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Eupafolin Suppresses P/Q-Type Ca^{2+} Channels to Inhibit Ca^{2+}/Calmodulin-Dependent Protein Kinase II and Glutamate Release at Rat Cerebrocortical Nerve Terminals

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Abstract

Eupafolin, a constituent of the aerial parts of *Phyla nodiflora*, has neuroprotective property. Because reducing the synaptic release of glutamate is crucial to achieving pharmacotherapeutic effects of neuroprotectants, we investigated the effect of eupafolin on glutamate release in rat cerebrocortical synaptosomes and explored the possible mechanism. We discovered that eupafolin depressed 4-aminopyridine (4-AP)-induced glutamate release, and this phenomenon was prevented in the absence of extracellular calcium. Eupafolin inhibition of glutamate release from synaptic vesicles was confirmed through measurement of the release of the fluorescent dye FM 1-43. Eupafolin decreased 4-AP-induced $[Ca^{2+}]_i$ elevation and had no effect on synaptosomal membrane potential. The inhibition of P/Q-type Ca^{2+} channels reduced the decrease in glutamate release that was caused by eupafolin, and docking data revealed that eupafolin interacted with P/Q-type Ca^{2+} channels. Additionally, the inhibition of calcium/calmodulin-dependent protein kinase II (CaMKII) prevented the effect of eupafolin on evoked glutamate release. Eupafolin also reduced the 4-AP-induced activation of CaMK II and the subsequent phosphorylation of synapsin I, which is the main presynaptic target of CaMKII. Therefore, eupafolin suppresses P/Q-type Ca^{2+} channels and thereby inhibits CaMKII/synapsin I pathways and the release of glutamate from rat cerebrocortical synaptosomes.

Key Words: Eupafolin, Glutamate release, P/Q-type Ca^{2+} channels, CaMKII, Synapsin I, Cerebrocortical synaptosomes

INTRODUCTION

Glutamate is a major excitatory neurotransmitter in the brain, and it plays vital physiological roles in neuronal development, synaptic plasticity, learning and memory (McEntee and Crook, 1993; Zhou and Danbolt, 2014). However, excess glutamate causes excessive activation of glutamate receptors, which results in calcium level elevation and subsequently invokes intracellular responses, including mitochondrial dysfunction, reactive oxygen species production, lipid peroxidation, and eventually cell death (Obrenovitch and Urenjak, 1997; Bano and Ankarcrona, 2018). Such glutamate-induced excitotoxicity is believed to be involved in numerous neurological diseases, such as ischemia, epilepsy, Alzheimer's disease, and Parkinson's disease (Choi, 1988; Lewerenz and Maher, 2015). Thus, the glutamatergic system is a promising target for treatment of these diseases. Some studies have suggested that the inhibition of glutamate release by nerve terminals can help to

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prevent excitotoxic damage (Wong *et al*., 2015; Chang *et al*., 2016; Lu *et al*., 2020).

Studies have reported plant-derived products to be potential therapeutic targets for neurological disorders (Parvez, 2018; Rehman *et al*., 2019). Eupafolin (Fig. 1A) is a major flavanone derived from the methanolic extract of *Phyla nodiflora* (Lin *et al*., 2014), a member of the family Verbenaceae. It has been used in traditional Chinese medicine to treat various inflammatory diseases, including hepatitis, bronchitis, and dermatitis (Abbasi *et al*., 2010; Lee *et al*., 2016). Several studies have reported anti-inflammatory, anticarcinogenic, and antioxidant activities of eupafolin (Lin *et al*., 2014; Ko *et al*., 2014; Lin *et al*., 2016; Zhang *et al*., 2017). In rats, eupafolin was reported to attenuate neurological deficits, neuroinflammation, and ischemia-induced brain damage (Chen *et al*., 2020), indicating that it may be promising as medicinal treatment for neurological diseases. Despite the neuroprotective effects of eupafolin, knowledge of its effect on glutamate release remains insufficient. Therefore, the present study examined the effect of eupafolin on endogenous glutamate release, which was stimulated using the potassium channel blocker 4-aminopyridine (4-AP) in prepared rat cerebrocortical synaptosomes; possible mechanism underlying this effect was also investigated.

