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Ginkgolide B, a constituent of Ginkgo biloba, facilitates glutamate exocytosis from rat hippocampal nerve terminals

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Abstract

Although previous studies have demonstrated that Ginkgo biloba extract has modest effects in the improvement of memory and cognitive function of the Alzheimer's disease patients, the mechanism(s) underlyling its beneficial effects remain(s) unclear. In this study, the effect of ginkgolide B, one of the major constituents of Ginkgo biloba extract, on the release of endogenous glutamate from rat hippocampal nerve terminals (synaptosomes) was studied. Ginkgolide B facilitated the Ca^{2+}-dependent release of glutamate evoked by 4-aminopyridine in a concentration-dependent manner. The facilitatory action of ginkgolide B was not due to it increasing synaptosomal excitability because ginkgolide B did not alter the 4-aminopyridine-evoked depolarization of the synaptosomal plasma membrane potential. Rather, examination of the effect of ginkgolide B on cytosolic free Ca^{2+} concentration revealed that the facilitation of glutamate release could be attributed to an enhancement of presynaptic voltage-dependent Ca^{2+} influx. Consistent with this, the ginkgolide B-mediated facilitation of glutamate release was significantly prevented in synaptosomes pretreated with a wide spectrum blocker of N-, P-, and Q-type Ca^{2+} channels, ω-conotoxin MVIIC. Moreover, the facilitation produced by ginkgolide B was completely abolished by the protein kinase A inhibitor, but not by the protein kinase C inhibitor. These results suggest that ginkgolide B effects a increase in protein kinase A activation, which subsequently enhances the Ca^{2+} entry through voltage-dependent N- and P/Q-type Ca^{2+} channels to cause a increase in evoked glutamate release from rat hippocampal nerve terminals. In addition, glutamate release elicited by Ca^{2+} ionophore (ionomycin) was also facilitated by ginkgolide B, which suggests that ginkgolide B may have a direct effect on the secretory apparatus downstream of Ca^{2+} entry. These actions of ginkgolide B may provide some information regarding the beneficial effects of Ginkgo biloba in the central nervous system.

Keywords: Ginkgolide B; Glutamate exocytosis; Hippocampal synaptosome; Presynaptic voltage-dependent Ca^{2+} influx; Protein kinase A

1. Introduction

Glutamate is one of the excitatory neurotransmitter of the central nervous system and exists in very high concentrations in the brain. In the central nervous system, glutamatergic activity is thought to participate in cognitive processing and other forms of synaptic plasticity such as learning and memory (Bliss and Collingridge, 1993; Menschik and Finkel, 1998). However, malfunction of glutamatergic transmission has also been shown to associate with cognitive impairment of Alzheimer's disease (Farber et al., 1998). Therefore, strengthening glutamatergic neurotransmission seems to be a new therapeutic strategy for memory dysfunction and cognitive disorders.

Extracts of the green leaves of Ginkgo biloba have antioxidative, vascular, and neuroprotective effects and are widely prescribed in Europe for the treatment of cerebrovascular and neurodegenerative disorders (Maclennan et al., 2002; Ahlemeyer and Krieglstein, 2003). Pre-clinical and clinical studies have demonstrated that Ginkgo biloba extract was effective in mild-to-moderate dementia of the Alzheimer's disease patients (Le Bars et al., 1997; Maurer et al., 1997). This suggests that Ginkgo biloba extract has modest therapeutic potential as a memory and cognition enhancing drug. However, the precise mechanism of action of Ginkgo biloba extract in cognitive function is still

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unclear. The extract of Ginkgo biloba contains multiple compounds, e.g., flavonoids and terpenoids, that are thought to contribute to its beneficial effects (Krieglstein et al., 1995). In this study, ginkgolide B was chosen because of it is one of the major constituents the of the terpenoid fraction of Ginkgo biloba extract, and the large number of studies that have evaluated its efficacy in the central nervous system (Li et al., 1999; Ahlemeyer et al., 1999; Kondratskaya et al., 2004). For example, several experimental studies have shown that ginkgolide B improves cerebral blood flow and inhibits the accumulation of fatty acid induced by electroconvulsive shock (Birkle et al., 1988; Oberpichler et al., 1990; Rodriguez de Turco et al., 1993).

Considering that proper glutamate transmission is required for normal neuronal function and synaptic plasticity such as learning and memory formation, clarification of the effect of ginkgolide B on glutamate release at the presynaptic level may give us new insight into the mechanism of memory and cognition enhancing effects by ginkgolide B and other components of Ginkgo biloba extract. Here, we therefore employed the isolated nerve terminal (synaptosome) model to assess the characteristics and mechanisms of action of ginkolide B in the regulation of glutamate exocytosis from nerve terminals purified from rat hippocampus, which is commonly used as a model to study synaptic plasticity since it is believed to be a crucial brain region for learning and memory. Using hippocampal synaptosomes, we found that ginkgolide B potently facilitates evoked glutamate release in the absence of any effect on synaptosomal excitability. Furthermore, examining the mechanism underlying the ginkgolide B-mediated facilitation of glutamate release, we found that this release facilitation seems to be through a cAMP-dependent mechanism involving protein kinase A, which modulates voltage-dependent Ca^{2+} influx as well as downstream events controlling synaptic vesicle recruitment and exocytosis.

