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Luteolin Inhibits the Release of Glutamate in Rat Cerebrocortical Nerve Terminals

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ABSTRACT: The present study investigated the effect and possible mechanism of luteolin, a food-derived flavonoid, on endogenous glutamate release in nerve terminals of rat cerebral cortex (synaptosomes). Luteolin inhibited the release of glutamate evoked by the K^+ channel blocker 4-aminopyridine (4-AP), and this phenomenon was concentration-dependent. The effect of luteolin on the evoked glutamate release was prevented by the chelation of the extracellular Ca^{2+} ions and by the vesicular transporter inhibitor, but was insensitive to the glutamate transporter inhibitor. Luteolin decreased the 4-AP-induced increase in $[Ca^{2+}]_C$, whereas it did not alter 4-AP-mediated depolarization. Furthermore, the effect of luteolin on evoked glutamate release was abolished by blocking the $Ca_v2.2$ (N-type) and $Ca_v2.1$ (P/Q-type) channels, but not by blocking the ryanodine receptors or the mitochondrial Na^+/Ca^{2+} exchange. In addition, the inhibitory effect of luteolin on evoked glutamate release was prevented by the mitogen-activated/extracellular signal-regulated kinase (MEK) inhibitors. Western blot analyses showed that luteolin decreased the 4-AP-induced phosphorylation of extracellular signal-regulated kinase 1 and 2 (ERK1/2) and synapsin I, the main presynaptic target of ERK. Thus, it was concluded that luteolin inhibits glutamate release from rat cortical synaptosomes through the suppression of presynaptic voltage-dependent Ca^{2+} entry and MEK/ERK signaling cascade.

KEYWORDS: luteolin, glutamate release, cerebrocortical nerve terminals, voltage-dependent Ca^{2+} channels, ERK

INTRODUCTION

Flavonoids represent the most common group of polyphenolic compounds in the human diet and are dietary components of a variety of fruits, vegetables, cereals, teas, and wines. Flavonoids have recently received considerable attention due to their beneficial effects on health, including prevention of cardiovascular disease, cancer, and neurodegenerative disease, as well as strengthening of the immune system.[1,2] Luteolin (3,4,5,7-tetrahydroxyflavone, Figure 1), a naturally occurring flavonoid,[3] has reported neuroprotective roles in various models, in vitro and in vivo. Previous studies have demonstrated that luteolin attenuates oxidative stress- or neurotoxin-induced neurotoxicity,[4−7] protects against ischemia-induced brain damage, and ameliorates β-amyloid-induced memory impairment.[8] However, the mechanisms involved in these neuroprotective effects have yet to be fully elucidated. Luteolin possesses potent antioxidant properties,[9,10] and it is generally assumed that its neuroprotective action is principally associated with these properties.[8]

In the mammalian central nervous system (CNS), glutamate is a major excitatory neurotransmitter, which plays important role in many brain functions such as synaptic plasticity, learning, and memory.[11,12] Excessive release of glutamate induces an increase in intracellular Ca^{2+} levels, which in turn triggers a cascade of cellular responses, including enhanced oxygen free radical production, disturbed mitochondrial function, and protease activation, ultimately leading to neuronal cell death.[13,14] Previous investigations have cited this type of overexcitation-induced neuronal damage as contributing to a number of neuropathological conditions, ranging from acute insults such as stroke, epileptic seizures, traumatic brain, and spinal cord injury, to chronic neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis.[15−17] Modulation of glutamate neurotransmission, such as inhibition of glutamate release at nerve terminals, may provide a potential target for neuroprotective action.

This study aimed to use isolated nerve terminals (synaptosomes) purified from the rat cerebral cortex to investigate the effects of luteolin on glutamate release and to characterize the underlying molecular mechanisms. The isolated presynaptic terminal represents a model system for direct investigation of 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 which might obscure interpretation of findings due to modulatory loci at non-neuronal, postsynaptic, or network levels.[18] The first series of experiments investigated the effects of luteolin on the release of endogenous glutamate, the synaptosomal plasma membrane potential, and the downstream activation of voltage-dependent Ca^{2+} channels (VDCCs). The second series of

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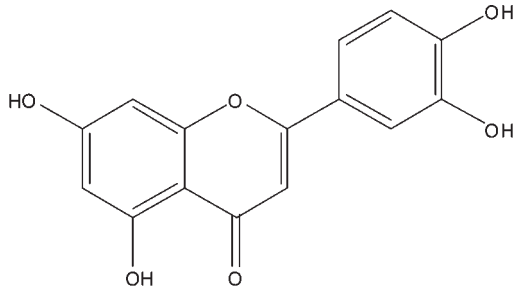

2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4*H*-chromen-4-one
(**luteolin**, flavone)

Figure 1. Chemical structure of luteolin.

experiments evaluated if the protein kinase signaling pathway participates in the regulation of glutamate release by luteolin.

MATERIALS AND METHODS

Chemicals. 3′,3′,3′-Dipropylthiadicarbocyanine iodide [$DiSC_3(5)$] and Fura-2-acetoxymethyl ester (Fura-2-AM) were obtained from Invitrogen (Carlsbad, CA). Luteolin, dantrolene, bafilomycin A1, 2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one (PD98059), 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene (U0126), DL-*threo*-*β*-benzyloxyaspartate (DL-TBOA), bisindolylmaleimide I (GF109203X), 7-chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzothiazepin-2(3*H*)-one (CGP37157), *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H89), and *ω*-conotoxin MVIIC (*ω*-CgTX MVIIC) were obtained from Tocris Cookson (Bristol, U.K.). Rabbit polyclonal antibodies directed against ERK1/2 and phospho-ERK1/2 was bought from Cell Signaling Technology (Beverly, MA). The anti-synapsin I phosphorylation state-specific rabbit polyclonal antibody directed against MAPK/ERK-phosphorylated sites 4 and 5 of synapsin I (Ser^{62}/Ser^{67}) was from Millipore (Bedford, MA). Horseradish peroxidase-conjugated anti-rabbit secondary antibodies were from Bio-Rad (Milan, Italy). Ethylene glycol bis(*β*-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), sodium dodecyl sulfate (SDS), and all other reagents were obtained from Sigma-Aldrich Co. (St. Louis, MO).

