Phosphorylation of Myristoylated Alanine-rich Protein Kinase C Substrate by Mitogen-activated Protein Kinase in Cultured Rat Hippocampal Neurons following Stimulation of Glutamate Receptors*

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Glutamate-induced phosphorylation of myristoylated alanine-rich protein kinase C substrate (MARCKS) was investigated in cultured rat hippocampal neurons. In 32P-labeled hippocampal neurons, exposure to 10 μM glutamate induced a long lasting increase in phosphorylation of MARCKS. The long lasting increase in MARCKS phosphorylation mainly required activation of the N-methyl-D-aspartate receptor. Unexpectedly, the MARCKS phosphorylation after the 10-min incubation with glutamate was not inhibited by treatment with calpactin C, a potent inhibitor for protein kinase C (PKC), or down-regulation of PKC but was largely prevented by PD098059, a selective inhibitor for mitogen-activated protein (MAP) kinase kinase. In contrast, the phosphorylation following the short exposure to glutamate was prevented by a combination of PD098059 and calpactin C. The phosphopeptide mapping and immunoblotting analyses confirmed that PKC-dependent phosphorylation of MARCKS was transient and the MAP kinase-dependent phosphorylation was relatively persistent. Investigations of the functional properties also showed that the MARCKS phosphorylation by MAP kinase regulates its calmodulin-binding ability and its interaction with F-actin as seen in the PKC-dependent phosphorylation. These results suggest that glutamate causes a long lasting increase in MARCKS phosphorylation through activation of the N-methyl-D-aspartate receptor and subsequent activation of MAP kinase in the hippocampal neurons.

The myristoylated alanine-rich protein kinase C substrate (MARCKS)1 with an apparent molecular mass of 80–87 kDa is a prototype of family members of prominent cellular substrates for protein kinase C (PKC). Comparison of known MARCKS sequences revealed three highly conserved regions: the N terminus, which contains a myristoylation consensus sequence, the MH2 domain, and a basic effector domain, which contains the PKC phosphorylation sites and calmodulin- and actin-binding sites (1–3). Within the basic internal domain of 25 residues, three or four serine residues are phosphorylated by PKC (4, 5). The potential to bind calcium/calmodulin and cross-links filamentous (F)-actin is regulated by PKC-dependent phosphorylation (4, 6). In addition, the PKC-dependent phosphorylation introduces negative charges into the basic cluster, reducing its electrostatic interaction with acidic lipids and results in translocation of MARCKS from membrane to cytoplasm (7–10). Genomic analysis with Southern blots and polymerase chain reactions revealed the presence of only the 87-kDa MARCKS gene in bovine and human genomes and only the 80-kDa MARCKS gene in the mouse and rat genomes. These are equivalent genes in the different species, and there is about 70% amino acid similarity between the 87-kDa and 80-kDa MARCKS (11). MacMARCKS, with a molecular mass of 48–60 kDa, is another member of the MARCKS family, which has been cloned from mouse macrophage (12) and mouse brain (13). MacMARCKS also has a myristoylated N terminus, a highly conserved MH2 domain, and a basic effector domain that contains PKC phosphorylation sites.

Protein and mRNA of MARCKS are widely distributed and are most abundant in brain, spinal cord, spleen, and lung (14). In the brain, MARCKS is widespread throughout the brain and is enriched in certain regions, including the piriform and entorhinal cortices, portions of the amygdaloid complex, the intralaminar thalamic nuclei, the hypothalamus, the nucleus of the solitary tract, nucleus ambiguus, and many catecholaminergic and serotonergic nuclei (15). In situ hybridization also revealed a high expression of mRNA in the hippocampal CA1 and dentate gyrus (16). Electron microscopic analysis revealed immunoreactivity in axons, axon terminals, small dendritic branches, and occasionally in dendritic spines. No immunoreactive product was observed in large dendrites, somata, or nuclei (15). Furthermore, disruption of the MARCKS gene in mice leads to abnormal brain development and perinatal death, with defects in neurulation, fusion of the cerebral hemispheres, formation of the great forebrain commissures, and retinal and cortical lamination (17). The observation suggests that expression of MARCKS during embryonic and fetal life in the mouse is necessary for normal brain development of the central nervous system.

The properties of MARCKS, including cross-linking F-actin and binding to plasma membrane, suggest that MARCKS reg-
ulates actin-membrane interaction and in turn maintains cell shape and motility. Consistent with this hypothesis, MARCKS is phosphorylated during chemotaxis, secretion, and phagocytosis in neutrophils and macrophages (18, 19), during neurosecretion (20, 21), and during mitogenesis (22, 23). PKC-dependent phosphorylation is possibly involved in functional roles of MARCKS. However, accumulating evidence has suggested that MARCKS is also an in vivo and in vitro substrate of proline-directed protein kinases, such as mitogen-activated protein (MAP) kinase and cycline-dependent protein kinase (cdk) 5. A mass spectroscopic analysis of intact MARCKS purified from bovine brain revealed at least 6 phosphorylation sites in the Ser-Pro motif in the N-terminal domain and upstream of the phosphorylation sites for PKC (24). Furthermore, the proline-directed protein kinases, such as MAP kinase (25) and cdc2 kinase or cdk5 (26), can phosphorylate recombinant mouse MARCKS and purified rat MARCKS, respectively.

