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Protein Kinase C Activation Modulates α-Calmodulin Kinase II Binding to NR2A Subunit of N-Methyl-D-Aspartate Receptor Complex*

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The N-methyl-D-aspartate (NMDA) receptor subunits NR2 possess extended intracellular C-terminal domains by which they can directly interact with a large number of postsynaptic density (PSD) proteins involved in synaptic clustering and signaling. We have previously shown that PSD-associated α-calmodulin kinase II (αCaMKII) binds with high affinity to the C-terminal domain of the NR2A subunit. Here, we show that residues 1412–1419 of the cytosolic tail of NR2A are critical for αCaMKII binding, and we identify, by site directed mutagenesis, PKC-dependent phosphorylation of NR2A-(Ser^{1416}) as a key mechanism in inhibiting αCaMKII-binding and promoting dissociation of αCaMKII·NR2A complex. In addition, we show that stimulation of PKC activity in hippocampal slices either with phorbol esters or with the mGluRs specific agonist *trans*-1-amino-1,3-cyclopentanedicarboxylic acid (t-ACPD) decreases αCaMKII binding to NMDA receptor complex. Thus, our data provide clues on understanding the molecular basis of a direct cross-talk between αCaMKII and PKC pathways in the postsynaptic compartment.

The excitatory glutamatergic synapse is thought to be controlled by a complex net of signaling proteins that regulate the postsynaptic ion channel activity. Electron microscopy and biochemical studies have identified, in the postsynaptic compartment of the glutamatergic synapse, the postsynaptic density (PSD)[1] fraction as a complex structure containing signaling proteins, cytoskeletal components, and ion channels (1, 2). Among ion channels, NMDA and 1-α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) glutamate receptors are particularly enriched in PSD (3–5). In particular, NMDA receptors are critical players in synaptogenesis, synaptic plasticity, as well as in the molecular pathogenesis of neurological disorders (6, 7). NMDA receptors are oligomeric complexes formed by the coassembly of members of three receptor subunit families: NR1, NR2 (NR2A–D; Ref. 6), and NR3A (8). The NR2 NMDA receptor subunits possess extended intracellular C-terminal domains by which they can interact with a large number of PSD proteins (9–13). Due to its anatomical localization and expression onset (14), NR2A is likely to play a major role in synaptic plasticity modulating long term potentiation and long term depression.

Although different groups identified NMDA receptor subunits NR2A/B as a target for αCaMKII in PSD (13, 15–18), the exact nature of these complex interactions requires further elucidation. In particular, the molecular mechanism(s) responsible for CaMKII dissociation from NMDA receptor complex is far from being clear. In the last 10 years, the hypothesis of a specific interaction between CaMKII and PKC pathways in the postsynaptic compartment has been put forward (19). The intracellular domains of NR2 subunits contain serine residues that can be phosphorylated by either CaMKII or protein kinase C (20–22). In addition and very recently, PKC has been shown to potentiate NMDA receptor activity even if the exact role of PKC phosphorylation on NMDA receptor complex function is still not completely understood (23, 24). Here we show that PKC-mediated phosphorylation on Ser^{1416} residue of NR2A C-terminal tail decreases its binding affinity for αCaMKII. Moreover we provide evidence that PKC stimulation in hippocampal slices either through phorbol esters or mGluR activation decreases the interaction between αCaMKII and NMDA receptor complex.

EXPERIMENTAL PROCEDURES

PSD Preparation—To isolate PSD from rat hippocampus, a modification of the method by Carlin *et al.* (25) was used, as described previously (16, 17).

Cloning, Expression, and Purification of GST Fusion Proteins—NR2A fragments were subcloned downstream of GST in the *Bam*HI and *Hin*dIII sites of the expression plasmid pGEX-KG by polymerase chain reaction using the *Pfu* polymerase (Stratagene, La Jolla, CA) on a cDNA containing plasmid for NR2A (kind gift from S. Nakanishi). GST-NR2A fusion constructs were expressed in *Escherichia coli* and purified on glutathione-agarose beads as described previously (17). Mutated GST-NR2A fusion proteins were produced by using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) or the ExSite™ PCR-Based Site-Directed Mutagenesis Kit (Stratagene). The inserts were fully sequenced with ABI Prism 310 Genetic analyzer (ABI Prisma).

