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Hippocampal Synaptic Plasticity Involves Competition between Ca^{2+}/Calmodulin-Dependent Protein Kinase II and Postsynaptic Density 95 for Binding to the NR2A Subunit of the NMDA Receptor

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NMDA receptor, Ca^{2+}/calmodulin-dependent protein kinase II (αCaMKII), and postsynaptic density 95 (PSD-95) are three major components of the PSD fraction. Both αCaMKII and PSD-95 have been shown previously to bind NR2 subunits of the NMDA receptor complex. The nature and mechanisms of targeting to the NMDA receptor subunits are, however, not completely understood. Here we report that the C-terminal NR2A(S1389-V1464) sequence was sufficient to guarantee the association of both native and recombinant αCaMKII and PSD-95. PSD-95(54–256) was able to compete with the binding of both native and recombinant αCaMKII to the NR2A C-tail. Accordingly, αCaMKII(1–325) competes with both the native PSD-95 and the native kinase itself for the binding to NR2A. In addition, Ser/Ala1289 and Ser/Asp1289 point mutations on the unique CaMKII phosphosite of NR2A did not significantly influence the binding of native αCaMKII and PSD-95 to the NR2A C-tail. Finally, the association–dissociation of αCaMKII and PSD-95 to and from the NR2A C-tail was significantly modulated by activation of NMDA receptor achieved by either pharmacological tools or long-term potentiation induction, underlining the importance of dynamic and reciprocal interactions of NMDA receptor, αCaMKII, and PSD-95 in hippocampal synaptic plasticity.

Key words: αCaMKII; LTP; NMDA; postsynaptic density; PSD-95; synaptic plasticity

A mature synapse is capable of modulating its efficacy by means of activity-dependent plasticity events. Such a process might in turn modulate the structural organization of specific synaptic compartment, i.e., the postsynaptic density (PSD), in which clustering of ligand-gated receptors to scaffolding proteins and to enzymes can be dynamically regulated (Ziff, 1997). PSD consists of a complex network of interacting proteins involved in the regulation of synaptic function and modulation of postsynaptic responses. PSDs are enriched in ionotropic AMPA and NMDA glutamate receptors (Ehlers et al., 1996; Kennedy, 1997, 1998). NMDA receptors are of major interest because they are involved in synaptogenesis, neuronal circuitry formation, synaptic plasticity, and learning and memory, as well as in the molecular pathogenesis of neurological disorders (Hollmann and Heinemann, 1994; During et al., 2000). NMDA receptors are oligomeric complexes formed by the coassembly of members of three receptor subunit families: NR1, NR2 subfamily (NR2A-D; Hollmann and Heinemann, 1994), and NR3A (Das et al., 1998). Among NR2 subunits, whose expression is developmentally regulated, NR2A is expressed in the adult rat brain in the large majority of synapses (Monyer et al., 1994). Because of its anatomical localization and expression onset, NR2A is likely to play a major role in synaptic plasticity modulating long-term potentiation (LTP) and long-term depression. In fact, animals with C-terminal truncation of postnatally expressed NR1/NR2A heteromeric receptors, but with an intact NR1/NR2B complex, exhibit impaired hippocampal LTP (Sprengel et al., 1998). In PSD, NR2A and NR2B subunits directly interact with PSD-95 (Kornau et al., 1995), chapsyn-110/PSD-93 (Kim et al., 1996), and other members of the membrane-associated guanylate kinase family (Lau et al., 1996) through their intracellular extended COOH sequence. In particular, NR2A C-terminal motif tSDV is mandatory for efficient binding to PSD-95 and synapse-associated protein 97 PDZ (PSD-95/Discs large/zona occludens 1) domains (Bassand et al., 1999). The interaction with the PSD-95 protein family induces the clustering of the channel proteins (Kim et al., 1996), thus playing an important role in the molecular organization of NMDA receptors, although more recent findings demonstrate that postsynaptic NMDA receptor clustering does not solely depend on the PSD-95 family (Migaud et al., 1998; Passafaro et al., 1999). In addition, PSD-95 appears to be important in coupling NMDA receptor to biochemical intracellular pathways controlling bidirectional synaptic plasticity (Tezuka et al., 1999; Yamada et al., 1999). Nevertheless, although it has been reported that the molecular interactions involving PSD-95 and the NMDA receptor are modified by pathological insults such as an ischemic challenge (Takagi et al., 2000), the physiological conditions influencing association–dissociation of specific proteins to and from NMDA receptor are not yet fully understood.

NR2 subunits are not solely associated to PSD-95; indeed, the NMDA receptor complex has been also shown to bind Ca^{2+}/calmodulin-dependent protein kinase II (αCaMKII) (Gardoni et al., 1998; Strack and Colbran, 1998; Leonard et al., 1999). We have demonstrated recently that the C-terminal domain 1349–

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1464 of NR2A subunit of NMDA receptor complex interacts with both PSD-95 and native αCaMKII and that receptor–kinase interaction occurs with unphosphorylated αCaMKII, but it is strengthened by kinase autophosphorylation (Gardoni et al., 1999).

Although all of these observations identify NMDA receptor subunits as a target for both PSD-95 and αCaMKII in PSD, the exact nature of these interactions requires further elucidation. In the present study, we address therefore the following questions: (1) is αCaMKII binding to NR2A directly and specifically antagonized by PSD-95?; and (2) can activity-dependent synaptic plasticity, i.e., LTP induction, modulate both αCaMKII and PSD-95 association to NMDA receptor complex?

Answering these questions will help clarify the importance of the reciprocal interaction of these three major components of PSD in synaptic function and synaptic plasticity.

MATERIALS AND METHODS

PSD preparation. To isolate PSD from rat hippocampus, a modification of the method by Carlin et al. (1980) was used as described by Gardoni et al. (1998, 1999).

Cloning, expression, and purification of glutathione S*-transferase fusion proteins.* NR2A, αCaMKII, and PSD−95 fragments were subcloned downstream of glutathione *S*-transferase (GST) in the *Bam*HI and *Hin*dIII site of the expression plasmid pGEX-KG by PCR using the Pfu polymerase (Stratagene, La Jolla, CA) on a cDNA containing plasmids forNR2A (kind gift from S. Nakanishi, Kyoto University, Kyoto, Japan), αCaMKII (kind gift from H. Schulman, Stanford University, CA) or PSD−95 cDNA (kind gift from M. Sheng, Massachusetts General Hospital, Boston, MA). The inserts were fully sequenced with ABI Prism 310 Genetic Analyser (Applied Biosystems, Foster City, CA).