Animals. Adult male Sprague−Dawley rats (150−200 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 Far-Eastern Memorial Hospital Institutional Animal Care and Utilization Committee.

Synaptosomal Preparation. Synaptosomes were prepared as described previously.[19] 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.) at 4 °C, and the supernatant was centrifuged again at 14500*g* (11000 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 of Percoll discontinuous gradient 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 32500*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 (HBM) 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 27000*g* (15000 rpm in a JA 25.5 rotor) for 10 min. The pellets thus formed were resuspended in 3 mL of HBM, and the protein content was determined using a Bradford Protein Assay Kit (Bio-Rad, Hercules, CA), according to the method of Bradford,[20] with BSA as a standard. Half a milligram of synaptosomal suspension was diluted in 10 mL of HBM 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 2−3 h.

Glutamate Release. Glutamate release from purified cerebrocortical synaptosomes was monitored online, with an assay that employed exogenous glutamate dehydrogenase (GDH) and $NADP^+$ to couple the oxidative deamination of the released glutamate to the generation of NADPH detected fluorometrically.[21] This method allows the detection of submicromolar concentrations of glutamate without interference from other amino acids.[22] Because an inherent delay is introduced by the time required for the reaction to occur, fluorometric traces do not reflect the actual glutamate concentration. Initial rates of glutamate release associated with depolarization may be underestimated as corrections for this delay were not incorporated.[23] Actually, this online enzyme-linked fluorescent detection from synaptosomes has long been considered an approach particularly appropriate to investigate the presynaptic regulation of neurotransmitter release by drugs.[21,24] In brief, synaptosomes (0.5 mg/mL) were resuspended in HBM that contained 16 *μ*M BSA and incubated in an LS-50B spectrofluorometer (Perkin-Elmer Life Sciences) at 37 °C with stirring. $NADP^+$ (2 mM), GDH (50 units/mL), and $CaCl_2$ (1.2 mM) were added after 3 min. In experiments that investigated Ca^{2+}-independent efflux of glutamate, EGTA (300 *μ*M) was added in place of $CaCl_2$. Other additions before depolarization were made as described in the figure captions. After a further 10 min of incubation, 4-AP (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 GDH. 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 of glutamate per milligram of synaptosomal protein (nmol/mg). Release values quoted in the text and expressed in bar graphs are levels attained at steady state after 5 min of depolarization (nmol/mg/5 min). Data were accumulated at 2 s intervals. Cumulative data were analyzed in Lotus 1-2-3 and MicroCal Origin. Estimation of the IC_{50} was based on a one-site model [inhibition = $(\text{inhibition}_{MAX} \times [\text{luteolin}]/(IC_{50} + [\text{luteolin}])$] using the nonlinear curve-fitting function in MicroCal Origin. Statistical analysis was performed by two-tailed Student's *t* test or one-way ANOVA, followed by post hoc two-tailed *t* test.

Synaptosomal Plasma Membrane Potential. The synaptosomal membrane potential can be monitored by positively charged, membrane potential-sensitive carbocyanine dyes such as $DiSC_3(5)$. $DiSC_3(5)$ 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.[25] Synaptosomes were preincubated and resuspended as described for the glutamate release experiments. After 3 min of incubation, 5 *μ*M $DiSC_3(5)$ was added and allowed to equilibrate before the addition of $CaCl_2$ (1 mM) after 4 min of incubation. Then, 4-AP (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. Cumulative data were analyzed in Lotus 1-2-3 and MicroCal Origin, and results are expresssed in fluorescence units. Statistical analysis was performed by two-tailed Student's *t* test or one-way ANOVA, followed by post hoc two-tailed *t* test.

Cytosolic Free Ca^{2+} Concentration ($[Ca^{2+}]_C$). $[Ca^{2+}]_C$ was measured using the Ca^{2+} indicator Fura-2-AM. Synaptosomes (0.5 mg/mL) were preincubated in HBM with 16 *μ*M BSA in the presence of 5 *μ*M 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

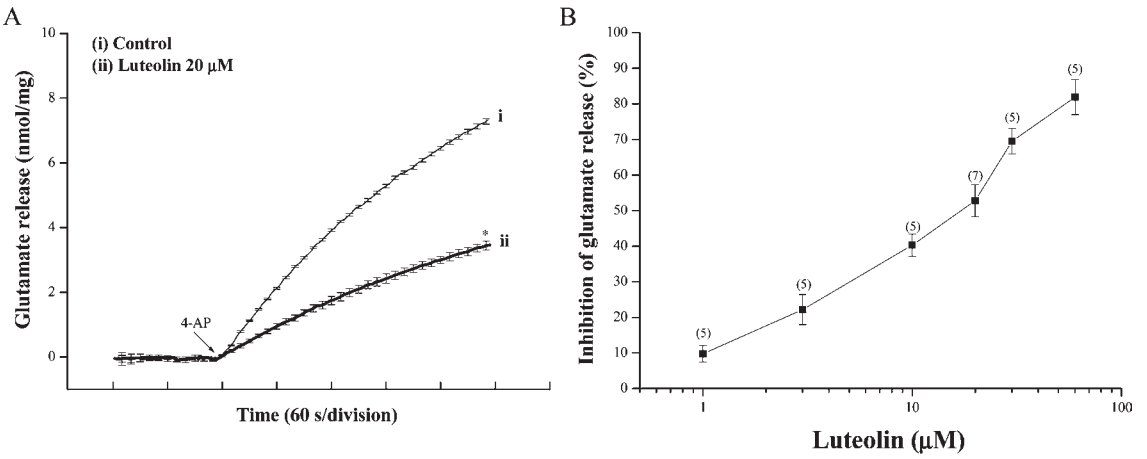

Figure 2. Luteolin exhibits a dose-dependent inhibition of 4-AP-evoked release of glutamate in rat cerebrocortical nerve terminals. Synaptosomes were resuspended in incubation medium at a final protein concentration of 0.5 mg/mL and incubated for 3 min before the addition of 1.2 mM $CaCl_2$. 4-AP (1 mM) was added after a further 10 min to effect depolarization (arrow). (A) Total glutamate release was measured under control conditions or in the presence of 20 μM luteolin added 10 min prior to the addition of 4-AP. (B) The dose−response curve for luteolin inhibition of 4-AP-evoked glutamate release shows percentage inhibition compared with controls. The numbers in parentheses indicate the number of experiments performed using independent synaptosomal preparations. Results are the mean ± SEM (∗, $P < 0.01$, Student's t test).

resuspended in HBM with BSA, and the synaptosomal suspension was stirred in a thermostated cuvette in a Perkin-Elmer LS-50B spectrofluorometer. $CaCl_2$ (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 of 505 nm) at 7.5 s intervals. Calibration procedures were performed as described previously,[26] using 0.1% SDS 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. $[Ca^{2+}]_C$ was calculated using equations described previously.[27] Cumulative data were analyzed in Lotus 1-2-3 and MicroCal Origin. Changes in $[Ca^{2+}]_C$, 5 min after the addition of 4-AP in the presence of luteolin, were calculated. Statistical analysis was performed by two-tailed Student's t test or one-way ANOVA, followed by post hoc two-tailed t test.

