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GABAergic modulation of hippocampal glutamatergic neurons: an in vivo microdialysis study

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Abstract

We have demonstrated the effects of activation of presynaptic γ-aminobutyric acid (GABA) receptors on glutamate release using in vivo brain microdialysis. A dialysis probe inserted into the hippocampus CA2 area of freely moving rats was perfused with Ringers solution containing 100 mM potassium chloride (KCl) or 0.05 mM veratridine for 20 min. Extracellular concentrations of amino acids were monitored by measuring their levels in dialysates by high performance liquid chromatography (HPLC) fluorometry. Perfusion with depolarizing agents, such as KCl or veratridine, increased extracellular glutamate levels in the hippocampus. Pretreatment with 1 mM GABA, before perfusion with depolarizing agents, significantly suppressed the depolarizing agent-induced increase in glutamate levels. The $GABA_B$ receptor agonist baclofen (1 mM) also significantly inhibited the depolarizing agent-induced increase in glutamate levels, whereas the $GABA_A$ receptor agonist, muscimol, had no affect. Similarly, baclofen (0.5 mM) decreased the KCl (13.5 mM)-induced $^{45}Ca^{2+}$ influx into cortical synaptosomes to 57% of the level induced in the absence of baclofen. On the other hands, GABA did not affect the increases in glycine and taurine level by depolarizing agents. These results suggest that GABA modulates depolarization-evoked glutamate release in the hippocampus by inhibiting Ca^{2+} entry into neurons, an effect mediated by presynaptic $GABA_B$ receptors.

Keywords: GABA (γ-Aminobutyric acid); Glutamate release; Hippocampus; Microdialysis, brain; Baclofen; Ca^{2+} influx

1. Introduction

Glutamate is the principal excitatory neurotransmitter in the mammalian central nervous system (Nadler et al., 1976). Recent reports have suggested that glutamate plays an important role in the induction of long-term potentiation, a phenomenon considered to underlie the neurochemical basis of learning and memory (Ghijsen et al., 1992; Kullmann et al., 2000). It has also been shown that an excessive release of other amino acids may be involved in the pathogenesis of epileptic seizures (Flavin et al., 1994), and in neuronal damage after ischemia (Kemp et al., 1987; Babcock et al., 2002). Therefore, it is important to identify the mechanisms that regulate glutamate release and whether glutamate release can be modified by other neurotransmitter system.

It is known that γ-aminobutyric acid (GABA) is a major inhibitory neurotransmitter in the brain, with action at three receptor subtypes, namely $GABA_A$, $GABA_B$ and $GABA_C$ (Olsen, 1981; Bowery et al., 1980; Feigenspan and Bormann, 1994). $GABA_A$ receptors are ligand-gated ion channels, which form a complex of GABA- and benzodiazepine-binding sites and Cl^- channel, and are sensitive to bicuculline (Olsen, 1981). In contrast, $GABA_B$ receptors are insensitive to bicuculline and modulate K^+ (Nicoll et al., 1990) or Ca^{2+} channels via guanine nucleotide-binding proteins (Dutar and Nicoll, 1988; Brody and Yue, 2000). Activation of presynaptic $GABA_A$ and $GABA_B$ receptors has an inhibitory effect on neurotransmitter release. It has been shown that the release of endogenous glutamate from the cerebrocortical synaptosomes of rats (Pende et al., 1993) and hippocampus slice (Wheeler et al., 1994) is inhibited when $GABA_B$ receptors are activated. However, there are a few reports on the potential in vivo effects of GABA on glutamate release (Burke and Nadler, 1988; Rowley et al., 1995; Freund and Buzsaki,

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1996). In vivo brain microdialysis is a useful technique to monitor neurotransmitter release from a population of nerve terminals. The in vivo effects of GABA receptor agonists were studied using this technique to determine whether GABAergic modulation of glutamatergic release occurs in the hippocampus. Clarification of the in vivo relationship between GABAergic and glutamatergic neurons will further our understanding of the mechanisms underlying the development of neurological disorders and may assist in the development of novel drug therapies for these conditions. In our previous experiments, we have observed the down-regulation of $GABA_A$ and $GABA_B$ receptors during the dependence of phenobarbital and withdrawal (Tanaka et al., 1991). Furthermore, we have demonstrated with in vivo brain microdialysis that extracellular concentrations of excitatory amino acids in the hippocampus and frontal cortex, such as glutamate and aspartate, significantly increase during the withdrawal from phenobarbital (Tanaka et al., 1996, 1997). These findings suggested possible interaction between GABA receptor and glutamate release. Therefore, in the present study, we studied whether GABAergic modulation of glutamate release occurs in the hippocampus.