GST-NR2A(1244–1464), GST-NR2A[(1244–1464)Ser1289/Asp], GST-NR2A[(1244–1464)Ser1289/Ala], GST-NR2A(1349–1464), GST-NR2A(1244–1389), GST-NR2A(1244–1461), GST-αCaMKII(315−478), and GST-PSD-95(54–256) fusion constructs were expressed in *Escherichia coli* and purified on glutathione agarose beads as described previously (Gardoni et al., 1999). GST fusion proteins were eluted with PBS, reduced 20 mM glutathione, 1% Triton X-100, and 0.1 mM PMSF overnight at 4°C. When indicated in the text, purified fusion proteins were incubated for protease cleavage, to cut the GST tags, for 1 hr at 25°C with 100 ng of human thrombin (Sigma, Deisenhofen, Germany) in elution buffer containing 150 mM NaCl and 2.5 mM $CaCl_2$. GST-NR2A[(1244–1464)Ser1289/Ala] and GST-NR2A[(1244–1464)Ser1289/Asp] were produced by using the QuikChange Site-Directed Mutagenesis kit (Stratagene).

"Pull-out" assay. Aliquots containing 5 μg of hippocampal PSD were diluted with PBS and 0.1 or 1% SDS to a final volume of 1 ml and incubated (1 hr, 37°C) with glutathione agarose beads saturated with GST fusion proteins or GST alone. After an incubation period, the beads were extensively washed with PBS and 0.1% Triton X-100. Bound proteins were resolved by SDS-PAGE and subjected to immunoblot analysis with a monoclonal anti-PSD-95 antibody, a monoclonal anti-αCaMKII antibody, and a polyclonal anti-GluR2/3 antibody.

GST-NR2A fusion protein phosphorylation. For CaMKII-dependent phosphorylation of NR2A, GST-NR2A purified fusion proteins were incubated with 50 U of αCaMKII(1–325) (New England Biolabs, Beverly, MA) for 30 min at 37°C, in the presence of 10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, 2.4 μM calmodulin, 2 mM $CaCl_2$, and 100 μM ATP (2 μCi/tube [γ-^{32}P]ATP; 5000Ci/mmol; Amersham Pharmacia Biotech, Little Chalfont, UK). The reaction was stopped by the addition of electrophoresis sample buffer (2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 62.5 mM Tris-HCl, pH 6.8). Proteins were separated by SDS-PAGE (running gel, acrylamide 11%), and phosphoproteins were revealed by autoradiography.

Preparation of hippocampal slices. Hippocampal slices were obtained as described previously (Caputi et al., 1997). 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 O_2 95%–CO_2 5% (v/v). Slices were then equilibrated at 37°C (O_2 95%–CO_2 5%) for 30 min. After the equilibration period, slices were incubated for 5 min in the absence or presence of 100 μM glutamate–1 μM glycine. KN-93 10^{-5} M was applied simultaneously to Glu/Gly, and the incubation was stopped after 5 min. After incubation, slices were transferred in <1 min (Suzuki et al., 1994) in the homogenization chamber and rapidly homogenized in 0.32 M cold sucrose containing 1 mM HEPES, 1 mM $MgCl_2$, 1 mM $NaHCO_3$, and 0.1 mM PMSF, pH 7.4 in the presence of a complete sets of proteases inhibitors (Complete; Boehringer Mannheim, Mannheim, Germany) and phosphatase inhibitors. The homogenized tissue was centrifuged at 1000 × *g* for 10 min. The resulting supernatant was centrifuged at 3000 × *g* for 15 min to obtain a fraction of mitochondria and synaptosomes. The pellet was resuspended in hypotonic buffer (in the presence of proteases inhibitors) in a glass–glass potter and centrifuged at 100,000 × *g* for 1 hr. The 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 hr. 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. This fraction is referred to as "Triton-insoluble fraction" (TIF).

Slice electrophysiology. Male Wistar rats, age of ~2 months (250–300 gm), were used. The animals were kept under a 12 hr light/dark regimen, with lights on at 7:00 A.M. The rats were decapitated after short period of inhalation anesthesia with isoflurane. The brains were rapidly removed and placed in ice-cold medium, and hippocampal slices of 450 μm were prepared. The slices were stored in artificial CSF (ACSF) of the following composition (in mM): 124 NaCl, 3.3 KCl, 1.2 KH_2PO_4, 1.3 $MgSO_4$, 2.5 $CaCl_2$, 20 $NaHCO_3$, and 10.0 glucose. After 1 hr at room temperature, the slices were transferred to the recording chamber and perfused with ACSF at a rate of 2 ml/min and at 30°C. Bipolar stainless steel electrodes of 100 μm placed on Schaffer collateral fibers of CA1 area were used as stimulation electrodes. Activity in the dendritic layer in the stratum radiatum was recorded by means of glass microelectrodes of 3–5 μm tip diameter and 0.5 MΩ resistance filled with ACSF.

A stimulus intensity that evoked half-maximum amplitude field EPSPs (fEPSPs), typically between 65 and 125 μA, was used. Only slices that displayed maximal fEPSP responses of >1 mV amplitude were included in the study. Baseline responses were recorded for at least 15 min with test stimuli given at a rate of 0.05 Hz. Only slices that showed stable baseline responses were used in the experiment. Three animals per group were used. From each animal, seven slices were included in the study. After 15 min of baseline recording, LTP was induced by a train of high-frequency stimulation (HFS) composed of 100 pulses given in 1 sec (100 Hz). The responses were then recorded for other 15 min using the test stimulation frequency of 0.05 Hz. The slices were then immediately frozen and stored in −80°C until use. For biochemical experiments, slices were thawed directly into homogenization chambers and immediately homogenized.

Immunoprecipitation. Triton-insoluble fraction proteins (50 μg) were incubated in buffer A containing: 200 mM NaCl, 10 mM EDTA, 10 mM Na_2HPO_4, 0.5% NP-40, and 0.1% SDS in a final volume of 200 μl with antibodies against NR2A/B as indicated in the text (dilution of 1:50) overnight at 4°C. 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 hr. 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 pelleted by centrifugation, and a volume of supernatants was applied to 6% SDS-PAGE.

CaMKII assay. For assay of CaMKII-dependent activity, Triton-insoluble fraction proteins were incubated in a medium containing: 50 mM HEPES, 10 mM Mg acetate, 100 μM [γ-^{32}P]ATP, 40 μM syntide-2, 1 mM $CaCl_2$, and 2.4 μM calmodulin, in a final volume of 30 μl. The reaction was stopped by spotting on phosphocellulose paper.

Antibodies. Monoclonal αCaMKII antibody was purchased from Boehringer Mannheim. Polyclonal antibodies against GluR2/3 and NR2A/B were purchased from Chemicon (Temecula, CA). A polyclonal antibody against GST was produced in rabbits using recombinant GST. Monoclonal antibody against PSD-95 was purchased from Affinity BioReagents Inc. Polyclonal antibody against p286-anti-active αCaMKII was purchased from Promega (Madison, WI).