MATERIALS AND METHODS

Materials

Eupafolin (purity>99%) was kindly provided by Professor Horng-Huey Ko. 7-chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzothiazepin-2(3*H*)-one (CGP37157), DL-threo-β-benzyloxyaspartate (DL-TBOA), dantrolene, N-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H89), and 1-[N,O-Bis (5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine (KN62) were purchased from Tocris (Bristol, UK). FM-43, 3,3,3-Dipropylthiadicarbocyanine iodide [$DiSC_3(5)$], and fura-2-acetoxymethyl ester (Fura-2-AM) were purchased from Thermo (Waltham, MA, USA). ω-conotoxin GVIA (ω-CgTX GVIA) and ω-agatoxin IVA (ω-Aga IVA) were purchased from Alomone lab (Jerusalem, Israel). CaMKII, p-CaMKII, synapsin I and anti-rabbit IgG horseradish peroxidase-conjugated antibodies were obtained from Cell Signaling Technologies (Beverly, MA, USA). p-synapsin I (Serine 603) antibody was purchased from GeneTex (CA, USA). 4-aminopyridine (4-AP) and all other reagents were purchased from Sigma (St. Louis, MO, USA).

Animals and synaptosomes preparation

Adult male Sprague–Dawley rats (150-200 g) were used in this study. Animal procedures were approved by the Fu Jen Institutional Animal Care and Utilization Committee (IACUC No. A10640). Animals were sacrificed via cervical dislocation and the cerebral cortex were rapidly removed. The brain tissue was homogenized in 320 mM sucrose solution and centrifuged at 5,000 rpm for 10 min. The supernatant was stratified on a Percoll discontinuous gradients and centrifuged at 16,500 rpm for 7 min. The synaptosomal fraction was collected and centrifuged for 10 min at 15,000 rpm. Protein concentration was determined using the Bradford assay. Synaptosomes were centrifuged in the final wash to obtain synaptosomal pellets with 0.5 mg protein, as previously described (Dunkley *et al*., 1986).

Glutamate release

For the glutamate release experiments, the synaptosomal pellet (0.5 mg protein) was resuspended in the hepes-buffered solution and glutamate release was assayed by on-line fluorimetry (Lu *et al*., 2019). $CaCl_2$ (1.2 mM), glutamate dehydrogenase (GDH, 50 units/mL) and $NADP^+$ (2 mM) were added at the start of incubation. Glutamate release was induced with 4-AP (1 mM) and monitored by measuring the increase of fluorescence (excitation and emission wavelengths of 340 and 460 nm, respectively) resulting from NADPH being produced by the oxidative deamination of released glutamate by GDH. Released glutamate was calibrated by a standard of exogenous glutamate (5 nmol) and expressed as nanomoles glutamate per milligram synaptosomal protein (nmol/mg).

FM1-43 release

Synaptic vesicle release was measured using release of the fluorescent dye FM1-43 (Baldwin *et al*., 2003). In brief, synaptosomes (0.5 mg/mL) were incubated in hepes-buffer medium containing FM1-43 (100 μM) and 1.2 mM $CaCl_2$ for 3 min at 37°C, followed by the addition of 30 mM KCl for 1 min to load FM1-43. Following loading, synaptosomes were pelleted by brief centrifugation followed by washing, repelleting, and resuspension in hepes-buffer medium containing 1.2 mM $CaCl_2$. FM1-43 release was induced by the addition of 1 mM 4-AP 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-s intervals, and data presented as the Ca^{2+}-dependent decrease in FM1-43 fluorescence.

Intrasynaptosomal Ca^{2+} concentration ($[Ca^{2+}]_i$)

Synaptosomes (0.5 mg protein) were incubated in the hepes-buffered solution containing Fura 2-AM (5 μM) and $CaCl_2$ (0.1 mM) for 30 min at 37ºC. Samples were centrifuged for 1 min at 5,000 rpm, and pellets were resuspended in hepes-buffered medium containing $CaCl_2$ (1.2 mM). Fura-2-Ca fluorescence was monitored at 5 s intervals for 5 min. $[Ca^{2+}]_i$ (nM) was calculated by using calibration procedures and equations described previously (Grynkiewicz *et al*., 1985).