"Pull-out" Assay—Aliquots of 5 μg of hippocampal PSD were diluted with phosphate-buffered saline, 1% SDS and incubated (1 h, 37 °C) with glutathione-agarose beads saturated with GST fusion proteins or GST alone. The beads were then extensively washed with phosphate-buffered saline, 0.2% Triton X-100. Bound proteins were resolved by SDS-PAGE and subjected to immunoblot analysis with a monoclonal anti-αCaMKII antibody.

Overlay Technique—An overlay procedure has been used to detect the binding of eluted GST-NR2A fusion proteins to either PSD-associated or purified αCaMKII. PSD proteins or purified αCaMKII were separated on a 6% SDS-PAGE gel and transferred overnight to nitrocellulose. The membrane was blocked for 1 h in 50 mM Tris-HCl, 200 mM

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[1] The abbreviations used are: PSD, postsynaptic density; PKC, protein kinase C; GST, glutathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis; t-ACPD, *trans*-1-amino-1,3-cyclopentanedicarboxylic acid; PDBu, phorbol 12,13-dibutyrate; TIF, Triton X-100-insoluble fraction; NMDA, *N*-methyl-D-aspartate; αCaMKII, α-caldmodulin kinase II.

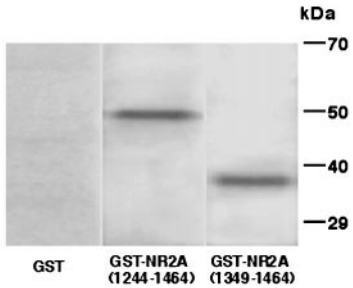

FIG. 1. ***In vitro* phosphorylation of GST-NR2A fusion proteins.** GST, GST-NR2A-(1244–1464), and GST-NR2A-(1349–1464) purified fusion proteins were incubated with purified protein kinase C in the presence of [γ-^{32}P]ATP as phosphate donor (2 μCi/tube; 3000 Ci/mmol). Proteins were separated by SDS-PAGE (running gel: 11% acrylamide) and phosphoproteins revealed by autoradiograph.

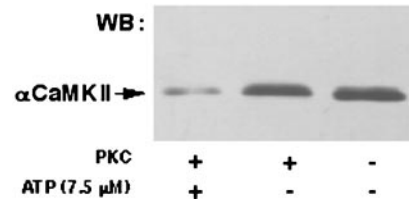

FIG. 2.**Effect of PKC-dependent phosphorylation on NR2A-αCaMKII association.** GST-NR2A-(1349–1464) fusion protein was phosphorylated in a PKC-dependent manner in absence of presence of ATP and then incubated in a pull-out assay with purified hippocampal PSD. Bound proteins were eluted from the beads with SDS sample buffer, separated by SDS-PAGE, and analyzed on Western blotting with anti-αCaMKII monoclonal antibody.

NaCl (TBS), pH 8.0, containing 1% Tween 20 (v/v) and nonfat powdered milk (5% w/v). The membrane was washed in TBS containing 0.1% v/v Tween 20 and nonfat powdered milk (5% w/v) and then incubated for 2 h at room temperature with constant shaking in overlay buffer TBS, 5% (w/v) nonfat powdered milk, 0.1% Tween 20 containing the fusion protein. After extensive washes, the membrane was subjected to immunoblot analysis using a polyclonal anti-GST antibody.

GST-NR2A Fusion Protein Phosphorylation—For PKC-dependent phosphorylation of NR2A, GST-NR2A-purified fusion proteins were incubated with 50 units of purified PKC (Sigma-Aldrich, Milan, Italy) for 30 min at 37 °C, in presence of 10 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 0.5 mM $CaCl_2$, and 10 μM ATP ([γ-^{32}P]ATP, 2 μCi/tube; 3000 Ci/mmol; Amersham Pharmacia Biotech, Little Chalfont, United Kingdom).

