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Ginsenosides Rg1 and Rb1 enhance glutamate release through activation of protein kinase A in rat cerebrocortical nerve terminals (synaptosomes)

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Abstract

We examined the effect of ginsenoside Rg1 or Rb1, the active ingredients of ginseng, on the release of endogenous glutamate from glutamatergic nerve terminals purified from rat cerebral cortex. Result showed that the Ca^{2+}-dependent release of glutamate evoked by 4-aminopyridine was facilitated by ginsenoside Rg1 or Rb1 in a concentration-dependent manner. Sequential experiments reveal that ginsenoside Rg1 or Rb1-mediated facilitation of glutamate release (i) results from an enhancement of vesicular exocytosis; (ii) is not due to an alternation of synaptosomal excitability; (iii) is associated with an increase in Ca^{2+} influx through presynaptic N- and P/Q-type voltage-dependent Ca^{2+} channels; (iv) appears to involve a protein kinase A pathway. These results conclude that ginsenoside Rg1 or Rb1 exerts their presynaptic facilitatory effect, likely through the activation of protein kinase A, which subsequently enhances Ca^{2+} entry to cause an increase in evoked glutamate release from rat cortical synaptosomes. This finding might provide important information regarding the action of ginseng in the central nervous system.

Keywords: Ginsenoside; Glutamate exocytosis; Voltage-dependent Ca^{2+} channel; Protein kinase A; Cerebrocortical synaptosomes

1. Introduction

Natural products and/or their synthetically developed active components have been used in medicine to prevent and treat a variety of disorders. Ginseng is one of the most commonly used natural products with a number of pharmacological effects (Gillis, 1997). Many pharmacological actions of ginseng are attributed to its active ingredient ginsenoside. Within more than 30 different ginsenosides, Rg1 and Rb1 have been shown to have beneficial effects on the central nervous system, especially cognitive function like learning and memory (Attele et al., 1999; Radad et al., 2004). It has been demonstrated, for example, that ginsenoside Rg1 or Rb1 administration is able to increase the performance in different animal models of learning/memory, such as passive avoidance and Morris water maze tasks (Benishin et al., 1991; Yamaguchi et al., 1995; Mook-Jung et al., 2001).

Although the cellular and molecular mechanisms that underlie the effect of ginsenosides on memory are not understood fully, studies in the literature suggest that the improving effect of ginsenosides on cognitive function could be related to an enhancement of central cholinergic function (Zhang et al., 1990; Benishin, 1992). Apart from facilitating central cholinergic neurotransmission, however, the increase of other neurotransmitter systems relating to cognitive function may be possibly involved in the beneficial effect of ginsenosides on learning and memory. For instance, glutamate, an excitatory neurotransmitter that exists in very high concentration in the mammalian brain, plays a crucial role in cognitive processing such as maintaining learning and memory functions (Izquierdo and Medina, 1997). There is evidence that the central glutamatergic transmission declines with age and that this decrease is associated with cognitive disturbances related to senility beyond its physiological role (Lipton and Rosenberg, 1994; Farber et al., 1998). Based on the above-studies, the memory improving effect of ginsenosides might be correlated with the regulation of brain glutamatergic neurotransmission. Indeed, data from electrophysiological experiments have shown that ginsenoside Rg1 can

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induce long-term potentiation (LTP) (Wang and Zhang, 2001), a physiological correlation of synaptic plasticity that is thought to underlie learning and memory (Bliss and Collingridge, 1993).

In the central nervous system, the regulation of glutamate release from the presynaptic neurons is one of the main mechanisms for altering synaptic efficiency that are considered to be necessary for learning and memory. Indeed, an increase of presynaptic glutamate release has been coupled with formation of several certain forms of cognitive function (Bekkers and Stevens, 1990; Reid et al., 2004). That is, there is a possibility that the presynaptic enhancement of central glutamate function is involved in the enhancing effects of ginsenoside on cognition and memory. To elucidate this possibility, we used isolated nerve terminals (synaptosomes) prepared from the rat cerebral cortex to investigate the effect of ginsenoside Rg1 or Rb1 on the release of glutamate. The synaptosome preparation provides a useful system for analysing the presynaptic action of ginsenosides on glutamatergic transmission given that synaptosomes, which are capable of accumulating, storing, and releasing neurotransmitters, do not contain intact neuronal circuits and thus are free of indirect effects mediated by functional glial or postsynaptic neurons, as what occur in more intact preparations, such as brain slices (Nicholls, 1993). Using an established method for looking at endogenous glutamate release, we found that ginsenoside Rg1 or Rb1 markedly facilitates the depolarization-evoked glutamate release from cerebrocortical synaptosomes. Also, this release facilitation seems do be mediated by positively modulating voltage-dependent Ca^{2+} channel activation through a signaling cascade involving protein kinase A.

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.). Bafilomycin A1, and ω-conotoxin MVIIC (ω-CgTX MVIIC) were obtained from Tocris Cookson (Bristol, U.S.A.). Cyclic 3, 5-(hydrogenphosphorothioate) triethylammonium (Sp-cAMPS), *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 cerebral cortex

All experiments were carried out in accordance with the guidelines established by the Fu Jen Institutional Animal Care and Utilization Committee. Synaptosomes were purified by discontinuous Percoll gradients as described previously (Nicholls et al., 1987). The cerebral cortex 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 g for 2 min at 4 °C. The supernatant fraction was collected and centrifuged at 14,500 g for 12 min. The resulting pellet was resuspended in 8 ml of 0.32 M sucrose, pH 7.4. 2 ml 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 containing 0.25 mM dithiothreitol and 1 mM ethylenediaminetetraacetic acid, pH 7.4. The gradients were centrifuged at 32,500 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 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 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

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, U.K.). $NADP^+$ (2 mM), glutamate dehydrogenase (50 units/ml) and $CaCl_2$ (1 mM) were added after 3 min. After a further 5 min of incubation, 4AP (1 mM), or KCl (15 mM) 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 2.2-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 administration 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).

