RELEASE OF \( \gamma \)-AMINOBUTYRIC ACID FROM ISOLATED BRAIN SYNAPTOSOMES DURING SEMICARBAZIDE-INDUCED CONVULSIONS

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(Received June 13, 1979)

Summary The in vivo effect of semicarbazide (SC) and aminoxyacetic acid (AOAA) upon \( \gamma \)-aminobutyric acid (GABA) levels in the synaptosomal fraction, and GABA release from the same fraction were studied in the mouse. The convulsive dose of SC reduced the GABA content in synaptosomes, and when the convulsions were protected by pretreatment with AOAA, the reduced GABA content in synaptosomes rose to or above the normal level. Moreover, the SC treatment decreased the GABA releases from synaptosomes both in a Ca\(^{2+}\)-free Ringer's solution and in a high K\(^+\) Ringer's solution. When the convulsions were protected by pretreatment with AOAA, the decreased GABA releases in both the conditions rose to or above the normal levels. Therefore, it is suggested that the decrease in GABA release from the nerve endings, because of the decrease of GABA content in the same compartment, is possibly an important factor in the onset of some kinds of convulsions.

Keywords K\(^+\), synaptosome, convulsion, antivitamin B\(_6\), GABA release

It is well known that many antivitamin B\(_6\) induce convulsions in animals and in many cases a vitamin B\(_6\) supplement prevents such convulsions. The biochemical mechanism involved in the production of convulsions, however, is not yet clear. Recent studies in this laboratory suggested that the convulsions induced by antivitamin B\(_6\) might be related to a decrease in \( \gamma \)-aminobutyric acid (GABA) level in nerve endings following an inhibition of glutamic acid decarboxylase (GAD)[EC 4.1.1.15] in the same compartment, while the cessation of the seizure activities by vitamin B\(_6\) or an anticonvulsant such as aminoxyacetic acid (AOAA) might be related to a recovery (or increase) in the synaptosomal GABA level (1–3).

On the other hand, isolated nerve endings (synaptosomes) have been shown to

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Abbreviations: SC, semicarbazide; AOAA, aminoxyacetic acid; GABA, \( \gamma \)-aminobutyric acid; AVA, \( \delta \)-amino-\( \eta \)-valeric acid.
be capable of a wide range of metabolic performance and provide useful system for the study of the release of transmitter substance in the central nervous system (4).

Synaptosomes appear to be able to generate a membrane potential and exposure of synaptosomes to high potassium concentrations or electrical pulses causes a release of excitatory and inhibitory amino acids (5).

The object of the present investigation is to study whether a reduction in the synaptosomal GABA content produced by semicarbazide (SC), an antivitamin B₆, might cause a decrease in the release of GABA from synaptosomes.

MATERIALS AND METHODS

Adult male white mice weighing about 20 g were used throughout this work. In all experiments SC and AOAA were injected intraperitoneally at a dose of 2.5 mmoles/kg and 0.27 mmole/kg, respectively. Solutions of the drugs were prepared daily in 0.9% NaCl and were adjusted to pH 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.

Preparation of synaptosomes. When animals treated with SC were having the first convulsions (about 1 hr after SC injection) they were decapitated and the brains were quickly removed, weighed and rinsed with cold 0.9% NaCl. A 10% homogenate in 0.32 M sucrose was prepared in a Teflon-glass homogenizer under gentle conditions (five strokes at about 1,000 rpm). Crude mitochondrial fractions containing synaptosomes were prepared by the method of Gray and Whittaker (6). Synaptosomes were separated from the crude mitochondrial fraction on a Ficoll-sucrose gradient according to Sellström et al. (7).

Perfusion of synaptosomes. The synaptosomes were pelleted at 90,000g for 20 min and resuspended in a Ca²⁺-free Ringer’s solution to give a protein concentration of 13 mg/ml. The solution is consisted of NaCl, 128 (mm); KCl, 5; KH₂PO₄, 1.2; MgSO₄, 1.3; NaHCO₃, 26; and glucose, 10.

A suspension (1.0 ml) was placed on prepared a filter unit that consisted of a 2.4-cm glass fiber filter (GF/C) and a 2.5-cm Millipore filter (pore diameter 0.45 μm) lying on the bottom of a 20-ml perfusion chamber (8). The chamber was then connected with a peristaltic pump and filled with Ca²⁺-free Ringer’s solution at 37°C. The perfusion rate was 0.5 ml/min, and normally 2-min fractions were collected into separate tubes and used for assay of GABA.

