Toxicology and Applied Pharmacology 263 (2012) 233–243

Contents lists available at SciVerse ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

Hispidulin inhibits the release of glutamate in rat cerebrocortical nerve terminals

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ARTICLE INFO

Article history:
Received 17 April 2012
Revised 15 June 2012
Accepted 22 June 2012
Available online 1 July 2012

Keywords:
Hispidulin
Glutamate release
Cerebrocortical nerve terminals
Voltage-dependent Ca^{2+} channels
ERK
Synapsin I

ABSTRACT

Hispidulin, a naturally occurring flavone, has been reported to have an antiepileptic profile. An excessive release of glutamate is considered to be related to neuropathology of epilepsy. We investigated whether hispidulin affected endogenous glutamate release in rat cerebral cortex nerve terminals (synaptosomes) and explored the possible mechanism. Hispidulin inhibited the release of glutamate evoked by the K^+ channel blocker 4-aminopyridine (4-AP). The effects of hispidulin on the evoked glutamate release were prevented by the chelation of extracellular Ca^{2+} ions and the vesicular transporter inhibitor bafilomycin A1. However, the glutamate transporter inhibitor DL-threo-beta-benzyl-oxyaspartate did not have any effect on hispidulin action. Hispidulin reduced the depolarization-induced increase in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_C$), but did not alter 4-AP-mediated depolarization. Furthermore, the effect of hispidulin 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 ryanodine receptors or mitochondrial Na^+/Ca^{2+} exchange. Mitogen-activated protein kinase kinase (MEK) inhibition also prevented the inhibitory effect of hispidulin on evoked glutamate release. Western blot analyses showed that hispidulin decreased the 4-AP-induced phosphorylation of extracellular signal-regulated kinase 1 and 2 (ERK1/2) and synaptic vesicle-associated protein synapsin I, a major presynaptic substrate for ERK; this decrease was also blocked by the MEK inhibitor. Moreover, the inhibition of glutamate release by hispidulin was strongly attenuated in mice without synapsin I. These results show that hispidulin inhibits glutamate release from cortical synaptosomes in rats through the suppression of presynaptic voltage-dependent Ca^{2+} entry and ERK/synapsin I signaling pathway.

Introduction

Epilepsy, which affects approximately 2% of people worldwide, is one of the most common brain disorder. Current antiepileptic drugs mainly affect transmitter receptors and ion channels. Unfortunately, because of unwanted side effects, approximately 30% of patients do not response to these drugs (Rogawski and Loscher, 2004). Therefore, seeking safe and effective antiepileptic drugs derived from natural products may enable development of novel treatments for epilepsy. Hispidulin is a naturally occurring flavone commonly found in *Saussurea involucrate* Kar. et Kir., a traditional Chinese medicinal herb. Several biological activities of hispidulin have emerged, for example, antioxidant, antifungal, anti-inflammatory, and antimutagenic properties (Gil et al., 1994; Tan et al., 1999). In addition to these properties, hispidulin has been confirmed to penetrate the blood–brain barrier (BBB) and possess antiepileptic activity (Kavvadias et al., 2004). However, the mechanisms involved in the antiepileptic effect of hispidulin have yet to be fully elucidated.

Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system (CNS), and evidence suggests that alterations in this neurotransmitter system may be associated with epilepsy. For instance, the experimental application of glutamate receptor agonists induces seizures in rats (Chapman, 1998; Loscher, 1998; Tizzano et al., 1995). Conversely, glutamate receptor antagonists exhibit antiepileptic-like properties and reduce seizure-induced brain damage in different animal models (Chapman et al., 2000; Clifford et al., 1990). Furthermore, a significant increase in glutamate level was observed in human epilepsy patients as well as in experimental models of epilepsy (Carlson et al., 1992; Chapman et al., 1996; During and Spencer, 1993; Millan et al., 1993; Smolders et al., 1997; Wilson et al., 1996). This evidence suggests that an overabundance of glutamatergic activity can occur in epilepsy. Thus, modulating central glutamatergic neurotransmission may provide a potential target for epilepsy treatment. Consequently, several glutamatergic modulators are being developed to treat epilepsy, including *N*-methyl-D-aspartic acid (NMDA) receptor antagonists, as well as metabotropic glutamate receptor agonists and antagonists. However, these drugs have been unsuccessful in

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doi:10.1016/j.taap.2012.06.015

clinical trials because of numerous side effects, such as ataxia, and memory impairment (Chapman, 1998; Moldrich et al., 2003).

Because excessive release of glutamate is known to be a critical factor in the neuropathology of epilepsy (Kaura et al., 1995; Meldrum, 1994), regulating its release may be an important mechanism of antiepileptic drugs. Some antiepileptic drugs have been revealed to decrease glutamate release in human and rat brain tissues (Kammerer et al., 2011; Sitges et al., 2007a, 2007b). Likewise, hispidulin has an antiepileptic-like effect and whether hispidulin has an effect on endogenous glutamate release should be evaluated. Thus, the present study used isolated nerve terminals (synaptosomes) purified from the rat prefrontal cortex as a model to investigate the effects of hispidulin on glutamate release and to characterize the underlying molecular mechanisms. In contrast to brain slices, synaptosomes do not suffer from any postsynaptic interactions and are, therefore, extensively used to evaluate presynaptic phenomena. The first series of experiments investigated the effects of hispidulin 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 experiments determined whether the protein kinase signaling pathway participates in the regulation of glutamate release by hispidulin. Finally, since it has been demonstrated that phosphorylation of the vesicle-associated protein synapsin I enhances vesicle mobilization and glutamate release (Jovanovic et al., 1996, 2000; Schenk et al., 2005; Yamagata et al., 2002), we examined if the regulation of glutamate release by hispidulin is linked to a decrease in synapsin I phosphorylation.

Materials and methods

Chemicals. 3′, 3′, 3′-Dipropylthiadicarbocyanine iodide [$DiSC_3(5)$] and Fura-2-acetoxy- methyl ester (Fura-2-AM) were obtained from Invitrogen (Carlsbad, CA, USA). Hispidulin, dantrolene, bafilomycin A1, 2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one (PD98059), N-(cyclopropylmethoxy)-3, 4, 5-trifluoro-2-[(4-iodo-2-methylphenyl) amino]-benzamide (PD198306), DL-*threo*-β-benzyloxyaspartate (DL-TBOA), bisindolylmaleimide I (GF109203X), 7-chloro-5-(2-chloroph enyl)-1,5-dihydro-4,1-benzothiazepin-2(3*H*)-one (CGP37157), N-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H89), isoguvacine, 6-imino-3-(4-methoxyphenyl)-1(6H)-pyridazinebutanoic acid hydrobromide (SR95531), ω-conotoxin GVIA (ω-CgTX GVIA), ω-agatoxin IVA (ω-AgTX IVA) and ω-conotoxin MVIIC (ω-CgTX MVIIC) were obtained from Tocris Cookson (Bristol, UK). Rabbit polyclonal antibodies directed against ERK1/2 and phospho-ERK1/2 was bought from Cell Signaling Technology (Beverly, MA, USA). The anti-$GABA_A$ receptor α1 and β3 rabbit polyclonal antibodies were bought from Novus (Littleton, USA). The anti-synapsin I phosphorylation state-specific rabbit polyclonal antibody directed against MAPK/ERK-phosphorylated sites 4, 5 of synapsin I (Ser^{62}/Ser^{67}) was from Millipore (MA, USA). Horseradish peroxidase-conjugated anti-rabbit secondary antibodies were from BioRad (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, USA).

Animals. Two-month old male Sprague–Dawley rats ($n=71$) or six-week old male wild-type mice ($n=6$) or synapsin I-deficient mutant mice ($n=6$) 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.

