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Effect of gabaculine on metabolism and release of γ-aminobutyric acid (GABA) formed from 4-aminobutyraldehyde in synaptosomes

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Previous work by the authors [1] has shown that peripherally administered 4-aminobutyraldehyde (ABAL*) readily penetrates the blood–brain barrier and is rapidly oxidized to γ-aminobutyric acid (GABA) by a brain enzyme showing ABAL dehydrogenase activity. Moreover, it has been shown that synaptosomes isolated from the brain oxidize ABAL to form GABA and release this GABA in response to stimulation.

It has also been reported by the authors [2] that net GABA uptake from the medium into synaptosomes is increased in the presence of gabaculine [an inhibitor of GABA transaminase (GABA-T)], whereas net synaptosomal synthesis of GABA from glutamate is not affected by this agent, and that the increase in GABA uptake results in an increase in GABA released by a Ca^{2+}-independent mechanism, but not by a Ca^{2+}-dependent one. These findings raise the possibility that GABA may exist in different compartments with different metabolic functions.

The aim of this study was to characterize the effect of gabaculine on the content and release of GABA synthesized from ABAL in synaptosomes and to compare it with the effect of this agent on GABA synthesized from glutamate and GABA captured in synaptosomes.

Materials and Methods

Materials. [2,3-^{3}H]GABA, [^{14}C(U)]glutamate and [2,3-^{3}H]putrescine dihydrochloride were purchased from the New England Nuclear Corp. ABAL diethylacetal was obtained from the Aldrich Chemical Co. Gabaculine was purchased from the Calbiochem Corp., and other reagents were from the Nakarai Chemical Corp.

Preparation of unlabeled ABAL. Water (4.3 mL) and 2 M HCl (0.3 mL) were added to 400 mg (2.23 mmol) of ABAL diethylacetal, and the solution was incubated for 10 min at room temperature. To remove ethylalcohol (one of the reaction products) from the hydrolysis mixture, a portion (1 mL) of the mixture was applied to a Dowex 50 (H^+) column (5 × 15 mm). The column was washed with 2 mL of water, and then ABAL was eluted with 7 mL of 1 M HCl [3]. The amount of ABAL was determined by the method of Jakoby and Fredericks [4]. Briefly, a portion of the eluate was mixed with 0.3 mL of 0.1% *o*-aminobenzaldehyde and 0.2 mL of 1 M potassium phosphate buffer (pH 7.2), and the volume was adjusted to 2.0 mL with water. The mixture was incubated at room temperature for 30 min. The formation of 2,3-trimethylene-1,2-dihydroquinazolium was measured at 435 nm ($\varepsilon_{435} = 1.86 \times 10^3$). The yield of ABAL was about 95%.

Preparation of [^{3}H]*ABAL.* [^{3}H]Putrescine dihydrochloride (25.8 nmol, 1 mCi) was incubated at pH 7.0 with 170 mUnits of diamine oxidase (0.5 units/mg protein) purified from pig kidneys by the procedure of Yamada *et al.* [5] for 60 min at 37° in a final volume of 1.2 mL. After incubation, the reaction mixture was acidified with 2 M HCl to a final concentration of 0.1 M in order to terminate the reaction, and applied to a Dowex 50 (H^+) column (5 × 10 mm). After washing with 2 mL of water, the [^{3}H]-ABAL that had formed was eluted with 7 mL of 1 M HCl, and then the remaining [^{3}H]putrescine was eluted with 2 mL of 6 M HCl. [^{3}H]ABAL was obtained from [^{3}H] putrescine at a yield of about 82% [3].

Preparation of synaptosomes from mouse brain. Adult male albino mice (DDY strain) weighing about 25 g were used throughout the experiments. Crude mitochondrial fraction of brain was prepared as described by Gray and Whittaker [6], and the synaptosomal fraction was subsequently separated from the crude mitochondrial fraction according to the method of Sellström *et al.* [7] with minor modification [8].