Membrane potential

The synaptosomal membrane potential was assayed with a positively charged membrane potential-sensitive carbocyanine dye $DiSC_3(5)$. $DiSC_3(5)$ fluorescence was monitored at 2 s intervals and data are expressed in fluorescence units (Akerman *et al*., 1987).

Molecular docking study

The molecular docking experiment was performed for eupafolin with LibDock in Discovery Studio 4.1 client (BIOVIA software Inc., CA, USA). Firstly, the 3D structure of eupafolin was created using Discovery Studio 4.1 client and the crystal structure of P/Q-type of calcium channel protein (PDB ID 3BXK) was downloaded from the RCSB Protein Data Bank, and then the docking study selects the probable binding poses of eupafolin in the active site in channel protein. Subsequently, the conformations of eupafolin was optimized using flexibly docked in a stepwise manner with the protocol of Dock ligands (Libdock).

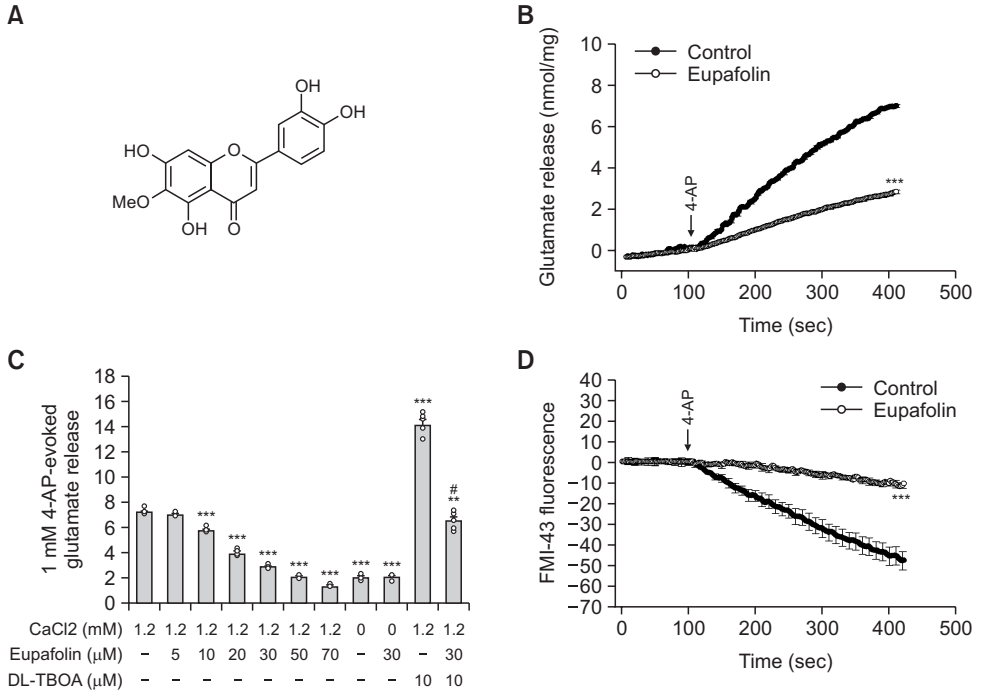

Fig. 1. Eupafolin inhibits 4-AP-evoked glutamate release from rat cerebrocortical nerve terminals through a decrease in vesicular exocytosis. (A) The chemical structure of eupafolin. (B) Glutamate release was measured under control conditions or in the presence of 30 μM eupafolin added 10 min prior to the addition of 4-AP. (C) Effect of eupafolin at different concentrations on 4-AP-evoked glutamate release and extracellular Ca^{2+}-free solution or glutamate transporter inhibitor DL-TBOA on the effect of eupafolin. (D) Effect of eupafolin on the release of FM1-43 evoked by 4-AP. Eupafolin or DL-TBOA was added 10 min before depolarization. Data are mean ± standard error of the mean (SEM) (n=5 per group). $^{**}p<0.05$, $^{***}p<0.001$ compared with the control group. $^{\#}p<0.001$ compared with the DL-TBOA-treated group.