Western Blotting. Synaptosomes (0.5 mg protein/mL) from control and luteolin-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 13000g at 4 °C for 10 min. Equal amounts of synaptosomal proteins were loaded on a SDS polyacrylamide gel and then transferred electrophoretically to nitrocellulose membranes. The membranes were blocked with Tris-buffered saline (TBS) that contained 5% low-fat milk and incubated with appropriate primary antibodies (antiphospho-ERK1/2, 1:2000, anti-ERK1/2, 1:1000, antiphospho-synapsin-I (Ser^{62}/Ser^{67}), 1:1000, β-actin, 1:500). Following three washes with TBS, the blots were incubated with the secondary horseradish peroxidase-conjugated antibody (1:3000) at room temperature for 1 h. The blots were washed again three times with TBS, and the immunoreactive bands were detected by using the enhanced chemiluminescence method. After immunoblotting, films were scanned at 600 dpi in transmittance mode by using the scanner. The level of phosphorylation was assessed by band density, which was quantified by densitometry.

RESULTS

Luteolin Inhibition of 4-AP-Evoked Glutamate Release from Rat Cerebrocortical Nerve Terminals. The depolarization of nerve terminals with the potassium channel blocker 4-aminopyridine (4-AP) has been shown to open voltage-dependent Ca^{2+} channels (VDCCs) and to induce the release of glutamate.[28] Using an online enzymatic assay for measuring glutamate, 4-AP (1 mM) evoked a glutamate release of 7.2 ± 0.1 nmol/mg/5 min from synaptosomes incubated in the presence of 1.2 mM $CaCl_2$. Application of luteolin (20 μM) for 10 min significantly reduced the 4-AP-evoked glutamate release (3.4 ± 0.1 nmol/mg/5 min; $n = 7$; $P < 0.01$), without altering the basal release of glutamate (Figure 2A). The luteolin-induced inhibition of 4-AP-evoked glutamate release was concentration dependent, and the IC_{50} value derived from a dose−response curve was approximately 20 μM (Figure 2B). Therefore, this concentration was used in subsequent experiments to evaluate the mechanisms that underlie the ability of luteolin to reduce glutamate release.

Effect of Ca^{2+} Chelation, DL-TBOA, or Bafilomycin A1 on the Inhibition of 4-AP-Evoked Glutamate Release by Luteolin. We next investigated whether the inhibition of 4-AP-evoked glutamate release by luteolin was mediated by an effect on exocytotic vesicular release or on Ca^{2+}-independent release attributable to cytosolic efflux via reversal of the glutamate transporter.[29] First, the Ca^{2+}-independent glutamate efflux was measured by depolarizing the synaptosomes with 1 mM 4-AP in extracellular-Ca^{2+}-free solution that contained 300 μM EGTA. Under these conditions, the release of glutamate evoked by 4-AP (1 mM) was 1.6 ± 0.02 nmol/mg/5 min. This Ca^{2+}-independent release evoked by 4-AP was, however, not affected by 20 μM luteolin (1.5 ± 0.3 nmol/mg/5 min) ($n = 5$; Figure 3A). Second, we used DL-TBOA, a nonselective inhibitor of all excitatory amino acid transporter (EAAT) subtypes, to examine the effect of luteolin on the 4-AP-evoked glutamate release in the normal Ca^{2+} concentration. In Figure 3B, the 4-AP (1 mM)-evoked glutamate release was inhibited by luteolin (20 μM) to about the same extent both in the absence and in the presence of DL-TBOA (10 μM). DL-TBOA by itself almost doubled the 4-AP (1 mM)-evoked glutamate release (because of inhibition of re-uptake of released glutamate) ($n = 5$; $P < 0.01$; Figure 3B). Finally, we examined the action of luteolin on the 4-AP-evoked glutamate release in the presence of bafilomycin A1, which causes the

