Phosphorylation of the α-Amino-3-hydroxy-5-methylisoxazole-4-propionic Acid Receptor GluR1 Subunit by Calcium/Calmodulin-dependent Kinase II*

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Modulation of α-amino-3-hydroxy-5-methylisoxazole-4-propionic Acid (AMPA) receptors in the brain by protein phosphorylation may play a crucial role in the regulation of synaptic plasticity. Previous studies have demonstrated that calmodulin (CaM) kinase II can phosphorylate and modulate AMPA receptors. However, the sites of CaM kinase phosphorylation have not been unequivocally identified. In the current study, we have generated two phosphorylation site-specific antibodies to analyze the phosphorylation of the glutamate receptor GluR1 subunit. These antibodies recognize GluR1 only when it is phosphorylated on serine residues 831 or 845. We have used these antibodies to demonstrate that serine 831 is specifically phosphorylated by CaM kinase II in transfected cells expressing GluR1 as well as in hippocampal slice preparations. Two-dimensional phosphopeptide mapping experiments indicate that Ser-831 is the major site of CaM kinase II phosphorylation on GluR1. In addition, treatment of hippocampal slice preparations with phorbol esters and forskolin increases the phosphorylation of serine 831 and 845, respectively, indicating that protein kinase C and protein kinase A phosphorylate these residues in hippocampal slices. These results identify the site of CaM kinase phosphorylation of the GluR1 subunit and demonstrate that GluR1 is multiply phosphorylated by protein kinase A, protein kinase C, and CaM kinase II in situ.

Long lasting changes in the efficiency of synaptic transmission are thought to underlie many forms of learning and memory. Two cellular models for learning and memory, long-term potentiation (LTP) and long-term depression (LTD), have recently been the subject of intense investigation (1–3). LTP is the long-term increase in excitatory synaptic transmission between neurons after a brief high frequency stimulus. In contrast, LTD is the long-term decrease in excitatory synaptic transmission following low frequency stimulation. The modulation of excitatory synaptic transmission during LTP and LTD has been reported to be due to changes in the presynaptic release of the neurotransmitter glutamate (4–6) or alternatively, to changes in the sensitivity of the postsynaptic glutamate receptors (7–9). Although the molecular mechanisms underlying LTP and LTD are not completely understood, postsynaptic calcium entry and protein phosphorylation and dephosphorylation have been shown to play essential roles in the induction and maintenance of LTP and LTD (1–3). For example, specific inhibitors of various protein kinases such as calcium/calmodulin-dependent (CaM) kinase II (10–12), PKC (10, 13), and protein tyrosine kinases (14) have been shown to block the induction of LTP, while protein phosphatase inhibitors have been shown to block the induction of LTD (15, 16). In addition, gene targeting techniques have demonstrated that mice lacking CaM kinase II (17), PKC-γ (18), or the tyrosine kinase fyn (19) do not have normal LTP and have certain deficits in spatial learning tasks. Recent experiments have focussed on a central role of CaM kinase II in the induction and maintenance of LTP (17, 20, 21). However, the substrates for the various kinases and phosphatases that mediate changes in synaptic transmission have not been identified.

Neurotransmitter receptors mediate signal transduction at the postsynaptic membrane of synapses in the central and peripheral nervous system. Protein phosphorylation of neurotransmitter receptors has been shown to be a major mechanism in the regulation of their function (22–24). Phosphorylation of the nicotinic acetylcholine receptor, GABA_A receptors, and glutamate receptors regulates a diverse set of properties including desensitization, ion channel properties, and subcellular targeting of the receptors (22–24). Thus, regulation of neurotransmitter receptor function by protein phosphorylation may play a critical role in the regulation of synaptic transmission (22–24). AMPA receptors mediate the majority of excitatory synaptic transmission in the mammalian central nervous system (25, 26). AMPA receptors are pentameric complexes of four homologous subunits GluR1–4 which combine to form various AMPA receptors subtypes. These receptors have been shown to be phosphorylated by several protein kinases including PKC (27–29), CAMP-dependent protein kinase (PKA) (28, 29), and CaM kinase II (27, 30, 31). Phosphorylation of AMPA receptors by all three of these kinases appears to potentiate their function (29–32), suggesting that AMPA receptor phosphorylation-dephosphorylation could, in part, mediate LTP and LTD. PKC and PKA have recently been shown to phosphorylate the AMPA receptor subunit GluR1 on serine residues 831 and 845, respectively, within its intracellular C-terminal domain and phosphorylation of these residues appears to potentiate GluR1 function (29). However, the site of CaM kinase II phosphorylation has not been unequivocally identified. In the present study we have generated two phosphorylation site-specific antibodies which recognize GluR1 only when Ser-831 (anti-S831-P) or Ser-845 (anti-S845-P) is phosphorylated. We use these antibodies to demonstrate that GluR1 is phosphorylated...
on Ser-831 by CaM kinase II in vitro, in transfected cultured cells, as well as in hippocampal slice preparations.

