Altered PLCβ-1 expression in the gerbil hippocampal complex following spontaneous seizure

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Although the phospholipase C (PLCβ-1) isoform is associated with spontaneous seizure and distinctively expressed in the telencephalon, the distribution of PLCβ-1 expression in the epileptic gerbil hippocampus remains controversial. Therefore, we determined whether PLCβ-1 is associated with spontaneous seizure in an animal model of genetic epilepsy. In the present study, PLCβ-1 immunoreactivity was down-regulated in seizure-sensitive (SS) gerbils more than in seizure-resistant (SR) gerbils. The expression of PLCβ-1 within calretinin (CR)-positive neurons was rarely detected within the dentate hilar region of SS gerbils. PLCβ-1 immunoreactivity in the hippocampus was significantly elevated as compared to that in pre-seizure SS gerbil 3 h post-ictal. These findings suggest that alterations in PLCβ-1 immunoreactivity in the SS gerbil hippocampus may be closely related to the epileptic state of the gerbil brain and transiently elevated PLCβ-1 protein levels following seizure episodes. Such alterations may be compensatory responses in the SS gerbil hippocampus. [BMB reports 2011; 44(9) : 566-571]

INTRODUCTION

Phosphoinositide (PI)-specific phospholipase C (PLC) hydrolyzes a minor component of cell membrane lipids to produce a pair of second messengers, inositol 1,4,5-trisphosphate (IP3) and 1,2-diacylglycerol (DAG), which activates IP3 receptors to mobilize Ca2+ from the smooth endoplasmic reticulum (sER) and stimulates protein kinase C (PKC) to phosphorylate various substrate proteins (1). These elements are also utilized for biosynthesis of endocannabinoid 2-arachidonoyl glycerol by DAG lipase. Thus, alterations in PI signaling are involved in cellular function, differentiation, and activity (2-4). In the brain, the PLCβ-1 isoform is distinctively expressed in the telencephalon, and especially in the hippocampal formation (1, 5, 6). Interestingly, in a previous study, the roles of PLCβ-1 in seizure prevention were demonstrated (7) to involve group I metabotropic glutamate receptor (mGluR)- and muscarinic acetylcholine receptor (mAChR)-dependent hippocampal oscillation (8, 9). Moreover, Kim et al. (7) suggested that recurrent attacks of generalized seizures occur in PLCβ-1 knock-out (KO) mice.

In contrast, epilepsy is a chronic condition that is characterized by the presence of spontaneous episodes of neuronal discharges. Some reports have suggested that this may, at least in part, result from mossy fiber sprouting, which is often accompanied by selective hippocampal loss of inhibitory interneurons (10-12), and is mainly discussed as the cause of hyperexcitatory epileptiform symptoms in human temporal lobe epilepsy (TLE; 13) and in experimental animal models for epilepsy (14). However, differences in the patterns of PLCβ-1 expression in the epileptic hippocampus remain to be clarified; specifically, whether altered PLCβ-1 expression relevant to seizure activity in Mongolian gerbils, as one of the animal models for genetic epilepsy. Therefore, in the present study, we provide the first comprehensive description of the immunohistochemical distribution of PLCβ-1 in the epileptic hippocampus of gerbils and the association between PLCβ-1 and different sequelae of spontaneous seizures.

RESULTS AND DISCUSSION

Regional-specific expression of PLCβ-1 immunoreactivity in the gerbil hippocampus

As shown Fig. 1A, PLCβ-1 immunoreactivity in the SR gerbil hippocampus was abundantly expressed in the principal neurons of CA1-3 and in the hilar neurons of the dentate gyrus (Fig. 1A, 2A, 3A). Additionally, the pattern of PLCβ-1 expression showed a somato-dendritic localization (Fig. 2A). PLCβ-1 immunoreactivity was detected in the stratum lacunosum-moleculare, which received the major excitatory input from
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Fig. 1. Immunoreactivity of PLCβ-1 in gerbil hippocampus (A-D). Panel A: SR gerbil hippocampus; panel B: pre-seizure; panel C: post-seizure 3 h; panel D: post-seizure 6 h. PLCβ-1 expression in SR gerbils is abundant (A), whereas the expression was reduced in pre-seizure SS gerbils (B). However, PLCβ-1 immunoreactivity in post-ictal SS gerbils was enhanced 3 h following a spontaneous seizure (C). Bar = 280 μm. Densitometry analysis of PLCβ-1 immunoreactivities in SR and SS gerbil hippocampi (E, mean ± S.E.M). Significant differences from SR gerbils, **P < 0.01.