2. Materials and methods

2.1. Materials

3′,3′,3′-Dipropylthiadicarbocyanine iodide ($DiSC_3(5)$) and Fura-2-acetoxymethyl ester (Fura-2-AM) were obtained from Molecular Probes (Eugene, or, U.S.A.). Percoll was obtained from Pharmacia. *S*-Adenosine,cyclic 3′,5′-(hydrogenphosphorothioate)-triethylammonium (Sp-cAMPS), bisindolylmaleimide XI (Ro32-0432), and ω-conotoxin MVIIC were obtained from Tocris Cookson (Bristol, USA). Ginkgolide B, *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride (H89) and all other reagents were obtained from Sigma (Poole, U.K.) or Merck (Poole, U.K.).

2.2. Isolation of synaptosomes from rat hippocampus

Synaptosomes were purified by discontinuous Percoll gradients as described previously (Sihra, 1997). The hippocampus from male Sprague–Dawley rats (2–3 months) was isolated and homogenized in a medium containing 0.32 M sucrose, pH 7.4. The homogenate was centrifuged at $3000 \times g$ for 2 min at 4 °C. The supernatant fraction was collected and centrifuged at $14\,500 \times g$ for 12 min. The resulting pellet was resuspended in 8 ml of 0.32 M sucrose, pH 7.4. Two milliliters of this synaptosomal suspension was loaded onto discontinuous gradients consisting of three 2 ml layers of filtered Percoll density gradient medium (23%, 10%, and 3%) in 0.32 M sucrose pulse 0.25 mM dithiothreitol and 1 mM ethylenediaminetetraacetic acid, pH 7.4. The gradients were centrifuged at $32\,500 \times g$ for 7 min at 4 °C. The synaptosomal fraction was collected from the 23%/10% Percoll interface and diluted in a volume of 30 ml of HEPES buffer medium consisting of 140 mM NaCl, 5 mM KCl, 5 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES (pH 7.4). The pellets were centrifuged at $27\,000 \times g$ for 10 min and resuspended in the appropriate HEPES buffer medium to remove Percoll. The protein concentration of the synaptosomal preparation was determined by the method of Bradford using bovine serum albumin as a standard. 0.5 mg of the synaptosomal suspension was diluted in 10 ml of HEPES buffer medium and centrifuged at $3000 \times g$ for 10 min. The supernatants were discarded and the pellets containing the synaptosomes were stored on ice and used within 4–6 h.

2.3. Measurement of glutamate release from synaptosomes

Glutamate release was assayed by on-line fluorimetry as described previously (Nicholls et al., 1987). Synaptosomal pellets (0.5 mg/ml) were resuspended in 2 ml of HEPES buffer medium containing 16 μM bovine serum albumin and incubated in a stirred and thermostated cuvette maintained at 37 °C in a Perkin-Elmer LS-50B spectrofluorimeter (Beaconsfield, UK). $NADP^+$ (2 mM), glutamate dehydrogenase (50 units/ml), and $CaCl_2$ (1 mM) were added after 3 min. After a further 5 min of incubation, 4-aminopyridine (1 mM), KCl (15 mM) or ionomycin (5 μM) was added to stimulate glutamate release. The oxidative decarboxylation of released glutamate, leading to the reduction of $NADP^+$, was monitored by measuring NADPH fluorescence at excitation and emission wavelengths of 340 nm and 460 nm, respectively. Data points were obtained at 2-s intervals. A standard of exogenous glutamate (5 nmol) was added at the end of each experiment and the fluorescence change produced by the standard addition was used to calculate the released glutamate as nmol glutamate/mg synaptosomal protein. Release traces are shifted vertically to align the point of depolarization as zero release. Unless otherwise indicated, release values quoted in the text are levels attained at steady-state after 5 min of depolarization (nmol/mg/5 min). Cumulative data were analysed using Lotus 1-2-3 and MicroCal Origin.

2.4. Cytosolic Ca^{2+} measurements using Fura-2

Synaptosomes (0.5 mg/ml) were preincubated in HEPES buffer medium with 16 μM bovine serum albumin in the presence of 5 μM Fura-2-acetoxymethyl ester and 0.1 mM $CaCl_2$ for 30 min at 37 °C in a stirred test tube. After Fura-2 loading, synaptosomes were centrifuged in a microcentrifuge for 30 s at $3000 \times g$. The synaptosomal pellets were resuspended in HEPES buffer medium with bovine serum albumin and the synaptosomal suspension stirred in a thermostatted cuvette in a Perkin-Elmer

LS-50B spectrofluorimeter. $CaCl_2$ (1 mM) was added after 3 min and further additions were made after an additional 5 min, as described in the legends to the figures. Fluorescence data were accumulated at excitation wavelengths of 340 nm and 380 nm (emission wavelength 505 nm) at data accumulated at 7.5-s intervals. Calibration procedures were performed as described by previously (Grynkiewicz et al., 1985), using 0.1% sodium dodecyl sulphate to obtain the maximal fluorescence with Fura-2 saturation with Ca^{2+}, followed by 10 mM EGTA (Tris buffered) to obtain minimum fluorescence in the absence of any Fura-2/Ca^{2+} complex. Cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_C$, nM) was calculated using equations described previously (Sihra et al., 1992). Cumulative data were analysed using Lotus 1-2-3 and MicroCal Origin.

