Cell type-specific upregulation of myristoylated alanine-rich C kinase substrate and protein kinase C- α , - β I, - β II, and - δ in microglia following kainic acid-induced seizures

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Abbreviations: CNPase, 2',3'-cyclic nucleotide 3'-phosphodiesterase; GFAP, glial fibrillary acidic protein; MARCKS, myristoylated alanine-rich C kinase substrate; PKC, protein kinase C

Abstract

Myristoylated alanine-rich C kinase substrate (MARCKS) is a widely distributed protein kinase C (PKC) substrate and has been implicated in actin cytoskeletal rearrangement in response to extracellular stimuli. Although MARCKS was extensively examined in various cell culture systems, the physiological function of MARCKS in the central nervous system has not been clearly understood. We investigated alterations of cellular distribution and phosphorylation of MARCKS in the hippocampus following kainic acid (KA)-induced seizures. KA (25 mg/kg, i.p.) was administered to eight to nine

week-old C57BL/6 mice. Behavioral seizure activity was observed for 2 h after the onset of seizures and was terminated with diazepam (8 mg/kg, i.p.). The animals were sacrificed and analyzed at various points in time after the initiation of seizure activity. Using double-labeling immunofluorescence analysis, we demonstrated that the expression and phosphorylation of MARCKS was dramatically upregulated specifically in microglial cells after KA-induced seizures, but not in other types of glial cells. PKC α , β I, β II and δ , from various PKC isoforms examined, also were markedly upregulated, specifically in microglial cells. Moreover, immunoreactivities of phosphorylated MARCKS were colocalized in the activated microglia with those of the above isoforms of PKC. Taken together, our *in vivo* data suggest that MARCKS is closely linked to microglial activation processes, which are important in pathological conditions, such as neuroinflammation and neurodegeneration.

Keywords: gene expression; kainic acid; microglia; myristoylated alanine-rich C kinase substrate; protein kinase C; seizures

Introduction

Myristoylated alanine-rich C kinase substrate (MARCKS) is a primary substrate of protein kinase C (PKC). The effector domain (ED) of MARCKS directly binds to PKC, calmodulin, filamentous actin and membrane phospholipid in a competitive manner (Blackshear, 1993). Therefore, MARCKS is proposed to be a major PKC substrate protein that integrates both PKC-mediated and calcium/calmodulin-mediated signals into the regulation of the actin cytoskeleton (Aderem, 1992; Blackshear, 1993).

MARCKS has been demonstrated to be widely distributed in several types of cells, which include fibroblasts (Allen and Aderem, 1995; Myat *et al.*, 1997), epithelial cells (Li *et al.*, 2001) and macrophages (Seykora *et al.*, 1991; Carballo *et al.*, 1999). Recently, several studies have been performed to explore the function of MARCKS in the central nervous system (CNS). Homozygous mice with targeted deletions of the Marcks gene die perinatally

and exhibit gross abnormalities in morphogenesis of the brain (Stumpo *et al.*, 1995). Heterozygous mutant adult mice exhibit deficits in hippocampal mossy fiber development, spatial learning (McNamara *et al.*, 1998), and hippocampal mossy fiber-CA3 long-term potentiation (LTP) (Hussain *et al.*, 2006).

Alterations of MARCKS expression have been previously studied *in vivo* (McNamara *et al.*, 2000a and 2000b; Kimura *et al.*, 2000; Higo *et al.*, 2002). McNamara and colleagues reported using *in situ* hybridization technique that MARCKS mRNA levels were not altered in the hippocampus after kainic acid (KA)-induced seizures (McNamara *et al.*, 2000b). Regarding the alteration of MARCKS expression in neurons, we previously demonstrated that levels of the MARCKS protein were not altered in mossy fiber terminals at hippocampal stratum lucidum following KA-induced seizures (Kim *et al.*, 2004). However, *in vivo* information of MARCKS regulation in glial cells has been limited yet.

