Differential Effects of a Calcineurin Inhibitor on Glutamate-induced Phosphorylation of Ca\(^{2+}\)/Calmodulin-dependent Protein Kinases in Cultured Rat Hippocampal Neurons*

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Calcium/calmodulin-dependent protein kinases (CaM kinases) are major multifunctional enzymes that play important roles in calcium-mediated signal transduction. To characterize their regulatory mechanisms in neurons, we compared glutamate-induced phosphorylation of CaM kinase IV and CaM kinase II in cultured rat hippocampal neurons. We observed that dephosphorylation of these kinases followed different time courses, suggesting different regulatory mechanisms for each kinase. Okadaic acid, an inhibitor of protein phosphatase (PP) 1 and PP2A, increased the phosphorylation of both kinases. In contrast, cyclosporin A, an inhibitor of calcineurin, showed different effects: the phosphorylation and activity of CaM kinase IV were significantly increased with this inhibitor, but those of CaM kinase II were not significantly increased. Cyclosporin A treatment of neurons increased phosphorylation of Thr\(^{196}\) of CaM kinase IV, the activated form with CaM kinase IV, which was recognized with an anti-phospho-Thr\(^{196}\) antibody. Moreover, recombinant CaM kinase IV was dephosphorylated and inactivated with calcineurin as well as with PP1, PP2A, and PP2C in vitro. These results suggest that CaM kinase IV, but not CaM kinase II, is directly regulated with calcineurin.

Calcium (Ca\(^{2+}\))/calmodulin (CaM)\(^{1}\) transduces increases in intracellular Ca\(^{2+}\) concentration. These increases activate Ca\(^{2+}\)-dependent molecules involved in signaling, such as calcium/calmodulin-dependent protein kinases (CaM kinases). Among the six types of known CaM kinases (namely, myosin light chain kinase, phosphorylase kinase, and CaM kinases I, II, III, and IV), CaM kinase II has been the most extensively characterized. It is abundantly expressed in the brain and plays an important functional role in hippocampal long-term potentiation (1–4). After activation by Ca\(^{2+}\)/CaM (5), CaM kinase II shows Ca\(^{2+}\)-independent activity, which prolongs its activity even after intracellular Ca\(^{2+}\) concentrations return to basal levels. CaM kinase IV is also abundant in the brain, including the cerebral cortex, the hippocampus, and particularly the nuclei of cerebellar granule cells (6–10). Because of its expression in the latter, this enzyme is also called CaM kinase Gr (granule). In addition, CaM kinase IV is found in the thymus and testis (7), suggesting functional importance not only in the brain but in the immune system and reproductive cells. The genomic structure of CaM kinase IV is quite unique: both CaM kinase IV and calsporin, a male germ cell-specific CaM-binding protein, are derived from a single CaM kinase IV gene (7, 11). CaM kinase IV has a broad substrate specificity and is thought to be multifunctional (8), as is CaM kinase II. Although less is known about the function of CaM kinase IV than that of CaM kinase II, recent work shows important biochemical and molecular differences between both kinases. CaM kinase IV functions as a monomer rather than an oligomer (8). CaM kinase IV requires phosphorylation at Thr\(^{196}\) with another CaM kinase, CaM kinase kinase, for full activation (12–15), rather than autophosphorylation, as typically observed in CaM kinase II. CaM kinase IV is localized in the nuclei of neurons (9), possibly associating with chromatin by the polyglutamate sequence in its C-terminal region, whereas the limited isoforms of CaM kinase II (\(\alpha_2\) and \(\delta_2\) isoforms) exist in the nuclei. The nuclear localization of CaM kinase IV suggests a role in the regulation of gene expression. CaM kinase IV can phosphorylate Ser\(^{133}\), the essential site for activation of CREB (16), which is essential for the activation of CRE-containing promoters. In an assay using cultured hippocampal neurons, it was reported that CREB phosphorylation induced by synaptic stimulations was mediated with CaM kinase IV (17). Moreover, Ca\(^{2+}\)-induced expression of brain-derived neurotrophic factor that has a CRE motif in its promoter region (18) was reported to be mediated by CaM kinase IV (19), suggesting that Ca\(^{2+}\)- and CRE-mediated gene expression is controlled with CaM kinase IV in vivo. Given the functional differences in these kinases, it is likely that CaM kinase IV and CaM kinase II are regulated by different mechanisms.

