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

Eupafolin Suppresses P/Q-Type Ca²⁺ Channels to Inhibit Ca²⁺/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 [Ca²⁺]i elevation and had no effect on synaptosomal membrane potential. The inhibition of P/Q-type Ca²⁺ channels reduced the decrease in glutamate release that was caused by eupafolin, and docking data revealed that eupafolin interacted with P/Q-type Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ channels, CaMKII, Synapsin I, Cerebrocortical synaptosomes
Animals and synaptosomes preparation

Adult male Sprague-Dawley rats (150-200 g) were used in this study. Animals were handled according to the guidelines of the animal care committee at the University of Michigan. Animals were sacrificed via cervical dislocation and the cerebral cortex was 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). CaCl2 (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 CaCl2 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, resuspension in hepes-buffer medium containing 1.2 mM CaCl2. 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 Ca2+-dependent decrease in FM1-43 fluorescence.

Intrasynaptosomal Ca2+ concentration ([Ca2+]i)

Synaptosomes (0.5 mg protein) were incubated in the hepes-buffered solution containing Fura-2-AM (5 µM) and CaCl2 (0.1 mM) for 30 min at 37°C. Samples were centrifuged for 1 min at 5,000 rpm, and pellets were resuspended in hepes-buffered medium containing CaCl2 (1.2 mM). Fura-2-Ca fluorescence was monitored at 5 s intervals for 5 min. [Ca2+]i (nM) was calculated by using calibration procedures and equations described previously (Gryniewicz et al., 1985).

Membrane potential

The synaptosomal membrane potential was assayed with a positively charged membrane potential-sensitive carbocyanine dye DiSC3(5). DiSC3(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).

Materials

Eupafolin (purity >99%) was kindly provided by Professor Horng-Huey Ko. 7-chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzoazepin-2(3H)-one (CGP37157), DL-threo-β-benzylxyaspartate (DL-TBOA), dantrolene, N-[2-(p-bromocinnamylamino)ethyl]-5-isooquinolinesulfonamide (H89), and 1-[N,O-Bis (5-isooquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine (KN62) were purchased from Tocris (Bristol, UK). FM-43, 3,3,3-Dipropylthiadicarbocyanine iodide [DiSC3(5)], and fura-2-acetoxymethyl ester (Fura-2-AM) were purchased from Thermo (Waltham, MA, USA). p-synapsin I (Serine 603) antibody was purchased from Cell Signaling Technologies (Beverly, MA, USA). p-synapsin I (Serine 603) antibody was purchased from GeneTex (CA, USA). 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).

Intracellular Ca2+ concentration ([Ca2+]i)

Synaptosomes (0.5 mg protein) were incubated in the hepes-buffered solution containing Fura-2-AM (5 µM) and CaCl2 (0.1 mM) for 30 min at 37°C. Samples were centrifuged for 1 min at 5,000 rpm, and pellets were resuspended in hepes-buffered medium containing CaCl2 (1.2 mM). Fura-2-Ca fluorescence was monitored at 5 s intervals for 5 min. [Ca2+]i (nM) was calculated by using calibration procedures and equations described previously (Gryniewicz et al., 1985).

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**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 Ca²⁺/calmodulin-dependent kinase II (CaMKII, 1:10,000), p-CaMKII (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/anti-body 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 Ca²⁺ 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 in the presence of 1.2 mM CaCl₂ (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 EC₅₀ of 17 μM and maximum inhibition of 59 ± 13%. In addition, 4-AP-evoked glutamate release was reduced in extracellular-Ca²⁺-free solution that contained 300 μM EGTA (p<0.001). This Ca²⁺-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 Ca²⁺-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 CaCl₂. Eupafolin (30 μM) also inhibited 4-AP-evoked Ca²⁺-dependent decrease in FM1-43 fluorescence (n=5, p<0.001 vs. control group).