Preparation of Hippocampal Slices—Hippocampal slices were obtained as described previously (26). Briefly, brains were removed and placed into chilled (4 °C) oxygenated Krebs buffer. After removal of meninges, hippocampal slices were prepared quickly with a McIlwain tissue chopper and placed in custom-made chambers equilibrated continuously with 95% O_2/5% CO_2 (v/v). Slices were then equilibrated at 37 °C (95% O_2/5% CO_2) for 30 min. After the equilibration period, slices were incubated for 15 min in the absence or presence of either t-ACPD (10 μm) or phorbol 12,13-dibutyrate (PDBu, 0.1 μM) or H-7 (10 μM). After incubation, slices were homogenized in cold 0.32 M sucrose containing 1 mM Hepes, 1 mM $MgCl_2$, 1 mM $NaHCO_3$, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4, in presence of a complete set of protease inhibitors (Complete™, Roche Molecular Biochemicals, Mannheim, Germany) and phosphatase inhibitors. The homogenized tissue was centrifuged at 1,000 × *g* for 10 min. The resulting supernatant was centrifuged at 3,000 × *g* for 15 min to obtain a fraction of mitochondria and synaptosomes. The pellet was resuspended in hypotonic buffer (in presence of proteases inhibitors) in a glass-glass potter and centrifuged at 100,000 × *g* for 1 h. The resultant pellet was resuspended in 1 ml of buffer containing 75 mM KCl and 1% Triton X-100 and centrifuged at 100,000 × *g* for 1 h. The final pellet was homogenized by 10 strokes in a glass-glass potter in 20 mM Hepes. An equal volume of glycerol was added and stored at −80 °C. The protein composition of this preparation was carefully tested for the absence of presynaptic markers and for the enrichment in the PSD proteins (αCaMKII, PSD-95, NMDA receptor subunits; Ref. 27). This fraction is referred to as "Triton-insoluble fraction."

Immunoprecipitation—Triton-insoluble fraction proteins (50 μg) were incubated overnight at 4 °C in buffer A containing: 200 mM NaCl, 10 mM EDTA, 10 mM Na_2HPO_4, 0.5% Nonidet P-40, 0.1% SDS in a final volume of 200 μl with antibodies against NR2A/B as indicated in the text (dilution 1:50). Protein A-agarose beads (5 mg/tube), or *Staphylococcus aureus* Cowan I cells (0.5%) washed in the same buffer, were added and incubation was continued for 2 h. The beads were collected by centrifugation and washed three times with buffer A. Sample buffer for SDS-PAGE was added, and the mixture was boiled for 3 min. Beads were pelletted by centrifugation, and supernatants were applied to 6% SDS-PAGE.

Antibodies—Monoclonal αCaMKII antibody was purchased from Roche Molecular Biochemicals; polyclonal antibody against anti-active p286-αCaMKII was purchased from Promega (Madison, WI); polyclonal antibody against NR2A/B was purchased from Chemicon International, Inc., (Temecula, CA); a polyclonal antibody against GST was produced in rabbits using recombinant GST (17).

RESULTS

We first investigated whether PKC was capable to phosphorylate in an *in vitro* assay the NR2A C-terminal tail. To this end, GST fusion proteins with different NR2A C-terminal domains were incubated with purified PKC in presence of [γ-^{32}P]ATP as a phosphate donor. Fig. 1 shows a representative autoradiograph of *in vitro* PKC-dependent phosphorylation of NR2A-(1244–1464) and NR2A-(1349–1464) GST fusion proteins; a radioactive band corresponding to both NR2A-(1349–1464) and NR2A-(1244–1464) GST fusion protein phosphorylation is clearly visible (Fig. 1), indicating the presence of several PKC phosphosites distributed in the NR2A C-terminal domain; the phosphorylation signal shown by the NR2A fragment is specific, since GST alone did not show any phosphoband (*leftmost lane*).

Previous studies of our laboratory (17) identified NR2A domain 1349–1464 as the binding region for both recombinant and PSD-associated αCaMKII; using the pull-out procedure we asked whether the PKC-dependent phosphorylation of this region could influence the NR2A-αCaMKII binding affinity. GST-NR2A-(1349–1464) fusion proteins, *in vitro* phosphorylated by purified PKC in presence or absence of ATP as phosphate donor, were incubated in a pull-out assay with solubilized hippocampal PSD (Fig. 2). PKC phosphorylation of GST-NR2A-(1349–1464) is able to decrease (−75.6% ± 9.3%, mean ± S.D.; $n = 6$, $p < 0.01$) the binding of PSD-associated αCaMKII to NR2 C-terminal tail (Fig. 2). Inspection of this NR2A region revealed the presence of at least two domains (YLR-S^{1403}-S^{1404}-LRS and SDR-S^{1416}-RGH) containing serine residues (Ser^{1403}-Ser^{1404}, Ser^{1416}) that might represent a putative phosphate acceptor site for PKC and that might be of relevance in αCaMKII binding to NR2A.