KA induces behavioral seizures and is also known to induce selective cell death of CA1 and CA3 pyramidal neurons, the consequent glial activation and synaptic reorganization of mossy fiber pathways (McNamara *et al.*, 2000b; Lee *et al.*, 2005). KA has been reported to induce differential alteration of PKC isozymes (McNamara *et al.*, 1999; Kaasinen *et al.*, 2002). Because MARCKS is a major molecular target of PKC (Blackshear, 1993), taken together, KA-induced seizure might be a good model to investigate the physiological function of MARCKS in CNS.

Therefore, in this study, we tried to further investigate alterations of cellular distribution of MARCKS and different PKC isozymes in KA-induced seizure model with immunohistochemistry and double-labeling immunofluorescence techniques, and focused on the determination of the type of glial cells in which the expression and phosphorylation of the MARCKS protein were regulated in the hippocampus after KA-induced seizures. It is important to determine the specific isoforms of PKC responsible for MARCKS phosphorylation since there exist different isozymes of PKC in the cellular environment (Mellor and Parker, 1998; Hayashi *et al.*, 1999). Taken together, all of this information about cell type-specific regulation of MARCKS and PKC isozymes following KA-induced seizures, might contribute to an understanding of the function of MARCKS in the CNS.

Materials and Methods

Animals

Kainic acid (KA, 25 mg/kg, *i.p.*, ICN), dissolved in sterile saline, was administered to eight to nine

week-old C57BL/6 mice. Behavioral seizure activity was recorded for 2 h after the onset of seizures, according to the scale of Racine (Racine, 1972), and was terminated with diazepam (8 mg/kg, *i.p.*, Myung In Pharm.). The animals were sacrificed at 4 h, 1 day, 4 days, 2 weeks, and 1 month after seizure termination; or 30 min, 1 h, 6 h, 1 day, and 4 days after KA injection. Control animals were treated with saline alone and sacrificed at the respective corresponding points in time. All experimental procedures were conducted and followed the approved institutional guidelines for the care and use of laboratory animals. After the experiments, the animals were anesthetized with pentobarbital (50 mg/kg, *i.p.*) and perfusion-fixed transcardially with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). The brains were then removed, post-fixed in 4% PFA in PBS for 6 h, and equilibrated serially in 10%, 20% and 30% sucrose in PBS at 4°C. The brains were immersed in a tissue-freezing medium, Tissue Tek O.C.T. compound (Sakura Finetek Japan), and were quickly frozen in isopentane on dry ice. Thirty μ m-coronal sections were cut on a cryostat, thawed onto gelatinized microscope slide glasses and stored with a desiccant at -70°C until use. Some brains were processed for paraffin-embedded tissue blocks, cut to six μ m-thick sections, and kept on slide glasses at room temperature until use.

Immunohistochemistry

Brain sections were washed in PBS, blocked with 5% normal goat serum for 30 min, and treated with 0.5% Triton X-100 in PBS for 20 min. The sections were incubated at 4°C overnight with primary antibodies, which included anti-MARCKS (1:2000, prepared as described previously [Scarlett *et al.*, 2003]), anti-Ser152/156 phosphorylated MARCKS (1:500, Chemicon), anti-F4/80 (1:100, Serotec) for a microglial marker, anti-GFAP (1:1000, Chemicon) for an astrocyte marker, anti-2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) for an oligodendrocyte marker (1:1,000, Sigma), PKC α (1:500, BD Biosciences), PKC β I (1:1,000, Chemicon), PKC β II (1:1,000, Chemicon), PKC δ (1:500, Chemicon), PKC ϵ (1:1,000, Upstate) and PKC ζ (1:1,000, Santacruz). Microglia were detected also with Alexa Fluor 594-conjugated isolectin IB₄ (1:500, Molecular Probes), as well as anti-F4/80. Since adequate Ca²⁺ was required for lectin bindings, PBS that contained 0.1 g/L CaCl₂ was used during all staining procedures with isolectin IB₄. After being rinsed in PBS, sections were incubated for 1 h with Alexa Fluor 488, 594-conjugated secondary antibodies, or biotinylated secondary antibodies. For the latter, the

immunoreactivities were detected by with a Vectastatin ABC kit (Vector Laboratories) and 0.2 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (DAB) as a color substrate in the presence of 0.3% hydrogen peroxide. Counter staining was accomplished with 0.5% Cresyl Violet solution (Sigma) or Hematoxylin solution (Sigma). The specificity of the antibodies was confirmed by non-specific rabbit IgG or mouse IgG. Especially in the case of MARCKS, several sections were incubated with antiserum that was pre-adsorbed with antigen as a negative control.