2.4. Cytosolic Ca^{2+} measurement

Synaptosomes (0.5 mg/ml) were preincubated in HEPES buffer medium with 16 μM bovine serum albumin (BSA) 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-AM loading, synaptosomes were centrifuged in a microcentrifuge for 30 s at 3000 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 of 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 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).

2.5. Synaptosomal membrane potential measurement

The synaptosomal membrane potential can be monitored by positively charged membrane potential-sensitive carbocyanine dyes such as $DiSC_3(5)$ (Akerman et al., 1987). The dye becomes incorporated into the synaptosomal plasma membrane lipid bilayer. Upon depolarization with 4AP, 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, U.K.). After 3 min incubation, 4 μM $DiSC_3(5)$ was added and allowed to equilibrate before the addition of $CaCl_2$ (1 mM) after 4 min incubation. Then, 4AP 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 2.2-s intervals. Cumulative data were analysed using Lotus 1-2-3 and the results are expressed in fluorescence units.

2.6. Statistical analysis

Cumulative data were analysed using Lotus 1-2-3 and MicroCal Origin. Data are expressed as mean±S.E.M. To compare the difference of the effect of the drug treated group from that of the control group, a two-tailed Student's *t*-test was used. When an additional comparison was required (such as whether a second treatment influenced the actions of ginsenoside Rg1 or Rb1), a two-way repeated-measures analysis of variance (ANOVA) was computed. $P<0.05$ was considered to represent a significant difference.

3. Results

3.1. Effect of ginsenoside Rg1 or Rb1 on 4-aminopyridine-evoked glutamate release from rat cerebrocortical synaptosomes

To investigate the influence of ginsenoside Rg1 or Rb1 on glutamate release, we purified nerve terminals (synaptosomes) from rat cerebral cortex. As this preparation is not amenable to electrical stimulation, a number of biochemical depolarization protocols have been applied, including the use of K^+ channel blockers like 4-aminopyridine, high external $[K^+]$ or Na^+ channel modulators like veratridine (Sihra, 1997). Among these depolarization agents, the stimulation produced by 4-aminopyridine closely simulates physiological stimulation and is therefore extensively used in synaptosomal studies examining neuromodulatory influences on transmitter release. Using an on-line enzymatic assay for measuring glutamate, we observed ginsenoside Rg1 or Rb1-mediated facilitation of glutamate release from rat cerebrocortical synaptosomes (Fig. 1A). We applied ginsenoside Rg1 or Rb1 at different concentrations to cerebrocortical synaptosomes in the presence of 1 mM $CaCl_2$. The application of 0.3, 3, 10 and 30 μM ginsenoside Rg1 or Rb1, all produced a clear and statistically significant facilitation of glutamate release evoked by 1 mM 4-aminopyridine (Fig. 1B). Given the robust 45.5±4.2% and 51.5±4.3% facilitation of 4AP-evoked glutamate release seen with 10 μM Rg1 and 3 μM Rb1, respectively, 10 μM Rg1 and 3 μM Rb1were used in subsequent experiments to evaluate the mechanisms underlying the ability of the ginsenosides to facilitate glutamate release.

3.2. Effect of calcium chelation or vesicular transporter inhibitor on facilitation of 4-aminopyridine-evoked glutamate release by ginsenosides

Glutamate release produced by the depolarization of isolated nerve terminals is known to have two components: a physiologically relevant Ca^{2+}-dependent component, which is produced by the exocytosis of synaptic vesicles containing glutamate, and a Ca^{2+}-independent component that results from prolonged depolarization causing a membrane potential-mediated shift of the glutamate transporter steady-state toward the outward direction to effect cytosolic glutamate efflux (Nicholls, 1989). To examine the degree to which the observed modulation of total release by ginsenoside Rg1 or Rb1 reflected the Ca^{2+}-independent efflux of glutamate, we analysed glutamate efflux by the addition of 0.1 mM EGTA to synaptosomes (incubated in the absence of external Ca^{2+}) prior to depolarization with 4-amniopyridine. This cytosolic release of glutamate amounted to less than 3 nmol/mg/5 min. However, this component of release was not affected by the prior addition of 10 μM Rg1 (2.9±0.2 nmol/mg/5 min) or 3 μM Rb1 (3.2±0.3 nmol/mg/5 min) (Fig. 1C), suggesting that the facilitation of glutamate release by ginsenosides reflects effect on the Ca^{2+}-dependent exocytosis of glutamate but not the Ca^{2+}-independent non-vesicular efflux. To further confirm this hypothesis, we examined the effect of ginsenoside Rg1 or Rb1 in the presence of bafilomycin A1, which depletes vesicle content by inhibiting the synaptic vesicle H^+-ATPase that drives glutamate uptake. As shown in Fig. 2A, in the presence of bafilomycin A1, although control 4-amniopyridine-evoked release of 6.9±0.1 nmol/mg/5 min was reduced by bafilomycin A1 (0.1 μM) to 3.9±0.4 nmol/mg/5 min, ginsenoside Rg1 or Rb1 failed to facilitate significantly the 4-aminopyridine-evoked glutamate release. On average, ginsenoside Rg1 or Rb1 resulted in a 7.7±2.3% and 5.1±2.8% facilitation on 4-aminopyridine-evoked glutamate release after treatment with bafilomycin A1, respectively, which were significantly different the facilitation of ginsenoside Rg1 or Rb1 alone ($n=5$; $P<0.05$; repeated-measures ANOVA; Fig. 2B).