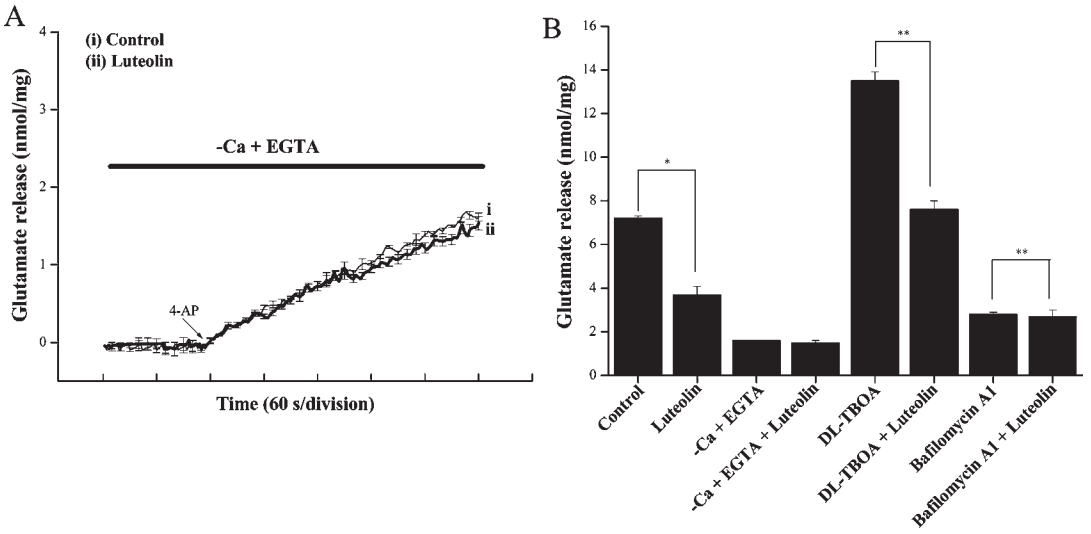

Figure 3. Luteolin effects on the Ca^{2+}-dependent, exocytotic component of 4-AP-evoked glutamate release. (A) Ca^{2+}-independent release was assayed by omitting $CaCl_2$ and adding 300 μM EGTA 10 min prior to depolarization and was evoked by 1 mM 4-AP under control conditions or in the presence of 20 μM luteolin added 10 min prior to the addition of 4-AP. (B) Glutamate release was evoked by 1 mM 4-AP in the absence (control) or presence of 20 μM luteolin, 300 μM EGTA (without $CaCl_2$), 300 μM EGTA (without $CaCl_2$), and 20 μM luteolin; 10 μM DL-TBOA, 10 μM DL-TBOA, and 20 μM luteolin; 0.1 μM bafilomycin A1; or 0.1 μM bafilomycin A1 and 20 μM luteolin. EGTA, DL-TBOA, or bafilomycin A1 was added 20 min before depolarization, whereas luteolin was added 10 min before depolarization. Results are the mean ± SEM of five independent experiments (*, $P < 0.01$, two-tailed Student's t test; **, $P < 0.05$, ANOVA followed by two-tailed Student's t test).

depletion of glutamate in synaptic vesicles. Figure 3B shows that bafilomycin A1 (0.1 μM) reduced the release of glutamate evoked by 4-AP (1 mM) to 2.8 ± 0.1 nmol/mg/5 min ($P < 0.01$), and completely prevented the inhibitory effect of luteolin on the 4-AP-evoked glutamate release (2.7 ± 0.3 nmol/mg/5 min; $n = 5$). All of these results suggest that the luteolin-mediated inhibition of glutamate release is due to a decrease in the Ca^{2+}-dependent, exocytotic component of glutamate release.

Effect of Luteolin on the Synaptosomal Membrane Potential and the Intrasynaptosomal Ca^{2+} Levels. We next addressed the potential mechanisms underlying the luteolin-mediated inhibition of glutamate release by assessing the effects of luteolin on the synaptosomal plasma membrane potential and the intracellular Ca^{2+} levels. Because inhibition of Na^+ channels or activation of K^+ channels is known to stabilize membrane excitability and consequently cause a reduction in the evoked entry of Ca^{2+} and neurotransmitter release,[30,31] we reasoned that the observed inhibitory effect of luteolin on 4-AP-evoked glutamate release could be due to an alteration of nerve terminal excitability. To test this possibility, we examined the effect of luteolin on the synaptosomal plasma membrane potential under resting conditions and on depolarization, with the membrane-potential-sensitive dye $DiSC_3(5)$. Figure 4A shows that 4-AP (1 mM) caused an increase in $DiSC_3(5)$ fluorescence by 11.7 ± 0.1 fluorescence units/5 min. Application of luteolin (20 μM) for 10 min before 4-AP addition did not alter the resting membrane potential and produced no significant change in the 4-AP-mediated increase in $DiSC_3(5)$ fluorescence (11.6 ± 0.1 fluorescence units/5 min) ($n = 5$). This result indicates that the observed inhibition of evoked glutamate release by luteolin is unlikely to have been caused by a hyperpolarizing effect of drug on the synaptosomal plasma membrane potential or attenuation of the depolarization produced by 4-AP. To confirm this, we examined the effect of luteolin on the release of glutamate evoked by an alternative secretagogue, high external $[K^+]$ (Figure 4A, inset). Elevated extracellular KCl depolarizes the plasma membrane by shifting the K^+ equilibrium potential above the threshold potential for activation of voltage-dependent ion channels. Whereas Na^+ channels are inactivated under these conditions, VDCCs are activated nonetheless to mediate Ca^{2+} entry, which supports neurotransmitter release.[32] Addition of 15 mM KCl led to a glutamate release of 8.9 ± 0.2 nmol/mg/5 min, which was reduced to 4.7 ± 0.3 nmol/mg/5 min in the presence of luteolin (20 μM) ($n = 5$; $P < 0.01$; Figure 4A, inset).

Downstream of membrane depolarization, presynaptic inhibition of neurotransmitter release can be mediated by a reduction of intraterminal Ca^{2+} levels. To investigate whether a reduction in $[Ca^{2+}]_C$ was responsible for the luteolin-mediated inhibition of release, we used a Ca^{2+} indicator Fura-2 to monitor intraterminal Ca^{2+} levels directly. In Figure 4B, after addition of 4-AP (1 mM), $[Ca^{2+}]_C$ in synaptosomes was elevated to a plateau level of 221.9 ± 5.3 nM. This 4-AP-evoked rise in $[Ca^{2+}]_C$ was decreased by application of luteolin (20 μM) to 190.5 ± 2.3 nM ($n = 5$; $P < 0.01$).

Effect of ω-CgTX MVIIC, Dantrolene, or CGP37157 on the Inhibition of 4-AP-Evoked Glutamate Release by Luteolin. In the adult rat cerebrocortical nerve terminal preparation, the release of glutamate evoked by depolarization is reported to be caused by Ca^{2+} influx through $Ca_v2.2$ (N-type) and $Ca_v2.1$ (P/Q-type) channels and Ca^{2+} release from internal stores.[33−35] Therefore, we next sought to establish which part of the Ca^{2+} source was involved in the effect of luteolin on the 4-AP-evoked glutamate release. To assess the role of $Ca_v2.2$ and $Ca_v2.1$ channels, synaptosomes were preincubated with 2 μM ω-conotoxin MVIIC (ω-CgTX MVIIC), a wide-spectrum blocker of $Ca_v2.2$ and $Ca_v2.1$ channels. In Figure 5A, glutamate release evoked by 1 mM 4-AP under control conditions was significantly decreased in the presence of 2 μM ω-CgTX MVIIC alone or 20 μM luteolin alone ($P < 0.01$). When ω-CgTX MVIIC (2 μM) and luteolin (20 μM) were applied simultaneously, the inhibition