EXPERIMENTAL PROCEDURES

Materials—GluR1 and actCaMKII cDNAs were generous gifts of Steve Heinemann (Salk Institute) and Howard Schulman (Stanford University), respectively. The generation of the GluR1 S831A and S845A mutants has been previously described (29). Other materials were purchased from the following sources: radioisotopes, Life Science Products; CaM kinase II, phorbol esters and forskolin, Calbiochem; cellulose TLC (thin layer chromatography) plates and X-AR photography film, Kodak; PVDF membrane, Millipore; nitrocellulose membrane, VWR; tissue culture media, sera, and supplies, Life Technologies, Inc. All other chemicals, unless otherwise noted, were from Sigma.

Synthesis of Phosphopeptides—Peptides were synthesized with an Applied Biosystems 430A Peptide Synthesizer as described previously (35) using the FastMoc procedures and reagents supplied by the Perkin-Elmer, Applied Biosystems division. Di-tert-butyl-N,N-diisopropylphosphoramidite and N-(9-fluorenylmethoxycarbonyl) serine (hydroxyl group not protected) were obtained from Novabiochem, La Jolla, CA. TOF MALDI mass spectrometry of the purified peptides was performed using a Compact MALDI III (Manchester, United Kingdom) in the Middle Atlantic Mass Spectrometry Laboratory, located at the Johns Hopkins University School of Medicine.

Generation of Anti-phosphopeptide Antibodies—The peptides LIPOQINSIAIK and KTLPRNSGAGS, corresponding to amino acids 826–836 and 840–850 of GluR1, respectively, were synthesized as described above with phosphoserines included at the Ser-831 and Ser-845 positions. Lysine residues were included to facilitate glutaraldehyde coupling to the carrier protein, thyroglobulin, and the resulting phosphopeptide/thyroglobulin mixtures were used to immunize New Zealand White rabbits. Sera were obtained periodically by Hazleton Research Products. Polyclonal antiphosphopeptide antibodies were purified from sera by sequential chromatography on Affi-Gel 15 (Bio-Rad) columns covalently linked to phosphorylated and unphosphorylated peptides. Antibodies were first eluted from the phosphorylated peptide affinity columns using 100 mM glycine (pH 2.7). The affinity purified antibodies were dialyzed against phosphate-buffered saline and then loaded onto unphosphorylated peptide affinity columns. The flowthrough was collected and used for immunoblot analysis. Anti-GluR1 C-terminal antibodies were described previously (34).

GluR1 Fusion Protein Phosphorylation—Bacterial fusion proteins containing the C-terminal region of GluR1 (amino acids 809–889) were covalently linked to phosphorylated and unphosphorylated peptides. Antibodies were first eluted from the phosphorylated peptide affinity columns using 100 mM glycine (pH 2.7). The affinity purified antibodies were dialyzed against phosphate-buffered saline and then loaded onto unphosphorylated peptide affinity columns. The flowthrough was collected and used for immunoblot analysis. Anti-GluR1 C-terminal antibodies were described previously (34).

Phosphorylation of the AMPA Receptor GluR1 Subunit

FIG. 1. Specificity of phosphorylation site-specific antibodies for phosphorylated GluR1 expressed in QT6 cells. QT6 cells expressing GluR1, GluR1 S831A, or GluR1 S845A were treated with control solution (control), 100 nM phorbol 12-myristate 13-acetate (PMA), or 10 μM forskolin and 100 μM isobutylmethylxanthine (FSK, IBMX) for 15 min as indicated. Membrane extracts were prepared and 3% of the total from each plate was resolved by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with the phosphorylation site-specific antibodies generated against phosphoserines 831 or 845, or an antibody to the C terminus of GluR1.