2.5. Synaptosomal membrane potential measurement

The synaptosomal membrane potential can be monitored by positively charged membrane potential-sensitive carbocyanine dyes such as $DiSC_3(5)$ (Rink et al., 1980; Akerman et al., 1987). The dye becomes incorporated into the synaptosomal plasma membrane lipid bilayer. Upon depolarization with 4-aminopyridine, the release of the dye from the membrane bilayer is indicated as an increase in fluorescence. Synaptosomes were resuspended in 2 ml HEPES-buffered incubation medium and incubated in a stirred and thermostated cuvette at 37 °C in a Perkin-Elmer LS-50B spectrofluorimeter (Beaconsfield, UK). After 3 min of incubation, 4 μM $DiSC_3(5)$ was added and allowed to equilibrate before the addition of $CaCl_2$ (1 mM) after 4 min of incubation. Then, 4-aminopyridine or KCl was added to depolarize the synaptosomes at 10 min, and $DiSC_3(5)$ fluorescence was monitored at excitation and emission wavelengths of 646 nm and 674 nm, respectively, and data points were collected at 2-s intervals. Cumulative data were analysed using Lotus 1-2-3 and results are expressed in fluorescence units.

2.6. Statistical analysis

Data are expressed as mean ± S.E.M. Statistical significance was determined using the Student's *t* test, and $P<0.05$ was considered statistically significant.

3. Results

To specifically investigate the presynaptic action of ginkgolide B on glutamate release, we purified nerve terminals (synaptosomes) from rat hippocampus. As this preparation is not amenable to electrical stimulation, a number of biochemical depolarization protocols have been developed, including K^+ channel blockers like 4-aminopyridine or high external $[K^+]$ (McMahon and Nicholls, 1991; Sihra, 1997). 4-Aminopyridine destabilizes the membrane potential and causes repetitive spontaneous of Na^+ channel-dependent depolarizations that closely approximate in vivo depolarization of the synaptic terminal and lead to activation of voltage-dependent Ca^{2+} channels and neurotransmitter release. In contrast to 4-aminopyridine, increased external K^+-concentration depolarizes the plasma membrane by shifting the K^+ equilibrium potential above the threshold potential for activation of voltage-dependent Ca^{2+} channels, which leads to Ca^{2+} entry and neurotransmitter release, while Na^+ channels are inactivated (Tibbs et al., 1989). The use of these stimulation protocols in release studies helps to distinguish whether the modulation of ion channels involved in the waveform of the action potentials or Ca^{2+} channels

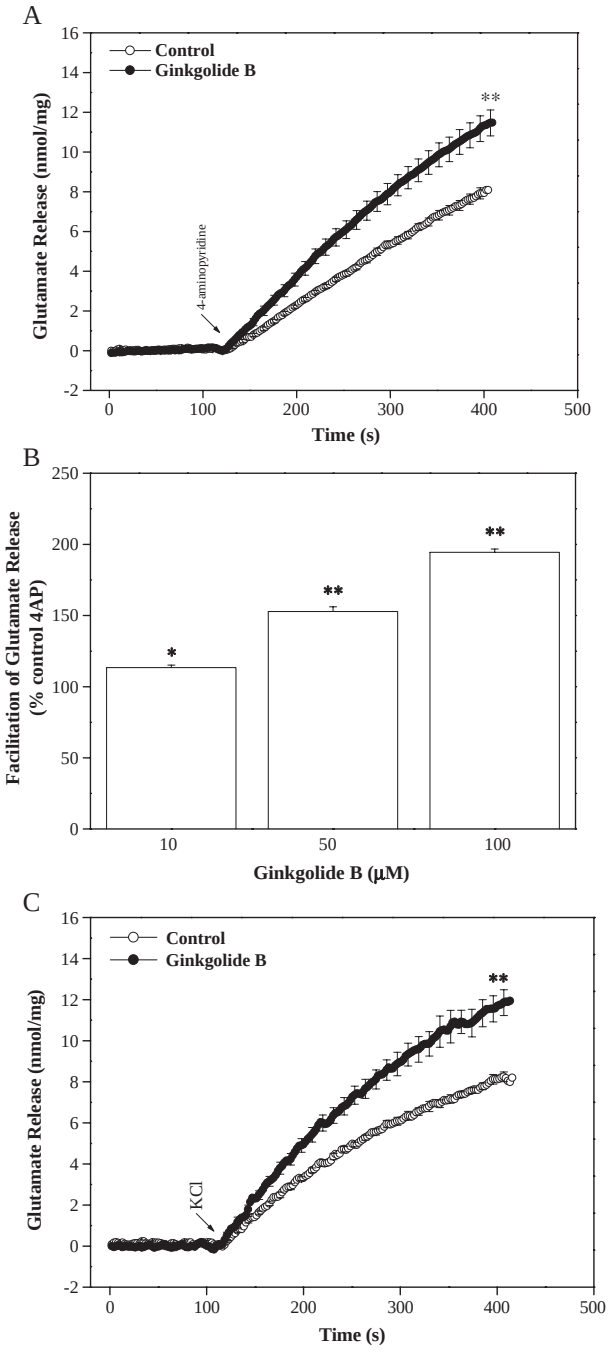

Fig. 1. Ginkgolide B facilitates the release of glutamate evoked by 4-aminopyridine and KCl in rat hippocampal synaptosomes. Glutamate release was evoked by (A) 1 mM 4-aminopyridine (arrow) or (C) 15 mM KCl (arrow) in the absence (control) or presence of ginkgolide B (50 μM) (added 10 min before depolarization). Data represent means ± S.E.M. of from experiments carried out with eight independent synaptosomal preparations. Means ± S.E.M. were calculated at each time-point (2 s), but error bars are only shown every 10 s for visual clarity. (B) Concentration-dependent potentiation of glutamate release by ginkgolide B. Results are means ± S.E.M. of five to eight independent experiments. ○ Control; ● 50 μM ginkgolide B, $*P<0.05$; $**P<0.01$; different from control.

