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Effects of bilobalide on γ-aminobutyric acid levels and glutamic acid decarboxylase in mouse brain

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Abstract

We have previously demonstrated that bilobalide, a constituent of the *Ginkgo biloba* extract, possesses anticonvulsant activity, and suggested that the mechanism of its anticonvulsant action involves modulation of γ-aminobutyric acid (GABA)-related neuronal transmission. This study examined the effects of bilobalide on the level of GABA and glutamate, the activity and the amount of glutamic acid decarboxylase (EC 4.1.1.15), and the function of $GABA_A$ receptors in the hippocampus, cerebral cortex and striatum of the mouse. GABA levels, glutamic acid decarboxylase activity, and the protein amount of 67 kDa glutamic acid decarboxylase in the hippocampus of mice treated with bilobalide (30 mg/kg, p.o., once a day for 4 days) were significantly higher than those in controls. However, there were no significant differences in glutamate levels or, the number and the dissociation constants of $GABA_A$ receptors in the hippocampus between control and bilobalide-treated mice. These results suggest that the anticonvulsant effect of bilobalide is due to elevation of GABA levels, possibly through potentiation of glutamic acid decarboxylase activity and enhancement of the protein amount of 67 kDa glutamic acid decarboxylase by bilobalide.

Keywords: Bilobalide; (*Ginkgo biloba* L.); γ-Aminobutyric acid; Glutamic acid decarboxylase; Anticonvulsant activity

1. Introduction

An extract of *Ginkgo biloba* L. leaves has been clinically used for the treatment of certain cerebral dysfunctions and neurological disorders (DeFeudis, 1991). Furthermore, it has been demonstrated recently that the extract exerts beneficial effects on dementia of the Alzheimer type and multi-infarct dementia (le Bars et al., 1997). Although the mechanism of action of the *G. biloba* extract in the central nervous system has not been established, several experimental studies have indicated that the extract improves cerebral blood flow (Oberpichler et al., 1988), prevents the hypoxia-induced decrease of ATP levels in endothelial cells (Janssens et al., 1995), and reduces brain edema induced by triethyltin in rats (Otani et al., 1986).

The extract contains multiple compounds, e.g., flavonoids and terpenoids (ginkgolides and bilobalide), that are thought to contribute to the neuroprotective effects and the modulation of neurotransmission, which may be the basis for the effects of the extract in the central nervous system (DeFeudis, 1991; Krištofiková and Klaschka, 1997).

Bilobalide is a main constituent of the terpenoid fraction of the *G. biloba* extract (Drieu, 1986). It has been demonstrated that bilobalide inhibits hypoxia-induced decreases of ATP levels in endothelial cells (Janssens et al., 1995) and hypoxia-induced choline release from rat hippocampal slices (Klein et al., 1997). These effects of bilobalide, as well as the effects of ginkgolides, which are potent platelet-activating factor antagonists (Braquet et al., 1991), may be related to the neuroprotective properties of the extract in post-hypoxic cell damage (Klein et al., 1997). In contrast, the effects of bilobalide on neurotransmission in the central nervous system have been studied less extensively.

Recently, we demonstrated that bilobalide possesses anticonvulsant activity, although with a lower potency than phenobarbital, against convulsions induced by isoniazid, pentylenetetrazol, and 4-*O*-methylpyridoxine (Sasaki et al., 1995, 1997). Since these chemical convulsants have been reported to affect γ-aminobutyric acid (GABA)-related

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neuronal transmission and induce convulsions (Horton et al., 1979; Mizuno et al., 1980; de Deyn and Macdonald, 1989), it was assumed that the mechanism of anticonvulsant action of bilobalide involves modulation of GABA-mediated neuronal transmission. However, it remains to be demonstrated how bilobalide modulates GABA-mediated neuronal transmission.

The aim of the present study is to investigate the effects of bilobalide on the GABAergic system in the mouse brain. This could also provide information regarding the modulatory role of bilobalide in neuronal transmission in the central nervous system. For this purpose, we examined alterations in the GABA level, the activity and amount of the GABA synthesizing enzyme glutamic acid decarboxylase (EC 4.1.1.15), and $GABA_A$ receptor characteristics in the hippocampus, cerebral cortex, and striatum of bilobalide-treated mice.

2. Materials and methods

2.1. Treatment of mice with drugs

All experiments were conducted in accordance with guidelines established by the Committee for the Care and Treatment of Laboratory Animals of the Institute of Animal Experimentation of Sapporo Medical University.

Four-week-old male ddY strain mice (weighing 26–30 g), obtained from Sankyo Labo Service (Tokyo), were provided with standard food pellets (Oriental Yeast, Tokyo) and tap water ad libitum. Mice were acclimated at least one week before experiments under a 12-h light/dark cycle.