Western blotting

Synaptosomal lysates (20 μg per lane) were separated on 10% SDS-PAGE and transferred onto nitrocellulose membranes, immunoblotted overnight at 4°C with antibodies to Ca^{2+}/calmodulin-dependent kinase II (CaMKII, 1:10,000), p-CMKII (1:2,000), synapsin I (1:30,000), and p-synapsin I (1:2,000). Horseradish peroxidase-conjugated secondary antibodies at a concentration of 1:5,000 were applied, and detection performed using chemiluminescence (GeneTex). The immunoblotting experiments were performed five times/antibody and were quantitatively analyzed using Image J software (Synoptics, Cambridge, UK).

Statistical analyses

Reported data were expressed as means ± SEM and was analyzed using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test to determine the difference. When only two groups were compared, the Student's *t* test was used. Differences were considered statistically significant at $p<0.05$.

RESULTS

Eupafolin depresses 4-AP-evoked glutamate release from rat cerebrocortical synaptosomes through suppression of exocytotic release

To investigate the effect of eupafolin on glutamate release, synaptosomes isolated from rat cerebral cortex were stimulated by 4-AP (1 mM), which opens voltage-dependent Ca^{2+} channels and induces the release of glutamate (Tibbs *et al*., 1989). As shown in Fig. 1B, preincubation with eupafolin (30 μM) for 10 min before 4-AP addition did not produce any significant effect on the basal release of glutamate, but markedly reduced the 4-AP-induced release of glutamate release in the presence of 1.2 mM $CaCl_2$ (n=5, $p<0.001$ vs. control group). At concentrations of 5-100 μM, the effects of eupafolin were concentration-dependent (Fig. 1C) with an EC_{50} of 17 μM and maximum inhibition of 59 ±1.3%. In addition, 4-AP-evoked glutamate release was reduced in extracellular-Ca^{2+}-free solution that contained 300 μM EGTA ($p<0.001$). This Ca^{2+}-independent glutamate release evoked by 4-AP was, however, not affected by eupafolin (30 μM) (n=5, $p=0.5$; Fig. 1C). On the other hand, we examined the effect of eupafolin on 4-AP-evoked glutamate release in the presence of 10 μM DL-TBOA, an inhibitor of the plasma membrane glutamate transporter, which blocks the Ca^{2+}-independent nonvesicular efflux by transporter reversal. With DL-TBOA present, eupafolin (30 μM) significantly inhibited the 4-AP-evoked glutamate release (n=5, $p<0.001$, Fig. 1C). We also examined the effect of eupafolin on 4-AP-triggered exocytosis by assaying the rate of destaining (loss of FM1-43 fluorescence intensity) (Murthy, 1999). As shown in Fig. 1D, 4-AP (1 mM) evoked a decrease in FM1-43 fluorescence in the presence of $CaCl_2$. Eupafolin (30 μM) also inhibited 4-AP-evoked Ca^{2+}-dependent decrease in FM1-43 fluorescence (n=5, $p<0.001$ vs. control group).

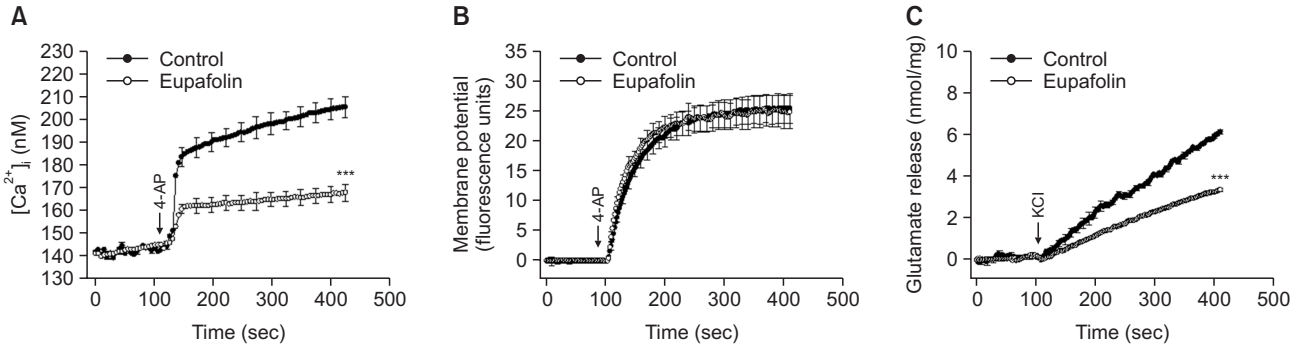

Fig. 2. Effect of eupafolin on $[Ca^{2+}]_i$ (A), the synaptosomal membrane potential (B), or the release of glutamate evoked by 15 mM KCl (C). Eupafolin (30 μM) was added 10 min before the addition of 4-AP or KCl. Data are mean ± standard error of the mean (SEM) (n=5 per group). ***p<0.001 compared with the control group.