RESULTS

Pull-out of αCaMKII and PSD-95 from purified hippocampal PSD by NR2A C-terminal domain

We have demonstrated previously that the C-terminal region 1349–1464 of NR2A subunit of NMDA receptor complex interacts with both native αCaMKII and PSD-95 from rat hippocampal PSD (Gardoni et al., 1999). To further confirm the specific

and direct association of αCaMKII and PSD-95 to NR2A, fusion proteins between GST and different amino acid stretches of NR2A cytoplasmic C-tail partially overlapping each other were prepared, immobilized on a glutathione affinity matrix, and used for a pull-out assay. The NR2A C-terminal region different fragments used were as follows: L1244-V1464, S1349-V1464, L1244-G1461, and L1244-V1389 (Fig. 1*A*). Solubilized PSD proteins purified from rat hippocampus were applied in native form batchwise to the affinity beads; the beads were extensively washed, and the bound material was resolved by SDS-PAGE and subjected to immunoblot analysis with antibodies raised against αCaMKII and PSD-95. GluR2/3, present in PSD preparation as described previously (Gardoni et al., 1998) and as shown by Western blot analysis performed in native solubilized PSD proteins (Fig. 1*B*, *Input*), was used as a negative control. Figure 1*B* shows that both αCaMKII and PSD-95 can associate with the C-terminal NR2A domains L1244-V1464 and S1349-V1464, confirming previous results (Gardoni et al., 1999) indicating that NR2A(1349–1464) was indeed sufficient to guarantee the association of both proteins. Deletion of 75 amino acids from the NR2A C-terminal side prevents the binding of both αCaMKII and PSD-95 to NR2A(L1244-V1389); in addition, the L1244-G1461 fusion protein that does not contain the NR2A C-terminal PDZ-binding domain tSDV binds αCaMKII but not PSD-95, indicating the region NR2A(1389–1461) as necessary for binding αCaMKII and that the tSDV binds PSD-95. The binding is specific because no αCaMKII and PSD-95 are pulled out using GST alone. Furthermore, the association of αCaMKII and PSD-95 to NR2A fusion proteins was not attributable to incomplete solubilization of the PSD offered to the beads, because another PSD protein (i.e., GluR2/3) (Fig. 1*B*, *top panel*) remained in the supernatant after the pull-out assay. Furthermore, the use of 1% SDS, a concentration detergent known to guarantee a higher PSD solubilization (McGlade-McCulloh et al., 1993), does not influence both αCaMKII and PSD-95 binding to NR2A (Fig. 1*B*, *rightmost lane*). These results demonstrate that both αCaMKII and PSD-95 binding sites reside in the NR2A(1389–1464) region but did not exclude that αCaMKII could be associated to the NMDA receptor complex through a previous binding with PSD-95. To exclude the hypothesis of a direct association between native αCaMKII and PSD-95, GST-PSD-95(54–256) fusion protein (Fig. 1*C*), containing the PDZ1 and PDZ2 domains (known to be responsible for NR2 binding) was prepared, immobilized on a glutathione affinity matrix, and used for pull-out assay with solubilized hippocampal PSD proteins. Figure 1*C* shows that native NR2A/B subunits bind to PSD-95(54–256), as expected (Kornau et al., 1995; Bassand et al., 1999), whereas no signal is detectable for native αCaMKII in the bound material (*bottom panel*), indicating no direct interaction between αCaMKII and PSD-95(54–256). To further support this hypothesis, recombinant αCaMKII did not bind PSD-95 in an overlay assay (data not shown), thus excluding that αCaMKII can bind to a PSD-95 domain other than PSD-95(54–256).

PSD-95 and CaMKII compete for NR2A binding

Because the αCaMKII binding domain resides in the NR2A(1389–1461) region, very close to the C-terminal tSDV domain of NR2A C-tail known to link specifically the PDZ2 domain of PSD-95 (Kornau et al., 1995; Bassand et al., 1999), one intriguing hypothesis was to study the possible αCaMKII/PSD-95 competition in the binding with the NR2A subunit of NMDA receptor complex.

To test this hypothesis, GST-NR2A(1244–1464) bound to glutathione agarose beads was incubated in an *in vitro* competition assay with a fixed concentration of recombinant αCaMKII(1–325) and increasing concentrations of PSD-95(54–256) fragment obtained by elution from glutathione beads by either reduced glutathione or thrombin cleavage (see Materials and Methods). After extensive washes, the association of αCaMKII(1–325) to NR2A C-tail was then evaluated in the bound material by immunoblot analysis with a monoclonal antibody raised against αCaMKII. Figure 2*A* shows that eluted GST-PSD-95(54–256) competes in a concentration dependent manner, in a range from 1 to 50 nM, for the binding of 10 nM αCaMKII(1–325) to NR2A. To verify whether the competition observed between the two proteins was not attributable to GST tags that might compete for binding sites, in a second set of experiments (Fig. 2*B*), the assay was performed using PSD-95(54–256) previously cut from GST tags by means of human thrombin (Smith and Johnson, 1988).

To test and confirm the specificity of PSD-95 competition on αCaMKII(1–325)/NR2A(1244–1464) binding, the experiment was repeated using solubilized PSD proteins (5 μg), containing a high amount of native αCaMKII (Kennedy, 1997) instead of recombinant αCaMKII(1–325). Figure 2*C* shows that native PSD-associated αCaMKII is also competed by PSD-95(54–256) (10 nM) on NR2A binding (Fig. 2*C*, *left panel*); a similar result was obtained incubating solubilized PSD in the presence of a monoclonal anti-NR2A/B antibody recognizing the last 20 amino acids of the NR2A C-terminal tail, thus occluding the PSD-95 binding site (Kornau et al., 1995). The same results were obtained when PSD proteins were cold-phosphorylated before the pull-out experiment in conditions known to promote αCaMKII autophosphorylation (Gardoni et al., 1998), thus suggesting that PSD-95(54–256) *in vitro* competes also with autophosphorylated αCaMKII (Fig. 2*D*). In parallel samples, αCaMKII autophosphorylation degree was tested by means of anti-p286-αCaMKII antibody in phosphorylated PSD when compared with native PSD not *in vitro* phosphorylated (Fig. 2*E*). As a further demonstration of the specificity of PSD-95/αCaMKII competition on NR2A C-tail binding, the pull-out assay was performed also on truncated GST-NR2A(1244–1461) fusion protein, not containing the tSDV domain and so not able to directly bind to native PSD-95 (Fig. 1*B*). Under these experimental conditions, no significant competition between PSD-associated αCaMKII and PSD-95(54–256) (10 nM) was observed (Fig. 2*C*, *right panel*), indicating that the association of PSD-95 to the three last amino acid tSDV of NR2A C-tail is necessary to directly antagonize the association of αCaMKII to NR2A. Similar results have been obtained with GST-NR2A(1349–1464) instead of GST-NR2A(1244–1464), further indicating that the region 1244–1348 is not essential for both αCaMKII and PSD-95 binding (data not shown).

