RhoA and Rho Kinase-dependent Phosphorylation of Moesin at Thr-558 in Hippocampal Neuronal Cells by Glutamate*

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When we were studying phosphorylated proteins in the rat brain after electroconvulsive shock (ECS), we observed the rapid phosphorylation of a 75-kDa protein, which cross-reacted with the anti-phospho-p70 S6 kinase antibody. The phosphorylated protein was purified and identified as moesin, a member of the ezrin/radixin/moesin (ERM) family and a general cross-linker between cortical actin filaments and plasma membranes. The purified moesin from rat brain was phosphorylated at serine and threonine residues. Moesin was rapidly phosphorylated at the threonine 558 residue after ECS in the rat hippocampus, peaked at 1 min, and returned to the basal level by 2 min after ECS. To investigate the mechanism of moesin phosphorylation in neuronal cells, we stimulated a rat hippocampal progenitor cell, H19–7/IGF-IR, with glutamate, and observed the increased phosphorylation of moesin at Thr-558. Glutamate transiently activated RhoA, and constitutively active RhoA increased the basal level phosphorylation of moesin. The inhibition of RhoA and its effector, Rho kinase, abolished increased Thr-558 phosphorylation by glutamate in H19–7/IGF-IR cells, suggesting that the phosphorylation of moesin at Thr-558 in H19–7/IGF-IR cells by glutamate is mediated by RhoA and Rho kinase activation.

Electroconvulsive shock (ECS)† has been used for the treatments of psychiatric disorders such as depression, schizophrenia, and manic-depressive illness. Although the mechanism of the treatment has not been elucidated, recent progress in the cellular and molecular biology of neuronal tissues has enabled neuronal functions to be linked to the regulation of gene expression and intracellular signal transduction (1). Previously, we reported (4) that ECS induces the expressions of various immediate early genes such as c-fos, junB, and Tie-S-8 and that it suppresses the expression of inositol 1,4,5-triphosphate kinase and inositol 1,4,5-triphosphate receptor genes. We also observed (2–5, 7) that ECS caused the phosphorylation of many signaling molecules, including those in the Pyk2-Ras-Raf-MEK-ERK pathway, and of stress-signaling pathways in various rat brain regions.

Earlier we reported that ECS induces the phosphorylation of CREB in the rat hippocampus (6). Several protein kinases have been suggested to be CREB kinases in neuronal tissues, and we looked for protein kinases potentially responsible for CREB phosphorylation. When we were studying the phosphorylation of one of the suggested CREB kinases, ribosomal S6 kinase (S6K), we observed that a 75-kDa protein in the rat hippocampus was rapidly phosphorylated after ECS. This was detected by an antibody specific to phospho-p70 S6K. The protein was not detected by the anti-p70 S6K antibody, suggesting that this protein was detected nonspecifically by an anti-phospho-p70-S6K antibody. So we purified and identified this protein as moesin (membrane-organizing extension spike protein), a member of the ezrin/radixin/moesin (ERM) family of proteins. ERM proteins consist of three domains: 1) a globular domain at the N-terminal half, which is conserved among the members of the band 4.1 superfamily and is referred to as the FERM (4.1 and ERM) domain, a membrane-binding domain; followed by 2) an extended α-helical domain; and 3) a charged C-terminal domain, which includes a consensus sequence motif for actin binding. Thus, ERM proteins have been suggested to function as cross-linkers between actin filaments and plasma membranes and to be involved in the formation of microvilli, cell adhesion sites, ruffling membranes, and cleavage furrows (8–13).

The ERM proteins are phosphorylated in growth factor-stimulated cells (14, 15). In thrombin-activated platelets, moesin is phosphorylated at a specific C-terminal 558 threonine residue (Thr-558), causing filopodia formation (16). In vitro functional analysis suggested that C-terminal threonine phosphorylation maintains ERM proteins in the active state by suppressing intramolecular interaction (17).

RhoA is a member of the Ras-like GTPase superfamily and has been shown to regulate the actin cytoskeleton and mitogenic signaling in response to extracellular signals (18). In fibroblasts, ERM proteins were phosphorylated and relocalized to apical membrane/actin protrusions in a RhoA-dependent manner (19), and in Swiss 3T3 cells at least one of the ERM...
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Experimental Procedures

Treatment of Animals and the Preparation of Hippocampal Lysates—Male Sprague-Dawley rats ranging from 150 to 200 g were used in this study. Animals were treated in accordance with National Institutes of Health guidelines for the care and use of laboratory animals. ECS was administered (150 V, 0.5 s) via an ear electrode. Control animals were treated in the same manner as the ECS-treated animals but without the electric current. The animals were decapitated at the prescribed times (0, 1, 2, 5, 10, 30, and 60 min after ECS), and hippocampi were removed onto ice and homogenized immediately with a glass Teflon homogenizer in 10 volumes of ice-cold homogenization buffer (25 mM Tris pH 7.5, 1 mM EDTA, 2 mM EGTA, 50 mM NaF, 1 mM Na3VO4, 10 mM sodium pyrophosphate, 0.2% Nonidet P-40, 1 mM PMSF, 1 mM DTT, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin) and centrifuged for 10 min at 10,000 × g, 4 °C. The protein samples were prepared by boiling the supernatant with 3X Laemmli’s sampling buffer. Protein was quantitatively assayed using bicinchoninic acid prepared by boiling the supernatant with 3 ml of 1% cupric sulfate and 1% sodium pyrophosphate, 0.2% Nonidet P-40, 1 mM PMSF, 1 mM DTT, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin in a ratio of 5 mlg by weight. The protein concentration was determined using bicinchoninic acid (Sigma) or Bradford reagent (Bio-Rad).

