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Hypericin, the active component of St. John's wort, inhibits glutamate release in the rat cerebrocortical synaptosomes via a mitogen-activated protein kinase-dependent pathway

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ABSTRACT

Changes in central glutamate neurotransmission are involved in the pathophysiology of depression and in the mechanism of antidepressants. In this study, the effect of hypericin, a major active constituent of St. John's wort that is widely used in the treatment of depression, on the release of glutamate from nerve terminals purified from rat cerebral cortex was examined. Result showed that hypericin inhibited the release of glutamate evoked by 4-aminopyridine in a concentration-dependent manner. Further experiments revealed that hypericin-mediated inhibition of glutamate release (i) results from a reduction of vesicular exocytosis, not from an inhibition of Ca^{2+}-independent efflux via glutamate transporter; (ii) is not due to an alternation of nerve terminal excitability; (iii) is associated with a decrease in presynaptic N- and P/Q-type voltage-dependent Ca^{2+} channel activity; and (iv) appears to involve the suppression of mitogen-activated protein kinase pathway. These results are the first to suggest that, in rat cerebrocortical nerve terminals, hypericin suppresses voltage-dependent Ca^{2+} channel and mitogen-activated protein kinase activity and in so doing inhibits evoked glutamate release. This finding may provide important information regarding the beneficial effects of St. John's wort in the brain.

1. Introduction

Depression is a prevalent psychiatric disorder that has been estimated to affect up to 21% of the world population (Schechter et al., 2005). At present, there are four main kinds of classical antidepressant drugs in clinical practice, including tricyclic antidepressants, selective serotonin reuptake inhibitors, monoamine oxidase inhibitors and norepinephrine-serotonin reuptake inhibitors (Adell et al., 2005). Unfortunately, these drugs have unwanted side effects and approximately 30% of patients do not respond to the therapy of these drugs (Millan, 2006). Therefore, seeking safe and effective antidepressant drugs from traditional herbs may enable us to uncover novel treatments for depression. Recently, a significant number of herbs have been claimed to improve mood and used in the treatment of depression. St. John's wort (*Hypericum perforatum*) is one of the most frequently prescribed herbal (Fugh-Berman and Cott, 1999; Beaubrun and Gray, 2000). Although the cellular and molecular mechanisms that underlie the effect of St. John's wort on mood are not understood fully, studies in the literature suggest that the antidepressive effect of St. John's wort could be related to an increase in concentration of brain monoamines (serotonin, noradrenaline and dopamine) (Muller et al., 1997; Neary and Bu, 1999; Muller, 2003). Apart from the central monoaminergic neurotransmitter system, however, the regulation of other neurotransmitter systems may be involved in the antidepressive effect of St. John's wort.

Glutamate, the major excitatory neurotransmitter in the brain, has been suggested to be important in the pathophysiology of depression and in the action of antidepressant drugs. This hypothesis stems from the following evidence: (1) high levels of glutamate both in plasma and the brain of depressed patients (Sanacora et al., 2004; Kendell et al., 2005); (2) antidepressant-like activity induced by glutamate receptor antagonists in animal models (Skolnick, 2002); (3) reduction of glutamate release and glutamate receptor function by antidepressants (Skolnick, 1999; Michael-Titus et al., 2000; Paul and Skolnick, 2003; Wang et al., 2003; Bonanno et al., 2005). All the evidence presented above indicates that a hyperfunction of the central glutamate system can occur in depression and that antidepressant effects can be achieved by the reduction of central glutamate neurotransmission.

Since excessive glutamate release has been considered to be part of the pathogenesis of depression (Sapolsky, 2000; Zarate et al., 2002), we explored whether a decrease in glutamate release is involved in the possible pharmacological mechanisms of St. John's wort. To address this issue, in the present study we used isolated nerve terminals (synaptosomes) purified from the rat cerebral cortex to investigate the influence

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of hypericin, an active constituent of St. John's wort, on glutamate release. The isolated presynaptic terminal represents a model system for investigating directly the molecular mechanisms underlying presynaptic phenomena. Specifically, this preparation is capable of accumulating, storing and releasing neurotransmitters, and is devoid of functional glial and nerve cell body elements that might obfuscate interpretation because of modulatory loci at non-neuronal, postsynaptic or network levels (Dunkley et al., 1986). The experiments were performed with synaptosomes by monitoring the effects of hypericin on the release of endogenous glutamate, synaptosomal plasma membrane potential, and downstream activation of voltage-dependent Ca^{2+} channels. In addition, in view of the demonstrated role of mitogen-activated protein kinase in presynaptic modulation (Grewal et al., 1999; Pereira et al., 2002), this study also examined if this signaling pathway was involved in the regulation of hypericin on glutamate release.

2. Materials and methods

2.1. Animals

Adult male Sprague–Dawley rats (200–250 g) were employed in these studies. All animal procedures were carried out in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, and were approved by the Fu Jen Institutional Animal Care and Utilization Committee.

2.2. Materials

Hypericin (purity>98%) was obtained from LKT Laboratories, Inc (U.S.A.). 3′,3′,3′-dipropylthiadicarbocyanine iodide [$DiSC_3(5)$], FM1-43 and fura-2-acetoxymethyl ester (Fura-2-AM) were obtained from Invitrogen (Carlsbad, CA). Bafilomycin A1, ω-conotoxin MVIIC, DL-threo-beta-benzyl-oxyaspartate (DL-TBOA), 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD98059) and bisindolylmaleimide I (GF109203X) were obtained from Tocris Cookson (Bristol, UK). 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid-acetoxymethyl ester (BAPTA-AM), and all other reagents were obtained from Sigma (Poole, Dorset, UK).