Heterozygous synapsin I knockout (B6; 129S-*Syn1*^{tm1Sud}/J) female (+/−) and wild type male (+/*y*) mice were purchased from the Jackson Laboratory (Stock no. 002444, Bar Harbor, ME, USA), and bred to produce an F1 population. Pups were weaned at 3 weeks of age and housed per gender at a maximum of four mice per cage. Due to the gene for synapsin I is located on the X chromosome, only male littermates were genotyped for wild-type (+/*y*) and synapsin I knockout (−/*y*) mice. All experiments were conducted on mice at 6–8 weeks of age. For genotyping, shortly after weaning, tail DNA was extracted and analyzed by a modified NEOTD standard polymerase chain reaction (PCR) protocol provided by the Jackson Laboratory. The primers used were as followings: oIMR6916: 5′-CTT GGG TGG AGA GGC TAT TC-3′; oIMR6917: 5′-AGG TGA GAT GAC AGG AGA TC-3′; oIMR8744: 5′-CAA ATG TTG CTT GTC TGG TG-3′; oIMR8745: 5′-GTC AGT CGA GTG CAC AGT TT-3′. The presence of the 280 bp PCR amplicon derived from the oIMR6916 and oIMR6917 primer combination indicated the knockout allele, whereas the presence of the ~200 bp PCR amplicon from the oIMR8744 and oIMR8745 primers symbolized the wild type allele. PCR was carried out with 200 ng template DNA, 1 μM each of oIMR6916, oIMR6917, oIMR8744 and oIMR8745 primers, 0.2 μM dNTP, 2.5 μl 10× buffer, and 1 unit DNA polymerase (*Takara Ex Taq*, Takara Biotechnology, Shiga, Japan) in a final volume of 25 μl. Following tail DNA extraction, DNA fragments were amplified for 30 cycles of 98 °C for 10 s, 55 °C for 30 s, and 72 °C for 1 min. Then, the mixture of 10 μl of PCR product and 2 μl 6× DNA loading dye (Protech Technology Enterprise Co., Ltd, Taipei, Taiwan) was run on an 1.5% agarose gel stained with ethidium bromide alongside a 1 kb DNA Ladder (Violet Bioscience In., Taipei, Taiwan). Bands were visualized with an ultraviolet light (Tseng Hsiang Life Science Ltd., Taipei, Taiwan) illumination.

Synaptosomal preparation. Animals were killed by decapitation and cerebral cortex rapidly dissected. Synaptosomes were prepared by homogenizing the tissue 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* (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. 2 ml 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 (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 27,000 ×*g* (15,000 rpm in a JA 25.5) 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, 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 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 4–6 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 (Nicholls and Sihra, 1986; Wang and Sihra, 2003). Synaptosomal pellets (0.5 mg protein) were resuspended in HBM and incubated in a stirred and thermostated cuvette maintained at 37 °C in a Perkin-Elmer LS-55 spectrofluorimeter (PerkinElmer Life and Analytical Sciences, Waltham, MA, USA). $NADP^+$ (2 mM), GDH (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 (4-AP; 1 mM), high external KCl (15 mM), or ionomycin (5 μM) 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. 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. Estimation of the IC_{50} was based on a one-site model [Inhibition = (Inhibition $_{MAX}$ × [hispidulin]/(IC_{50} + [hispidulin])] using the nonlinear curve-fitting function in MicroCal Origin.

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 (Akerman et al., 1987). 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. 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 wave lengths of 646 nm and 674 nm, respectively. Results are expressed in fluorescence units.

Cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_C$). $[Ca^{2+}]_C$ was measured using the Ca^{2+} indicator Fura-2. Synaptosomes (0.5 mg/ml) were preincubated in HBM with 16 μM BSA in the presence of 5 μM Fura-2 and 0.1 mM $CaCl_2$, for 30 min at 37 °C in a stirred test tube. After Fura-2 loading, synaptosomes were centrifuged in a microcentrifuge for 30 s at 3000 ×*g* (5000 rpm). The synaptosomal pellets were resuspended in HBM with BSA, and the synaptosomal suspension was stirred in a thermostated cuvette in a Perkin-Elmer LS-55 spectrofluorimeter (PerkinElmer Life and Analytical Sciences, Waltham, MA, USA). $CaCl_2$ (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% 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 (Grynkiewicz et al., 1985).

Western blot analysis. Synaptosomes 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 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 (anti-$GABA_A$ receptor α1, 1:1000, anti-$GABA_A$ receptor β3, 1:1000, anti-phospho-ERK1/2, 1:2000, anti-ERK1/2, 1:1000, anti-phospho-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 for three times by 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.

Data analysis. Cumulative data were analyzed in Lotus 1-2-3 (IBM, White Plains, NY) and MicroCal Origin (OriginLab Corporation, Northampton, MA, USA). Data are expressed as mean ± SEM. To test the significance of the effect of a drug versus control, an unpaired Student's *t* test was used. When an additional comparison was required (such as whether a second treatment influenced the action of hispidulin), a one-way analysis of variance (ANOVA) was used followed by a post hoc LSD comparison. Analysis was completed via software SPSS (17.0; SPSS Inc., Chicago, IL).

Results

Hispidulin inhibits 4-AP-evoked glutamate release from rat cerebrocortical synaptosomes, and this phenomenon is mediated by a reduction in the Ca^{2+}-dependent exocytotic component of glutamate release

To examine the influence of hispidulin on glutamate release, isolated nerve terminals were depolarized with the potassium channel blocker 4-aminopyridine (4-AP) which has been shown to open voltage-dependent Ca^{2+} channels (VDCCs) and to induce the release of glutamate (Nicholls, 1998). In synaptosomes incubated in the presence of 1 mM $CaCl_2$, 4-AP evoked a glutamate release of 7.2 ± 0.1 nmol/mg/5 min. Application of hispidulin (30 μM) produced an inhibition of 4-AP-evoked glutamate release to 3.4 ± 0.3 nmol/mg/5 min ($n = 7$; $P < 0.001$), without altering the basal release of glutamate ($P > 0.05$; Fig. 1A). A maximal inhibition of 80% occurred with 100 μM hispidulin. The IC_{50} value for hispidulin inhibition of 4-AP-evoked glutamate release, derived from a log dose–response curve, was 22 μM (Fig. 1A, inset).

The 4-AP-evoked release of glutamate from synaptosomes can be sustained by different mechanisms, including exocytosis (Ca^{2+}-dependent release) and reversal of the transporter (Ca^{2+}-independent release) (Nicholls, 1998; Nicholls et al., 1987). To discriminate the effect of hispidulin on these two components of endogenous glutamate release evoked by 4-AP, we performed a series of experiments as follows. First, the Ca^{2+}-independent glutamate efflux was measured by depolarizing the synaptosomes with 4-AP (1 mM) in extracellular-Ca^{2+}-free solution that contained 300 μM EGTA. Under these conditions, the release of glutamate evoked by 4-AP (1.3 ± 0.2 nmol/mg/5 min) was not affected by 30 μM hispidulin (1.2 ± 0.2 nmol/mg/5 min) ($n = 5$; $P > 0.05$; Fig. 1B). Second, we investigated the action of hispidulin in the presence of DL-TBOA, a non-selective inhibitor of all excitatory amino acid transporter (EAAT) subtypes, or bafilomycin A1, which causes the depletion of glutamate in synaptic vesicles. In Fig. 1C, DL-TBOA (10 μM) increased control 4-AP-evoked glutamate release (7.3 ± 0.1 nmol/mg/5 min) to 10.1 ± 0.3 nmol/mg/5 min (because of inhibition of reuptake of released glutamate) ($P < 0.001$). In the presence of DL-TBOA, hispidulin (30 μM) was able to produce a 50% inhibition on 4-AP-evoked glutamate release, which was similar to that observed for the 56% inhibition produced by hispidulin (30 μM) alone ($n = 5$; Fig. 1C). In contrast to DL-TBOA, bafilomycin A1 (0.1 μM) reduced control 4-AP-evoked glutamate release to 2.1 ± 0.2 nmol/mg/5 min ($P < 0.001$), and completely prevented the inhibitory effect of hispidulin (30 μM) on 4-AP-evoked glutamate release (1.8 ± 0.3 nmol/mg/5 min; $n = 6$; Fig. 1C).

Hispidulin reduces depolarization-induced increase in intraterminal Ca^{2+} levels but does not change the synaptosomal membrane potential

We next addressed the potential mechanisms underlying the hispidulin-mediated inhibition of glutamate release by assessing the effects of hispidulin on intracellular Ca^{2+} levels and synaptosomal plasma membrane potential. Fig. 2A shows that 4-AP (1 mM) evoked a rise in $[Ca^{2+}]_C$, from 157.1 ± 1.2 nM to a plateau level of 236.7 ± 5.5 nM.

