Autophosphorylation-dependent Targeting of Calcium/Calmodulin-dependent Protein Kinase II by the NR2B Subunit of the N-Methyl-d-aspartate Receptor*

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Activation and Thr<sup>286</sup> autophosphorylation of calcium/calmodulin-dependent kinase II (CaMKII) following Ca<sup>2+</sup> influx via N-methyl-d-aspartate (NMDA)-type glutamate receptors is essential for hippocampal long term potentiation (LTP), a widely investigated cellular model of learning and memory. Here, we show that NR2B, but not NR2A or NR1, subunits of NMDA receptors are responsible for autophosphorylation-dependent targeting of CaMKII. CaMKII and NMDA receptors colocalize in neuronal dendritic spines, and a CaMKII/NMDA receptor complex can be isolated from brain extracts. Autophosphorylation induces direct high-affinity binding of CaMKII to a 50 amino acid domain in the NR2B cytoplasmic tail; little or no binding is observed to NR2A and NR1 cytoplasmic tails. Specific colocalization of CaMKII with NR2B-containing NMDA receptors in transfected cells depends on receptor activation, Ca<sup>2+</sup> influx, and Thr<sup>286</sup> autophosphorylation. Translocation of CaMKII because of interaction with the NMDA receptor Ca<sup>2+</sup> channel may potentiate kinase activity and provide exquisite spatial and temporal control of postsynaptic substrate phosphorylation.

CaMKII is a multifunctional, calcium-activated kinase (1, 2), whose α and β isoforms are particularly abundant in brain cytosol and in postsynaptic densities (PSDs),<sup>1</sup> submembranous scaffolds for receptors, ion channels, and signal transducers (3, 4). Postsynaptic calcium influx triggers autophosphorylation of CaMKII at a threonine residue in the autoinhibitory domain (Thr<sup>286</sup> in CaMKII<sub>α</sub>) (5), which renders the kinase persistently active and causes a translocation of soluble CaMKII to the PSD (6). Multiple lines of evidence indicate Thr<sup>286</sup> autophosphorylation of postsynaptic CaMKII is necessary for NMDA receptor-dependent LTP (7–11), a cellular model of learning and memory. PSD-associated CaMKII phosphorylates ionotropic glutamate receptors (6, 12–14), providing a mechanism for increased synaptic strength during LTP (15).

Mechanisms by which CaMKII is targeted to its postsynaptic substrates are poorly understood. Previous gel overlay analyses revealed a candidate PSD-associated CaMKII-anchoring protein, p190, that binds selectively to the Thr<sup>286</sup>-autophosphorylated kinase (IP-T286)CaMKII<sub>α</sub> (16). The NR2A and NR2B subunits of the NMDA receptor share several properties with this CaMKII-binding activity, including apparent size, enrichment in PSDs, and regional and developmental expression profiles (17). Here, we demonstrate a direct and specific interaction between IP-T286CaMKII<sub>α</sub> and NR2B and show that NR2B targets CaMKII in intact cells.

**Experimental Procedures**

**Immunoprecipitations—PSD isolation and immunoprecipitation of sodium dodecyl sulfate (SDS)-solubilized PSD proteins were carried out as described (6) using 2 μg/ml NR2A/B antibodies (Chemicon) and protein phosphatase 1 antibodies (18). CaMKII/NMDA receptor coimmunoprecipitation, PSDs (1 mg/ml) were cross-linked (45 min, 4 °C) with 0.25 mM dithiobis(succinimidyl suberate), dissolved by sonication in 2% SDS, and diluted 15-fold in 1% SDS, 50 mM NaCl, 0.5 mM EDTA, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1 μM microcin-LR. The supernatant after ultracentrifugation (30 min, 100,000 × g) was immunoprecipitated with 3 μg/ml goat anti-CaMKII (16) or preimmune IgG (19). The cross-linker was cleaved and proteins eluted from the beads by boiling in reducing SDS sample buffer.