Fig. 2. PLCβ-1 expression in the CA1 region of SR and SS gerbils (A-D). Panel A: SR gerbil CA1; panel B: pre-seizure SS gerbil; panel C: post-seizure 3 h SS gerbil; panel D: post-seizure 6 h SS gerbil. PLCβ-1 immunoreactivity in SR gerbils was mainly detected in the soma and dendrites of CA1 principal neurons (high-magnification in panel A); however, the pattern of expression in SS gerbils was decreased (high-magnification in panel B), although PLCβ-1 immunoreactivity was enhanced at 3 h in SS gerbils following spontaneous seizures (C). Rectangles in panels A and B indicate the high-magnifications of panels A and B. Bar = 50 μm (panels A-D) and 25 μm (high-magnifications of panels A and B).

the entorhinal cortex (arrows, Fig. 1A), and PLCβ-1 expression in the hilar neuron was observed in the calretinin (CR)-positive neurons (Fig. 3E).

The major finding of the present study is that PLCβ-1 immunoreactivities were abundantly observed in the pyramidal cell layer of CA1-3 and in the dentate hilar neurons, as previously described patterns of expression of PLC β subtypes in the hippocampus (1, 5, 6, 15, 16). In addition, Fukaya et al. (1) reported that the expression of PLCβ-1 is mainly observed in somato-dendritic components of principal neurons within the excitatory synapses of the hippocampus, which is similar to our results. However, PLCβ-1 immunoreactivity was detected in the CR-positive hilar neurons of the gerbil hippocampus. In agreement with this finding, a previous investigation suggested that CR-positive neurons are excitatory neurons which project to the basal part of the granule cell dendrites and are mainly labeled for PLCβ-1, whereas somatostatin-positive interneurons express PLCβ-4 (1, 14, 17). Thus, the results in the present study are in close agreement with those from previous investigations. Therefore, our findings confirm and extend the regional specific distribution of PLCβ-1 immunoreactivity in the gerbil hippocampus.

As shown Fig. 1B, PLCβ-1 immunoreactivity within the CA1-3 and the CR-positive hilar neurons was markedly down-regulated in pre-seizure SS gerbils (Fig. 1B, 2B, 3B, 3F). Indeed, the expression of PLCβ-1 in the stratum lacunosum-moleculare was reduced as compared to that in the SR gerbil hippocampus (arrows, Fig. 1B).

Because some investigators have suggested that the definite down-regulation of PLCβ-1 expression in the hippocampus is consistent with a hippocampal origin of the hyperexcitatory phenotype in PLCβ-1 transgenic mice (18) and the hyperexcitatory behaviors in this animal model, such as convulsions and seizures, are well-described phenomena in experimentally-induced epilepsy animal models (19-21), our findings reveal that decreased PLCβ-1 expression may be closely correlated with spontaneous seizure activity in the epileptic gerbil hippocampus. In addition, seizure activity in PLCβ-1 mutant mice is due to an enhancement of N-methyl-D-aspartic acid (NMDA)-evoked currents in CA1 pyramidal neurons (22), and these events cause an excessive increment of excitatory amino acid receptors, which can have a neurotoxic effect on distinct neuronal cell populations in the hippocampus (18). Therefore, the down-regulation of PLCβ-1 immunoreactivity in the pre-seizure SS gerbil may be correlated with impaired control of hyperexcitability of the epileptic hippocampus because the unique expression of PLCβ-1 in excitatory hippocampal neu-
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Fig. 3. Effects of seizure activity within PLCβ-1 expression in the dentate gyrus in SR and SS gerbils (A-D). Panel A: SR gerbil; panel B: pre-seizure SS gerbil; panel C: post-seizure 3 h SS gerbil; panel D: post-seizure 6 h SS gerbil. PLCβ-1 immunoreactivity is detected in hilar neurons in SR gerbils (A), whereas the expression was reduced in SS gerbils (B). However, 3 h following seizure onset (C), PLCβ-1 expression was enhanced in post-ictal SS gerbils. Rectangles in panels A and B indicate the high-magnification of panels A and B. Bar = 50 μm (panels A-D) and 25 μm (high-magnification in panels A and B). Double immunofluorescent staining for PLCβ-1 (red)/CR (green) in SR and SS gerbils (E and F). In SR gerbils (E), PLCβ-1 immunoreactivity was detected within the CR-positive neurons, although the immunoreactive expression of PLCβ-1 was reduced as compared with that in SR gerbils (F). The blue counter staining was dyed with DAPI. Bar = 25 μm.