3.3. Effect of ginsenoside Rg1 or Rb1 on Ca^{2+} influx

A presynaptic facilitation of neurotransmitter release can be mediated by an increase of Ca^{2+} influx into the nerve terminals.

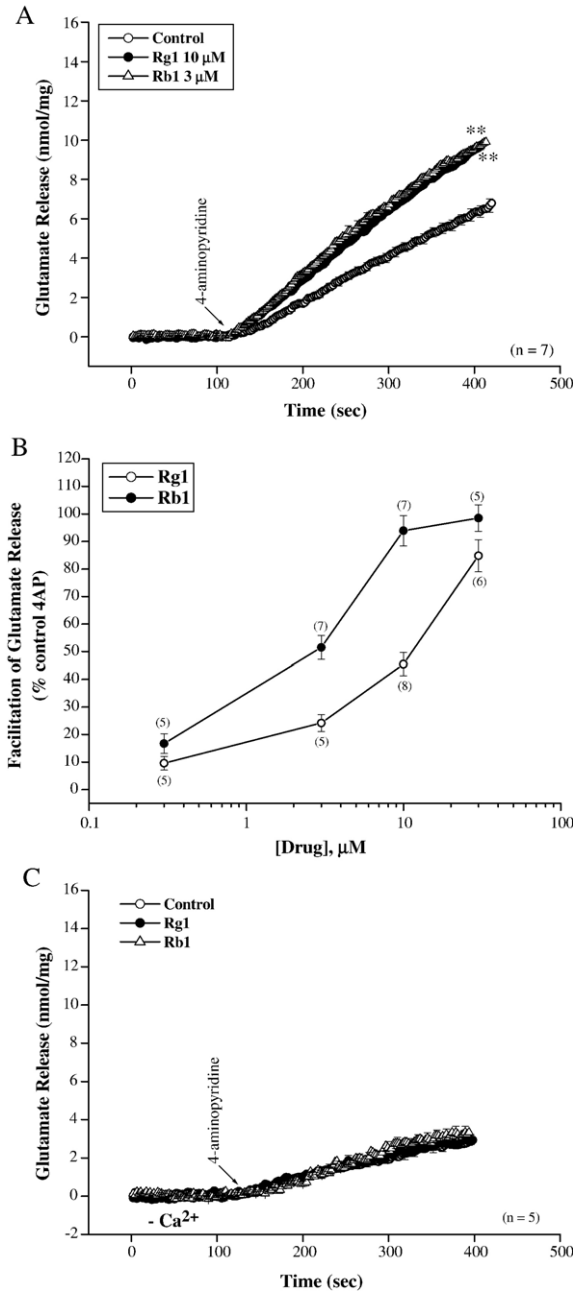

Fig. 1. Ginsenoside Rg1 or Rb1 facilitates 4-aminopyridine-evoked glutamate release from rat cerebrocortical nerve terminals in concentration-dependent manner. Synaptosomes were resuspended in incubation medium at a final protein concentration of 0.5 mg/ml and incubated for 3 min before the addition of 1 mM $CaCl_2$. 4-aminopyridine (1 mM) was added after further 10 min to effect depolarization (arrow). Ca^{2+}-independent release was assayed by omitting $CaCl_2$ and adding 0.2 mM EGTA 30 s prior to depolarization. Glutamate release was assayed by on-line fluorometry, as described in Materials and methods. Total glutamate release (A) and Ca^{2+}-independent glutamate release (-Ca^{2+}; C) were measured under control conditions (open circles) or in the presence of 10 μM Rg1 (solid circles) or 3 μM Rb1 (open triangles) added 10 min prior to the addition of 4-aminopyridine. B: Dose–response curve for ginsenoside Rg1 or Rb1 facilitation of 4-aminopyridine-evoked total glutamate release. Results are the mean±S.E.M. values of independent experiments, using synaptosomal preparations from five to eight animals. Mean and S.E.M. were calculated at each time-point (2.2 s), but error bars are shown only every 10 s for clarity. Total glutamate release (A) evoked by 4-aminopyridine, in the presence of ginsenoside Rg1 or Rb1, was significantly different from control condition (**$P<0.01$, two-tailed Student's t-test).