To characterize the differences between CaM kinase IV and CaM kinase II in the central nervous system and focus on the differences in the regulatory mechanisms of these kinases, we investigated glutamate-induced phosphorylation and the effects of protein phosphatase (PP) inhibitors, using primary cultured rat hippocampal pyramidal neurons. We also examined the susceptibility of recombinant CaM kinase IV to protein phosphatases.
**Regulation of CaM Kinase IV with Calcineurin**

**EXPERIMENTAL PROCEDURES**

**Materials**—The following chemicals and materials were obtained from the indicated sources: [γ-32P]ATP and [32P]orthophosphate, ICN; calycin A, okadaic acid, and cyclosporin A, Wako; CNQX, AP3, and MK801, Tocris Cookson; protein A-Sepharose CL-4B and glutathione-Sepharose 4B, Pharmacon Biotech; recombinant human protein phosphatase 1 catalytic subunit, Boehringer Mannheim; rat brain protein phosphatase 2A α holoenzyme, purified according to the method of Barnes et al. (20) in our laboratory; bovine purified calcineurin, UBI; and recombinant rat protein phosphatase 2C, a generous gift from Dr. S. Tamura (Tohoku University, Institute of Development, Aging and Cancer, Sendai, Japan) (21).

**Preparation of Polyclonal Antibodies**—The following peptides derived from rat CaM kinase IV were synthesized commercially by Fujiya and sequenced: mouse homolog of the C terminus of rat brain protein phosphatase 1A holoenzyme, (PQQDAILPEY), (b) Thr196-phosphorylated peptides that correspond to amino acids 184–197 (LSKIVEHQVLMKT(p)V), (b) Thr196-phosphorylated CaM kinase IV antibody (anti-pT196) was purified using an affinity peptide column using the SulfoLink kit (Pierce) according to the manufacturer’s protocol. Preparation of the anti-CaM kinase IV polyclonal antibody was reported previously (22). Cultures of hippocampal pyramidal neurons were grown and transfected into C6 cells using a calcium phosphate method. Antibodies were raised against peptides corresponding to amino acids 184–197, 185–196, 195–206, and 194–205 of CaM kinase IV. The antibodies were purified on the immobilized peptide.

**Cell Stimulation with Glutamate**— Cultures were stimulated using the following protocol. Culture medium was aspirated, and the neurons were preincubated with KRH buffer (23) for 30 min and then stimulated with 10 μM glutamate and 10 μM glycine for indicated periods. In the experiments using the phosphatase inhibitors, 5 μM cyclosporin A (CsA) was added throughout the preincubation and stimulation or 1 μM okadaic acid was added for 15 min of the preincubation and throughout the stimulation. Cells were frozen in liquid nitrogen immediately after stimulation, harvested with 1X-100, 10 mM EDTA, and 1 mM dithiothreitol, and homogenized with brief bursts of ultrasonication, followed by centrifugation at 4 °C for 10 min with gentle tapping. Reactions were terminated by centrifugation of the immunocomplex. Aliquots of the supernatants were spotted onto Whatman P81 P-cellulose paper. The papers were washed in 75 mM phosphoric acid buffer, and the radioactivity on the paper was measured by scintillation spectrometry.

**Preparation of GST-Fusion Proteins**—cDNAs encoding rat CaM kinase IV α (7) and CaM kinase IV α (15) were amplified from rat brain total RNA by reverse transcription-polymerase chain reaction using Tth XL DNA polymerase (Perkin-Elmer) and expressed as fusion proteins with GST as described below. The primer sequences are as follows (5’ to 3’): (a) CaM kinase IV 5′ primer, GAGTCTCGAGGCGAGGAAGGCTGGGTTAGTACTCTGG; (b) CaM kinase IV 3′ primer, CCTGGGCCCCTCGAGGAAGCTGGGTTAGTACTCTGG. CaM kinase 5′ primer, ACGAGGAATTCCTCCACGGAGCTGAAGTGATGGAGCG; and (c) CaM kinase IV 3′ primer, ATTCGAATTCGCTCCCTGGTCATGCGCTGCG.