Fig. 4. (A) Western blot analysis of PLCβ-1 antibody in gerbil hippocampus. Lane 1, SR gerbil; Lane 2, pre-seizure SS gerbil; Lane 3, post-ictal 3 h SS gerbil; Lane 4, post-ictal 6 h SS gerbil. (B) Optical density (O.D.) analyses of PLCβ-1 protein levels in the hippocampus (mean ± SEM). Significant differences from SR gerbils, *P < 0.05, **P < 0.01.

rons is closely related to the normal functioning of excitatory neuronal circuitry (1).

On the other hand, Kim et al. (7) demonstrated that PLCβ-1 is essential for controlling the inhibitory neuronal circuitry and a deficiency of PLCβ-1 selectively impairs mAChR signaling in the hippocampus. In addition, the seizure activity in PLCβ-1 mutant mice is closely related with the impairment of cholinergic signaling because axotomy of cholinergic input in the septohippocampal pathway triggers chronic seizures (23). In contrast, cholinergic stimulation in hippocampal slices decreases excitability by activating inhibitory interneurons (24). This is important because the diverse GABAergic interneurons are involved in the functional and anatomical specializations evolved to control distinct network operations (25, 26). Indeed, the controlling function of PLCβ-1 for excitatory neuronal circuitry mainly affects the inhibitory interneurons to generate rhythmic and synchronous network activities, which may in turn provide powerful feedback suppression to excitatory networks (3). In this regard, alterations of PLCβ-1 immunoreactivity within CR-positive hilar neurons, which are called “Mossy cells” as one of the glutamatergic neurons (27), in the pre-seizure SS gerbil seem to be involved in the imbalance of GABAergic inhibitory functions. Because these mossy cells activate hilar interneurons, reduced PLCβ-1 expression in CR cells of pre-seizure gerbils indicates reduced GABAergic inhibition as compared to SR gerbils. In addition, the functional cholinergic impairment causes a loss of GABAergic inhibition (24) and this functional cholinergic abnormality in PLCβ-1 transgenic mice concerning seizure activity (23). However, the relationship between cholinergic signaling and spontaneous seizure initiating and/or spreading in the epileptic gerbil hippocampus is controversial. Therefore, to identify the relationship between cholinergic signaling and spontaneous seizure activity in this animal model for epilepsy, it needs to be determined how these signaling and epileptic seizures are associated.

Alterations of PLCβ-1 immunoreactivity in the SS gerbil hippocampus after seizure activity
As shown Fig. 1C, PLCβ-1 immunoreactivity was enhanced in the CA1 region 3 h post-ictal (Fig. 2C). In this time window, the immunodensity of PLCβ-1 was enhanced as compared with that in the pre-seizure SS gerbil group (Fig. 1E). In addition, PLCβ-1 expression in the hilar neurons was increased (Fig. 3C), whereas immunoreactivity was reduced 6 h following seizure onset (Fig. 1D). Moreover, immunodensity and immunoblot analyses of PLCβ-1 expression showed results similar to those of immunohistochemical data (Fig. 1E, 4).

In this study, PLCβ-1 expression 3 h post-ictal was enhanced in the gerbil hippocampus. Previously, Buckmaster (28) suggested that spontaneous seizure activity in the SS gerbil induces alterations of various channels and/or enzyme expressions, and thus these alterations induced by spontaneous seizure tend to be reinforcements and/or normalizations of inhibitory signaling, which may play an important role in main-
taining refractory periods (1-2 days) of seizure activity in this animal model. Some investigators have described that an enhancement of PLCβ-1 expression is a necessary downstream signal for the occurrence of mGluR agonist-induced prolonged epileptiform activities (8). Moreover, the generation of rhythmic single cell bursts and the synchronized epileptiform discharges by activation of group I mGluRs are PLCβ-1-dependent in the hippocampal neurons (8, 29). With respect to these reports, our findings suggest that enhanced PLCβ-1 expression in the post-ictal SS gerbil hippocampus may be a compensatory response to neuronal hyperexcitability due to abnormal control of excitatory neuronal circuits. Furthermore, enhanced PLCβ-1 expression in CR cells activates GABAergic interneurons, which increase inhibitory neuronal circuits as a compensatory phenomenon in response to spontaneous seizure.

In conclusion, the alterations in PLCβ-1 expression in the SS gerbil hippocampus may involve seizure generation and spreading due to the abnormality of inhibitory signaling for controlling excitatory neuronal circuits. Additionally, the enhanced PLCβ-1 immunoreactivity in the post-ictal SS gerbil may be a compensatory response to the hyperexcitability of principal neurons and the activation of GABAergic interneurons in the epileptic gerbil hippocampus for controlling seizure activity.