Double-labeling immunofluorescent analysis

In order to identify glial cell types with the marker antibodies, brain sections were incubated at 4°C overnight at the same time in a double mixture of primary antibodies, which had been produced in different animal species. One component was either anti-MARCKS or anti-phosphorylated MARCKS (pMARCKS), and the other component was one of the glial marker antibodies. Alexa Fluor 488 or 594-conjugated secondary antibodies were used to visualize immunoreactivity. Some brain sections were incubated at the same time in the mixture of anti-MARCKS, anti-GFAP and isolectin IB₄. In this experiment, Alexa Fluor 488 signals indicated MARCKS and Alexa Fluor 594 signals indicated both glial markers. Prolong kit (Molecular Probes) was used for an anti-fading mounting solution.

Image analysis

Confocal laser scanning microscopy (Zeiss LSM 510, Carl Zeiss) or conventional fluorescent microscopy (Zeiss Axioskop, Carl Zeiss) was used to investigate the double-fluorescent immunoreactivity. Overlapped images with two fluorochromes were analyzed at consecutive focal planes, which were spaced at 0.5-1 μ m intervals by use of a Z-stack function. Some immunofluorescent images were analyzed by a digital imaging system, which included a charge-coupled device (CCD) camera (AxioCam HRm, Carl Zeiss) and AxioVision 3.0 software (Carl Zeiss). Non-fluorescent images were obtained by a digital imaging system, which included a CCD camera (DC350F, Leika) and Image-Pro Plus software (Media Cybernetics).

Results

Basal expression profiles of MARCKS in the hippocampus

Regional and cellular distribution of the MARCKS protein was examined in 6 μ m-thick paraffin-embedded brain sections or 30 μ m-thick frozen brain sections. Some glia-like cells were immunoreactive for the MARCKS protein in the control hippocampus, which was not subjected to seizure. Regarding neuronal expression of MARCKS, the MARCKS protein was barely detectable in neuronal cell body layers in the hippocampus (Figure 1A), except punc-

Figure 1. Alteration of MARCKS expression in a subpopulation of activated glial cells after KA-induced seizures. KA (25 mg/kg, i.p.) was administered to eight to nine week-old C57BL/6 mice. Behavioral seizure activity was observed for 2 h after the onset of seizures and was terminated with diazepam (8 mg/kg, i.p.). The animals were sacrificed and analyzed at various points in time after the initiation of seizure activity. Regional and cellular alterations of MARCKS expression were examined in 6 μ m-thick paraffin-embedded hippocampal sections following KA-induced seizures. The representative images were obtained from control hippocampus which was not subjected to seizure (A) and the hippocampus section processed 4 days after seizures (B). The immunoreactivities were detected by with a Vectastatin ABC kit and 3,3'-diaminobenzidine tetrahydrochloride (DAB). Neuronal cells were counterstained with 0.5% Cresyl Violet solution. Scale bar: 50 μ m.