2.3. Synaptosomal preparation

Synaptosomes were prepared as described previously (Wang and Sihra, 2004). Briefly, the cerebral cortex from male Sprague–Dawley rats was isolated and homogenized in a medium that contained 320 mM sucrose, pH 7.4. The homogenate was spun for 2 min at 3000×*g* (5000 rpm in a JA 25.5 rotor; Beckman Coulter, Inc., USA) at 4 °C, and the supernatant was spun again at 14 500×*g* (11 000 rpm in a JA 25.5 rotor) for 12 min. The pellet was gently resuspended in 8 ml of 320 mM sucrose, pH 7.4. Two milliliters of this synaptosomal suspension was added to 3 ml Percoll discontinuous gradients that contained 320 mM sucrose, 1 mM EDTA, 0.25 mM DL-dithiothreitol, and 3, 10 and 23% Percoll, pH 7.4. The gradients were centrifuged at 32 500×*g* (16500 rpm in a JA 20.5 rotor) for 7 min at 4 °C. Synaptosomes placed between the 10 and 23% Percoll bands were collected and diluted in a final volume of 30 ml of HEPES buffer medium that consisted of 140 mM NaCl, 5 mM KCl, 5 mM $NaHCO_3$, 1 mM $MgCl_2 \cdot 6H_2O$, 1.2 mM Na_2HPO_4, 10 mM glucose, and 10 mM HEPES (pH 7.4), before centrifugation at 27 000×*g* (15 000 rpm in a JA 25.5) for 10 min. The pellets thus formed were resuspended in 3 ml of HEPES buffer medium, and the protein content was determined using a Bradford Protein Assay Kit (Bio-Rad, Hercules, CA, USA), based on the method of Bradford (1976), with BSA as a standard. 0.5 mg of synaptosomal suspension was diluted in 10 ml of HEPES buffer medium and spun at 3000×*g* (5000 rpm in a JA 20.1 rotor) for 10 min. The supernatants were discarded, and the synaptosomal pellets were stored on ice and used within 4–6 h.

2.4. Glutamate release

Glutamate release from purified cerebrocortical synaptosomes was monitored online, with an assay that employed exogenous glutamate dehydrogenase and $NADP^+$ to couple the oxidative deamination of the released glutamate to the generation of NADPH detected fluorometrically (Nicholls, 1993). Synaptosomal pellets were resuspended in HBM that contained 16 μM bovine serum albumin and incubated in a stirred and thermostated cuvette maintained at 37 °C in a Perkin-Elmer LS-50B spectrofluorimeter. $NADP^+$ (2 mM), glutamate dehydrogenase (50 units/ml) and $CaCl_2$ (1 mM) were added after 3 min. In experiments that investigated Ca^{2+}-independent efflux of glutamate, EGTA (200 μM) was added in place of $CaCl_2$. Other additions before depolarization were made as described in the figure legends. After a further 10 min of incubation, 4-aminopyridine (1 mM), or KCl (15 mM) was added to stimulate glutamate release. Glutamate release was monitored by measuring the increase of fluorescence (excitation and emission wavelengths of 340 and 460 nm, respectively) caused by NADPH being produced by oxidative deamination of released glutamate by glutamate dehydrogenase. Data were accumulated at 2-s intervals. A standard of exogenous glutamate (5 nmol) was added at the end of each experiment, and the fluorescence response used to calculate released glutamate was expressed as nanomoles glutamate per milligram synaptosomal protein (nmol/mg). Values quoted in the text and expressed in bar graphs represent levels of glutamate cumulatively release after 5 min of depolarization.

2.5. Styryl dye release assay

Synaptic vesicle fusion with the plasma membrane was measured using release of the fluorescent dye FM1-43, as described previously (Baldwin et al., 2003). In brief, synaptosomes (0.5 mg/ml) were incubated in HEPES buffer medium with 1.2 mM $CaCl_2$ for 2 min at 37 °C in a stirred test tube. FM1-43 (100 μM) was added 1 min before stimulation with 30 mM KCl. After 3 min of stimulation to load FM1-43, synaptosomes were washed twice in HEPES buffer medium that contained 1.2 mM $CaCl_2$ and 1 mg/ml bovine serum albumin to remove non-internalized FM1-43. Synaptosomes were then resuspended in 2 ml of HEPES buffer medium (plus 1.2 mM Ca^{2+}), and incubated in a stirred and thermostated cuvette maintained at 37 °C in a Perkin-Elmer LS-50B spectrofluorimeter. Release of accumulated FM1-43 was induced by the addition of 1 mM 4-aminopyridine, and measured as the decrease in fluorescence upon release of the dye into solution (excitation 488 nm, emission 540 nm). Data points were obtained at 2.2-s intervals, and data presented as the Ca^{2+}-dependent decrease in FM1-43 fluorescence. Any drugs were added after the dye-loading procedure, and the synaptosomes were preincubated with hypericin for 10 min before depolarization with 4-aminopyridine.

2.6. Synaptosomal plasma membrane potential measurement

The synaptosomal membrane potential can be monitored by positively charged, membrane potential-sensitive carbocyanine dyes such as $DiSC_3(5)$. Synaptosomes were preincubated and resuspended as described for the glutamate release experiments. After 3 min incubation, 5 μM $DiSC_3(5)$ was added and allowed to equilibrate before the addition of $CaCl_2$ (1 mM) after 4 min incubation. 4-aminopyridine (1 mM) was added to depolarize the synaptosomes at 10 min, and $DiSC_3(5)$ fluorescence was monitored at excitation and emission wavelengths of 646 and 674 nm, respectively.

2.7. Cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_C$) measurement

$[Ca^{2+}]_C$ was measured using the Ca^{2+} indicator Fura-2-AM. 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 (Fura-2-AM) 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* (5000 rpm). The synaptosomal pellets were resuspended in HEPES buffer medium with bovine serum albumin, and the synaptosomal suspension was stirred in a thermostated 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 10 min. Fluorescence data were accumulated at excitation wavelengths of 340 and 380 nm (emission wavelength 505 nm) at 7.5-s intervals. Calibration procedures were performed as described previously (Sihra et al., 1992), using 0.1% sodium dodecyl sulphate (SDS) to obtain the maximal fluorescence with Fura-2-AM 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. $[Ca^{2+}]_C$ was calculated using equations described previously (Grynkiewicz et al., 1985).

2.8. Western blotting analysis

Synaptosomes (0.5 mg protein/ml) from control and hypericin-treated groups were lysed in ice-cold Tris–HCl buffer solution, pH 7.5, that contained 20 mM Tris–HCl, 1% Triton, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM phenylmethanesulfonyl fluoride, 1 mM sodium orthovanadate and 1 μg/ml leupeptin. The lysates were sonicated for 10 s and then centrifuged at 13 000×*g* at 4 °C for 10 min. Equal amounts of synaptosomal proteins were loaded on a SDS polyacrylamide gel and transferred electrophoretically to nitrocellulose membranes. The membranes were blocked with Tris-buffered saline that contained 5% low-fat milk and incubated with monoclonal antibody (phospho-p44/42 MAPK; 1:2000; Cell Signaling, MA) or polyclonal antibody (p44/42 MAPK; 1:1000; Cell Signaling, MA) in appropriate dilutions. After incubation with appropriate secondary antibodies (1:3000), protein bands were detected by using the ECL chemiluminescence system (Amersham, Buckinghamshire, UK). Films were scanned using a scanner and the level of phosphorylation was assessed by band density, which was quantified by densitometry.