Purification of the 75-kDa Protein—The resulting antibody reacted with only phosphorylated ERM proteins, the moesin DNA sequence was amplified by polymerase chain reaction and subcloned into vector pEGX-4T-1 (Amersham Biosciences). The construct was confirmed by DNA sequencing. GST fusion proteins were expressed in DH5α cells and purified on glutathione-agarose beads. A mouse polyclonal antibody was produced in rabbit against purified GST-fused full-length mouse moesin protein. The antibody reacted only with moesin, not with ezrin or radixin, and we have named the antibody TM2. A polyclonal antibody against Thr-558-phosphorylated ERM proteins was raised in rabbits against a KLIH-conjugated synthetic phosphopeptide (KRYRtLILQCCCCCC, where pT is phosphothreonine- phosphorylated from GenScript; KLIH from Pierce), which corresponded to the mouse moesin sequence from amino acids 555–561. The resulting antibody reacted with only phosphorylated ERM proteins but not unphosphorylated ERM proteins.

Cell Culture and Transfection—H19–7/IGF-IR cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum, 200 μg/ml G418, and 1 μg/ml puromycin at 34 °C under 5% CO2. H19–7/IGF-IR cells were seeded at 2 × 105 in a 6-well plate coated with poly-l-lysine. For glutamate treatment, the cells were cultured in serum-free DMEM for 18 h and then stimulated with or without 100 μM glutamate for various periods (2, 5, 10, 30, and 60 min).

The transfection of plasmids (3.2 μg of pDNA C3, pCMV-Myc RhoaV14, or empty vectors) into H19–7/IGF-IR cells was carried out using LipofectAMINE 2000 reagent (Invitrogen) according to the manufacturer’s instructions. Transient overexpression of the pCMV-Myc RhoaV14 proteins was verified by immunoblotting cell lysates with an anti-Myc monoclonal antibody.

Production of Antibodies and Recombinant Proteins—A plasmid (pSK moesin) encoding full-length mouse moesin protein was obtained from Dr. S. Tsukita (Kyoto University). To construct the GST fusion proteins, the moesin DNA sequence was amplified by polymerase chain reaction and subcloned into vector pEGX-4T-1 (Amersham Biosciences). The construct was confirmed by DNA sequencing. GST fusion proteins were expressed in DH5α cells and purified on glutathione-agarose beads. A mouse polyclonal antibody was produced in rabbit against purified GST-fused full-length mouse moesin protein. The antibody reacted only with moesin, not with ezrin or radixin, and we have named the antibody TM2. A polyclonal antibody against Thr-558-phosphorylated ERM proteins was raised in rabbits against a KLIH-conjugated synthetic phosphopeptide (KRYRtLILQCCCCCC, where pT is phosphothreonine- phosphorylated from GenScript; KLIH from Pierce), which corresponded to the mouse moesin sequence from amino acids 555–561.

The resulting antibody reacted with only phosphorylated ERM proteins but not unphosphorylated ERM proteins.
fetal bovine serum. For lysoosphatidic acid (LPA, Avanti Polar Lipids) treatment, Rat1 cells were seeded at 4 × 10^5 cells in a 6-well plate. The next day, the cells were serum-starved for 18 h in serum-free DMEM. After adding LPA to a concentration of 1 μM, the cells were incubated for various periods (0, 2, 5, 10, and 30 min). For phosphorylation analysis, H19–7/IGF-IR and Rat1 cells were fixed with 10% trichloroacetic acid, washed with phosphate-buffered saline three times, and then solubilized with 100 μl of 1 × Laemmli’s sample buffer. Rhodamine Red-E, Rac1, and Cdc42 Activity Assays—Rhodamine Red-E, Rac1, and Cdc42 activities were measured according to the modified method of Ren et al. (23). The H19–7/IGF-IR cells (2 × 10^5 cells) were seeded in 100-mm culture dishes and serum-starved in serum-free DMEM for 18 h before the lysis. The cells were then treated with or without 100 μM glutamate. For a RhoA activity assay, the cells were lysed with ice-cold buffer (25 mM Tris, pH 7.5, 250 mM NaCl, 5 mM MgCl₂, 1 mM sodium orthovanadate, 1 mM sodium deoxycholate, 0.25% Nonidet P-40, and 1 μM DTT). The bound proteins were eluted in Laemmli buffer (25 mM Tris, pH 7.5, 10 mM MgCl₂, 2% Nonidet P-40, 1 μM sodium orthovanadate, 1 mM PMSF, 1 μM aprotinin, and 1 μM leupeptin) for 15 min on ice. The cell lysates were then passed 10 times through a 23-gauge needle and centrifuged at 10,000 × g for 10 min at 4 °C. The supernatants were incubated with 30 μg of glutathione-Sepharose beads bound to the GST-RhoA-binding domain of mouse rhodotin for 60 min at 4 °C. For Rac1 or Cdc42 activity assays, the cells were resuspended in 200 μl of NS buffer (25 mM Tris, pH 7.5, 1.5 mM MgCl₂, 1 mM sodium orthovanadate, 1 mM PMSF, 1 μM aprotinin, and 1 μM leupeptin) for 10 min and then passed 10 times through a 25-gauge needle. After the addition of 200 μl of 2× no-sample buffer (50 mM Tris, pH 7.5, 10 mM MgCl₂, 2% Nonidet P-40, 1 mM sodium orthovanadate, 1 mM PMSF, 1 μM aprotinin, and 1 μM leupeptin), the cell lysate was passed through a 23-gauge needle 10 times and placed on ice for 5 min, and then 400 μl of high salt-binding buffer (25 mM Tris, pH 7.5, 30 mM MgCl₂, 100 mM NaCl, 0.5% Nonidet P-40, 1 mM sodium orthovanadate, 1 mM PMSF, 1 μM aprotinin, and 1 μM leupeptin) were added, and the cell lysates were centrifuged at 10,000 × g for 10 min at 4 °C. The supernatants were incubated with 30 μg of glutathione-Sepharose beads bound to the GST-Rac/Cdc42-binding domain of rat PAK3 (GST-PBD) for 60 min at 4 °C. The beads were washed three times with wash buffer (25 mM Tris, pH 7.5, 40 mM NaCl, 30 mM MgCl₂, 1% Nonidet P-40, and 1 mM DTT). The bound proteins were eluted in Laemmli’s sample buffer separated by 15% SDS-PAGE, and analyzed by immunoblotting with mouse monoclonal anti-RhoA, anti-Rac1, and anti-Cdc42 antibodies.