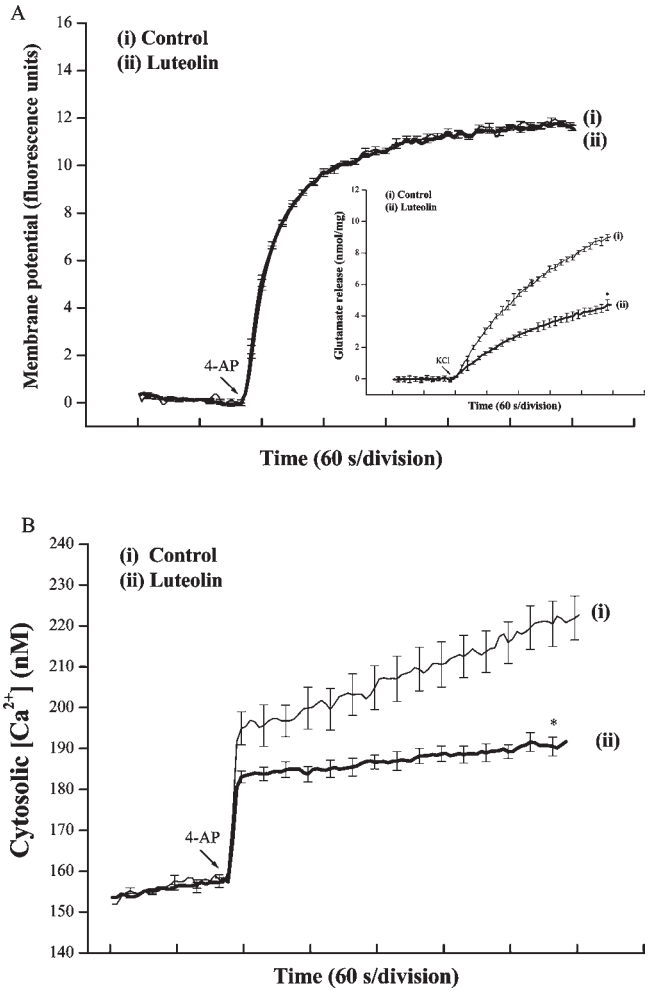

Figure 4. Luteolin does not change the synaptosomal membrane potential but attenuates the 4-AP-induced increase in cytosolic Ca^{2+} levels. (A) Synaptosomal membrane potential monitored with $DiSC_3(5)$ in the absence (control) and in the presence of 20 μM luteolin, added 10 min before depolarization with 1 mM 4-AP. (Inset) Glutamate release was induced by 15 mM KCl in the absence (control) or presence of 20 μM luteolin, added 10 min before depolarization. (B) Cytosolic free Ca^{2+} concentration (nM) was monitored using Fura-2. Synaptosomes were stimulated with 1 mM 4-AP in the absence (control) and in the presence of 20 μM luteolin, added 10 min before depolarization. Results are the mean $\pm$ SEM of five independent experiment ($*$, $P < 0.01$, Student's t test).

of glutamate release following 4-AP depolarization was not significantly different from the effect of ω-CgTX MVIIC alone ($n = 6$; Figure 5B). Dantrolene is known to inhibit Ca^{2+} release from intracellular stores by acting on ryanodine receptors on the endoplasmic reticulum.[36] Treatment with dantrolene (50 μM) caused a reduction in glutamate release evoked by the same stimulus ($P < 0.01$; Figure 5B). In the presence of dantrolene, however, luteolin (30 μM) could still effectively inhibit 4-AP-evoked glutamate release ($n = 5$; $P < 0.05$; Figure 5B). Similar to dantrolene, CGP37157 (100 μM), a membrane-permeable blocker of mitochondrial Na^+/Ca^{2+} exchange, decreased the release of glutamate evoked by 4-AP (1 mM) ($P < 0.01$), but had a nonsignificant effect on the luteolin-mediated inhibition of 4-AP-evoked glutamate release ($n = 5$; $P < 0.05$; Figure 5B).

Involvement of a MAPK/ERK Pathway in the Luteolin-Mediated Inhibition of Glutamate Release. Because various kinases including mitogen-activated protein kinase (MAPK), protein kinase C (PKC), and protein kinase A (PKA) have been shown to regulate glutamate release at the presynaptic level,[37−39] we sought to establish which type of protein kinase signaling pathway participated in the inhibition of 4-AP-evoked glutamate release mediated by luteolin. To this aim, we performed occlusion experiments with protein kinase inhibitors. First, we examined the effect of PD98059, an inhibitor of mitogen-activated/extracellular signal-regulated kinase kinase (MEK), the immediate upstream regulator of MAPK. Figure 6A shows that control glutamate release evoked by 1 mM 4-AP was reduced by 50 μM PD98059 ($P < 0.01$), reflecting an inhibition of the reported basal MAPK activity present in nerve terminals.[37] Although luteolin (20 μM) reduced the 4-AP-evoked glutamate release ($P < 0.01$), this effect was abolished by the pretreatment with PD98059, with the release measured in the presence of PD98059 and luteolin being similar to that obtained in the presence of PD98059 alone. Similar results were observed with another MEK inhibitor, U0126. As with PD98059, although U0126 (50 μM) attenuated control 4-AP (1 mM)-evoked glutamate release ($P < 0.01$), it also occluded the inhibitory effect of luteolin on the 4-AP (1 mM)-evoked glutamate release (Figure 6B). In contrast, the PKC inhibitor GF109203X (10 μM) and the PKA inhibitor H89 (100 μM) reduced control 4-AP (1 mM)-evoked glutamate release ($P < 0.01$), but they had no effect on the luteolin-mediated inhibition of 4-AP-evoked glutamate release (Figure 6B).