2.9. Data analysis

Cumulative data were analyzed in Lotus 1-2-3 and MicroCal Origin. Data are expressed as mean ± S.E.M. To test the significance of the effect of a drug versus control, a two-tailed Student's *t* test was used. When an additional comparison was required (such as whether a second treatment influenced the action of hypericin), a one-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 hypericin on 4-aminopyridine-evoked glutamate release from rat cerebral cortex nerve terminals

To investigate whether hypericin could directly influence the release of glutamate, isolated cerebrocortical nerve terminals were depolarized with the K^+-channel blocker 4-aminopyridine. 4-aminopyridine destabilizes the membrane potential and is thought to cause repetitive spontaneous Na^+-channel-dependent depolarization that closely approximates *in vivo* depolarization of the synaptic terminal, which leads to the activation of voltage-dependent Ca^{2+} channels and neurotransmitter release (Tibbs et al., 1989). Using an online enzymatic assay for measuring glutamate, we observed 4-aminopyridine evoked a glutamate release of 8.4 ± 0.3 nmol/mg/5 min in synaptosomes incubated in the presence of 1 mM $CaCl_2$. Preincubation of synaptosomes with 10 μM hypericin produced an inhibition of 4-aminopyridine-evoked glutamate release to 5.2 ± 0.1 nmol/mg/5 min ($n=8$; $P<0.01$), without altering the basal release of glutamate (Fig. 1A). The hypericin-induced inhibition of 4-aminopyridine-evoked glutamate release was concentration dependent, with an IC_{50} value derived from a dose–response curve of about 13.3 μM (Fig. 1B).

3.2. Effect of calcium chelation, glutamate transporter inhibitor or vesicular transporter inhibitor on the hypericin-mediated inhibition of 4-aminopyridine-evoked glutamate release

We next performed a series of experiments to examine whether the effect of hypericin on 4-aminopyridine-evoked glutamate release reflected an effect on physiological exocytotic vesicular release or on Ca^{2+}-independent release of glutamate attributable to cytosolic efflux via the reversal of the glutamate transporter (Nicholls et al., 1987). First, the Ca^{2+}-independent glutamate efflux was measured by depolarizing the synaptosomes with 1 mM 4-aminopyridine in extracellular-Ca^{2+}-free solution that contained 50 μM BAPTA-AM, a cell-permeable Ca^{2+} chelator. Under these conditions, the release of glutamate evoked by 4-aminopyridine was 3.2 ± 0.1 nmol/mg/5 min. This Ca^{2+}-independent glutamate release evoked by 4-aminopyridine was, however, not affected by the prior addition of 10 μM hypericin (3.3 ± 0.1 nmol/mg/5 min) ($n=6$; Fig. 2A). The same result was also obtained with 30 μM hypericin (2.9 ± 0.4 nmol/mg/5 min; $n=3$). Second, we examined the effect of hypericin on 4-aminopyridine-

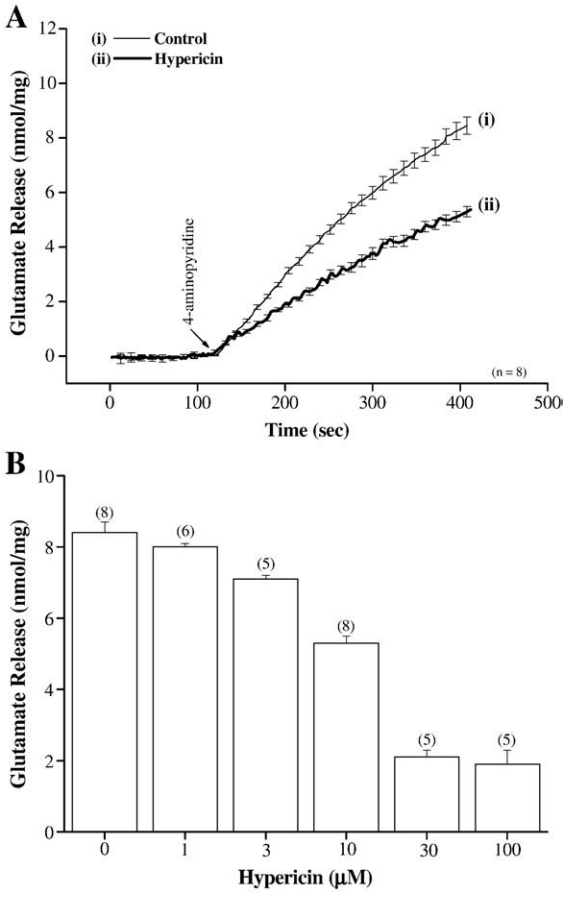

Fig. 1. Hypericin inhibits 4-aminopyridine-evoked glutamate release in cerebrocortical synaptosomes. A. Glutamate release was evoked by 1 mM 4-aminopyridine in the absence (i) or presence of 10 μM hypericin (ii) (added 10 min before the addition of 4-aminopyridine). B. Concentration dependency of effect of hypericin on 4-aminopyridine-evoked glutamate release. Results are the mean ± S.E.M. values of independent experiments, using synaptosomal preparations from five to eight animals.

evoked glutamate release in the presence of DL-threo-beta-benzyl-oxyaspartate (DL-TBOA), a non-selective inhibitor of all excitatory amino acid transporter (EAAT) subtypes, which should block or severely reduce the Ca^{2+}-independent nonvesicular efflux by transporter reversal (Dunlop, 2006). In the presence of 10 μM DL-TBOA, although 4-aminopyridine-evoked glutamate release (8.6 ± 0.4 nmol/mg/5 min) was increased by the inhibitor (because of inhibition of reuptake of released glutamate) (12.7 ± 0.9 nmol/mg/5 min; $P<0.01$), hypericin (10 μM) still inhibited significantly 4-aminopyridine-induced release of glutamate (6.1 ± 0.3 nmol/mg/5 min) ($n=5$; $P<0.05$; Fig. 2B). This indicates that a major proportion of the inhibition produced by hypericin reflects an effect on the physiologically relevant, exocytotic pool of glutamate release. Third, the effect of hypericin on 4-aminopyridine-evoked glutamate release was examined in the presence of bafilomycin A1, which causes the depletion of glutamate in synaptic vesicles (Cavelier and Attwell, 2007). In contrast to DL-TBOA, bafilomycin A1 (0.1 μM) reduced 1 mM 4-aminopyridine-evoked glutamate release from 8.6 ± 0.5 to 2.3 ± 0.3 nmol/mg/5 min ($P<0.01$), and completely prevented the inhibitory effect of hypericin (10 μM) on 4-aminopyridine-evoked glutamate release (2.1 ± 0.1 nmol/mg/5 min; $n=7$; Fig. 3A). In the presence of bafilomycin A1, therefore, hypericin induced a statistically insignificant inhibition