We have demonstrated previously that recombinant αCaMKII(1–325) catalytic domain but not αCaMKII(315–478) associative domain is able to directly associate to NR2A C-tail and to compete for the binding with the native PSD-associated kinase in a pull-out assay (Gardoni et al., 1999). As a direct consequence of these results, we investigated which αCaMKII domain was able to antagonize the binding of native PSD-95 to NR2A C-terminal tSDV domain. To answer this question, GST-NR2A(1244–1464) was incubated in a pull-out assay with a fixed amount of solubilized PSD proteins (5 μg) in the absence or presence of the same concentration of recombinant αCaMKII(1–325) catalytic domain (10 nM) or αCaMKII(315–478) associative domain (10 nM). Using these experimental conditions, in the

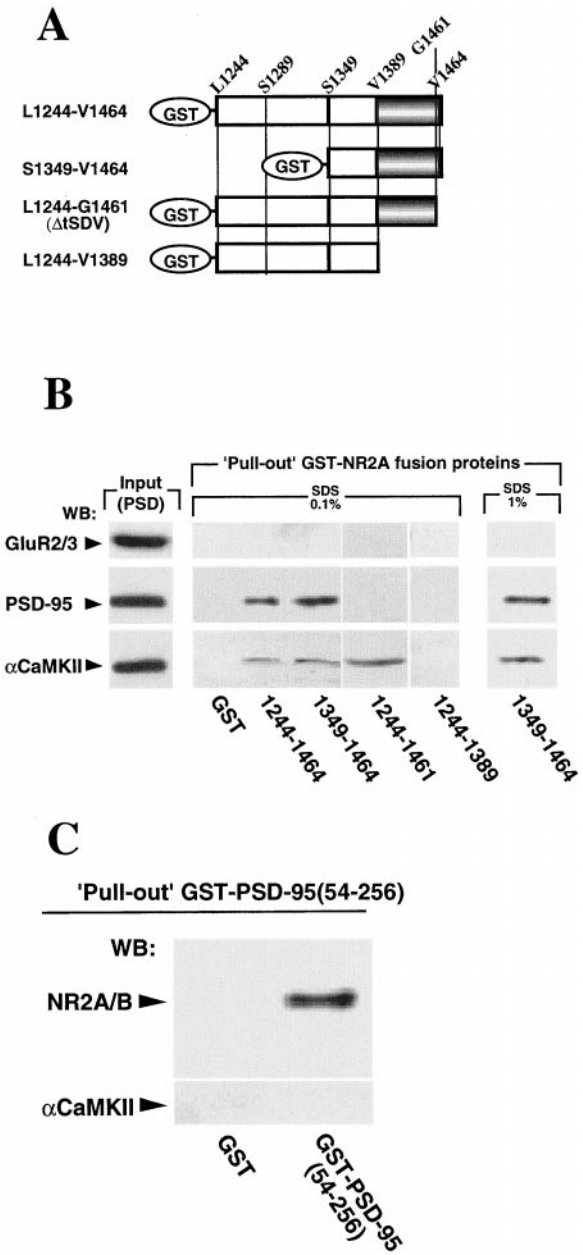

Figure 1. αCaMKII and PSD-95 bind NR2A C-terminal domain. *A*, Fusion proteins of GST with different fragments of the NR2A C-terminal domain, partially overlapping each other, were prepared. *B*, Pull-out of αCaMKII, PSD-95, and GluR2/3 from hippocampal PSD by GST, GST-NR2A(1244–1389), GST-NR2A(1244–1464), GST-NR2A(1349–1464), and GST-NR2A(1244–1461). *Input* (hippocampal PSD) is 20%. Fusion proteins were purified from bacterial extracts on glutathione agarose beads and incubated for 1 hr at 37°C with purified hippocampal PSD previously solubilized in 0.1 or 1% SDS 1 (*rightmost lane*); after extensive washes, the bound proteins were eluted from the beads with SDS sample buffer, separated by SDS-PAGE, and analyzed by Western blotting. Data are representative of three independent experiments performed on different PSD preparations and replicated three times in each PSD preparation. *C*, PSD-95(54–256) directly binds to PSD-associated NR2A but not to αCaMKII; pull-out of αCaMKII and NR2A/B from hippocampal PSD by GST and GST-PSD-95(54–256) purified fusion proteins. Data are representative of three independent experiments performed on different PSD preparations and replicated four times in each PSD preparation.

presence of αCaMKII(1–325), the amount of native αCaMKII ($-72.2 \pm 5.4\%$; $p < 0.01$ vs control) bound to NR2A(1244–1464) was significantly lower. On the other hand, incubation with 10 nM αCaMKII(315–478) did not significantly influence the amount of native αCaMKII associated, further indicating that αCaMKII(1–325) but not αCaMKII(315–478) was capable of binding NR2A. Furthermore, preincubation with αCaMKII(1–325) significantly decreased the amount of native PSD-95 bound to NR2A ($-86.6 \pm 6.3\%$; $p < 0.01$ vs control), thus indicating that the exogenously added αCaMKII(1–325) competed with PSD-95 for NR2A binding (Fig. 3, *left panel*). Experiments performed with αCaMKII(315–478) associative domain did not significantly affect the binding of native kinase but also of PSD-95 to the NMDA receptor subunit (Fig. 3, *right panel*).

Phosphorylation of NR2A (Ser1289) does not affect either αCaMKII or PSD-95 binding

NR2A and NR2B subunits have been shown to be substrates for different protein kinases (Moon et al., 1994; Omkumar et al., 1996; Strack and Colbran, 1998; Gardoni et al., 1999), playing a significant role in modulating channel activity and in synaptic plasticity processes (Yu et al., 1997). Accordingly, it was of interest to determine whether CaMKII-dependent phosphorylation of NR2A Ser1289 (Gardoni et al., 1999) might influence the interaction of the kinase itself and/or of PSD-95 to the NMDA receptor subunit. To study the direct effects of NR2A CaMKII-dependent phosphorylation, a point mutation on Ser1289 was introduced to produce GST-NR2A[(1244–1464)Ser1289/Asp] and GST-NR2A[(1244–1464)Ser1289/Ala], and subsequently control and mutated fusion proteins were phosphorylated by recombinant kinase in the presence of [γ-^{32}P]ATP as phosphate donor. Figure 4*A* shows a representative autoradiograph of *in vitro* CaMKII-dependent phosphorylation of GST-NR2A fusion proteins; a radioactive band at 50 kDa corresponding to native NR2A(1244–1464) GST fusion protein is clearly visible (*right lane*). The phosphorylated *bottom bands* correspond to degradation products of the primary fragment because they are recognized by the anti-GST antibody (data not shown). Both GST-NR2A[(1244–1464)Ser1289/Asp] and GST-NR2A[(1244–1464)Ser1289/Ala] did not show any phosphorylation, thus confirming the presence in Ser1289 of a unique CaMKII phosphosite in the NR2A region comprised between 1244 and 1464 (Strack and Colbran, 1998; Gardoni et al., 1999).

To test the influence of Ser1289 phosphorylation on αCaMKII and PSD-95 association, the same amount of control and mutated GST-NR2A(1244–1464) fusion proteins were subjected to pull-out experiments with hippocampal PSD, and bound proteins were eluted and immunoblotted for αCaMKII and PSD-95. Figure 4*B* shows that both Ala1289 and Asp1289 mutation in NR2A C-terminal tail do not significantly influence the interaction of the NR2A fusion proteins to both native αCaMKII and PSD-95.