Assay of GABA synthesized from ABAL in synaptosomes. Formation of GABA from ABAL in synaptosomes was estimated by measuring the [^{3}H]GABA formed from [^{3}H] ABAL. Synaptosomes (200 mg of original tissue) were suspended in 0.9 mL of Ca^{2+}-free Krebs–Ringer bicarbonate buffer (Ca^{2+}-free KRB) consisting of 128 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 1.3 mM $MgSO_4$, 26 mM $NaHCO_3$, 10 mM glucose, and 0.1 mM ethyleneglycolbis(aminoethylether)tetra-acetate (EGTA) at pH 7.4 (aeration with 5% CO_2 and 95% O_2 at 37°). The suspension was incubated with 0.1 mL of 10 μM [^{3}H]ABAL (0.2 μCi) in the same buffer at 37° for the intervals shown in each figure (up to 60 min). After incubation, the synaptosomes were pelleted by centrifugation at 20,000 *g* for 10 min. Radioactive substances in the pellet were extracted with 1 mL of 1 M perchloric acid. The extract was adjusted to pH 4 with KOH and applied to a Dowex 50 (Na^+) column (5 × 15 mm) in order to isolate the [^{3}H]GABA that had formed. After washing with 3 mL of 10 mM acetate buffer, [^{3}H]GABA was eluted with 3 mL of 0.1 M sodium phosphate buffer (pH 6.5), and then [^{3}H]ABAL was eluted with 2 mL of 6 M HCl. [^{3}H]GABA was assayed by counting the eluate in a scintillation spectrometer [3].

Assays of GABA synthesized from glutamate and taken up in synaptosomes. The synthesis of GABA in synaptosomes was estimated by measuring [^{14}C]GABA formed from [^{14}C]glutamate [2]. Synaptosomes (200 mg of original tissue) were suspended in 0.9 mL of Ca^{2+}-free KRB, and the suspension was incubated with 0.1 mL of 10 μM [^{14}C]glutamate (0.5 μCi) in the same buffer at 37° for the intervals shown in each figure. After incubation, the mixture was poured into 4 mL of ice-cold Ca^{2+}-free KRB, and the synaptosomes were pelleted by centrifugation at 20,000 *g* for 10 min. Radioactive substances in the pellet were extracted with 1 mL of 1 M perchloric acid. The extract was neutralized with KOH to about pH 7 and subjected to a Dowex 1 (CH_3COO^-) column (5 × 40 mm) to remove the substrate [^{14}C]glutamate and [^{14}C]acidic products. The [^{14}C]GABA that had formed was initially eluted with 3 mL of water and assayed by counting the eluate in a scintillation spectrometer.

Uptake of GABA into synaptosomes was assayed using [^{3}H]GABA [3]. Synaptosomes (200 mg of original tissue) suspended in 0.9 mL of Ca^{2+}-free KRB were incubated with 0.1 mL of 10 μM [^{3}H]GABA (0.2 μCi) in the same buffer at 37° for the intervals shown in each figure. After incubation, the mixture was poured into 4 mL of ice-cold Ca^{2+}-free KRB, and the synaptosomes were pelleted by centrifugation at 20,000 *g* for 10 min. Radioactive substances in the pellet were extracted with 1 mL of 1 M perchloric acid. The extract was adjusted with KOH to pH 4 and subjected to a Dowex 50 (H^+) column (5 × 15 mm) to

* Abbreviations: ABAL, 4-aminobutyraldehyde; GABA, γ-aminobutyric acid; GABA-T, γ-aminobutyric acid transaminase; KRB, Krebs–Ringer bicarbonate; and GAD, glutamate decarboxylase.