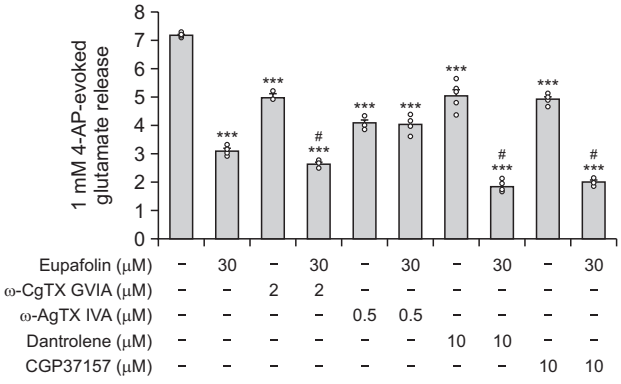

Fig. 3. Effect of eupafolin on 4-AP-evoked glutamate release in the presence of N-type Ca^{2+} channel blocker ω-CgTX GVIA, P/Q-type Ca^{2+} channel blocker ω-AgTX IVA, ryanodine receptor inhibitor dantrolene, or mitochondrial Na^+/Ca^{2+} exchanger inhibitor CGP37157. Eupafolin was added 10 min before the addition of 4-AP, and other drugs were added 10 min before this. Data are mean ± standard error of the mean (SEM) (n=5 per group). ***p<0.001 compared with the control group. #p<0.001 compared with the dantrolene- or CGP37157-treated group.

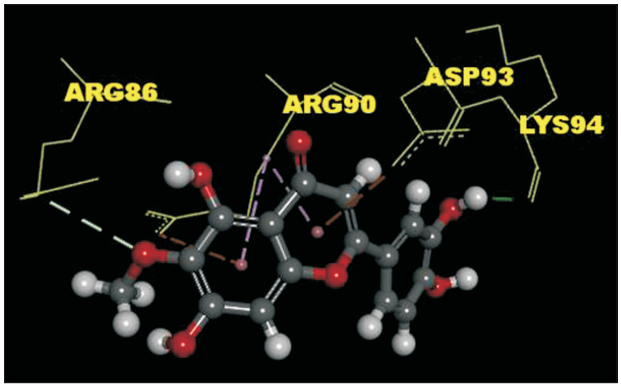

Fig. 4. Predicted the interaction of eupafolin to P/Q-type of Ca^{2+} channel protein using molecular docking. Molecular docking modeling of eupafolin with the active site of monomeric P/Q-type of calcium channel protein (PDB ID 3BXK) was performed using the Discovery Studio program. Protein–ligand hydrogen-bonding interactions are displayed as green and gray-green dashed lines, and the π–π interactions of the core ring are depicted as red and pink dashed lines.

Eupafolin decreases 4-AP-induced $[Ca^{2+}]_i$ elevation

Fig. 2A shows that 4-AP (1 mM) elicited a rise in $[Ca^{2+}]_i$ and eupafolin (30 μM) princubation reduced the 4-AP-induced $[Ca^{2+}]_i$ increase by 36 ± 2.5 % (n=5, p<0.001 vs. control group). Eupafoin (30 μM) had no significant effect on the basal $[Ca^{2+}]_i$ (p=0.96). In addition, 4-AP (1 mM) evoked $DiSC_3(5)$ fluorescence increase and this phenomenon was not affected by eupafolin (30 μM) preincubation (n=5, p=0.9, Fig. 2B). Furthermore, eupafolin (30 μM) preincubation efficiently decreased 15 mM KCl-evoked glutamate release (n=5, p<0.001 vs. control group, Fig. 2C), a process that involves Ca^{2+} influx primarily through voltage-dependent Ca^{2+} channel opening (Barrie *et al*., 1991).