**CaMKII Gel Overlays—** Purified recombinant CaMKII<sub>α</sub> was autophosphorylated with γ<sup>32</sup>P-ATP (8,000–40,000 cpm/pmol) in the presence of calcium/calmodulin or EGTA at Thr<sup>286</sup> or Thr<sup>305/306</sup>, respectively, and desalted (16). Stoichiometries ranged between 0.17 and 0.39 (Thr<sup>286</sup>) and 0.24 and 0.47 (Thr<sup>305/306</sup>). Protein blots to be analyzed for CaMKII binding were blocked and incubated with 100–200 μM [γ<sup>32</sup>P]CaMKII<sub>α</sub> in 5% milk for 3 h, washed extensively, and autoradiographed.

**Immunofluorescence—** 18-Day-old cultures of dissociated neonatal rat cortex were fixed in acetone/methanol (1:1), blocked, and incubated 10–14 h in 1:500 dilutions of goat anti-CaMKII (16), rabbit anti-NR1 (20), and mouse anti-synaptophsyn (Boehringer Mannheim) in 1% normal donkey serum, 10 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Triton X-100. Cultures were treated with species-specific donkey antibodies conjugated to Cy3, Cy2, and Cy5 (Jackson Laboratories) and imaged on a Zeiss laser scanning confocal microscope.

**Generation and Analysis of NMDA Receptor Fusion Proteins—** The entire cytoplasmic domains (C terminus starting immediately after transmembrane region IV) of NR1 (splice variant A containing both C1 and C2 exon cassettes), NR2A, and NR2B subunits, as well as shorter NR2B constructs, were subcloned from full-length cDNAs by polymerase chain reaction using Pfu polymerase and primers containing restriction sites or by restriction digestes. Fragments were sequenced and ligated into pRSSET-A His<sub>6</sub>-tag fusions were expressed, and GST fusions were expressed and purified.

**APV, 2-amino-5-phosphonovaleric acid; GST, glutathione S-transferase; LTP, long term potentiation.**

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1 The abbreviations used are: PSD, postsynaptic density; CaMKII, calcium/calmodulin-dependent protein kinase II; CaMKII<sub>α/β</sub>, α/β isoform of CaMKII; [P-T286]CaMKII<sub>α</sub>, CaMKII<sub>α</sub> autophosphorylated at threonine 286; [P-T305/306]CaMKII<sub>α</sub>, CaMKII<sub>α</sub> autophosphorylated at threonine 305 and/or threonine 306; NMDA, N-methyl-d-aspartate; APV, 2-amino-5-phosphonovaleric acid; GST, glutathione S-transferase; LTP, long term potentiation.

fied according to the manufacturers` instructions. His₄ tag fusion protein lysates were subjected to CaMKII overlay (see above) or immunoprecipitated with anti His₄ tag antibodies (CLONTECH) and ¹²⁵I-labeled secondary antibodies for expression levels, followed by PhosphorImager quantification.

Microtiter Plate Solution Binding—Ni²⁺-coated 96-well plates (HisSorb strips, Qiagen) were adsorbed for 2 h with soluble His₄ tag NR2B fusion protein expressing or nonexpressing bacterial extracts (0.25 mg/ml) in blocking buffer (5 mg/ml bovine serum albumin, 200 mM NaCl, 50 mM Tris, pH 7.5, 0.1% Tween 20, 5 mM β-mercaptoethanol). After extensive washes, [²⁵³P-T286]CaMKIIα diluted in blocking buffer (200 μl) was allowed to bind to the tethered fusion protein for 2 h, followed by 10–12 more washes. Bound CaMKII was solubilized in 1% SDS, 0.2% NaOH, 50 mM EDTA, and quantified by liquid scintillation counting. Nonspecific binding to control bacterial extracts was subtracted from total binding to obtain specific binding. No specific binding was observed using [²⁵³P-T306]CaMKIIα.

GST Pull-down Analysis—GST fusion proteins were incubated (1 h, 4 °C) with either purified CaMKIIα (Fig. 2D, see caption) or with a freshly prepared rat brain cytosolic extract (~3 mg/ml extract protein, 10 μg/ml GST fusion protein) containing 2 μM microcystin-LR and 0.5% Triton X-100, precipitated with glutathione-agarose, washed extensively, and eluted with SDS sample buffer. CaMKIV antibodies were from Transduction Laboratories.