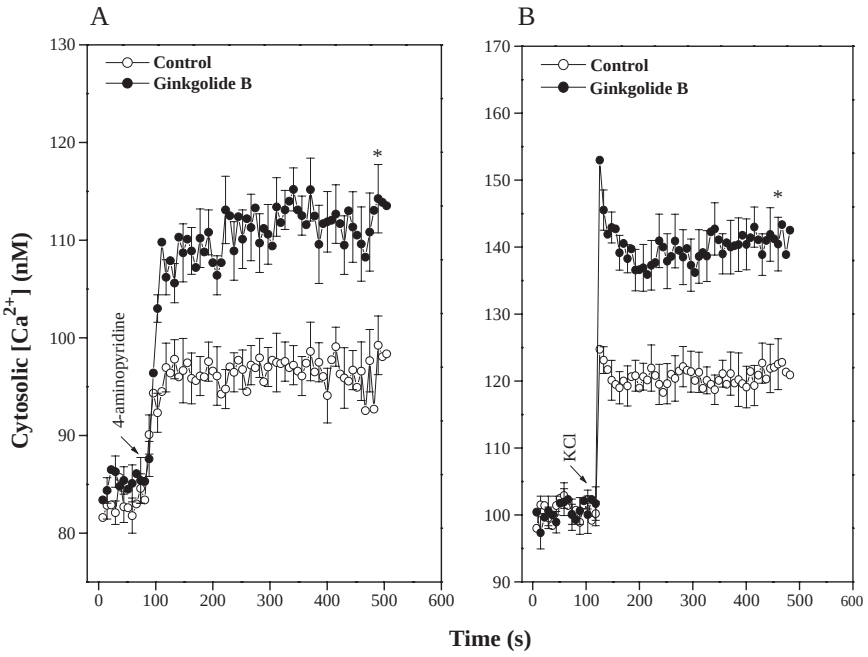

Fig. 2. Ginkgolide B enhances the rise in $[Ca^{2+}]_C$ evoked by 4-aminopyridine and KCl. Cytosolic free Ca^{2+} concentration (nM) was monitored before and after the addition of (A) 1 mM 4-aminopyridine (arrow) or (B) 15 mM KCl (arrow), in the absence (control) or presence of 50 μM ginkgolide B, added 10 min before depolarization. Results are means±S.E.M. of seven independent experiments. Means±S.E.M. were calculated at each time-point (7.5 s), but error bars are shown for every fourth reading for clarity. ○ Control; ● 50 μM ginkgolide B, $*P<0.05$; different from control.

coupled to glutamate release is involved in the regulation of neurotransmitter glutamate release by drugs (Barrie and Nicholls, 1993).

First, we therefore determined the effect of ginkgolide B on the release of glutamate evoked by 4-aminopyridine. On-line fluorometric determination of glutamate release from rat hippocampal synaptosomes revealed a 4-aminopyridine (1 mM)-evoked control release of 8.0±0.2 nmol glutamate per mg synaptosomal protein after 5 min of depolarization (Fig. 1A). This 4-aminopyridine-evoked release was significantly enhanced by ginkgolide B, at 50 μM, to 11.4±0.6 nmol/mg/5 min ($n=8$). The ginkgolide B-mediated facilitation of glutamate release was concentration-

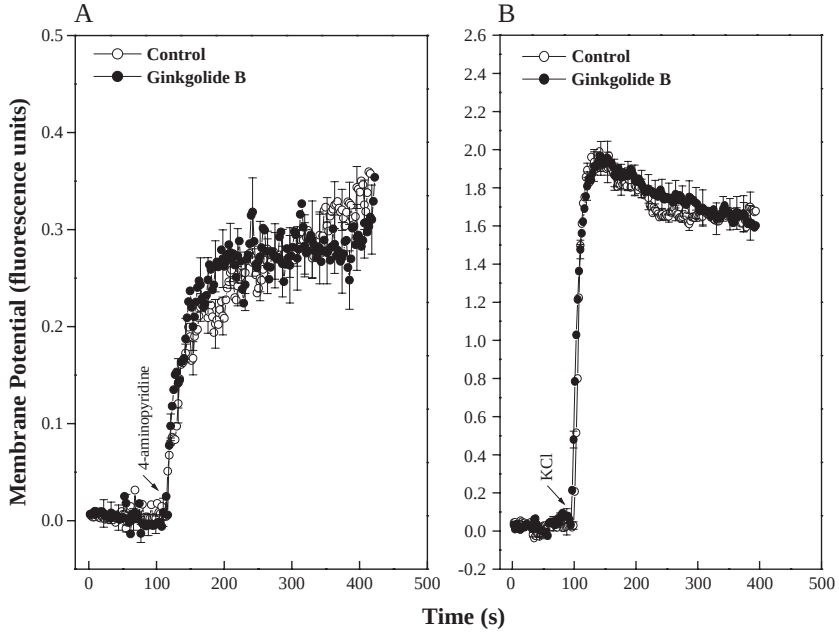

Fig. 3. Ginkgolide B does not change the synaptosomal membrane potential. Synaptosomal membrane potential was monitored with $DiSC_3(5)$ on depolarization with (A) 1 mM 4-aminopyridine (arrow) or (B) 15 mM KCl (arrow) in the absence (control) or presence of 50 μM ginkgolide B, added 2 min before depolarization. Results are means±S.E.M. of six independent experiments. Means±S.E.M. were calculated at each time-point (2 s), but error bars are only shown every 10 s for visual clarity. ○ Control; ● 50 μM ginkgolide B.

dependent, with 10, 50, and 100 μM ginkgolide B increasing glutamate release to 113.4±1.8%, 142.5±3.3%, and 194.4±2.4% of control values respectively (Fig. 1B). Similarly, the release evoked by 15 mM KCl (8.2±0.2 nmol/mg/5 min) was also facilitated when synaptosomes incubated in the presence of 50 μM ginkgolide B (11.9±0.7 nmol/mg/5 min; $n=8$; Fig. 1C). Because 4AP-induced depolarization involves the action of both Na^+ channels and Ca^{2+} channels, whereas KCl-induced depolarization involves only Ca^{2+} channels. Thus, these results suggest that the facilitation by ginkgolide B primarily result from an increase in the activity of the release-coupled Ca^{2+} channels.

