

# $\alpha$ CaMKII binding to the C-terminal tail of NMDA receptor subunit NR2A and its modulation by autophosphorylation

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**Abstract**  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II (CaMKII), a multifunctional, widely distributed enzyme, is enriched in post-synaptic densities (PSDs). Here, we demonstrate that CaMKII binds to a discrete C-terminal region of the NR2A subunit of NMDA receptors and promotes the phosphorylation of a Ser residue of this NMDA receptor subunit. Glutathione *S*-transferase (GST)-NR2A(1349-1464) binds native CaMKII from solubilised hippocampal PSDs in 'pull-out' and overlay experiments and this binding is competed by recombinant  $\alpha$ CaMKII(1-315). The longer GST-NR2A(1244-1464), although containing the CaMKII phosphosite Ser-1289, binds the kinase with a lower efficacy. CaMKII association to NR2A(1349-1464) is positively modulated by kinase autophosphorylation in the presence of  $\text{Ca}^{2+}$ /calmodulin. These data provide direct evidence for a mechanism modulating the synaptic strength.

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**Key words:** Rat; Hippocampus; Phosphorylation; Post-synaptic density; CaMKII; GST fusion protein

## 1. Introduction

$\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II (CaMKII) is a multifunctional, widely distributed kinase which is particularly enriched in neuronal tissues, where it represents up to 2% of the total protein [1]. Within the neuronal compartment, CaMKII is highly concentrated in the post-synaptic density (PSD: [2]), where it phosphorylates ionotropic glutamate receptors [3,4], providing a mechanism for modulating the synaptic strength [5,6].

The organisation of the PSD has been studied and described over the past few years [7]. It is now known that the PSD is a fibrous specialisation of the cytoskeleton located beneath the post-synaptic membrane and that it contains: cytoskeletal proteins, such as actin, spectrin, tubulin [8,9], members of the PSD-95/SAP family [10-12], membrane proteins like ionotropic glutamate receptors [13-15] and signal transduction molecules such as CaMKII [16], src [17] and SynGap [18,19].

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**Abbreviations:** CaMKII, calcium/calmodulin-dependent protein kinase II; GST, glutathione *S*-transferase; LTD, long-term depression; LTP, long-term potentiation; NMDA, *N*-methyl-D-aspartate; PMSF, phenyl-methyl-sulfonyl fluoride; PSD, post-synaptic density; SDS, sodium dodecyl sulfate

The characteristic insolubility and electrondense properties of the PSD structure is due to the formation of a tight net of interacting proteins. Among these, *N*-methyl-D-aspartate (NMDA) receptor subunits have been described to interact directly with PSD-95 [20], chapsyn 110 [21], SAP102 [22], spectrin [23] and  $\alpha$ -actinin-2 [24]. Moreover, the NMDA receptor complex was demonstrated to co-precipitate with  $\alpha$ CaMKII in the PSD [4]. In vitro overlay and cross-linking experiments suggested a direct interaction between  $\alpha$ CaMKII and NR2A/B subunits of NMDA receptors in native PSD [4]. In line with this, Strack and Colbran [25] demonstrated that recombinant CaMKII in vitro binds to the C-terminal domain of NR2B and with a lower affinity to NR2A.

Both the nature of CaMKII association to the NR2A subunits of NMDA receptor in PSD and its modulation by post-translational modifications (i.e. phosphorylation) are not yet fully understood. NR2A is by far the most expressed subunit in the mature forebrain [26,27] and therefore, it is most likely involved in transduction mechanisms at the mature synapse where  $\text{Ca}^{2+}$  influx through a NMDA channel can lead to either long-term potentiation (LTP) or long-term depression (LTD). In the present study, we investigated therefore the following questions: (i) is an association domain for CaMKII present in the C-terminal region of NR2A subunits of NMDA receptors? and (ii) is CaMKII autophosphorylation a necessary prerequisite for such an association?

## 2. Materials and methods

### 2.1. PSD preparation

To isolate PSD from rat hippocampus, a modification of the method by Carlin et al. [9] was used. Rats (Sprague-Dawley) were killed and brain areas were dissected within 2 min. If a longer time was required, the tissue was discarded [28]. Hippocampi (from at least 25 animals) were dissected according to the criteria described above and pooled. Homogenisation was carried out by 10 strokes in a Teflon-glass homogeniser (clearance 0.25 mm, 700 rpm) in 4 ml/g wet weight cold 0.32 M sucrose (Fluka, Buchs, Switzerland) containing 1 mM HEPES pH 7.4 (Sigma, St. Louis, MO, USA), 1 mM  $\text{MgCl}_2$  (Fluka, Buchs, Switzerland), 1 mM  $\text{NaHCO}_3$  (Fluka, Buchs, Switzerland), 0.1 mM phenyl-methyl-sulfonyl fluoride (PMSF) (Sigma, St. Louis, MO, USA) in the presence of a complete set of protease inhibitors (Complete, Boehringer Mannheim, Mannheim, Germany). The homogenised tissue was centrifuged at  $1000\times g$  for 10 min. The resulting supernatant was centrifuged at  $3000\times g$  for 15 min to obtain a fraction of mitochondria and synaptosomes. The pellet was resuspended in 2.4 ml/g of 0.32 M sucrose containing 1 mM HEPES pH 7.4, 1 mM  $\text{NaHCO}_3$ , 0.1 mM PMSF, layered on a sucrose gradient (0.85-1.0-1.2 M) and centrifuged at  $82\,500\times g$  for 2 h. The fraction between 1.0 and 1.2 M sucrose was removed, diluted with an equal volume of 1% Triton X-100 in 0.32 M sucrose containing 1 mM HEPES pH 7.4, 1 mM  $\text{NaHCO}_3$ , 0.1 mM PMSF and stirred at  $4^\circ\text{C}$  for 15 min followed by centrifugation at  $82\,500\times g$  for 30 min. The

