changes were accompanied by ultrastructural alterations of certain nerve endings of the cerebral cortex which were mainly isolated in the nonaminergic or GAD-rich fraction of nerve endings.

EXPERIMENTAL

Enzyme determinations. In the experiments *in vivo*, DL-C-allylglycine (Sigma Chemical Co.) was injected i.p. into Wistar adult rats at a dose of 150 mg/kg. After a short period of considerable excitation with running and jumping by the animal, convulsions followed by rigidity were observed between 2 and 2·5 hr. At this time, the rats were decapitated, the cerebral cortex was removed in the cold, and a 10% homogenate (w/v) in distilled water was made. Control homogenates were obtained in a similar way from untreated animals. The enzyme determinations were done the same day or the next; in the latter case, the homogenates were kept frozen. In all cases, the determinations were made simultaneously in the control and the experimental homogenates. In the experiments *in vitro*, allylglycine was added in the buffer–substrate mixture to each homogenate in an equimolar concentration with respect to the corresponding substrate.

GAD was determined according to the technique of Lowe *et al.*[8] by incubating 20 μl of buffer–substrate mixture with 20 μl of a homogenate containing 2 mg fresh tissue. The final concentration of the components of the incubation medium were: potassium phosphate buffer, 0·1 M, pH 6·4; pyridoxal phosphate, 0·5 mM; and L-glutamate, 25 mM. Unless otherwise stated, after 60 min at 37°, 20 μl trichloroacetic acid, 10% (w/v) was added; after centrifugation, measurement of GABA was made on 10-μl aliquots as described by Lowe *et al.*[8] The blank tissue fluorescence for each homogenate was determined by adding the trichloroacetic acid before incubating the homogenate. The quenching of fluorescence induced by allylglycine was subtracted by preparing a standard curve for GABA with allylglycine. With a final concentration of 10 mM allylglycine, the quenching was less than 8 per cent. The enzyme was expressed in units per gram of fresh tissue. One unit is the amount of tissue which produces μmole GABA/hr at 37°.

GABA-aminotransferase (GABA-AT) was determined according to Salvador and Albers[9] with adaptation to a microscale; the unit of activity was the amount of tissue which produces 1 μmole of succinic semialdehyde per hour at 37°.

Glutamine synthetase (GS) was assayed by using the technique of Sellinger and De Balbian Verster[10] with adaptation to a microscale; the unit of activity corresponds to the formation of 1 μmole of glutamohydroxamate per hour at 37°. The method of Tonhazy[11] was used for aspartate- and alanine-aminotransferase (Asp-AT and Alan-AT), with either aspartate or alanine as substrate; each unit of activity represents

the production of 1 μmole/hr at 37° of oxalacetate or pyruvate respectively. All enzymatic determinations were made in triplicate.

Assay of free amino acids. At the onset of convulsions, rats weighing 100 g were immersed for 2 min in an acetone–dry ice mixture. The cerebral cortex was removed and a 10% homogenate in 10 N ClO_4H was made and the extracts were processed as described by Lindsay and Bachelard,[12] using a column of Dowex 50W × 8.* The descending chromatography, carried on for 30 hr, was made on Schleicher and Schüll paper 2043b. The chromatographic solvent was phenol–ethanol–ammonia (65:20:2·3, v/v); prior to the preparation of the mixture, the phenol was saturated with water containing 0·02% 8-hydroxyquinoline. The chromatograms were dried for 48 hr in air and dipped in acetone containing 1% lactic acid. After 20–30 min at room temperature, they were heated at 105° in an oven for 10 min. The spots were eluted by shaking for 30 min with ethanol–water–1% $CdSO_4$ (94:6:2·5, v/v) and the optical density was determined at 510 mμ in a PMQ II Zeiss spectrophotometer, using a paper blank for zero setting. With every chromatogram, standard amino acid mixtures containing 0·06 μmole of each amino acid were run. The cerebral cortex of normal rats, processed in the same way, was used as control for the amino acid content in the nonconvulsant state.