To investigate whether the facilitatory effect of ginsenosides on glutamate release reflected an increase in Ca^{2+} influx, we used the Ca^{2+} indicator Fura-2 to assess the effect of ginsenosides on the 4-aminopyridine-evoked increase of $[Ca^{2+}]_c$. As shown in Fig. 4, 4-aminopyridine caused a rise in $[Ca^{2+}]_c$ to a plateau level of 133.4±4.2 nM. This 4-aminopyridine-evoked rise in $[Ca^{2+}]_c$ was increased by 23 nM and 30 nM with ginsenoside Rg1 (10 μM) or Rb1 (3 μM), respectively ($n=7$; $P<0.01$; Fig. 3). In addition, the release of glutamate evoked by depolarization from adult rat cerebrocortical nerve terminal preparation is supported by the entry of Ca^{2+} through the N- and P/Q-type Ca^{2+} channels (Turner and Dunlap, 1995; Vazquez and Sanchez-Prieto, 1997). To further determine whether the

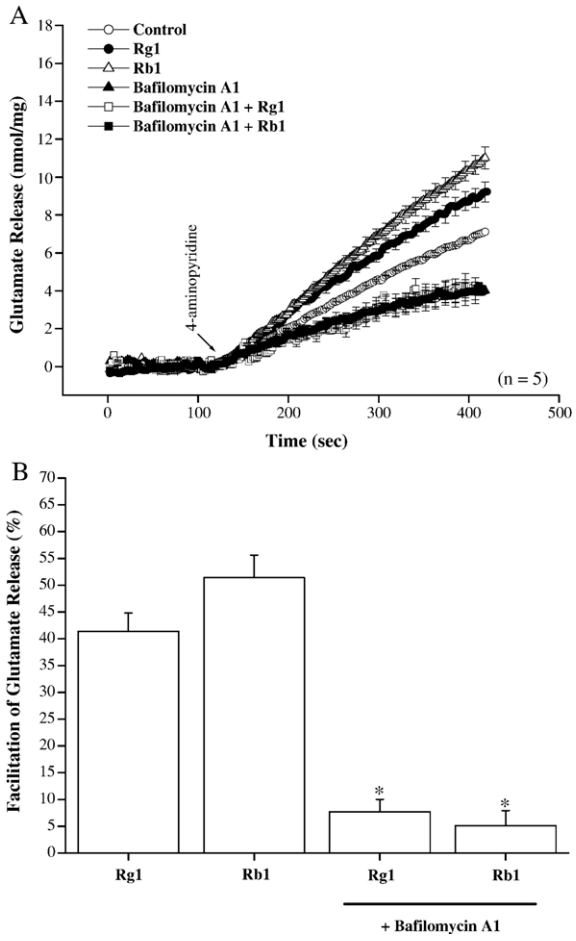

Fig. 2. Ginsenoside Rg1 or Rb1-mediated facilitation of 4-aminopyridine-evoked glutamate release is inhibited by vesicle transporter inhibitor bafilomycin A1. A: Glutamate release was evoked by 1 mM 4-aminopyridine in the absence (open circles) or presence of 10 μM Rg1 (solid circles), 3 μM Rb1 (open triangles), 0.1 μM bafilomycin A1 (solid triangles), 0.1 μM bafilomycin A1+10 μM Rg1 (open squares) or 0.1 μM bafilomycin A1+3 μM Rb1 (solid squares). Rg1 or Rb1 was added 10 min before depolarization and, bafilomycin A1, 10 min prior to this. Results are the mean±S.E.M. values of independent experiments, using synaptosomal preparations from five animals. Mean and S.E.M. were calculated at each time-point (2.2 s), but error bars are only shown every 10 s for clarity. B: Bar plots showing the average percent facilitation of evoked release produced by Rg1 or Rb1 in synaptosomes from control, and bafilomycin A1-treated groups. Asterisks denote conditions under which release facilitation was significantly different from that obtained with Rg1 or Rb1 alone ($P<0.05$, ANOVA followed by two-tailed Student's t-test).