Bilobalide (30 mg/kg) or a vehicle (aqueous 2% gum Arabic solution) was administered orally to mice once a day for 4 successive days. Twenty-four hours after the last treatment, mice treated with the vehicle were administered valproate (300 mg/kg, i.p.) (valproate-treated group) or physiological saline (as a vehicle)(control group) and bilobalide-treated mice were administered physiological saline (bilobalide-treated group). They were killed 30 min later.

In some experiments, isoniazid (250 mg/kg, i.p.), a chemical convulsant, was administered to bilobalide-treated or vehicle-treated mice and mice were killed 40 min later.

2.2. Assay of GABA and glutamic acid levels

To prevent postmortem increases in GABA levels, the mice were injected with 3-mercaptopropionic acid (100 mg/kg, i.p.) (van der Heyden and Korf, 1978) and sacrificed 2.5 min later. The brain was quickly removed and the hippocampus, cortex, and striatum were dissected on ice as described by Heffner et al. (1980), and stored at −80°C until assayed.

On the day of the assay, brain slices were homogenized in ice-cold 0.2 N perchloric acid. The homogenate was centrifuged at $15{,}000 \times g$ for 10 min at 4°C and the supernatants containing GABA and glutamate were analyzed using high performance liquid chromatography (HPLC) (Model LA-6A chromatographic system, Shimadzu Seisaku-sho, Kyoto; Eicompak MA-5ODS reverse phase column, Eicom, Kyoto) and fluorescent detection (RF-535 spectro-flurometric detector, Shimadzu Seisakusho)after precolumn derivatization with *o*-phthaldialdehyde as described by Lindroth and Mopper (1979) with some modifications.

Two eluents were used with a flow rate of 1.0 ml/min: 0.1 M sodium phosphate (pH 6.75) containing 50% methanol for the GABA elution and 0.1 M sodium phosphate (pH 6.75) containing 47% methanol for the glutamate elution. The excitation and emission wavelengths were 330 and 440 nm, respectively.

2.3. Assay of glutamic acid decarboxylase activity

Brain tissue was homogenized in 10 volumes of ice-cold 50 mM potassium phosphate (pH 6.4), containing 1 mM aminoethylisothiouronium bromide and 0.2 mM pyridoxal 5′-phosphate. Glutamic acid decarboxylase activity in homogenates was measured according to the methods described by Lowe et al. (1958). Homogenate (0.2–0.4 mg protein) was incubated in 0.2 ml of medium containing 0.1 M potassium phosphate (pH 6.4), 25 mM glutamic acid, and 0.2 mM pyridoxal phosphate. After incubation at 37°C for 60 min, the reaction was quenched by the addition of 0.1 ml of ice-cold 10% trichloroacetic acid, and the mixture was kept on ice. The precipitated proteins were removed by centrifugation, and the GABA formed in the glutamic acid decarboxylase reaction was measured by the HPLC method described above. For tissue blanks, 0.1 ml of ice-cold 10% trichloroacetic acid was added prior to incubation.

2.4. Immunoblotting of 67 and 65 kDA glutamic acid decarboxylase

Hippocampus was homogenized in 10 volumes of ice-cold 20 mM HEPES (pH 7.4) containing 0.3 mM phenylmethylsulfonyl fluoride and 1 mM dithiothreitol. Hippocampal homogenates (20 μg of protein/lane) were dissolved in 3% Laemmli sample buffer with 50 mM dithiothreitol and subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Protein was transferred to nitrocellulose filters as described by Towbin et al. (1979). After transfer, filters were blocked by a 2-h incubation with 3% bovine serum albumin in a buffer of 10 mM Tris (pH 7.5), 500 mM NaCl, and 0.1% Tween 20 (TBS-T). Nitrocellulose filters were then incubated with

TBS-T containing 0.1% bovine serum albumin and polyclonal rabbit antiserum against 67 kDa glutamic acid decarboxylase (AB-108, Chemicon Int.) at 1:2000 dilution or a monoclonal mouse antibody against 65 kDa glutamic acid decarboxylase (GC3208, Affiniti Res. Prod.) at 1:5000 dilution. After incubation for 2 h at room temperature, filters were washed in TBS-T three times and then incubated with TBS-T containing 0.1% bovine serum albumin and horseradish peroxidase-linked anti-rabbit immunoglobulin($F(ab')_2$) (Amersham) at 1:5000 dilution for 2 h at room temperature. Filters were washed three times with TBS-T and immunoreactivity was detected with an enhanced chemiluminescence (ECL) system (Amersham) using ECL HYPER film (Amersham). The immunoreactive bands were quantified with the Macintosh-based image analysis program NIH Image (version 1.58) using a Macintosh computer (Power Macintosh 6100/66) and a flat-bed scanner.