The critical determinant of neurotransmitter release is an increase in the intraneuronal calcium concentration following invasion of the nerve terminal by the action potential. A number of different types of voltage-gated calcium channels have been characterized, but surprisingly, little is known about their distribution and functional importance. Several types of Ca^{2+} channels in neurons have been shown to be involved in neurotransmitter release. For example, both ω-conotoxin GVIA, a selective and irreversible inhibitor of N-type voltage-sensitive calcium channels (VSCCs) (Doolley et al., 1987), and ω-agatoxin IVA, an inhibitor of P-type calcium channels (Mintz et al., 1992), have been reported to inhibit glutamate release from hippocampal slices (Burke et al., 1993), and synaptosomes (Luebke et al., 1993). Recently, ω-conotoxin MVIIC has been shown to block VSCCs and to inhibit synaptic transmission resistant to inhibition by either ω-conotoxin GVIA or ω-agatoxin IVA (Hillyard et al., 1992). However, it is not clear which type of Ca^{2+} channel is involved in glutamate release in the hippocampus. A possible role for the involvement of $GABA_B$ receptor-mediated modulation of Ca^{2+} channels in glutamate release remains to be established. In order to elucidate further the interaction between GABAergic and glutamatergic systems, the effects of GABA receptor agonists on depolarization-induced $^{45}Ca^{2+}$ influx in synaptosomes were studied.

2. Materials and methods

2.1. Materials

The following drugs were obtained from commercial sources: glutamate, glycine, taurine and GABA from Wako (Osaka, Japan); *o*-phthalaldehyde from Merck (Darmstadt, Germany); muscimol and baclofen from Sigma (St. Louis, MO); veratridine from Funakoshi (Tokyo, Japan). All other chemicals were of analytical grade or the highest grade commercially available.

2.2. In vivo brain microdialysis

All of the animal experiments were conducted under the Showa University Animal Experiment and Welfare Regulations. Three days before the perfusion experiment, rats were anesthetized with pentobarbital sodium (40 mg/kg, i.p.). A guide cannula (G-8, Eicom, Kyoto, Japan) was implanted into the right hippocampus (P, 5.8; L, 5.0; V, 5.0 mm from the bregma) and fixed to the skull with dental cement. On the day before the experiment, each rat was lightly anesthetized with diethyl ether, and an I-shaped microdialysis probe (2 mm dialysis membrane, 220 μm diameter, A-I-8-02, Eicom) filled with Ringers solution (147 mM NaCl, 4 mM KCl, 2.5 mM $CaCl_2$, 1 mM sodium phosphate buffer, pH 7.4) was inserted into the guide. On the day of the experiment, the probe was perfused with Ringers solution at 2.0 μl/min. After a 2-h equilibration period, dialysate samples were collected every 20 min. After the baseline collection period of 100 min, Ringers solution containing 100 mM KCl or 0.05 mM veratridine was perfused for 20 min. GABA, muscimol and baclofen were applied via the probe for a total of 40 min, 20 min before KCl or veratridine stimulation, and concomitantly, for a further 20 min in the presence of these depolarizing agents. The dialysis experiments were carried out in freely moving rats that were placed in $30 \times 30 \times 35$ cm Plexiglas cages.

