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Hispidulin inhibits the release of glutamate in rat cerebrocortical nerve terminals

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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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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,