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γ-AMINOBUTYRIC ACID IN SUBCELLULAR FRACTIONS OF MOUSE BRAIN AND ITS RELATION TO CONVULSIONS

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Abstract—The *in vivo* effects of convulsant drugs (penicillamine, semicarbazide and isonicotinic acid hydrazide) on the γ-aminobutyric acid (GABA) levels in subcellular fractions were studied in mice. All the drugs reduced the concentration of GABA in the synaptosomal fraction; it was unlikely that this decrease in the GABA concentration was due to redistribution or degradation during the separating procedures. When the convulsions were prevented by simultaneous pyridoxine injection, the GABA level in synaptosomes rose to normal. It is suggested that the decrease of GABA concentration in nerve endings may be an important factor in the onset of some kinds of convulsions.

The presence of γ-aminobutyric acid (GABA) in mammalian brain [1, 2] and its possible role as an inhibitory neurotransmitter substance [3, 4] have led to speculation that compounds which decrease the concentration of the amino acid in brain might act as convulsant agents [5]. However, various workers later demonstrated a lack of correlation between the onset of convulsions and the decrease in content of brain GABA [6–9]. In fact, convulsions occurred even in animals in which brain concentrations of GABA were maintained at elevated levels by the use of an agent such as hydrazine [7]. However, these observations could not be considered to rule out conclusively a role for GABA in the etiology of convulsions, since the drug-induced elevation in the GABA level might be in the wrong subcellular location to counteract the induced decreases in the concentration of the amino acid.

Alternative hypotheses have been proposed in an attempt to implicate GABA metabolism in the etiology of convulsions. For example, it has been shown that the excitable state of the brain can be related to a function embodying changes in both glutamic decarboxylase (GAD) (EC 4.1.1.15) activity and GABA levels [9]. But, again, the changes in these parameters of GABA metabolism may not accurately reflect the events at a critical subcellular location, since the parameters, in most cases, were measured using preparations from whole brain tissue.

An aspect of GABA metabolism in the brain which inordinately complicates the interpretation of the above experimental results is its compartmentalization. Previous studies have shown that GAD is located mainly in nerve endings, and GABA transaminase (EC 2.6.1.19) mainly in mitochondria [10–12]. On the basis of the distribution of the two enzymes, it can be postulated that the critical site of GABA synthesis is probably the nerve endings, and that a decrease of GABA concentration in the nerve endings, because of the GAD inhibition, may result in increased cerebral excitability (convulsion). In the present work, we have studied the levels of GABA in subcellular fractions from the brains of mice treated with various convulsant drugs (antivitamin B_6), which change GABA metabolism in whole brain [7, 13, 14].

MATERIALS AND METHODS

Animals. Adult male albino mice (local strain) weighing about 20 g were used throughout this work. They were allowed free access to food and water.

Administration of drugs. The convulsant agents used were DL-penicillamine (PeA), semicarbazide (SC) and isonicotinic acid hydrazide (INAH) (all from Nakarai Chemicals, Kyoto). Solutions of the drugs were prepared daily in 0.9% NaCl, the pH being adjusted to 7 with NaOH before use. The final concentration of each drug was adjusted so that the required dosage was administered in a volume equivalent to 1 per cent of the body weight of the animal. The solution of pyridoxine (PN; Nakarai Chemicals, Kyoto) was also similarly prepared. All injections were intraperitoneal; the injected animals were kept in a laboratory with minimal background noise.

Subcellular fractionation. Animals were decapitated, and the brains were quickly removed and pooled in a chilled homogenizer until enough tissue (usually five to six brains) had been collected for subcellular fractionation. A 10% homogenate in 0.32 M sucrose was prepared in a Teflon–glass homogenizer under mild conditions (five strokes at approximately 1000 rev/min). The primary fractions (nuclei, crude mitochondria, microsomes and supernatant) and the subfractions (myelin, synaptosomes and mitochondria) of the crude mitochondrial fraction were obtained by the procedure of Gray and Whittaker [15]. In some cases where the synaptosomes were used for *in vitro* experiments taking a long time, the subfractionation of crude mitochondria was carried out by the method of Sellström *et al.* [16], which requires less time to prepare the synaptosomes than the procedure of Gray and Whittaker. Pellets containing each fraction or subfraction were resuspended in cold water and used for assay of GABA. Care was taken to maintain all material and tissue at 0–4° throughout all the fractionations.