Figure 2. Double-labeling immunofluorescent analysis for determination of the cell type of glia showing upregulation of MARCKS expression after KA-induced seizures. (A) The MARCKS-IR non-neuronal cells were not double-labeled in the control hippocampus with a microglial marker [isolelectin IB₄] (A1), an astrocyte marker [anti-GFAP] (A2), and an oligodendrocyte marker [anti-CNPase] (A3). Alexa Fluor 488 (green) signals indicated MARCKS; and Alexa Fluor 594 (red) signals indicated three glial markers. There were few thin processes of resting glial cells stained in the control brain sections attached on slide glasses. (B1). Brain sections at 4 days after seizure-time point were incubated at the same time in the mixture of anti-MARCKS, anti-GFAP and isolelectin IB₄. Alexa Fluor 488 signals indicated MARCKS; and Alexa Fluor 594 signals indicated both glial markers. From Alexa Fluor 594-labeled cells, morphologically astrocyte-like cells never demonstrated any co-localization with MARCKS, as indicated by the arrowheads. However, most of the morphologically microglia-like and Alexa Fluor 594-labeled cells were double-immunostained with MARCKS, as indicated by the white thick arrows. Their morphology often resembled long worms or bushes. A few MARCKS-IR cells were not double-immunostained with isolelectin IB₄, and a few isolelectin IB₄-positive microglial cells were not double-immunostained with MARCKS antibody, as indicated by the black thick arrows. (B2-3) No co-localization signals were observed between Alexa Fluor 488-labeled MARCKS and Alexa Fluor 594-labeled GFAP (B2), and between Alexa Fluor 488-labeled MARCKS and Alexa Fluor 594-labeled CNPase (B3). Confocal images were obtained from the CA1 region in the hippocampus. Scale bar: 50 μ m.

ta expression in stratum lucidum (Kim *et al.*, 2004). The MARCKS-immunoreactive (IR) non-neuronal cells in the control hippocampus, were not double-labeled with any glial markers, including a microglial marker [isolelectin IB₄] (Figure 2A1), an astrocyte marker [anti-GFAP] (Figure 2A2), and an oligodendrocyte marker [anti-CNPase] (Figure 2A3) in immunofluorescent analysis. These cells were not located in any neuronal layers in the hippocampus. We observed in Cresyl Violet staining that these cells morphologically resembled glial cells, in which the cytoplasm was relatively smaller and darker than that of neurons. They sometimes demonstrated a pattern of enwrapping neuronal cell bodies like glial cells, consistent with the previous reports on the expression profiles of MARCKS (Ouimet *et al.*, 1999). They were not double-labeled with the interneuron marker, anti-glutamate decarboxylase

(GAD) 67 (data not shown).

Microglia-specific upregulation of MARCKS expression after KA-induced seizures

In the control hippocampus, there were thin processes of resting glial cells that were stained in brain sections attached to slide glasses. KA-induced status epilepticus activity strongly induced typical glial cell activation. Glial cells were morphologically transformed to the activated forms with thick glial processes. The number of glial cells was markedly increased following seizure activity (Figure 1B). We observed that glial activation was demonstrated rarely 4 h after seizures, and became moderate 1 day after seizures. The glial activation was accelerated to a maximal state 4 days after seizures and then returned to a basal level 2 weeks after

seizures. The glial cells, which dramatically appeared and demonstrated strong MARCKS-immunoreactivity following seizure activity, were morphologically similar to the activated microglial cells. They often exhibited bush-like or long worm-like appearances (Figure 1B).

Astrocytes, microglia and oligodendrocytes exert different functions in CNS. Therefore, it is important to determine the cell types of glia in which MARCKS expression was strongly induced following seizure activity. Because it is somewhat difficult to distinguish astrocytes and microglia based on morphological criteria alone, we utilized double-labeling immunofluorescence analysis with confocal laser scanning microscopy to address this issue. Double-labeling data demonstrated that strong MARCKS expression was found in most of the activated microglial cells that demonstrated extremely severe morphological transformation 4 days after seizure (Figure 2B1), while MARCKS immunoreactivity was barely detectable in resting microglia in the saline-treated control animals (Figure 1A1). A few MARCKS-IR cells were not double-immunostained with isolectin IB₄, and these cells were not morphologically transformed even after seizure activity (Figure 2B1). These cells seemed originally to be MARCKS-IR non-neuronal cells, which were not stained by general glial markers (data not shown). Under the conditions employed in this study, no co-localization signals were found between MARCKS and anti-GFAP (Figure 2A2 and B2), and between MARCKS and anti-CNPase (Figure 2A3 and B3). Taken together, the results indicated that MARCKS expression was dramatically upregulated specifically

in microglia during glial activation processes following seizures, but not in other types of glial cells.