To further authenticate the role of MAPK in the observed inhibition of glutamate release by luteolin, we performed Western blotting to examine the effect of luteolin on the phosphorylation of MAPK/extracellular signal-regulated kinases 1 and 2 (ERK1/2). Figure 7 shows the results of analysis performed on extracts of synaptosomes depolarized with 4-AP in the presence of external Ca^{2+} and treated with luteolin. The results show that depolarization of synaptosomes with 4-AP (1 mM) markedly increased the phosphorylation of ERK1/2 ($P < 0.01$). When synaptosomes were pretreated with luteolin (20 μM) for 10 min before depolarization with 4-AP, a significant decrease in the 4-AP (1 mM)-induced ERK1/2 phosphorylation was observed ($n = 5$; $P < 0.05$; Figure 7A). In addition, the present study found that the MEK inhibitor PD98059 (50 μM) reduced the 4-AP (1 mM)-induced ERK1/2 phosphorylation ($P < 0.05$). This is consistent with that observed for the reduction of release (Figure 6A). In the presence of PD98059, the action of luteolin on the 4-AP-induced ERK1/2 phosphorylation was completely abolished ($n = 4$; Figure 7A). This study also examined the effect of luteolin on the phosphorylation of synaptic vesicle-associated protein synapsin I, which is the major presynaptic substrate for MAPK/ERK.[40] Figure 7B shows 4-AP (1 mM) increased the phosphorylation of synapsin I at MAPK-specific sites 4 and 5 ($P < 0.01$), and this phenomenon was reduced after luteolin (20 μM) treatment ($P < 0.05$; $n = 3$).

DISCUSSION

The excessive release of glutamate is known to be a critical factor in a variety of CNS diseases including cerebral ischemia, epilepsy, and neurodegenerative disorders.[41−43] Therefore, one feasible strategy to prevent and treat this overexcitation-induced neuronal damage is to reduce the amount of glutamate in the synaptic cleft. Luteolin is a naturally occurring flavonoid with a neuroprotective profile.[4,5,8,10] However, the mechanisms underlying the neuroprotective effects of luteolin are not fully clarified. Therefore, the aim of

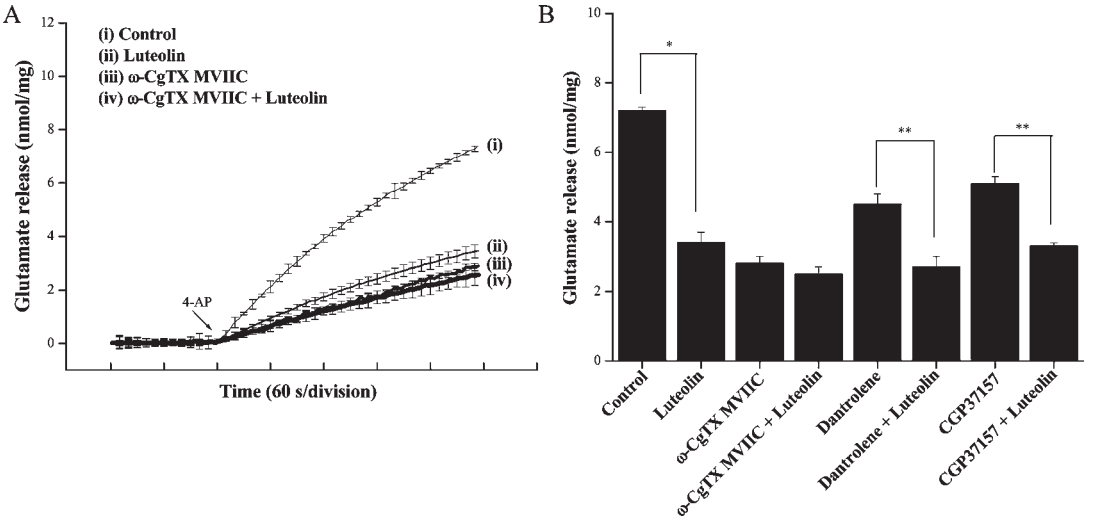

Figure 5. Blockade of $Ca_v2.2$ and $Ca_v2.1$ channels abolishes luteolin inhibition of glutamate release. (A) Glutamate release was evoked by 1 mM 4-AP in the absence (control) or presence of 20 μM luteolin, 2 μM ω-CgTX MVIIC, 2 μM ω-CgTX MVIIC, and 20 μM luteolin. (B) Quantitative comparison of the extent of glutamate release by 1 mM 4-AP in the absence or presence of 20 μM luteolin, 2 μM ω-CgTX MVIIC, 2 μM ω-CgTX MVIIC and 20 μM luteolin; 50 μM dantrolene, 50 μM dantrolene and 20 μM luteolin; 100 μM CGP37157; or 100 μM CGP37157 and 20 μM luteolin. Luteolin was added 10 min before depolarization, whereas the other drugs were added 30 min before depolarization. Results are the mean ± SEM of five or six independent experiments (∗, $P < 0.01$, two-tailed Student's t test; ∗∗, $P < 0.05$, ANOVA followed by two-tailed Student's t test).