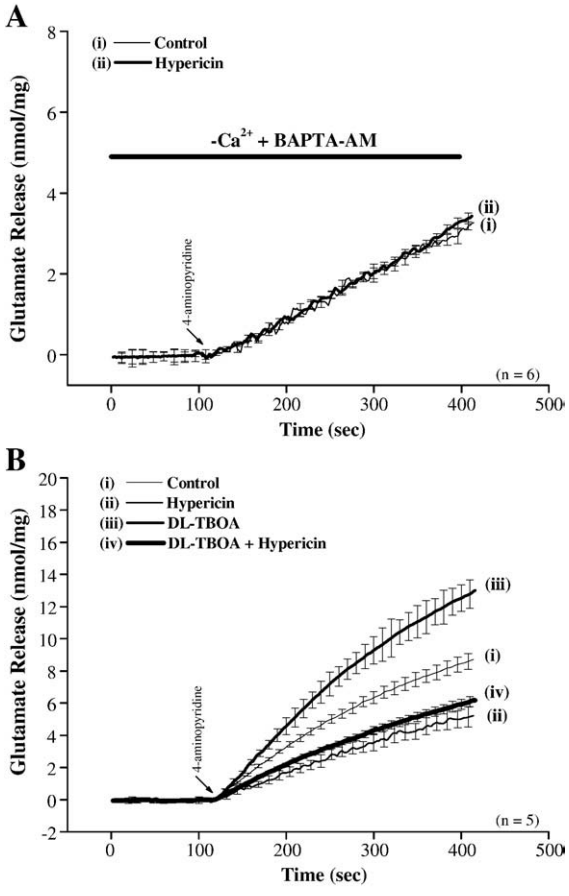

Fig. 2. Hypericin does not affect the 4-aminopyridine-evoked Ca^{2+}-independent glutamate release. A. Ca^{2+}-independent release was assayed by omitting $CaCl_2$ and adding 50 μM BAPTA-AM 10 min prior to depolarization, and was evoked by 1 mM 4-aminopyridine in the absence (i) and presence of 10 μM hypericin (ii) (added 10 min before the addition of 4-aminopyridine). B. Glutamate release was evoked by 1 mM 4-aminopyridine in the absence (i) or presence of 10 μM hypericin (ii), 10 μM DL-TBOA (iii), or 10 μM DL-TBOA + 10 μM hypericin (iv). Hypericin was added 10 min before depolarization and, DL-TBOA, 10 min prior to this. Results are the mean ± S.E.M. values of independent experiments, using synaptosomal preparations from five to six animals.

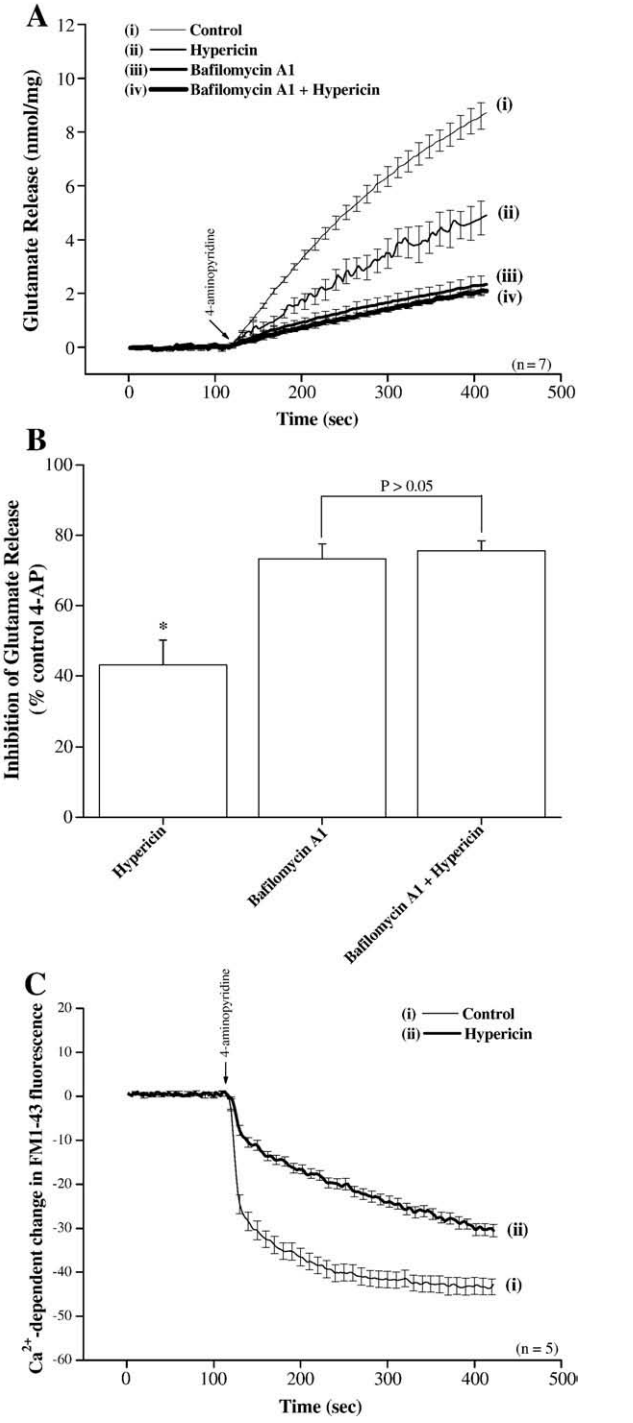

Fig. 3. Hypericin effects on the Ca^{2+}-dependent, exocytotic component of 4-aminopyridine-evoked glutamate release. A. Glutamate release was evoked by 1 mM 4-aminopyridine in the absence (i) or presence of 10 μM hypericin (ii), 0.1 μM bafilomycin A1 (iii), or 0.1 μM bafilomycin A1 + 10 μM hypericin (iv). Hypericin was added 10 min before depolarization and, bafilomycin A1, 10 min prior to this. B. Quantification of modulation using release levels achieved 5 min post 4-aminopyridine. C. The release of FM1-43 was evoked by 1 mM 4-aminopyridine in the absence (i) or presence of 10 μM hypericin (ii). Results are the mean ± S.E.M. values of independent experiments, using synaptosomal preparations from five to seven animals (*$P<0.01$, two-tailed Student's t test.