MAP kinase, especially ERK 2, was found to be widely expressed in whole rat brain and enriched in the hippocampal formation. In addition, neurotransmitters and neurotrophic factors have been seen to activate MAP kinase in neurons (27, 28). For example, activation of glutamate receptors, especially the NMDA receptor, causes a large increase in MAP kinase in the hippocampal neurons (29, 30), and stimulation of AMPA/kainate receptors elevated MAP kinase activity in cultured cortical neurons (31). Similarly, basic fibroblast growth factor, epidermal growth factor, and brain-derived neurotrophic factor stimulate MAP kinase activity to the same extent as seen with glutamate (30, 32–34). Taken together, MAP kinase is a possible candidate for phosphorylation of MARCKS in vivo in the central nervous system. We now report that a long lasting increase in MARCKS phosphorylation in rat hippocampal neurons following stimulation of the glutamate receptor was mainly though the NMDA receptor activation and is due to activation of MAP kinase rather than PKC. Furthermore, a transient increase in the PKC-dependent phosphorylation of MARCKS was observed following stimulation of the glutamate receptors.

EXPERIMENTAL PROCEDURES

Materials—The following chemicals and reagents were obtained from the indicated sources: Nu serum, Collaborative Research; [γ-32P]ATP, [γ-32P]-labeled GTP, 1 l-cystine; 5,5′-dithio-bis(2-nitrobenzoic acid); [35S]methionine, ICN Biochemicals; l-glutamate, AP3, and phorbol 12-myristate 13-acetate (PMA), Sigma; l-A5P, CNXQ, NMDA, ACPD, and AMPA, Tocris Neuramin; calphostin C, Kyowa Medex Co. Ltd.; PD098059, Research Biochemicals International; llysyl endopeptidase (Pseudomonas aeruginosa), Seikagaku Co.; actin (rabbit skeletal muscle), Sigma; monoclonal antibody against microtubule associated protein 2, Amersham Pharmacia Biotech; and purified MAP kinase (sea star Pseudomus sp. P. sp.) Upstate Biotechnology (its specific activity is 0.5 μmol of phosphate/min/mg using myelin basic protein as substrate). The polyclonal antibody against CaM kinase II, which recognized both subunits, was prepared as described (35). PKC was partially purified based on the procedures of Wooten et al. (36). The procedures contained sequential chromatography on DEAE-Sephacel, Sephacryl S-100, and phenyl-Sepharose CL-4B columns. The specific activity was 3.0 μmol of phosphate/min/mg using myelin basic protein as substrate.

Preparation of MARCKS Antibodies—MARCKS was purified from rat brains according to the method by Patel and Kligman (37), except that a DEAE-cellulose column was used instead of a Mono Q column. MARCKS purified by this procedure was subjected to SDS-PAGE, and the MARCKS protein band was excised from the gel. The gels were emulsified in complete Freund’s adjuvant and used to raise antisera in rabbits. The IgG fraction from the antisera was prepared and used in the present study. To prepare a phospho-specific antibody against PKC phosphorylation sites in MARCKS, the phosphopeptide KRFSP/FFKKSIp/FKLSG, which contains two PKC phosphorylation sites, Ser-152 and Ser-156, was synthesized and used to raise antisera in rabbits. The phospho-specific antibody was affinity-purified from the serum by sequential chromatography on the nonphosphorylated peptide-conjugated and the phosphopeptide-conjugated columns. Further characterization of the phospho-specific antibody will be described elsewhere by Yamamoto et al. (manuscript in preparation). The specificity of both antibodies is shown in Figs. 1 and 7.

Cell Culture—Neonatal hippocampal cell cultures were prepared as described (38). Briefly, hippocampi were removed from Wistar rats on postnatal day 1 and placed in growth medium consisting of Eagle’s minimum essential medium (Life Technologies, Inc.) containing 10% fetal calf serum, 10% horse serum, 2% Nu serum, 12 ng/ml nerve growth factor, and 30 mg/liter kanamycin. Cells were mechanically dissociated by fire-polished Pasteur pipettes and seeded at a density of 3.5 × 10^5 cells/35-mm dish pretreated with calf skin collagen (Sigma type III). One day after plating the neurons, cultures were treated with 5 μM cytosine-β-arabinofuranoside to prevent the replication of nonneuronal cells. The culture medium was replaced by growth medium lacking 10% fetal calf serum at 2 and 6 days of culture. The cells were maintained in humidified 95% air and 5% CO2 at 37 °C for 8–10 days before use.