P/Q-type Ca^{2+} channels involved in the eupafolin-mediated inhibition of glutamate release

Either voltage-gated Ca^{2+} channels (VGCCs) or intracellular Ca^{2+} stores is responsible for the release of glutamate evoked by depolarization (Vázquez and Sánchez-Prieto, 1997; Berridge, 1998). As shown in Fig. 3, 4-AP-evoked glutamate release was reduced by 2 μM ω-CgTX GVIA (p<0.001 vs. control group) and 0.5 μM ω-AgTX IVA (p<0.001 vs. control group), which selectively block N- and P/Q-type Ca^{2+} channels, respectively (Millan and Sánchez-Prieto, 2002). With ω-CgTX GVIA present, 4-AP-evoked glutamate release was further inhibited by eupafolin (30 μM) (n=5, p<0.001 vs. ω-CgTX GVIA-treated group). However, the inhibitory action of eupafolin was abolished in the presence of ω-Aga IVA. The release measured in the presence of ω-Aga IV and eupafolin being similar to that obtained in the presence of ω-Aga IV (n=5, p=0.9 vs. ω-Aga IVA-treated group). In addition, 4-AP-evoked glutamate release was reduced by 10 μM dantrolene, an inhibitor of intracellular Ca^{2+} release from endoplasmic reticulum (p<0.001 vs. control group), and 10 μM CGP37157, an inhibitor of mitochondrial Na^+/Ca^{2+} exchange (p<0.001 vs. control group). With dantrolene or CGP37157 present, eupafolin (30 μM) was able to reduce 4-AP-evoked glutamate release (n=5, p<0.001 vs. dantrolene- or CGP37157-treated group). Furthermore, the binding mode of eupafolin to the active site of P/Q-type of calcium channel protein (PDB ID 3BXK) observed four key interactions: two hydrogen-bonding interaction with the amino

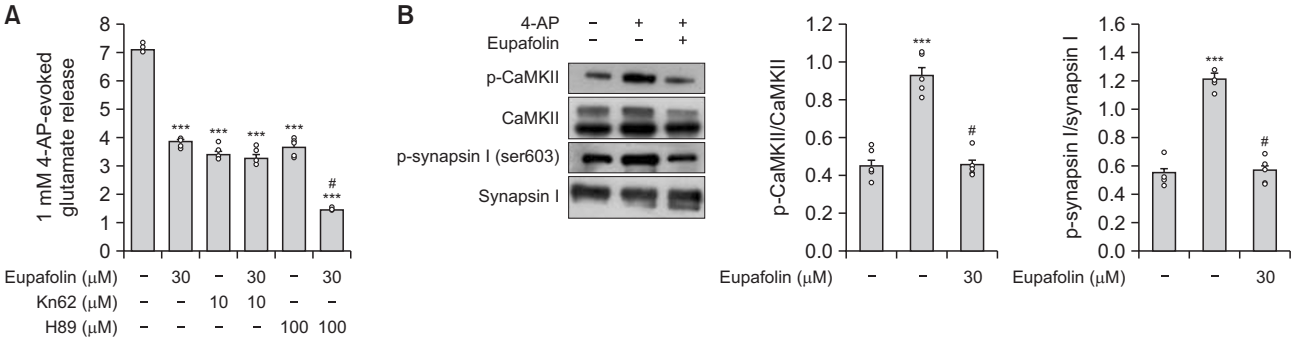

Fig. 5. (A) Effect of the CaMKII inhibitor KN62 or PKA inhibitor H89 on the eupafolin-mediated inhibition of 4-AP-evoked glutamate release. (B) Effect of eupafolin on CaMKII and synapsin I phosphorylation evoked by 4-AP. Eupafolin or KN62 was added 10 min before the addition of 4-AP. Data are mean ± SEM (n=5-7 per group). ***p<0.001 compared with the control group. $^{\#}p$<0.001 compared with the H89- or 4-AP-treated group.

acid residues Arg86 and Lys94, and the π–π interaction of the core ring with Arg90 and ASP93 of P/Q-type of calcium channel protein in the docking analysis (Fig. 4).