2.5. $GABA_A$ receptor binding

Synaptic membrane fractions were prepared from the hippocampus as described by Zukin et al. (1974) with some modifications. Hippocampus was homogenized in 10 volumes of ice-cold 0.32 M sucrose buffer, and the homogenates were centrifuged at $1000 \times g$ for 10 min at 4°C. Supernatants were collected and centrifuged at 20,000 $\times g$ for 20 min at 4°C. Pellets were resuspended in distilled water and centrifuged at $8000 \times g$ for 20 min at 4°C. The supernatant and pellet upper layer were collected and centrifuged at $30{,}000 \times g$ for 30 min at 4°C. The pellet was resuspended in 3 ml of 50 mM Tris-citrate buffer (pH 7.1) and centrifuged two times at $30{,}000 \times g$ for 30 min at 4°C and stored at −80°C for at least 18 h. The frozen pellets were resuspended in 50 mM Tris-citrate buffer (pH 7.1) and centrifuged at $30{,}000 \times g$ for 30 min at 4°C. The final pellet was resuspended in the same buffer and used for the following $GABA_A$ receptor binding assay.

[^{3}H]Muscimol agonist binding to $GABA_A$ receptors in hippocampal membranes was performed as described previously (Toki et al., 1996). Membranes (~200 μg protein) were incubated with [^{3}H]muscimol (5–300 nM) at 2°C for 30 min in 50 mM Tris-citrate buffer (pH 7.1) in a total volume of 0.25 ml. The reaction was quenched by the addition of ice-cold 50 mM Tris-citrate buffer. Bound and free ligands were separated by rapid vacuum filtration (Brandel Cell Harvester M 24R) on Whatman GF/B filters. The filters were washed three times with 3 ml of the same ice-cold buffer, and bound radioactivity was quantified in a Beckman LS 5801 counter. Specific binding was defined as the difference between [^{3}H]muscimol binding in the absence and presence of 0.1 mM muscimol. Binding parameters were analyzed to obtain the values of K_d and B_{max} by Scatchard transformation of the saturation curves adjusted using LIGAND (Munson and Rodbard, 1980).

2.6. Protein determination and statistics

Protein was determined according to the method of Bradford (1976) with bovine serum albumin as a standard. Statistical analyses were performed by the Tukey–Kramer test. P values of less than 0.05 were considered statistically significant.

2.7. Materials

[Methylene-^{3}H]Muscimol (740 GBq/mmol) was purchased from New England Nuclear (Tokyo). Bilobalide was isolated from *Ginkgo* extract provided by Japan Greenwave (Tokyo), as described previously (Wada et al., 1993) and identification was made by mass ^{1}H-nuclear magnetic resonance and ^{13}C-nuclear magnetic resonance spectral analyses. Bilobalide was suspended in aqueous 2% gum Arabic solution. Valproate and isoniazid were from RBI (Natick) and Sigma (St. Louis), respectively. Valproate, and isoniazid were dissolved in physiological saline. Muscimol hydrobromide was from Sigma. All other reagents used were of analytical or HPLC grade.

3. Results

3.1. Alteration in GABA and glutamate levels

Valproate has been shown to increase the GABA level in the brain in mice and rats, and in plasma of humans (Löscher, 1993). Treatment of mice with valproate resulted in significant elevation of GABA levels in the hippocampus, cerebral cortex, and striatum to 122, 153, and 127% of the control levels, respectively (Fig. 1). Similar to the results obtained with valproate, bilobalide significantly elevated GABA levels in the hippocampus and cerebral cortex to 111 and 118%, respectively, but not in the striatum. In contrast to the increase in GABA levels induced by bilobalide and valproate, there were no significant differences in the levels of glutamate in the three brain regions tested among control, bilobalide-treated, and valproate-treated mice (Fig. 1). The glutamate/GABA ratio was significantly reduced in the hippocampus and cerebral cortex of bilobalide-treated and valproate-treated mice compared with controls. Although no significant difference in the ratio of glutamate/GABA was observed in the striatum with the various treatments, the ratio in valproate-treated mice declined to 83% of that in the control.