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**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 Ca²⁺-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.
Eupafolin decreases 4-AP-induced \([\text{Ca}^{2+}]\) elevation

Fig. 2A shows that 4-AP (1 mM) elicited a rise in \([\text{Ca}^{2+}]\), and eupafolin (30 \(\mu\)M) preincubation reduced the 4-AP-induced \([\text{Ca}^{2+}]\) increase by 36 \(\pm\) 2.5% \((n=5, p<0.001\) vs. control group). Eupafolin (30 \(\mu\)M) had no significant effect on the basal \([\text{Ca}^{2+}]\) \((p=0.96)\). In addition, 4-AP (1 mM) evoked DiSC3(5) fluorescence increase and this phenomenon was not affected by eupafolin (30 \(\mu\)M) preincubation \((n=5, p=0.9, \text{Fig. 2B})\). Furthermore, eupafolin (30 \(\mu\)M) preincubation efficiently decreased 15 mM KCl-evoked glutamate release \((n=5, p<0.001\) vs. control group, Fig. 2C), a process that involves \(\text{Ca}^{2+}\) influx primarily through voltage-dependent \(\text{Ca}^{2+}\) channel opening \((\text{Barrie et al., 1991})\).

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

Either voltage-gated \(\text{Ca}^{2+}\) channels (VGCCs) or intracellular \(\text{Ca}^{2+}\) stores is responsible for the release of glutamate evoked by depolarization \((\text{Vázquez and Sánchez-Prieto, 1997; Ber-ridge, 1998})\). As shown in Fig. 3, 4-AP-evoked glutamate release was reduced by 2 \(\mu\)M ω-CgTX GVIA \((p<0.001\) vs. control group) and 0.5 \(\mu\)M ω-AgTX IVA \((p<0.001\) vs. control group), which selectively block N- and P/Q-type \(\text{Ca}^{2+}\) channels, respectively \((\text{Millan and Sánchez-Prieto, 2002})\). With ω-CgTX GVIA present, 4-AP-evoked glutamate release was further inhibited by eupafolin (30 \(\mu\)M) \((p<0.001\) 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 \(\mu\)M dantrolene, an inhibitor of intracellular \(\text{Ca}^{2+}\) release from endoplasmic reticulum \((p<0.001\) vs. control group), and 10 \(\mu\)M CGP37157, an inhibitor of mitochondrial Na\(^+\)/Ca\(^{2+}\) exchange \((p<0.001\) vs. control group). With dantrolene or CGP37157 present, eupafolin (30 \(\mu\)M) 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 acid π–π interactions of the core ring are depicted as red and pink dashed lines.
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 (Serine603, 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.

The processes by which 4-AP induces the release of glutamate from neurons are Ca2+-dependent exocytotic release from glutamate-containing synaptic vesicles and Ca2+-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 Ca2+ (Ca2+-independent release), indicating its dependence on extracellular Ca2+. 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 Ca²⁺-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 Ca²⁺ 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 Ca²⁺ channels, whereas that induced by 15 mM external KCl involved only Ca²⁺ 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 Ca²⁺ and thus reduce glutamate release.

However, in our study eupafolin reduced the 4-AP-evoked increase in intracellular Ca²⁺ levels. Because both extracellular Ca²⁺ influx through VDCCs and intracellular Ca²⁺ release from stores can mediate a depolarization-induced increase in [Ca²⁺]; 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 Ca²⁺ channels were blocked. However, neither dan-trolene, an inhibitor of the release of intracellular Ca²⁺ from the endoplasmic reticulum, nor CGP37157, a mitochondrial Na⁺/ Ca²⁺ 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 Ca²⁺ channels. Our docking observation that eupafolin interacted with P/Q-type Ca²⁺ 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 Ca²⁺ 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 (Linhas 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 (Ser603). 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 Ca²⁺ 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 antixcitotoxic drug for the treatment of neurological disorders because glutamate excitotocixity is a common mechanism of neuronal death.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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