Electron microscopy. Rats injected with allylglycine were decapitated during convulsions. Slices of the cortex were fixed in a mixture of glutaraldehyde and paraformaldehyde, then "refixed" in 1% osmium tetroxide in phosphate buffer, pH 7·4, and embedded in Epon 812. From the cerebral cortex, the crude mitochondrial fraction and the aminergic and nonaminergic nerve endings were separated as previously described.[13] Pellets of the various layers brought to 0·32 M sucrose were centrifuged at 100,000 *g* for 30 min and were fixed as indicated above. Sections of the cerebral cortex and of the various fractions were observed under a Siemens Elmiskop I electron microscope. Controls from untreated animals were prepared for each experiment.

TABLE 1. ACTION *IN VIVO* OF ALLYLGLYCINE ON ENZYMES*

Enzyme	Control	Allylglycine	% Variation
GAD	68·0 ± 4·24 (3)	50·3 ± 2·25 (8)	− 25
GABA-AT	122·4 ± 4·70 (2)	113·6 ± 8·80 (9)	− 8
GS	65·9 ± 3·30 (3)	55·1 ± 4·32 (9)	− 16
Asp-AT	194·0 ± 0·31 (3)	229·0 ± 21·2 (10)	+ 18
Alan-AT	32·8 ± 3·25 (6)	37·2 ± 2·29 (5)	+ 13

* The enzymes were determined in individual homogenates from the cerebral cortex of untreated controls and in convulsant rats 2 hr after the administration of 150 mg/kg of allylglycine. Results are expressed as units/g and as per cent variation with respect to the control. In parenthesis is the number of experiments. For abbreviations, see text.

RESULTS

Effects of allylglycine on enzymes and amino acids. Tables 1 and 2 show the results of the action *in vivo* and *in vitro* of allylglycine on several enzymes related to the metabolism of amino acids in the cerebral cortex. Both GAD and GABA-AT show a

* The slight modifications introduced were recommended by A. A. Ramírez de Guglielmone (personal communication).

reduction of activity *in vivo*, GAD being somewhat more inhibited that GABA-AT. The inhibition of GAD *in vitro* is even more pronounced (58 per cent), while GABA-AT does not change with respect to the control. The small inhibition of GS observed *in vivo* was not observed in the experiments *in vitro*. Asp-AT and Alan-AT showed

TABLE 2. ACTION *IN VITRO* OF ALLYLGLYCINE ON ENZYMES*

Enzyme	Substrate (mM)	Control	Allyglycine	% Variation
GAD	10	25·8 ± 2·82 (6)	10·9 ± 1·91 (6)	− 58
GABA-AT	31	108·0 ± 0·10 (5)	108·0 ± 0·11 (5)	0
GS	30	51·9 ± 0·85 (5)	56·1 ± 6·40 (5)	+ 8
Asp-AT	100	204·0 ± 31·05 (3)	202·0 ± 32·17 (3)	0
Alan-AT	90	39·4 ± 4·55 (4)	25·9 ± 1·84 (4)	− 34

* The enzymes were determined in individual homogenates of the cerebral cortex. Allylglycine was added in equimolar concentration with respect to that of the substrate, which is indicated (mM). Results are expressed as units/g and per cent variation with respect to the control. In parenthesis is the number of experiments. For abbreviations, see text.

some increase in activity *in vivo*, but Asp-AT was unaffected *in vitro* and Alan-AT was inhibited. The assay of the free amino acids showed practically no change for aspartate, glutamate, glycine, glutamine and alanine, while the content of GABA appeared to be reduced by 40 per cent. The concentration of GABA in μmoles/g fresh tissue was 1·93 ± 0·20 in the 5 controls and 1·16 ± 0·28 in the 8 convulsant rats.

Table 3 shows the activity of GAD at different times after preincubation of the homogenate with the buffer substrate. The results obtained indicate that the inhibition

TABLE 3. EFFECT OF PREINCUBATION WITH ALLYLGLYCINE ON GAD*

Preincubation time (min)	Control	Allylglycine	% Variation
0	22·5	12·6	− 44
10	18·9	10·2	− 47
20	12·0	8·7	− 32
30	15·9	13·5	− 15

* The homogenate of the rat cerebral cortex was preincubated at 37° for different times in the presence of a buffer–substrate mixture containing 10 mM glutamate. Aliquots were also assayed in the presence of 10 mM allylglycine. Results are expressed in units/g of GAD activity and in per cent variation.