Modulation of αCaMKII/PSD-95 binding to NMDA receptor complex in acute hippocampal slices

It has been shown that interaction between the NR2 C-terminal tail and native PSD-associated αCaMKII occurs with unphosphorylated αCaMKII but is strengthened by kinase autophosphorylation (Strack and Colbran, 1998; Gardoni et al., 1999; Leonard et al., 1999). However, the mechanism(s) that regulate targeting of αCaMKII to NMDA in physiological conditions are not yet fully understood. To further investigate the modulation of αCaMKII-NR2A/B association mediated by CaMKII activa-

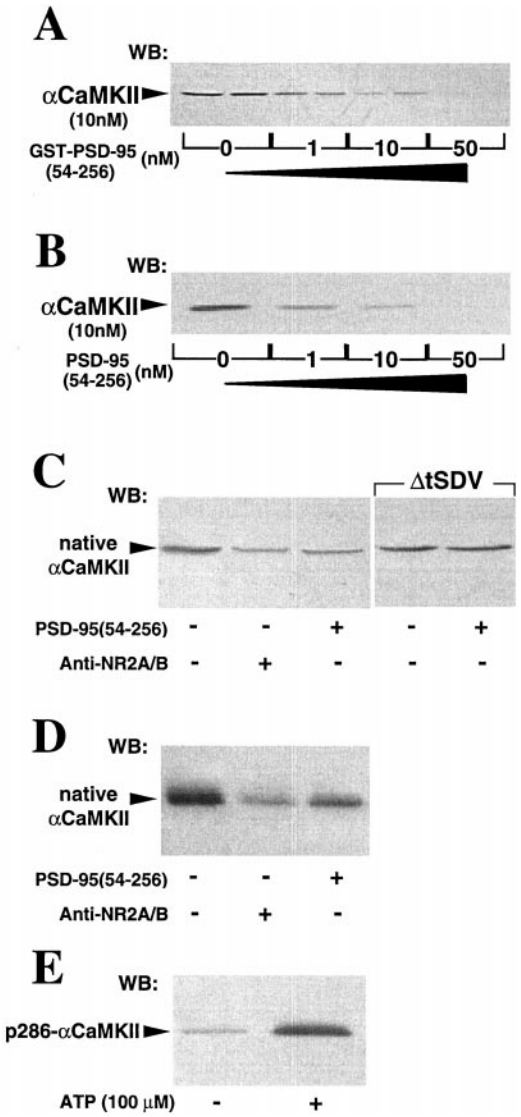

Figure 2. Competition between recombinant αCaMKII(1–325) and PSD-95(54–256) for binding to the NR2A C-terminal tail. *A*, GST-NR2A(1244–1464) fusion proteins (5 μg) bound to glutathione agarose beads were incubated with a fixed amount of recombinant αCaMKII(1–325) (10 nM) (New England Biolabs) and increasing concentrations (0–50 nM) of eluted GST-PSD-95(54–256). Bound proteins were eluted and immunoblotted for αCaMKII using a monoclonal anti-αCaMKII antibody. *B*, GST-NR2A(1244–1464) fusion proteins (5 μg) bound to glutathione agarose beads were incubated with a fixed amount of recombinant αCaMKII(1–325) (10 nM) and increasing concentrations (0–50 nM) of thrombin-cut PSD-95(54–256). *C*, GST-NR2A(1244–1464) and GST-NR2A(1244–1461), not containing the tSDV domain (Δ*tSDV*), bound to glutathione agarose beads (5 μg), were incubated in a pull-out assay with a fixed amount of hippocampal PSD (5 μg) in the absence or presence of PSD-95(54–256) (10 nM) or anti-NR2A/B polyclonal antibody (Chemicon). *D*, GST-NR2A(1244–1464) was incubated in a pull-out assay with a fixed amount of cold phosphorylated hippocampal PSD (5 μg) in the absence or presence of PSD-95(54–256) (10 nM) or anti-NR2A/B polyclonal antibody. *E*, Western blotting analysis performed with anti-p286-αCaMKII in native [ATP (100 μM); indicated by −] or *in vitro* phosphorylated PSD [ATP (100 μM); indicated by +]. Data are representative of three independent experiments performed on different PSD preparations and replicated four times in each PSD preparation.

tion, we used hippocampal slices as described previously (Caputi et al., 1997); slices were incubated with 100 μM glutamate–1 μM glycine in the absence or presence of the specific CaMKII inhibitor KN-93 (10^{-5} M) or with vehicle alone. After incubation, a TIF was obtained, and CaMKII-activity and NMDA receptor coprecipitation studies were performed. The TIF had to be used in these experiments instead of the classical PSD preparation because the amount of the starting material from hippocampal slices was very limited (15 mg wet weight). Nevertheless, the protein composition of this preparation was carefully tested for the absence of presynaptic markers (i.e., synaptophysin and synaptotagmin were absent) and for the enrichment in the PSD proteins (αCaMKII, PSD-95, NMDA, and AMPA receptor subunits) (Caputi et al., 1999). Figure 5*A* shows that CaMKII activity, measured as phosphorylation of syntide-2, is highly increased (+252.7 ± 21.7% of control value; $^{*}p < 0.01$ vs control value) after treatment of hippocampal slices with 100 μM glutamate–1 μM glycine, whereas concomitant treatment with the CaMKII-specific inhibitor KN-93 (10^{-5} M) in the presence of 100 μM glutamate–1 μM glycine is able to reduce kinase activity to values even lower than control [−28.7 ± 5.6% of control value; $^{**}p < 0.01$ glutamate–glycine versus glutamate–glycine plus KN-93 (10^{-5} M)]. In parallel, glutamate–glycine and KN-93 treatment is able to similarly modulate CaMKII-dependent phosphorylation of NR2A/B (data not shown). Western blotting analysis performed in the TIF fraction with anti-αCaMKII and anti-PSD-95 (Fig. 5*B*) monoclonal antibodies shows that the incubation with 100 μM glutamate–1 μM glycine in the absence or presence of KN-93 (10^{-5} M) does not influence the concentration of both αCaMKII and PSD-95 in the TIF compartment. Coimmunoprecipitation experiments were then performed in the same slices with a polyclonal anti-NR2A/B, and the presence of αCaMKII, PSD-95, and NR2A/B was evaluated in the immunoprecipitated material by Western blotting (Fig. 5*C*,*D*). In basal conditions, both αCaMKII and PSD-95 coimmunoprecipitate with the NMDA receptor complex; 100 μM glutamate–1 μM glycine, a treatment that activates CaMKII, induced a significant increase of αCaMKII coprecipitation with NMDA receptor complex and a parallel decrease in PSD-95 coprecipitation (data are expressed as the ratio of the relative amount of αCaMKII and PSD-95 present in the immunocomplex: ratio αCaMKII/PSD-95, +173.5 ± 18.3% of control value; $^{*}p < 0.05$ glutamate–glycine versus control). Treatment with KN-93, able to abolish CaMKII activation, restores both αCaMKII and PSD-95 coprecipitation with NMDA receptor complex to values not significantly different from control slices [ratio αCaMKII/PSD-95, −28.4 ± 19.1%; 100

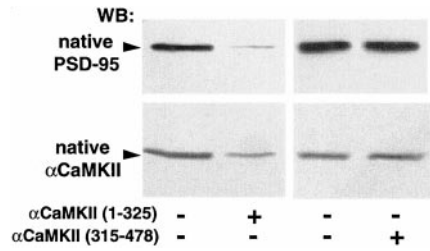

Figure 3. αCaMKII(1–325) competition with both native αCaMKII and PSD-95 for NR2A binding. GST-NR2A(1244–1464) fusion proteins bound to glutathione agarose beads were incubated in a pull-out assay with a fixed amount of hippocampal PSD (5 μg) in the absence or presence of 10 nM αCaMKII(1–325) or 10 nM αCaMKII(315–478); after extensive washes, the bound proteins were eluted, separated by SDS-PAGE, and analyzed on Western blotting with monoclonal anti-αCaMKII and monoclonal anti-PSD-95 antibodies. Data are representative of three independent experiments performed on different PSD preparations and replicated four times in each PSD preparation.