Analysis of glutamate, aspartate, glycine and taurine levels were carried out as previously described (Tanaka et al., 1996). Aliquots (30 μl) of the sample were derivatized with *o*-phthalaldehyde and injected into the high performance liquid chromatography system. Amino acids were separated on a reversed-phase column (C_{18} column 3-120A, 80×4.6 mm, Niko Bioscience, Tokyo, Japan). The elution buffer was 0.1 M sodium phosphate buffer (pH 6.0) containing 25% methanol and 0.46 mM EDTA. Fluorescence (FS-8010, Tohso, Tokyo, Japan) was monitored with excitation at 355 nm and emission at 450 nm.

The amino acid level, averaged from five consecutive basal samples, was defined as 100% and changes in amino acid levels are expressed as percentages of the corresponding basal values.

2.3. Isolation of synaptosomes and ^{45}Ca influx experiments

Synaptosomes were prepared as previously described (Dunkley et al., 1988). Male Wistar rats were decapitated and cerebral cortices were homogenized in 4 volumes of 0.32 M sucrose containing 1 mM EDTA and 0.25 mM dithiothreitol. The homogenate was centrifuged at $1000 \times g$ for 10 min and the supernatants were subjected to Percoll gradient centrifugation. The pelleted synaptosomes were resuspended

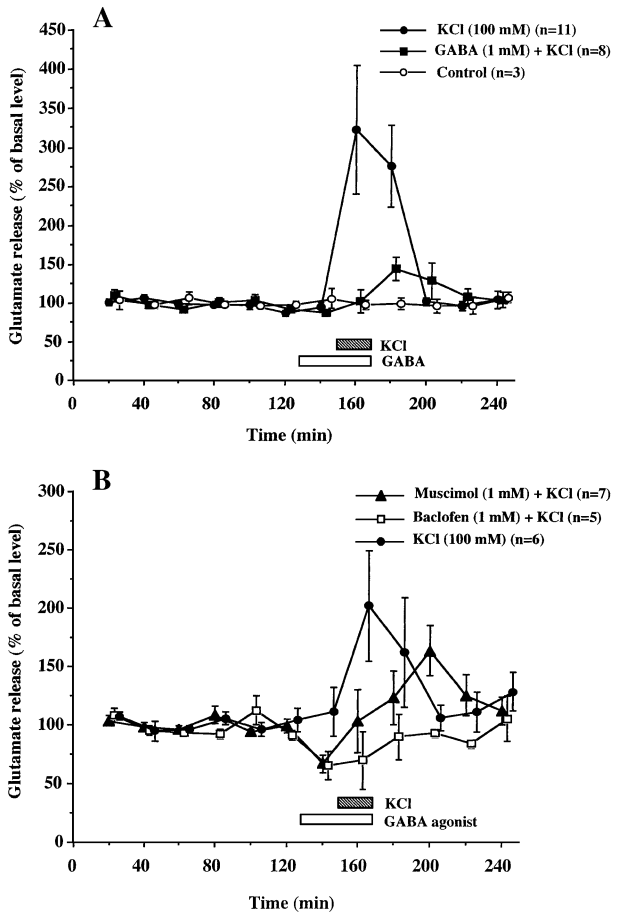

Fig. 1. Effects of GABA receptor agonists on the KCl-induced increase in glutamate levels in rat hippocampus. Changes in glutamate level in dialysates collected from the rat hippocampus were expressed as percentages of the basal level and represented as the means ± S.E.M. (A) and (B) showed effects of GABA and GABA receptor agonists, respectively. Statistical analysis was assessed with repeated measure ANOVA followed by post hoc Scheffe *F*-test. Statistical differences were obtained between the KCl-stimulated group and control group ($P<0.05$), the KCl-stimulated group and GABA-treated group ($P<0.05$ (A), and the KCl-stimulated group and baclofen-treated group (B).

and recentrifuged (19,000 × *g* for 10 min) in 20 mM Tris–HCl buffer (pH 7.4) containing 143 mM NaCl, 4.7 mM KCl, 1 mM $CaCl_2$, 1.2 mM $MgCl_2$, 24.9 mM $NaHCO_3$ and 10 mM glucose, pregassed with 95% O_2/5% CO_2.