HEK293 Cell Colocalization—HEK293 cells were seeded on coverslips in 35-mm dishes, transfected with a total of 3 μg/dish DNA (1 μg of Srα promoter-CaMKIIα expression plasmid, 2 μg of cytomegalovirus promoter plasmids with NMDA receptor subunits at a mass ratio of 1:3 NR1a and NR2A/B subunits), and grown for 48 h as described (21). Robust expression of NMDA currents was verified by patch-clamp recording of parallel cultures.² Cells were washed and incubated in Mg²⁺/Ca²⁺/free Hanks' balanced saline containing 2 mM CaCl₂ and either the NMDA receptor antagonist 2-amino-5-phosphonovaleric acid (APV, 50 μM) or NMDA/glycine (100/10 μM) for 15 min. Cultures were fixed and processed for immunofluorescence (see above) using 1:500 antibody dilutions of goat anti-CaMKII (16), mouse anti-NR1 (PharMingen), and rabbit anti-NR2A/B (Chemicon). Between 2 and 5% of cells were strongly positive for at least one label; only those cells expressing high levels of at least 50% of transfected cells) were included in the analyses. Under basal conditions, CaMKIIα expression was diffusely cytoplasmic. Irrespective of agonist treatment, NR1 and NR2A/B strictly colocalized (mean scores >3.4, see below) in a patchy or reticular, often perinuclear pattern as seen previously in heterologous cells (22). Cultures were randomized prior to sampling digital images on a confocal microscope to prevent operator bias. Coded images (as in Fig. 3) were assigned a colocalization score by a second, naive observer: 0, mutual exclusion; 1, coincidental overlap; 2 or 3, increasing degrees of colocalization, 4, complete overlap of labels. For reference, the cells in Fig. 3 scored a 0, 1, 2, 2, and a 3 (from left to right, top to bottom).

RESULTS AND DISCUSSION

To determine whether NR2 subunits contribute to the previously characterized “p190” overlay binding activity (16), we analyzed immunoprecipitated NR2A/B by gel overlay with [²⁵³P-T286]CaMKIIα (Fig. 1A). A CaMKII-binding activity comigrating with NR2A and NR2B was immunoprecipitated with NR2A/B antibodies, but not control antibodies, indicating that NR2A and/or NR2B are CaMKII-binding proteins.

This interaction may be physiologically relevant, because triple immunofluorescent labeling of cultured cortical neurons demonstrated that CaMKII colocalizes with NMDA receptors in many punctaæ along dendritic shafts, identified as synapses by the adjacent or overlapping presence of synaptophysin (Fig. 1B). Higher magnification revealed a mostly postsynaptic localization of CaMKII in dendritic spines (Fig. 1C). Moreover, a complex of CaMKII with NMDA receptor subunits can be immunoprecipitated from PSDs using CaMKII antibodies, but not preimmune IgG (Fig. 1D). NR2B was more efficiently coprecipitated than NR1, likely because association of CaMKII with NR1 is indirect (i.e. via NR2B, see below). Recovery of the receptor-kinase complex required pretreatment of PSDs with a reversible cross-linker prior to essentially complete PSD solubilization in 2% SDS, indicating that the interaction of CaMKII with NMDA receptors is not stable in harsh detergents. The specificity of the cross-linking procedure was demonstrated by the absence of other abundant PSD proteins in the immunoprecipitate, including the catalytic subunit of protein phosphatase 1 (Fig. 1D).

NMDA receptor subunits have a common transmembrane topology with three membrane-spanning regions and a C-terminal tail of variable length, which forms the intracellular portion of the receptor (Fig. 2A, diagram). Bacterial lysates expressing the cytoplasmic domains of the predominant forebrain NMDA receptor subunits, NR1, NR2A, and NR2B, as His₄ tag fusion proteins were screened for [²⁵³P-CaMKIIα binding by overlay (Fig. 2A). The NR2B cytoplasmic domain bound about six times more [²⁵³P-T286]CaMKIIα than the corresponding region of NR2A; neither NR1 nor any endogenous bacterial proteins showed detectable binding. Interactions with NR2A and NR2B were specific for autonomously active CaMKII, as

³ R. L. Popp and D. M. Lovinger, personal communication.

