Identification of the Ca\(^{2+}\)/Calmodulin-dependent Protein Kinase II Regulatory Phosphorylation Site in the α-Amino-3-hydroxy-5-methyl-4-isoxazole-propionate-type Glutamate Receptor*  

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Ca\(^{2+}\)/CaM-dependent protein kinase II (CaM-KII) can phosphorylate and potentiate responses of α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate-type glutamate receptors in a number of systems, and recent studies implicate this mechanism in long term potentiation, a cellular model of learning and memory. In this study we have identified this CaM-KII regulatory site using deletion and site-specific mutants of glutamate receptor 1 (GluR1). Only mutations affecting Ser\(^{831}\) altered the \(^{32}\)P peptide maps of GluR1 from HEK-293 cells co-expressing an activated CaM-KII. Likewise, when CaM-KII was infused into cells expressing GluR1, the Ser\(^{831}\) to Ala mutant failed to show potentiation of the GluR1 current. The Ser\(^{831}\) site is specific to GluR1, and CaM-KII did not phosphorylate or potentiate current in cells expressing GluR2, emphasizing the importance of the GluR1 subunit in this regulatory mechanism. Because Ser\(^{831}\) has previously been identified as a protein kinase C phosphorylation site (Roche, K. W., O’Brien, R. J., Mammen, A. L., Bernhardt, J., and Huganir, R. L. (1996) Neuron 16, 1179–1188), this raises the possibility of synergistic interactions between CaM-KII and protein kinase C in regulating synaptic plasticity.

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Brief trains of high frequency stimulation to monosynaptic excitatory pathways in the hippocampus and several other brain regions cause an abrupt and sustained increase in the efficiency of synaptic transmission. This phenomenon, known as long term potentiation (LTP), is a cellular model for learning and memory (1–3). LTP induction requires activation of ionotropic N-methyl-D-aspartate-type glutamate receptors, Ca\(^{2+}\) influx into the postsynaptic dendritic spine, and activation of postsynaptic protein kinases (1–3). Ionotropic α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate glutamate receptors (AMPA-Rs) in the postsynaptic densities of excitatory synapses are primarily responsible for basal rapid excitatory transmission and for the enhancement of synaptic current in LTP (4, 5). AMPA-Rs are heterocomplexes composed of subunits GluR1–GluR4 (6), and these channels can be modulated by phosphorylation. Infusion of activated Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaM-KII) potentiates AMPA-R responsiveness in Xenopus oocytes and HEK-293 cells expressing GluR1 (7) or of native AMPA-Rs in hippocampal cultured neurons (8), dorsal root ganglion cells (9), and hippocampal slice CA1 neurons (10, 11). In the latter system, expression or infusion of CaM-KII not only increases AMPA-R responsiveness, but it also increases synaptic current and occludes subsequent induction of LTP, suggesting common mechanisms for potentiation. A crucial role for CaM-KII in the induction of LTP is strongly indicated (12), and we recently demonstrated phosphorylation of AMPA-Rs by CaM-KII after LTP induction in hippocampal slices (13). Although phosphorylation of AMPA-Rs by CaM-KII appears to be a key event in LTP (12), this regulatory phosphorylation site has not been positively identified. Previous studies have implicated Ser\(^{827}\) in GluR1 (7), Ser\(^{896}\) in GluR2 (14), and Ser\(^{884}\) and/or Ser\(^{866}\) in GluR6 (15, 16) as regulatory phosphorylation sites by various kinases, but the revised membrane topology model places these sites extracellular (17, 18). PKA and PKC can also phosphorylate AMPA-Rs in cortical neurons and GluR1 expressed in 293 cells (19), and recently the PKA (Ser\(^{845}\)) and PKC (Ser\(^{831}\)) phosphorylation sites in GluR1 were identified in the intracellular COOH terminus (20). In this paper we have re-examined the CaM-KII regulatory phosphorylation site using site-specific mutagenesis and electrophysiology of recombinant AMPA-type glutamate receptors.