Synaptosomes were resuspended in physiological buffer to approximately 0.7 mg protein/100 μl. After addition of 100 μl volumes of synaptosomes, reaction mixtures (1 ml) containing a GABA receptor agonist and/or a Ca^{2+} channel blocker were preincubated for 15 min at 37 °C. Then, high KCl solution (final concentration 13.5 mM) containing $^{45}Ca^{2+}$ (1 μCi/tube) was applied and incubated for a further 2 min. Incubation was terminated by rapid vacuum filtration through prewashed glass fiber filters (GF/B, Whatman International, Maidstone, England) using a Cell Harvester (Brandel, Gaithersburg, MD). Filters were washed four times with 4 ml of ice-cold, Ca^{2+}-free, EGTA buffer and the radioactivity of filters was determined in a scintillation counter. Each experiment was performed in triplicate.

2.4. Statistical analysis

Statistical analysis was performed using repeated-measures analysis of variance (ANOVA) followed by the post hoc Scheffe *F*-test. In $^{45}Ca^{2+}$ influx experiments, the non-parametric Mann–Whitney *U*-test was applied.

3. Results

3.1. Effect of GABA and GABA receptor agonists on KCl- or veratridine-induced increases in glutamate levels in rat hippocampus

Perfusion with Ringers solution containing 100 mM KCl increased the extracellular concentration of glutamate up to

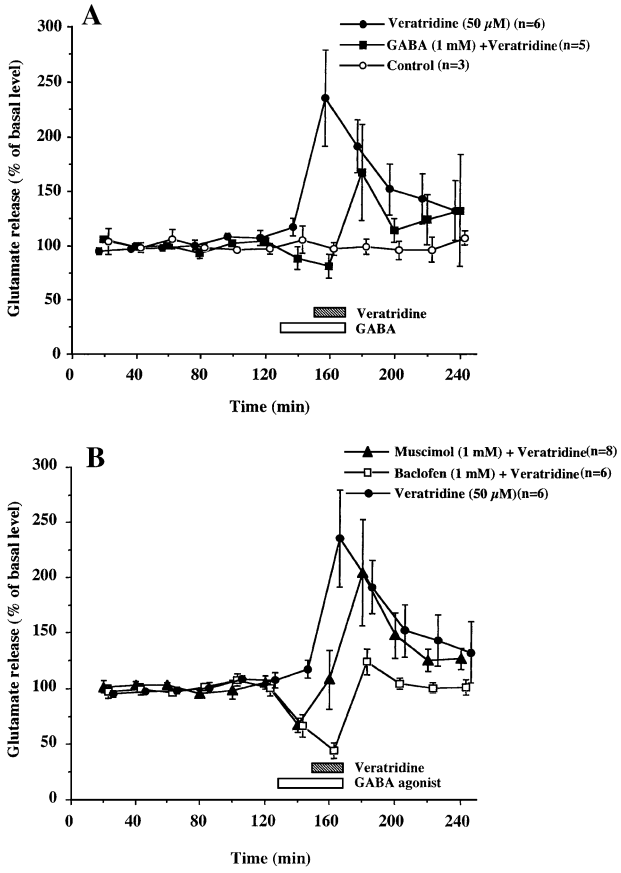

Fig. 2. Effects of GABA receptor agonists on the veratridine-induced increase in glutamate levels in rat hippocampus. Data represented as the means ± S.E.M. (A) and (B) showed effects of GABA and GABA receptor agonists, respectively. See the legend of Fig. 1 for details. Statistical differences were obtained between the veratridine-stimulated group and control group ($P<0.05$), the veratridine-stimulated group and GABA-treated group ($P<0.05$, A), and the veratridine-stimulated group and baclofen-treated group ($P<0.05$, B).