Subcellular Fractionation—To obtain cytosolic and membranous fractions, Rat1 cells were rinsed once in cold phosphate-buffered saline and then lysed in detergent-free buffer (25 mM Tris, pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 1 mM sodium orthovanadate, 1 mM PMSF, 1 mM DTT, 1 μg/ml aprotinin, and 1 μg/ml leupeptin) by passing 10 times through a 25-gauge needle. The lysed cells were pelleted at 100,000 × g for 1 h at 4 °C. The supernatants were used as cytosol, and the pellets as crude membrane. The samples were added with sample buffer (3×) and incubated at 100 °C for 5 min.

RESULTS

Purification and Identification of the 75-kDa Phosphoprotein in the Rat Hippocampus—Previously we reported that ECS induces CREB phosphorylation in the rat hippocampus (6). To identify the kinases responsible for the phosphorylation of CREB in the rat hippocampus after ECS, we studied the phosphorylation of Rsk, a cytosolic protein kinase for ribosomal S6, which was reported as a CREB kinase in glial cell progenitors (24). Initially, we examined the mobility shifts of Rsk after ECS in the rat hippocampus and could observe the mobility shifts (data not shown). We then examined the phosphorylation status of S6K in the rat brain after ECS using an anti-phospho-p70 S6K antibody. S6K was highly phosphorylated in the rat hippocampus before stimulation, and we were unable to find any further S6K phosphorylation (Fig. 1, arrowhead). However, four antibodies detected with five subbands (Fig. 1), and three of these showed density changes after ECS (upper, middle, and lower arrows). The antibody against S6K itself did not detect any protein bands coincident with these three proteins, indicating that these proteins might not be directly related to S6K. Because the phospho-specific antibody was generated against the serine- and threonine-phosphorylated peptide of S6K, we presumed that the increase in band intensities suggested the increased phosphorylation of serine and/or threonine residues in these proteins. Of the three bands, we focused on the middle protein, which was a doublet of 75 and 80 kDa. The phosphorylation of the doublet was so rapid that it only lasted 1 min after ECS in the rat hippocampus (Fig. 1). To identify the protein, we purified it from the brain lysates of ECS-treated rats (1 min after ECS) using the six-step procedure described under “Experimental Procedures.” During the purification steps, we lost the 80-kDa protein and, therefore, were only able to purify the 75-kDa protein to homogeneity (Fig. 2A, left panel). Because we traced the 75-kDa protein during the purification procedures by immunoblotting, we were unable to estimate purification fold and yield. Coomassie staining of the final preparation revealed two protein bands, a major band with molecular size of 75 kDa and a minor smaller molecular size band. These two proteins both reacted with the anti-phospho-p70 S6K antibody, and their band intensities coincided with the protein amounts (Fig. 2A, right panel), suggesting that the smaller protein might be a proteolytic product of the major protein. The major band was eluted from the gel and digested with trypsin. The masses of the tryptic peptides were measured by MALDI-TOF. The masses of 10 peptides were analyzed using a data base search program (NCBInr 02.15.00), and it was found that seven of these peptides matched those of rat moesin (data not shown). To confirm whether the purified 75-kDa protein was really a moesin, the purified protein was immunoblotted with a commercially available monoclonal antibody against human moesin (Figs. 2 and 4, α-ME). This antibody reacted with the lower band as well as the upper band, and the intensities corresponded well with the amount of purified protein (Fig. 2B, left panel). Although we did not identify the lower band of purified proteins by peptide analysis, these results imply that the lower band may have been caused by a cleavage of moesin during the purification.