pellet was resuspended, layered on a sucrose gradient (1.0–1.5–2.1 M) and centrifuged at  $100\,000\times g$  at  $4^{\circ}\text{C}$  for 2 h. The fraction between 1.5 and 2.1 M sucrose was collected and diluted with an equal volume of 1% Triton X-100 and 150 mM KCl. PSDs were finally obtained by further centrifugation at  $100\,000\times g$  at  $4^{\circ}\text{C}$  for 30 min and stored at  $-80^{\circ}\text{C}$  in 50% glycerol.

## 2.2. Cloning, expression and purification of GST fusion protein

The NR2A subunit and  $\alpha\text{CaMKII}$  fragments were subcloned downstream of glutathione *S*-transferase (GST) in the *Bam*HI and *Hind*III sites of the expression plasmid pGEX-KG by PCR using the Pfu polymerase (Stratagene) on a NR2A cDNA template (kind gift from S. Nakanishi) or on a  $\alpha\text{CaMKII}$  cDNA template (kind gift from H. Schulman). The inserts were fully sequenced with a ABI Prism 310 Genetic Analyser (ABI Prisma).

In particular, GST-NR2A(1049–1464), GST-NR2A(1244–1464), GST-NR2A(1349–1464) and GST- $\alpha\text{CaMKII}$ (315–478) fusion proteins were expressed in *Escherichia coli* and purified on glutathione-agarose beads (Sigma, St. Louis, MO, USA) as previously described [29]. Briefly, overnight cultures from single colonies of *E. coli* transformed with the plasmid were grown in 50 ml of Luria-Bertani medium (Sigma, St. Louis, MO, USA) containing 100  $\mu\text{g}/\text{ml}$  ampicillin (Sigma, St. Louis, MO, USA) at  $37^{\circ}\text{C}$ , diluted 1:10 with Luria-Bertani medium containing 100  $\mu\text{g}/\text{ml}$  ampicillin and incubated under the same conditions for 2 h. Synthesis of recombinant proteins was induced by 0.1 mM isopropyl- $\beta$ -D-thiogalactopyranoside (Sigma, St. Louis, MO, USA), the bacteria were grown for another 4 h and harvested by centrifugation. Bacterial pellets were resuspended with ice-cold PBS (8.4 mM  $\text{Na}_2\text{HPO}_4$ , 1.9 mM  $\text{NaH}_2\text{PO}_4$ , 150 mM NaCl, pH 7.4) containing 5 mM dithiothreitol (DTT), 100  $\mu\text{g}/\text{ml}$  lysozyme, 0.1 mM PMSF and incubated on ice for 15 min. Lysis was achieved by the addition of 1.5% *N*-laurylsarcosine (sarkosyl) from a 10% stock in PBS. Bacteria were sonicated on ice for 1 min and the lysate was clarified by centrifuging at  $10\,000\times g$  (5 min,  $4^{\circ}\text{C}$ ) in a SS-34 rotor (Sorvall). Supernatants were adjusted to 2% Triton X-100 and incubated with glutathione-agarose beads (50% v/v in PBS) for 2 h at room temperature. The beads were then extensively washed with ice-cold PBS.

## 2.3. Overlay technique

An overlay procedure has been used to detect the binding of GST-NR2A(1049–1464), GST-NR2A(1244–1464) and GST-NR2A(1349–1464) to native PSD. PSD proteins were separated on a 6% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred overnight to nitrocellulose. The membrane was blocked for 1 h in 50 mM Tris-HCl, 200 mM NaCl (TBS) pH 8.0, containing 1% Tween-20 (v/v) and non-fat powdered milk (5% w/v). The membrane was washed in TBS containing 0.1% v/v Tween-20 and non-fat powdered milk (5% w/v) and then incubated for 2 h at room temperature with constant shaking in overlay buffer TBS/non-fat powdered milk 5% (w/v)/0.1% Tween-20 containing GST-NR2A fusion proteins. The membranes were extensively washed and subjected to immunoblot analysis using a polyclonal anti-GST antibody.