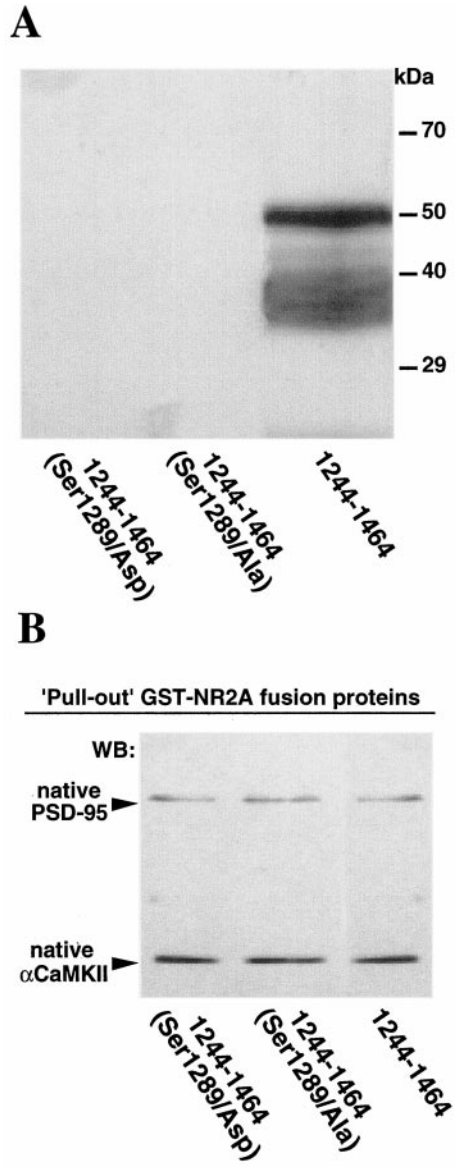

Figure 4. Influence of NR2A(Ser1289) *in vitro* CaMKII-dependent phosphorylation on αCaMKII/PSD-95 binding to the NR2A C-terminal tail. *A*, *In vitro* CaMKII-dependent phosphorylation of GST-NR2A fusion proteins. GST-NR2A(1244–1464), GST-NR2A[(1244–1464)Ser1289/Asp], and GST-NR2A[(1244–1464)Ser1289/Ala] purified fusion proteins were incubated with 50 U of αCaMKII(1–325) (New England Biolabs) for 30 min at 37°C. Proteins were separated by SDS-PAGE (running gel, acrylamide 11%), and phosphoproteins were revealed by autoradiography. *B*, GST-NR2A(1244–1464), GST-NR2A[(1244–1464)Ser1289/Asp], and GST-NR2A[(1244–1464)Ser1289/Ala] purified fusion proteins were incubated in a pull-out assay with a fixed amount of hippocampal PSD (5 μg); after extensive washes, the bound proteins were eluted, separated by SDS-PAGE, and analyzed by Western blotting with monoclonal anti-αCaMKII and monoclonal anti-PSD-95 antibodies. Data are representative of three independent experiments performed on different PSD preparations and replicated four times in each PSD preparation.

μM glutamate–1 μM glycine plus KN-93 (10^{-5} M) compared with untreated control slices; $^{**}p < 0.05$ 100 μM glutamate–1 μM glycine plus KN-93 (10^{-5} M) versus 100 μM glutamate–1 μM glycine]. In the presence of KN-93 (10^{-5} M), there is a nonsignificant decrease of CaMKII and a parallel increase of PSD-95 coprecipitation with NMDA receptor complex, probably because of the inhibition of CaMKII basal activity (Fig. 5*A*) in KN-93-