Analysis of GABA. GABA in synaptosomes was measured by a colorimetric method (9). The suspension of synaptosomes obtained as above was homogenized in the some volume of 2 N perchloric acid and centrifuged at 17,000g for 15 min. The extract was adjusted with 5 N KOH to about pH 4 and passed through a Dowex-50-H⁺ column (0.6 x 4 cm). The column was washed with 10 ml of water and the amino acids were eluted with 6 ml of 2 N NH₄OH. 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 to Toyo filter paper No. 51 (40 x 40 cm).
After development in 80% phenol, the paper was dried and fully sprayed with 0.2% ninhydrin in 95% ethanol. The ninhydrin color was developed by heating the paper in an oven at 70°C for 30 min. The spot corresponding to GABA was cut out and extracted with 5 ml of 60% ethanol; the absorbance of the extract was determined at 570 nm.

GABA in the perfusate obtained was measured using the gas chromatographic method described by Schmid and Karobath (10), with a minor modification. Each fraction was acidified with 0.1 N HCl to about pH 4 and passed through a Dowex-50-H⁺ column (0.6 × 4 cm). The column was washed with 30 ml of water and GABA was eluted with 6 ml of 2 N NH₄OH. After δ-amino-n-valeric acid (AVA), a structural analog of GABA, was added as an internal standard, the eluate was evaporated to dryness under vacuum. To the residue, 0.15 ml of a mixture of trifluoroacetic anhydride and 1,1,1,3,3,3-hexafluoropropan-2-ol (2:1) was added for chemical modification. The tubes were stoppered and kept for 70 min at room temperature. The reagents were removed under a stream of dry nitrogen and the residue was dissolved in 1.0 ml of ethyl acetate. The tube was capped with silicone-rubber septum and the sample was ready for gas chromatographic injection. A Shimadzu GC 4B M gas chromatograph equipped with a 63Ni electron-capture detector was used. The separation was usually carried out on a 2 m × 3 mm siliconized glass column packed with 1.5% OV 17 and 2.0% QF-1 on Chromosorb W HP (80–100 mesh) under the following routine conditions: injector temperature = 150°C; column temperature = 135°C; detector temperature = 300°C; nitrogen flow rate = 30 ml/min. GABA was estimated by measuring the ratio of the peak areas of GABA and the internal standard AVA.

Protein was measured by the method of Lowry et al. (11).

RESULTS AND DISCUSSION

GABA contents in synaptosomes

Figure 1 shows the effect of SC, SC+AOAA or AOAA on GABA content in synaptosomes. Convulsant dose of SC (2.5 mmoles/kg) caused a marked decrease of the synaptosomal GABA content. When the convulsion by SC was prevented by injection of AOAA (0.27 m mole/kg) 1 hr prior to SC treatment (SC+AOAA), the decreased GABA content in synaptosomes was elevated to or above the normal levels. The animals treated with AOAA alone exhibited higher levels in synaptosomal GABA than did the SC+AOAA-treated animals. The onset of convulsions by SC treatment is accompanied by the fall in synaptosomal GABA levels, and in contrast, the cessation of the convulsions by AOAA pretreatment is accompanied by the recovery (or preferably the increase) in GABA levels. These results suggest the existence of a correlation between the decrease in synaptosomal GABA level and the onset of convulsions by SC, which is in agreement with our previous reports (3,12). The decrease of GABA by SC is probably due to an inhibition of synaptosomal GAD activity by SC while the recovery (or increase) by
AOAA is probably due to a severe inhibition of GABA transaminase by AOAA (12). The fact that there is a simple relationship between the onset of seizures and the decrease in synaptosomal GABA led us to postulate that the convulsant action of SC may be ascribed to a reduction of GABA levels in the nerve endings, followed by a decrease of GABA release from the same compartment.

**Spontaneous efflux of GABA**

When synaptosomes were washed with Ca²⁺-free Ringer's solution as described in MATERIALS AND METHODS, a small portion of GABA was released in the perfusate (Fig. 2). An initial rapid release of GABA was followed by a slower efflux. After perfusing for 54 min (27 fractions) about 90 per cent of GABA remained in the synaptosomes.

**Effect of high potassium concentrations on GABA efflux**

After 12 min (6 fractions) of washing with the Ca²⁺-free Ringer's solution, continuous perfusion of the synaptosomes with a high K⁺ Ringer's solution caused a large increase in GABA efflux (Fig. 2); the high K⁺ Ringer's solution consisted of NaCl, 73 (mm); KCl, 56; CaCl₂, 2.54; KH₂PO₄, 1.2; MgSO₄, 1.3; NaHCO₃, 26; and glucose, 10. The maximum release, which was about six times the spontaneous release, occurred at 4-6 min after perfusing the synaptosomes with the high K⁺ Ringer's solution; the release rate then decreased to a relatively constant level for about 40 min. Continuous perfusion with high K⁺ Ringer's solution released about 25 per cent of the total GABA in synaptosomes in the first 40 min. When the
Fig. 2. GABA efflux from synaptosomes of normal mice. ○—○ indicates the spontaneous release of GABA, and •—• and ○—○ indicate the GABA releases observed after perfusion with a high potassium (56 mM) solution. Perfusion periods with high potassium are indicated by shaded rectangles.

synaptosomes were exposed twice to the high K+ Ringer’s solution, two responses could be elicited by the respective exposures, though the second response was small (Fig. 2). These results indicate that the synaptosomes used in this experiment can maintain an ability to respond to the depolarizing concentration of K+ and Ca2+ for a long period.