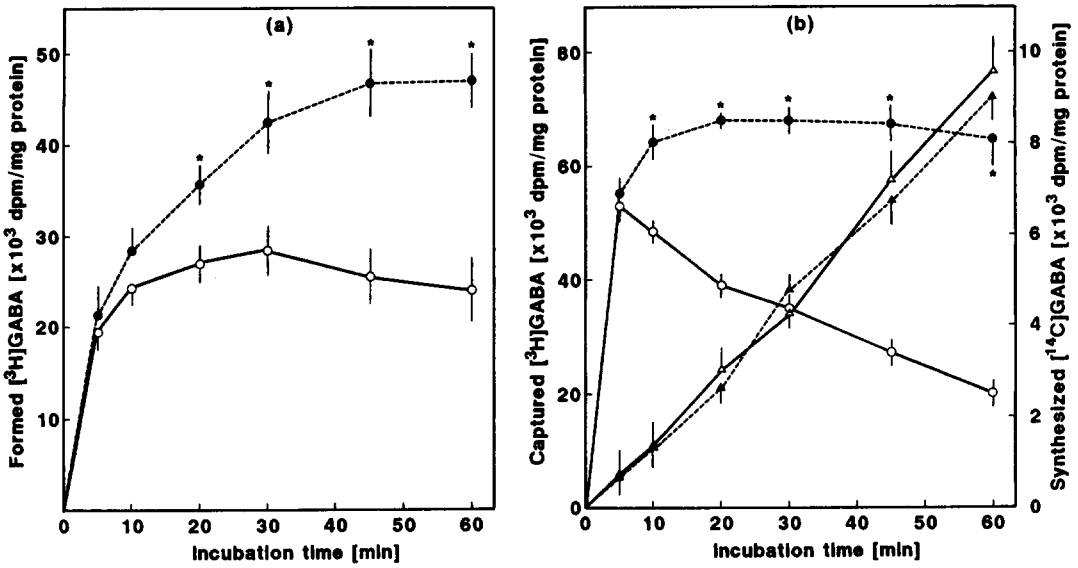

Fig. 1. Effect of gabaculine (1 μM) on the metabolism of GABA formed from ABAL (a), GABA captured from the medium and GABA synthesized from glutamate (b) by synaptosomes. Key: (a) [^{3}H]-GABA formed from [^{3}H]ABAL (0.2 μCi) in synaptosomes treated with (●) or without (○) gabaculine; (b) [^{3}H]GABA taken up from a medium containing [^{3}H]GABA (0.2 μCi) into synaptosomes treated with (●) or without (○) gabaculine, and [^{14}C]GABA synthesized from [^{14}C]glutamate (0.5 μCi) in synaptosomes treated with (▲) or without (△) gabaculine. The amount of synaptosomal protein used for the experiment was 4.71 ± 0.35 mg. Bars show ± SD of three or four independent experiments. An asterisk (*) indicates a statistically significant difference from the control ($P < 0.01$).

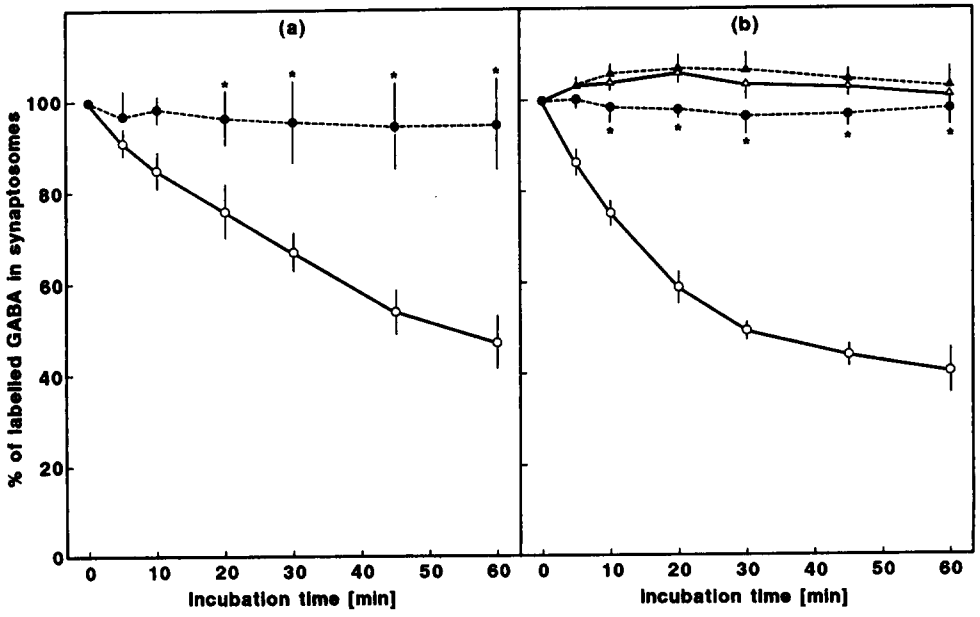

Fig. 2. Effect of gabaculine (1 μM) on the metabolism of preloaded GABAs in synaptosomes. (a), Gabaculine was added (●) or was not added (○) to synaptosomes preloaded with [^{3}H]GABA formed from [^{3}H]ABAL at 37° for 10 min. (b) Gabaculine was added (●, ▲) or was not added (○, △) to synaptosomes containing [^{3}H]GABA taken up from the medium or [^{14}C]GABA synthesized from [^{14}C]-glutamate at 37° for 10 min, respectively. The amount of synaptosomal protein used for the experiment was 4.63 ± 0.33 mg. Bars show ± SD of three independent experiments. An asterisk (*) indicates a statistically significant difference from the control ($P < 0.01$).

remove [^{3}H]acidic products. After washing with 3 mL of water, [^{3}H]GABA was eluted with 3 mL of 2 M ammonia solution and assayed by counting the eluate in a scintillation spectrometer.