Microglia-specific phosphorylation of MARCKS after KA-induced seizures

We utilized the antibody against a phosphorylated form of MARCKS (pMARCKS) to examine the seizure-induced phosphorylation of MARCKS. Anti-pMARCKS antibody, purchased from Chemicon, was generated with a synthetic peptide that corresponded to amino acids surrounding the phosphorylated-Ser152/156. Phosphorylation of MARCKS was examined after a KA-induced seizure in the hippocampus at 30 min, 1 h, 6 h, 1 day and 4 days after treatment with KA. Phosphorylation signals were observed rarely at the control hippocampal sections under the experimental conditions. However, we observed that phosphorylation was induced in certain activated glial cells 6 h after KA treatment. It became maximal 1 day after KA treatment and decreased slightly, but was almost sustained 4 days after KA treatment. No phosphorylation signals were demonstrated in neuronal cell body layers at any of the above points in time. Double-labeling immunofluorescence analysis was employed to determine the type of glial cell in which MARCKS was phosphorylated following seizure activity. The results revealed that most of the pMARCKS-IR glial cells were overlaid with F4/80-IR microglia in the hippocampus (Figure 3A). Under the conditions employed in this study, we did not observe any co-localization signals between pMARCKS and GFAP (Figure 3B), or between pMARCKS and CNPase (Figure 3C),

Figure 3. Double-labeling immunofluorescent analysis for determination of the cell type of glia showing MARCKS phosphorylation after KA-induced seizures. Phosphorylation of MARCKS was induced strongly in certain glial cells 1 day after KA treatment. In double-labeling immunofluorescent analysis, most of the Alexa Fluor 488-labeled pMARCKS-IR cells were overlaid with Alexa Fluor 594-labeled F4/80-IR microglia (A). Co-localization signals between MARCKS and F4/80 are demonstrated in yellow. No co-localization signals were observed between pMARCKS and Alexa Fluor 594-labeled GFAP (B), and between pMARCKS and Alexa Fluor 594-labeled CNPase (C). Confocal images were obtained from the stratum radiatum in the hippocampal CA3 region. Scale bar: 50 μ m.

Figure 4. Double-labeling immunofluorescent analysis for determination of the cell type of glia showing PKC α and δ expression after KA-induced seizures. Double-labeling immunofluorescence data demonstrated that Alexa Fluor 488-labeled PKC α (A4) and δ (B4) were strongly induced, specifically, in most of the Alexa Fluor 594-labeled F4/80-IR activated microglia 1 day after KA treatment. There was no co-localization signal between PKC α , δ and Alexa Fluor 594-labeled GFAP (A2 and B2). Basal immunoreactivities of PKC isozymes were detectable to a minimal degree in resting glial cells in a saline-treated control hippocampus (A1, A3, B1 and B3). Co-localization signals between MARCKS and F4/80 are demonstrated in yellow. Confocal images were obtained from the stratum radiatum in the hippocampal CA3 region. Scale bar: 50 μ m.

(Figure 3C). The results indicated that phosphorylation of MARCKS was strongly induced specifically in microglial cells following seizure activity, whereas it was not induced either in astrocytes or in oligodendrocytes.

Microglia-specific upregulation of PKC α , β I, β II, δ expression after KA-induced seizures

Regional and cellular expressions of PKC isozymes were examined following KA-induced seizures. PKC α , β I, β II, δ , ϵ and ζ were examined. PKC α , β I, β II and δ were induced in certain glial cells 6 h after KA treatment, although the signals were weak. The expression levels became maximal 1 day after KA treatment, and were slightly decreased but almost sustained 4 days after KA treatment. Double-labeling immunofluorescence data demonstrated that PKC α , β I, β II and δ were strongly induced, specifically, in most of the F4/80-IR activated microglia from the PKC isozymes utilized (Figures 4 and 5). PKC ϵ and ζ were constitutively expressed in mossy fiber terminals and pyramidal cell layers in the hippocampus, respectively. No induction of PKC ϵ and ζ was found in the activated microglia (data not shown). There was no co-localization signal between PKC α , β I, β II, δ and anti-GFAP (Figure 4 and 5), or between PKC α , β I, β II, δ and anti-CNPase

(data not shown). Basal immunoreactivities of PKC isozymes in resting glia were detectable to a minimal degree in a saline-treated control hippocampus.