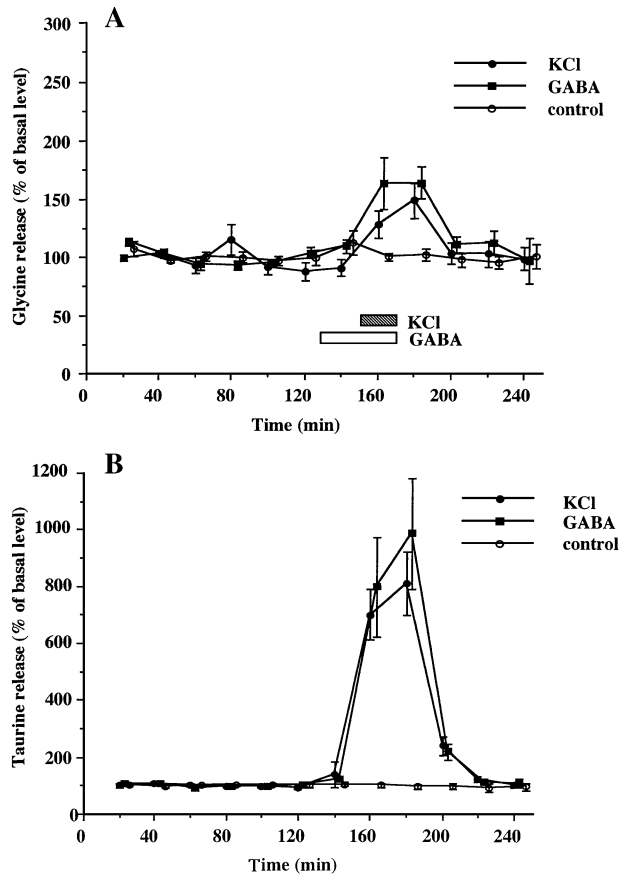

Fig. 3. Effect of GABA on the KCl-induced glycine and taurine release in rat hippocampus. (A) showed the change in glycine levels in dialysates was expressed as percentages of the basal level and represented as the means ± S.E.M. (B) The change in taurine level in dialysates collected from the rat hippocampus.

3.2-fold compared to the basal level (Fig. 1A). Perfusion of 1 mM GABA for 20 min before KCl stimulation followed by 20 min perfusion with 1 mM GABA during KCl stimulation significantly decreased the KCl-induced increase in glutamate level. Subsequently, the effects of selective $GABA_A$ or $GABA_B$ receptor agonists on the KCl-induced increase in glutamate levels were investigated. Perfusion with muscimol (1 mM) for 20 min, a $GABA_A$ receptor agonist, before KCl stimulation produced a transient decrease in the basal level of glutamate, and only partially suppressed the KCl-induced increase in glutamate level but there was a delay in the time taken to reach the peak of the increase (Fig. 1B). On the other hand, baclofen (1 mM), a $GABA_B$ receptor agonist, decreased the basal level of glutamate and abolished the KCl-induced increase in glutamate levels (Fig. 1B).

The effects of GABA and GABA receptor agonists on veratridine-induced glutamate release in rat hippocampus were also investigated. Veratridine induces an influx of sodium into nerves by opening tetrodotoxin-sensitive, voltage-dependent sodium channels. The veratridine-induced release of neurotransmitter appears to involve a calcium-sensitive process. Perfusion with 50 μM veratridine increased the extracellular glutamate level by up to 2.4-fold (Fig. 2A). Pretreatment with 1 mM GABA, before veratridine stimulation and the subsequent perfusion of GABA during veratridine stimulation, partially suppressed the increase in glutamate concentration, with a delayed peak. Treatment with muscimol decreased the basal level of glutamate, but the veratridine-induced increase in glutamate levels was only marginally inhibited, again with a delayed peak (Fig. 2B). However, baclofen strongly suppressed both the basal level of glutamate and the veratridine-induced increase.

3.2. Effect of GABA and GABA receptor agonists on the KCl-induced increase in glycine and taurine levels in rat hippocampus

Perfusion with Ringers solution containing 100 mM KCl increased the extracellular glycine level up to 1.7-fold (Fig. 3A), and increased the taurine level in dialysate up to 9-fold (Fig. 3B). However, the increases in glycine and taurine levels were not affected by pretreatment with 1 mM GABA (Fig. 3A and B). Perfusion with veratridine increased extracellular taurine levels in the hippocampus, though glycine levels were not affected by veratridine (data not shown). Perfusion with 1 mM GABA, before veratridine stimulation, did not affect the veratridine-induced increase in taurine levels (data not shown).