Hippocampi were dissected and chilled in ice-cold ACSF solution (126 mM NaCl, 5 mM KCl, 1.25 mM NaH2PO4, 25 mM NaHCO3, 10 mM d-glucose, 2 mM CaCl2, 2 mM MgCl2, saturated with 95% O2, 5% CO2). Slices (300 μM) were prepared by sonication of the slices on ice with 1 ml of RB for 30 s. The homogenates were centrifuged at 12,000 × g for 5 min, the membrane pellets resuspended in 200 μl total volume. Phosphorylation reaction buffer contained 10 mM HEPES (pH 7.5), 1 mM MgCl2, 800 μM CaCl2, 50 μM γ-32P-ATP (2200 cpm/pmol), and 10 μg/ml calmodulin. Reactions were quenched by the addition of 50 μl of 3 X SDS-PAGE sample buffer and the phosphorylated GluR1 fusion proteins were excised from gels for phosphopeptide mapping.

Cell Culture, Transfection, and Metabolic Labeling—QT6 cells were maintained and transfected with a total of 20 μg of DNA using calcium phosphate coprecipitation as described previously (33). For metabolic labeling studies the cells were labeled 48 h after transfection in phosphate-free minimal essential medium (Life Technologies, Inc.), for 4 h with 4 μCi/ml 32Porthophosphate and treated for 15 min in 100,000 × g for 5 min with 10 μM phorbol 12-myristate 13-acetate or vehicle solution as indicated. The cells were then harvested, the GluR1 protein was isolated by immunoprecipitation using anti-GluR1 antibodies as described previously (34, 35), and the immunoprecipitate resolved by SDS-PAGE on 7.5% acrylamide gels. Slices containing 32P-labeled GluR1 were excised from gels for phosphopeptide mapping.

For immunoblot analysis, cells were treated with drugs or vehicle solutions as indicated 48 h after transfection, rinsed in phosphate-buffered saline, scraped into 1 ml of resuspension buffer (RB) consisting of 10 mM sodium phosphate (pH 7.0), 100 mM NaCl, 10 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 5 mM EDTA, 5 mM EGTA, 1 μM okadaic acid, 1 μM microcystin-LR, and 10 units/ml aprotinin and then sonicated and centrifuged at 100,000 × g for 1 h. The membrane pellets were resuspended in 200 μl of RB including 1% SDS and then diluted with 800 μl of RB including 1% Triton X-100. The resulting extracts were spun at 14,000 × g for 15 min, the supernatants loaded onto SDS-PAGE gels as membrane preparations, and the resulting gels transferred to PVDF membranes.

Hippocampal Slice Preparation—The hippocampi of 6–8-week-old male Sprague-Dawley rats were prepared as described (33). Briefly, the
blasts which had been transfected with wild-type GluR1 (Fig. 1). Both of these antibodies detected only low levels of GluR1 in GluR1-transfected QT6 cells, consistent with a low basal level of phosphorylation of GluR1 in these cells. However, when the QT6 cells were treated with phorbol esters or forskolin to activate PKC or PKA, respectively, the anti-S831-P and anti-S845-P antibodies specifically recognized GluR1 (Fig. 1). Mutation of serines 831 or 845 to alanine residues eliminated recognition of GluR1 by the appropriate antibody, demonstrating their specificity. In addition, phosphatase treatment of the PVDF membrane prior to immunoblotting abolished immunorecognition by both antibodies demonstrating the phosphorylation dependence of the anti-S831-P and anti-S845-P antibodies. In addition to the 106-kDa GluR1 receptor, the phosphorylation-site specific antibodies each recognized a low molecular mass protein that was present in non-transfected cells. The GluR1 phosphorylation-site specific antibodies did not detect other AMPA receptor subunits (GluR2–4) expressed under similar conditions, demonstrating the specificity of these antibodies for GluR1 (Fig. 2).

Because of the central role of CaM kinase II in the induction and maintenance of LTP, we have used the phosphorylation specific antibodies to examine whether CaM kinase II can phosphorylate Ser-831 and Ser-845. Treatment of the QT6 cells with the calcium ionophore A23187 increased phosphorylation of GluR1 on Ser-831 with little effect on phosphorylation of Ser-845 (see Fig. 3). The specific CaM kinase II inhibitor KN-62 (37) antagonized this ionophore-induced phosphorylation. Furthermore, cotransfection of a constitutively active form of CaM kinase II (actCaMKII) with GluR1 resulted in a dramatic increase in the phosphorylation of Ser-831 without significant phosphorylation of Ser-845. These results suggest that CaM kinase II, in addition to PKC, can specifically phosphorylate Ser-831 in the GluR1 subunit.