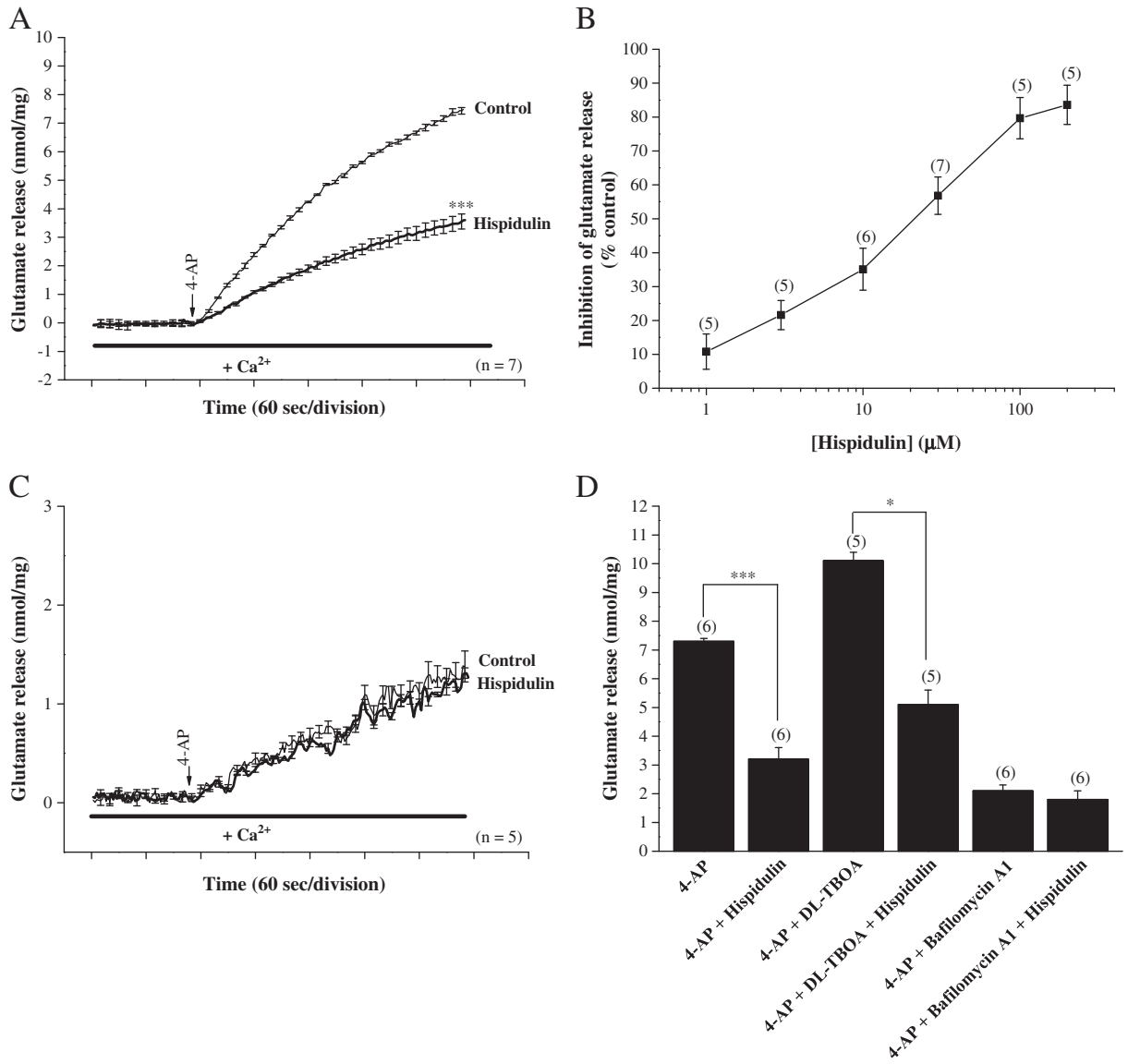


Fig. 1. Hispidulin inhibits 4-AP-induced glutamate release from rat cerebrocortical nerve terminals; this effect is due to a decrease in vesicular exocytosis. Synaptosomes were resuspended in incubation medium at a final protein concentration of 0.5 mg/ml and incubated for 3 min before the addition of 1 mM $CaCl_2$. 4-AP (1 mM) was added after a further 10 min to effect depolarization (arrow). Ca^{2+}-independent release was assayed by omitting $CaCl_2$ and adding 300 μM EGTA 10 min prior to depolarization. Total glutamate release ($+Ca^{2+}$; A) and Ca^{2+}-independent glutamate release ($-Ca^{2+}$; B) was measured under control conditions or in the presence of 30 μM hispidulin added 10 min before the addition of 4-AP. Inst. Dose–response curve for hispidulin inhibition of 4-AP-evoked glutamate release, showing percentage inhibition compared with controls. (C) Quantitative comparison of the extent of glutamate release by 1 mM 4-AP in the absence and presence of 30 μM hispidulin and absence and presence of 10 μM DL-TBOA, or 0.1 μM bafilomycin A1. Hispidulin was added 10 min before depolarization and other drugs 10 min before this. Results are the mean ± SEM values of independent experiments carried out with individual synaptosomal preparations from the indicated number of animals. ***$P<0.001$, unpaired Student's t test. *$P<0.05$, one-way ANOVA.

Application of hispidulin (30 μM) did not affect basal Ca^{2+} levels, but caused a ~24% decrease in the 4-AP-evoked rise in $[Ca^{2+}]c$ (196.5 ± 3.1 nM; $n=5$; $P<0.001$; Fig. 2A). Similarly, KCl (15 mM) depolarization caused a rise in $[Ca^{2+}]_C$ to a plateau level of 262.5 ± 4.1 nM. This KCl-evoked rise in $[Ca^{2+}]_C$ was also reduced by 50 nM with 30 μM hispidulin (212.2 ± 3.1 nM) ($n=5$; $P<0.001$; Fig. 2B). Fig. 2C shows that 4-AP (1 mM) caused an increase in $DiSC_3(5)$ fluorescence by 10.9 ± 0.2 fluorescence units/5 min. Application of hispidulin (30 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 (10.6 ± 0.3 fluorescence units/5 min) ($n=5$). In addition, we confirmed the hispidulin-mediated inhibition of glutamate release using a alternative secretagogue, KCl. In Fig. 2D, control glutamate release evoked by 15 mM KCl (9.4 ± 0.2 nmol/mg/5 min) was potently inhibited by 30 μM hispidulin (4.5 ± 0.1 nmol/mg/5 min; $n=5$; $P<0.001$).

A reduction of calcium influx through $Ca_v2.2$ (N-type) and $Ca_v2.1$ (P/Q-type) channels is involved in the action of hispidulin on glutamate release

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 such as endoplasmic reticulum (ER) and mitochondria (Berridge, 1998; Millan and Sanchez-Prieto, 2002). Therefore, we next sought to establish which part of the Ca^{2+} source was involved in the hispidulin-mediated inhibition of glutamate release. Fig. 3B shows glutamate release evoked by 1 mM 4-AP was significantly decreased in the presence of 2 μM ω-conotoxin GVIA (ω-CgTX GVIA) or 500 nM ω-agatoxin IVA (ω-AgTX IVA), which selectively blocks $Ca_v2.2$ and $Ca_v2.1$ channels, respectively (Leenders et al., 2002; Turner et al., 1993; Vazquez and Sanchez-