3.2. Alteration in the activity and the protein amount of glutamic acid decarboxylase

GABA is synthesized from glutamate by glutamic acid decarboxylase, and the enzyme exists as two isoforms with

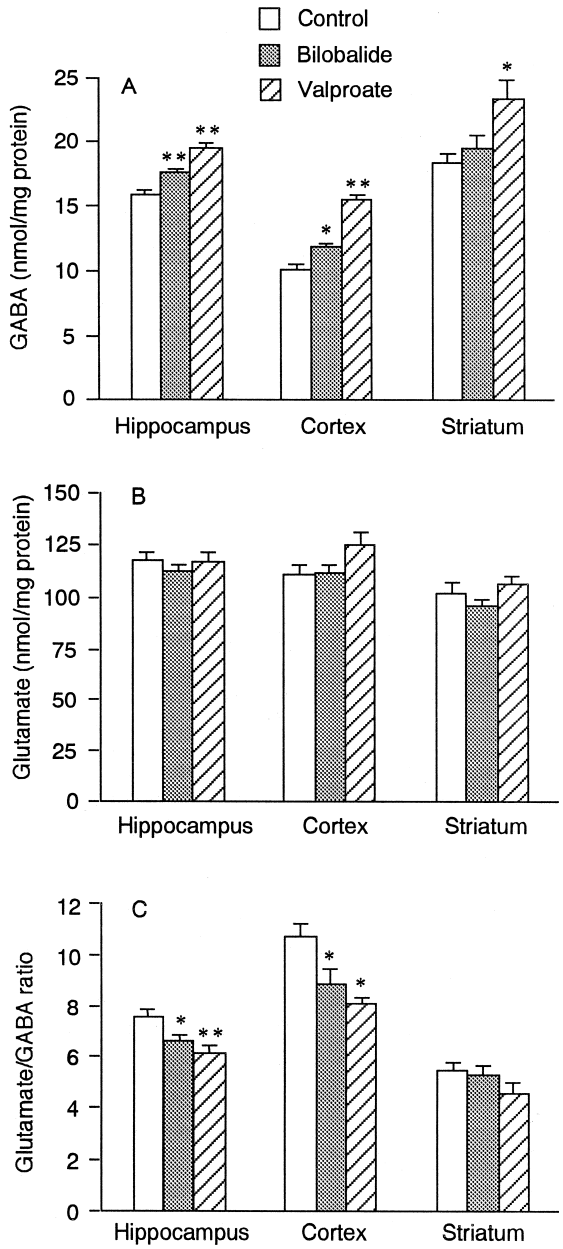

Fig. 1. The levels of GABA (A) and glutamate (B), and the ratio of glutamate/GABA (C) in the hippocampus, cerebral cortex, and striatum of control, bilobalide-treated, and valproate-treated mice. Mice were killed 24 h and 30 min after bilobalide (30 mg/kg, p.o., once a day for 4 days) and valproate (300 mg/kg, i.p.) administration, respectively. Results are means ± S.E. of seven to 11 separate experiments. Significant differences are indicated as follows: $* P < 0.05$ and $** P < 0.01$ compared with control.

molecular masses of 65 and 67 kDa (Erlander and Tobin, 1991; Erlander et al., 1991). As shown in Fig. 2, bilobalide treatment caused a significant enhancement of the total glutamic acid decarboxylase activity in the hippocampal and cerebral cortical homogenates to 118 and 112% of the control activity, respectively, while it did not potentiate the activity in the striatum. On the other hand, when bilobalide (0.1–100 μM) was added in vitro to hippocampal homogenates, it had no significant effect on the total glutamic acid decarboxylase activity (bilobalide 0 μM, 123.8 ± 6.3 nmol/mg protein/h; 0.1, 121.4 ± 7.4; 1.0, 122.9 ± 5.1; 10, 119.1 ± 6.5; 100, 127.9 ± 7.3). Valproate significantly enhanced the total glutamic acid decarboxylase activity in the hippocampus and striatum, but not in the cerebral cortex.

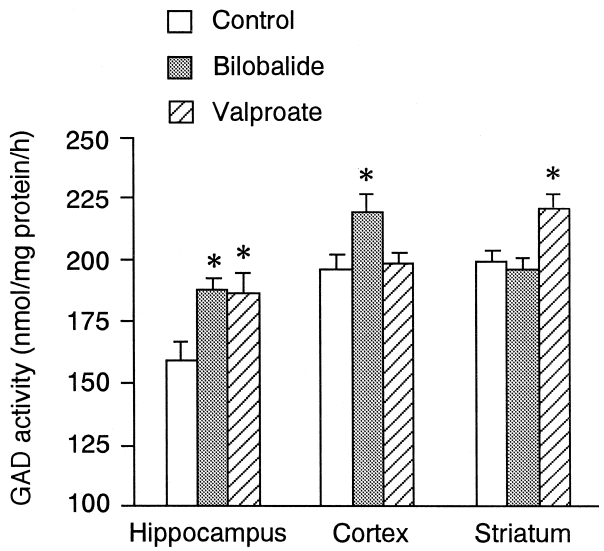

Fig. 2. Total glutamic acid decarboxylase activity in hippocampal, cerebral cortical, and striatal homogenates from control, bilobalide-treated, and valproate-treated mice. Results are means ± S.E. of six to nine separate experiments. Significant differences are indicated as follows: $* P < 0.05$ compared with control.