**Immuno blot Analysis**—The cell lysate, brain lysate, or fusion proteins were mixed with Laemmli’s SDS-sample buffer (24) and boiled for 5 min. Samples were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a nitrocellulose membrane at 70 V for 3.5 h using a Trans Blot Cell (Bio-Rad) in a cold room (4 °C). After blocking for 1 h at room temperature with TBS-T solution containing 2.5% bovine serum albumin, the membranes were incubated overnight at 4 °C with the first antibody diluted with TBS-T and bovine serum albumin (1:1000 for the anti-CaM kinase IV C-terminal antibody and 1:5 for the anti-pT196 antibody). Bound antibodies were visualized with 1/1,000 dilution of protein A-HRP (1.0 μg/ml) in TBS-T and 0.05% Tween 20, followed by exposure to X-ray film after development with Enhanced Phosphorimager (Amersham). The exposed film was then prepared as a sample for SDS-PAGE. After SDS-PAGE, the gels were dried and analyzed by a Bio-Imaging Analyzer (FLA-2000, Fuji Film).

**Assay for Immunoprecipitated CaM Kinase IV**—Neurons were stimulated and homogenized, and the immunoprecipitation was performed with the anti-CaM kinase IV antibody as described above, except for the following: After CaM kinase IV immunoprecipitation, the immunocomplex was washed four times with buffer containing of 50 mM HEPES, pH 7.5, 1 mM EDTA, and 1 mM dithiothreitol. Immunoprecipitated CaM kinase IV was assayed using peptide-γ (8) (synthesized commercially by Fujuya) in 100 μl of a total volume of the reaction mixture containing 50 mM HEPES (pH 7.5), 10 mM Mg(OAc)2, 0.5 mM CaCl2, 0.3 μM CaM, 40 μM peptide-γ, and 1 μM [γ-32P]ATP (100–5000 cpm/pmol) at 30 °C for 4 min. A 5% of total reaction volume was used as a substrate.

**Phosphorylation of Fusion Proteins**—Approximately 250–500 μg of recombinant CaM kinase IV were incubated at 30 °C for 60 min with approximately 10 μM of recombinant CaM kinase IV in buffer containing 50 mM HEPES (pH 7.5), 10 mM Mg(OAc)2, 0.5 mM CaCl2, 0.3 mM CaM, 0.3 mg of bovine serum albumin, and 0.1 mM [γ-32P]ATP (3000–5000 cpm/pmol) in a total volume of 50 μl. The reactions were terminated by the addition of 20 μl of SDS-sample buffer (24) followed by centrifugation at 10,000 × g for 10 min to exclude insoluble materials. 5% of total reaction volume was used as a substrate.
RESULTS

Characterization of Anti-CaM Kinase IV Polyclonal Antibodies—We prepared a rabbit anti-rat CaM kinase IV polyclonal antibody. This antibody recognized a 63-kDa protein (CaM kinase IVa) found in a rat hippocampal lysate and a 90-kDa GST-fusion protein of rat CaM kinase IV (Fig. 1A). Using the antibody, we performed immunoprecipitation of the cell lysates obtained from 32P-labeled hippocampal neurons (Fig. 1B). In our previous study (23), we demonstrated that the activation of CaM kinase II by glutamate stimulation was mediated by a NMDA-type glutamate receptor. We examined whether this observation was also applicable to the activation of CaM kinase IV. The incorporation of phosphate into the 63-kDa protein was significantly increased by stimulation with glutamate for 3 min (182.6 ± 10%; n = 4) compared with a control and was reduced by the application of 20 μM MK801 (a noncompetitive inhibitor of NMDA-type glutamate receptor; 92.7 ± 2% of control) (Fig. 1B), indicating that phosphorylation of CaM kinase IV depended on NMDA-type glutamate receptors. The application of MK801 without glutamate did not show significant changes in the phosphorylation levels of CaM kinase II and CaM kinase IV compared with controls (89% and 87%, respectively). Other inhibitors of glutamate receptors (20 μM CNQX (a specific inhibitor of AMPA-type glutamate receptors; 212.7 ± 11%) and 1 mM AP5 (an inhibitor of metabotropic glutamate receptors; 191.5 ± 7%) did not show inhibitory effects on CaM kinase IV phosphorylation.