Immunofluorescence Analysis—Immunofluorescence analysis of cultured hippocampal cells was carried out as described (38). In short, cells in a 35-mm dish were fixed for 10 min at –20 °C with cold methanol. After air drying the dishes, cells were washed in phosphate-buffered saline (PBS) and permeabilized in 0.05% Triton X-100 in PBS for 10 min. A phospho-specific antibody was blocked by 5% goat serum in PBS (blocking solution) for 20 min. Anti-MARCKS polyclonal (1 mg of IgG/ml) and anti-MAP2 monoclonal (Amersham Pharmacia Biotech) antibodies were diluted 1:100 and 1:50, respectively, in the blocking solution. Cells were incubated with the primary antibodies overnight at 4 °C. The cells were then washed in PBS and incubated in fluorescein-conjugated goat anti-rabbit IgG (Cappel) and rhodamine-conjugated goat anti-mouse IgG (Tago, Inc.). Negative controls were immunostained with the MARCKS antibody preabsorbed with an excess of purified MARCKS. Following treatment with secondary antibodies, the cultures were washed with PBS and covered with a 22-mm coverslip.

Labeling of Cells—Eight- to 10-day cultured hippocampal cells were washed once with phosphate-free and serum-free minimum essential medium and labeled in 1.0 ml of this medium containing carrier-free [32P]orthophosphate (0.25 mCi/ml), as described (39). After labeling for 5 h, the cells were incubated with Krebs-Ringer HEPES (KRH) solution, which contained 128 mM NaCl, 5 mM KCl, 1 mM NaHPO4, 2.7 mM CaCl2, 1.2 mM MgSO4, 10 mM glucose, and 20 mM HEPES (pH 7.4). After incubation for 30 min in KRH, cells were incubated at 37 °C for the specified times (with or without specific test agents) or for 1 h with the MgCl2 without controls). Following incubation for the indicated period, the medium was quickly aspirated, and the cells were frozen in liquid N2.

Immunoprecipitation and Quantitation of 32P-MARCKS and 32P-CaM kinase II—Cells were harvested and homogenized in 0.4 ml of the solubilization solution containing 50 mM Tris-HCl (pH 7.5), 0.5 mM CaCl2, 0.5% Triton X-100, 10 mM EDTA, 1 mM Na3VO4, 30 mM sodium pyrophosphate, 50 mM NaF, 100 μM calyculin A, 0.1% SDS, 0.1 mM leupeptin, 75 μM pepstatin A, and 0.1 mg/ml aprotinin. Sonication and centrifugation were performed as described (38). Aliquots (20 μl) of the supernatant were used to determine protein content and 32P incorporation into total proteins by trichloroacetic acid precipitation. To determine the 32P incorporation, Whatman 3MM filter papers on which the aliquots (20 μl) were spotted were washed with trichloroacetic acid, acetone, and ethanol. After drying, the radioactivity was measured by a liquid scintillation counter. The radioactivities of the 32P incorporation into the total proteins were not different between the control and the stimulated cells (data not shown). The supernatant fraction containing the same amount of radioactivity was incubated at 4 °C for 4 h with antibodies to MARCKS (10 μg of IgG protein) and/or CaM kinase II (10 μg of IgG protein) and 75 μl of protein A-Sepharose CL-4B suspension (50% v/v). The immunocomplex immobilized on protein A was washed three times with solubilization solution. After immunoprecipitation of 32P-MARCKS and 32P-CaM kinase II, the immunoprecipitates were eluted from protein A-Sepharose CL-4B by treatment with the SDS sample buffer (40) and boiled for 4 min. Supernatants were subjected to SDS-PAGE (40) followed by autoradiography. A Bio-Imaging Analyzer (BA100, Fuji Film) was used to quantify the amount of 32P incorporation into MARCKS and CaM kinase II subunits.

Analysis of Phosphopeptide Mapping by HPLC—The phosphorylation sites of MARCKS were determined as described (24) using limited

2 H. Yamamoto, manuscript in preparation.
proteolysis of 32P-labeled MARCKS in gel pieces with 1 or 2 μg of lysyl endopeptidase (P. aeruginosa). In control experiments, purified rat brain MARCKS was phosphorylated by MAP kinase or PKC in appropriate conditions for each kinase. MARCKS (2 μg) was incubated for 30 min with 20 mM MAP kinase in the presence of 50 mM HEPES buffer, pH 7.5, 10 mM MgCl₂, 1 mM EGTA, 0.1 mM (γ-32P)ATP, and 1 mM dithiothreitol or with 20 mM PKC in the presence of 50 mM HEPES buffer, pH 7.5, 10 mM MgCl₂, 1 mM CaCl₂, 0.1 mM (γ-32P)ATP, 1 mM dithiothreitol, and 5 μg/ml phosphatidylserine, and 5 μg/ml 1,3-diolein. An additional control was prepared by phosphorylation of MARCKS with both MAP kinase and PKC under the conditions used in PKC-dependent phosphorylation. The in vitro phosphorylated MARCKS and in situ phosphorylated MARCKS immunoprecipitated with the antibody were separated by SDS-PAGE and cut from the gel. After incubation in gel pieces for 10 h at 35 °C with lysyl endopeptidase, the reaction was terminated by addition of 0.1% of trifluoroacetic acid, at a final concentration. After the gel pieces were removed by centrifugation, the supernatant was applied to a C18 column (4 × 150 mm) in an HPLC apparatus (Hitachi L-6000). The MARCKS peptides were eluted with a linear gradient of 0.2 M ammonium formate in 60% acetonitrile in the presence of 0.1% trifluoroacetic acid at a flow rate 1 ml/min. The eluate was collected by a fraction collector, and the radioactivity of each fraction was counted by liquid scintillation spectrometry.