MATERIALS AND METHODS

Experimental animals
These studies utilized the progeny of Mongolian gerbils (Meriones unguiculatus) obtained from the Medical College of Soonchunhyang University, Cheonan, Republic of Korea. Animals were provided with a commercial diet and water ad libitum under conditions of controlled temperature, humidity and lighting (22 ± 2°C, 55 ± 5%, and a 12:12 light/dark cycle, respectively). For immunohistochemistry, all animals were anesthetized with urethane [1.5 g/kg IP] and perfused via the ascending aorta with 200 ml of 4% paraformaldehyde in phosphate buffer. Brains were removed, post-fixed in the same fixative for 4 h and rinsed in phosphate buffer (PB) containing 30% sucrose at 4°C for 2 days. Thereafter, tissues were frozen and sectioned using a cryostat (CM3050S Cryostat Micromite) at 30 μm, and consecutive sections were collected in 6-well plates containing phosphate-buffered saline (PBS). These free-floating sections were first incubated with 10% normal goat serum for 30 minutes at room temperature, and then incubated with rabbit anti-PLCβ-1 antibody (diluted 1:100; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) in PBS containing 0.3% Triton X-100 and 2% normal goat serum overnight at room temperature. After washing 3 times for 10 minutes with PBS, the sections were sequentially incubated in goat anti-rabbit IgG (Vector, Burlingame, CA, USA) and ABC complex (Vector, Burlingame, CA, USA), diluted 1:200 in the same solution used for the primary antiserum. Between the incubations, the tissues were washed with PBS 3 times for 10 minutes each. The sections were visualized with 3,3′-diaminobenzidine (DAB) in 0.1 M Tris buffer and mounted on gelatin-coated slides.

Double immunofluorescence
Based on the results of the immunohistochemical study, we performed double immunofluorescent staining for rabbit anti-PLCβ-1 (1:50; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and mouse anti-calretinin (CR) IgG (Chemicon, Billerica, MA, USA) to confirm the cell type. Brain tissues were incubated in the mixture of primary antiserum overnight at room temperature. After washing 3 times for 10 minutes with PBS, sections were also incubated in a mixture of Cy2-conjugated donkey anti-rabbit IgG (Vector, Burlingame, CA, USA) and Cy3-conjugated donkey anti-mouse IgG (1:200; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) West Grove and Cy3-conjugated donkey anti-rabbit IgG (1:200; Jackson ImmunoResearch Laboratories Inc., West Grove, USA) for 1 hr at room temperature.

Western blot
Based on the immunohistochemical results, the expression of PLCβ-1 protein in the gerbil hippocampus was quantified and shown to be similar to expression in a previous study (31, 32). For tissue preparation, animals were decapitated and the hippocampi were removed; then each tissue was homogenized in 10 mM PB containing 0.1 mM EDTA, 1 mM 2-mercaptoethanol, and 1 mM PMSF. After centrifugation, the protein concentrations in the supernatants were determined using a Micro BCA protein assay kit (Pierce Chemical, Rockford, IL, USA) with bovine serum albumin as the standard. Aliquots containing 30 μg of total protein were boiled with an equal volume of 2× SDS sample buffer for 3 min, and then each mixture was loaded onto a 10%-polyacrylamide gel. After electrophoresis, the gels were transferred to nitrocellulose transfer membranes (Schleicher and Schuell, Keene, NH, USA). To reduce back-
Densitometry analysis of data
For densitometry analysis, we performed an optical density analysis, as previously described (34, 35). Briefly, the images of each section on the monitor were captured (15 sections per each animal). The mean gray value and the standard deviation were obtained from selected images using Adobe Photoshop (version-8.0). Each image was normalized by assessing the mean gray value. After the regions (CA1-3, subiculum and dentate granule cell layer) were outlined, 10 areas/gerbil (500 μm²/area) were selected from the hippocampus and the gray values were measured. Intensity measurements were represented as the mean number of a 256-gray scale (using NIH Image 1.59 software). Values for background staining were obtained from the corpus callosum. Optical density values were then corrected by subtracting the average values of the background noise obtained from 15 image inputs.

Statistical analysis
All data were analyzed using one-way ANOVA to determine statistical significance. Bonferroni’s test was used for post-hoc comparisons. P values < 0.01 or 0.05 were considered statistically significant (30-32, 36).

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22. Lu, W. Y., Jackson, M. F., Bai, D., Orser, B. A. and MacDonald, J. F. (2000) In CA1 pyramidal neurons of the hippocampus protein kinase C regulates calcium-dependent ground staining, the filters were incubated with 5% non-fat dry milk in PBS containing 0.1% Tween 20 for 45 min, sequentially incubated with primary antisera (1 : 200) and peroxidase-conjugated anti-rabbit IgG (Sigma, St. Louis, MO, USA), and then with an ECL kit (Amersham, Pittsburgh, PA, USA). The optical density was measured using NIH Image 1.59 software.