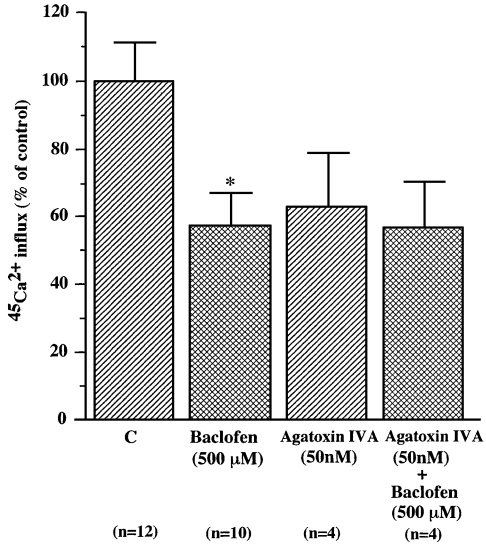

Fig. 4. Effect of baclofen and Ca^{2+} antagonists on the KCl-induced $^{45}Ca^{2+}$ influx into synaptosomes. KCl (13.5 mM)-induced $^{45}Ca^{2+}$ influx for 2 min were expressed as 100% and results are represented as the mean ± S.E.M. Statistical analysis was performed using Kruskal–Wallis test followed nonparametric Mann–Whitney *U*-test. $*P < 0.05$ compared to the control group.

3.3. Effect of GABA, baclofen and Ca^{2+} channel blockers on KCl-induced $^{45}Ca^{2+}$ influx into synaptosomes

When synaptosomes from the cerebral cortex were depolarized with 13.5 mM KCl for 2 min, $^{45}Ca^{2+}$ influx increased up to 2.2-fold compared with basal influx (data not shown). Baclofen (0.5 mM) decreased the KCl-induced $^{45}Ca^{2+}$ influx to 57% of basal influx (Fig. 4). ω-agatoxin IVA decreased the KCl-induced $^{45}Ca^{2+}$ influx to 62% of basal influx. The combined effect of baclofen and ω-agatoxin IVA on the KCl-induced $^{45}Ca^{2+}$ influx was no greater than the effect of each treatment alone.

4. Discussion

We have conducted in vivo brain microdialysis in order to examine the effects of GABA receptor agonists on extracellular glutamate levels in the rat hippocampus. In the present study, perfusion with Ringers solution containing 100 mM KCl or 0.05 mM veratridine increased the release of endogenous glutamate in the hippocampus. Perfusion with GABA (1 mM) through a microdialysis probe inhibited the depolarizing agent-induced increase in glutamate release. This suppressive effect of GABA was mimicked by the $GABA_B$ receptor agonist baclofen, but not by the $GABA_A$ receptor agonist muscimol. It has been reported that the highest density of $GABA_B$ binding is observed in the CA2 and CA3 areas of the hippocampus whereas $GABA_A$ binding sites are highly distributed in the CA1 area (Knott et al., 1993). The difference in distribution between $GABA_A$ and $GABA_B$ receptors in the hippocampus is compatible with our present results: Baclofen, but not muscimol, mimicked the effect of GABA on glutamate release induced by depolarizing agents. These in vivo observations are in agreement with in vitro data indicating that the release of glutamate from rat cerebrocortical synaptosomes (Pende et al., 1993) and cultured cells (Wheeler et al., 1994) can be inhibited when GABA receptors are activated.

In cultured hippocampal neurons, $GABA_B$ receptor activation markedly reduces voltage-activated calcium currents and this reduction is mediated by PTX-sensitive G proteins. These findings suggest that inhibition of voltage-gated Ca^{2+} channels could be the mechanism that produces presynaptic inhibition of the evoked excitatory postsynaptic currents (Pfrieger et al., 1994). Furthermore, $GABA_B$ receptors are also responsible for the inhibition of the potassium current. However, Thompson and Gahwiler (1992) concluded that the $GABA_B$ receptor-induced potassium current did not cause presynaptic inhibition of excitatory synaptic transmission. The modulation of glutamate release via $GABA_B$ receptors may be mediated directly by presynaptic inhibition of glutamatergic neurons in agreement with the findings of Pfrieger et al. (1994), Scanziani et al. (1992) and Travagli et al. (1991). Veratridine induces an influx of sodium into nerves by opening tetrodotoxin-sensitive, voltage-dependent sodium channels, and the veratridine-induced release of neurotransmitter appears to involve a Ca^{2+}-sensitive process. Suppression of glutamate release by GABA was also observed in veratridine-stimulated glutamate release experiments (Fig. 2). This observation supports the view that regulation of glutamate release through GABA receptors can occur under various physiological conditions, an effect mediated mainly by $GABA_B$ receptors.