To confirm that Ser-831 is directly phosphorylated by CaM kinase II, we performed in vitro phosphorylations using purified CaM kinase II with purified fusion proteins corresponding to the C terminus of GluR1. As shown in Fig. 4, the GluR1 C-terminal fusion protein was phosphorylated by CaM kinase II. However, mutation of Ser-831 completely eliminated this phosphorylation. To further analyze this phosphorylation, the CaM kinase II-phosphorylated fusion protein was cut from the gel, digested with trypsin, and subjected to two-dimensional phosphopeptide map analysis (Fig. 4). Three major phosphopeptides were detected which were eliminated by mutation of Ser-831. These three phosphopeptides (phosphopeptides 3, 4, and 6; numbering is based on previous studies (28, 29)) have been previously characterized to be partial tryptic phosphopeptides containing Ser-831 (29).

We also performed two-dimensional phosphopeptide map analysis on GluR1 isolated from transfected QT6 cells which had been prelabeled with [32P]orthophosphate. As has been described, control cells contained three major phosphopeptides.
(numbers 1, 3, and 7). Phosphopeptide number 3, as discussed above, contains Ser-831 while phosphopeptide numbers 1 and 7 are unidentified phosphorylation sites. Stimulation of cells with phorbol esters resulted in increased phosphorylation of phosphopeptide numbers 3 and 6 with a small increase in phosphorylation of phosphopeptide number 1 (Fig. 3). Cotransfection of GluR1 or GluR1 and actCaMKII were prelabeled with [32P]orthophosphate and treated as indicated. GluR1 was immunoprecipitated, resolved by SDS-PAGE, excised from the gel, and digested with trypsin. Phosphopeptide map analysis of each sample was performed (B). In both A and B, the phosphopeptides were numbered according to our previously published scheme (29). Phosphopeptide numbers 3, 4, and 6 contain Ser-831 (29).

Previous studies have suggested that Ser-627 is the major CaMKII phosphorylation site on GluR1 (31). However, recent studies have suggested that this serine residue is located on the extracellular region of GluR1 and should not be accessible to protein kinases (38). We therefore tested to see if mutation of Ser-627 to an alanine residue effected CaMKII phosphorylation of GluR1. As shown in Fig. 5, phosphopeptide maps of the S627A mutant GluR1 subunit were identical to wild-type and phosphorylation of phosphopeptide numbers 3 and 6 by CaMKII was still observed.

To analyze phosphorylation of GluR1 in vivo we have used the phosphorylation site-specific antibodies to examine hippocampal slice preparations. Both the anti-S831-P and anti-S845-P antibodies recognize GluR1 on immunoblots of hippocampal slices, suggesting that Ser-831 and Ser-845 are basally phosphorylated. As in the QT6 cell experiments, pre-treating the immunoblots with phosphatase abolished staining with the phosphospecific antibodies (data not shown). Similarly, preblocking the phosphospecific antibodies with the appropriate phosphorylated peptide abolished antibody recogni-
tion of GluR1. In contrast, incubation of phosphospecific antibodies with the equivalent non-phosphorylated peptides did not block antibody recognition of GluR1 (data not shown).

To quantitate the relative degree of GluR1 phosphorylation in control slices and in slices that had been treated with drugs to regulate the various kinases, we calculated the ratio of the intensity of GluR1 labeling with the phosphorylation site-specific antibodies over the intensity of labeling with a C-terminal phosphorylation-independent GluR1 antibody. Treatment of the slices with phorbol esters and forskolin increased the phosphorylation of Ser-831 (intensity ratio = 2.09 ± 0.73 of control; n = 3) and Ser-845 (intensity ratio = 2.72 ± 0.14 of control; n = 3), respectively, confirming that PKC and PKA phosphorylate these sites in vivo (Fig. 6). Phorbol ester treatment of the slices also significantly (p < 0.05) and reproducibly increased the phosphorylation of Ser-845. As shown in Fig. 1, this effect was not observed in transfected cells. Treatment of the slices with the calcium ionophore A23187 caused a decrease in the phosphorylation of Ser-831 and Ser-845 (data not shown), presumably due to the activation of calcium-regulated protein phosphatases. However, treatment of the slices with the CaM kinase II inhibitor KN-62 significantly (p < 0.05) inhibited phosphorylation of Ser-831 (intensity ratio = 0.71 ± 0.04 of control; n = 4), with little effect on Ser-845 phosphorylation, strongly suggesting that CaM kinase II phosphorylates Ser-831 in vivo.