($P>0.05$; one-way repeated-measures ANOVA; Fig. 3B). Furthermore, we examined the effect of hypericin on the release of FM1-43, a lipophilic but membrane impermeable fluorescent styryl dye. The

fluorescent dye FM1-43 has proven a valuable tool to monitor exocytosis at presynaptic terminals. Thus, when neuronal terminals are stimulated, a depolarization occurs that results in endocytosis of FM1-43 dye. This dye is released on subsequent exocytosis and, therefore, the loss of fluorescence can be used to monitor synaptic vesicle release (Baldwin et al., 2003). In Fig. 3C, 4-aminopyridine (1 mM) caused a decrease in FM1-43 fluorescence in the presence of $CaCl_2$. Notably, this 4-aminopyridine-evoked Ca^{2+}-dependent decrease in FM1-43 fluorescence was also affected by the prior addition of 10 μM hypericin ($n=5$; $P<0.01$). All results together demonstrate that a major proportion of the inhibition produced by hypericin reflects an effect on the physiologically relevant, exocytotic pool of glutamate release.

3.3. Effect of hypericin on synaptosomal membrane potential and Ca^{2+} influx

To further understand the mechanism responsible for the hypericin-mediated inhibition of glutamate release, we used a membrane potential-sensitive dye, $DiSC_3(5)$, to determine the effect of hypericin on the synaptosomal plasma membrane potential. $DiSC_3(5)$ is a positively charged carbocyanine that accumulates in polarized synaptosomes that are negatively charged on the inside. At high concentrations, the dye molecules accumulate and the fluorescence is quenched. Upon depolarization, the dye moves out and hence the fluorescence increases (Akerman et al., 1987). As shown in Fig. 4A, 4-aminopyridine (1 mM) caused an increase in $DiSC_3(5)$ fluorescence of 1.38 ± 0.13 fluorescence units/5 min. Preincubation of synaptosomes with 10 μM hypericin for 10 min before 4-aminopyridine addition did not alter the resting membrane potential, and produced no significant change in the 4-aminopyridine-mediated increase in $DiSC_3(5)$ fluorescence (1.35 ± 0.11 fluorescence units/5 min; $P>0.05$; $n=6$). This indicates that the observed inhibition of evoked glutamate release by hypericin is unlikely to have been caused by a hyperpolarizing effect of the drug on the synaptosomal plasma membrane potential, or attenuation of the depolarization produced by 4-aminopyridine. Confirmation that hypericin 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). KCl (15 mM) effected a control glutamate release of 10.5 ± 0.2 nmol/mg/5 min, which was decreased to 5.5 ± 0.7 nmol/mg/5 min in the presence of 10 μM hypericin ($n=8$; Fig. 4A, inset).

Downstream of membrane depolarization, presynaptic inhibition of neurotransmitter release can be mediated by a reduction of Ca^{2+} influx into nerve terminals. To investigate this possibility, we used the Ca^{2+} indicator Fura-2 to assess the effect of hypericin on the 4-aminopyridine-induced increase in $[Ca^{2+}]_C$. As shown in Fig. 4B, after addition of 4-aminopyridine (1 mM), $[Ca^{2+}]_C$ in synaptosomes was elevated to a plateau level of 185.8 ± 3.1 nM. This 4-aminopyridine-evoked rise in $[Ca^{2+}]_c$ was decreased by 28 nM with 10 μM hypericin (157.4 ± 2.2 nM; $n=5$; $P<0.01$).