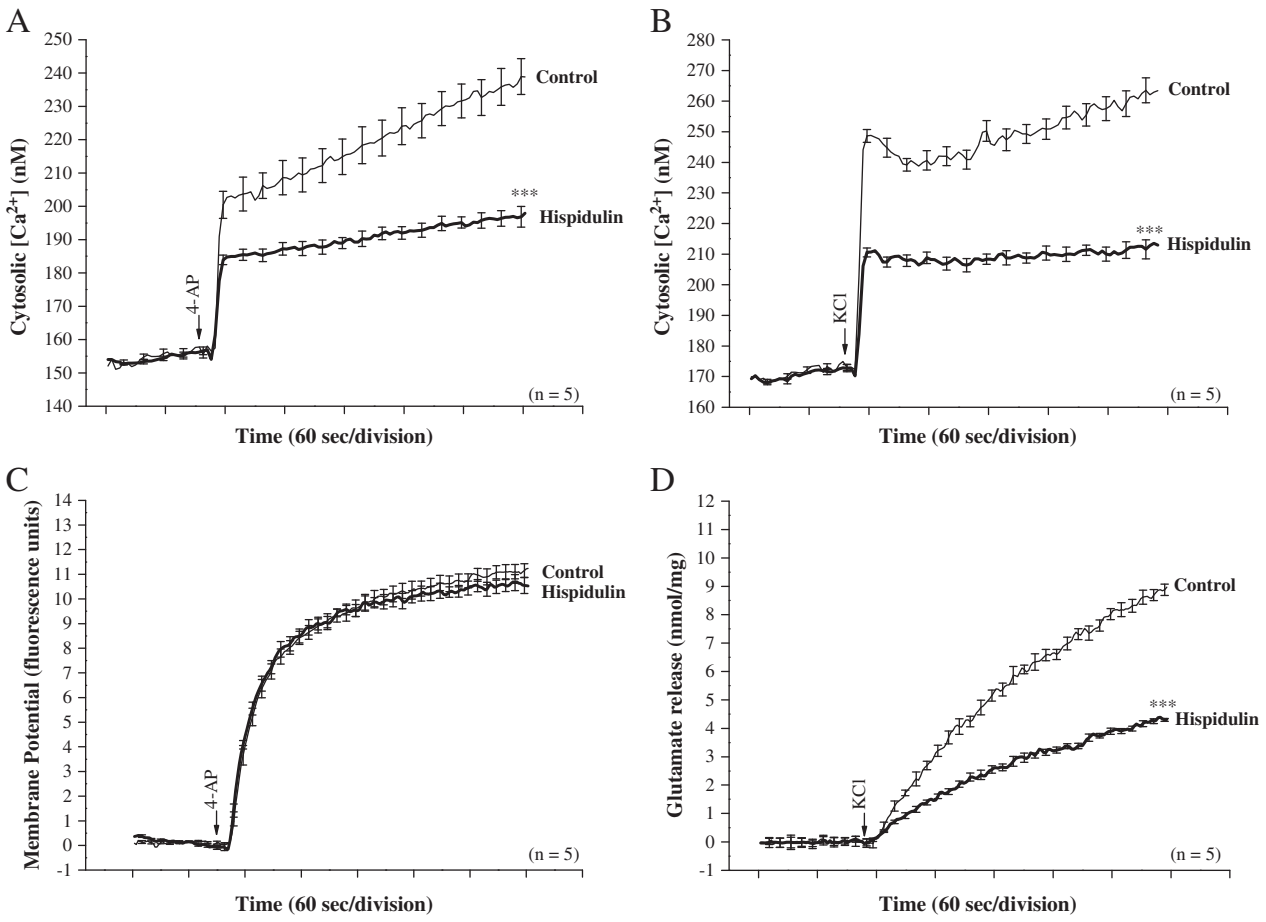


Fig. 2. Hispidulin attenuates the 4-AP- and KCl-induced increase in cytosolic Ca^{2+} levels but does not change the synaptosomal membrane potential. Cytosolic free Ca^{2+} concentration (nM) was monitored using Fura-2 in the absence (control) and in the presence of 30 μM hispidulin, added 10 min before depolarization with 1 mM 4-AP (A) or 15 mM KCl (B). (C) Synaptosomal membrane potential monitored with $DiSC_3(5)$ in the absence (control) and in the presence of 30 μM hispidulin, added 10 min before depolarization with 1 mM 4-AP. (D) Glutamate release was induced by 15 mM KCl in the absence (control) or presence of 30 μM hispidulin, added 10 min before depolarization. Results are the mean ± SEM values of independent experiments carried out with individual synaptosomal preparations from the indicated number of animals. ***$P<0.001$, unpaired Student's *t* test.

Prieto, 1997). In the presence of either ω-CgTX GVIA or ω-AgTX IVA alone, application of 30 μM hispidulin was still able to reduce 4-AP-evoked glutamate release by a further 30% and 32%, respectively ($n=5$; $P<0.05$). Thus, the individual blockade of $Ca_v2.2$ and $Ca_v2.1$ by ω-CgTX GVIA and ω-AgTX IVA did not completely prevent the action of hispidulin (Fig. 3B). To test the possibility that hispidulin mediates its effects through the combined inhibition of $Ca_v2.2$ and $Ca_v2.1$, the effect of hispidulin was tested in the combined application of ω-CgTX GVIA and ω-AgTX IVA. 2 μM ω-CgTX GVIA and 500 nM ω-AgTX IVA together reduced 4-AP-evoked glutamate release by 64% ($n=7$; $P<0.001$) (Fig. 3A). In the combined presence of Ca^{2+} channel inhibitors, application of hispidulin (30 μM) only reduced glutamate release by a further 4% ($n=7$), indicating significant reduction compared with that obtained when hispidulin was applied alone (56%; $n=5$; $P<0.05$) (Fig. 3A). Similar results were observed with 2 μM ω-conotoxin MVIIC (ω-CgTX MVIIC), a wide-spectrum blocker of $Ca_v2.2$ and $Ca_v2.1$ channels ($n=6$; Fig. 3B).

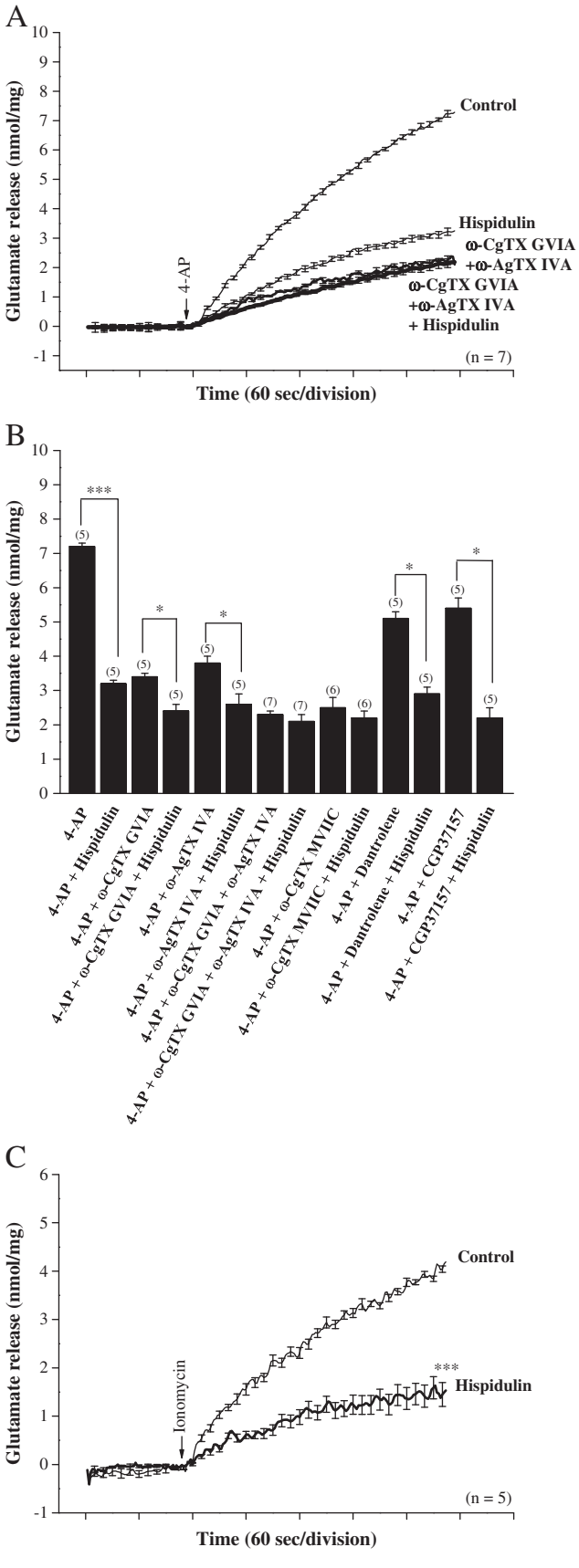
In addition, a potential role of intracellular Ca^{2+} release in hispidulin-mediated inhibition of glutamate release was tested in the presence of dantrolene, an inhibitor of intracellular Ca^{2+} release from endoplasmic reticulum, and CGP37157, a membrane-permeant blocker of mitochondrial Na^+/Ca^{2+} exchange. Fig. 3B shows 4-AP (1 mM)-evoked glutamate release was reduced by dantrolene (50 μM) ($P<0.01$), indicating that Ca^{2+} release from ER ryanodine receptors contributes significantly to the 4-AP-evoked glutamate release. In the presence of dantrolene, however, hispidulin (30 μM) still effectively inhibited 4-AP-evoked glutamate release ($n=5$; $P<0.05$; Fig. 3B). Similar to dantrolene, CGP37157 (100 μM), a membrane-permeable blocker of mitochondrial Na^+/Ca^{2+} exchange, decreased 4-AP-evoked glutamate release ($P<0.01$), but it had no effect on the hispidulin-mediated inhibition of 4-AP-evoked glutamate release ($n=5$; Fig. 3B).