A point mutation strategy was used to determine the effects of PKC site-specific phosphorylation of NR2A: GST-NR2A-(1349–1464)S1416A, GST-NR2A-(1349–1464)S1403A/S1416A, and GST-NR2A-(1349–1464)S1403A/S1404A/S1416A fusion proteins were generated and *in vitro* phosphorylated by PKC in presence of [γ-^{32}P]ATP as phosphate donor. As shown in Fig. 3, the single-mutated S1416A product displays *per se* a decrease of 63.7% ± 5.3% (mean ± S.D.; $n = 6$, $p < 0.01$) of the ^{32}P incorporation as compared with the nonmutated protein, thus indicating that this site represents a major phosphate consensus site for PKC in the NR2A C-terminal tail. The concomitant S1403A/S1404A/S1416A mutations lead to the complete inability of NR2A fusion protein to be *in vitro* PKC-phosphorylated.

To analyze whether PKC-mediated NR2A(Ser^{1416}) phosphorylation in the C-terminal region was sufficient to affect αCaMKII binding, control and mutated GST-NR2A-(1349–1464)S1416A fusion proteins were cold-phosphorylated by PKC and then subjected to pull-out assay in the presence of hippocampal PSD previously radiolabeled in conditions known to activate αCaMKII autophosphorylation. It is well known that

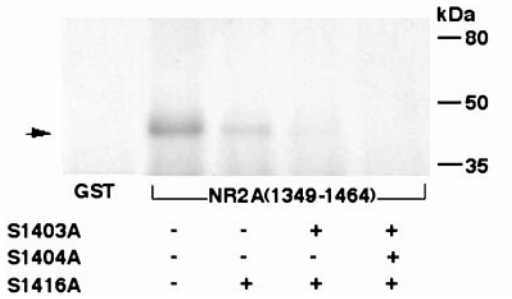

FIG. 3. **In Vitro PKC-dependent phosphorylation of GST-NRA2-(1349–1464) fusion proteins.** GST, GST-NR2A-(1349–1464), GST-NR2A-(1349–1464)S1416A, GST-NR2A-(1349–1464)S1403A-/S1416A, and GST-NR2A-(1349–1464)S1403A/S1404A/S1416A were phosphorylated by purified PKC in presence of [γ-^{32}P]ATP as phosphate donor (2 μCi/tube; 3000 Ci/mmol). Proteins were separated by SDS-PAGE (running gel: 11% acrylamide) and phosphoproteins revealed by autoradiograph.

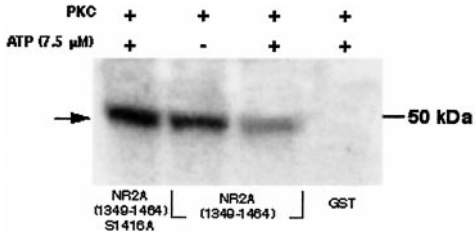

FIG. 4. **Effect of NR2A (S1416A) point mutation on NR2A-αCaMKII association.** Control and GST-NR2A-(1349–1464)S1416A fusion proteins were phosphorylated in a PKC-dependent manner in the presence or absence of ATP and then incubated in a pull-out assay with purified radiolabeled hippocampal PSD; following extensive washes, the bound proteins were eluted from the beads with SDS sample buffer, separated by SDS-PAGE, and autoradiographed.

this condition fosters maximal αCaMKII/NR2A association (17). Fig. 4 shows a representative autoradiograph of this pull-out assay. A phosphorylated band at 50 kDa corresponding to bound ^{32}P-αCaMKII is present. S1416A mutation (*first lane*) is able to prevent the modulation of αCaMKII binding to NR2A-(1349–1464) induced by PKC phosphorylation when compared with the nonmutated NR2A fusion protein (*middle lanes*; −59.6% ± 8.3%, mean ± S.D.; $n = 6$, $p < 0.05$).

The binding of PKC-phosphorylated NR2A C-terminal tail to either PSD-associated αCaMKII or purified αCaMKII was confirmed by means of the overlay procedure using control and mutated GST-NR2A-(1349–1464) fusion proteins. PSD proteins or purified αCaMKII were separated in SDS-PAGE and electroblotted; blocked nitrocellulose was incubated with eluted control and mutated GST-NR2A-(1349–1464) fusion proteins previously phosphorylated in a PKC-dependent manner. As shown in Fig. 5*A* (*first* and *second lanes*), PKC phosphorylation of eluted control fusion products is sufficient to decrease significatively the binding of NR2A-(1349–1464) to PSD-associated αCaMKII (*upper panel*; −77.2% ± 7.3%, mean ± S.D.; $n = 8$, $p < 0.01$) and to purified αCaMKII (*lower panel*; −70.3% ± 9.3%, mean ± S.D.; $n = 8$, $p < 0.01$), confirming the results obtained using the pull-out assay (Fig. 2). S1416A mutation restore αCaMKII-NR2A binding to control levels (*third lane*); additional S1403A and S1404A mutations do not further increase the αCaMKII/GST-NR2A association (*fourth* and *fifth lanes*). In addition, S1416D mutant qualitatively and quantitatively mimics the binding of the PKC phosphorylated S1416-NR2A to αCaMKII both in pull-out (Fig. 5*C*) and overlay assays using either PSD (Fig. 5*B*, *left panel*) or purified αCaMKII (Fig. 5*B*, *right panel*).