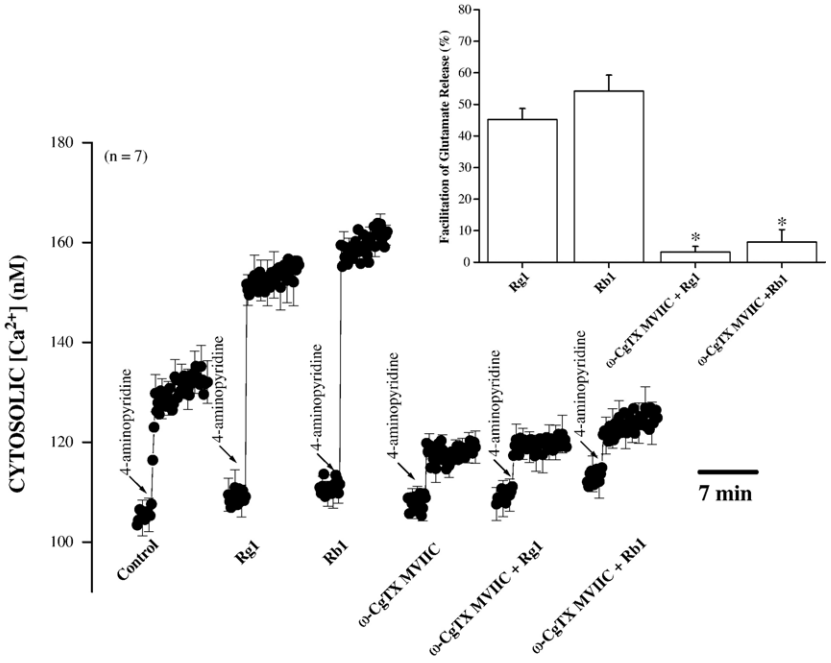

Fig. 3. Release facilitation by ginsenoside Rg1 or Rb1 is associated with an increase in Ca^{2+} influx through voltage-dependent N- and P/Q-type Ca^{2+} channels. Synaptosomes (0.5 mg/ml) were incubated as described under Materials and methods, and cytosolic $[Ca^{2+}]$ was monitored using Fura-2. Synaptosomes were stimulated with 1 mM 4-aminopyridine (arrow) in the absence (control) or presence of Rg1 (10 μM), Rb1 (3 μM), ω-CgTX MVIIC (2 μM), ω-CgTX MVIIC (2 μM)+Rg1 (10 μM) or ω-CgTX MVIIC (2 μM)+Rb1 (3 μM). Rg1 or Rb1 was added 10 min before depolarization and, ω-CgTX MVIIC, 30 min prior to this. Results are the mean±S.E.M. values of independent experiments, using synaptosomal preparations from seven animals. Mean and S.E.M. were calculated at each time-point (7.5 s), but error bars are only shown every 30 s for clarity. Synaptosomal $[Ca^{2+}]$ in the presence of Rg1 or Rb1 was significantly different from control ($P<0.01$, two-tailed Student's *t*-test). Inset: Comparison of the facilitation of glutamate release with Rg1 or Rb1 alone and in the presence of ω-CgTX MVIIC. Results are the mean±S.E.M. values of experiments carried out with six independent synaptosomal preparations. Release facilitation (percentage) of Rg1 or Rb1 in the presence of ω-CgTX MVIIC was significantly different from that obtained with Rg1 or Rb1 alone (*$P<0.05$, ANOVA followed by two-tailed Student's *t*-test).

enhancement of this Ca^{2+} channel activity contributed to the increase of Ca^{2+} influx observed with ginsenosides, the effect of ginsenoside Rg1 or Rb1 on the 4-aminopyridine-evoked increase of $[Ca^{2+}]_c$ was compared before and after the application of ω-conotoxin MVIIC (ω-CgTX MVIIC), a wide-spectrum blocker of N-, P-, and Q-type Ca^{2+} channels. As shown in Fig. 3, ω-CgTX MVIIC (2 μM) reduced 4-aminopyridine-evoked rise in $[Ca^{2+}]_c$ by 67.3±5.2% ($n=7$; $P<0.01$). In the presence of ω-CgTX MVIIC, application of ginsenoside Rg1 (10 μM) or Rb1 (3 μM) only increased 4-aminopyridine-evoked increase of $[Ca^{2+}]_c$ by further 3.4±2.1% and 5.1±2.7%, respectively, indicating a significant reduction compared with that obtained when ginsenoside Rg1 or Rb1 was applied alone ($P<0.05$; repeated-measures ANOVA). In addition, the facilitation of 4-aminopyridine-evoked glutamate release by ginsenoside Rg1 or Rb1 was also significantly suppressed by ω-CgTX MVIIC ($n=6$; Fig. 3, inset). In the treated-ω-CgTX MVIIC (2 μM) synaptosomes, ginsenoside Rg1 or Rb1 only produced a 3.2±1.8% or 6.4±3.9% increase in the 4-aminopyridine-evoked glutamate release after the application ω-CgTX MVIIC, respectively, which was significantly different from the facilitation produced by ginsenoside Rg1 or Rb1 alone ($n=8$; $P<0.05$; repeated-measures ANOVA). Therefore, these results indicate that an increase of Ca^{2+} influx through presynaptic N- and P/Q-type Ca^{2+} channels is involved in the ginsenosides Rg1 and Rb1-mediated facilitation of 4-aminopyridine-evoked glutamate release.

3.4. Effect of ginsenoside Rg1 or Rb1 on nerve terminal excitability

Because activation of Na^+ channels or inhibition of K^+ channels is known to increase membrane excitability and consequently cause an enhancement in the evoked entry of Ca^{2+} and neurotransmitter release (Rehm and Tempel, 1991; Pongs et al., 1999), we reasoned that the observed facilitatory effect of ginsenosides on 4-aminopyridine-evoked Ca^{2+} influx and glutamate release could be due to an alteration of nerve terminal excitability. To test this possibility, we examined the effects of ginsenoside Rg1 or Rb1 on synaptosomal plasma membrane potential under resting conditions and on depolarization, with the membrane potential-sensitive dye $DiSC_3(5)$. As shown in Fig. 4, 4-aminopyridine (1 mM) caused an increase in $DiSC_3(5)$ fluorescence of 1.32±0.04 fluorescence units/5 min. Preincubation of synaptosomes with ginsenoside Rg1 (10 μM) or Rb1 (3 μM) for 10 min before 4-aminopyridine addition did not alter the resting plasma membrane potential and had no significant effect on the 4-aminopyridine-mediated elevation of $DiSC_3(5)$ fluorescence [(fluorescence units/5 min): Rg1+4-aminopyridine, 1.26±0.07; Rb1+4-aminopyridine, 1.38±0.09] ($n=8$; $P>0.01$; two-tailed Student's *t*-test). This indicates that the observed