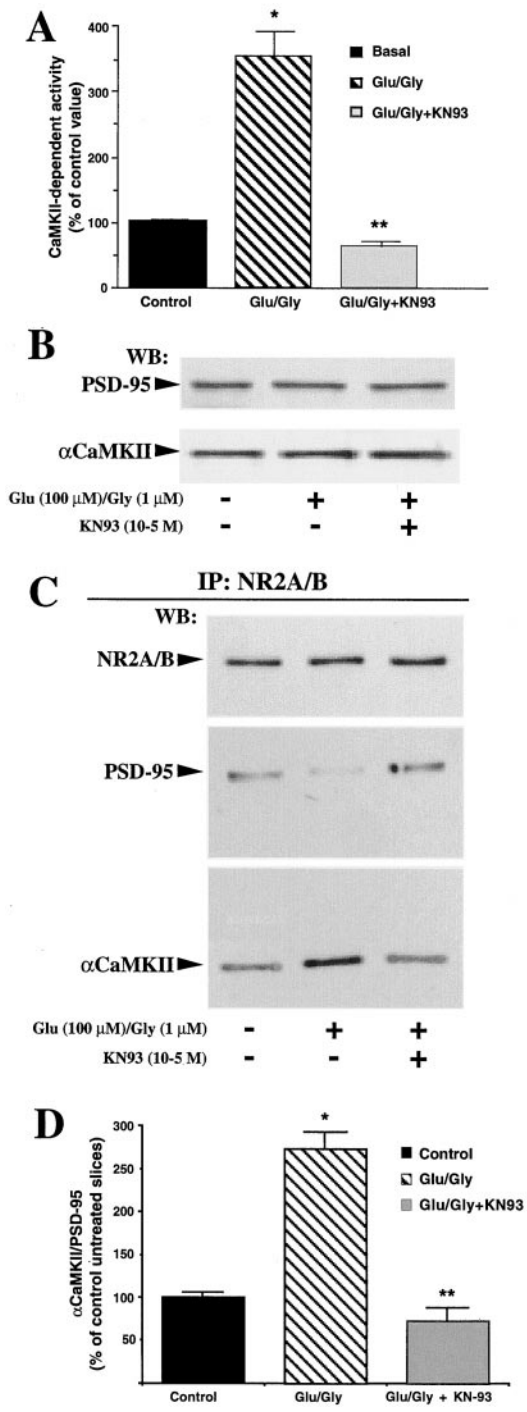

Figure 5. Modulation of αCaMKII/PSD-95 binding to NMDA receptor complex in acute hippocampal slices. *A*, CaMKII activity in TIF obtained from hippocampal slices treated in the absence or presence of 100 μM glutamate–1 μM glycine and KN-93 (10^{-5} M) [$^{*}p < 0.01$ 100 μM glutamate–1 μM glycine vs control slices; $^{**}p < 0.01$ 100 μM glutamate–1 μM glycine vs 100 μM glutamate–1 μM glycine plus KN-93(10^{-5} M)]. *B*, Western blotting analysis performed in the TIF fraction with anti-αCaMKII and anti-PSD-95 monoclonal antibodies. C, Proteins from TIF were immunoprecipitated with a polyclonal antibody raised against NR2A/B subunits of NMDA receptor complex; Western blot analysis was performed in the immunoprecipitated material with anti-NR2A/B, anti-αCaMKII, and anti-PSD-95. *D*, Diagram illustrating statistical analysis of Western blotting experiments performed on immunoprecipitated material. Data are expressed as the ratio αCaMKII/PSD-95 ± SEM of three independent experiments performed on different TIF preparations and replicated four times in each TIF preparation.

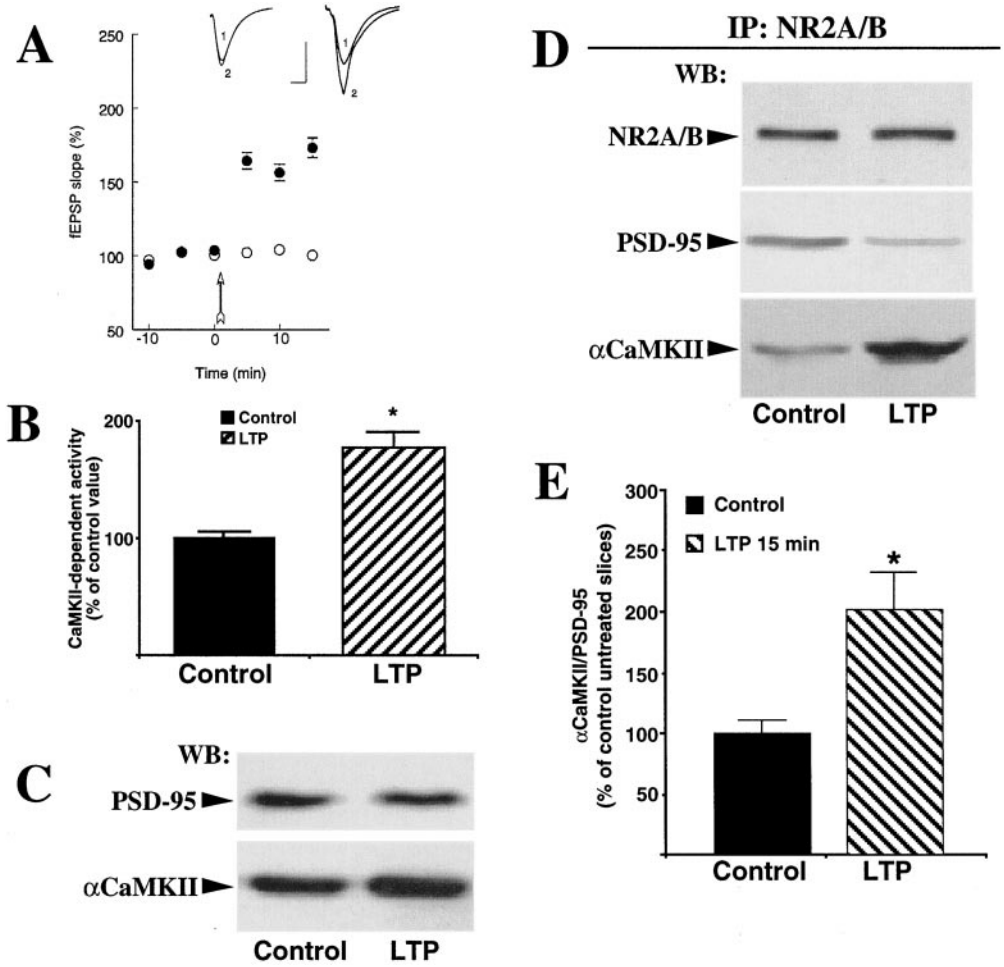

Figure 6. Modulation of αCaMKII/PSD-95 binding to NMDA receptor complex is mediated by LTP induction. *A*, Long-term potentiation in the CA1 field of the hippocampus. HFS (*open arrow*) significantly potentiated the fEPSP slopes in 21 slices taken from three animals (*filled circles*; Wilcoxon match pair-pairs signed test; $p < 0.05$). Three control slices taken from three animals (*open circles*) were not subjected to the conditioning stimulation. Data are mean ± SEM. *Inset*, *Left*, Control slices; *traces* represent 15 averaged fEPSPs recorded at the beginning (*1*) and the end (*2*) of the experiment. *Right*, Tetanized slices; *traces* represent 15 averaged fEPSPs recorded before (*1*) and 15 min after (*2*) HFS. Calibration: 5 min, 1 mV. *B*, CaMKII activity in TIF in control and HFS slices. *C*, Western blotting analysis performed in the TIF fraction with anti-αCaMKII and anti-PSD-95 monoclonal antibodies. *D*, proteins from TIF were immunoprecipitated with a polyclonal antibody raised against NR2A/B subunits of NMDA receptor complex; Western blot analysis was performed in the immunoprecipitated material with anti-NR2A/B, anti-αCaMKII, and anti-PSD-95. *E*, Quantitative analysis of Western blotting experiments performed on immunoprecipitated material. Data are expressed as the ratio αCaMKII/PSD-95 ± SEM of three independent experiments performed on different TIF preparations and replicated four times in each TIF preparation.

treated slices. In all of the experiments, the NMDA receptor immunoprecipitation was quantitative, because no signal for both NR1 and NR2A/B subunits was found in the supernatant after immunoprecipitation reaction.

In a second set of experiments, LTP was induced in hippocampal slices by HFS on Schaffer collateral fibers of CA1 area (Fig. 6). After 15 min of LTP induction, CaMKII activity as well as the relative concentration of αCaMKII and PSD-95 associated to NMDA receptor complex were evaluated in a coimmunoprecipitation assay (anti NR2A/B), as described above. The amount of LTP achieved by HFS is reported in Figure 6*A*, being that the average fEPSP slope in HFS slices was +70% compared with control stimulated slices. Figure 6*B* shows results of αCaMKII activity measured in tetanized hippocampal slices and in control slices subjected to low-frequency stimulation (LFS). In LTP-potentiated slices, αCaMKII activity was increased +78.2 ± 12.1% (*$p < 0.05$, HFS vs LFS slice) when compared with LFS slices, as expected and previously described by others (Liu et al., 1999). Fifteen minutes after LTP induction, slices were snap frozen in liquid nitrogen, and Western blotting analysis was performed in the TIF fraction with anti-αCaMKII and anti-PSD-95 (Fig. 6*C*). Representative Western blotting in Figure 6*C* shows a slight but not significant increase of αCaMKII concentration in the TIF pool in LTP-potentiated slices (+26.4 ± 10.3%; $p > 0.05$ LTP-established slices vs control LFS slices); furthermore, no differences are observed in the concentration of PSD-95 in the TIF compartment. In the same slices, NMDA receptor complex and associated proteins were coimmunoprecipitated from TIF with anti-NR2A/B. Figure 6*D* shows a representative Western blot analysis of NR2A/B, αCaMKII, and PSD-95 present in the immunocomplex after precipitation with an anti-NR2A/B antibody in LFS slices (*left lane*) and in LTP-potentiated slices (*right lane*); LTP increased the association of αCaMKII to NR2A/B subunit and concomitantly decreased the association of PSD-95 [ratio αCaMKII/PSD-95, +102.2 ± 29.1% of LFS slices; *$p < 0.05$ LTP-established slices versus control