Contribution of decreased CaMKII/synapsin I pathway to the eupafolin-mediated inhibition of glutamate release

The involvement of CaMKII in the eupafolin-mediated inhibition of glutamate release was studied using the selective CaMKII inhibitor KN62 (10 μM) (León *et al*., 2008). As shown in Fig. 5A, KN62 (10 μM) reduced the glutamate release induced by 4-AP (p<0.001 vs. control group). With KN62 present, eupafolin (30 μM) did not produce any significant inhibition of glutamate release (n=7, p=0.6). We also examined the effect of eupafolin on glutamate release in the presence of H89, a protein kinase A (PKA) inhibitor. With H89 present, eupafolin was able to reduce the 4-AP-evoked glutamate release (n=5, p<0.001 vs. H89-treated group, Fig. 5A). In addition, we determined the effect of eupafolin on the phosphorylation of CaMKII and synapsin I (Serine^{603}, a substrate site of CaMKII) in synaptosomes. Compared with the control group, a statistically significant increase in the phosphorylation of CaMKII and synapsin I was observed in the 4-AP group (p<0.001). After pretreatment with eupafolin (30 μM), no significant increase in CaMKII and synapsin I phosphorylation was observed after exposure to 1 mM 4-AP compared with the control group (n=6, p=0.9, Fig. 5B).

DISCUSSION

The glutamatergic system is involved in the pathophysiology of many brain disorders, which are accompanied by increased levels of glutamate (Obrenovitch and Urenjak, 1997; Bano and Ankarcrona, 2018). A reduction in synaptic glutamate levels is a critical step in a series of cascade responses that lead to neuroprotective activity (Mdzinarishvili *et al*., 2012; Wong *et al*., 2015; Lazarevic *et al*., 2018). Eupafolin has been demonstrated to mediate neuroprotective effects in the central nervous system (Chen *et al*., 2020); however, no studies have determined its effect on glutamatergic transmission, especially at the presynaptic level. Using purified synaptosomal preparations, we demonstrated for the first time that eupafolin reduces 4-AP-evoked glutamate release in the rat cerebral cortex.

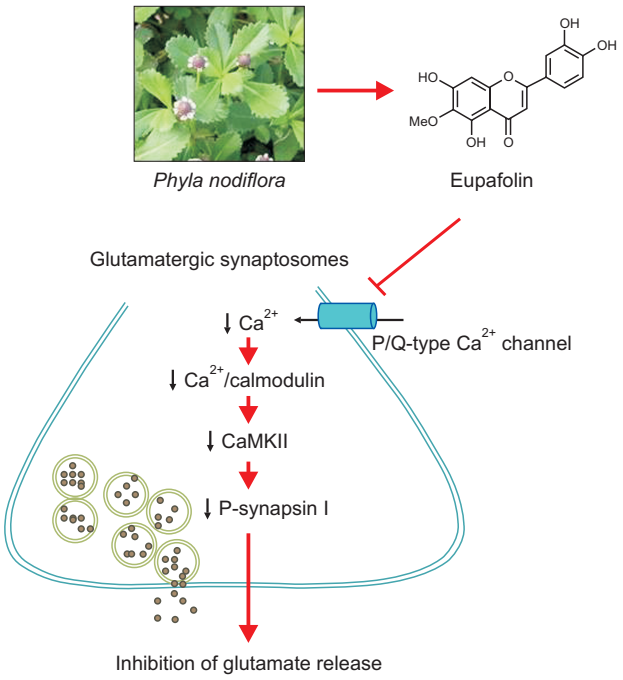

Fig. 6. A proposed mechanism underlying the inhibition of glutamate release by eupafolin in rat cerebrocortical synaptosomes. Eupafolin suppresses P/Q-type Ca^{2+} channels, which in turn inhibits CaMKII/synapsin I pathway, thus decreasing the amounts of glutamate release.

The processes by which 4-AP induces the release of glutamate from neurons are Ca^{2+}-dependent exocytotic release from glutamate-containing synaptic vesicles and Ca^{2+}-independent release, which is facilitated by the reversal of the operation of the plasma membrane glutamate uptake carrier caused by prolonged depolarization (Nicholls *et al*., 1987). In the current study, eupafolin failed to inhibit the 4-AP-induced release of glutamate in the absence of extracellular Ca^{2+} (Ca^{2+}-independent release), indicating its dependence on extracellular Ca^{2+}. In addition, the inhibitory effect of eupafolin on the induced glutamate release was not prevented in the pres-

ence of DL-TBOA, an inhibitor of glutamate transporters. Furthermore, eupafolin inhibition of 4-AP-evoked FM1-43 release was also observed through the use of an FM1-43 exocytosis assay. Our data indicate that eupafolin-mediated inhibition of glutamate release is ascribable mainly to a reduction in Ca^{2+}-dependent exocytosis.