Fig. 3 shows representative immunoblots of 65 and 67 kDa glutamic acid decarboxylase in hippocampal homogenates from control, bilobalide-treated and valproate-treated mice, and Fig. 4 summarizes the results of the densitometric evaluation of the assay. The protein level of 67 kDa glutamic acid decarboxylase was significantly higher in bilobalide-treated mice than that in control mice, and tended to increase in valproate-treated mice, although not significantly. On the other hand, there were no signifi-

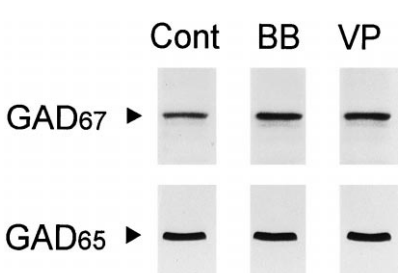

Fig. 3. Immunoblotting of 67 kDa glutamic acid decarboxylase (GAD_{67}) and 65 kDa glutamic acid decarboxylase (GAD_{65}) in hippocampal homogenates from control (Cont), bilobalide-treated (BB), and valproate-treated (VP) mice. The results are representative of five to six similar experiments. Immunoblotting was performed using antibodies AB-108 and GC3208 against 67 kDa glutamic acid decarboxylase and 65 kDa glutamic acid decarboxylase, respectively, as described in Section 2.

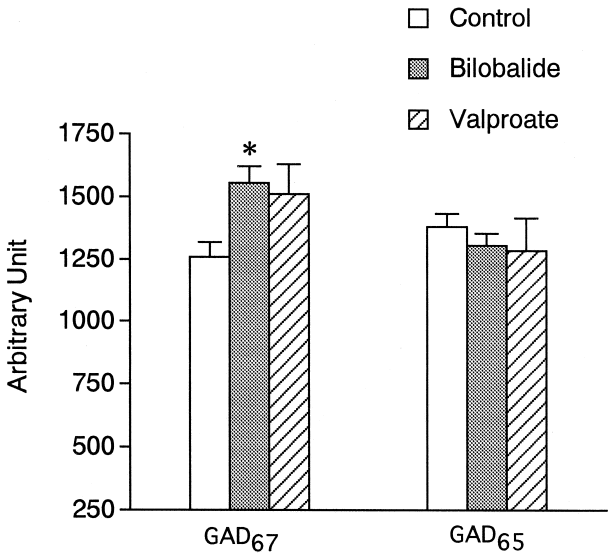

Fig. 4. Comparative levels of 67 kDa glutamic acid decarboxylase (GAD_{67}) and 65 kDa glutamic acid decarboxylase (GAD_{65}) protein in hippocampal homogenates from control and bilobalide-treated mice. Data are expressed as optical density values (arbitrary units), and are shown as means ± S.E. of five to six separate experiments. Significant differences are indicated as follows: $* P < 0.05$ compared with control.

cant differences in the protein level of 65 kDa glutamic acid decarboxylase among control, bilobalide-treated, and valproate-treated mice.

3.3. $GABA_A$ receptor binding

To investigate whether bilobalide affects the functions of $GABA_A$ receptors, [^{3}H]muscimol binding was performed in hippocampal membranes from control and bilobalide-treated mice. Analysis of [^{3}H]muscimol binding to the $GABA_A$ receptor with Scatchard transformation revealed two binding sites with high and low affinities. Both in high- and low-affinity sites of [^{3}H]muscimol binding, no significant differences were found in the maximum number of binding sites (B_{max}) or in the dissociation constant (K_d) between control and bilobalide-treated mice (Table 1).

Table 1
[^{3}H]Muscimol binding to $GABA_A$ receptors in hippocampal membranes from control and bilobalide-treated mice

Group	High affinity		Low affinity	
	B_{max}	K_d	B_{max}	K_d
Control	2.46 ± 0.19	8.41 ± 1.18	10.56 ± 0.78	374.1 ± 10.1
Bilobalide	2.54 ± 0.68	8.10 ± 3.62	10.51 ± 1.38	349.5 ± 10.7

B_{max} and K_d values were determined by Scatchard transformation of the saturation curves adjusted using LIGAND. B_{max} and K_d are expressed as pmol/mg protein and nM, respectively. The values are means ± S.E. of three experiments using separate membrane preparations, each of which used pooled hippocampal membranes from four to five mice.