LFS slices] (Fig. 6*E*), confirming results obtained by chemical stimulation of NMDA receptor in hippocampal slices shown above (Fig. 5*D*).

DISCUSSION

In the last few years, binding of various PSD proteins to NMDA receptor subunits has been extensively described (Lau et al., 1996; Wyszynski et al., 1997; Gardoni et al., 1998; Wechsler and Teichberg, 1998). In particular, a large number of studies identified the NMDA receptor complex as a target for specific enzymes, i.e., αCaMKII (Gardoni et al., 1998, 1999; Strack and Colbran, 1998; Leonard et al., 1999) and for scaffolding proteins, i.e., the PSD-95 family (Kornau et al., 1995; Kim et al., 1996; Lau et al., 1996; Bassand et al., 1999), underlining the crucial role played by NMDA receptor in building up the complex network of PSD proteins.

The results reported here demonstrate that both αCaMKII and PSD-95 are associated to the NR2A C-terminal region in amino acid domains comprised between 1389 and 1464. Furthermore, αCaMKII association to NR2A 1389–1464 can be affected by activation of the NMDA receptor *in vitro* by either pharmacological tools or induction of LTP. The increased αCaMKII binding to the receptor entails the detachment of PSD-95 from tSDV-NR2A both *in vitro* (Figs. 2, 3), using purified and recombinant proteins, and *ex vivo* in chemically and electrically stimulated hippocampal slices (Figs. 5,6). In fact, we show here that PSD-95(54–256) competes with both native and recombinant αCaMKII(1–325) on the binding to the NR2A C-tail. The displacement is specific because, using an NR2A truncated protein lacking the tSDV region [NR2A(1244–1461)], we completely abolished the competition (Fig. 2*C*). αCaMKII can bind the NR2A subunit C-tail also in the absence of the tSDV domain (Fig. 1*B*). These data support the hypothesis that the tSDV domain of NR2A is mandatory but not sufficient for interacting with PSD-95, suggesting a more complex regulation of PSD-95/NR2A anchoring, involving perhaps domains upstream to the NR2A tSDV motif (Bassand et al., 1999). Moreover, the interaction observed between αCaMKII/PSD-95 and NR2A was found not to be correlated with CaMKII-dependent phosphorylation of the NR2A subunit on Ser1289, because Ser/Ala1289 and Ser/Asp1289 point mutations did not significantly influence the binding of both proteins to the NR2A C terminus.

It is becoming increasingly evident that targeting of αCaMKII in specific subcellular structures is likely to play an important role in defining specific physiological roles of the kinase. Our data showing that LTP can foster higher association of αCaMKII to NMDA receptor complex adds further value to previous observations reported by us and others, suggesting a role of NMDA receptor activation in promoting αCaMKII translocation in the postsynaptic compartment (Strack et al., 1997; Gardoni et al., 1998; Strack and Colbran, 1998; Shen and Meyer, 1999). In addition, we provide here direct evidence that αCaMKII recruitment during activity-dependent synaptic plasticity entails partial dissociation of PSD-95 from NMDA receptor complex. This observation further expands our knowledge on the molecular mechanism(s) underlying synaptic plasticity because it suggests that not only modulation of glutamate receptor distribution in PSD but also the dynamic regulation of proteins clustered to ionotropic glutamate receptors in PSD is critical for regulating synaptic efficacy.

The association of PSD-95 to and the dissociation from the NR2A C terminus mediated by αCaMKII is particularly relevant in a physiological context because it enables NMDA receptors to couple to different signal transduction proteins. Indeed, it has been reported that the PSD-95 family is not solely implicated in NMDA receptor anchoring and localization (Kornau et al., 1995; Lau et al., 1996), but it is capable of binding different signaling proteins (Migaud et al., 1998; Yamada et al., 1999), i.e., nonreceptor tyrosine kinases (Tezuka et al., 1999). Therefore, a dissociation of PSD-95 from the NR2A C terminus might separate nonreceptor tyrosine kinases from NMDA receptors, making them available for phosphorylating other substrates differentially located in the postsynaptic compartment. It is known that both CaMKII and Src family enzymes play a fundamental role in LTP expression. However, they might intervene in temporally and spatially distinct phases of the process, thus mediating a different biochemical cascade. In our experiments, we choose a relatively short time interval after LTP induction to evaluate CaMKII recruitment to NMDA receptor complex. The rationale for this time schedule is based on previous observations by Barria et al. (1997) who observed an increased CaMKII activity in the early stages after LTP induction and CaMKII-dependent AMPA subunits phosphorylation after 25 min of LTP induction. Our data suggest that, in the very early stage of LTP, CaMKII first gets autophosphorylated, as reported by other authors (Fukunaga et al., 1995), and it is then recruited to and phosphorylates NMDA receptor subunits, triggering a biochemical event that further sustains synaptic activity.

Based on these observations, it appears clear that a shuttling of αCaMKII and PSD-95 to and from the NMDA receptor complex might be of great relevance for coupling NMDA receptor to different signal transduction pathways. On this line, emerging evidence suggests that phosphorylation of NMDA receptor subunits by specific kinases as well as association of specific proteins to NMDA receptor complex are altered in an animal model of neurological disorders (Di Luca et al., 1999; During et al., 2000). For instance, CaMKII has been shown to translocate toward the PSD very rapidly after ischemia (Aronowski and Grotta, 1996; Domanska-Janik et al., 1999). On the other hand, coimmunoprecipitation experiments demonstrated an ischemia-induced decrease in the association between PSD-95 and NR2A/B (Takagi et al., 2000), probably because of the interaction of NMDA receptor complex with a different interacting proteins present in the PSD fraction.

In conclusion, dynamic interactions between three of the major components of the PSD in the mature synapse (αCaMKII, PSD-95, and NR2A) could represent a molecular mechanism involved in the regulation of postsynaptic function in response to NMDA receptor channel activation. Our data demonstrating a functional αCaMKII/PSD-95 competition on NR2A C-tail both *in vitro* and in hippocampal slices after LTP induction add a tile on the knowledge of the molecular changes occurring in the postsynaptic compartment during activity-dependent synaptic plasticity.

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