EXPERIMENTAL PROCEDURES

Phosphorylation of AMPA-Rs and Two-dimensional Peptide Mapping—HEK-293 cells were transfected with AMPA-Rs (wild type or mutants) and CaM-KII H282R in a ratio 2:1. At confluency, cells were incubated in phosphate-free Dulbecco’s modified Eagle’s medium for 4 h and then labeled with \(^{32}\)Porthophosphate during 90 min. After labeling, cells were rinsed with cold phosphate-buffered saline and harvested in homogenization buffer containing 500 mm NaCl, 30 mm Na\(_3\)P\(_2\)O\(_7\), 50 mm NaF, 50 mm Tris-HCl (pH 7.5), 200 mm EDTA, 200 mm EGTA, 100 mm Na\(_2\)V\(_3\), 0.1 mm leupeptin, 1 mm phenylmethylsulfonyl fluoride, 20 μg/ml soybean trypsin inhibitor, 1 μl microcystine-LR, 0.5% Triton X-100, and 0.1% deoxycholate. Samples were sonicated and centrifuged at 10,000 rpm for 15 min. The receptor was immunoprecipitated from the supernatant and analyzed by SDS-PAGE and autoradiography. Two dimensional peptide mapping was performed as in Ref. 13 except that the electrophoresis step was increased from 70 to 90 min. This results in the resolution of two distinct peptides, 1 and 2. All shown peptide maps were reproduced at least three times.

Kines Assays—HEK-293 cells were transfected (CaM-KII H282R or mock) and homogenized as above, and PKC was assayed in the supernatant at 30 °C using 50 mm HEPES (pH 7.5), 10 mm magnesium acetate, 1 mm dithiothreitol, 0.1 mm \(^{32}\)P/ATP, 0.1 mm myristoylated alanine-rich C kinase substrate protein, with or without 0.3 mm CaCl\(_2\), polyacrylamide gel electrophoresis; GST, glutathione S-transferase; TPA, 12-O-tetradecanoylphorbol-13-acetate.
Ser627 is not the CaM-KII regulatory phosphorylation site on GluR1. A, phosphorylation of wild type (WT) or mutant S627A GluR1 expressed in HEK-293 cells with or without coexpression of the CaM-KII mutant H282R, which has 20% constitutive activity (21). Two days after transfection, cells were 32P-labeled and GluR1 was immunoprecipitated as described previously (13). Equal amounts of the receptor were separated by SDS-PAGE and analyzed by autoradiography (left top panel) and for GluR1 immunoreactivity (left bottom panel). The right panel compares constitutive (−Ca2+) and total (+Ca2+) CaM-KII activities from mock transfected or CaM-KII (H282R mutant) transfected cells. B, the 32P-GluR1 was excised from the gel and subjected to complete tryptic digestion followed by two-dimensional peptide mapping: the two main phosphopeptides are circled and labeled. C, effect of CaM-KII on whole cell currents (−80 mV, insets), in response to a 100-ms application of 10 mM glutamate (13), recorded from HEK-293 cells expressing wild type (left panel, n = 9–10 cells) or mutant S627A (right panel, n = 4–5 cells) GluR1. Peak currents were normalized to zero time for cells infused with activated (CaM-KII) or with heat-inactivated CaM-KII (HI CaM-KII) (13).

20 mol % phosphatidylserine, and 5 mol % 1,2-diacylglycerol. CaM-KII was assayed (21) using 40 μM Syntide-2 as a substrate, and 1 mM CaCl2 and 2 mM CaM for total activity or 2 mM EGTA for Ca2+-independent activity.

Expression and Phosphorylation of GST-GluR1816–889: The COOH terminus of GluR1 (residues 816–889) was cloned into pGEX-4T-2 (Pharmacia Biotech Inc.), and the fusion protein was expressed and purified according to company protocols. The fusion construct was phosphorylated in medium containing 50 mM Tris-HCl (pH 7.5), 10 mM magnesium acetate, 1 mM CaCl2, 2 mM CaM, 0.1 mM [32P]ATP, and 1 mM CaM-KII. At different times the reaction was stopped with SDS-sample buffer and resolved by SDS-PAGE for analysis by autoradiography. Quantification of the radioactivity was done using Phosphor Screen from Molecular Dynamics.

AMPA Receptor Currents (13)—Wild type or mutant GluR1 were expressed in HEK-293 cells using Lipofectin (Life Technologies, Inc.) and seeded at low density. Whole cells recording were made 16–20 h after transfection as has been reported previously. When added to intracellular solution, activated CaM-KII truncated at residue 316 and autothiophosphorylated (11) was at 0.4 μM. Glutamate was delivered to single cells by rapid application (7).