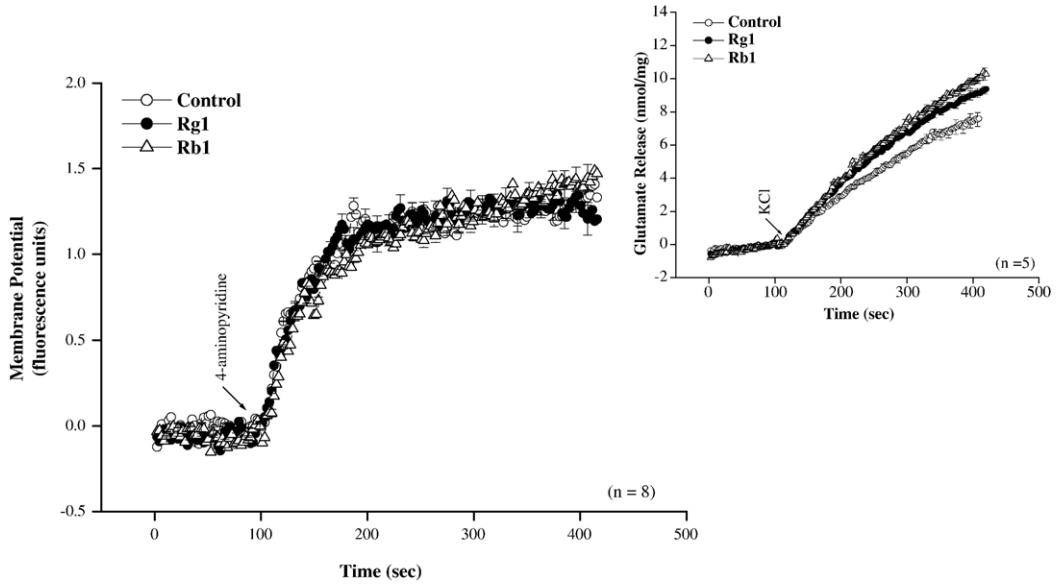

Fig. 4. Ginsenoside Rg1 or Rb1 does not alter the synaptosomal membrane potential. Synaptosomal membrane potential was monitored with 5 μM $DiSC_3(5)$ on depolarization with 4-aminopyridine (1 mM) in the absence (open circles) or in the presence of 10 μM Rg1 (solid circles) or 3 mM Rb1 (open triangles) added 10 min before depolarization. Inset: Rg1 or Rb1 modulation of KCl (15 mM)-evoked glutamate release. Experiments were carried out as described in previous release figure except for the addition of 15 mM KCl as secretagogue instead of 4-aminopyridine. Each trace is the mean±S.E.M. values of independent experiments, using synaptosomal preparations from five to eight animals. Mean and S.E.M. were calculated at each time-point (2.2 s), but error bars are only shown every 10 s for clarity. Asterisk indicates the difference in the release of glutamate evoked by KCl was significantly different in the absence and presence of Rg1 or Rb1 ($P<0.01$, two-tailed Student's *t*-test).

facilitatory effect of ginsenosides on 4-aminopyridine-evoked glutamate release is unlikely to be due to either a depolarizing effect of drug on the synaptosomal plasma membrane potential or an increase in synaptosomal depolarization produced by 4-aminopyridine. Confirmation that ginsenosides effect did not impinge on synaptosomal excitability was obtained with experiments using high external $[K^+]$-mediated depolarization, which "clamps" the membrane potential according to the imposed K^+ electrochemical gradient and thereby activates voltage-dependent Ca^{2+} channels (McMahon and Nicholls, 1991). Addition of 15 mM KCl effected a control release of 7.5±0.4 nmol/mg/5 min, which was increased to 9.3±0.2 and 10.1±0.4 nmol/mg/5 min by ginsenosides Rg1 (10 μM) or Rb1 (3 μM), respectively ($n=5$; $P<0.01$; two-tailed Student's *t*-test) (Fig. 4, inset).

3.5. Effect of protein kinase A activator or inhibitor on facilitation of 4-aminopyridine-evoked glutamate release by ginsenosides

What intracellular signaling cascade is involved in the regulation of ginsenosides on glutamate release from nerve terminals? It has been reported that ginsenoside Rg1 increases rat brain cAMP levels (Stancheva and Alova, 1993). Because most effects of cAMP in the central nervous system are mediated by activation of protein kinase A, we sought to examine whether activation of cAMP/protein kinase A signaling cascade participated in the release facilitation of ginsenosides. To test this possibility, the effect of ginsenoside Rg1 or Rb1 on 4-aminopyridine-evoked glutamate release was compared in the presence and absence of the protein kinase A activator cyclic 3, 5-(hydrogenphosphorothioate) triethylammonium (Sp-cAMPS). As shown in Fig. 5, 4-aminopyridine (1 mM) evoked a glutamate release of 6.6±0.1 nmol/mg/5 min, which was facilitated to 9.5±0.6 nmol/mg/5 min in synaptosomes pretreated with Sp-cAMPS (25 μM) ($n=5$; $P<0.01$; two-tailed