Moesin Phosphorylation at Serine and Threonine in the Rat Hippocampus After ECS—After the purified protein had been identified as moesin, we examined the phosphorylation of the purified moesin. Because the anti-phospho-p70 S6K antibody we used was produced against a synthetic peptide dually phosphorylated at the Thr-421 and Ser-424 residues of S6K, we examined the phosphorylation of the serine and threonine residues of the purified moesin with the anti-phosphoserine and anti-phosphothreonine antibodies. Both the anti-phosphoserine and anti-phosphothreonine antibodies reacted with the purified moesin, indicating that the purified moesin was phosphorylated at both the serine and threonine residues (Fig. 2B, middle and right panels). Because the brain lysate used for the purification of the 75-kDa protein was prepared from rats 1 min after ECS, the results suggested that ECS might increase the phosphorylation of moesin in the rat hippocampus. To
determine whether this was the case, the moesin in the hippocampal lysates of sham and ECS-treated (1 min) rats were immunoprecipitated with anti-moesin monoclonal antibody and immunoblotted with polyclonal anti-phosphoserine and anti-phosphothreonine antibodies. We confirmed that the phosphorylation of moesin at both the serine and threonine residues indeed increased in the rat hippocampus 1 min after ECS (Fig. 2C).

**Phosphorylation of Moesin at Thr-558 in the Rat Hippocampus after ECS**—Having confirmed that the purified moesin from rat brain was phosphorylated at its serine and threonine residues, we examined the phosphorylation of moesin at Thr-558 in the rat hippocampus after ECS. Thr-558 is a well known phosphorylation site of moesin and is shared by other ERM protein family members (Thr-567 in ezrin and Thr-564 in radixin). The rat hippocampal lysates after ECS were examined with the phospho-Thr-558-specific antibody 297S (kindly provided by Professor Tsukita), which can detect all three ERM phosphopeptide (KYKpTLRQCCCCC) corresponding to the mouse moesin sequence from amino acids 555–561, which is shared by all three ERM proteins.

In addition, we produced a phospho-ThrERM (p-ThrERM)-specific antibody. The polyclonal antibody p-ThrERM was raised in rabbits against the KLH-conjugated synthesized phosphopeptide (KYKpTLRQCCCCC) corresponding to the mouse moesin sequence from amino acids 555–561, which is shared by all three ERM proteins.

To test the reactivity and specificity of this antibody, the lysates of Rat1 cells were immunoblotted after LPA stimulation. A protein band detected by the anti-p-ThrERM antibody increased in intensity 2 min after LPA stimulation, and this was sustained up to 30 min (Fig. 4C). The molecular size of the detected band was 75 kDa, and this coincided well with moesin when the blot was reprobed with the moesin antibody TM2. The temporal pattern of increased moesin phosphorylation was similar to that reported previously (17, 19), indicating that the phospho-specific antibody detected the Thr-558-phosphorylated form of moesin. To ensure that this phospho-Thr-558-specific antibody was specific for Thr-558-phosphorylated moesin and did not cross-react with unphosphorylated moesin, we immunoblotted the membrane and the cytosolic fractions of Rat1 cell lysates with p-ThrERM and TM2 antibodies. It was found that the phosphorylated moesin localized only to the

**FIG. 2. Purification and identification of the 75-kDa protein as moesin.**

A, purified 75-kDa protein was electrophoresed in 8% SDS-PAGE and stained with Coomassie Blue (left panel) or immunoblotted with anti-phospho-p70 S6 kinase antibody (right panel). B, purified protein was electrophoresed, transferred to a membrane, and immunoblotted with anti-moesin monoclonal (α-ME), antiphosphoserine (α-p-Ser) or anti-phosphothreonine (α-p-Thr) antibodies. C, 1 min after ECS or sham treatment the rats were sacrificed, and moesin was immunoprecipitated from the hippocampal lysates with the anti-moesin monoclonal antibody. The immunoprecipitates were fractionated by SDS-PAGE, immunoblotted with anti-phosphoserine and anti-phosphothreonine antibodies, and then stripped and reprobed with an anti-moesin polyclonal antibody (α-MR) as a protein content control. S, sham-treated animal; E1, 1 min after ECS. The results are representative of three independent experiments.

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**Production of Anti-moesin and Thr-558-phosphorylated Moesin-specific Antibodies**—To obtain the moesin-specific antibody, the purified full length of mouse moesin fused to GST was injected subcutaneously into two rabbits. As shown in Fig. 4A, the polyclonal antibody raised against whole moesin (designated as TM2) reacted only with moesin even in MDCK cells (which expressed large amounts of ezrin) and did not cross-react with ezrin or radixin, whereas the monoclonal antibody moesin (Figs. 2 and 4, α-ME) detected two protein bands with molecular sizes of 75 and 85 kDa, which were moesin and ezrin, respectively (Fig. 4A).

Because moesin was the major phosphorylated ERM protein in the rat hippocampus and the phosphoprotein we had purified from rat brain after ECS, we decided to focus on moesin phosphorylation.

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(4-fold that of the basal level) and returning to the base line 2 min after ECS (Fig. 3B). Because moesin was the major phosphorylated ERM protein in the rat hippocampus and the phosphoprotein we had purified from rat brain after ECS, we decided to focus on moesin phosphorylation.
through the release of neurotransmitters, so we studied whether glutamate could induce the phosphorylation of moesin in H19–7/IGF-IR-immortalized rat hippocampal progenitor cells. The anti-p-ThrERM antibody detected only one band in the lysate of the H19–7/IGF-IR cells, and this band corresponded to moesin. Like Rat1 cells, moesin was the predominant ERM protein in H19–7/IGF-IR cells, and the levels of ezrin and radixin were very low (Fig. 4A). The Thr-558 of moesin was phosphorylated in H19–7/IGF-IR cells even without glutamate stimulation, as has been described previously (16), but glutamate treatment rapidly increased moesin phosphorylation at Thr-558. This phosphorylation level was doubled in 2 min, was sustained to 30 min, and then declined slowly to reach the baseline 60 min after glutamate treatment (Fig. 5, A and B).