To determine whether the PKC phosphorylation consensus site surrounding NR2A-Ser^{1416} was necessary for αCaMKII binding, site-directed mutagenesis was applied to introduce

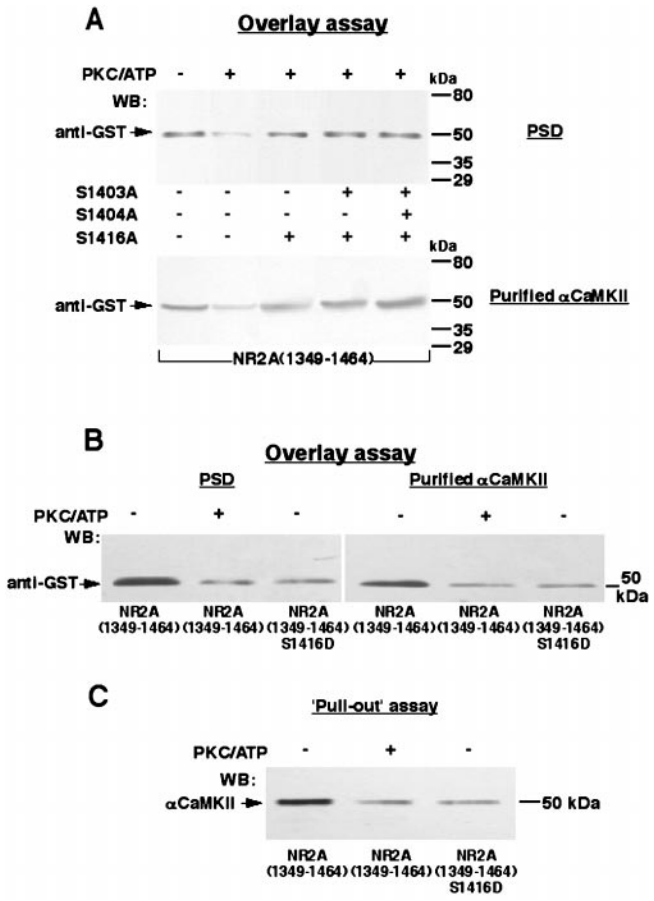

FIG. 5. **Overlay (*A* and *B*) and pull-out assay *(C)*.** *A*, GST-NR2A-(1349–1464), GST-NR2A-(1349–1464)S1416A, GST-NR2A-(1349–1464)S1403A/S1416A and GST-NR2A-(1349–1464)S1403A/S1404A/S1416A bind in overlay experiments both PSD-associated (*upper panel*; 50-kDa band) and purified αCaMKII (*lower panel*). Either PSD proteins or purified αCaMKII were subjected to SDS-PAGE and electroblotted; blocked nitrocellulose was incubated with eluted control and mutated GST-NR2A-(1349–1464) fusion proteins previously phosphorylated in a PKC-dependent manner. The nitrocellulose sheet is revealed by Western blotting (*WB*) using anti-GST polyclonal antibody. *B*, control and S1416D mutant GST-NR2A-(1349–1464) fusion proteins binding in overlay experiments to both PSD-associated (*left panel*, 50-kDa band) and purified αCaMKII (*right panel*). *C*, control and S1416D mutant GST-NR2A-(1349–1464) fusion proteins binding in pull-out assay to αCaMKII.

deletions in the NR2A C-terminal tail to produce GST-NR2A-(1349–1411) and GST-NR2A-(1349–1464)Δ1412–1419 fusion proteins. Deletion of the NR2A-(1411–1464) C-terminal domain completely prevents the binding to αCaMKII both in pull-out (Fig. 6*B*) and overlay assays using either PSD (Fig. 6*A*, *left panel*) or purified αCaMKII (Fig. 6*A*, *right panel*). Δ1412–1419 deletion is not sufficient to preclude but significatively decreases the association of NR2A to αCaMKII both in pull-out (Fig. 6*B*) and overlay assays (Fig. 6*A*) using either PSD (*left panel*; −67.2% ± 6.3%, mean ± S.D.; $n = 8$, $p < 0.01$) and purified αCaMKII (*right panel*; −62.1% ± 5.9%, mean ± S.D.; $n = 8$, $p < 0.01$), thus suggesting an involvement of both NR2A-(1412–1419) and the downstream NR2A-(1420–1464) domain in the association with αCaMKII.