To assess further whether ginkgolide B increases the influx of Ca^{2+}, we determined the $[Ca^{2+}]_C$ using the Ca^{2+} indicator Fura-2. Stimulation of synaptosomes with 1 mM 4-aminopyridine caused a rise in $[Ca^{2+}]_C$ to a plateau level of 96.6±3.7 nM. Ginkgolide B 50 μM did not significantly affect basal Ca levels (control, 82.7±2.1 nM; 50 μM ginkgolide, 85.4±1.8 nM; Fig. 2A), but caused an 16.9% increase in the 4-aminopyridine-evoked rise in $[Ca^{2+}]_C$ (113.5±4.6 nM) ($n=7$). This potentiatory effect of ginkgolide B was also evident with 15 mM KCl-evoked Ca^{2+} influx, which facilitated 15.8% from 122.2±3.8 nM in control conditions to 141.5±4.1 nM in the presence of 50 μM ginkgolide B ($n=7$; Fig. 2B). In principle, the facilitatory effect of ginkgolide B on the elevation of $[Ca^{2+}]_C$ might be attributed either to a direct increase in the entry of Ca^{2+} through voltage-dependent Ca^{2+} channels or secondary effects resulting from, for example, modulation of K^+ channels with the consequent alteration in plasma membrane potential. In order to distinguish between these two possibilities, the effect of ginkgolide B on the synaptosomal membrane potential under resting conditions and on depolarization was examined with the membrane potential-sensitive dye $DiSC_3(5)$. As shown in Fig. 3A, 4-aminopyridine (1 mM) application caused an increase in $DiSC_3(5)$ fluorescence of 0.36±0.02 fluorescence units/3 min. Preincubation of synaptosomes with 50 μM ginkgolide B did not alter the resting plasma membrane potential and produced no significant change in the 4-aminopyridine-mediated increase in $DiSC_3(5)$ fluorescence (0.33±0.04 fluorescence units/3 min; $n=6$). With KCl (15 mM) depolarization (1.7±0.08 fluorescence units/3 min), preincubation with ginkgolide B again did not affect depolarization (1.65±0.09 fluorescence units/3 min; $n=6$; Fig. 3B). These experiments indicate that the observed facilitation of evoked glutamate release by ginkgolide B is likely to be due to a direct increase in the activity of voltage-dependent Ca^{2+} channels in the hippocampal nerve terminal.

Because glutamate release is triggered by the entry of Ca^{2+} through both N- and P/Q-type Ca^{2+} channels (Wheeler et al., 1994; Turner and Dunlap, 1995; Vazquez and Sanchez-Prieto, 1997), we also examined whether the modulation of N- and P/Q-type Ca^{2+} channel activity involves in the ginkgolide B-mediated facilitation of glutamate release. We studied this issue in a group of synaptosomes ($n=5$) where ginkgolide B was applied in the presence of 1 μM ω-conotoxin MVIIC, a wide spectrum blocker of N-, P-, and Q-type Ca^{2+} channels. As shown in Fig. 4A, application of 1 μM ω-conotoxin MVIIC caused a 46.2±2.1% inhibition on 4-aminopyridine-evoked glutamate release, from 10.4±0.1 nmol/mg/5 min in control conditions to 5.6±0.4 nmol/mg/5 min in the presence of ω-conotoxin MVIIC. In the presence of 1 μM ω-conotoxin MVIIC, ginkgolide B (50 μM) facilitated 4-aminopyridine-evoked glutamate release by only 17.8±3.4% (6.6±0.5 nmol/mg/5 min) compared with control after the application of ω-conotoxin MVIIC (Fig. 4B; $n=5$), which significantly less than that when ginkgolide B was applied alone (49.5±2.6%; $n=7$). These results suggest that ginkgolide B-mediated facilitation of glutamate release is most probably result from a increase of Ca^{2+} influx through presynaptic N- and P/Q-type Ca^{2+} channels.