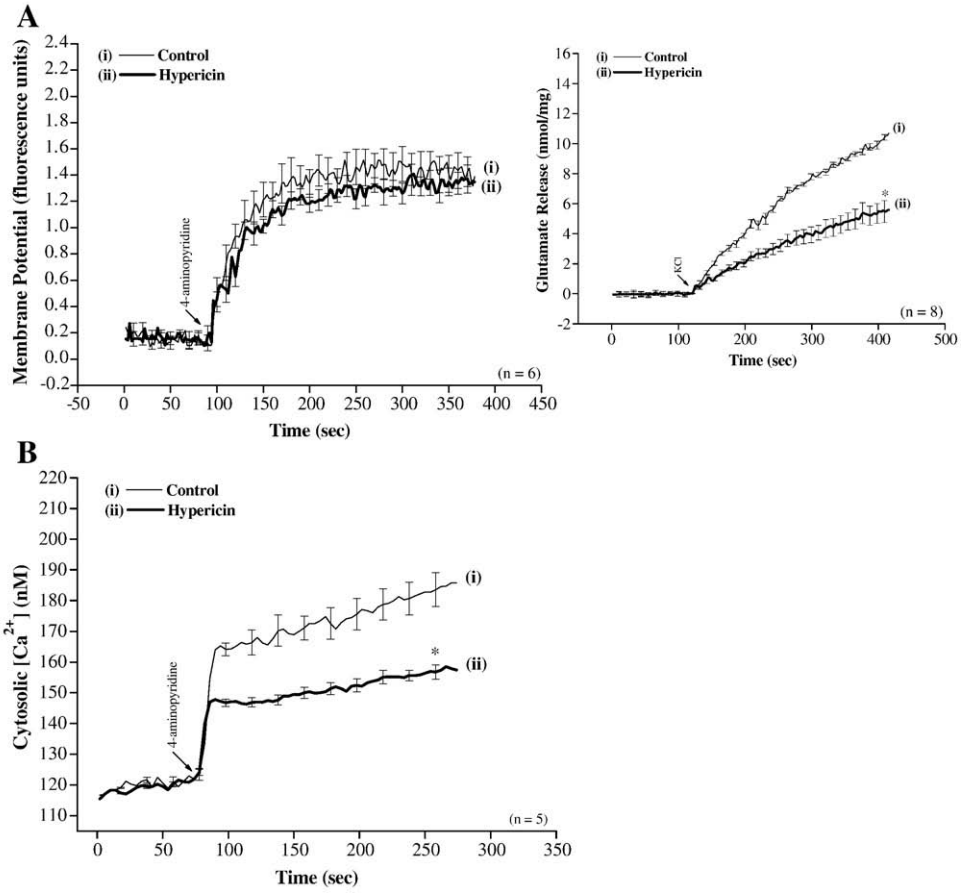

Fig. 4. Hypericin does not alter the synaptosomal membrane potential but significantly attenuates 4-aminopyridine-induced voltage-dependent Ca^{2+} influx. A. Synaptosomal membrane potential was monitored with 5 μM $DiSC_3(5)$ on depolarization with 1 mM 4-aminopyridine, in the absence (i) or presence of 10 μM hypericin (ii) (added 10 min before depolarization). Inset: Glutamate release was evoked by 15 mM KCl in the absence (i) or presence of 10 μM hypericin (ii), added 10 min before depolarization. B. $[Ca^{2+}]_c$ was monitored by using Fura-2. Synaptosomes were stimulated with 1 mM 4-aminopyridine in the absence (i) or presence of 10 μM hypericin (ii) (added 10 min before stimulation). Results are the mean ± S.E.M. values of independent experiments, using synaptosomal preparations from five to eight animals (*$P<0.01$, two-tailed Student's t test).

3.4. Effect of presynaptic N- and P/Q-type Ca^{2+} channel blocker on the hypericin-mediated inhibition of 4-aminopyridine-evoked glutamate release

In the adult rat cerebrocortical nerve terminal preparation, the release of glutamate evoked by depolarization relies on the entry of Ca^{2+} through N- and P/Q-type voltage-dependent Ca^{2+} channels (Millan and Sanchez-Prieto, 2002). Therefore, we sought to examine whether the regulation of these Ca^{2+} channel was involved in the hypericin-mediated inhibition of 4-aminopyridine-evoked glutamate release, by performing occlusion experiments with ω-conotoxin MVIIC, a wide spectrum blocker of N-, P- and Q-type Ca^{2+} channels. Application of 1 μM ω-conotoxin MVIIC reduced 4-aminopyridine-evoked glutamate release from 7.8 ± 0.1 to 2.5 ± 0.3 nmol/mg/5 min. In the presence of ω-conotoxin MVIIC, 10 μM hypericin caused a marginal further decrease to 2.3 ± 0.1 nmol/mg/5 min ($P<0.01$; Fig. 5A). This represented a statistically insignificant change in 4-aminopyridine-evoked glutamate release that resulted from hypericin in the presence of N- and P/Q-type VDCC block ($n=5$; $P>0.05$; Fig. 5B). These data point to the possibility that the effect of hypericin on glutamate release is manifested through the attenuation of N- and P/Q-type VDCC activity.

3.5. Involvement of mitogen-activated protein kinase pathway in the hypericin-mediated inhibition of 4-aminopyridine-evoked glutamate release

Since mitogen-activated protein kinase signaling pathway is one of the major second messenger systems regulating glutamate release at the presynaptic level (Grewal et al., 1999; Pereira et al., 2002), we examined the effect of the inhibition of this pathway on hypericin-mediated inhibition of glutamate release by using the selective mitogen-activated protein kinase inhibitor PD98059 (Pereira et al., 2002). PD98059 (50 μM) itself produced an inhibition of 4-aminopyridine-evoked glutamate release of $65.3 \pm 2.6\%$, but subsequent application of 10 μM hypericin inhibited 4-aminopyridine-evoked glutamate release by $69.1 \pm 3.8\%$ (i.e. to a level of release comparable to control). In the presence of PD98059, therefore, hypericin induced a statistically insignificant inhibition of glutamate release of 4% compared with the 37% inhibition produced by hypericin alone

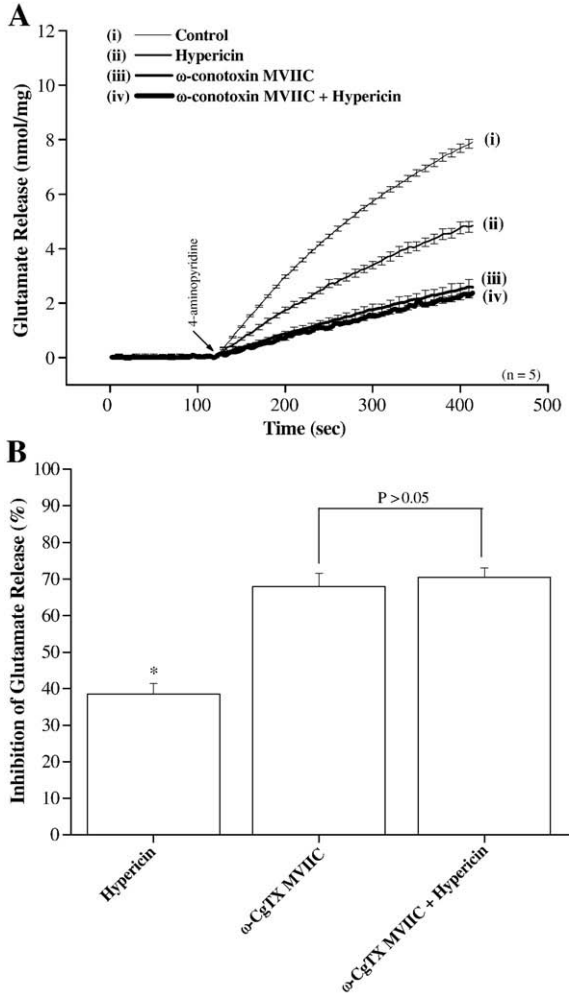

Fig. 5. Blockade of N- and P/Q-type Ca^{2+} channels abolishes hypericin inhibition of glutamate release. A. Glutamate release was evoked by 1 mM 4-aminopyridine in the absence (i) or presence of 10 μM hypericin (ii), 2 μM ω-conotoxin MVIIC (iii), or 2 μM ω-conotoxin MVIIC + 10 μM hypericin (iv). B. Quantification of modulation using release levels achieved 5 min post 4-aminopyridine. Hypericin was added 10 min before depolarization and other drugs 20 min prior to this. Results are the mean ± S.E.M. values of independent experiments, using synaptosomal preparations from five animals (*$P<0.01$, two-tailed Student's t test; $P<0.05$, ANOVA followed by two-tailed Student's t test).