Metabolism of radioactive GABAs. Synaptosomes preloaded with [^{3}H]GABA formed from [^{3}H]ABAL, [^{14}C]GABA synthesized from [^{14}C]glutamate, or [^{3}H]GABA taken up from the medium were pelleted by centrifugation at 20,000 *g* for 10 min. The pellets (200 mg of original tissue) were resuspended in 1 mL of Ca^{2+}-free KRB and incubated at 37° in the presence or absence of 1 μM gabaculine, and the amount of radioactive GABA remaining without degradation was assayed by the above procedures.

Perfusion experiment for release of GABA from synaptosomes. This experiment was carried out following the method of Raiteri *et al.* [9] with minor modification [2, 10]. Synaptosomes (200 mg of original tissue) containing radioactive GABA ([^{3}H]GABA formed from [^{3}H]ABAL or [^{14}C]GABA synthesized from [^{14}C]glutamate, or [^{3}H]GABA taken up from the medium) were used for the perfusion experiments [2]. The perfusion rate was 0.5 mL/min. At 16 min after the beginning of perfusion, the synaptosomes were stimulated by either 2 mL of Ca^{2+}-free high K^+ KRB (Ca^{2+}-free KRB replaced with 51 mM KCl for an equivalent amount of NaCl) to estimate K^+-stimulated, Ca^{2+}-independent GABA release, or 2 mL of high K^+ KRB (same as Ca^{2+}-free high K^+ KRB except that EGTA was omitted and 2.5 mM $CaCl_2$ substituted for an equivalent amount of NaCl) to estimate K^+-stimulated, Ca^{2+}-dependent GABA release. The radioactivity of GABA in perfusate collected for 16 min after the beginning of stimulation was assayed.

Determination of protein. Protein was measured by the method of Lowry *et al.* [11].

Results and Discussion

Effect of gabaculine on the metabolism of various GABAs in synaptosomes. Figure 1 shows that gabaculine clearly increased the net amount of synaptosomal GABA synthesized from ABAL or taken up from the medium, but had no effect upon that synthesized from glutamate.

When synaptosomes preloaded with radioactive GABA synthesized from ABAL or glutamate or taken up from the medium were incubated in the absence of gabaculine, GABA synthesized from ABAL and GABA taken up from the medium were rapidly degraded, though the degradation rate of the former GABA was somewhat less than that of the latter. In the presence of 1 mM gabaculine, however, both degradations were inhibited completely (Fig. 2). In contrast to these GABAs, GABA synthesized from glutamate showed little degradation, not only in the presence of gabaculine, but also in its absence (Fig. 2b).

Since gabaculine is known to be a specific irreversible inhibitor of GABA-T [12, 13], the above results may be explained by presuming that both GABA synthesized from ABAL and GABA captured from the medium are in a compartment exposed to the action of GABA-T, whereas GABA synthesized from glutamate is in a compartment not so exposed.

A previous study in this laboratory [14] showed that there are probably at least two GABA pools in synaptosomes. One pool (GABA-T pool) contains GABA captured from the medium and is associated with GABA-T, while the other pool (GAD pool) contains GABA synthesized from glutamate by glutamate decarboxylase (GAD) and is not associated with GABA-T. The results of the present study suggest that ABAL is taken up from the medium into synaptosomes and rapidly converted to the GABA that is present in the GABA-T pool.

ABAL, unlike GABA, may be taken up into various kinds of synaptosomes and be converted to GABA by the ABAL dehydrogenase in them. Such a process occurring in synaptosomes that do not contain GABA-T, as well as in those that do, might explain the slower rate of degradation of GABA derived from ABAL as compared with that of captured GABA (Fig. 2).