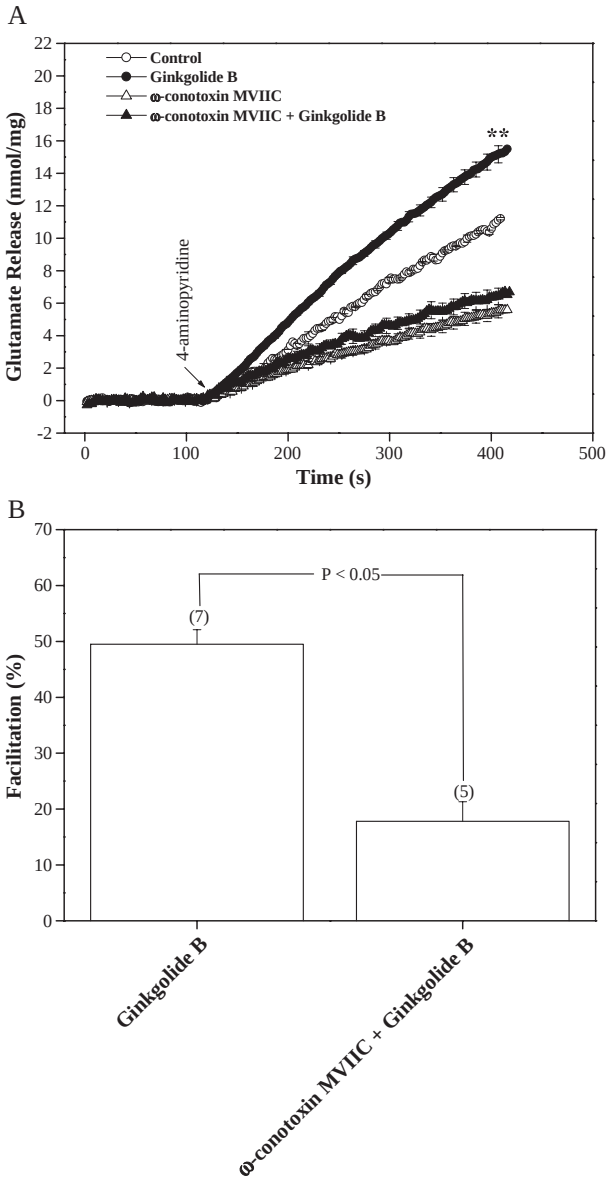

Fig. 4. Suppression of ginkgolide B-mediated facilitation of 4-aminopyridine evoked glutamate release by ω-conotoxin MVIIC. (A) Glutamate release was evoked by 1 mM 4-aminopyridine (arrow) in the absence (control) or presence of 50 μM ginkgolide B, 1 μM ω-conotoxin MVIIC or 1 μM ω-conotoxin MVIIC+50 μM ginkgolide B. Ginkgolide B was added 10 min before depolarization and, ω-conotoxin MVIIC, 20 min prior to this. (B) Bar plots showing average percentage facilitation of the 4-aminopyridine-evoked glutamate release by ginkgolide B in the control group or in the presence of ω-conotoxin MVIIC. Data represent means±S.E.M. of from experiments carried out with five independent synaptosomal preparations. Means±S.E.M. were calculated at each time-point (2 s), but error bars are only shown every 10 s for visual clarity. ○ Control; ● 50 μM ginkgolide B; △ 1 μM ω-conotoxin MVIIC; 1 μM ω-conotoxin MVIIC+50 μM ginkgolide. $^{**}P<0.01$; different from control.

To determine whether the regulation of glutamate release by ginkgolide B also reflected putative actions of Ca^{2+} influx downstream, we triggered neurotransmitter release using ionomycin. This Ca^{2+}-selective ionophore is able to mediated direct Ca^{2+} influx into nerve terminals, independently of depolarization and voltage-dependent Ca^{2+} channel activation, and thus obviates the involvement of regulation at these loci (Sihra et al., 1992). Ionomycin (5 μM) caused a control glutamate release of 7.9±0.6 nmol/mg/5 min. In the presence of 50 μM ginkgolide B, ionomycin-induced release of glutamate was potentiated to 13.9±0.3 nmol/mg/5 min (Fig. 5), indicating that ginkgolide B may have direct effects on the processes leading up to and including the exocytosis of synaptic small vesicles.

Protein phosphorylation of voltage-dependent Ca^{2+} channels subunits by protein kinases (protein kinase A or protein kinase C) has been shown to represent a key regulatory influence on Ca^{2+} influx (Catterall, 2000). If activation of protein kinase A or protein kinase C is involved in the ginkgolide B-mediated facilitation of glutamate release, pharmacological inhibition of these protein kinase activity should block the effect of ginkgolide B. We test this prediction by application of H89 (10 μM for 30 min), a protein kinase A inhibitor, and Ro32-0432 (10 μM, for 30 min), a protein kinase C inhibitor. As shown in Fig. 6, 4-aminopyridine (1 mM) evoked a glutamate release of 7.4±0.5 nmol/mg/5 min, which was facilitated by 45.9% (10.8±0.6 nmol/mg/5 min) in the presence of 50 μM ginkgolide B. Application of H89 (10 μM) alone had no significant effect on control 4-aminopyridine-evoked glutamate release (6.9±0.3 nmol/mg/5 min), but the ginkgolide B-induced facilitation of glutamate release was effectively prevented by the presence of 10 μM H89, there being no statistical difference between the release after H89 alone and after H89+ginkgolide B treatment (7.1±0.4 nmol/mg/5 min; $n=6$). In contrast to H89, Ro32-0432 (10 μM) did not block the ginkgolide B-induced facilitation of glutamate release. In seven synaptosomes tested, ginkgolide B (50 μM) was still able to produce a 43.4±4.5% increase in the 4AP-evoked glutamate release after the application of Ro32-0432 (10.9±0.5 nmol/mg/5 min), which was not significantly different from the potentiation produced by ginkgolide B alone (45.9±4.1%; $n=7$; $P>0.05$). We also determined whether exogenous cyclic AMP could abrogate the facilitatory effect induced by ginkgolide B. Fig. 6 shows that preincubation of synaptosomes with the membrane-permeable cyclic AMP analogue Sp-cAMPS (100 μM) produced a 46.1±3.2% facilitation on the release of glutamate evoked by 4-aminopyridine, form 7.6±0.5 nmol/mg/5 min in control conditions to 11.1±0.3 nmol/mg/5 min in the presence of 100 μM Sp-cAMPS, and completely occluded the ability of ginkgolide B to facilitate glutamate release evoked by 4-aminopyridine (12.2±0.5 nmol/mg/5 min). These results suggest that a cAMP/protein kinase A-dependent pathway is possibly involved in the observed effects of ginkgolide B on 4-aminopyridine-evoked glutamate release from hippocampal synaptosomes.