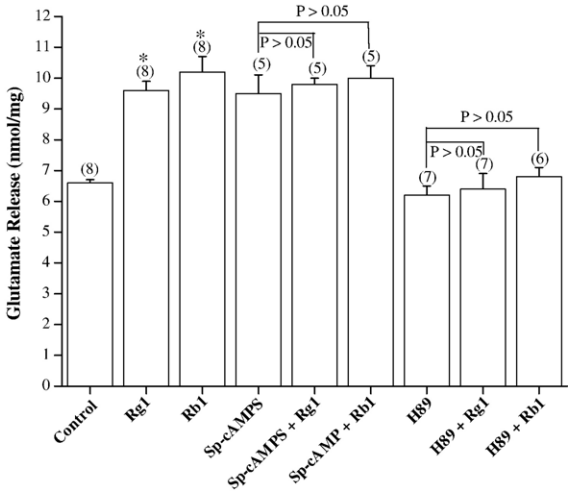

Fig. 5. Activation of protein kinase A is involved in the facilitation of 4-aminopyridine-evoked glutamate release by ginsenoside Rg1 or Rb1. The bar diagrams showing the glutamate release evoked by 1 mM 4-aminopyridine in the absence (control) or presence of Rg1 (10 μM), Rb1 (3 μM), Sp-cAMPS (25 μM), Sp-cAMPS (25 μM)+Rg1 (10 μM), Sp-cAMPS (25 μM)+Rb1 (3 μM), H89 (1 μM), H89 (1 μM)+Rg1 (10 μM) or H89 (1 μM)+Rb1 (3 μM). H89 and Sp-cAMPs were added 30 min before depolarization with 4-aminopyridine and, Rg1 or Rb1 was added 10 min after the first addition of H89 or Sp-cAMPS. Data represent mean±S.E.M. values of independent experiments, using synaptosomal preparations from five to eight animals. Asterisks denote conditions under which release was significantly different from control 4-aminopyridine-evoked release ($P<0.01$, two-tailed Student's *t*-test).

Student's *t*-test). Ginsenoside Rg1 (10 μM) or Rb1 (3 μM) alone facilitated the 4-aminopyridine-evoked release of glutamate to 9.6± 0.3 and 10.2±0.5 nmol/mg/5 min, respectively. However, the release facilitation produced by these two ginsenosides were occluded by pretreatment with Sp-cAMPS (25 μM), and there is no statistical difference between the release after Sp-cAMPS alone and after Sp-cAMPS+ginsenoside Rg1 or Rb1 treatment ($n=5$; $P>0.05$; repeated-measures ANOVA). In addition, to further confirm that a protein kinase A signaling pathway was activated by ginsenosides in their facilitation of glutamate release, we performed experiments to test the effect of ginsenoside Rg1 or Rb1 following inhibition of protein kinase A. Fig. 5 shows that preincubation of synaptosomes with the protein kinase A inhibitor *N*-[2-(p-bromocinnamylamino)-ethyl]-5-isoquinolinesulfonamide dihydrochloride (H89) (1 μM) for 30 min, caused a small inhibition on control 4-aminopyridine-evoked glutamate release (6.2±0.3 nmol/mg/5 min), but this effect was statistically insignificant ($n=6$; $P>0.01$; two-tailed Student's *t*-test). Under this condition, ginsenoside Rg1 (10 μM) or Rb1 (3 μM) failed to produce a significantly facilitation on 4-aminopyridine-evoked glutamate release.

4. Discussion

Ginseng and several of its active components have been reported to have a positive impact on brain aging to retard the impairment of cognitive function and memory, but the mechanism of this beneficial effect is not entirely clear. Although some experimental evidence suggests that the beneficial effects of ginseng on learning/memory could be related to an improving effect on central cholinergic function (Benishin et al., 1991; Benishin, 1992; Yamaguchi et al., 1996), the participation of other neurotransmitter systems cannot be ruled out. For instance, the central glutamatergic system is also important for learning, memory and cognitive function as reported by many authors (Bliss and Collingridge, 1993; Izquierdo and Medina, 1997). To our knowledge, however, fewer studies have focused on the relation between ginseng or its active components and brain glutamate function. In the current study, by using isolated glutamatergic nerve terminals from rat cerebral cortex, our results demonstrate for the first time that ginsenosides Rg1 and Rb1, the active ingredients of ginseng, facilitate the release of glutamate evoked by 4-aminopyridine, a potassium channel blocker that depolarizes nerve terminals *in vitro*, in a manner corresponding to *in vivo* depolarization (Tibbs et al., 1989). As memory deficits have been considered to be associated with impairment of glutamatergic system, our finding that ginsenosides strengthen glutamatergic neurotransmission by increasing glutamate release might explain the cognition enhancing effect observed in the previous *in vivo* studies (Attele et al., 1999).

4.1. Mechanisms of presynaptic facilitation of glutamate release by ginsenosides Rg1 and Rb1

A key issue to address is the nature of the mechanism responsible for ginsenosides-facilitated 4AP-evoked glutamate release from rat cerebrocortical synaptosomes. In principle, 4-aminopyridine-evoked release of glutamate from nerve terminals can be sustained by different mechanisms, including exocytosis, supported by both external Ca^{2+} influx and mobilization of Ca^{2+} from internal stores, and reversal of the transporter (Ca^{2+}-independent release) (Raiteri et al., 2002). In the present study, we found that, in the presence of calcium-free medium containing 200 μM EGTA, either ginsenoside Rg1 or Rb1 failed to produce a significant facilitatory effect on 4-aminopyridine-evoked glutamate release. Furthermore, suppression of ginsenoside Rg1 or Rb1-mediated facilitation of 4-aminopyridine-evoked glutamate release by the vesicular transporter inhibitor bafilomycin A1 was observed. Therefore, these data confirmed that the observed facilitation of glutamate release produced by ginsenoside Rg1 or Rb1 originates from synaptic vesicle exocytosis rather than reversal of the plasma membrane glutamate transporter.