3.4. Effects of bilobalide on GABA and glutamate levels in isoniazid-treated mice

Isoniazid is known to reduce the GABA concentration in the mouse brain through its inhibition of glutamic acid

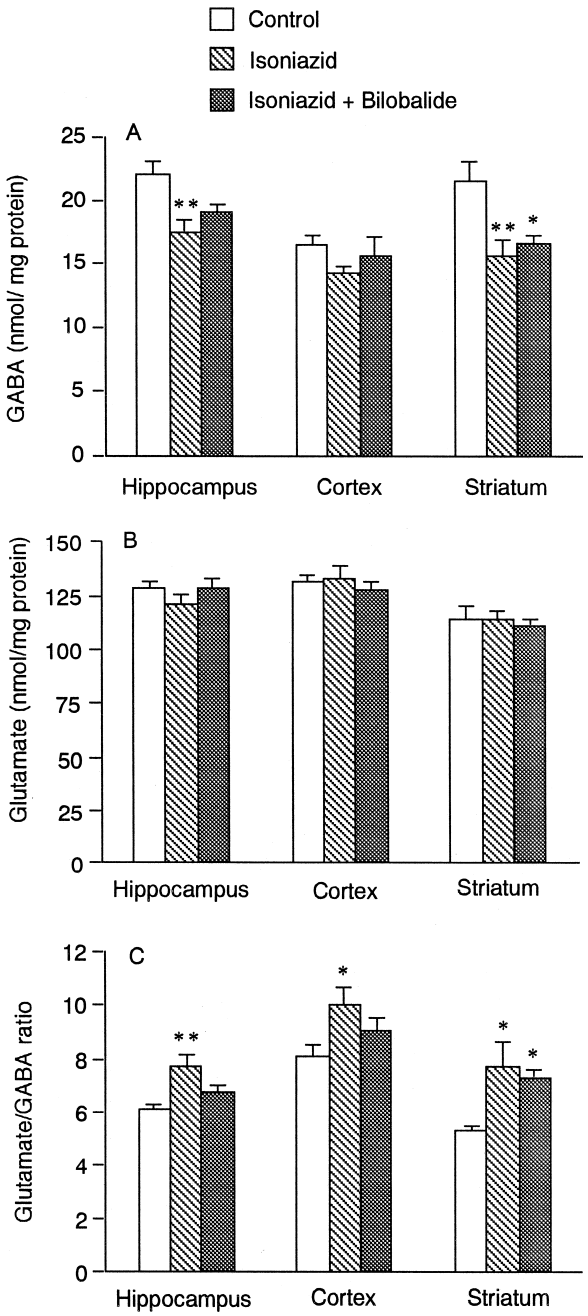

Fig. 5. Effects of the bilobalide pretreatment on the isoniazid-induced changes in the levels of GABA (A) and glutamate (B), and the ratio of glutamate/GABA (C) in the hippocampus, cerebral cortex, and striatum. Isoniazid (250 mg/kg, i.p.) was injected into mice 24 h after the administration of bilobalide (30 mg/kg, p.o.) or vehicle once a day for 4 days. In the control, mice were treated with vehicle once a day for 4 days and injected with vehicle instead of isoniazid. Mice were killed 40 min after isoniazid or vehicle administration. Results are means ± S.E. of five to nine separate experiments. Significant differences are indicated as follows: $* P < 0.05$ and $** P < 0.01$ compared with control.

decarboxylase and induce tonic-clonic convulsions (Horton et al., 1979). Fig. 5 shows the effects of bilobalide pretreatment on isoniazid-induced changes in GABA and glutamate levels in the hippocampus, cerebral cortex, and striatum of mice. Consistent with the results in Fig. 1, treatment of mice with bilobalide alone caused significant elevation of GABA levels and reduction in the glutamate/GABA ratio in the hippocampus and cerebral cortex, but not in the striatum (data not shown). Isoniazid caused a significant reduction in GABA levels of the hippocampus and striatum, to 79 and 72% of the control level, respectively. The cortical GABA level was also decreased by isoniazid (87%), although not significantly. Glutamate levels were not changed in control and isoniazid-treated mice, and consequently, the glutamate/GABA ratio was raised in isoniazid-treated mice compared with that in the control. Pretreatment of mice with bilobalide effectively prevented the isoniazid-elicited decrease in the GABA levels in the hippocampal and cerebral cortex toward control levels. However, bilobalide failed to prevent the reduction in the striatal GABA level in isoniazid-treated mice. On the other hand, bilobalide did not affect glutamate levels in the three brain regions compared with either control or isoniazid-treated mice. Consequently, in the hippocampus and cerebral cortex of isoniazid-treated mice, the increased ratio of glutamate/GABA was effectively suppressed toward control levels by bilobalide.