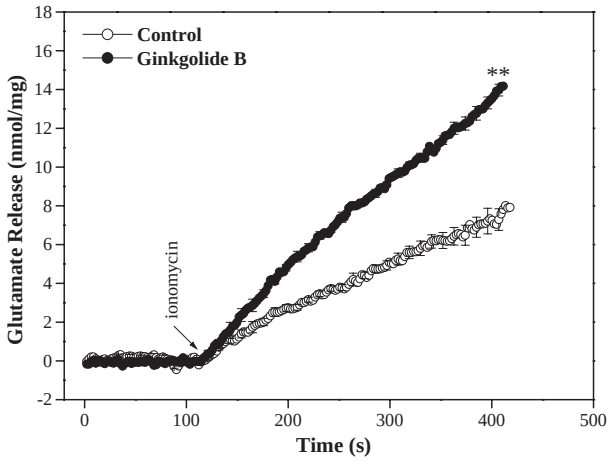

Fig. 5. Ginkgolide B facilitates ionomycin-evoked glutamate release. Glutamate release was evoked by 5 μM ionomycin (arrow) in the absence (control) or presence of 50 μM ginkgolide B, added 10 min before depolarization. Data represent means±S.E.M. of from experiments carried out with six independent synaptosomal preparations. Means±S.E.M. were calculated at each time-point (2 s), but error bars are only shown every 10 s for visual clarity. **$P<0.01$; different from control. ○ Control; ● 50 μM ginkgolide B.

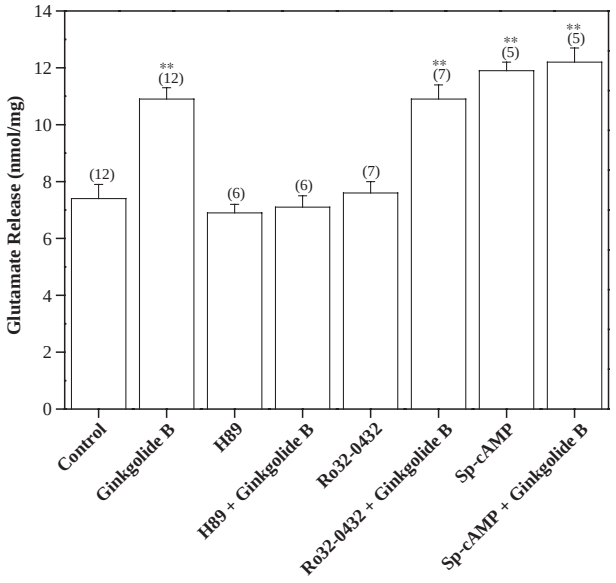

Fig. 6. Ginkgolide B-mediated facilitation of glutamate release is blocked by protein kinase A inhibitor H89 but not by protein kinase C inhibitor Ro32-0432, and is occluded by cAMP analog Sp-cAMPS. The bar diagrams showing the glutamate release evoked by 1 mM 4-aminopyridine (arrow) in the absence (control) or presence of 50 μM ginkgolide B, 10 μM H89, 10μM H89+50 μM ginkgolide, 10 μM Ro32-0432, 10μM Ro32-0432+50 μM ginkgolide, 100 μM Sp-cAMPS or 100 μM Sp-cAMPS+50 μM ginkgolide. Ginkgolide B was added 10 min before depolarization and, H89, Ro32-0432 or Sp-cAMP, 30 min prior to this. Data represent means±S.E.M. of from experiments carried out with five to twelve independent synaptosomal preparations. Means±S.E.M. were calculated at each time-point (2 s), but error bars are only shown every 10 s for visual clarity. **$P<0.01$; different from control.

4. Discussion

The release of neurotransmitter from presynaptic sites is a complex phenomenon and the regulation of this release process represents an important mechanism for the modulation of excitability and synaptic transmission in central neurons. By using a preparation of nerve terminals from rat hippocampus and by measuring the release of glutamate,

this study provide a new finding is that ginkgolide B, one of the major components of Ginkgo biloba extract, significantly enhances the release of glutamate evoked by 4-aminopyridine. This effect seems to be through a protein kinase A-dependent signaling cascade to produce a positive modulatory influence on neurotransmitter glutamate release.

4.1. Mechanisms of presynaptic facilitation of glutamate release by ginkgolide B

Where does ginkgolide B act in potentiating glutamate release from hippocampal synaptosomes? In principle, neurotransmitter release can be modulated at several loci in the stimulus–exocytosis cascade, including ion-channels modulating nerve terminal excitability (Dascal and Lotan, 1991; Roeper and Pongs, 1996), voltage-dependent Ca^{2+} channels and, downstream of Ca^{2+} entry, components of the synaptic vesicle trafficking and exocytotic apparatus (Thompson et al., 1993; Sihra and Nichols, 1993). Our observation, using Fura-2, that the depolarization-induced rise in $[Ca^{2+}]_C$ was enhanced by ginkgolide B, suggested that ginkgolide B facilitates glutamate release by increasing Ca^{2+} influx into nerve terminal. The increment in intracellular Ca^{2+} mediated by ginkgolide B could be due to Ca^{2+} entry through voltage-dependent Ca^{2+} channels, and Ca^{2+} release from intracellular stores. In the presence of ω-conotoxin MVIIC, which largely reduced the potentiatory effect of ginkgolide on 4-aminopyridine-evoked glutamate release. Thus, it is likely that ginkgolide B facilitates glutamate release by the activation of a pathway leading to an enhancement of N- and P/Q-type Ca^{2+} channel activity and in the subsequent increase in the influx of Ca^{2+} in the nerve terminal. However, other unidentified types of Ca^{2+} channels or intracellular stores released Ca^{2+} cannot be completely excluded because ω-conotoxin MVIIC could not block the action of ginkgolide B fully.