**RhoA and Rho kinase Are Involved in the Phosphorylation of Moesin by Glutamate**—The Rho family proteins RhoA, Rac1, and Cdc42 have been implicated in the phosphorylation of moesin in various cell lines. To determine whether the activation of RhoA, Rac1, or Cdc42 is involved in the phosphorylation of moesin at Thr-558, we examined the activities of RhoA, Rac1, or Cdc42 in H19–7/IGF-IR cells after glutamate treatment by measuring the amounts of RhoA bound to the GST-fused RhoA-binding domain of rho-kinase (GST-RBD) or Rac1/Cdc42 bound to the GST-fused Rac/Cdc42-binding domain of pak3 (GST-PBD). Glutamate induced a rapid increase in the amount of cellular GTP-bound RhoA in H19–7/IGF-IR cells but did not increase in the amount of cellular GTP-bound Rac1 (Fig. 6, A and B). The amount of GTP-bound Cdc42 was barely detectable in H19–7/IGF-IR cells even after glutamate treatment, suggesting that the activity of Cdc42 in this hippocampal progenitor cell line was very low and not increased by glut-
The phosphorylation of moesin at Thr-558 in H19–7/IGF-IR cells. A, serum-starved H19–7/IGF-IR cells were unstimulated or stimulated with 100 μM glutamate, and at 2, 5, 10, 30, or 60 min of incubation whole cell lysates were electrophoresed in SDS-PAGE and analyzed by immunoblotting with anti-p-ThrERM (α-p-ThrERM) or TM2 (α-TM2) antibodies. B, the intensity of phosphorylation and total moesin bands in A was quantitated by densitometric analysis, and the amounts of phosphorlated moesin were normalized to the amounts of total moesin. The data represent the means ± S.E. of four independent experiments. C, unstimulated H19–7/IGF-IR cells.

Among the downstream effectors of RhoA, Rho kinase has been reported to be involved in the phosphorylation of moesin in various non-neuronal cell lines. Therefore, we examined glutamate-induced moesin phosphorylation in H19–7/IGF-IR cells after pretreating with the Rho kinase inhibitor Y-27632. The moesin phosphorylation induced by glutamate treatment was completely suppressed in the presence of 30 μM Y-27632 (Fig. 8, A and B), suggesting that Rho kinase was involved in the phosphorylation of moesin at Thr-558. Although the inhibition of RhoA and Rho kinase abolished the phosphorylation of moesin at Thr-558 in H19–7/IGF-IR cells induced by glutamate, its inhibitory effect did not change the basal level of moesin phosphorylation, suggesting that there might be pathways of moesin phosphorylation not involving RhoA and Rho kinase. This was the same picture that emerged in the brain. ECS rapidly induced the phosphorylation of moesin in the rat hippocampus, but we also observed a basal phosphorylation of moesin. Further studies will be needed to elucidate the pathways involved in the basal phosphorylation of moesin at Thr-558 in H19–7/IGF-IR cells.
In the present study, we demonstrate that ECS induced the phosphorylation of moesin at Thr-558 in the rat hippocampus.

The phosphorylation and activation of signaling molecules in situ is not easy to study because it is difficult to find effective stimuli for tissues or organs. We have used ECS as a unique stimulus for the study of signaling molecule activation and the induction of genes in brain tissues. We have reported previously the activation of the Pyk2-Ras-Raf-MEK-ERK cascade and the activation of other signaling molecules such as CREB and stress kinases in the rat hippocampus after ECS (2, 3, 5–7). However, the mechanism of activation of these signaling molecules remains unknown. We assumed that glutamate, known to be released after ECS and/or depolarization induced by electricity, may be involved in the activation of these signaling molecules. Based on this assumption, we used a hippocampal progenitor cell H19–7/IGF-IR to study the effects of glutamate on the phosphorylation of moesin. Indeed, the glutamate treatment did induce the phosphorylation of moesin at Thr-558 in H19–7/IGF-IR cells, suggesting the possibility that ECS may induce the phosphorylation of moesin by the release of glutamate in the rat hippocampus. However, the temporal pattern of moesin phosphorylation by ECS in the rat hippocampus was quite different from that found for glutamate in H19–7/IGF-IR cells. Moesin phosphorylation was very rapid and transient in the rat hippocampus; it immediately increased after ECS and returned to the basal level after 2 min, whereas the phosphorylation lasted for 30 min after glutamate treatment in H19–7/IGF-IR cells. This kind of discrepancy was observed even in the activation of other signaling molecules such as ERKs. The phosphorylation of ERKs in the rat hippocampus by ECS was rapid and transient and returned to a basal level 5 min after ECS, whereas the activation of ERKs in rat hippocampal slice cultures lasted longer (3, 27). The reasons for these discrepancies are unknown, but they indicate that the activation and inactivation of signal transduction systems in the live brain are more dynamic than that in in vitro cultured cells.

ERM proteins are activated by C-terminal threonine phosphorylation, which maintains ERM proteins in an active state.