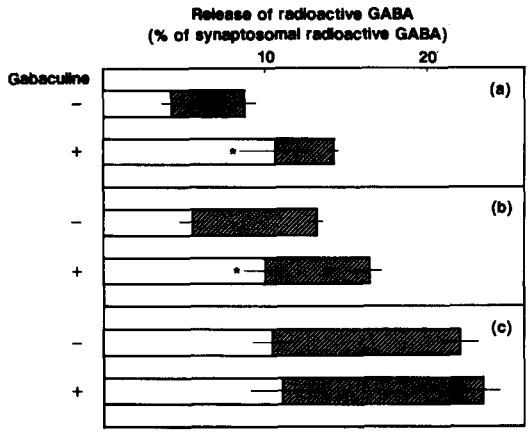

Fig. 3. Effect of treatment with gabaculine (1 μM) on Ca^{2+}-independent (open columns) and Ca^{2+}-dependent (hatched columns) release of [^{3}H]GABA formed from [^{3}H]ABAL (a), [^{3}H]GABA captured from the medium (b), and [^{14}C]GABA synthesized from [^{14}C]glutamate (c). The amount of Ca^{2+}-independent release was calculated by the difference between K^+-stimulated Ca^{2+}-independent release and spontaneous release, and the amount of Ca^{2+}-dependent release was calculated by the difference between K^+-stimulated Ca^{2+}-dependent release and K^+-stimulated Ca^{2+}-independent release, each value measured in a perfusate collected for 16 min after the beginning of stimulation. Levels of GABA formed from ABAL, captured GABA, and GABA synthesized from glutamate in synaptosomes before perfusion experiments were as follows (dpm/mg protein); (a) control, 45,500 ± 4,400; +gabaculine, 61,900 ± 6,200; (b) control, 37,100 ± 2,700; +gabaculine, 64,000 ± 5,600; (c) control, 14,600 ± 700; +gabaculine, 14,400 ± 1,800; (mean ± SD of three experiments). Bars show ± SD of three experiments. The amount of synaptosomal protein used for the experiment was 4.63 ± 0.33 (a) and 4.85 ± 0.38 mg (b, c). An asterisk (*) indicates a statistically significant difference from the control ($P < 0.01$).

Influence of gabaculine on release of various GABAs from synaptosomes. Synaptosomes that had been preincubated at 37° for 10 min in the presence or absence of 1 μM gabaculine and further incubated for 10 min with 1 μM [^{3}H]ABAL (0.5 μCi), 1 μM [^{14}C]glutamate (2 μCi) or 1 μM [^{3}H]GABA (0.5 μCi) were used as synaptosome beds for perfusion experiments. Ca^{2+}-independent release and Ca^{2+}-dependent release of radioactive GABAs were assayed and calculated according to the procedure described in the legend to Fig. 3. The experimental results are summarized in Fig. 3. The Ca^{2+}-independent release of GABA synthesized from ABAL and GABA captured from the medium was increased greatly by gabaculine treatment, whereas the same release of GABA synthesized from glutamate was not affected by this treatment. Gabaculine did not influence the Ca^{2+}-dependent release of any type of GABA.

If total (Ca^{2+}-dependent plus Ca^{2+}-independent) release was calculated as a percent of the radioactive GABA in the synaptosomes before perfusion, the values obtained on GABA derived from ABAL, from the medium and from glutamate were about 8.8, 13.2 and 22.5%, respectively.

A previous report by the authors [2] suggested that GABA in the GABA-T pool was degraded by GABA-T and released by a Ca^{2+}-independent mechanism, whereas GABA in the GAD pool was not in contact with GABA-T and was released by both a Ca^{2+}-independent and a Ca^{2+}-dependent mechanism. Furthermore, we argued that any movement of GABA between the pools was from the GABA-T pool to the GAD pool, with the movement unaffected by the size of the GABA-T pool. The results of the present study indicate that, like GABA captured from the medium, GABA derived from ABAL in the GABA-T pool is also subjected to active degradation by GABA-T, to release by a Ca^{2+}-independent mechanism, and to transport into the GAD pool.

The slightly lower release of GABA derived from ABAL compared with that of GABA taken up from the medium may be explained in the same way as its slightly lower degradation rate, that is, by presuming that ABAL is oxidized to GABA not only in synaptosomes that release GABA but also in synaptosomes that do not.

In summary, we report here that GABA formed from ABAL in synaptosomes was accumulated in a GABA-T pool and released in response to stimulation in the same way as GABA taken up from the medium, whereas GABA synthesized from glutamate went into a different pool. The physiological actions of GABA formed from ABAL in brain may be similar to those of GABA taken up from synaptic clefts.

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Protective effect of α-tocopherol, ascorbic acid and rutin against peroxidative stress induced by oxidized lipoproteins on lymphoid cell lines

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Lipid peroxides and oxygen reactive species are involved in major physiological or pathological events. The oxidative stress against unsaturated phospholipids can result in severe structural and functional damages in cell membranes [1] as demonstrated in the case of ischemia and vascular diseases [2–4].

Various antioxidants have been used for a long time in medical therapy of chronic venous insufficiency. The