Cross-linking of 125I-CaM to MARCKS—Purified MARCKS was cross-linked by the method of Graff (42) as described (26). MARCKS (1.5 μg) was incubated for 30 min at 30 °C without or with PKC or MAP kinase in the presence of 0.5 mM ATP. The MARCKS protein (1.5 μg) were incubated for 1 h at 25 °C with 125I-CaM (0.2 μCi/3 ng) in 38 mM HEPES (pH 7.5) and 2.5 mM CaCl₂ in a final volume of 50 μl. After addition of disuccinimidyl suberate in a final concentration of 0.25 mM, the sample was further incubated for 20 min at 25 °C. The reaction was terminated by addition of Tris-HCl (pH 7.5) to a final concentration of 5 mM. The sample was treated with the SDS-sample buffer and boiled for 2 min. The sample was subjected to SDS-PAGE, followed by autoradiography. The cross-linking of 125I-CaM to MARCKS was totally abolished by inclusion of an excess amount of nonlabeled CaM in the medium (data not shown).

Interaction of MARCKS with F-actin—The interaction of MARCKS with F-actin was determined by a co-sedimentation assay as described (42). After preparation of F-actin by polymerization of G-actin, the interaction of MARCKS with F-actin was determined by a co-sedimentation assay. The samples were then centrifuged for 15 min at 100,000 × g. The pellet containing F-actin and co-sedimented MARCKS was resuspended with SDS-sample buffer and subjected to SDS-PAGE. After electrophoresis, gels were stained with Coomassie Brilliant Blue. Immunoblot analysis using anti-MARCKS antibody could specifically immunoprecipitate the 80-kDa MARCKS from cell extracts of unstimulated or stimulated hippocampal cells with glutamate.

Using 32P-labeled hippocampal neurons, the antibody could specifically immunoprecipitate the phosphorylated 80-kDa MARCKS from cell extracts of cultured hippocampal neurons incubated in the presence or absence of glutamate (Fig. 1). Although MacMARCKS, a MARCKS-related protein with a molecular mass with 48–52 kDa, was seen to be present in the rat brain (45) and was even cloned from mouse brain (13), the 48-kDa protein does not seem to be MacMARCKS, because the antibody did not recognize the MacMARCKS protein in rat peritoneal macrophages (data not shown).

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cant increase was observed only at the 3-min incubation. In separate experiments with cultured cortical astrocytes, the concentration of glutamate required to potentiate MARCKS phosphorylation was much higher, and 50 ± 10% increase in the phosphorylation was observed with 500 μM glutamate. Ten μM glutamate had no effect on phosphorylation in the cultured astrocytes (data not shown). Therefore, the effect of glutamate observed in the present study was considered to occur in the neurons. Exposure of cultured neurons to over 100 μM glutamate or 100 μM NMDA seemed to be toxic in the cultured hippocampal neurons, as we reported earlier (46), and the maximum effect on the MARCKS phosphorylation was obtained with 10 μM glutamate in these cultures. In addition, the 10-min exposure to glutamate did not cause a significant neuronal toxicity, such as cell death within the next 24 h (data no shown). We therefore examined MARCKS phosphorylation using 10 μM glutamate to clarify its physiological roles in the cultured hippocampal neurons. However, because the persistent phosphorylation of MARCKS was observed in the continued presence of glutamate or NMDA in the medium, it may also be involved in pathological events such as the glutamate-induced neuronal cell death.