**GABA efflux from synaptosomes of mouse treated with SC**

The spontaneous GABA efflux from synaptosomes of a mouse convulsed with SC is shown in Fig. 3: the synaptosomes from the mice treated with SC were referred to as SC-synaptosomes. The spontaneous GABA release was significantly decreased in the SC-synaptosomes compared with the control ones, although the GABA-releasing patterns were similar in both the SC- and control synaptosomes. When the Ca2+-free Ringer’s solution was quickly and thereafter continuously substituted with a depolarizing concentration of K+ and Ca2+ (the high K+ Ringer’s solution), the profile of GABA release from the SC-synaptosomes was very similar to that of control synaptosomes but the initial rate of GABA release and the amount of released GABA were significantly smaller than those of the control synaptosomes (Fig. 3). These results suggest that the decrease in spontaneous GABA release and in the K+, Ca2+-stimulated release of GABA may be caused by a reduction in GABA content in synaptosomes induced by SC.

Since the administration of AOAA 1 hr prior to the SC administration prevented the seizures induced by SC alone, the GABA efflux of the animals treated with SC+AOAA was studied; the synaptosomes from mice treated with
SC+AOAA were referred to as SC-AOAA-synaptosomes. Figure 3 shows the GABA efflux from the SC-AOAA-synaptosomes measured in the same manner as described above. The spontaneous GABA release markedly increased and then declined very rapidly, compared to that of SC-synaptosomes or control synaptosomes. The GABA release stimulated by the depolarizing concentration of K⁺ and Ca²⁺ also was significantly higher than that of SC- or control synaptosomes. These results also show that both the spontaneous and the K⁺, Ca²⁺-stimulated GABA releases vary with the GABA contents of synaptosomes as shown in Fig. 1.

The GABA effluxes from the SC-, SC-AOAA-synaptosomes and control synaptosomes were quantitatively compared. Net amount of K⁺, Ca²⁺-stimulated GABA efflux (c in Fig. 4 A) was calculated by subtracting a part of spontaneous GABA efflux (b in Fig. 4 A) from apparent K⁺, Ca²⁺-stimulated GABA efflux (shaded area in Fig. 4 A). The effects of these drug administrations on the spontaneous and the net K⁺, Ca²⁺-stimulated GABA efflux are summarized in Fig. 4 B. The spontaneous GABA releases (a+b in Fig. 4 A) and the net K⁺, Ca²⁺-stimulated GABA releases from SC-synaptosomes decreased to 60–70 per cent of those of the control, being accompanied by the production of convulsions by SC. In contrast, both the spontaneous GABA releases and the net K⁺, Ca²⁺-stimulated GABA releases of SC-AOAA-synaptosomes rose to almost twice those of the control, being accompanied by the lack of convulsions. These results suggest a correlation between the decrease in spontaneous or K⁺, Ca²⁺-stimulated GABA...
Fig. 4. GABA released from synaptosomes of control mice and of mice treated with semicarbazide (SC), SC plus aminooxyacetic acid (SC+AOAA), and AOAA, respectively. Each result is the mean of at least three experiments ± standard deviation. Open histograms show spontaneous GABA efflux (corresponding to a+b in Fig. 4 A) and shaded histograms show K⁺, Ca²⁺-stimulated GABA efflux (corresponding to c in Fig. 4 A).

release from synaptosomes and the onset of convulsions by SC, and between the recovery in these GABA releases and the cessation of convulsions by AOAA.

The fact that there is a simple relationship leads to the assumption that a decrease in GABA release from the nerve endings, probably because of the reduction of GABA levels caused by the GAD inhibition, may result in an increased cerebral excitability (convulsion). Since it has been postulated that GABA is released into the synaptic cleft in a depolarized state of the GABA-dependent inhibitory nerve endings (13), the GABA release from a synaptosome, especially the K⁺, Ca²⁺-stimulated GABA release as described above, may well reflect the physiological synaptic events. The results described in the present paper, therefore, lead us to conclude that the convulsant effect of some drugs results from a decrease of GABA release from the nerve endings, where GABA is involved in the tonic or clonic inhibition on the postsynaptic neuron.

This work was supported by a grant from the Ministry of Education, Science and Culture of Japan.

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