RESULTS AND DISCUSSION

We have reported that mutagenesis of Ser627 eliminates the effect of CaM-KII on AMPA currents in Xenopus oocytes expressing GluR1 (7). Because the revised membrane topological model places this site extracellular (17, 18), we examined whether if Ser627 is phosphorylated by CaM-KII. Wild type GluR1 or mutant GluR1 S627A were expressed in HEK-293 cells, metabolically 32P-labeled, and then immunoprecipitated with GluR1 antibody and separated by SDS-PAGE (13). Low basal phosphorylation was observed, but co-expression of GluR1 with a mutant (H282R) of CaM-KII, which has 20% constitutive activity, significantly increased the 32P labeling of either GluR1 wild type or the S627A mutant (Fig. 1A, upper left panel). Transfection with CaM-KII gave a 2–3-fold increase in total CaM-KII activity and a large increase in constitutive activity (Fig. 1A, right panel) that catalyzed the phosphorylation of co-expressed GluR1. Because CaM-KII might phosphorylate GluR1 at several sites, two-dimensional phosphopeptide mapping was performed. The mapping conditions were modified (see “Experimental Procedures”) and resulted in resolution of two major phosphopeptides 1 and 2 (Fig. 1B) rather than a single, broad spot as previously reported (13). In the absence of transfected CaM-KII, basal phosphorylation of wild type GluR1 was predominantly in peptide 1 (Fig. 1B, left panel), whereas co-expressed CaM-KII produced a large increase in peptide 2 phosphorylation (Fig. 1B, middle panel). Because the maps of wild type GluR1 and mutant GluR1 S627A appeared identical (Fig. 1B, middle and right panels), this demonstrated that the
CaM-KII phosphorylation site was not Ser627. Wild type GluR1 expressed in HEK-293 cells shows a small run-down of current over 30 min, and infusion of activated CaM-KII through the patch pipette potentiates wild type GluR1 current by 50–75% (13). Mutant GluR1 S627A did not exhibit run-down but exhibited some spontaneous potentiation, and CaM-KII failed to further potentiate the current (Fig. 1 right panel), similar to what we observed in Xenopus oocytes (7). The mutation of Ser627 may alter the normal conformation of the receptor, preventing its modulation by phosphorylation. Interestingly, another mutant, consensus CaM-KII site Ser593 to Ala in intracellular loop 2, showed similar behavior; it had a 32P peptide map identical to wild type GluR1, indicating that this site was not phosphorylated by CaM-KII, but infusion of CaM-KII did not prevent receptor phosphorylation in 293 cells (data not shown).

These results directed our strategy to identify mutations that lacked both the CaM-KII 32P labeling and potentiation. A previous study identified phosphorylation sites for PKC (Ser831) and PKA (Ser845) in the intracellular COOH terminus of GluR1 (20), so we examined whether a fusion construct of GST and GluR1 COOH-terminal residues 816–889 was phosphorylated by CaM-KII. GST itself was not phosphorylated by CaM-KII (not shown), but the fusion construct was slowly phosphorylated (Fig. 2A, left panel), and its peptide map contained predominantly phosphopeptide 2 (Fig. 2B). Therefore, we concentrated on mutations of consensus CaM-KII sites (Arg-Xaa-Xaa (Ser/Thr)) within the COOH terminus of GluR1. Triple mutant GluR1 S814A,S816A,S818A and single mutant GluR1 S863A were phosphorylated in HEK-293 cells by co-expressed CaM-KII to a similar extent as wild type GluR1, and their two-dimensional maps were identical to that of wild type GluR1 (data not shown). Deletion of amino acids 829–844 (GluR1 Δ829–844) eliminated CaM-KII phosphorylation, whereas deletion of amino acids 845–859 (GluR1 Δ845–859) did not prevent receptor phosphorylation in 293 cells (Fig. 3A). These experiments indicated that the CaM-KII phosphoryla-

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**FIG. 3.** Mutation of Ser831, but not Ser845, blocks CaM-KII phosphorylation of GluR1 and potentiation of its current. A, autoradiography of immunoprecipitated GluR1 mutants lacking residues 829–844 or 845–859 32P-labeled by co-expressed CaM-KII in HEK-293 cells. Arrows indicate the position of the receptor. B, phosphorylation of GluR1 single mutants S831A or S845A by co-expressed CaM-KII. The top panel is the autoradiograph of the SDS-PAGE; the 32P-GluR1 was cut from the gel and subjected to two-dimensional tryptic peptide mapping in the bottom panel. C, whole cell recordings of GluR1 mutants expressed in HEK-293 cells. Peak currents were normalized to zero time for cells expressing GluR1 mutants Ser831Ala (n = 4–6 cells) or S845A (n = 3–4 cells) after infusion of activated or heat-inactivated CaM-KII (13).