Determination of GABA. The homogenate or the suspensions of subcellular fractions were homogenized in the same volume of 2 N perchloric acid and centrifuged at 17,000 *g* for 15 min. The extract was treated with 5 N KOH to about pH 4 and passed through a

Dowex-50-H$^+$ column (0.6 × 4 cm). The column was washed with 10 ml of water and the amino acids were eluted with 6 ml of 2 N NH_4OH. The eluate was evaporated to dryness under vacuum. The residue was dissolved in 0.1 ml of water, and 40 μl of the solution was spotted on Toyo filter paper No. 51. The chromatograms were run (ascending chromatography, 80% phenol), dried for 24 hr and fully treated with 0.2% ninhydrin. The ninhydrin color was developed by heating the paper in an oven at 70° for 30 min. The spot corresponding to GABA was cut out and extracted with 5 ml of 60% ethanol, and the absorbance of the extract was determined at 570 nm [17].

Other biochemical analyses. Succinic acid dehydrogenase (EC 1.3.99.1) was assayed by the method of Slater and Bonner [18], as modified by De Robertis *et al.* [19]. Acetylcholinesterase (EC 3.1.1.7) was assayed according to Ellman *et al.* [20]. Protein was measured according to the method of Lowry *et al.* [21].

RESULTS AND DISCUSSION

The primary fractionation, as well as the separation of the crude mitochondrial fraction, in this experiment gave results similar to those reported previously [11, 19, 22–24] from biochemical viewpoints: (1) acetycholinesterase (a marker enzyme for synaptosomes) was localized mainly in the crude mitochondrial and the synaptosomal fractions; and (2) succinic acid dehydrogenase (a marker enzyme for mitochondria) was in the crude and the purified mitochondrial fractions. The distribution of protein in these experiments was also similar to that reported previously [11, 19, 22–24].

Subcellular distribution of GABA. In normal mouse brain, the content of GABA in the whole homogenate was 2.6 μmoles/g. Among the primary fractions, GABA was distributed mainly in the crude mitochondrial fraction (30 per cent) and in the supernatant fraction (50 per cent); the nuclear and the microsomal fractions contained only about 8 per cent, respectively (recovery was 95 per cent). When the crude mitochondrial fraction was subfractionated according to Gray and Whittaker, 65 per cent of the recovered GABA was found in the synaptosomal band, and about 20 per cent was found in the myelin band and in the mitochondrial pellet (recovery was 75 per cent).

Since GABA is a low molecular weight substance, which theoretically could easily diffuse out of, or into, the subcellular particles, several experiments were carried out to assess its possible redistribution during the separating procedures. When a 10% homogenate in 0.32 M sucrose was centrifuged at 100,000 *g* for 30 min, 45 per cent of the GABA was obtained in the pellet (three experiments). This value did not differ from that observed in the particulate fractions obtained after separation of the primary fractions (sum of nuclear, crude mitochondrial and microsomal fractions; 47 per cent). In the following experiments, the homogenate was maintained for several hours at 0°, and the primary fractions were prepared from portions taken at 1, 2, 4 and 6 hr, and used for determination of GABA. No significant changes in the contents of GABA in the crude mitochondrial and the microsomal pellets were observed during the experimental period, although the GABA contents in other fractions rose steadily with incubation time (Fig. 1). When the crude mitochondria

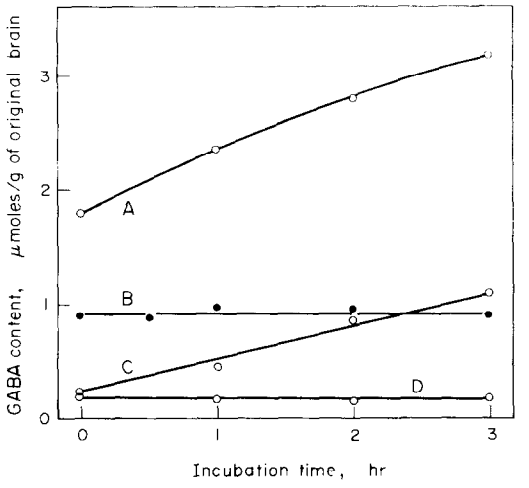

Fig. 1. Relation between incubation time of homogenate and changes of GABA contents in subcellular fractions obtained from the homogenate. A 10% brain homogenate in 0.32 M sucrose was incubated at 0°, and at various intervals the subcellular fractions were prepared from the homogenate and were used for the GABA assay. (A) 10^5 *g* supernatant; (B) crude mitochondria; (C) nuclei; and (D) microsomes.