Although the above-mentioned experiments indicate a correlation of the inhibitory effect of hispidulin on glutamate release with a suppression of VDCCs, the possibility remains that hispidulin could inhibit glutamate release by directly affecting the release machinery, downstream of Ca^{2+} influx. To examine this possibility, we examined the effect of hispidulin on glutamate release induced by ionomycin. The Ca^{2+} ionophore ionomycin causes a direct increase in intrasynaptosomal Ca^{2+} levels without previous depolarization and VDCC activation. Thus, ionomycin-induced release reflects the modulation of release machinery downstream of Ca^{2+} entry (Sihra et al., 1992). In Fig. 3C, the release triggered by ionomycin (5 μM, 3.9 ± 0.1 nmol/mg/5 min) was inhibited by hispidulin (30 μM, 1.6 ± 0.2 nmol/mg/5 min; $n=5$; $P<0.001$).

Hispidulin-mediated inhibition of glutamate release is prevented by the MEK inhibitors

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 (Grilli et al., 2004; Hung et al., 2011; Millan et al., 2003; Pereira et al., 2002; Wang and Sihra, 2003), we sought to establish which type of protein

kinase signaling pathway participated in the inhibition of 4-AP-evoked glutamate release mediated by hispidulin. First, we used PD98059 to specifically prevent the activation of mitogen-activated/extracellular signal-regulated kinase kinase (MEK) (Alessi et al., 1995), the protein kinase upstream of MAPK. Fig. 4A shows that control glutamate release evoked by 1 mM 4-AP was reduced by 50 μM PD98059 ($P<0.001$), reflecting an inhibition of the reported basal MAPK activity present in nerve terminals (Pereira et al., 2002). Although hispidulin (30 μM) reduced the 4-AP-evoked glutamate release ($P<0.001$), this effect was abolished by the pretreatment with PD98059, with the release measured in the presence of PD98059 and hispidulin being similar to that obtained in the presence of PD98059 alone ($n=6$; $P>0.05$; Figs. 4A and B). Similar results were observed with the another MEK inhibitor PD198306 ($n=5$; Fig. 4B). In contrast, the PKC inhibitor GF109203X (10 μM) and the PKA inhibitor H89 (100 μM) reduced control 4-AP (1 mM)-evoked glutamate release ($P<0.001$), but they had no effect on the hispidulin-mediated inhibition of 4-AP-evoked glutamate release ($n=5$; Fig. 4B).



Hispidulin decreases the phosphorylation of ERK1/2 and synapsin I

Fig. 5A shows that depolarization of synaptosomes with 4-AP (1 mM) markedly increased the phosphorylation of extracellular signal-regulated kinase 1 and 2 (ERK1/2) (121.9 ± 4.5%; $P<0.01$). When synaptosomes were pretreated with hispidulin (30 μM) or PD98059 (50 μM) alone for 10 min before depolarization with 4-AP, 4-AP (1 mM)-enhanced ERK1/2 phosphorylation was decreased to 65.8 ± 6.9% and 108.4 ± 5.2%, respectively ($n=5$; $P<0.05$; Fig. 5). Furthermore, the action of hispidulin on the 4-AP-induced ERK1/2 phosphorylation was completely abolished in the presence of PD98059 (112.5 ± 4.7%; $n=5$; Fig. 5A). Similar results were obtained from analysis of phosphorylation of synaptic vesicle-associated protein synapsin I, which is the major presynaptic substrate for ERK (Jovanovic et al., 2000) ($n=4$; Fig. 5).

Hispidulin-mediated inhibition of evoked glutamate release is attenuated in synapsin I-deficient mice

To further authenticate the role of synapsin I in the observed inhibition of glutamate release by hispidulin, we compared synaptosomes derived from wild-type and synapsin I-deficient mice. We tested the effect of deleting the synapsin I gene on the evoked release of glutamate, in the absence or presence of hispidulin (Fig. 6). In the absence of hispidulin, both 4-AP- and ionomycin-evoked glutamate release were attenuated in synapsin I-deficient mice (Fig. 6). In wild-type mice, hispidulin caused a inhibition of 4-AP-evoked glutamate release of 3.4 ± 0.3 nmol/mg/5 min (55 ± 6%%; $n=6$; Fig. 6A). In contrast, hispidulin caused no significant inhibition of 4-AP-evoked glutamate release in synapsin I-deficient mice (Fig. 6B). In the case of ionomycin-evoked glutamate release, hispidulin caused a inhibition in wild-type mice of 1.9 ± 0.1 nmol/mg/5 min (53 ± 3%%; $n=6$; Fig. 6C). This inhibition was reduced to 0.3 ± 0.1 nmol/mg/5 min in synapsin I-deficient mice (Fig. 6D).

Fig. 3. Hispidulin-mediated inhibition of 4-AP-evoked glutamate release is abolished by $Ca_v2.2$ and $Ca_v2.1$ channels blockade. (A) Glutamate release was evoked by 1 mM 4-AP in the absence (control) or presence of 30 μM hispidulin, 2 μM ω-CgTX GVIA + 500 nM ω-AgaTX IVA, or 2 μM ω-CgTX GVIA + 500 nM ω-AgaTX IVA + 30 μM hispidulin. (B) Quantitative comparison of the extent of glutamate release by 1 mM 4-AP in the absence and presence of 30 μM hispidulin, and absence and presence of 2 μM ω-CgTX GVIA, 500 nM ω-AgaTX IVA, 2 μM ω-CgTX GVIA + 500 nM ω-AgaTX IVA, 2 μM ω-CgTX MVIIC, 100 μM dantrolene, or 100 μM CGP37157. (**c**) Glutamate release was induced by 5 μM ionomycin, in the absence (control) and in the presence of 30 μM hispidulin. Hispidulin was added 10 min before depolarization, whereas the other drugs were added 30 min before depolarization. Results are the mean ± SEM values of independent experiments carried out with individual synaptosomal preparations from the indicated number of animals. ***$P<0.001$, *$P<0.05$, one-way ANOVA.

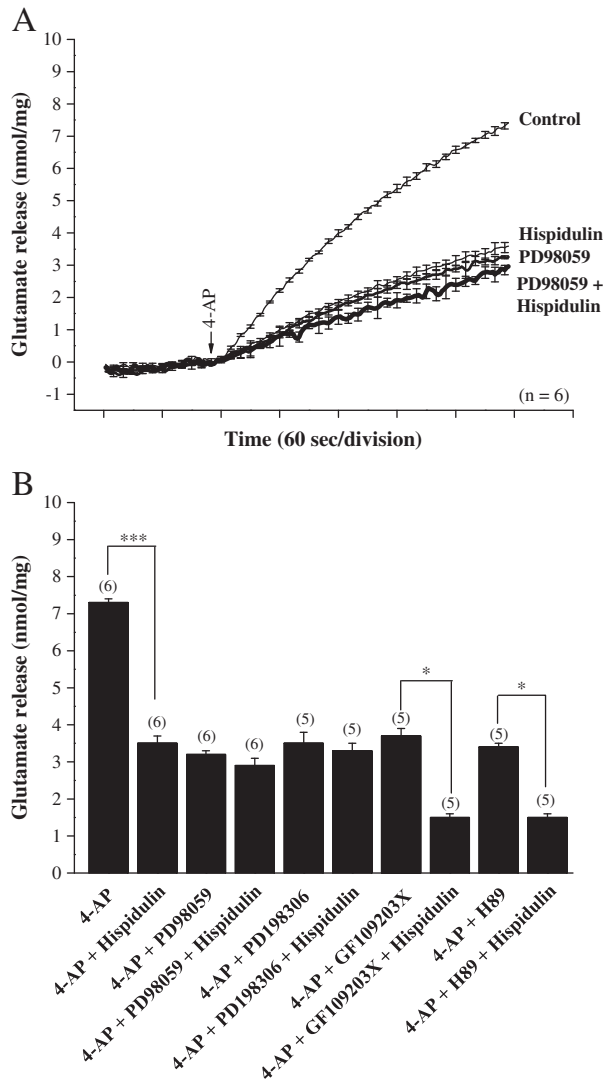


Fig. 4. MEK inhibitors occlude hispidulin-mediated inhibition of 4-AP-evoked glutamate release. (A) Glutamate release was evoked by 1 mM 4-AP in the absence (control) or presence of 30 μM hispidulin, 50 μM PD98059, or 50 μM PD98059 + 30 μM hispidulin. (B) Quantitative comparison of the extent of glutamate release by 1 mM 4-AP in the absence and presence of 30 μM hispidulin, and absence and presence of 50 μM PD98059, 50 μM PD198306, 1 μM GF109203X, or 100 μM H89. PD98059, PD198306, GF109203X, or H89 was added 40 min before depolarization, whereas hispidulin was added 10 min before depolarization. Results are the mean ± SEM values of independent experiments carried out with individual synaptosomal preparations from the indicated number of animals. ***$P<0.001$, *$P<0.05$, one-way ANOVA.