Using Fura-2, we found that both ginsenosides Rg1 and Rb1 significantly enhanced 4-aminopyridine-evoked increase in $[Ca^{2+}]_c$ and these effects were largely suppressed in synaptosomes pretreated with a wide-spectrum blocker of the N- and P/Q-type voltage-dependent Ca^{2+} channels, ω-CgTX MVIIC. These results, together with the blockade of ginsenoside Rg1 or Rb-mediated facilitation of 4-aminopyridine-evoked glutamate release by ω-CgTX MVIIC, suggest that the observed ginsenosides-mediated facilitation of glutamate release is indeed related to an increase in Ca^{2+} influx through N- and P/Q-type voltage-dependent Ca^{2+} channels. Given these results, the question remained of whether the enhancement of release-coupled voltage-dependent Ca^{2+} channels by ginsenosides action reflected a direct effect on voltage-dependent Ca^{2+} channel function or resulted from the increment of synaptosomal excitability. To address this issue, we compared the relative effects of ginsenoside Rg1 or Rb1 on the 4-aminopyridine- vs. KCl-evoked glutamate release. Both of these depolarizing treatments are thought to activate N- and P/Q-type voltage-dependent Ca^{2+} channels coupled to glutamate similarly and thus should reflect this by qualitatively similar modulation, if this occurs at the level of voltage-dependent Ca^{2+} channels. Where the two depolarizing paradigms differ is in that, whereas 4-aminopyridine-evoked glutamate release involves the action of Na^+ channels, and Ca^{2+} channels, 15 mM external KCl-evoked release involves only Ca^{2+} channels (Barrie et al., 1991; Nicholls, 1998). Comparison of the two depolarizing agents revealed that both 4-aminopyridine- and KCl-evoked glutamate release were facilitated by ginsenoside Rg1 or Rb1. Based on the mechanistic differences between the 4-aminopyridine- and the KCl-mediated depolarization discussed above, the data presented indicate that the observed facilitatory effect of ginsenoside Rg1 or Rb1 on Ca^{2+} influx and glutamate release is unlikely to be due to an indirect effect through modulation of ion-channel activities (e.g., Na^+ or K^+ channel) determining synaptosomal excitability. In line with this, 4-aminopyridine-mediated synaptosomal membrane potential depolarization, probed using the membrane potential-sensitive dye $DiSC_3(5)$, was unaffected by ginsenoside Rg1 or Rb1. However, our finding is inconsistent with previous electrophysiological data showing that ginsenoside Rb1 inhibits Na^+ channel activity in tsA201 cells transfected with cDNA expressing human $brain_{2a}$ sodium channel α subunit (Liu et al., 2001). The discrepancy is not clear but may be due to

the difference in experimental model investigated. Although there is lack of direct evidence for ginsenosides action on presynaptic ion channels, these results clearly indicate the influence of Ca^{2+} channel activity as a potential mechanism underlying the facilitation of glutamate release by ginsenosides.

How come ginsenosides facilitate Ca^{2+} and glutamate release from cerebrocortical nerve terminals? Ginsenoside has been shown to inhibit brain cAMP phosphodiesterase activity, resulting in the increase of cAMP levels and the activation of protein kinase A (Stancheva and Alova, 1993). Consistently with this, in the present study, a cAMP/protein kinase A pathway seems to be involved in the facilitatory action of ginsenoside Rg1 or Rb1 on 4-aminopyridine-evoked glutamate release from cerebrocortical synaptosomes. Two lines of evidence support this notion. First, the facilitatory effect of ginsenoside Rg1 or Rb1 on 4-aminopyridine-evoked glutamate release was completely occluded by Sp-cAMPS, a membrane permeant cAMP analog. Second, in synaptosomes treated with the specific protein kinase A inhibitor H89, ginsenoside Rg1 or Rb1-mediated facilitation of glutamate release was substantially reduced. In the mammalian brain, cAMP-mediated signaling cascade has been implicated in the modulation of many critical physiological processes, such as learning and memory acquisition (Sunahara et al., 1996; Wong et al., 1999). Thus, it is possible that activation of cAMP/protein kinase A pathway causes the increase in glutamate release mediated by ginsenoside Rg1 or Rb1, contributing to the potential therapeutic benefits of ginseng in the context of learning and memory defects. Here, a question arises how activation of protein kinase A might be involved in the ginsenoside Rg1 or Rb1-mediated facilitation of glutamate release. In fact, synaptosomal voltage-dependent Ca^{2+} channels and several synaptic proteins involved in the synaptic vesicle trafficking/recruitment and exocytosis, including synapsin I, rabphilin 3A, syntaxin, SNAP 25 and synaptobrevin, have been shown to be phosphorylated by protein kinase A (Greengard et al., 1993; Fykse et al., 1995; Lonart et al., 1998; Risinger and Bennett, 1999; Catterall, 2000; Jarvis and Zamponi, 2001). Thus, future studies will be required to determine which downstream substrate of protein kinase A is involved in the ginsenoside Rg1 or Rb1-mediated facilitation of glutamate release.

4.2. Conclusion and therapeutic implications

The major finding from this analysis is that ginsenosides Rg1 or Rb1 facilitates evoked glutamate release from rat cerebral cortex nerve terminals by increasing Ca^{2+} influx through N- and P/Q-type Ca^{2+} channels, and this release facilitation is likely to depend, at least in part, on the activation of cAMP/protein kinase A pathway. Even the functional role of ginsenosides-facilitated glutamate exocytosis explored here is not clear; this effect of ginsenosides could be of physiological importance in the aging-related decrements in cognitive function, as this decline has been shown to associate with a significant decrease in glutamate release (Aprikyan and Gekchyan, 1988). Although the detailed mechanisms remain to be determined, the present report provides important novel information regarding the central effects of ginseng and offers a useful therapeutic choice in the treatment of age-related cognitive decline.

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