\[ \text{CaMKII Binding to NR2B} \]
CaMKII binding to NR2B

Fig. 2. Identification of a CaMKII-binding domain in NR2B. A, full-length cytosolic domains of NR1 (splice variant A, 834-T), NR2A (838-T), and NR2B (839-T) (where T indicates terminus) and the diagrammed NR2B constructs were screened for overlay binding of \(^{32}P\)CaMKII \(\alpha\) phosphorylated at either Thr\(^{286}\) (T286) or Thr\(^{305}\) (T306). Data were corrected for expression levels and autophosphorylation stoichiometries, normalized to NR2B-(839-T) and expressed as means ± S.E. of four to eight experiments. B, a blot of 0.5 \(\mu\)g of the indicated NR2A or NR2B residues fused to GST or 1.5 \(\mu\)g of GST alone was first stained for protein with Ponceau S (top) and then analyzed for \(^{32}P\)CaMKII \(\alpha\) binding (bottom). C, indicated soluble His\(_{6}\) tag NR2B fusion proteins were affinity-tethered to a microtiter plate and incubated with the indicated concentrations of \(^{32}P\)T286CaMKII \(\alpha\). Shown are means ± S.D. of duplicate determinations from one experiment representative of three. Inset, linear fit of Scatchard plot of same data. D, CaMKII \(\alpha\) and GST-NR2B-(1260–1309) fusion protein (0.5 \(\mu\)M) each with or without calcium/calmodulin (0.5 mM/3 \(\mu\)M) and Thr\(^{286}\) autophosphorylation were sedimented with glutathione-agarose and analyzed by Ponceau S staining of protein blots. E, a rat brain cytosolic extract was incubated with GST-NR2B or GST alone, purified with glutathione-agarose, and immunblotted with the indicated antibodies. Data (D, E) are representative of three experiments.

CaMKII \(\alpha\) phosphorylated in the absence of calcium/calmodulin at Thr\(^{286}\)/Thr\(^{305}\) ([P-T306]CaMKII \(\alpha\)) bound only weakly (<5%). Because NR2B displayed the most robust interaction with CaMKII, we mapped its CaMKII-binding domain by creating a series of truncation and internal deletion constructs. Only constructs containing NR2B residues 1260–1309 showed CaMKII binding similar to the full-length cytoplasmic tail. Fusion of NR2B-(1260–1309) to GST demonstrated that this domain is also sufficient for interaction with autonomous CaMKII (Fig. 2B).

A solution interaction assay was employed to examine binding of CaMKII to NR2B that had not undergone denaturation/renaturation for gel overlay analysis. \(^{32}P\)T286CaMKII \(\alpha\) bound saturably to a His\(_{6}\) tag NR2B fusion protein containing residues 1260–1309, but not to a construct that starts at residues 1310, C-terminal of this domain (Fig. 2C). Scatchard analysis indicated that binding involves a simple bimolecular interaction with a \(K_d\) of 138 ± 60 nM (n = 3) (Fig. 2C, inset). This \(K_d\) is ~100 times lower than the average concentration of CaMKII in forebrain (16, 23), suggesting that the interaction can readily occur in neurons.

The CaMKII-binding domain in NR2B contains a high-affinity phosphorylation site, Ser\(^{1309}\), which is phosphorylated by CaMKII \(\alpha\) in vitro and is also phosphorylated in vivo (13). However, three lines of evidence indicate that the binding of CaMKII to NR2B-(1260–1309) is not dependent on a substrate interaction. First, the model peptide substrate syntide-2 only weakly inhibits CaMKII binding (~30%) at concentrations of ~100-fold the \(K_m\) for phosphorylation (not shown). Second, even though NR2A residues 1255–1298 are 36% identical to NR2B-(1260–1309), and sequences surrounding the phosphorylation site are almost perfectly conserved (NR2B, LRRQH-SYD; NR2A, INRQHSYD) (13), CaMKII binding to NR2A-(1255–1298) is ~10-fold weaker under our overlay conditions (10.7 ± 1.8%, n = 3, Fig. 2B), suggesting that nonconserved residues in NR2B-(1260–1309) are important for high-affinity CaMKII binding. Third, “pull-down” experiments, in which GST-NR2B fusion protein was purified with glutathione-agarose, showed that calcium/calmodulin alone did not promote CaMKII interaction with NR2B, but that stoichiometric interaction was instead strictly dependent on CaMKII \(\alpha\) autophosphorylation at Thr\(^{286}\) (Fig. 2D). On the other hand, calcium/...
CaMKII Binding to NR2B

calmodulin binding is sufficient for full CaMKII activation, and Thr286 autophosphorylation stabilizes the active conformation of the kinase in the absence of calcium/calmodulin (1, 2). Thus, CaMKII residues outside the substrate binding site are involved in the interaction with NR2B.