In addition, while the elevation of $[Ca^{2+}]_C$ by ginkgolide B could occur by increased nerve terminal excitability, this hypothesis appears unlikely for the following reasons. First, the facilitatory effect of ginkgolide B on glutamate release is also observed when KCl is used as a depolarizing agent, a condition in which transient channels that require repolarization of the plasma membrane to rest are inactivated. Many of the K^+ channels of the nerve terminal are of this transient nature. Second, the plasma membrane potential measured using a membrane potential-sensitive dye $DiSC_3(5)$ is unaffected by the addition of ginkgolide B before depolarization with either 4-aminopyridine or KCl. These data suggest that the facilitatory effect of ginkgolide B on glutamate release does not come about as a result of an indirect effect through modulation (blockade) of K^+ channels with the consequent alteration in synaptosomal excitability. However, since activation of Na^+ channels is known to depolarize the presynaptic neuronal membrane and enhance the amount of transmitter release by increasing the opening of Ca^{2+} channels linked to neurotransmitter release, we cannot rule out the possibility that the effect of ginkgolide B on glutamate release is due to a direct enhancement of Na^+ channel activity. On the other hand, the signaling through this release facilitation by ginkgolide B seems to involve protein kinase A but not protein kinase C. With respect to this conclusion, we can give two evidences to support it. First, the protein kinase A inhibitor H89 could successfully antagonize the ginkgolide B-mediated facilitation of glutamate release, whereas the protein kinase C inhibitor Ro32-0432 is without effect. Secondly, the effect of ginkgolide B on glutamate release is occluded by Sp-cAMPS, a membrane permeant cAMP analog. These results, together with the fact that phosphorylation of presynaptic voltage-dependent Ca^{2+} channel subunits by protein kinase A represents a key regulatory influence on Ca^{2+} influx (Catterall, 2000), suggesting that a direct regulation of voltage-dependent Ca^{2+} channels linked to glutamate release by cAMP/protein kinase A-dependent phosphorylation, may underlie, at least in part, the observed facilitation of Ca^{2+} influx and glutamate release by ginkgolide B.

In addition to the regulation of glutamate release by controlling Ca^{2+} influx, there are multiple potential loci for the regulation of exocytosis downstream of ionic events. To delineate the action of ginkgolide B following Ca^{2+} entry, we obviated the involvement of Ca^{2+} channel activation by using the Ca^{2+} ionophore, ionomycin, which induces glutamate release by a direct increase intrasynaptosomal Ca^{2+} levels, without previous depolarization and Ca^{2+} channel activation (Sihra et al., 1992). Our observation that ionomycin-induced glutamate release was also enhanced after ginkgolide B treatment, indicates that ginkgolide B may have a direct effect on the sites downstream of Ca^{2+} entry at the levels of synaptic vesicle recruitment, docking, or exocytosis. In fact, a number of synaptic proteins, associated with moving, docking and fusion of synaptic vesicles, including synapsin I, rabphilin 3A, syntaxin, SNAP 25 and synaptobrevin, have been reported to be phosphorylated by protein kinase A (Greengard et al., 1993; Fykse et al., 1995; Lonart et al., 1998; Risinger and Bennett, 1999). Since our finding that the facilitation of glutamate release from hippocampal synaptosomes produced by ginkgolide B occurred through a cAMP/protein kinase A-dependent cascade, it remains to be determined whether protein kinase A-mediated phosphorylation of synaptic vesicle proteins is also involved in the action of ginkgolide B. Overall, the present study demonstrates, for the first time, that ginkgolide B facilitates glutamate release from hippocampal nerve terminals not only possible by an upregulation of voltage-dependent Ca^{2+} entry but also by a direct effect on the exocytotic machinery itself.

4.2. Therapeutic implications

As stated in the introductory remarks, Ginkgo biloba extract is claimed to reduce memory loss and the symptoms

of mild cognitive disorders including Alzheimer's disease (Oken et al., 1998; Le Bars et al., 2002). Although the precise mechanisms by which Ginkgo biloba extract exerts its beneficial effects on cognition and memory function is not yet clear, one of the major modes of action of Ginkgo biloba extract is thought to stem from its ability to increase cerebral blood flow and enhance utilization of nutrient (Krieglstein et al., 1986; Kleijnen and Knipschild, 1992). In addition, in view of evidence that the expression of learning and memory is considered to be related to an increase in neurotransmitter release, particularly glutamate (Bekkers and Stevens, 1990; Bliss and Collingridge, 1993; Reid et al., 2004), the ability of ginkgolide B to facilitate glutamate release in hippocampal nerve terminals suggests that an increase in glutamate levels by ginkgolide B that provides a mechanism for strengthening excitatory synapses during learning and memory processes is likely to be responsible for its cognition and memory enhancing effects. However, whether such this effect contributes to the apparent therapeutic potential of Ginkgo biloba extract in Alzheimer's disease and dementia remains to be explored by further research.

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