To evaluate the glutamate receptor types associated with MARCKS phosphorylation, several glutamate receptor antagonists were tested. When hippocampal neurons cultured for 8–10 days were used, the basal MARCKS phosphorylation did not change by treatments with AP-3, CNQX, or MK801 alone, or a combination of CNQX and MK801. However, the basal MARCKS phosphorylation was inhibited by 20% by treatment with MK801 but not with CNQX or AP3 in neurons cultured for 14–21 days, due to the inhibition of MARCKS phosphorylation stimulated by spontaneous synaptic activity observed in mature neurons in culture. The increase in MARCKS phosphorylation following a 10-min incubation with glutamate was weakly but significantly inhibited by CNQX, a non-NMDA receptor antagonist, and strongly by MK801, a specific inhibitor of the NMDA receptor, whereas AP3, a metabotropic receptor inhibitor, had little inhibitory effect (Fig. 4 B). The combination of CNQX and MK801 abolished the glutamate-induced MARCKS phosphorylation as well as the increased autophosphorylation of CaM kinase II (Fig. 4, A and B). This finding is consistent with the observation that treatments with NMDA or AMPA significantly potentiated MARCKS phosphorylation at the 10-min incubation, as shown in Fig. 3. These observations suggest that the long lasting increase in MARCKS phosphorylation by exposure to glutamate was mainly through the NMDA receptor and more weakly through the kainate or AMPA type receptor. The metabotropic glutamate receptor did not contribute to the persistent MARCKS phosphorylation in hippocampal neurons.

Inhibition of MARCKS Phosphorylation by PD098059—To study the involvement of PKC in glutamate-induced MARCKS phosphorylation, we stimulated cells by glutamate in the presence of 200 nM calphostin C, a relatively specific and potent

Fig. 2. Immunofluorescence with the anti-MARCKS antibody in cultured hippocampal cells. Hippocampal cells were examined by phase contrast (A and D) and immunofluorescence (B, C, and E) microscopy after being stained with the anti-MARCKS antibody (B) and anti-MAP2 antibody (C). The immunofluorescence was strong in the cell bodies and neurites in stellate and pyramidal-like neurons. In the neurite, punctate-staining was observed, whereas weak immunofluorescence was observed in glial cells. The immunofluorescence in glial cells, as well as neurons, disappeared by preabsorption of antibodies with purified MARCKS (D and E).

Fig. 3. Increased phosphorylation of MARCKS by stimulation of glutamate receptors. The time course of agonist-induced MARCKS phosphorylation. The cultured hippocampal cells (8–10 days in culture) were prelabeled with [32P]orthophosphate for 5 h and washed once in KRH solution. After incubation at 37 °C for 30 min in KRH, cells were incubated without the agonist (control) in KRH or with the indicated agonists: 10 μM glutamate, 50 μM NMDA in Mg2⁺-free KRH with 1 mM glycine, 50 μM AMPA, or 500 μM ACPD in KRH. After incubation for the indicated times, the cells were frozen on liquid N2, and homogenized. The 32P-labeled MARCKS was immunoprecipitated. The 32P-incorporation into MARCKS was analyzed using a Bio-Imaging analyzer (BA100, Fuji Film). In control, cells were incubated under the same conditions without an agonist, and 32P-incorporation into MARCKS was determined at each time point. Values are expressed as a percentage when the 32P-incorporation into MARCKS from control cells was taken as 100% at each time point. Values are means ± S.E. (n = 4–6). The changes in MARCKS phosphorylation were statistically significant versus control; *, p < 0.05; **, p < 0.01.
inhibitor for PKC, or after down-regulation of PKC by pretreatment with PMA for 16 h. Unexpectedly, treatment with calphostin C did not abolish the glutamate-induced phosphorylation after 10-min stimulation (Fig. 5A and B). Similarly, the down-regulation of PKC did not inhibit the glutamate-induced phosphorylation. Calphostin C and down-regulation of PKC were working to inhibit PKC in these conditions, because the PKC-induced MARCKS phosphorylation was largely prevented by treatment with 200 ng calphostin C as well as by the down-regulation of PKC (Fig. 5B). Interestingly, treatment with 50 μM PD098059, a specific inhibitor for MAP kinase kinase inhibitor (47, 48) largely inhibited glutamate-induced MARCKS phosphorylation (Fig. 5A and B). When the MAP kinase activity was measured by in-gel kinase assay using SDS-polyacrylamide gel containing myelin basic protein, as reported (30), the MAP kinase activity increased to 336 ± 10% by 10-min stimulation with glutamate. Inclusion of 50 μM PD098059 totally inhibited glutamate-induced MAP kinase activation to near control levels (108 ± 6% as compared with the control). In contrast, the increased phosphorylation by 2-min stimulation with glutamate was partly abolished by PD098059 and calphostin C and totally abolished by a combination of PD098059 and calphostin C (Fig. 5C). The down-regulation of PKC also significantly inhibited the MARCKS phosphorylation. Thus, the glutamate-induced MARCKS phosphorylation for a longer period was mainly due to activation of MAP kinase in the cultured hippocampal neurons.