by allylglycine is reduced as the time of preincubation is increased, suggesting some kind of protection by the buffer–substrate, which was particularly effective after 30 min. This time was adopted for all the experiments shown in Table 4. By studying the effect of temperature on the preincubation of GAD with allylglycine, it was found that at 0° only 10 per cent inhibition was obtained, while at 37° it increased to 47 per cent. When allylglycine was added together with the buffer–substrate, there was practically no inhibition of GAD at both temperatures, indicating that some protection of the enzyme occurred. The strongest inhibition of GAD (80 per cent) was

observed when the homogenate was preincubated with allylglycine without buffer–substrate and then assayed in the presence of an equimolar concentration of glutamate (10 mM). This result should be compared with the 58 per cent inhibition obtained without preincubation shown in Table 2. When the preincubation with allylglycine was carried out in the presence of the buffer–substrate, 50 per cent inhibition was

TABLE 4. GAD INHIBITION BY 10 mM ALLYLGLYCINE UNDER VARIOUS CONDITIONS OF PREINCUBATION*

Temperature	Glutamate (mM)	Preincubation	Addition after preincubation	Units/g	% activity
0°	25	H	BS	61·5	100
0°	25	H + A-gly	BS	55·3	90
0°	25	H + A-gly + BS		61·5	100
37°	25	H	BS	47·4	100
37°	25	H + A-gly	BS	24·9	53
37°	25	H + A-gly + BS		40·8	86
37°	10	H	BS	28·8	100
37°	10	H + A-gly	BS	5·7	20
37°	10	H + BS		26·6	100
37°	10	H + BS + A-gly		13·3	50
37°	10	H + BS	A-gly	21·8	82
37°	10	H + PP	B-Ph + Glut	43·8	100
37°	10	H + PP + A-gly	B-Ph + Glut	17·5	40
37°	10	H + PP	B-Ph + Glut + A-gly	15·1	35

* Fresh homogenates from rat cerebral cortex were preincubated for 30 min under conditions indicated. Results are expressed in absolute values and as percentage variation with respect to the corresponding control. H, homogenate; BS, buffer–substrate mixture; PP, pyridoxal phosphate; B-Ph, buffer phosphate; A-gly, allylglycine; Glut, glutamate.

obtained, while only 28 per cent inhibition was observed when the homogenate was preincubated in the buffer–substrate and the inhibitor was added later, prior to the assay. These results suggest that allylglycine and the substrate mixture compete for the active site of GAD and the final result depends not only on the relative concentrations of the inhibitor and substrate but also on the time sequence in which they reach the enzyme.

Table 4 also demonstrates that preincubation with pyridoxal phosphate had no protective effect on the inhibition of GAD by allylglycine.

Ultrastructural changes in nerve endings induced by allylglycine. Electron microscope observation of neuropile regions of the cerebral cortex of convulsant rats showed a striking alteration of a certain number of nerve endings. These are distinguished from the normal-looking nerve endings by the clearer matrix, indicating swelling of the axoplasm, by reduction in the number of synaptic vesicles, by some swelling of the intraterminal mitochondria, and frequently by an extensive vacuolization of the nerve ending (Fig. 1). In the mitochondrial fraction of the cerebral cortex, altered nerve endings, with the changes described above, may be recognized among isolated nerve endings having a normal appearance (Fig. 2). When the mitochondrial fraction was subfractionated on a sucrose gradient as described by De Robertis *et al.*,[13] fraction D of the GAD-rich (nonaminergic) nerve ending contained mainly

altered terminals of the cerebral cortex (Fig. 3). (The corresponding normal controls of Figs. 1 and 2 may be found in reference 4 and those of Fig. 3 in ref. 13.)

DISCUSSION

Microphysiological experiments have demonstrated that, when applied on the neuronal surface, some aminoacids cause excitation while others result in an inhibition of neuronal activity. The excitatory effect of L-aspartic and L-glutamic acids,[14] as well as the inhibitory action of GABA[15] and glycine,[16] have been reported. Of these amino acids, GABA is of particular interest because it is found only in the CNS. Furthermore, it is synthesized irreversibly by way of GAD, an enzyme that is specifically located in gray matter. Salganicoff and De Robertis[17] found that this enzyme is concentrated in the nonaminergic type of nerve endings isolated in submitochondrial fraction D.[13] De Robertis[18] has recently summarized the data suggesting that this fraction contains the inhibitory nerve endings of the cerebral cortex.