Table 1. Effects of concentration of GABA in brain homogenate on GABA levels in synaptosomes and supernatant fraction obtained from the homogenate*

	GABA content (nmoles/mg protein)			
	Control	+ GABA†	SC	SC + GABA‡
Supernatant	51.5 ± 0.8 (22)	92.7 (2)	32.8 (2)	50.7 (2)
Synaptosomes	15.5 ± 0.4 (24)	16.0 (2)	10.4 (2)	10.6 (2)

* Each value is the mean ± S.E.M. or the mean for the number of experiments given in parentheses. Synaptosomes were prepared by the method of Gray and Whittaker [15].

† Brains were homogenized in 0.32 M sucrose containg 0.2 mM GABA.

‡ Brains were homogenized in 0.32 M sucrose containg 0.1 mM GABA.

were resuspended at a concentration of 5 mg protein/ml in 0.32 (or 0.8) M sucrose, these suspensions were maintained in the same manner as above and were centrifuged at 30, 60 and 150 min; again, no significant changes in the contents of GABA in the pellets were found during this period (data not shown). Moreover, when a 10% homogenate was prepared in 0.32 M sucrose containing 0.2 mM GABA and the homogenate was subjected to the centrifugation procedure to obtain the synaptosomal fraction, the content of GABA in this fraction was not affected by the addition of GABA, in spite of a considerable (expected) increase of GABA content in the 10^5 *g* supernatant fraction (Table 1).

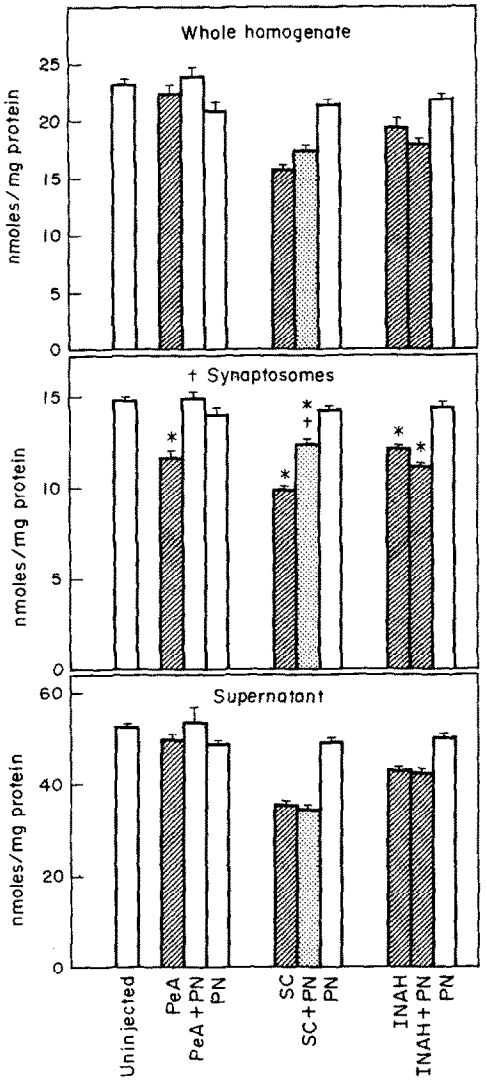

Fig. 2. Effect of PeA (3.3 m-moles/kg), SC (4.0 m-moles/kg) and INAH (2.2 m-moles/kg), with and without PN (1.8 m-moles/kg), on GABA content in whole brain, and in synaptosomal and supernatant fractions. Synaptosomes were prepared by the method of Gray and Whittaker [15]. Each histogram shows the mean ± S.E.M. for at least six experiments. Open histograms indicate that no seizures were observed after drug treatment; lightly shaded and darkly shaded histograms indicate that seizures were sometimes (No. of convulsed mice/No. of treated mice = 7/15) and always observed after treatment. Mice with convulsions were decapitated at the onset of convulsions, and mice without convulsions were decapitated at the mean time to convulsions after each convulsant alone treatment (PeA, 130 min; Sc, 55 min; INAH, 29 min). The single asterisk (*) indicates $P < 0.001$, compared with control (uninjected) mice, and the single dagger (†) indicates $P < 0.001$, compared with convulsant alone treated mice, that the values occurred by chance (Student's *t*-test).