**FIG. 4.** GluR2 is not phosphorylated or potentiated by CaM-KII. A, HEK-293 cells were transfected with GluR2 with or without CaM-KII H282R and 32P-labeled, and the receptor was immunoprecipitated (13) using a commercial GluR2 antibody (Upstate Biotechnology). The arrow indicates the position of the receptor after SDS-PAGE. B, normalized peak currents from whole cell recordings of HEK-293 cells expressing GluR2 and infused with active (●, n = 5 cells) or heat inactivated (▲, n = 4 cells) CaM-KII. C, activity of PKC in HEK-293 cells transfected without (MOCK) or with CaM-KII H282R.
tion site may be located within residues 829–844, so we tested the effect of mutating Ser$^{831}$ to Ala in the GluR1 COOH terminus of the GST fusion construct. This mutation blocked phosphorylation of the GST fusion protein (Fig. 2A, right panel), implicating Ser$^{831}$ as a likely candidate for the CaM-KII phosphorylation site (Fig. 2A). The S831A mutant of full-length GluR1 was not phosphorylated by CaM-KII (Fig. 3B, upper panel) when co-expressed in 293 cells; it was devoid of major phosphopeptide 2 in its two-dimensional map (Fig. 3B, lower panel), and its current was not potentiated by CaM-KII (Fig. 3C, left panel). In contrast to the S831A mutant, the adjacent PKA site mutant (GluR1 S845A) was still phosphorylated by CaM-KII; its two-dimensional map was comparable with the wild type (Fig. 3B, right panel); and its current was potentiated (Fig. 3C, right panel).

These results (Fig. 3, B and C) demonstrate that Ser$^{831}$ was the regulatory CaM-KII phosphorylation site. This site is unique to GluR1 because GluR2–6 subunits do not contain sites homologous to Ser$^{831}$. Therefore, one would predict that co-expression of CaM-KII with GluR2 might not enhance its phosphorylation, and this was observed (Fig. 4A). Likewise, infusion of CaM-KII into cells expressing GluR2 did not potentiate its current (Fig. 4B). This specificity of CaM-KII for GluR1 would implicate a unique role for GluR1 in potentiating synaptic current through its phosphorylation. This may account for a major role of CaM-KII in mediating LTP in region CA1 of hippocampus because most native AMPA-Rs should contain GluR1 because only GluR1 and GluR2 are strongly expressed (22).

Consistent with the previous report (20), the S831A mutant failed to show enhanced phosphorylation upon stimulation of the $^{32}$P-labeled cells with phorbol ester (not shown). The two-dimensional phosphopeptide map of the wild type GluR1 phosphorylated upon stimulation of the cells with TPA was indistinguishable from the map of wild type GluR1 phosphorylated by co-expressed CaM-KII (not shown). One possible interpretation of these data would be that activation of PKC leads to an increase in the endogenous CaM-KII activity through an increase in free calmodulin as has been demonstrated in PC12 cells (23). The activated CaM-KII could then catalyze phosphorylation of the AMPA-R. However, in the present study TPA treatment (1 $\mu$M for 15 min) did not increase the activity of CaM-KII in 293 cells co-expressing CaM-KII and GluR1 (not shown), as assessed by immunoprecipitated $^{32}$P-CaM-KII (i.e. autophosphorylation), just as was previously observed in cultured hippocampal neurons treated with TPA (24). There is no established mechanism by which CaM-KII can activate PKC, and we did not see any increase in PKC activity comparing cells without (mock) and with transfected CaM-KII H282R (Fig. 4C).

Because CaM-KII and PKC phosphorylate the same regulatory site in GluR1, this raises the possibility that both kinases might regulate synaptic plasticity through this mechanism. Both kinases can phosphorylate GluR1 in isolated rat brain postsynaptic densities and cultured hippocampal neurons (24). In the postsynaptic density CaM-KII is several orders of magnitude stronger as a catalyst, perhaps because of its high concentration in this synaptic organelle compared with PKC. Both CaM-KII (13, 25, 26) and PKC (27–31) are activated for a prolonged period upon LTP induction, and it has been reported that only a combination of inhibitors of both kinases can turn off established LTP (32). Although the role of CaM-KII in LTP through phosphorylation of AMPA-Rs has extensive experimental support (12, 13), an additional role for PKC should also be considered. It is also interesting that Ser$^{831}$ is not a “consensus” phosphorylation site for either CaM-KII or PKC, presumably explaining its slow rate of phosphorylation in the GST fusion construct (Fig. 2A) and the slow potentiation of GluR1 current in 293 cells (Fig. 1B), which correlate with the slow phosphorylation of AMPA-Rs by CaM-KII after induction of LTP (13). The fact that Ser$^{831}$ is not a good substrate for CaM-KII may be an important physiological checkpoint in that it would prevent AMPA-R phosphorylation and synaptic potentiation by weak synaptic input. Only intensive synaptic activity would be able to elevate CaM-KII activity for a sufficient time to slowly phosphorylate the AMPA-R and thereby enhance postsynaptic current. These results implicate Ser$^{831}$ as a “memory locus” on the AMPA-R and make its mutation a prime candidate for testing on learning and memory in animal models.

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