The results described here demonstrate that the convulsant drug, allylglycine, produces an inhibition of GAD *in vivo*, which is further enhanced by treatment *in vitro*. Approximately 60 per inhibition is obtained when equimolar concentrations of substrate and allylglycine are used *in vitro*. The inhibition is even greater if allylglycine is preincubated with the homogenate before the assay. A protective effect can be observed by adding the buffer–substrate mixture before the allylglycine. It may be demonstrated that the inhibition of GAD by allylglycine is not by way of the cofactor, pyridoxal phosphate. This finding agrees with the fact that pyridoxine given *in vivo* before or simultaneously with allylglycine has no protective effect.[6] The inhibition of GAD by pyridoxal phosphate antagonists has been extensively studied. Such drugs generally produce GAD inhibition at low concentrations.[19] The relatively high concentration of allylglycine needed to inhibit GAD may be due to competition for the substrate and not for pyridoxal phosphate.

The other enzymes related to the metabolism of glutamate, such as glutamine synthetase and the aspartate- and alanine-aminotransferases, showed no appreciable changes with allylglycine. The only variation was a certain inhibition of Alan-AT *in vitro*. Since the level of free glutamate and alanine in the cerebral cortex remained unchanged after allylglycine, the effect upon this enzyme seems to be of little importance. Furthermore, the formation of glutamate by transamination with alanine and aspartate from ketoglutarate may be probably more dependent on Asp-AT than on Alan-AT. As already described by Salganicoff and De Robertis,[7] this may be a consequence of the large difference in the absolute values (see also Table 1).

To interpret the mechanism of action of allylglycine, a decisive finding is the considerable reduction observed in the GABA level of the cerebral cortex during convulsions; this is at variance with the levels of the other free amino acids which remain practically unchanged. The level of GABA in brain is mainly determined by the activities of GAD and GABA-aminotransferase, the latter enzyme being localized in mitochondria.[7] In our experiments, the convulsant rats had practically no inhibition of GABA-aminotransferase and *in vitro* no changes were found. The fact that GAD is inhibited both *in vivo* and *in vitro* by allylglycine suggests that the reduction in GABA is directly determined by the effect of the convulsant on the synthesizing enzyme. This neurochemical finding should be correlated with the remarkable ultrastructural changes observed in certain nerve endings of the cerebral cortex changes