These observations suggest that the GABA levels in the synaptosomal fraction are scarcely affected by concentrations of GABA in the medium surrounding the synaptosomal particles during the subcellular fractionation procedures, and may accurately reflect the GABA concentration in the nerve endings, even if GABA is somewhat lost from the nerve endings by damage during homogenization.

Effects of administration of various convulsant drugs on GABA level in synaptosomes. Figure 2 shows the effects of PeA, SC and INAH on GABA contents in the homogenate, synaptosomal and 10^5 *g* supernatant fractions. Convulsant doses of these drugs (PeA 3.3 m-moles/kg, SC 4.0 m-moles/kg, INAH 2.2 m-moles/kg) commonly produced a decrease of GABA in the synaptosomes, although the decrease in GABA content varied with the different drugs used. When the convulsions by PeA were prevented by PN supplement (1.8 m-moles/kg), the decrease in synaptosomal GABA level was completely prevented. When the same amount of PN was administered with SC, PN only partially prevented the convulsions by SC, and slightly, but significantly, lessened only the decrease in synaptosomal GABA level by SC alone. In the case of INAH, the PN supplement could not prevent the decrease in synaptosomal GABA level, consonant with the lack of prevention of the convulsions by INAH. Mice treated with PN alone were also included in the study for comparative purposes. The effect of PN treatment (1.8 m-moles/kg) *per se* was minimal, the trend being a very small decrease during each experimental period (Fig. 2). In summary, the onset of seizure activities induced by these convulsants coincides with the fall in synaptosomal GABA levels; in contrast, the cessation of the seizure activities by PN administration coincides with the recovery in synaptosomal GABA levels. Moreover, the effects of SC on the synaptosomal GABA levels were determined at various times after administration of the drug. When the convulsant dose of SC (4.0 m-moles/kg) was administered, the GABA level gradually decreased, and the decrease continued until the onset of convulsions (Fig. 3). These results suggest the existence of a correlation between the decrease in synaptosomal GABA level and the onset of convulsions by PeA, SC and INAH.

This correlation would indicate that some drug-induced convulsions may be associated with a decrease of synaptosomal GABA level. However, since the changes in synaptosomal GABA level may be caused by several factors during preparation of subcellular fractions, (such as the rates of release, uptake, synthesis and degradation of the amino acid), several experiments were carried out to assess these possibilities.

First, the brains of SC-treated mice were homogenized in 0.32 M sucrose containing 0.1 mM GABA, which was adjusted so that the GABA concentration in the supernatant fraction was similar to that of control mice, and the synaptosomal fraction was prepared by

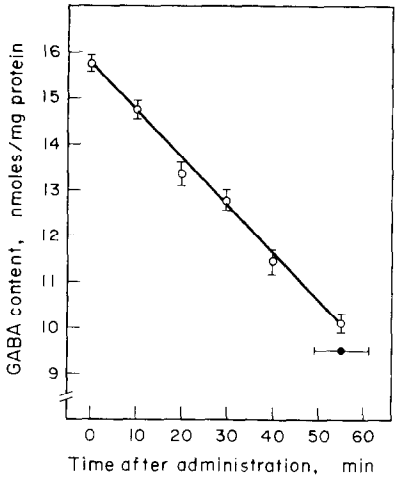

Fig. 3. Changes with time of synaptosomal GABA levels after injection of convulsant dose of SC (4.0 m-moles/kg). Synaptosomes were prepared by the method of Gray and Whittaker [15]. Each value is the mean ± S.E.M. for three experiments. The horizontal bracket indicates the convulsion time ± S.D. after the treatment (ten animals).

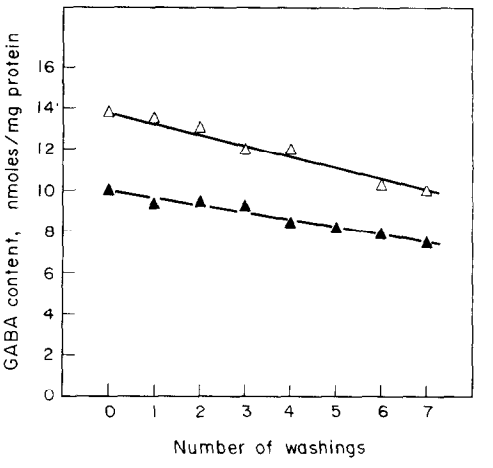

Fig. 5. Changes of GABA contents in synaptosomes with the number of times the synaptosomes were washed. Synaptosomes prepared by the method of Sellström *et al.* [16] were washed by resuspension (8 mg protein/ml of 0.32 M sucrose) and centrifugation (100,000 *g* for 20 min) at 0°. All points are the mean of duplicate determinations. Key: (△—△) synaptosomes from control mice; and (▲—▲) synaptosomes from SC-treated (4 m-moles/kg) mice at seizures.