Further evidence for specific association of CaMKII with NR2B was obtained by performing GST-NR2B pull-downs from brain cytosolic extracts. α and β isoforms of CaMKII were isolated following incubation with GST-NR2B-(1260–1309), but not GST alone. Affinity-purified CaMKIIs displayed an upward electrophoretic mobility shift characteristic of autophosphorylation (Fig. 2E). CaM kinase IV, a related kinase with a similar phosphorylation site preference (24), as well as other kinases and phosphatases tested, were not detected in the precipitated material, strongly indicating that NR2B-(1260–1309) binds selectively to CaMKII.

The NR2B subunit of the NMDA receptor was shown to target Thr286 autophosphorylated CaMKII in HEK293 cells. CaMKII was coexpressed with various NMDA receptor subunit combinations, and their distributions were compared by immunofluorescence (Fig. 3). Whereas NR1 alone does not form functional NMDA receptors in HEK293 cells, activation of both NR1/NR2A and NR1/NR2B receptors leads to massive calcium influx (25). Coexpression of CaMKIIα and NR1 alone resulted in low colocalization scores that were unaffected by acute treatment with the receptor agonists NMDA/glycine (Fig. 3A). Perhaps reflecting the low but detectable CaMKII binding activity of NR2A (Fig. 2, A and B), additional expression of the NR2A subunit led to a small increase in CaMKIIα and NR1/NR2A colocalization, which was not significantly increased by NMDA/glycine treatment (Fig. 3D). In cells expressing NR2B with CaMKIIα and NR1, we observed a similarly modest increase in colocalization in the absence of agonist treatment compared with CaMKIIα and NR1 alone (Fig. 3, C and D). In contrast to NR2A-containing NMDA receptors, activation of NR1/NR2B receptors with NMDA/glycine caused a highly significant redistribution of CaMKIIα into receptor-positive patches (Fig. 3, C and D), strongly suggesting that receptor activation induced the formation of a CaMKII-NR2B complex. Replacing extracellular calcium with barium, which is receptor-permeable but binds only poorly to calmodulin, completely blocked the effect of NMDA (Fig. 3D). Thus, opening of NMDA receptors is not sufficient for complex formation, but calcium influx is essential, presumably to stimulate calcium/calmodulin-dependent autophosphorylation of CaMKII. Consistent with this interpretation, an autophosphorylation-incompetent form of the kinase, T286A-CaMKIIα (26, 27), expressed at similar levels of wild-type CaMKIIα failed to show activity-induced colocalization with NR1/NR2B containing NMDA receptors (Fig. 3D). Thus, NR2B mediates targeting of CaMKII to NMDA receptors in a calcium- and Thr286 autophosphorylation-dependent manner in intact cells.

Our data support a model in which dendritic calcium influx induced by synaptic activity triggers CaMKII autophosphorylation at Thr286 and subsequent binding to residues 1260–1309 in the NR2B subunit of the NMDA receptor. What are the functional consequences of this interaction? Autonomous CaMKII in the PSD is inactivated by PSD-associated serine/threonine phosphatases (18, 28, 29). Once dephosphorylated at Thr286, CaMKII positioned near the mouth of the NMDA receptor calcium channel is likely to undergo rapid re-autophosphorylation even during periods of low level NMDA receptor activation. Thus, an interaction of CaMKII with NMDA receptors is predicted to boosts autonomous kinase activity, leading to enhanced phosphorylation of nearby downstream effectors of synaptic plasticity (15). Furthermore, recruitment of CaMKII into the PSD structure (6), possibly via association with NR2B, may play a role in the rapid ultrastructural changes of synapses that undergo LTP (30, 31). The developmental appearance of NR2A and down-regulation of NR2B in the mammalian visual system correlate with the end of the "critical period" of synapse maturation (32, 33). Preferential association of CaMKII with NR2B over NR2A may therefore provide a mechanism by which NMDA receptor subunit composition can impact developmental plasticity.

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