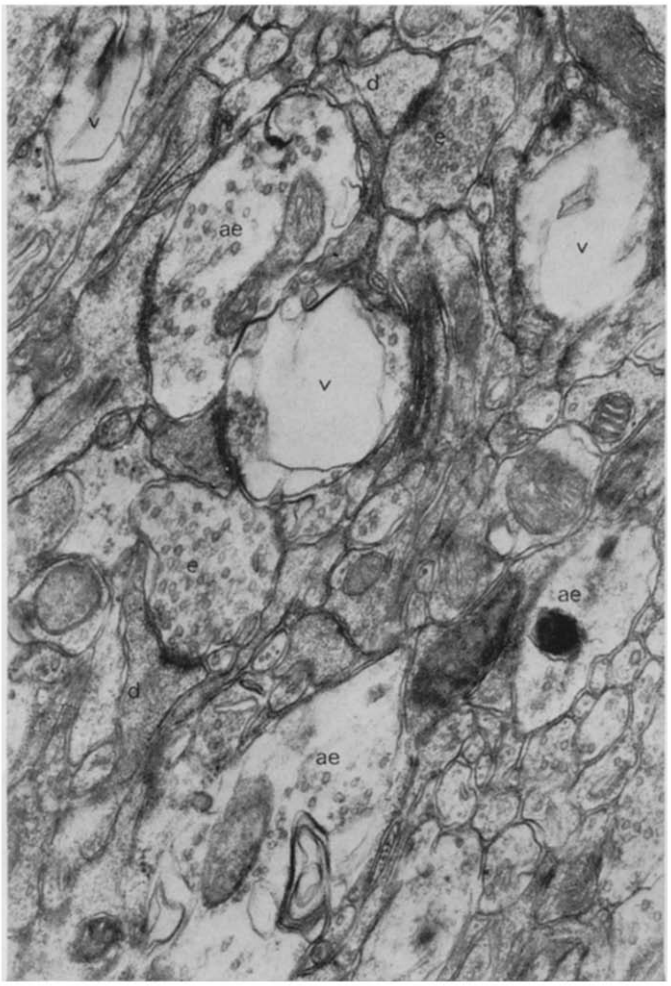

FIG. 1. Electronmicrograph of the neuropile of the cerebral cortex of a rat undergoing convulsions induced by allyglycine. Several normal nerve endings (e) making synaptic contacts with dendrites (d) are observed. Others show different degrees of alteration, consisting of swelling, loss of synaptic vesicles or vacuolization (v) of the ending; ae = altered nerve ending (× 54,000).

B.P.—*facing page 142*

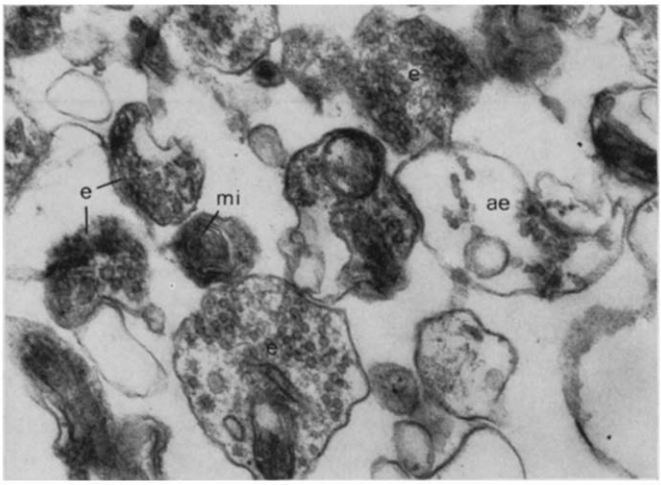

FIG. 2. Electron micrograph of the crude mitochondrial fraction of the cerebral cortex of a rat undergoing convulsions. In addition to free mitochondria (mi), normal (e) and altered (ae) nerve endings are observed ($\times$ 55,000).

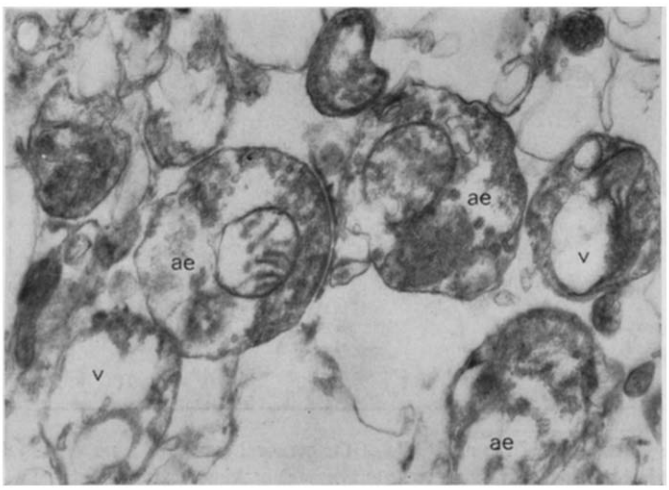

FIG. 3. Submitochondrial fraction D containing the GAD-rich (non-aminergic) nerve endings. Most of them appear altered (ae) ,showing vacuolization (v), swelling and loss of synaptic vesicles. Damage of the intraterminal mitochondria is also apparent ($\times$ 45,000).

which consist of swelling, reduction in the number of synaptic vesicles, and vacuolization. Such alterations affect only a certain number of nerve endings while the others appear completely normal. These findings were corroborated by observations of the isolated nerve endings in the mitochondrial fraction of the cerebral cortex and in the submitochondrial fractions. The fact that more altered nerve endings were found in our D fraction of GAD-rich nerve endings (nonaminergic) also supports the conclusion that the mechanism of allylglycine is by its action on this enzyme, which is contained in the inhibitory nerve endings of the cortex. The specific alteration of these nerve endings produced by allylglycine may be a tool to study, at the neuroanatomical level, the localization of these inhibitory synapses. Studies of this sort are now being planned.

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