the procedure of Gray and Whittaker to determine its GABA content. The synaptosomal GABA content was not influenced by the addition of GABA (Table 1), showing that the decrease in synaptosomal GABA level of drug-treated mice was not due to the change of amino acid level in the other subcellular fractions, especially in the supernatant fraction. In the next experiment, the synaptosomes, prepared according to the method of Sellström *et al.* from the brains of SC-treated and control mice, were resuspended in 0.32 M sucrose (9 mg protein/ml), and maintained for several hours at 0°, and the high-speed pellets (at 100,000 *g* for 20 min) were obtained from portions taken at 0, 1, 2 and 3 hr to assay its GABA content. No significant differences between the two time-courses of the synaptosomal GABA contents were observed during the experimental period (Fig. 4), showing that there is no evidence that the synaptosomal GABA of convulsant-treated mice decreased more rapidly during the synaptosome-separating procedure than the GABA of control mice. Furthermore, the same synaptosomes were similarly resuspended (8 mg protein/ml) and, after an adequate volume of the suspension was taken out for the assay of synaptosomal GABA, the remaining suspension was centrifuged at 100,000 *g* for 20 min. The pellet was again suspended in an adequate volume of 0.32 M sucrose so that the concentration of synaptosomes was equivalent to that of the original suspension. Such a procedure was repeated several times and the GABA content in each of these suspensions was assayed. The synaptosomal GABA contents of the SC-treated and the control mice decreased gradually with the number of times the synaptosomes were washed, and the decreasing rates were similar in both cases, showing that there is no evidence that the synaptosomal GABA of convulsant-treated mice leaked more rapidly during the synaptosome-separating procedure than that of the control mice (Fig. 5).

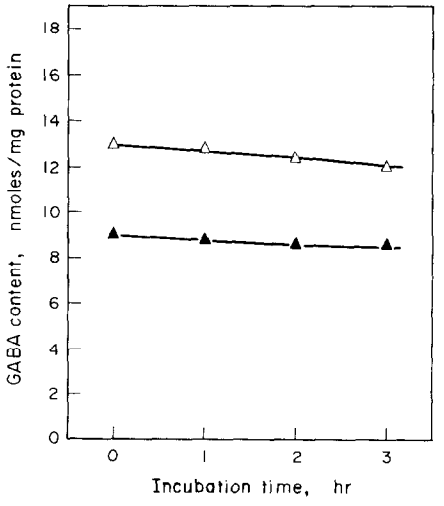

Fig. 4. Changes of GABA contents in synaptosomes with incubation time. Synaptosomes prepared by the method of Sellström *et al.* [16] were incubated in 0.32 M sucrose at 0° as suspensions (9 mg protein/ml). All points are the mean of duplicate determinations. Key: (△—△) synaptosomes from control mice; and (▲—▲) synaptosomes from SC-treated (4 m-moles/kg) mice at seizures.

All these experimental results suggest that the changes in synaptosomal GABA levels may not be due to redistribution or metabolism during the separating procedures, but may accurately reflect the events at the nerve endings. The fact that there is a simple relationship between the onset of seizures and the decrease in synaptosomal GABA level, as described above (Fig. 2), has led us to postulate that a decrease of GABA concentration in the nerve endings, probably because of the GAD inhibition [23], results in increased cerebral excitability (convulsion). Since it has been postulated

that GABA is released into the synaptic cleft at the GABA-dependent inhibitory synapses, where GABA exerts a tonic inhibition on the post synaptic membrane, we conclude that the convulsant effects of some drugs could be due to a decrease of GAD activity in the nerve endings, with a consequent reduction of GABA levels in the same compartment, followed by a decrease of GABA release from the nerve endings, with a consequent deficiency in the tonic inhibition exerted on the post-synaptic neuron.

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