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Potential role of pyridoxal-5′-phosphate phosphatase/chronopin in epilepsy

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Abstract

Changes in actin dynamics and pyridoxal-5′-phosphate (PLP) metabolisms are closely related to the pathophysiological profiles of the epileptic hippocampus. Recently, it has been reported that PLP phosphatase/chronophin (PLPP/CIN) directly dephosphorylates actin-depolymerizing factor (ADF)/cofilin as well as PLP. In the present study, therefore, we have investigated whether PLPP/CIN is linked to the dynamics of actin filament assembly and the excitability in the rat hippocampus. In control animals, pyridoxine chloride (PNP) treatment increased PLPP/CIN immunoreactivity only in astrocytes, which did not affect electrophysiological properties. Following status epilepticus, the PLPP/CIN protein level increased in granule cells and reactive astrocytes. These changes in PLPP/CIN protein level showed an inverse correlation with phospho-ADF (pADF)/cofilin levels and F-actin content. These changes were also accompanied by alterations in the excitability ratio and paired-pulse inhibition. Transduction of PLPP/CIN by Tat-PLPP/CIN showed similar effects on pADF/cofilin levels, F-actin content and excitability ratio in normal animals. These findings suggest that PLPP/CIN-mediated actin dynamics may play an important role in the changes of morphological properties and excitability of the epileptic hippocampus.

Keywords: Epilepsy; Pyridoxal-5′-phosphate (PLP)-phosphatase/chronophin; Actin-depolymerizing factor (ADF)/cofilin; Vitamin B_6 metabolism; Actin dynamics

Introduction

Pyridoxal-5′-phosphate (PLP) plays a role as a cofactor in the synthesis of various neurotransmitters, including γ-amino-butyric acid (GABA) (Fukuda et al., 1998; Esclapez and Houser, 1999; Bahn et al., 2002). PLP synthesis is regulated by the phosphorylation of pyridoxal using pyridoxal kinase (EC 2.7.1.35) or by the oxidation of pyridoxine-5′-phosphate or pyridoxamine-5′-phosphate using pyridoxine-5′-phosphate oxidase (EC 1.4.3.5.) (Wada and Snell, 1961; Lumeng and Li, 1975; Spector and Shikuma, 1978; Cash et al., 1980; Meisler and Thanassi, 1980; Choi et al., 1987). In addition, PLP concentration was regulated by PLP phosphatase (PLPP, Jang et al., 2003). Interestingly, PLPP was also identified as chronophin (CIN), which is a phosphatase for actin-depolymerizing factor (ADF)/cofilin (Gohla et al., 2005). ADF/cofilin is the best characterized stimulus-responsive mediator of actin dynamics (Theriot, 1997; Bamburg, 1999; Bamburg et al., 1999), which is a fundamental factor in the construction and remodeling of a variety of polarized subcellular structures, cell growth, differentiation, division, membrane organization, motility and neurotransmission *in vivo* (Wiggan et al., 2005). ADF/cofilin undergoes rapid dephosphorylation in response to several extracellular stimuli (Moon and Drubin, 1995). Dephosphorylation of ADF/cofilin accelerates filament-severing and monomer-binding activities of F-actin (Arber et al., 1998; Yang et al., 1998; Maekawa et al.,

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1999; Sumi et al., 1999; Amano et al., 2001), and ADF/cofilin activity is negatively regulated by phosphorylation at a single site (Ser-3).

Epilepsy is a chronic neurological disease that is characterized by the periodic occurrence of seizures. Extensive experimental data indicate that PLP plays an important role in the pathophysiology of epilepsy, since vitamin B_6 deficiency has been established as a cause of seizure activity due to the reduction of GABA concentrations (Gospe et al., 1994; Glenn et al., 1995; Ishioka et al., 1995; Waymire et al., 1995). In addition, changes in actin dynamics are closely related to the pathophysiological profiles of the epileptic hippocampus, including reactive gliosis, aberrant axonal reorganization and synaptogenesis (Cavalheiro et al., 1991; Mello et al., 1993; Mathern et al., 1995; Wittner et al., 2001). However, much less is known whether PLPP/CIN is linked to the dynamics of actin filament assembly and the excitability in the hippocampus *in vivo*. Recently, we have reported that carrier peptides derived from the HIV-1 Tat protein effectively delivers various exogenous enzymes (ex., Cu^{2+}/Zn^{2+} superoxide dismutase) into intracellular regions, and that the biological functions of the transduced enzymes are preserved *in vitro* and *in vivo* (Jin et al., 2001; Kim et al., 2005b; Choi et al., 2006; Kim et al., 2006). This technique provides an opportunity for investigators to identify the biological function of macromolecules, such as enzymes, *in vivo*. Therefore, during the course of this study, we investigate the roles of PLPP/CIN in ADF/cofilin-mediated actin dynamics and neurotransmissions in the rat hippocampus following status epilepticus (SE) and transduction of PLPP/CIN by Tat-PLPP/CIN.