Hispidulin-inhibited glutamate release is unlikely to involve interaction with GABA$_A$ receptors

Hispidulin has been shown to modulate the function of $GABA_A$ receptor (Kavvadias et al., 2004). $GABA_A$ receptor activation inhibits voltage-dependent Ca^{2+} influx and glutamate release from nerve terminals (Long et al., 2009). For these reasons, we test whether $GABA_A$ receptor was involved in the observed hispidulin-mediated inhibition of glutamate release. Initially, the expression of endogenous $GABA_A$ receptor was checked in our system. In Fig. 7A, immunoreactivity of $GABA_A$ receptor α1 and β3 subunits was observed ($n=3$). Next, we tested the effect of isoguvacine (a agonist of $GABA_A$ receptor) on the hispidulin-mediated inhibition of glutamate release. Fig. 7B shows that control 4-AP-evoked glutamate release (7.3 ± 0.1 nmol/mg/5 min) was reduced by hispidulin (30 μM) or isoguvacine (300 μM) to 3.6 ± 0.4 nmol/mg/5 min and 5.5 ± 0.1 nmol/mg/5 min, respectively. In the presence of isoguvacine, hispidulin resulted in a 49 ± 6% inhibition of the 4-AP-evoked release, which was similar with the inhibition produced by hispidulin alone (51 ± 5%; $n=5$; Fig. 7B). Furthermore, the $GABA_A$ receptor antagonist SR95531 (100 μM) had no effect on either control 4-AP-evoked glutamate release or inhibition thereof by hispidulin ($n=5$; Fig. 7B).

Discussion

Epilepsy is a common neurological disorder affecting people worldwide, and obtaining antiepileptic-like medicine is highly crucial in treating this disease. Hispidulin is a naturally occurring flavonoid and has been reported to have an anticonvulsant profile (Kavvadias et al., 2004). However, the exact mechanism of its antiepileptic activity remains to be explored. To help address this, this study used isolated cerebrocortical nerve terminals to examine the effect of hispidulin on glutamate release because excessive glutamate release has been proposed to be involved in the pathophysiology of epilepsy (Kaura et al., 1995; Meldrum, 1994). In this work, for the first time, we obtained clear evidence that hispidulin inhibits the depolarization-evoked release of glutamate. Hispidulin did not affect the basal release of glutamate from the nerve terminals, suggesting that hispidulin might work

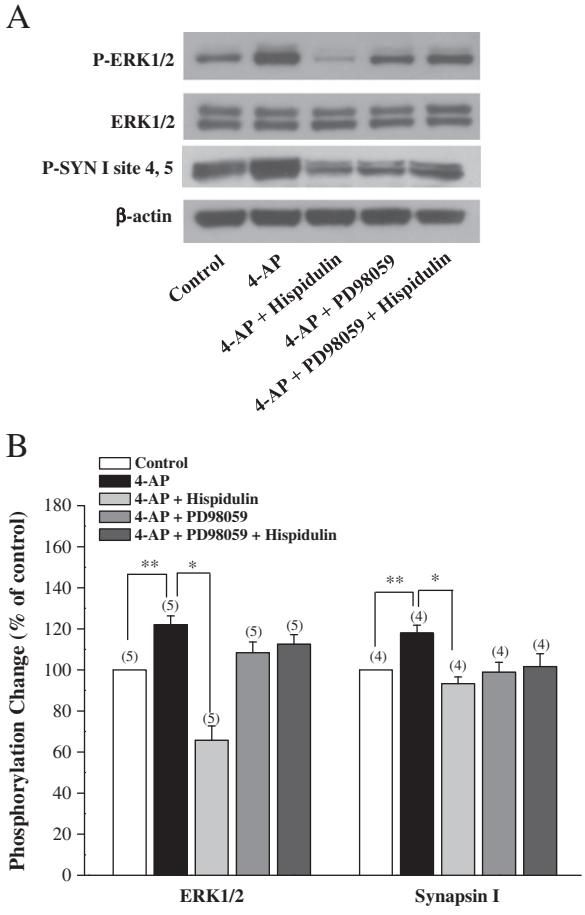


Fig. 5. Hispidulin decreases 4-AP-induced phosphorylation of ERK1/2 and synapsin I, and this effect is prevented by PD98059. (A) Phosphorylation of ERK1/2 and synapsin I at MAPK-specific sites 4, 5 (P-SYN I site 4, 5) was detected in synaptosomal lysates by western blotting using phosphorylation state-specific 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 + 30 μM hispidulin, 1 mM 4-AP + 50 μM PD98059, or 1 mM 4-AP + 50 μM PD98059 + 30 μM hispidulin. PD98059 was added 40 min before 4-AP addition, whereas hispidulin was added 10 min before depolarization. (B) 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 values of independent experiments carried out with individual synaptosomal preparations from the indicated number of animals. ***$P<0.001$, *$P<0.05$, one-way ANOVA.

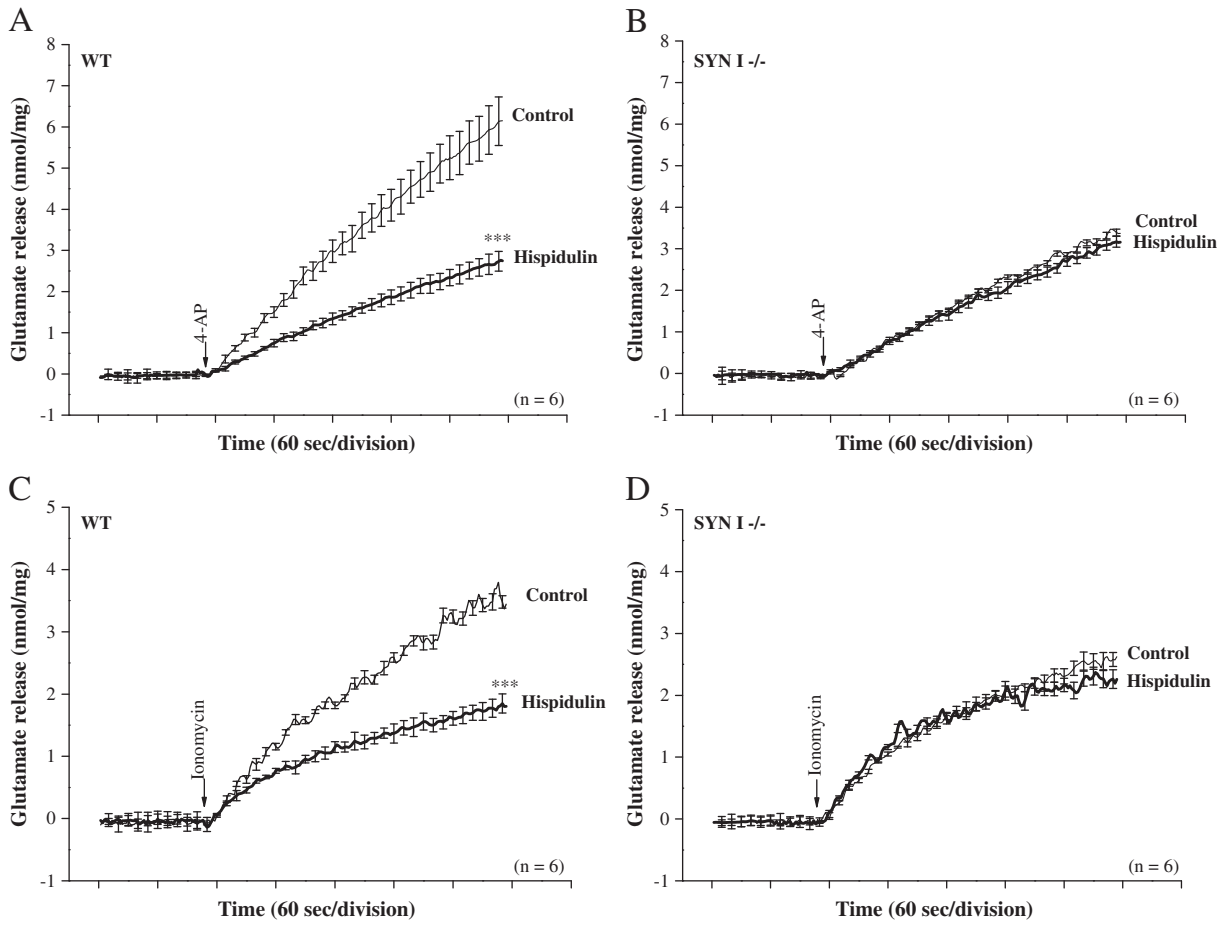


Fig. 6. Hispidulin-mediated inhibition of evoked glutamate release is attenuated in synaptosomes from synapsin I-deficient mice. Glutamate release evoked by 1 mM 4-AP (A, B) or by 5 μM ionomycin (C, D). Synaptosomes from wild-type (WT) and synapsin I-deficient (SYN I−/−) mice were preincubated for 10 min under standard conditions in the absence (control) or presence of hispidulin (30 μM) followed by the addition of a stimulator of secretion. Results are the mean ± SEM values of independent experiments carried out with individual synaptosomal preparations from the indicated number of animals. ***$P<0.001$, unpaired Student's t test.

by reducing the release of glutamate triggered by neuronal activation. Several possible mechanisms for this effect are discussed as follows.