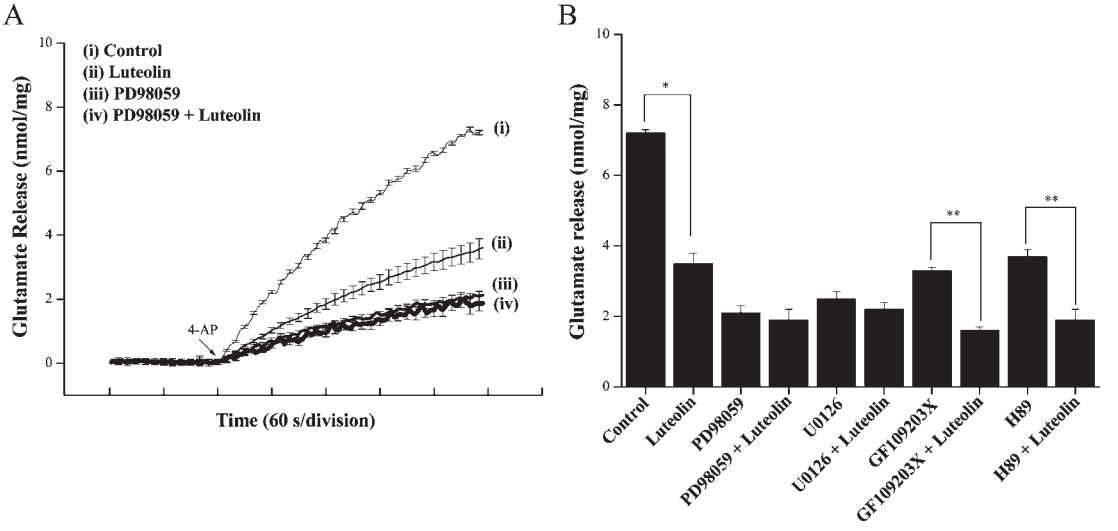

Figure 6. MEK inhibitors occlude luteolin-mediated inhibition of glutamate release. (A) Glutamate release was induced by 1 mM 4-AP in the absence (control) or presence of 20 μM luteolin, 50 μM PD98059, or 50 μM PD98059 and 20 μM luteolin. (B) Quantitative comparison of the extent of glutamate release by 1 mM 4-AP in the absence (control) or presence of 20 μM luteolin, 50 μM PD98059, 50 μM PD98059, and 20 μM luteolin; 50 μM U0126, 50 μM U0126, and 20 μM luteolin; 10 μM GF109203X, 10 μM GF109203X, and 20 μM luteolin; 100 μM H89; or 100 μM H89 and 20 μM luteolin. PD98059, U0126, GF109203X, or H89 was added 40 min before depolarization, whereas luteolin was added 10 min before depolarization. Results are the mean ± SEM of five or six independent experiments (∗, $P < 0.01$, two-tailed Student's t test; ∗∗, $P < 0.05$, ANOVA followed by two-tailed Student's t test).

this study was to investigate the relationship between luteolin and presynaptic modulation of glutamate release and to determine the underlying molecular mechanisms. Using a preparation of nerve terminals from a rat cerebral cortex to examine the release of endogenous glutamate, this study demonstrated that luteolin rapidly and dose-dependently inhibits the depolarization-evoked glutamate release. This is the first study assessing the possible influence of luteolin on glutamate release at the presynaptic level.

In principle, neurotransmitter release can be modulated at several loci in the stimulus−exocytosis cascade, including ion channels (Na^+ channels or K^+ channels) modulating nerve terminal excitability, voltage-dependent Ca^{2+} channels (VDCCs), the downstream of Ca^{2+} entry, and components of the synaptic vesicle trafficking and exocytotic apparatus.[27,44,45] Therefore, when addressing the mechanism responsible for the luteolin-mediated inhibition of Ca^{2+}-dependent glutamate release, this study considered two scenarios that might be involved: (1) alteration of the plasma membrane potential and/or (2) direct inhibition of the Ca^{2+} channel coupled to glutamate exocytosis. The first possibility is unlikely for three reasons. First, luteolin did not significantly affect

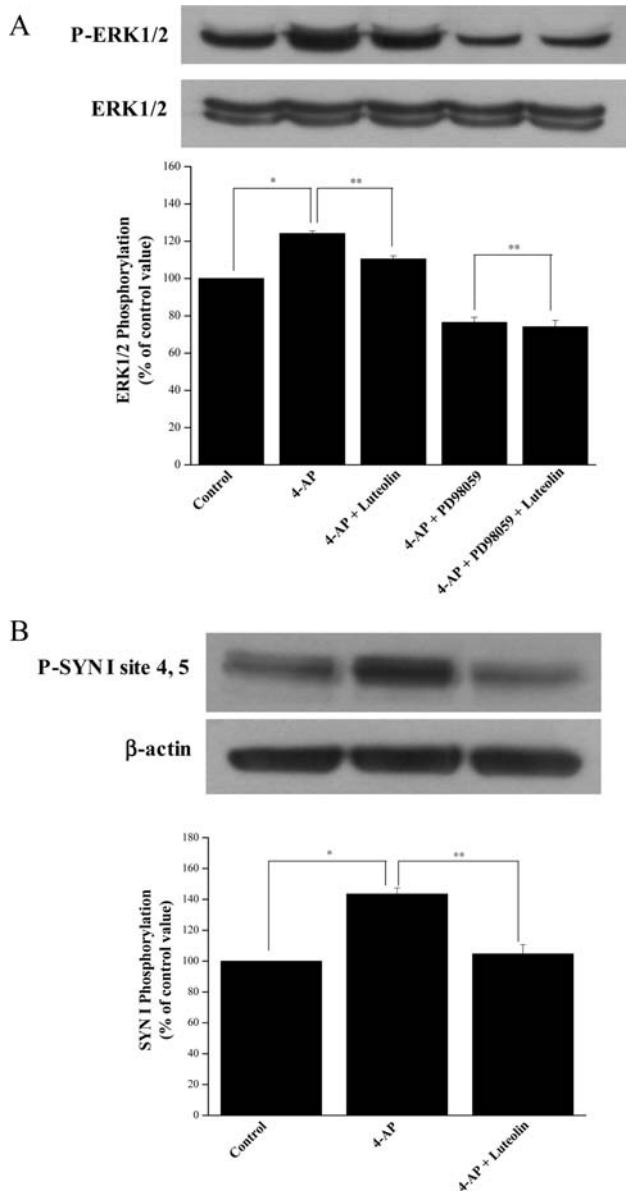

Figure 7. Luteolin decreases 4-AP-induced phosphorylation of ERK1/2 and synapsin I. Phosphorylation of ERK1/2 (A) and synapsin I at MAPK-specific sites 4 and 5 (P-SYN I site 4, 5) (B) was detected in synaptosomal lysates by Western blotting using phosphorylation state-specific antibodies. Purified synaptosomes were incubated at 37 °C for 2 min in HBM that contained 1.2 mM $CaCl_2$ in the absence (control) or presence of 1 mM 4-AP, 1 mM 4-AP, and 20 μM luteolin; 1 mM 4-AP and 50 μM PD98059; or 1 mM 4-AP, 50 μM PD98059, and 20 μM luteolin. PD98059 was added 40 min before 4-AP addition, whereas luteolin was added 10 min before depolarization. Data are expressed as a percentage of the phosphorylation obtained in the controls in the absence of 4-AP stimulation. Results are the mean ± SEM of three to five independent experiments (*, $P < 0.01$, two-tailed Student's t test; **, $P < 0.05$, ANOVA followed by two-tailed Student's t test).

the synaptosomal plasma membrane potential either in the resting state or after depolarization with 4-AP, indicating a lack of effect on K^+ conductance. Second, the inhibitory effect of luteolin was observed when KCl was used as a depolarizing agent. Because 4-AP-evoked glutamate release involves Na^+ and Ca^{2+} channels, whereas KCl-evoked glutamate release involves only Ca^{2+} channels,[28,32] it is unlikely that Na^+ channels are involved in the modulation of glutamate release by luteolin. Third, luteolin did not affect the 4-AP-evoked Ca^{2+}-independent glutamate release, which depends only on the membrane potential,[46] suggesting that luteolin does not affect glutamate release by reversing the direction of the plasma membrane glutamate transporter. Additionally, luteolin-mediated inhibition of 4-AP-evoked glutamate release was found to be prevented by the vesicular transporter inhibitor bafilomycin A1, but was insensitive to the glutamate transporter inhibitor DL-TBOA. These results clearly suggest that the luteolin-mediated decrease of evoked glutamate release is not due to a reduction in synaptosomal excitability caused by the modulation of Na^+ or K^+ ion channels.