Co-localization of pMARCKS and PKC α , β I, β II, δ in the activated microglia

We demonstrated that both pMARCKS and PKC α , β I, β II, δ were induced after KA-induced seizures, specifically, in F4/80-IR activated microglial cells (Figures 3-5). Finally, we observed that pMARCKS and PKC α , β I, β II, δ were co-localized in the same activated glial cells in the hippocampus. Representative image data on PKC α , β I and δ were demonstrated in Figure 6 (Image of β II not demonstrated here). The results indicated that phosphorylation of MARCKS might be induced by PKC α , β I, β II, δ from PKC isozymes during microglial activation processes.

Discussion

In this study, we demonstrated that MARCKS expression was dramatically upregulated, specifically, in microglial cells after KA-induced seizures, where it was not in other types of glial cells (Figures 1 and 2). A strong MARCKS expression was observed in most

of the activated microglial cells that demonstrated severe morphological transformations (Figure 2B1). Phosphorylation of the MARCKS protein was also induced strongly in microglial cells, but not in other types of glial cells (Figure 3). In addition, PKC α , β I, β II and δ , from the various PKC isoforms examined,

were markedly upregulated; specifically, in microglial cells following KA-induced seizure activity (Figures 4 and 5). Moreover, immunoreactivities of pMARCKS in the activated microglial cells were co-localized with those of the above isoforms of PKC (Figure 6), which suggested MARCKS phosphorylation by

Figure 5. Double-labeling immunofluorescent analysis for determination of the cell type of glia showing PKC β_1 and β_2 expression after KA-induced seizures. Double-labeling immunofluorescence data demonstrated that Alexa Fluor 488-labeled PKC β_1 (A4) and β_2 (B4) were induced strongly and specifically in most of the Alexa Fluor 594-labeled F4/80-IR microglia 1 day after KA treatment. There was no co-localization signal between PKC β_1 , β_2 and Alexa Fluor 594-labeled GFAP (A2 and B2). Basal immunoreactivities of PKC isozymes were detectable to a minimal degree in resting glial cells in a saline-treated control hippocampus (A1, A3, B1 and B3). Co-localization signals between MARCKS and F4/80 are demonstrated in yellow. Confocal images were obtained from the stratum radiatum in the hippocampal CA3 region. Scale bar: 50 μ m.

Figure 6. Double-labeling immunofluorescent analysis for co-localization of pMARCKS and PKC isozymes in the activated glial cells after KA-induced seizures. Double-labeling immunofluorescent data demonstrated that Alexa Fluor 488-labeled pMARCKS and Alexa Fluor 594-labeled PKC α (A), β_1 (B), δ (C) were co-localized in the same activated glial cells in the hippocampus 1 day after KA treatment. Co-localization signals between pMARCKS and PKC isozymes are demonstrated in yellow. Conventional fluorescent microscopic images were obtained from the stratum radiatum in the hippocampal CA3 region. Scale bar: 50 μ m.

these specific isoforms of PKC. Taken together, it was concluded that MARCKS was dramatically upregulated and phosphorylated after KA-induced seizures, specifically in microglial cells out of glial cells, and the MARCKS phosphorylation might be related to PKC α , β I, β II and δ .