Phosphopeptide Mapping Analysis of Phosphorylated MARCKS—To further confirm the involvement of MAP kinase in the glutamate-induced MARCKS phosphorylation by 10-min incubation, phosphopeptide mapping analysis was carried out after limited proteolysis with lysyl endopeptidase, according to the method of Taniguchi et al. (24). To clarify the in situ phosphorylation sites of MARCKS, the purified rat brain MARCKS was in vitro phosphorylated by purified PKC and MAP kinase in initial experiments and was separated by SDS-PAGE. After cutting out gel bands corresponding to MARCKS, MARCKS was digested in gel pieces with 1 or 2 μg of lysyl endopeptidase. After the digestion, the MARCKS peptides were separated from the gel pieces and analyzed using a conventional high performance liquid chromatography apparatus, as described under “Experimental Procedures.” When MARCKS peptides were eluted with a linear gradient of acetonitrile-H2O in the presence of 0.1% trifluoroacetic acid, three major 32P-labeled radioactive peaks were detected by PKC phosphorylation (Fig. 6A). Each peak was eluted in fractions of 26, 39, and 41 (Fig. 6A). In contrast, one major peak in the fraction of 32, a small shoulder in the fraction of 30, and several minor peaks were detected in MARCKS phosphorylated by MAP kinase (Fig. 6B). As an additional control, the MARCKS phosphorylated by both MAP kinase and PKC was analyzed (Fig. 6C). Three major peaks originated from PKC- and MAP kinase-dependent phosphorylation sites were separated, but the first peak of PKC peptides eluted in fraction 26 was shifted to fraction 28 after the phosphorylation with both kinases. Although the reason for the shifting of the first peak of PKC peptides is unclear, dually phosphorylated peptides may be produced under the phosphorylation conditions. Next, MARCKS that was in situ phosphorylated by 2- or 10-min stimulation with glutamate was analyzed using the same procedures. The elution patterns of in situ phosphopeptides were apparently similar to that of in vitro phosphorylation as shown in Fig. 6C. At 2 min, phosphopeptides of MARCKS of the control cells showed major peaks in fractions of 29, 31, and 36 and several minor peaks (Fig. 6D). Stimulation with glutamate for 2 min mainly produced phosphopeptides in fractions 29 and 31, which corresponded to PKC- and MAP kinase-dependent phosphorylation sites, respectively, as shown in Fig. 6C. In contrast, following 10-min stimulation with glutamate, a peak of fraction 32 with a shoulder in fraction 30 largely increased with minor peaks around the fractions 37–41 (Fig. 6E). However, changes in other minor peaks were not consistent in repeated experiments. The increased phosphorylation of PKC- and MAP kinase-dependent sites were also evident in the PMA-stimulated MARCKS phosphorylation, because PMA is known to be a strong activator for MAP kinase as well as PKC in the hippocampal neurons (30). These results suggest that the PKC-dependent phosphorylation in MARCKS is transient and that the persistent glutamate-induced phosphorylation in MARCKS is made through MAP kinase rather than PKC.
Furthermore, as shown in Fig. 6, D–F, the phosphorylation by MAP kinase and PKC already occurred in the basal conditions in cultured hippocampal neurons.

Phosphorylation of MARCKS by PKC—MARCKS is originally known to be a substrate for PKC. We further confirmed the transient increase in phosphorylation of MARCKS by PKC following stimulation with glutamate. We developed a method to detect sites phosphorylated by PKC. A specific antibody that recognizes the phosphorylation sites Ser-152 and Ser-156 in MARCKS was produced by immunization of the phosphopeptide of MARCKS. In control experiments, the purified MARCKS was phosphorylated by PKC in the presence of non-radioactive ATP and was subjected to immunoblot analysis. The antibody to the phosphopeptide of MARCKS could detect only the phosphorylated form by PKC, as shown in the last two lanes of Fig. 7A. In addition, PMA could stimulate the phosphorylation of PKC-dependent sites (Fig. 7A), and its effect was abolished by addition of 200 nM calphostin C. Consistent with the results in Fig. 6, the increased phosphorylation of PKC-dependent sites by stimulation with glutamate was transient, reaching a maximum between 1 and 3 min, followed by a decline to the basal levels within 10 min (Fig. 7B). The amount of MARCKS protein detected with nonselective antibody did not change during the incubation with glutamate (Fig. 7B). These results confirm that activation of PKC following glutamate stimulation is transient and PKC can primarily phosphorylate MARCKS during the early period by stimulation with glutamate. In addition, basal phosphorylation of PKC sites was observed in all these preparations. The basal phosphorylation by PKC is possibly due to endogenous release of glutamate and/or other stimulants that stimulate PKC in cultured hippocampal neurons.