To further confirm the specific role of NR2A domain amino acids 1412–1419 in αCaMKII binding, phospho-SRD**S(p)**RGH and nonphosphorylated SRD**S**RGH peptides corresponding to NR2A-(1412–1419) were used in *in vitro* competition experiments with GST-NR2A-(1349–1464) and hippocampal PSD. The competition experiment with phosphopeptide SRD-**S(p)**RGH (Fig. 7*B*) does not result in a change of NR2A-(1349–

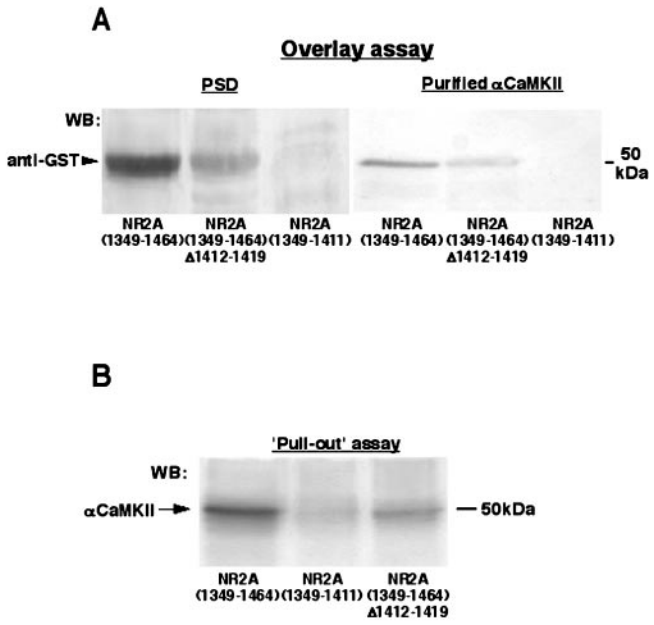

FIG. 6. **Overlay (*A*) and pull-out assay (*B*).** Deletion of NR2A-(1411–1464) C-terminal domain completely prevents the binding to αCaMKII; Δ1412–1419 deletion is not sufficient to preclude, but significatively decreases ($p < 0.01$), the NR2A-αCaMKII association in all experimental conditions.

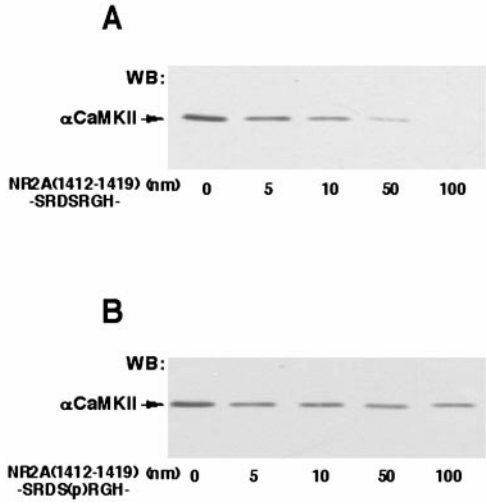

FIG. 7. **NR2A-(1412–1419) Competes with GST-NR2A-(1349–1464) in the binding with PSD-associated αCaMKII.** In vitro competition experiments between GST-NR2A-(1349–1464) and hippocampal PSD in the presence of either nonphosphorylated SRDSRGH (A: 0, 5, 10, 50, and 100 nm) or phospho-SRDS(p)RGH peptides (B: 0, 5, 10, 50, and 100 nm) corresponding to NR2A-(1412–1419); following extensive washes, the bound proteins were eluted, separated by SDS-PAGE and analyzed on Western blotting with anti-**αCaMKII monoclonal antibodies.**

1464)/αCaMKII binding even at high concentrations. On the other hand, the nonphosphorylated peptide SRD**S**RGH (Fig. 7*A*) competes with NR2A-(1349–1464) for αCaMKII binding in a concentration-dependent manner (0–100 nM), indicating a role of the NR2A-(1412–1419) domain in the modulation of αCaMKII binding (SRD**S**RGH, 50 nM: −78.4% ± 8.9%, mean ± S.D.; $n = 8$ $p < 0.01$). In addition, the competition experiment strengthens the relevance of Ser^{1416} phosphorylation in αCaMKII binding modulation.