In synaptic terminals, the inhibition of Na^+ channels or activation of K^+ channels results in presynaptic inhibition due to nerve terminal hyperpolarization. This inhibition causes a subsequent decrease in voltage-dependent presynaptic Ca^{2+} entry into neurons and a consequent decrease in transmitter release (Nicholls, 1998). Tibbs *et al*. (1989) demonstrated that glutamate release induced by 1 mM 4-AP involved both Na^+ and Ca^{2+} channels, whereas that induced by 15 mM external KCl involved only Ca^{2+} channels. In our study, eupafolin significantly inhibited 4-AP- and KCl-induced glutamate release. Thus, Na^+ channels were not involved in the inhibition of glutamate release by eupafolin. Furthermore, no substantial effect of eupafolin on synaptosomal membrane potential was observed either in the resting condition or during depolarization with 4-AP, thus indicating no effect on K^+ conductance. Thus, eupafolin does not reduce synaptosomal excitability, which would in turn decreases the influx of Ca^{2+} and thus reduce glutamate release.

However, in our study eupafolin reduced the 4-AP-evoked increase in intracellular Ca^{2+} levels. Because both extracellular Ca^{2+} influx through VDCCs and intracellular Ca^{2+} release from stores can mediate a depolarization-induced increase in $[Ca^{2+}]_C$ coupled with a release of glutamate (Berridge, 1998; Millan and Sánchez-Prieto, 2002), we sought to determine whether eupafolin's inhibition of glutamate release is due to either of these mechanisms. We observed that eupafolin's inhibitory effect on glutamate release was prevented when P/Q-type Ca^{2+} channels were blocked. However, neither dantrolene, an inhibitor of the release of intracellular Ca^{2+} from the endoplasmic reticulum, nor CGP37157, a mitochondrial Na^+/Ca^{2+} exchange blocker, affected the inhibitory effect of eupafolin on 4-AP-evoked glutamate release. Thus, we conclude that eupafolin inhibits glutamate release by suppressing presynaptic P/Q-type Ca^{2+} channels. Our docking observation that eupafolin interacted with P/Q-type Ca^{2+} channels supported this conclusion.

Exocytosis includes the activation of some signaling pathways, such as that of CaMKII, a serine/threonine kinase that is activated by Ca^{2+} and calmodulin (Hudmon and Schulman, 2002). Several studies have noted that CaMKII increases glutamate release by phosphorylating numerous synaptic proteins, including synapsin I (Nichols *et al*., 1990; Hinds *et al*., 2003; León *et al*., 2008). The phosphorylation of synapsin I at the site of Serine 603 by CaMKII causes synaptic vesicles to dissociate from the cytoskeleton, thereby increasing the proportion of nerve terminal vesicles that are available for release (Llinas *et al*., 1991; Chi *et al*., 2003; Leenders and Sheng, 2005). In the present study, we found that the effect of eupafolin on glutamate release was eliminated by the CaMKII inhibitor KN62 not by PKA inhibitor H89. Moreover, eupafolin inhibited the 4-AP-induced phosphorylation of CaMKII and synapsin I (Ser^{603}). These data suggest that the suppression of CaMKII activity and inhibition of synapsin I phosphorylation play roles in the eupafolin-mediated inhibition of glutamate release. In addition to synapsin I, however, several other presynaptic proteins, including syntaxin, synaptophysin, and SNAP 25, are phosphorylated by CaMKII (Ohyama *et al*., 2002; Wang, 2008). The involvement of decreased phosphorylation of these proteins in eupafolin's inhibitory effect cannot be ruled out at this time.

As illustrated in Fig. 6, eupafolin suppressed P/Q-type Ca^{2+} channels, and this suppression inhibited the CaMKII/synapsin I pathway, thus reducing the glutamate release from the rat cerebrocortical synaptosomes. Although the relevance of our finding to clinical situations is undetermined, our findings suggest that eupafolin is promising as an antiexcitotoxic drug for the treatment of neurological disorders because glutamate excitotoxicity is a common mechanism of neuronal death.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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