**Fig. 7.** RhoA is involved in the phosphorylation of moesin by glutamate. A, the suppression of glutamate-induced moesin Thr-558 phosphorylation by C3 transferase. Empty vector DNA (Vector)- or pcDNA C3 (C3)-transfected serum-starved cells were unstimulated or stimulated with 100 μM glutamate for 10 min, and whole cell lysates were electrophoresed in SDS-PAGE and analyzed by immunoblotting with anti-p-ThrERM (α-p-ThrERM) or TM2 (α-TM2) antibodies. Densitometric analysis was performed, and the data shown represent the means ± S.E. of four independent experiments. B, immunoblot detection of RhoA (lower arrow) and ADP-ribosylated RhoA (upper arrow) with the anti-RhoA antibody (α-RhoA) in lysates of H19–7/IGF-IR cells transfected with pcDNA empty vector or pcDNA C3 plasmids. C, an increase in the level of phosphorylated moesin by RhoAV14 is shown. H19–7/IGF-IR cells were transfected with pCMV-Myc RhoAV13 DNA (RhoAV14) or empty vector (Vector). Cells were then serum-starved and whole cell lysates were electrophoresed in SDS-PAGE and analyzed by immunoblotting with anti-p-ThrERM or TM2 (α-TM2) antibodies. The presence of RhoAV14 increased the basal level of moesin Thr-558 phosphorylation 2-fold in H19–7/IGF-IR cells. To ensure RhoAV14 expression, the blot was immunoblotted with an anti-Myc antibody (bottom panel). The results shown are representative of three independent experiments.

**Fig. 8.** Inhibition of moesin Thr-558 phosphorylation by Rho kinase inhibitor. A, serum-starved H19–7/IGF-IR cells were preincubated with the Rho kinase inhibitor Y-27632 (60 min, 30 μM) and then unstimulated or stimulated with 100 μM glutamate for 10 min. Whole cell lysates were electrophoresed by SDS-PAGE and analyzed by immunoblotting with anti-p-ThrERM (α-p-ThrERM) or TM2 (α-TM2) antibodies. A Rho kinase inhibitor suppressed the glutamate-induced moesin phosphorylation. B, densitometric analysis was performed, and the data shown represent the means ± S.E. of four independent experiments.

**DISCUSSION**

In the present study, we demonstrate that ECS induced the phosphorylation of moesin at Thr-558 in the rat hippocampus.
by suppressing intramolecular interactions (28). These proteins play a crucial role in the formation of microvilli, cell-to-cell adhesion, the maintenance of cell shape, cell motility, and membrane trafficking. Recent analyses have shown that ERM proteins are not only involved in the cytoskeletal organization but are also involved in signaling pathways. In neuronal cells, moesin and radixin are reported to be important for growth cone development and maintenance (29). However, the functional role of ERM proteins as well as their phosphorylation in mature neurons has not been elucidated. In this study, for the first time, we showed that the ERM protein moesin was phosphorylated in the rat hippocampus by ECS and that glutamate can induce the phosphorylation of moesin at Thr-558 by activating RhoA and Rho kinase. However, we have not studied the functional role of phosphorylated moesin. Thus, it remains to be elucidated whether phosphorylated moesin is also involved in the cytoskeletal organization of neuronal cells and/or other signaling pathways in neuronal tissues.

Although many cultured cells express all three ERM proteins, the amounts of ERM proteins widely vary from cell to cell. In epithelial cells, ezrin expression is much higher than moesin or radixin, and moesin is reported to predominate among ERM proteins in endothelial cells. In neuronal cells, moesin is highly expressed in PC12 cells and glioma cells, whereas the three ERM proteins are equally expressed in astrocytes (30–32). In the rat hippocampus and hippocampal progenitor cells, moesin predominated (Figs. 2A and 4A). Thus, moesin is the major phosphorylated ERM protein in the rat hippocampus after ECS and in H19–7/IGF-IR cells after glutamate treatment. In primary cultured neurons, moesin and radixin but not ezrin regulate growth cone development and cell motility (29). Thus, it seems likely that moesin and radixin are needed for neuronal development and that moesin may be the major ERM protein in terms of neuronal development.

It has been reported that the expression of constitutively active mutants of members of the small GTPase family, Rho, Rac, and Cdc42, induce phosphorylation on the C-terminal threonine residue of ERM proteins (33). In the present study, we found that glutamate transiently activates only RhoA and not Rac1 or Cdc42 in H19–7/IGF-IR cells and that the inhibition of RhoA resulted in the suppression of moesin phosphorylation induced by glutamate. Our results suggest that RhoA activation is involved in the phosphorylation of moesin at Thr-558 in hippocampal progenitor cells. Previously, LPA-induced RhoA activation was reported to induce the threonine phosphorylation of moesin and changes in the actin-based cytoskeleton of fibroblast cells (17). However, we did not observe any changes in either the morphology or the actin-based cytoskeleton in H19–7/IGF-IR cells after glutamate treatment (data not shown), which suggests that the activated RhoA in H19–7/IGF-IR cells may not induce cytoskeletal changes. In neuronal cells, the Rho signaling pathway has classically been implicated in axonal outgrowth, dendrogenesis, cell migration during neural development, exocytosis, and endocytosis (34). In addition, the Rho effector protein citron forms a heteromeric complex with PSD-95 and N-methyl-D-aspartate receptors and is concentrated at postsynaptic sites in hippocampal neurons (35). Although the functional roles of citron have not been defined, previous reports suggest its involvement in the synaptic function. Taken together, it is possible that glutamate-induced RhoA activation and activated RhoA-mediated phosphorylation of moesin in the rat hippocampus and neuronal cells may affect cellular functions other than cytoskeleton changes, such as synaptic vesicle recycling or glutamate receptor endocytosis. Thus, studies on the exact intracellular localizations of moesin and its phosphorylated form and on the proteins bound to them are needed to elucidate the function of moesin in neuronal cells.