Regulation of Functional Properties of MARCKS by MAP Kinase—Finally, we addressed question whether the functional properties of MARCKS is regulated by phosphorylation by MAP kinase. We then investigated effects of MARCKS phosphorylation by MAP kinase on its CaM-binding ability assessed by cross-linking with [125I]-CaM and interaction with F-actin determined by a co-sedimentation assay. The effects were compared with the changes by PKC-dependent phosphorylation. The purified rat brain MARCKS was incubated without or with protein kinases in the presence of 0.5 mM ATP (Fig. 8). Under
In these conditions, the total phosphate incorporated into MARCKS were 0.9 and 2.6 mol of phosphate/mol of MARCKS by MAP kinase and PKC, respectively. There was no incorporation of phosphate without each protein kinase. As reported previously (4), the CaM-binding ability of MARCKS was totally abolished by PKC-dependent phosphorylation, as shown in Fig. 8. The phosphorylation by MAP kinase slightly but significantly reduced its CaM-binding ability to 75% of control. In contrast, the interaction between MARCKS and F-actin was largely affected by the MAP kinase-dependent phosphorylation to the same extent as seen for the PKC-dependent phosphorylation (Fig. 9). These results suggest that MAP kinase can functionally regulate the properties of MARCKS, especially in its interaction with F-actin.

**DISCUSSION**

In neutrophils and macrophages, MARCKS is phosphorylated during chemotaxis, secretion, and phagocytosis (18, 19); during neurosecretion (20, 21, 49, 50); and during mitogenesis (22, 23). Because this phosphorylation seems to be closely associated with activation of PKC and its ability to bind calcium/calmodulin and cross-link actin filaments are directly regulated by the PKC-dependent phosphorylation (4, 6), the physiological functions of MARCKS would appear to be mainly regulated by PKC in vivo. However, mass spectrometrical analysis using purified bovine MARCKS demonstrated six novel phosphorylation sites in addition to the known PKC phosphorylation sites (24). The endogenous phosphorylation by PKC was a minor portion and all the novel sites were serine residues, followed immediately by proline residues, which means that MARCKS is also a good substrate for proline-directed protein kinase, including MAP kinase and cdk5, in vivo.

We reported activation of PKC and MAP kinase, as well as CaM kinase II, in cultured rat hippocampal neurons (30, 38). We focused on activation of the protein kinase cascades following the activation of NMDA receptors in the hippocampal neurons, because NMDA receptor activity was exclusively associated with synaptic plasticity in the developing brain as well as in the adult brain. For example, activation of CaM kinase II in cultured hippocampal neurons was predominantly regulated by the activity of the NMDA receptor, because glutamate-induced activation of CaM kinase II was only inhibited by addition of the NMDA receptor antagonist but not by antagonists of AMPA/kainate and the metabotropic receptors (38). The NMDA receptor-dependent activation of CaM kinase II was also evident in the long term potentiation (LTP) in the hippocampal CA1 regions (51). The potentiation of CaM kinase II was closely associated with the induction of LTP, in an NMDA receptor-dependent manner (51, 52). Similarly, activation of MAP kinase by stimulation with glutamate was primarily due to activation of the NMDA receptor (30). The activation of MAP kinase may be related to the induction of LTP in the CA1 region, because PD98059 attenuated the induction of LTP (53). Thus, MAP kinase became an attractive candidate related to the underlying the molecular basis for expressing a
In the present study, we demonstrated that MARCKS is one in vivo substrate for MAP kinase following stimulation of glutamate receptors. MARCKS phosphorylation was sustained during more than 30 min after glutamate stimulation, a long lasting increase predominantly due to activation of the NMDA receptor. In addition, the long lasting glutamate-induced MARCKS phosphorylation was largely prevented by PD908059, a MAP kinase kinase inhibitor but not by a PKC inhibitor. When examining our previous results in terms of MAP kinase activation by stimulation of the glutamate receptors, the time course of glutamate-induced MARCKS phosphorylation was closely related to that of MAP kinase activation, in which the NMDA receptor activation was mainly involved (30). Treatment with PD908059 totally inhibited the glutamate-induced MAP kinase activation, as shown in the present study. Consistent with these observations, the major site for MAP kinase detected by HPLC analysis was mainly potentiated after a 10-min exposure to glutamate. The phosphorylation of the major site for MAP kinase was elevated even after a 30-min incubation (data not shown). The finding of one major peak in MARCKS phosphorylated in vitro by MAP kinase is consistent with findings that Ser-113 in mouse MARCKS and Ser-116 in bovine MARCKS were mainly phosphorylated by the purified MAP kinase in vitro (24, 25). Furthermore, Schönwaßer et al. (25) demonstrated that the mutation of Ser-113 to alanine in MARCKS largely abolished the phosphorylation by MAP kinase. However, Ser-113 was not phosphorylated in permeabilized Swiss 3T3 cells after stimulation with platelet-derived growth factor as well as PMA (25). Because platelet-derived growth factor activates MAP kinase in a variety of cells, the phosphorylation of MARCKS by MAP kinase may not generally occur. In hippocampal neurons, MARCKS may be an important substrate for MAP kinase, because stimulation with brain-derived neurotrophic factor in hippocampal neurons could also elicit an increase in MARCKS phosphorylation by MAP kinase.