To investigate in a more physiological context the modulation of αCaMKII·NMDA receptor complex association mediated by PKC activation, we used hippocampal slices prepared as described previously (26); in a first set of experiments, slices

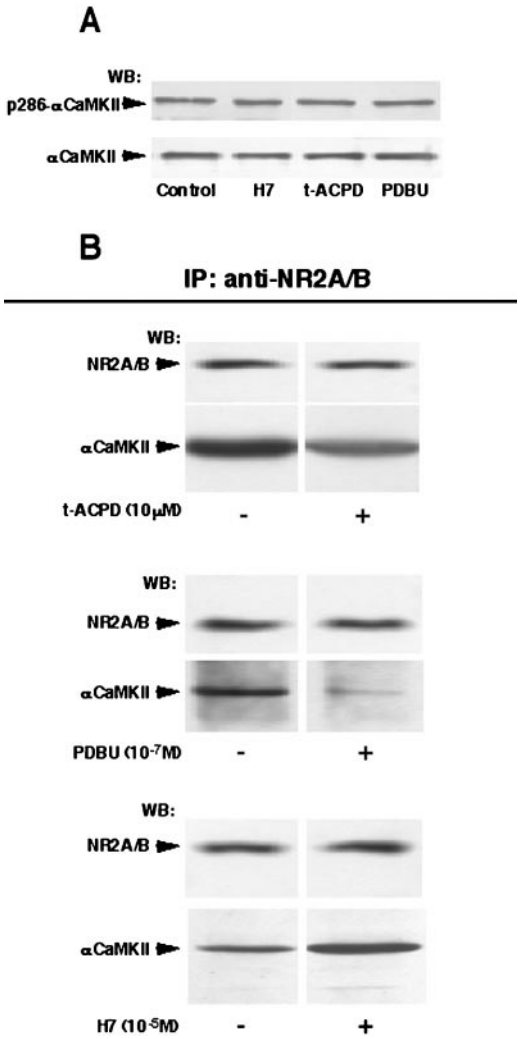

FIG. 8. **Modulation of αCaMKII binding to NMDA receptor complex in acute hippocampal slices is modulated by PKC activation.** *A*, Western blotting analysis performed in the TIF fraction with anti-αCaMKII and anti-active p286-αCaMKII; *B*, proteins from the TIF were immunoprecipitated with a polyclonal antibody raised against NR2A/B subunits of NMDA receptor complex; Western blot analysis was performed in the intraperitoneal material with anti-NR2A/B, anti-αCaMKII.

were incubated with PKC activator PDBu (10^{-7} M), with the PKC inhibitor H-7 (10^{-5} M), and with vehicle alone. After incubation, a Triton X-100-insoluble fraction (TIF) was obtained, and Western blotting analysis and NMDA receptor coprecipitation studies were performed (27). Western blotting analysis performed in the TIF fraction with anti-αCaMKII and anti-active p286-αCaMKII (Fig. 8*A*) monoclonal antibodies shows that the incubation with either PDBu or H-7 does not influence both the concentration and the autophosphorylation degree of αCaMKII in the TIF compartment. On the other hand, when TIF proteins were immunoprecipitated with a polyclonal antibody raised against NR2A/B subunits of NMDA receptor complex, Western blot analysis performed in the cointraperitoneal material (Fig. 8*B*) shows a significative increase of αCaMKII·NMDA receptor association in presence of the PKC inhibitor (+82.7% ± 9.2%, mean ± S.D.; $n = 6$, $p < 0.01$) and a decrease of αCaMKII cointraperitoneal in presence of the PKC activator (−84.7% ± 11.2%, mean ± S.D.; $n = 6$, $p < 0.01$). In a second set of experiments hippocampal slices were incubated in presence of t-ACPD to induce PKC activation mediated by mGluR; also under these experimental conditions, immunoprecipitation of NMDA receptor complex leads to a

significant reduction in αCaMKII cointraperitoneal, even if at a lower extent when compared with the results obtained in presence of PDBu (−54.7% ± 9.2%, mean ± S.D.; $n = 6$, $p < 0.05$).