We also raised an antibody against CaM kinase IV phosphorylated at Thr196 with CaM kinase kinase designated anti-pT196. This antibody recognized the 63-kDa protein in rat hippocampal lysate (Fig. 1A, lane 1), 0.1 μg of GST-CaM kinase IV (lanes 2 and 7), 50 μg of rat hippocampal lysate treated with 1 mM EGTA for 5 min (lane 3), 50 μg of rat hippocampal lysate treated with Ca2+/CaM for 1 min (lane 4), 3 min (lane 5), and 10 min (lanes 6 and 9) and 0.1 μg of GST-CaM kinase IV phosphorylated with GST-CaM kinase kinase (lanes 8 and 10). The minor bands below CaM kinase IV may be degradation products because these bands disappeared when the anti-pT196 antibody was preabsorbed with antigen (lane 9). Left, markers (MW) indicate a molecular mass of 116, 97.4, 66, and 45 kDa (from top to bottom).
ward. In contrast, the glutamate-induced phosphorylation of CaM kinase II was relatively sustained without CsA after phosphorylation (Fig. 2B and inset). The difference in the phosphorylation level of CaM kinase IV between 3 and 10 min was significant \((p < 0.01, \text{Student's } t \text{ test})\), whereas that of CaM kinase II was not significant. These results suggest the existence of different dephosphorylation mechanisms for CaM kinase IV and CaM kinase II.

The Effects of CsA on Phosphorylation—To test the above-mentioned idea, we examined the effects of protein phosphatase inhibitors using these points of the time in the following experiments. After a 30-min pretreatment of cells with 5 \(\mu M\) CsA, an inhibitor of calcineurin, glutamate-induced phosphorylation of CaM kinase IV increased significantly (Fig. 2A), whereas CaM kinase II phosphorylation was not significantly affected (Fig. 2B). The addition of CsA alone had no effect on the phosphorylation of either CaM kinase IV (Fig. 2A) or CaM kinase II (Fig. 2B). These results suggest that calcineurin is

![Fig. 2](image-url)
The phosphorylation of Thr196 of CaM kinase IV is regulated with calcineurin and protein phosphatase 1 (Fig. 2, C and D), showing that both enzymes are regulated with protein phosphatase 1 and/or 2A. The effects of CsA on CaM kinase IV activity has not been reported previously. We examined whether increased phosphorylation of both CaM kinase IV and CaM kinase II (Fig. 2, C and D), showing that both enzymes are regulated with protein phosphatase 1 and/or 2A. Protein phosphatase 2A has been reported to regulate both CaM kinase II (25) and CaM kinase IV (13, 26) and to be associated with CaM kinase IV (27). The increased level of CaM kinase IV phosphorylation after treatment with okadaic acid was about 2-fold higher than that seen after treatment with CsA alone. It seems that CaM kinase IV is more strictly regulated with okadaic acid-sensitive protein phosphatases than with CsA-sensitive protein phosphatases.

Effects of CsA on the Activity of CaM Kinase IV—Although the regulation of CaM kinase IV with an okadaic acid-sensitive protein phosphatase (e.g. PP2A) has been reported previously (26), the effect of CsA on CaM kinase IV activity has not been reported previously. We examined whether increased phosphorylation of CaM kinase IV by CsA treatment resulted in an increase in CaM kinase IV activity. As shown in Fig. 3, a 3-min application of glutamate to neurons significantly increased CaM-dependent CaM kinase IV activity. In comparison, cells stimulated for 10 min showed decreased activity. Moreover, CaM kinase IV activity after stimulation with glutamate plus CsA for both 3 and 10 min were significantly increased compared with those of cells stimulated for the same periods with glutamate or CsA alone. Because the phosphorylation of Thr196 of CaM kinase IV with CaM kinase kinase significantly increased both CaM-dependent and -independent activities in vitro (15, 28), the results indicate that phosphorylation of Thr196 of CaM kinase IV and subsequent autophosphorylation in the N terminus may occur. Thus, calcineurin is involved in the dephosphorylation of at least Thr196.