Thr-558 of moesin was reported to be effectively phosphorylated in vitro by Rho kinase, phosphatidylinositol-4-phosphate 5-kinase, PKC-θ, and myotonic dystrophy kinase-related Cdc42-binding kinase (17, 21, 33, 36). However, the kinases for moesin phosphorylation in vivo remain controversial. Originally, Rho kinase was suggested to be a downstream kinase for the phosphorylation of ERM proteins by a constitutively active form of RhoA in Swiss 3T3 cells (37), and this was supported by the phosphorylation of moesin by the dominant Rho kinase in transfected COS cells (21). However, Rho kinase phosphorylated moesin light chain and not ERM proteins in glioma cells (38), and Matsui et al. (28) reported that the inhibition of phosphatidylinositol-4-phosphate 5-kinase, rather than Rho kinase, eliminated the threonine phosphorylation of ERM proteins in NIH 3T3 cells by LPA stimulation. It is not clear whether this discrepancy was caused by differences in the cell lines used for these studies. In the present study, the phosphorylation of moesin at Thr-558 by glutamate treatment in hippocampal progenitor cells was completely suppressed by the inhibitory effect of Rho kinase, which indicates that the phosphorylation is mediated by Rho kinase in this neuronal cell line.

Taken together, our results show that the phosphorylation of moesin at Thr-558 in hippocampal progenitor cells (H19–7/IGF-IR) by glutamate treatment is mediated by RhoA and Rho kinase. However, we do not exclude the possibility that kinases other than RhoA and Rho kinase may be involved in the phosphorylation of moesin in hippocampal neuronal cells. We observed a basal level of moesin phosphorylation in H19–7/IGF-IR cells and in the rat hippocampus. Moreover, this basal level of phosphorylation in H19–7/IGF-IR cells was not affected by the inhibition of RhoA or Rho kinase, suggesting that kinases other than Rho kinase are involved in the phosphorylation of moesin and that the activities of these other kinases are not affected by glutamate treatment. We will continue studying to identify those kinases responsible for the basal level of moesin phosphorylation in hippocampal neuronal cells.

In the present study, we identified the phosphorylation of moesin in the rat hippocampus after ECS with the anti-phospho-S6K antibody, which suggests a possible sequence homology between the amino acid sequences around the Thr-558 of moesin and those around the dual phosphorylation site, Thr-421 and Ser-424 of S6K. However, the examination of amino acid sequences revealed no homology between these two sequences, indicating that the detection of moesin phosphorylation by anti-phospho-S6K antibody was nonspecific and not amino acid sequence related.

We also observed the phosphorylation of serine residue(s) in the purified moesin from the rat brain. The phosphorylation of ERM proteins at the serine residue(s) has been reported but not extensively studied. As yet, the phosphorylated serine residue(s) of ERM proteins and the signaling system involved in the phosphorylation of the serine residue(s) remain unknown. Although ECS increased the phosphorylation at the serine residue(s) of moesin, we did not observe serine phosphorylation in moesin in H19–7/IGF-IR cells after glutamate treatment (data not shown). The intensities of the stimuli may explain the difference. The increase of moesin phosphorylation in the rat hippocampus by ECS was at least twice that observed in H19–7/IGF-IR cells by glutamate. Therefore, glutamate stimulation may not be strong enough to induce the phosphorylation of moesin at serine residue(s), and a second stimulus such as depolarization may be needed to activate the signaling system for the phosphorylation of moesin at serine residue(s) in the rat.
brain. Further studies on the phosphorylation of ERM proteins at serine residue(s) are needed to elucidate the mechanism of serine phosphorylation in moesin.

In conclusion, we demonstrate the phosphorylation of moesin at Thr-558 by ECS in the rat hippocampus and by glutamate treatment in the hippocampal progenitor cells H19–7/IGF-IR. Glutamate also activated RhoA in H19–7/IGF-IR cells, and this moesin phosphorylation was mediated by RhoA and Rho kinase. To our knowledge, this is the first report on the phosphorylation of moesin in neuronal tissues. However, the signaling pathways involved in the activation of RhoA by glutamate remain to be elucidated.