The present study demonstrated that stimulation of hippocampal neurons with glutamate elicits biphasic increases in phosphorylation of MARCKS: that is, phosphorylation with

3 M. Kanahori, K. Fukunaga, and E. Miyamoto, unpublished data.
PKC through the metabotropic glutamate receptor in a short period and with MAP kinase through activation of the NMDA receptor over a long period. This was confirmed by using a specific antibody that recognizes PKC-phosphorylation sites. These results are consistent with previous observations that glutamate-induced translocation of PKC from the cytosol to the membrane fraction was transient and were reverted to basal levels in 5 min (38). The translocation of PKC was only inhibited by addition of an inhibitor for the metabotropic glutamate receptor (38). Transient increase in phosphorylation of MARCKS was noted in hippocampal neurons (54), cerebellar granule cells (55), and hippocampal slices from adult rats (56). It is important to determine which kinase is involved in the phosphorylation described in previous works (54–56).

The possibility remains that other proline-directed protein kinases, such as cdc2 kinase and cdk5, are involved in glutamate-induced MARCKS phosphorylation. The phosphopeptides eluted in fractions 38–42 were not identified. It has not been elucidated how cdc2 kinase and cdk5 are activated by external stimuli for receptors, although these kinases are expressed in neurons (57–60) and have the potential to stoichiometrically phosphorylate MARCKS in vitro (26, 61).

The present study is apparently the first finding to demonstrate the in situ phosphorylation of MARCKS through MAP kinase following extracellular stimuli. MAP kinase-induced phosphorylation of synapsin I was noted in cultured cortical neurons following stimulation with brain-derived neurotrophic factor (41). Synapsin I is predominantly located in presynaptic regions and regulates the binding between synaptic vesicles and actin filaments. In vitro phosphorylation of synapsin I by MAP kinase reduced the ability of cross-linking of actin filaments but had no evident effect on the binding ability to synaptic vesicles. The regulation of bundling of actin filaments by synapsin I phosphorylation through MAP kinase suggests that MAP kinase is implicated in neurite extension rather than exocytosis in the phosphorylation of synapsin I. On the other hand, MARCKS, which is also enriched in nerve terminals, regulates interactions between actin filaments and plasma membranes. In this context, we investigated the physiological significance of MARCKS phosphorylation by MAP kinase. The phosphorylation by MAP kinase significantly regulated the interaction between MARCKS and F-actin rather than its CaM-binding ability. The results suggest a potential role of MAP kinase in the regulation of F-actin-membrane interaction. The site for MAP kinase, Ser-113, is located in the near upstream site for the central PKC phosphorylation domain that includes Ser-152, Ser-156, and Ser-163 and is conserved in all members of the MARCKS family and MacMARCKS. The inhibitory effect of phosphorylation of Ser-113 by MAP kinase on binding to calmodulin may be due to conformational changes in the MARCKS structure. In cerebellar granule cells, involvement of MARCKS phosphorylation in NMDA-stimulated neurite outgrowth was suggested as MARCKS was present in neurites and growth cones (55). The present study demonstrated that MARCKS is also present in neurites and the varicosity-like structure, as based on light microscopic observations of cultured hippocampal neurons (Fig. 2). Further investigation of localization of MARCKS phosphorylated by MAP kinase is needed to clarify the roles of MARCKS in the central nervous system. MARCKS may serve as a good substrate for MAP kinase during the LTP expression, because MARCKS can provide a reversible cross-bridge between the actin cytoskeleton and the plasma membrane, which may contribute to reorganization or morphological changes in synapses in the hippocampus during LTP expression.

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Fig. 9. Effect on the binding to F-actin of MARCKS phosphorylation by PKC and MAP kinase. F-actin (10 µg) was incubated with MARCKS that had been preincubated without (None) or with PKC or MAP kinase (MAPK) as in Fig. 8. After centrifugation, the pellet with F-actin and co-sedimented MARCKS was separated by SDS-PAGE and stained with Coomassie Blue (A). In order to detect the co-sedimented MARCKS, the gel was subjected to immunoblotting analysis with the anti-MARCKS antibody, followed by autoradiography (B). The position of MARCKS is indicated by an arrow head. The results from six independent experiments were examined statistically (C). Values represent means ± S.E. The changes were statistically significant versus without protein kinase (None); **, p < 0.01.

In Situ Phosphorylation of MARCKS by MAP Kinase

FIG. 9. Effect on the binding to F-actin of MARCKS phosphorylation by PKC and MAP kinase. F-actin (10 µg) was incubated with MARCKS that had been preincubated without (None) or with PKC or MAP kinase (MAPK) as in Fig. 8. After centrifugation, the pellet with F-actin and co-sedimented MARCKS was separated by SDS-PAGE and stained with Coomassie Blue (A). In order to detect the co-sedimented MARCKS, the gel was subjected to immunoblotting analysis with the anti-MARCKS antibody, followed by autoradiography (B). The position of MARCKS is indicated by an arrow head. The results from six independent experiments were examined statistically (C). Values represent means ± S.E. The changes were statistically significant versus without protein kinase (None); **, p < 0.01.
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