Examination of the Phosphorylation of Thr196 of CaM Kinase IV—Phosphorylation of CaM kinase IV by treatment with glutamate alone and glutamate plus CsA increased the kinase activity, as described above (Fig. 3). Because this finding indicates that Thr196 of CaM kinase IV is phosphorylated, we asked whether the phosphorylation level of Thr196 of CaM kinase IV increases after treatment with glutamate and/or CsA using the anti-pThr196 antibody. CsA alone had no effect on the phosphorylation of Thr196 of CaM kinase IV. Treating neurons with glutamate significantly increased phosphorylation, and an additional increase was observed when neurons were treated with glutamate plus CsA (Fig. 4). The total protein levels of CaM kinase IV were not altered by stimulation with glutamate and/or CsA. These results show that Thr196 of CaM kinase IV is phosphorylated with CaM kinase kinase in response to glutamate and/or CsA.

In Vitro Dephosphorylation of Recombinant CaM Kinase IV with Protein Phosphatases—To further examine whether CaM kinase IV is phosphorylated with CaM kinase kinase and dephosphorylated with protein phosphatases, recombinant GST fusion of CaM kinase kinase and CaM kinase IV were used (Fig. 5). GST-CaM kinase IV phosphorylated with CaM kinase kinase with an incorporation of 43 pmol phosphate/µg CaM kinase IV was dephosphorylated with protein phosphatase 1, protein phosphatase 2A, calcineurin, and protein phosphatase 2C in a time-dependent manner (Figs. 5, A and B). To quantitate the relative potency of each protein phosphatase activity, we adjusted the protein levels of protein phosphatases to that which can dephosphorylate an equivalent amount of phosphate from microtubule-associated protein 2. Among the protein phosphatases tested, protein phosphatase 1 was the most active in dephosphorylating CaM kinase IV phosphorylated with CaM kinase kinase, followed by calcineurin and protein phosphatases 2A and 2C (Fig. 5B).
phosphorylation of Thr\textsuperscript{196} with CaM kinase kinase, these results show that protein phosphatase 1, protein phosphatase 2A, calcineurin, and protein phosphatase 2C can each dephosphorylate this site and regulate kinase activity.

**DISCUSSION**

In previous reports, we have investigated the activation of CaM kinase II by autophosphorylation in response to neurotransmitters, growth factors, and hormones in various cells (rat embryo fibroblasts (29), hippocampal neurons (23), cerebellar granule cells (5), NG 108-15 cells (30), hippocampal slices (1), rat cortical astrocytes (31), MIN 6 cells (32), and bovine adrenal chromaffin cells (33)). This study describes the activation of CaM kinase IV in hippocampal neurons by stimulation with an excitatory neurotransmitter, glutamate. In this connection, Bito \textit{et al.} (17) reported that CREB phosphorylation caused by synaptic stimulation or by high potassium-induced depolarization was mediated by CaM kinase IV activation in hippocampal neurons. Furthermore, Park and Soderling (26) reported activation of CaM kinase IV in Jurkat T lymphocytes by stimulation of the antigen receptor CD3. Thus, CaM kinase IV as well as CaM kinase II in living cells is activated in response to neurotransmitters. This is the first report to show directly that CaM kinase IV is activated in neurons by glutamate stimulation, an observation lending support to the idea that CaM kinase IV is involved in \(\text{Ca}^{2+}\)-mediated gene expression.

Although CsA alone had no effect on the phosphorylation of CaM kinase IV in cultured hippocampal neurons, the combination of glutamate and CsA greatly increased its phosphorylation compared with the increases observed with glutamate alone (Fig. 2A). In contrast, no difference in phosphorylation of CaM kinase II was observed when stimulation with glutamate alone was compared with stimulation with glutamate plus CsA. This finding suggested that calcineurin could dephosphorylate CaM kinase IV but not CaM kinase II. The CaM kinase II results were consistent with \textit{in vitro} observations made in a previous report (34). Recombinant GST-CaM kinase IV phosphorylated with GST-CaM kinase kinase using cold ATP was dephosphorylated with control (none), PP1, PP2A, calcineurin, and PP2C for 30 min. After incubation, GST-CaM kinase IV was immunoprecipitated with the anti-CaM kinase IV antibody, and the immunocomplexes were assayed for CaM kinase IV activity as described under "Experimental Procedures." \(n = 4, * \), \(p < 0.01\) (Student’s \(t\) test).