DISCUSSION

In the last few years, it has been shown that CaMKII shuttles between the cytosol and the postsynaptic compartment and that CaMKII localization is under the dynamic regulation of neuronal activity (28). Although in the resting state CaMKII is highly localized in the cytosol and partially bound to actin, glutamate stimulation translocates the kinase to the PSD region. Once bound to the PSD, CaMKII still remains active, suggesting that PSD-trapped CaMKII can serve as a molecular tag involved in synapse potentiation for subsequent long term modifications (1). Different groups have supported the idea that αCaMKII translocation to the PSD fraction is probably due to a high affinity binding interaction with NMDA receptor subunits (13, 16–18).

We show here that the activation of PKC pathway in the postsynaptic compartment promotes αCaMKII dissociation from NMDA receptor complex. The mechanism shown in this study is not mediated by alteration of the autophosphorylation state of the kinase (Fig. 8) and does not affect the total amount of αCaMKII present in the Triton-insoluble fraction (that mimics the PSD; Fig. 8), suggesting a mechanism intrinsic to this cellular compartment and therefore involving αCaMKII translocation to the cytosolic fraction.

Moreover, we show here that stimulation of mGluR through t-ACPD, inducing NMDA receptor potentiation (29), decreases the affinity of αCaMKII for the NMDA receptor complex. Since stimulation of mGluR1 and mGluR5 leads to activation of PKC, and since PKC-mediated phosphorylation of NR2A decreases the affinity of NR2A for αCaMKII, it is likely that phosphorylation of NR2A by activated PKC through mGluR results in inhibition of αCaMKII-NR2A interaction.

In particular, we identify NR2A-Ser^{1416} as PKC-dependent phosphosite partially responsible of αCaMKII-NR2A binding modulation. Either deletion of NR2A-(1412–1419) or PKC-mediated phosphorylation of NR2A-Ser^{1416} are sufficient to decrease, but not to prevent, the αCaMKII-NR2A association. On the other hand, deletion of the C-terminal tail NR2A-(1411–1464) renders the receptor subunit completely unable to bind the kinase, suggesting the presence of at least two domains involved in αCaMKII-NR2A binding.

In the last decade different experimental approaches have suggested a functional cross-talk between Ca^{2+}/CaM and PKC pathways during long term potentiation induction (19, 30), even if the precise interaction between CaMKII and PKC during synaptic potentiation were not elucidated. PKC activity has been indicated to be required for Ca^{2+}/CaM-induced potentiation, as it has been shown for tetanus-induced long term potentiation (19), suggesting a positive communication between the CaMKII and PKC pathways. On the other hand, other data suggest that CaMKII and PKC pathways can run in parallel: these findings are supported by the observation that PKC or CaMKII activation can be independent and sufficient for synaptic potentiation (31). Very recently, the group of MacDonald and coworkers (24) demonstrated a PKC-mediated modulation of NMDA receptor assembly, suggesting a PKC phosphorylation-dependent regulation of the interactions occurring between NMDA receptor subunits, CaM and PSD proteins. Therefore, the role of PKC in modulating the NMDA receptor can be viewed as independent from a simple mechanism of channel potentiation due to subunits phosphorylation (23).

The role of PKC-dependent phosphorylation of NR2A in affecting the clustering between NMDA channel and other PSD proteins and the functional consequences of CaMKII dissociation on NMDA channel activity needs further elucidation. However, our data showing a dissociation of the complex CaMKII·NMDA receptor after PKC activation without affecting the total amount of CaMKII localized in the Triton-insoluble fraction shed light on a new fine biochemical mechanism intrinsic to this specialized postsynaptic compartment. Recent studies indicating αCaMKII binding to other PSD proteins, *i.e.* NR2B and Densin-180 (32), suggest a dynamic modulation of αCaMKII localization inside the PSD fraction of relevance in modulating NMDA-dependent potentiation of the postsynaptic compartment. The dissociation of αCaMKII·NMDA complex modulated by t-ACPD increases our knowledge on the mechanisms regulating metabotropic and ionotropic glutamate receptors in the postsynaptic compartment.

In conclusion, our results demonstrate a fine tuning of NMDA receptor complex assembly mediated by PKC and propose new molecular aspects of the cross-talk occurring between CaMKII and PKC in a specialized postsynaptic compartment involved in synaptic potentiation.

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Protein Kinase C Activation Modulates α-Calmodulin Kinase II Binding to NR2A Subunit of N-Methyl-D-Aspartate Receptor Complex

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