**DISCUSSION**

Protein phosphorylation is an important mechanism for the regulation of AMPA receptor function (22–24). For example, we have previously shown that the AMPA receptor GluR1 subunit is phosphorylated by PKC and PKA on Ser-831 and Ser-845, respectively, and that phosphorylation of Ser-845 by PKA potentiates AMPA receptor function (29). Additional studies have demonstrated that AMPA receptors are phosphorylated by CaM kinase II (27, 30) and have suggested that CaM kinase II phosphorylation potentiates AMPA receptor function (30, 31). However, the site of CaM kinase II phosphorylation has not been unequivocally determined (29, 31). Recent studies using site-specific mutagenesis and electrophysiological techniques have suggested that serine 627 is phosphorylated by CaMKII (31). However, this region of the GluR1 subunit is now believed to be located in an extracellular region of the GluR1 subunit where it would not be accessible to the CaMKII (38). In addition, previous studies in our laboratory have found no evidence that CaMKII phosphorylates serine 627 (29). Interestingly, in our previous study we found that cotransfection of actCaMKII with GluR1 in HEK-293 cells did not increase phosphorylation of GluR1 (29). This was most likely due to the high basal phosphorylation of GluR1 on serine 831 in these cells (29). In the current study we have used QT6 cells which have a relatively low basal phosphorylation of serine 831, which has allowed us to study the phosphorylation of this site by CaMKII. Using phosphorylation site-specific antibodies we have demonstrated that CaM kinase II phosphorylates Ser-831 in vitro and in vivo. Cotransfection of GluR1 and constitutively active CaM kinase II in QT6 cells leads to the specific phosphorylation of Ser-831. In addition, purified CaM kinase II specifically phosphorylates a fusion protein of the C-terminal domain of GluR1 on Ser-831. Furthermore, we have shown that Ser-831 is likely to be phosphorylated by CaM kinase II in vitro, since hippocampal slices incubated with the specific CaM kinase II inhibitor KN-62 have reduced levels of Ser-831 phosphorylation. Anti-S831-P and anti-S845-P were also used to demonstrate that treatment of hippocampal slices with phorbol esters and forskolin leads to increased phosphorylation of Ser-831 and Ser-845, respectively. Interestingly, we also found that Ser-831 and Ser-845 were both basally phosphorylated in hippocampal slices. In contrast, these residues are only phosphorylated following synaptic activity or stimulation with phorbol esters or forskolin in

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**FIG. 6. Phosphorylation of GluR1 in hippocampal slices.** A, phosphorylation of Ser-831 in hippocampal slices. Rat hippocampal slices (400 μm) were treated with phorbol dibutyrate (PDBu) or forskolin and isobutylmethylxanthine (FSK), and the membrane fractions were prepared from each pool of slices. The phosphorylation of GluR1 Ser-831 in hippocampal slices membrane fractions were detected by anti-S831P, and total amount of GluR1 on the PVDF membrane was detected by a GluR1 C-terminal antibody. The ratios of intensity of the signal (intensity of anti-S831P antibody labeling/intensity of C-terminal antibody labeling) were calculated and normalized to the control slices. The averages from three independent experiments were plotted. Error bars are S.D. B, phosphorylation states of Ser-845 in hippocampal slices. The phosphorylation of Ser-845 was detected by anti-GluR1 S845P antibody and the changes of phosphorylation were determined as described above. C, effect of CaMKII inhibitor KN-62 on basal GluR1 phosphorylation. The amounts of phosphorylation of Ser-831 and Ser-845 were estimated as described above (n = 4).

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neuronal cortical cultures (28). Although the amino acid sequence surrounding Ser-845 (LPRRNSGAGA) is consistent with the known consensus sites for PKA (RXS) (39), the sequence surrounding Ser-831 (LIPQQSINEA) does not fit the consensus sites for CaMKII and PKC, which usually contain basic residues close to the phosphorylated serine residue (39). However, Ser-831 contains hydrophobic residues at the −5 (Leu) and +1 (Ile) positions and recent studies have suggested that hydrophobic residues at these positions are very important for substrate recognition by CamKII (40). These sites are not conserved on the other AMPA receptor subunits (GluR2–4), however, it is possible that GluR2–4 are phosphorylated by PKA, PKC, or CaMKII on other sites.

Since CaM kinase II is required for the induction and maintenance of LTP (10–12, 17), it has been proposed that direct phosphorylation of glutamate receptors by CaM kinase II may underlie the postsynaptic component of this potentiation. The phoshospecific antibodies we have characterized here should prove useful in monitoring the phosphorylation state of GluR1 to see if it correlates with synaptic plasticity during models of learning and memory such as LTP and LTD.

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