**FIG. 5.** \textit{In vitro} dephosphorylation of GST-CaM kinase IV and correlation of activity and dephosphorylation. A, 1 \(\mu\text{g}\) of GST-CaM kinase IV phosphorylated with \(^{32}\text{P}\)ATP by GST-CaM kinase was dephosphorylated with protein phosphatases as indicated for 0, 3, 10, and 30 min. CaMKIV, CaM kinase IV. After incubation, the reaction mixtures were subjected to SDS-PAGE as duplicate samples, followed by autoradiography. Minor bands with molecular masses lower than that of GST-CaM kinase IV may be degradation products. B, protein phosphatase activities were assayed for \(^{32}\text{P}\)-labeled phosphorylated GST-CaM kinase IV with protein phosphatase 1 (●), protein phosphatase 2A (▲), calcineurin (■), and protein phosphatase 2C (■) during a time course. C, a sample incubated without protein phosphatases. Values represent the means of triplicate determinations. Similar experiments were performed at least three times, and a representative experiment is shown. C, effects of dephosphorylation on phosphorylated GST-CaM kinase IV activity. One \(\mu\text{g}\) of GST-CaM kinase IV phosphorylated with GST-CaM kinase kinase using cold ATP was dephosphorylated with control (none), PP1, PP2A, calcineurin, and PP2C for 30 min. After incubation, GST-CaM kinase IV was immunoprecipitated with the anti-CaM kinase IV antibody, and the immunocomplexes were assayed for CaM kinase IV activity as described under "Experimental Procedures." \(n = 4, * \), \(p < 0.01\) (Student’s \(t\) test).
may be due to the difference in the isoforms of protein phosphatase 1; they used an α-isofrom, whereas we used a human γ-isofrom that was purchased commercially. Because both of the catalytic subunits of protein phosphatase 1 are recombinant, the preparations have no contamination by other protein phosphatases.

CaM kinase IV has multiple autophosphorylation sites in the N-terminal region (37) and in a calmodulin-binding domain in the C-terminal region (38) in addition to the Thr196 phosphorylated with CaM kinase IV. Although the relationship between kinase activity and N-terminal autophosphorylation is not yet clearly understood, C-terminal autophosphorylation was reported to cause inactivation of CaM kinase IV (38). Because the physiological significance of this phosphorylation is still unclear, it is important to determine the protein phosphatases responsible for the dephosphorylation of the auto-phosphorylated C-terminal because dephosphorylation of this site may reactivate CaM kinase IV.

Because it was shown in this study that CaM kinase IV was dephosphorylated with calcineurin (Fig. 5), the interaction between CaM kinase IV and calcineurin is predicted to occur in neurons. CaM kinase IV, which is primarily localized to the nuclei of neurons (17), has also been seen in the axons of neurons (9). Although calcineurin is thought to be localized to the cytoplasm, it has been reported that calcineurin may translocate to the nucleus with NF-AT after immunycin treatment (39), which shows Ca2+-mediated translocation of calcineurin to the nucleus. It is unclear how the phosphorylation state of nuclear CaM kinase IV compares with that of the putative cytoplasmic form and whether translocation of calcineurin with glutamate stimulation occurs in neurons. How these enzymes interact should be elucidated in a further study.

This study focused on the dephosphorylation of CaM kinase IV in cultured hippocampal neurons. Because CaM kinase IV is activated with CaM kinase in a Ca2+-CaM-dependent manner, CaM kinase IV is also activated in response to glutamate. Because it was reported that CaM kinase IV showed strong autophosphorylation in the presence of Ca2+/CaM (40), we do not exclude the possibility that activation of CaM kinase IV may be controlled by the regulation of CaM kinase IV with protein phosphatases.

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