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REFERENCES
1. Fochtmann, L. J. (1994) *Psychopharmacol. Bull.* 30, 321–444
2. Kang, U. G., Jeon, S. H., Lee, J. E., Joo, Y.-H., Yi, J. S., Park, J.-B., Juhn, Y.-S., and Kim, Y. S. (2000) *Neuropharmacology* 39, 703–706
3. Kang, U. G., Hong, K. S., Jung, H. Y., Kim, Y. S., Seong, Y. S., Yang, Y. C., and Park, J. B. (1994) *J. Neurochem.* 63, 1979–1982
4. Lee, Y. H., Ryu, S. H., Suh, P. G., Park, J. B., Ahn, Y. M., and Kim, Y. S. (1993) *Biochem. Biophys. Res. Commun.* 194, 665–670
5. Oh, S. W., Abu, Y. M., Kang, U. G., Kim, Y. S., and Park, J. B. (1999) *Neurosci. Lett.* 271, 101–104
6. Jeon, S. H., Seong, Y. S., Juhn, Y. S., Kang, U. G., Ha, K. S., Kim, Y. S., and Park, J. B. (1997) *Neuropsychopharmacology* 30, 411–414
7. Jeon, S. H., Oh, S. W., Kang, U. G., Ahn, Y. M., Bae, C. D., Park, J. B., and Kim, Y. S. (2001) *Biochem. Biophys. Res. Commun.* 282, 1026–1030
8. Bretscher, A., Recek, D., and Berryman, M. (1997) *J. Cell Sci.* 110, 3011–3018
9. Tsukita, S., and Yonemura, S. (1997) *Curr. Opin. Cell Biol.* 9, 70–75
10. Yabuki, T., Carpen, O., Heiska, L., Hander, T. S., Jaaskelainen, J., Majander-Nordenswan, P., Sainio, M., Timonen, T., and Turunen, O. (1997) *Curr. Opin. Cell Biol.* 9, 659–666
11. del Pozo, M. A., Nieto, M., Serrador, J. M., Sancho, D., Vicecante-Manzanares, M., Martinez, C., and Sanchez-Madrid, F. (1999) *Cell Adhes. Commun.* 6, 125–133
12. Mangeat, P., Roy, C., and Martin, M. (1999) *Trends Cell Biol.* 9, 187–192
13. Bretscher, A. (1999) *Curr. Opin. Cell Biol.* 11, 109–116
14. Bretscher, A. (1989) *J. Cell Biol.* 108, 921–930
15. Krieg, J., and Hunter, T. (1992) *J. Biol. Chem.* 267, 19258–19265
16. Nakamura, F., Amiura, M. R., and Furthmayr, H. (1995) *J. Biol. Chem.* 270, 31377–31385
17. Matsu, T., Maeda, M., Doi, Y., Yonemura, S., Amano, M., Kaibuchi, K., Tsukita, S., and Tsukita, S. (1998) *J. Cell Biol.* 140, 647–657
18. Mackay, D. J., and Hall, A. (1998) *J. Biol. Chem.* 273, 20685–20688
19. Shaw, R. J., Hary, M., Solomon, F., and Jacks, T. (1998) *Mol. Biol. Cell* 9, 403–419
20. Mackay, D. J., Ech, F., Furthmayr, H., and Hall, A. (1997) *J. Cell Biol.* 138, 927–938
21. Oshiro, N., Fukata, Y., and Kaibuchi, K. (1998) *J. Biol. Chem.* 273, 34663–34666
22. Gonzalez-Agosti, C., and Solomon, F. (1996) *Cell Motil. Cytoskeleton* 34, 122–136
23. Ren, X. D., Kiess, W. B., and Schwartz, M. A. (1999) *EMBO J.* 18, 378–385
24. Pende, M., Fisher, T. L., Simpson, P. B., Russell, J. T., Blenis, J., and Gallo, V. (1997) *J. Neurosci.* 17, 1291–1301
25. Rowley, H. L., Martin, K. F., and Marsden, C. A. (1995) *Neuroscience* 68, 415–422
26. Aktories, K., and Hall, A. (1989) *Trends Pharmacol. Sci.* 10, 415–418
27. Baron, C., Benes, C., Van Tan, H., Fagard, R., and Roisin, M. P. (1996) *J. Neurochem.* 66, 1005–1010
28. Matsu, T., Yonemura, S., and Tsukita, S. (1999) *Curr. Biol.* 9, 1259–1262
29. Paglini, G., Kunda, P., Quiroga, S., Kosik, K., and Caceres, A. (1998) *J. Cell Biol.* 143, 443–455
30. Franck, Z., and Bretscher, A. (1999) *Curr. Biol.* 9, 1025–1043.
31. Schwartz-Albiez, R., Merling, A., Spring, H., Moller, P., and Koretz, K. (1995) *Europ. J. Cell Biol.* 67, 189–198
32. Amieva, M. R., and Furthmayr, H. (1995) *Exp. Cell Res.* 219, 180–196
33. Nakamura, N., Oshino, N., Fukata, Y., Amano, M., Fukata, M., Kuroda, S., Matsuura, Y., Leung, T., Lim, L., and Kaibuchi, K. (2000) *Genes Cells* 7, 557–561
34. Van Aelst, L., and D'Souza-Schorey, C. (1997) *Genes Dev.* 11, 2295–2322.
35. Zhang, W., Vazquez, L., Apperson, M., and Kennedy, M. B. (1999) *J. Neurosci.* 19, 96–108
36. Pietromonaco, S. F., Simons, P. C., Altman, A., and Elias, L. (1998) *J. Biol. Chem.* 273, 7594–7603
37. Fukita, Y., Kimura, K., Oshiro, N., Saya, H., Matsuura, Y., and Kaibuchi, K. (1999) *J. Cell Biol.* 141, 409–418
38. Kosako, H., Yoshida, T., Matsuura, F., Ishizaki, T., Narumiya, S., and Inagaki, M. (2000) *Oncogene* 19, 6659–6666

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