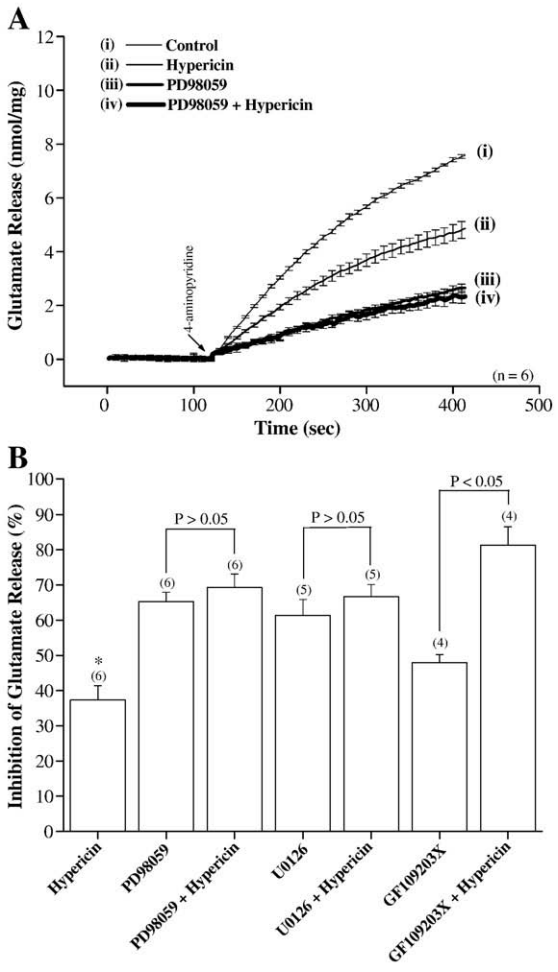

Fig. 6. Hypericin-mediated inhibition of glutamate release is prevented by the mitogen-activated protein kinase inhibitors, PD98059 and U0126, but not by the protein kinase C inhibitor GF109203X. A. Glutamate release was evoked by 1 mM 4-aminopyridine in the absence (i) or presence of 10 μM hypericin (ii), 100 μM PD98059 (iii), or 100 μM PD98059 + 10 μM hypericin (iv). B. Quantification of modulation using release levels achieved 5 min post 4-aminopyridine. Hypericin was added 10 min before depolarization and PD98059, U0126 or GF109203X 30 min prior to this. Results are the mean ± S.E.M. values of independent experiments, using synaptosomal preparations from four to six animals (*$P<0.01$, two-tailed Student's t test; $P<0.05$, ANOVA followed by two-tailed Student's t test).

($n=6$; Fig. 6A and B). This inhibition of the effect of hypericin by PD98059 was also obtained with another mitogen-activated protein kinase inhibitor, U0126 (Pereira et al., 2002). As with PD98059, although U0126 (50 μM) attenuated 4-aminopyridine-evoked glutamate release by 61.2 ± 4.6% ($P<0.01$; two-tailed Student's t test), it also suppressed the inhibitory effect of hypericin (10 μM) on 4-aminopyridine-evoked glutamate release to 66.7 ± 3.4%, a statistically insignificant change ($n=5$; Fig. 6B). In contrast to the effect of MAPK inhibitors, the protein kinase C inhibitor GF109203X (Wang and Sihra, 2004) failed to influence the ability of hypericin to inhibit 4-aminopyridine-evoked release of glutamate ($n=4$; Fig. 6B). These results indicate that hypericin-mediated decrease in glutamate release involves the mitogen-activated protein kinase cascade in cerebrocortical synaptosomes.

To further confirm that the mitogen-activated protein kinase pathway was suppressed by hypericin during its inhibition of 4-aminopyridine-evoked glutamate release, we performed Western blotting to examine the effect of hypericin on the phosphorylation of mitogen-activated protein kinase in cerebrocortical synaptosomes. Fig. 7 shows that depolarization of synaptosomes with 1 mM 4-aminopyridine in the presence of 1.2 mM $CaCl_2$ significantly increased mitogen-activated protein kinase phosphorylation (127.1 ± 2.6%; $n=5$; $P<0.01$). When synaptosomes were pretreated with 10 μM hypericin for 10 min before depolarization with 1 mM 4-aminopyridine, 4-aminopyridine-enhanced phosphorylation of mitogen-activated protein kinase was markedly decreased to 111.4 ± 3.3% ($n=5$; $P<0.05$; one-way repeated-measures ANOVA).

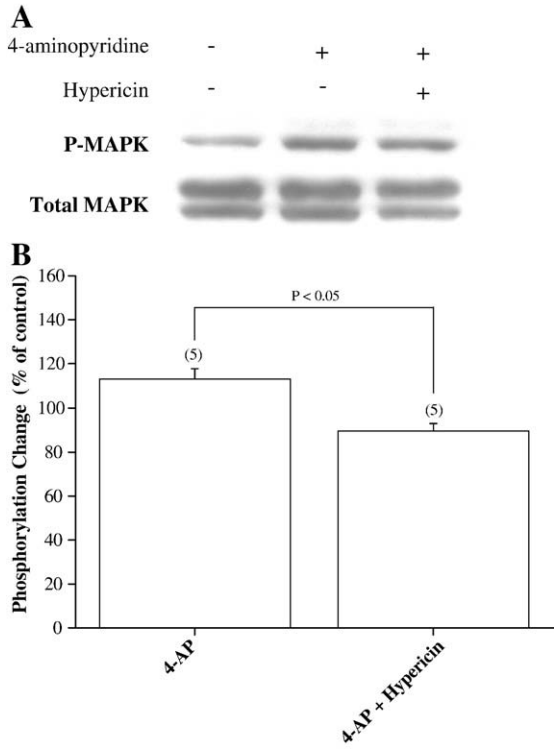

Fig. 7. Hypericin decreases 4-aminopyridine-evoked phosphorylation of mitogen-activated protein kinase. A. Phosphorylation of mitogen-activated protein kinase (MAPK) was detected in synaptosomal lysates by Western blotting using an antibody that specifically recognizes the phosphorylated form of MAPK. Purified synaptosomes were incubated for 2 min in HEPES buffer medium that contained 1.2 mM $CaCl_2$ at 37 °C in the absence (control) or presence of 1 mM 4-aminopyridine, or 1 mM 4-aminopyridine + 10 μM hypericin. Hypericin was added 10 min before the addition of 4-aminopyridine. B. Data are expressed as a percentage of the control phosphorylation obtained in the absence of 4-aminopyridine stimulation. Results are the mean ± S.E.M. values of independent experiments, using synaptosomal preparations from five animals ($P<0.05$, ANOVA followed by two-tailed Student's t test).