4. Discussion

It is thought that the generation of seizure activity results from an imbalance between excitatory and inhibitory processes, specifically, a reduction in inhibitory GABAergic transmission and an enhancement of glutamate-mediated excitation (McNamara, 1992). Glutamatergic transmission mediated through both *N*-methyl-D-aspartate (NMDA)-sensitive and non-NMDA receptors has been implicated in the induction of convulsions (Morimoto and Sato, 1992).

GABA is the major inhibitory neurotransmitter in the central nervous system and GABA synapses are present in virtually all brain structures. GABA may act to counter the glutamate-induced excitation. Thus, it seems generally accepted that impairment of GABAergic inhibitory neurotransmission can lead to convulsions, whereas potentiation of GABAergic transmission results in anticonvulsant effects (Löscher, 1989). Administration of drugs that antagonize GABA receptors often results in focal or generalized seizure, whereas drugs that elevate GABA levels in the brain, such as valproate, are used to treat various forms of epilepsy (de Deyn et al., 1990; Löscher, 1998).

Bilobalide is a sesquiterpene lactone that constitutes 3% of the standardized *G. biloba* extract (Chatterjee et al., 1986). In a previous study, we suggested that bilobalide possesses anticonvulsant activity and that the mechanism of its anticonvulsant action underlies GABA-mediated neurotransmission (Sasaki et al., 1995). In the present study, we demonstrated that treatment of mice with bilobalide increased GABA levels in the hippocampus and cerebral cortex, but not in the striatum (Fig. 1). Increased GABA levels were also found in the three brain regions of valproate-treated mice. Alterations in GABAergic function in brain regions such as the hippocampus and cerebral cortex, as well as the substantia nigra, are thought to be involved in epilepsy and anticonvulsant effects (Bradford and Peterson, 1987; Gale, 1989; Houser, 1991). It seems likely, therefore, that elevation of hippocampal and cortical GABA levels by bilobalide is involved in anticonvulsant effects of bilobalide, as has been suggested for the mechanism of anticonvulsant effects of valproate (Löscher, 1993).

In contrast to the results on GABA levels, there were no significant differences in glutamate levels in the three brain regions among control, bilobalide-treated, and valproate-treated mice (Fig. 1). Thus, glutamatergic excitatory neurotransmission in three brain regions did not seem to be affected by bilobalide and valproate. Since glutamate is a principle GABA precursor, a reduction in glutamate levels was expected due to increased GABA levels. The reason for the apparent absence of significant changes in glutamate levels may be the high concentration of glutamate relative to that of GABA (Fig. 1) and the complex nature of the glutamate metabolism and compartmentation (Martin and Rimvall, 1993). It has been previously shown that valproate has no significant effect on the glutamate level in the rat hippocampus (Biggs et al., 1992).

As mentioned above, the balance between excitatory and inhibitory transmitters, as well as their absolute concentrations in the brain, may be an important factor in the generation of convulsions. The present study demonstrated that the glutamate/GABA ratio was reduced in the hippocampus and cerebral cortex of bilobalide-treated and valproate-treated mice compared with controls (Fig. 1). These results may indicate that in the hippocampus and cerebral cortex, bilobalide elicits an increase in the activity of GABAergic inhibitory transmission relative to glutamatergic excitatory transmission, providing favorable conditions for prevention of convulsions. Furthermore, bilobalide counteracted the isoniazid-induced reduction in GABA levels and elevation in the ratio of glutamate/GABA toward the control levels in the hippocampus and cerebral cortex. This seems to be consistent with our previous findings that bilobalide effectively prevented isoniazid-induced convulsions in mice (Sasaki et al., 1995). In addition, reduction in GABA levels and elevation in the glutamate/GABA ratio elicited by 4-*O*-methylpyridoxine, which induces convulsions by reducing the GABA concentration (Mizuno et al., 1980), was effectively suppressed by the pretreatment of mice with bilobalide (Sasaki et al., unpublished observation). Thus, these results further support the idea that the increase in GABA levels by bilobalide contributes to its anticonvulsive action.