Materials and methods

Experimental animals

This study utilized the progeny of male Sprague–Dawley (SD) rats (7 weeks old) obtained from Experimental Animal Center, Hallym University, Chunchon, South Korea. The animals were provided with a commercial diet and water *ad libitum* under controlled temperature, humidity and lighting conditions (22±2 °C, 55±5% and a 12:12 light/dark cycle with lights). Procedures involving animals and their care were conducted in accord with our institutional guidelines that comply with NIH Guide for the Care and Use of Laboratory Animals.

Molecular gene cloning of Tat-PLPP/CIN

To evaluate directly correlation between PLPP/CIN expression level and F-actin contents in the normal hippocampus, we cloned Tat-PLPP/CIN. On the basic of the human brain PLPP/CIN cDNA sequence by using the following primers: the sense, 5′-CTCGAGGGATCCGGCTGCATGGCGCGCTGCGA-3′ contains an Xho I site, and the antisense, 5′-GGATCCGGATCCGGGGGTGGGCCTGTGGCTGCAG-3′. The cDNA of PLP phosphatase was amplified via PCR. The PCR product was partially digested with Xho I–BamH I and subcloned into the Xho I–BamH I sites of pET-15b vector. The reaction mixture was made up in a 50 μl siliconized reaction tube and heated at 95 °C for 5 min. The program for PCR consisted of 33 cycles of extension at 94 °C for 40 s, denaturation at 61 °C for 40 s, annealing at 72 °C for 2 min, and the final extension at 72 °C for 20 min, 37 °C for 1 min. The PCR products were purified preparative agarose gel electrophoresis. The purified products were ligated into a pGEM-T cloning vector and then transformed into a competent cell. The purified pGEM-T vector containing PLPP/CIN cDNA was partially digested with Xho I and BamH I, and then subcloned into a pET15b expression vector. The host *E. coli* JM109 (DE3) was transformed with pET-PLP-P, and then the transformants were selected on a LB plate containing ampicillin. The clones with the expected insert were selected using Xho I–BamH I restriction analysis and then analyzed by sequencing.

Functional expression and purification of Tat-PLPP/CIN

BL21 *E. coli* transformed with plasmids encoding the PLPP/CIN protein was grown overnight at 37 °C in LB broth supplemented with 100 μg/ml ampicillin. The overnight cultures were diluted fifty-fold with fresh LB media and cultured at 30 °C while shaking at 250 rpm until $O.D_{600}=0.6$. Protein expression was induced by the addition of IPTG to a final concentration of 1.0 mM for 12 h at 30 °C. The induced cells were harvested and lysed by French Press Cell and by sonication in a binding buffer (5 mM imidazole, 500 mM NaCl, and 20 mM Tris–HCl, pH 7.9). After the removal of the cell debris by centrifugation, the clarified cell extracts were then loaded onto a Ni^{2+}-IDA column. The column was washed first with the binding buffer and then with a wash buffer (30 mM imidazole, 500 mM NaCl, and 20 mM Tris–HCl, pH 7.9). The proteins were eluted by an elute buffer (500 mM imidazole, 500 mM NaCl and 20 mM Tris–HCl, pH 7.9), followed by desalting with a PD10 column (Amersham). The purified recombinant was confirmed by immunoblot analysis. The purified PLPP/CIN fusion proteins dissolved in PBS containing 20% glycerol were then aliquoted and stored at −80 °C.

Seizure induction and compound treatment

Rats were treated with pilocarpine (380 mg/kg, i.p.) at 20 min after atropine methylbromide (5 mg/kg, i.p.). Diazepam (10 mg/kg, i.p.) was administered 2 h after onset of status epilepticus (SE) and repeated, as needed. Some animals were injected intraperitoneally with the following agents: Tat-PLPP/CIN (200 μg/kg), pyridoxine chloride (PNP, Sigma, USA, 30 mg/kg) or saline. Since PLP causes seizures in adult and young animals due to direct disruption of $GABA_A$ receptor-mediated inhibition (Kouyoumdjian and Ebadi, 1981; Gospe et al., 1994; Ishioka et al., 1995; Salazar and Tapia, 2001), we had chosen PNP as vitamin B_6 derivative.

Extracellular recording

Animals were anesthetized (urethane, 1.5 g/kg, I.P.) and placed in stereotaxic frames. Holes were drilled through the skull for introducing electrodes. The coordinates (in mm) referenced