4. Discussion

Although St. John's wort and several of its active components have been reported to have antidepressant activity, there are still many open questions about its mechanism of action. Therefore, the main aim of this study was to obtain better knowledge about the mechanism of action of St. John's wort and its active constituents in depression. In the present study, by using a preparation of nerve terminals from rat cerebral cortex, we provide the novel finding that hypericin, one of the major components of St. John's wort, potently inhibits evoked glutamate release. To the best of our knowledge, this study represents the first examination of the effect of St. John's wort on endogenous glutamate release at the presynaptic level.

4.1. Mechanism of action of hypericin in the inhibition of glutamate release

A key issue to address is the nature of the mechanism responsible for hypericin-inhibited 4-aminopyridine-evoked glutamate release from rat cerebrocortical nerve terminals. In principle, 4-aminopyridine-evoked glutamate release from isolated nerve terminals is known to have two components. The first is a physiologically relevant Ca^{2+}-dependent component, which is produced by exocytosis of synaptic vesicles that contain glutamate. The second is a Ca^{2+}-independent component that results from prolonged depolarization that causes a membrane potential-mediated shift of the glutamate transporter steady-state toward the outward direction, to effect cytosolic glutamate efflux (Nicholls et al., 1987). In the present study, we found that: (1) in the presence of Ca^{2+}-free medium that contained BAPTA-AM, hypericin failed to inhibit significantly 4-aminopyridine-evoked glutamate release (Ca^{2+}-independent release); (2) the inhibitory effect of hypericin on 4-aminopyridine-evoked glutamate release was effectively abolished by bafilomycin A1, which depletes the glutamate content of synaptic vesicles, but not by DL-TBOA, a non-selective inhibitor of all excitatory amino acid transporter subtypes; and (3) using an exocytosis assay with FM1-43, inhibition of 4-aminopyridine-evoked FM1-43 release by hypericin was observed. All these data suggest that the inhibition of 4-aminopyridine-evoked glutamate release attributable to the hypericin treatment is accounted for only by a reduction of the Ca^{2+}-dependent, exocytotic component of glutamate release.

An inhibition of the Ca^{2+}-dependent glutamate release by hypericin could be ascribed to an alteration of plasma membrane potential, and/or a direct inhibition of the exocytosis-coupled VDCCs. The former possibility, that hypericin may effect synaptosomal plasma membrane hyperpolarization or decrease in synaptosomal excitability, is untenable on the basis of three reasons. Firstly, no significant effect of hypericin on synaptosomal plasma membrane potential, measured with a membrane potential-sensitive dye, $DiSC_3(5)$, was observed either resting conditions or on depolarizing with 4-aminopyridine. Secondly, apart from 4-aminopyridine-evoked glutamate release, KCl-evoked glutamate release was also inhibited by hypericin. This indicates that a direct effect on voltage-dependent Ca^{2+} channel function seems to be involved in the inhibitory effect of hypericin on glutamate release, because 4-aminopyridine-evoked glutamate release involves the action of Na^+ and Ca^{2+} channels, 15 mM external KCl-evoked glutamate release involves only Ca^{2+} channels (Barrie et al., 1991; Nicholls, 1998). Thirdly, hypericin did not affect the 4-aminopyridine-evoked Ca^{2+}-independent glutamate release, a component of glutamate release that is dependent only on membrane potential (Attwell et al., 1993). In addition, using the Ca^{2+} indicator Fura-2-AM, we directly demonstrate here that hypericin indeed significantly reduced the 4-aminopyridine-evoked increase in $[Ca^{2+}]_C$. Moreover, the inhibitory effect of hypericin on 4-aminopyridine-evoked glutamate release was prevented completely when the release-coupled N- and P/Q-type voltage-dependent Ca^{2+} channels had been blocked indicates that hypericin effectively suppressed glutamate release components supported by N- and P/Q-

type voltage-dependent Ca^{2+} channels. Therefore, these results clearly indicate that hypericin is able to inhibit glutamate release specifically and directly via the reducing activity of the voltage-dependent Ca^{2+} channels, although there is lack of direct evidence for hypericin actions on presynaptic Ca^{2+} channels.

In synaptic terminals, mitogen-activated protein kinase activation is triggered by cytosolic calcium elevations, which leads to the facilitation of glutamate release (Grewal et al., 1999; Pereira et al., 2002). Moreover, this signaling pathway has been implicated in the mechanism of action of antidepressants (Duman et al., 2007). Thus, it is reasonable to speculate that the inhibitory effect of hypericin on Ca^{2+} entry observed here may decrease mitogen-activated protein kinase activity and, in turn, glutamate release. In elucidating this, we found that the inhibitory effect of hypericin on 4-aminopyridine-evoked glutamate release was substantially prevented by the mitogen-activated protein kinase inhibitors. Furthermore, hypericin significantly decreased 4-aminopyridine-induced phosphorylation of mitogen-activated protein kinase. These data suggest that an intracellular cascade involving the suppression of mitogen-activated protein kinase-dependent pathways is linked to inhibition of 4-aminopyridine-evoked glutamate release by hypericin. Moreover, the observed inhibitory effect of hypericin on 4-aminopyridine-evoked glutamate release was not affected by the protein kinase C inhibitor, which demonstrated some specificity for hypericin action on the mitogen-activated protein kinase pathway. How mitogen-activated protein kinase suppression involves in the hypericin-mediated inhibition of glutamate release is an open question. It was shown previously that mitogen-activated protein kinase increases the phosphorylation of synapsin I, a synaptic vesicle-associated phosphoprotein involved in regulating vesicle availability for release, resulting to an increase of glutamate release (Greengard et al., 1993; Jovanovic et al., 1996, 2000; Yamagata et al., 2002; Schenk et al., 2005). Therefore, it is possible that hypericin inhibits glutamate release through decreasing synapsin I phosphorylation and synaptic vesicle availability, although further studies are required.

5. Conclusion

The main finding of the present study is that hypericin, through a decrease in the voltage-dependent Ca^{2+} influx and mitogen-activated protein kinase activity, depresses evoked glutamate release from rat cerebrocortical nerve terminals. This finding is of particular significance and represents an additional explanation for the antidepressant activity of St. John's wort, because of the proposed role of excessive glutamate release in the pathophysiology of depression (Sapolsky, 2000; Zarate et al., 2002). However, whether reducing glutamate release from nerve endings contributes to the apparent therapeutic potential of St. John's wort in depression remains to be explored by further research.

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