It was of interest that the MARCKS-IR non-neuronal cells, observed in the control hippocampus, were not double-labeled with a microglial marker [isolectin IB₄] (Figure 2A1), an astrocyte marker [anti-GFAP] (Figure 2A2), or an oligodendrocyte marker [anti-CNPase] (Figure 2A3) in immunofluorescent analysis. These cells were not located in any neuronal cell layers in the hippocampus. We observed that these cells morphologically resembled glial cells, in which the cytoplasm was relatively smaller and darker in Cresyl Violet staining than in neurons. They demonstrated occasionally, a pattern of enwrapping neuronal cell bodies like glial cells. They were not double-labeled with an interneuron marker, anti-GAD 67 (data not shown). These unusual MARCKS-IR cells might be related to the few MARCKS-IR cells which were not double-immunostained with isolectin IB₄, even after seizures (Figure 2B1). Therefore, it was concluded that there were conceptually two separate results derived from this study on MARCKS expression, before and after KA-induced seizures. One is that MARCKS was dramatically upregulated in microglial cells following seizure activity, while it was detectable minimally in the resting microglia. The other is that there were originally MARCKS-IR non-neuronal cells which were not stained with general glial markers in the resting state, regardless of seizure activity. In this study, we focused on the former issue of the microglia-specific upregulation of MARCKS following seizures. The latter issue was not fully examined here. Therefore, further characterization and identification of originally MARCKS-IR non-neuronal cells needs to be addressed in order to understand the physiological function of MARCKS.

Based on our observations, the time courses of PKC induction and MARCKS phosphorylation were similar to each other. PKC induction and MARCKS phosphorylation were apparent 6 h after KA treatment, even though the signals were weak. Both became maximal 1 day after KA treatment and were slightly decreased but almost sustained 4 days after seizures. On the other hand, KA-induced microglial cell activation, characterized by an increase in the number of microglial cells and the hypertrophic transformation (Deckert *et al.*, 2006), and microglia-specific MARCKS upregulation were observed to be somewhat delayed, compared to PKC induction and MARCKS phosphorylation. Considering their time courses, it could be concluded that the expression

and phosphorylation of MARCKS might be induced almost simultaneously in resting microglia within several h after KA-induced seizures and continued to be increased during the microglial cell activation process.

Alterations of MARCKS gene expression have been studied *in vivo* by others (Kimura *et al.*, 2000; McNamara *et al.*, 2000a and 2000b; Higo *et al.*, 2002). McNamara and colleagues previously reported that MARCKS mRNA levels were not altered in the hippocampus after KA-induced seizures (McNamara *et al.*, 2000b); however, after a facial nerve injury, MARCKS mRNA levels were increased at neurons, microglia and astrocytes in the facial motor nucleus (FMN) (McNamara *et al.*, 2000a). MARCKS phosphorylation was detected at microglial processes within amyloid plaques in human brains with Alzheimer's disease (Kimura *et al.*, 2000). With respect to MARCKS expression, our *in vivo* data demonstrated here that MARCKS is specifically linked to microglia in the hippocampus following KA-induced seizures and might play an important role during the microglial activation process including morphogenesis, proliferation, cell motility, cytokine release, and phagocytosis (Min *et al.*, 2004).

MARCKS has been implicated in the function of macrophages, which are inflammatory cells. Reduced phagocytic activity was demonstrated previously in MARCKS-deficient macrophages that were generated from the fetal liver of MARCKS knockout mice (Carballo *et al.*, 1999). Treatment of bacterial lipopolysaccharide (LPS) or amyloid β increased MARCKS mRNA levels or induced MARCKS phosphorylation in both macrophages and cultured microglial cells (Seykora *et al.*, 1990; Rosé *et al.*, 1996; Nakai *et al.*, 2001). These previous reports could support our *in vivo* data that MARCKS might be specifically linked to the function of microglia. Since microglial cells are considered as brain macrophages, the role of MARCKS in the function of macrophages in the peripheral system (Seykora *et al.*, 1990; Carballo *et al.*, 1999) could be expected to be similar to that in microglial cells in the CNS. Nonetheless, the expression and function of MARCKS demonstrated in macrophages needs to be elucidated in brain microglial cells, because the environments of the brain and the peripheral blood system are quite different.

Considering that MARCKS has been proposed as a major PKC substrate protein to integrate both PKC-mediated and calcium/calmodulin-mediated signals into the regulation of the actin cytoskeleton (Aderem, 1992; Blackshear, 1993), the role of MARCKS in microglia might be the rearrangement of the actin cytoskeleton, which is essential for the microglial activation process under pathological conditions,

such as neuroinflammation and neurodegeneration. However, in order to corroborate the present *in vivo* findings, it needs to be determined whether the microglial activation process could be directly disturbed by manipulating the expression and phosphorylation of MARCKS.

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