GABA is synthesized principally from glutamate by glutamic acid decarboxylase (Martin and Rimvall, 1993), which uses pyridoxal 5′-phosphate (PLP) as a cofactor. Glutamic acid decarboxylase exists in at least two isoforms with molecular weights of 65 and 67 kDa, which are encoded by separate genes (Erlander et al., 1991). The expression of the two glutamic acid decarboxylase genes and consequently the activity and function of the two enzyme proteins appear to be controlled by different mechanisms. It has been suggested that 67 kDa glutamic acid decarboxylase is distributed throughout the cell body and in nerve terminals, whereas 65 kDa glutamic acid decarboxylase is preferentially located in nerve terminals (Erlander et al., 1991; Kaufman et al., 1991; Rimvall and Martin, 1994). Recent studies using mutant mice, whose 65 or 67 kDa glutamic acid decarboxylase is inactivated by targeted mutagenesis, have suggested that 65 kDa glutamic acid decarboxylase plays an important role in the susceptibility to seizures, whereas 67 kDa glutamic acid decarboxylase controls the level of GABA, which is required for the basal function of inhibitory neurotransmission (Asada et al., 1996, 1997; Kash et al., 1997).

Bilobalide treatment produced a significant potentiation in the total activity of glutamic acid decarboxylase in hippocampal and cortical homogenates, whereas it had no effect on the striatal total activity of the enzyme (Fig. 2). These results correlate well with bilobalide-induced elevation in GABA levels observed in the hippocampus and cerebral cortex. In addition, the protein amount of 67 kDa glutamic acid decarboxylase was found to be increased in the hippocampus of bilobalide-treated mice, although bilobalide exerted no significant effect on the amount of 65 kDa glutamic acid decarboxylase (Fig. 4). Taken together, these findings suggest that the increases in GABA levels induced by bilobalide may be due to the potentiation of glutamic acid decarboxylase activity and the enhancement of the 67 kDa glutamic acid decarboxylase protein level. Bilobalide added in vitro to hippocampal homogenates caused no significant effect on glutamic acid decarboxylase activity (Section 3). In addition, bilobalide required at least 24 h to exert significant anticonvulsant activity (Sasaki et al., 1997). Thus, potentiation of glutamic acid decarboxylase activity by bilobalide appears to be a secondary effect rather a direct action of bilobalide on the enzyme, although the mechanisms are not clear at present. Further investigation will be required to achieve a more complete understanding of this.

Brain GABA level is controlled not only by glutamic acid decarboxylase, but also GABA transaminase (EC 2.6.1.19), which catalyzes the degradation of GABA to succinic semialdehyde. In our preliminary experiments, it was observed that bilobalide treatment resulted in an inhibition of GABA transaminase activity in the hippocampus, but not in the cerebral cortex and striatum (Sasaki et al., unpublished observation). The inhibition of GABA transaminase activity caused by bilobalide treatment might in part be responsible for the increase in hippocampal GABA levels.

Valproate potentiated the total glutamic acid decarboxylase activity in the hippocampus and striatum, where it caused a significant increase in GABA levels. On the other hand, valproate had no significant effect on the cortical glutamic acid decarboxylase activity (Fig. 2) consistent with a previous report (Phillips and Fowler, 1982). Thus, the increase in the cortical GABA level induced by valproate may result from inhibition of GABA transaminase (Phillips and Fowler, 1982; Löscher, 1993).

It was previously shown that chronic treatment with the *G. biloba* extract increased the numbers of 5-hydroxytryptamine$_{1A}$ receptors, α_2-adrenoceptors, and muscarinic receptors in the brain in aged rats, but not in young rats (Taylor, 1986; Huguet and Tarrade, 1992; Huguet et al., 1994). However, there has been no report with respect to the effect of bilobalide on the function of neurotransmitter receptors. The present results demonstrated that treatment of mice with bilobalide for 4 days did not alter either the number (B_{max}) or the dissociation constant (K_d) of $GABA_A$ receptors in the hippocampus of mice (Table 1). This suggests that the function of the $GABA_A$ receptor itself was not affected by bilobalide treatment.

In summary, the present study demonstrates, for the first time, that bilobalide elevates GABA levels in the hippocampus and cerebral cortex of mice, possibly through potentiation in glutamic acid decarboxylase activity and enhancement in the protein amount of 67 kDa glutamic acid decarboxylase by bilobalide. Such an increase in GABA levels by bilobalide is likely to be responsible for its anticonvulsant effects against certain types of convulsions. Some studies have suggested that enhancement of GABAergic transmission in the hippocampus is beneficial for protection against ischemic neuronal cell damage (Johansen and Diemer, 1991; Schwartz et al., 1994). Thus, potentiation of GABAergic neurotransmission by bilobalide could also contribute to the neuroprotective properties of the *G. biloba* extract.

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