Using Fura-2, this study demonstrated that luteolin significantly reduced the 4-AP-evoked increase in $[Ca^{2+}]_C$. In synaptic terminals, a depolarization-induced increase in $[Ca^{2+}]_C$ coupled to glutamate release is mediated by an extracellular Ca^{2+} influx through $Ca_v2.2$ and $Ca_v2.1$ channels and Ca^{2+} release from intracellular stores such as endoplasmic reticulum and mitochondria.[33,34] In this study, the inhibition of glutamate release by luteolin was highly sensitive to the inhibition of $Ca_v2.2$ and $Ca_v2.1$ channels, which are involved in triggering glutamate release from synaptosomes.[34,35] This suggested that $Ca_v2.2$ and $Ca_v2.1$ are involved in the modulation of glutamate release by luteolin. In contrast, the reduced release of stored Ca^{2+} from the ER ryanodine receptors and mitochondria during the luteolin-mediated inhibition of glutamate release could be excluded from participating. This was because the inhibitory effect of luteolin on 4-AP-evoked glutamate release was not affected by the ER ryanodine receptor inhibitor dantrolene or the mitochondrial Na^+/Ca^{2+} exchange inhibitor CGP37157. Therefore, in the presence of dantrolene or CGP37157, luteolin still induced a 44 or 40% decrease in the glutamate release, respectively. The additive relationship between dantrolene or CGP37157 and luteolin indicates that, although intracellular Ca^{2+} release contributes significantly to the 4-AP-evoked glutamate release, they appear not to mediate the effect of luteolin on glutamate release. Although we have no direct evidence that luteolin acts on presynaptic Ca^{2+} channels, our results imply that the inhibition of glutamate release by luteolin occurs primarily via a suppression of Ca^{2+} influx through $Ca_v2.2$ and $Ca_v2.1$ channels. However, because the blockade of $Ca_v2.2$ and $Ca_v2.1$ channel activity did not completely eliminate the inhibitory effect of luteolin on the 4-AP-evoked glutamate release (approximately 6% of the activity remained), this study cannot discount the involvement of other Ca^{2+} channel types.

This study suggests a role for the MAPK/ERK pathway in the luteolin-mediated inhibition of glutamate release based on the following results: (1) the inhibitory effects of luteolin on the 4-AP-evoked glutamate release were prevented by the MEK (MAP kinase kinase) inhibitors PD98059 and U0126, but not by PKC and PKA inhibitors, demonstrating some specificity for luteolin action on the MAPK pathway; (2) luteolin significantly decreased 4-AP-induced phosphorylation of MAPK/ERK1/2, and this phenomenon was also prevented by the MEK inhibitor PD98059; and (3) 4-AP-induced phosphorylation of synapsin I at MAPK-specific site 4 was reduced by luteolin. MAPK/ERK is a vital intracellular signaling system that is known to be present at the presynaptic level and to play a crucial role in neurotransmitter exocytosis.[37,40,47] Depolarization-stimulated Ca^{2+} entry has been proven to lead to MAPK/ERK activation and to phosphorylation of synapsin I at sites 4 and 5. This phosphorylation reaction

promotes dissociation of synaptic vesicles from the actin cytoskeleton, thereby making more vesicles available at the active zone for neurotransmitter exocytosis, resulting in increased glutamate release.[40,48,49] Accordingly, the results imply that the suppression of MAPK/ERK-dependent synapsin I phosphorylation and the consequent decreased availability of synaptic vesicles is involved in the observed luteolin-mediated inhibition of glutamate release. Besides a participation of synapsin I in the inhibitory effect of luteolin on glutamate release, the possible involvement of other synaptic proteins should be considered. Synapsins II and III, for example, are reported to be phosphorylated by MAPK.[40,50]

In conclusion, the results described in this paper demonstrate that luteolin inhibits glutamate release from rat cerebrocortical synaptosomes by suppressing presynaptic $Ca_v2.2$ and $Ca_v2.1$ channels and MAPK/ERK activity. This finding supports the hypothesis that luteolin may function as a potential source for brain therapeutics against glutamate-induced neurotoxicity because excessive glutamate release is proposed to be involved in the pathophysiology of several neurological states, including ischemic brain damage and neurodegenerative diseases.[42,43] Although the relevance of our finding to in vivo clinical situations remains to be determined, the present finding may provide further understanding of the mode of luteolin action in the brain.

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ABBREVIATIONS USED

4-AP, 4-aminopyridine; $[Ca^{2+}]_C$, cytosolic free Ca^{2+} concentration; CNS, central nervous system; DiSC3(5), 3′,3′,3′-dipropylthiadicarbocyanine iodide; VDCCs, voltage-dependent Ca^{2+} channels; $Ca_v2.2$ and $Ca_v2.1$ channels, $Ca_v2.2$ (N-type) and $Ca_v2.1$ (P/Q-type) channels; DL-TBOA, DL-*threo*-β-benzyloxy aspartate; Fura-2-AM, fura-2-acetoxymethyl ester; GDH, glutamate dehydrogenase; HBM, HEPES buffer medium; BSA, bovine serum albumin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; H89, *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide; SDS, sodium dodecyl sulfate; CGP37157, 7-chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzothiazepin-2(3*H*)-one; ω-CgTX MVIIC, ω-conotoxin MVIIC; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated protein kinase; PKA, protein kinase A; PKC, protein kinase C; PD98059, 2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one); GF109203X, bisindolylmaleimide I; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)-butadiene.

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