Neurotransmitter release is a complex phenomenon and can be modulated at several putative sites in the nerve terminal, including Na^+ channels, K^+ channels, Ca^{2+} channels, and the release process itself (Nicholls, 1998; Wu and Saggau, 1997). Activation of Na^+ channels or inhibition of K^+ channels is known to regulate membrane excitability and consequently the amount of transmitter release (Li et al., 1993; Pongs et al., 1999; Rehm and Tempel, 1991). Therefore, when addressing the mechanism responsible for the hispidulin-mediated inhibition of glutamate release, this study considers 2 scenarios that might be involved: (1) alteration of the synaptosomal plasma membrane potential and downstream modulation of Ca^{2+} influx into the terminal, and (2) direct regulation of VDCCs affecting Ca^{2+} entry. The first possibility is unlikely, based on several observations. First, in addition to 4-AP-evoked glutamate release, the inhibitory effect of hispidulin on glutamate release appeared when KCl was used as a depolarizing agent. Because 4-AP-evoked glutamate release involves the action of Na^+ and Ca^{2+} channels, whereas KCl-evoked glutamate release only involves Ca^{2+} channels (Barrie et al., 1991; Nicholls, 1998), it is unlikely that Na^+ channels modulate glutamate release by hispidulin. Second, no significant effect of hispidulin on synaptosomal plasma membrane potential appeared either in the resting condition or on depolarization with 4-AP (indicating a lack of effect on K^+ conductance). Third, hispidulin did not affect the 4-AP-evoked Ca^{2+}-independent glutamate release, which depends only on the membrane potential (Nicholls et al., 1987). This indicates that hispidulin does not affect glutamate release by reversing the direction of the plasma membrane glutamate transporter. The vesicular transporter inhibitor bafilomycin A1 (but not the glutamate transporter inhibitor DL-TBOA) completely abolished the inhibitory effect of hispidulin on 4-AP-evoked glutamate release, supporting this suggestion. These results clearly suggest that the hispidulin-mediated inhibition of 4-AP-evoked glutamate release is mediated by a decrease in the Ca^{2+}-dependent exocytotic component of glutamate release. Moreover, this phenomenon is not because of a reduction in synaptosomal excitability caused by the modulation of Na^+ or K^+ ion channels.

Using the Ca^{2+} indicator Fura-2, this study demonstrates that hispidulin reduces the depolarization-evoked increase in $[Ca^{2+}]_C$, indicating the inhibitory effect of hispidulin on glutamate release by decreasing intracellular Ca^{2+} levels. In synaptic terminals, extracellular Ca^{2+} influx through VDCCs and intracellular store Ca^{2+} release mediates a depolarization-induced increase in $[Ca^{2+}]_C$, coupled with glutamate release (Berridge, 1998; Millan and Sanchez-Prieto, 2002). In the present study, the inhibition of glutamate release by hispidulin was abolished only under conditions in which all release-coupled $Ca_v2.2$ and $Ca_v2.1$ channels had been blocked, suggesting the involvement of $Ca_v2.2$ and $Ca_v2.1$ channels. Conversely, the reduced release of stored Ca^{2+} from the ER ryanodine receptors and mitochondria during the hispidulin-mediated inhibition of glutamate release can be excluded. This is because the inhibitory effect of hispidulin on 4-AP-evoked glutamate release was insensitive to both the ER ryanodine receptor inhibitor dantrolene and the mitochondrial

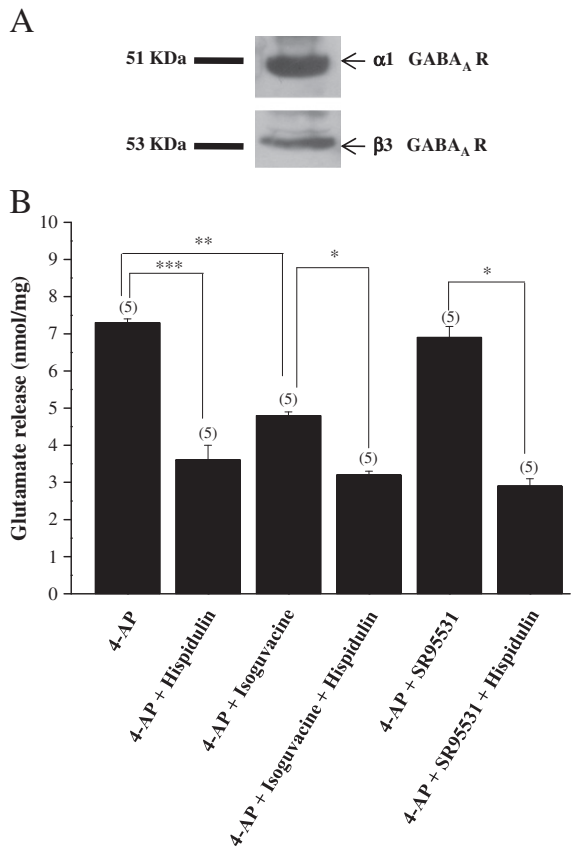


Fig. 7. Nerve terminal $GABA_A$ receptor activity is not involved in the Hispidulin-mediated inhibition of evoked glutamate release. (A) Immunodetection of $GABA_A$ receptor subunits in purified rat cerebrocortical synaptosomes. (B) Glutamate release was evoked by 1 mM 4-AP in the absence (control) or in the presence of 30 μM hispidulin, 300 μM isoguvacine (a agonist of $GABA_A$ receptor), 300 μM isoguvacine + 30 μM hispidulin, 100 μM SR95531 (a antagonist of $GABA_A$ receptor), or 100 μM SR95531 + 30 μM hispidulin. Hispidulin, isoguvacine or SR95531 was added 10 min before depolarization. Results are the mean ± SEM values of independent experiments carried out with individual synaptosomal preparations from the indicated number of animals. ***$P<0.001$, **$P<0.01$, *$P<0.05$, one-way ANOVA.

Na^+/Ca^{2+} exchange inhibitor CGP37157. Although there is no direct evidence that hispidulin acts on presynaptic Ca^{2+} channels, these data implicate that a reduction in Ca^{2+} influx mediated by $Ca_v2.2$ and $Ca_v2.1$ channels is associated with the inhibition of glutamate release by hispidulin. However, the blockade of $Ca_v2.2$ and $Ca_v2.1$ channel activity did not completely eliminate the inhibitory effect of hispidulin on the 4-AP-evoked glutamate release (approximately 4 % of the activity remained), raising the possibility that the $Ca_v2.2$ and $Ca_v2.1$-resistant Ca^{2+} channel types are involved in the action of hispidulin. In addition, the present study directly determined whether hispidulin might affect glutamate release through a mechanism independent of the Ca^{2+} entry using a Ca^{2+} ionophore to stimulate release. Hispidulin inhibited ionomycin-induced glutamate release without any modulatory effects on ion channels, suggesting, therefore, that at least one locus of hispidulin action lies downstream of the Ca^{2+} entry. Collectively, these data suggest that hispidulin contributes to the decrease in glutamate release, not only by attenuating voltage-dependent Ca^{2+} entry, but also by directly interfering with the exocytotic machinery release itself.