Ca^{2+} influx serves as a trigger for depolarization–secretion coupling in nerve terminals. Previous reports have shown that the K^+-evoked release of glutamate from the hippocampus is largely Ca^{2+}-dependent (Burke et al., 1993; McMahon and Nicholls, 1990) and that glutamatergic synaptic transmission in the hippocampus is regulated by multiple Ca^{2+} channel types (Luebke et al., 1993), although the subtypes of Ca^{2+} channels involved remained unclear. Therefore, we looked for a possible involvement of $GABA_B$ receptor-associated Ca^{2+} channels by investigating the inhibitory effects of various Ca^{2+} channel blockers on $^{45}Ca^{2+}$ influx into synaptosomes. In in vitro experiments of $^{45}Ca^{2+}$ influx to synaptosomes, we used synaptosomes purified with parcoll gradients methods from the cerebral cortex and hippocampus. It is very difficult to get enough amount of synaptosomes only from the hippocampus, because of small size of the tissue. Therefore, the in vitro data could be suggestive, but not a direct evidence for the in vivo microdialysis data. We found that baclofen and ω-agatoxin IVA suppressed the KCl-induced $^{45}Ca^{2+}$ influx to 57% and 62% of controls, respectively. The addition of baclofen plus ω-agatoxin IVA did not produce any further decrease in $^{45}Ca^{2+}$ influx. These results indicate that P-type Ca^{2+} channels are expressed in the cerebral cortex, and that the suppressive effect of baclofen on $^{45}Ca^{2+}$ influx may be associated with inhibition of P-type Ca^{2+} channel activity. However, other types of Ca^{2+} channels may be involved in depolarization-induced $^{45}Ca^{2+}$ influx in addition to the P-type Ca^{2+} channels associated with $GABA_B$ receptors. It has been reported that baclofen inhibits both P- and N-type Ca^{2+} channels in spinal cord (Mintz and Bean, 1993) and hippocampal pyramidal neurons (Scholz and Miller, 1991). There is also a report that glutamate release evoked by KCl or veratridine is blocked by daurisoline, an N-type Ca^{2+} channel blocker (Lu et al., 1991).

Our findings suggest that the suppression of glutamate release produced by $GABA_B$ receptor activation in vivo is due to the inhibition of Ca^{2+} channels regulated by the $GABA_B$ receptor. However, it remains to be established unequivocally which Ca^{2+} channel linked to the $GABA_B$ receptor regulates glutamate release in the hippocampus.

We have also studied other amino acid neurotransmitters using in vivo brain microdialysis. Glycine and taurine levels in dialysates were enhanced by perfusing with Ringers solution containing 100 mM KCl. The effect on taurine levels was particularly marked, with an increase of up to 9-fold. However, the increase in glycine and taurine levels was

not affected by pretreatment with GABA. On the other hand, perfusion with veratridine increased the extracellular concentration of taurine but not glycine in the hippocampus (data not shown). Perfusion with 1 mM GABA, before the veratridine treatment, did not change the veratridine-induced increase in taurine concentration. Therefore, it is likely that the suppressive effect of GABA on amino acid neurotransmitters in the hippocampus is specific to glutamatergic neurons.

In conclusion, our in vivo results suggest that the depolarization-evoked release of endogenous glutamate in rat hippocampus can be modulated through GABAergic transmission. The inhibition of glutamate is mediated mainly by presynaptic $GABA_B$ receptors which, when activated, inhibits Ca^{2+} influx into nerve terminals.

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