Some protein kinases, such as MAPK, PKC, and PKA, have been shown to be involved in the regulation of presynaptic VDCC function and glutamate release (Grilli et al., 2004; Millan et al., 2003; Pereira et al., 2002; Wang and Sihra, 2003). This study suggests a role for the MAPK/ERK pathway in the hispidulin-mediated inhibition of glutamate release based on the following results: (1) the MEK (MAP kinase kinase) inhibitors abolished the inhibitory effects of hispidulin on the 4-AP-evoked glutamate release; (2) neither the PKC inhibitor GF109203X nor the PKA inhibitor H89 altered release inhibition; and (3) hispidulin decreased 4-AP-induced phosphorylation of ERK1/2 and synapsin I at MAPK-specific sites 4 and 5; this phenomenon was also prevented by the MEK inhibitor. MAPK/ERK is a vital intracellular signaling system that is present at the presynaptic level and plays a crucial role in neurotransmitter exocytosis (Chi et al., 2003; Grewal et al., 1999; Pereira et al., 2002). 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 the dissociation of synaptic vesicles from the actin cytoskeleton. This in turn makes more vesicles available at the active zone for neurotransmitter exocytosis, resulting in an increased glutamate release (Jovanovic et al., 1996, 2000; Schenk et al., 2005; Yamagata et al., 2002). 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 hispidulin-mediated inhibition of glutamate release. Such a pathway is further supported by the observation that the inhibition of glutamate release by hispidulin was strongly attenuated in mice lacking synapsin I. However, apart from synapsin I, the possible involvement of other synaptic proteins should be considered. Synapsin II and synapsin III, for example, are reported to be phosphorylated by MAPK (Hilfiker, 2006; Jovanovic et al., 2000).

Hispidulin possesses high affinities to $GABA_A$ receptor and enhances the GABA-induced current (Kavvadias et al., 2003, 2004). $GABA_A$ receptors are expressed in the brain and localize both pre- and postsynaptically (Kullmann et al., 2005; Sieghart, 2006). At the presynaptic level, $GABA_A$ receptors have been shown to inhibit Ca^{2+} influx and glutamate release (Long et al., 2009). Therefore, the question arises as to whether $GABA_A$ receptors play any role in the hispidulin-mediated inhibition of glutamate release. In the present study, western blotting data confirmed the existence of $GABA_A$ receptors in cerebrocortical nerve terminals. Furthermore, the $GABA_A$ receptor agonist isogvacine reduced 4-AP-evoked glutamate release. These findings are consistent with previous study (Long et al., 2009). However, the inhibitory effect of hispidulin on 4-AP-evoked glutamate release does not seem to involve $GABA_A$ receptors because it was obtained in the presence of the $GABA_A$ receptor agonist and antagonist.

Generally, currently available antiepileptic drugs exert their effect by antagonizing Na^+ or Ca^{2+} channels, facilitating GABAergic inhibition, or reducing glutamatergic neurotransmission (Mula, 2009; Rogawski and Loscher, 2004). Regarding the exact mechanism responsible for the antiepileptic effect of hispidulin remains to be elucidated. Previous research had already demonstrated that hispidulin could enhance the GABA-induced chloride currents at recombinant $GABA_A$ receptors expressed by *Xenopus laevis* oocytes (Kavvadias et al., 2004). The chloride channel of the $GABA_A$ receptor is responsible for the rapid hyperpolarization, and activation of $GABA_A$ receptor induces opening of chloride channel and subsequent hyperpolarization, which can result in a decrease of neuronal excitability. The ability of hispidulin to increase $GABA_A$ receptor activation may explain, in part, its antiepileptic mechanism. In addition to the dysfunction of the GABAergic systems, however, a current hypothesis proposes that elevated extracellular glutamate causes excessive glutamatergic neurotransmission that is damaging to neuronal function and contributes to epilepsy (Kaura et al., 1995). As a result, reducing glutamate release may have critical consequences and may be a potential mechanism of antiepileptic drugs. Accordingly, several clinically used antiepileptic drugs, such as carbamazepine, phenytoin, lamotrigine and gabapentin, have been demonstrated to reduce glutamate release in human and rat brain tissues, including the cerebral cortex (Kammerer et al., 2011; Quintero et al., 2011; Sitges et al., 2007a, 2007b). Such a mechanism may lead to decreased glutamatergic neurotransmission, an effect

similar to that induced by glutamate receptor antagonists, which are known to produce antiepileptic-like actions (Chapman et al., 2000; Clifford et al., 1990; Loscher, 1998). Consequently, it may be reasonable to assume that reduced glutamate release from nerve terminals contributes to the antiepileptic activity of hispidulin.

Kavvadias et al. (2004) revealed that hispidulin can cross the blood–brain barrier and enter the brain by rapid kinetics. Conducting electrophysiological studies at recombinant $GABA_A$ receptors expressed in *Xenopus laevis* oocytes, they demonstrated that hispidulin at 10 μM enhances the GABA-activated current. Furthermore, administering hispidulin to animals at a dose of 10 mg/kg reduces the number of animals suffering from seizures. In this study, hispidulin-mediated inhibition of glutamate release is dose dependent, maximal at 100 μM, with a IC_{50} of 22 μM. Although the dose of hispidulin (30 μM) used in this study to produce the effect was higher than the concentration used in previous studies, the action of hispidulin has specific. The observation supporting this statement revealed the following: (1) the effect of hispidulin on the evoked glutamate release was prevented by chelating the extracellular Ca^{2+} ions, and by the vesicular transporter inhibitor, but was insensitive to the glutamate transporter inhibitor; (2) hispidulin decreased the depolarization-induced increase in $[Ca^{2+}]_C$, whereas it did not alter 4-AP-mediated depolarization; (3) hispidulin-mediated inhibition of glutamate release was abolished by the N-, P- and Q-type Ca^{2+} channel blocker, but not by the ryanodine receptor blocker, or the mitochondrial Na^+/Ca^{2+} exchanger blocker; (4) MEK inhibitors blocked the effect of hispidulin, but PKA or PKC inhibitor did not have such an effect; (5) the effect of hispidulin was significantly attenuated in mice without synapsin I; and (6) the $GABA_A$ receptor agonist and antagonist failed to influence the effect of hispidulin.

In conclusion, the results of this study demonstrate that hispidulin inhibits glutamate release from rat cerebrocortical synaptosomes by suppressing presynaptic $Ca_v2.2$ and $Ca_v2.1$ channels, as well as ERK activity. This effect of hispidulin action might account for some of its antiepileptic activity. The relevance of our finding to in vivo clinical situations remains to be determined. However, this finding may provide further understanding of the mode of hispidulin action in the brain, thereby emphasizing the therapeutic potential of this compound in treating epilepsy.

Abbreviations

4-AP 4-aminopyridine
$[Ca^{2+}]_C$ cytosolic free Ca^{2+} concentration
$DiSC_3(5)$ 3′,3′-dipropylthiadicarbocyanine iodide
DL-TBOA DL-threo-beta-benzyl-oxy aspartate
Fura-2-AM fura-2-acetoxymethyl ester
GDH glutamate dehydrogenase
HBM HEPES buffer medium
BSA bovine serum albumin
ω-CgTX GVIA ω-conotoxin GVIA
ω-AgTX IVA ω-agatoxin IVA
ω-CgTX MVIIC ω-conotoxin MVIIC
MAPK mitogen-activated protein kinase
MEK mitogen-activated/extracellular signal-regulated kinase kinase
ERK1/2 extracellular signal-regulated kinase 1 and 2
PKC protein kinase C
PKA protein kinase A
SR95531 6-imino-3-(4-methoxyphenyl)-1(6H)-pyridazinebutanoic acid hydrobromide
GF109203X bisindolylmaleimide I
CGP37157 7-chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzothiazepin-2(3H)-one
PD98059 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one)
PD198306 N-(cyclopropylmethoxy)-3, 4, 5-trifluoro-2-[(4-iodo-2-methylphenyl)amino]-benzamide
H89 N-[2-(p-bromocinnamylamino)-ethyl]-5-iso-quinolinesulfonamide dihydrochloride
VDCC voltage-dependent Ca^{2+} channel
$Ca_v2.2$ and $Ca_v2.1$ channel $Ca_v2.2$ (N-type) and $Ca_v2.1$ (P/Q-type) channel
ER endoplasmic reticulum
CNS central nervous system

Conflict of interest statement

There is no conflict of interest to disclose for any of the authors.

Acknowledgments

This work was supported by grants from the Far-Eastern Memorial Hospital (FEMH-2012-D-006) and the National Science Council (NSC 100-2320-B-030-006-MY3), Taiwan.

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