## 2.4. 'Pull-out' assay

Aliquots of PSD containing 10  $\mu\text{g}$  proteins were diluted with TBS, 0.1% SDS to a final volume of 200  $\mu\text{l}$  and incubated (1 h,  $37^{\circ}\text{C}$ ) with glutathione-agarose beads saturated with GST fusion proteins or GST alone. The beads were extensively washed with TBS, 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- $\alpha\text{CaMKII}$  antibody and a polyclonal anti-GluR2/3 antibody as previously described [4]. In some experiments, GST-NR2A(1349–1464) was incubated with 100 ng of  $\alpha\text{CaMKII}$ (1–315) (New England BioLabs) for 30 min at  $37^{\circ}\text{C}$ , in 50 mM Tris-HCl (pH 7.5), 10 mM  $\text{MgCl}_2$ , 1 mM DTT, 2.4  $\mu\text{M}$  calmodulin, 2 mM  $\text{CaCl}_2$  and subsequently, the 'pull-out' assay was performed.

## 2.5. GST-NR2A fusion protein phosphorylation

For CaMKII-dependent phosphorylation of NR2A, GST-NR2A(1244–1464) and GST-NR2A(1349–1464) purified fusion proteins were incubated with 50 U  $\alpha\text{CaMKII}$ (1–315) (New England BioLabs) for 30 min at  $37^{\circ}\text{C}$ , in the presence of 20 mM HEPES pH 7.4, 10 mM  $\text{MgCl}_2$ , 1 mM DTT, 2.4  $\mu\text{M}$  calmodulin, 2 mM  $\text{CaCl}_2$  with 100  $\mu\text{M}$  ATP ( $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  2  $\mu\text{Ci}/\text{tube}$ , 5000 Ci/mmol; Amersham, Little Chal-

font, UK) as previously described [3,4]. Proteins were separated by SDS-PAGE (running gel: acrylamide 11%) and phosphoproteins were revealed by autoradiography.

## 2.6. PSD proteins phosphorylation

10  $\mu\text{g}$  of PSDs purified from hippocampus was incubated in a buffer of 20 mM HEPES pH 7.4, containing 10 mM  $\text{MgCl}_2$ ,  $10^{-5}$  M okadaic acid (Sigma, St. Louis, MO, USA), 100  $\mu\text{M}$  PMSF, 1 mM DTT, 100  $\mu\text{M}$  ATP in the presence of 2 mM  $\text{CaCl}_2$  and 2.4  $\mu\text{M}$  calmodulin as previously described [4]. Reactions were carried out at  $37^{\circ}\text{C}$  for 30 min and subsequently, the 'pull-out' assay with GST-NR2A(1349–1464) was performed.

## 2.7. Antibodies

Antibody against  $\alpha\text{CaMKII}$  was purchased from Boehringer Mannheim, Mannheim, Germany. A polyclonal antibody against GST was produced in rabbits using recombinant GST. Antibody against PSD-95 was purchased from Affinity BioReagents, USA. Antibodies against NR2A/B and GluR2/3 were purchased from Chemicon (Temecula, CA, USA).

## 3. Results

### 3.1. 'Pull-out' of $\alpha\text{CaMKII}$ and PSD-95 from purified PSDs by the NR2A cytoplasmic domain

We have previously demonstrated that CaMKII and NR2A subunit of NMDA receptors co-precipitate in native hippocampal PSD [4]. To further confirm the specific and direct association of  $\alpha\text{CaMKII}$  to NR2A, fusion proteins between GST and the cytoplasmic C-tail of the NR2A subunit partially overlapping each other or GST alone were prepared, immobilised on a glutathione affinity matrix and used for a 'pull-out' assay. This procedure allows for analysis of proteins that directly bind to the purified fusion protein. The different fragments of the C-terminal region of NMDA receptor subunit NR2A were: R-1049–V-1464, L-1244–V-1464, S-1349–V-1464.

PSD proteins purified from rat hippocampus in their native form were applied 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  $\alpha\text{CaMKII}$ , PSD-95 and GluR2/3. Fig. 1A shows that both  $\alpha\text{CaMKII}$  and PSD-95 can associate with the C-terminal NR2A cytoplasmic domain. Both proteins associated with all the NR2A fragments assayed, thus leading to the conclusion that the sequence S-1349–V-1464 is sufficient to guarantee the association of the kinase and PSD-95 to the NMDA receptor. The binding is specific since no  $\alpha\text{CaMKII}$  and PSD-95 are pulled-out using GST alone. The association of  $\alpha\text{CaMKII}$  and PSD-95 to NR2A(1349–1464) was not due to incomplete solubilisation of the PSD proteins offered to the beads, since another PSD protein (i.e. GluR2/3, upper panel) remained in the supernatant in the pull-out assay. The association of NR2A C-tail with  $\alpha\text{CaMKII}$  is further confirmed by overlay experiments in which native PSD proteins purified from rat hippocampus were separated on 6% SDS-PAGE, transferred to nitrocellulose and overlaid with eluted GST-NR2A fusion proteins. Fig. 1B shows the results of a representative Western blot after probing with the GST antibody after the overlay procedure performed with the GST-NR2A(1349–1464), GST-NR2A(1244–1464) and GST-NR2A(1049–1464) fusion proteins. A positive signal at 50 kDa indicates that all the fusion proteins assayed bind to  $\alpha\text{CaMKII}$  in native PSD. In addition, multiple signals between 90–105 kDa are present, showing that all the NR2A

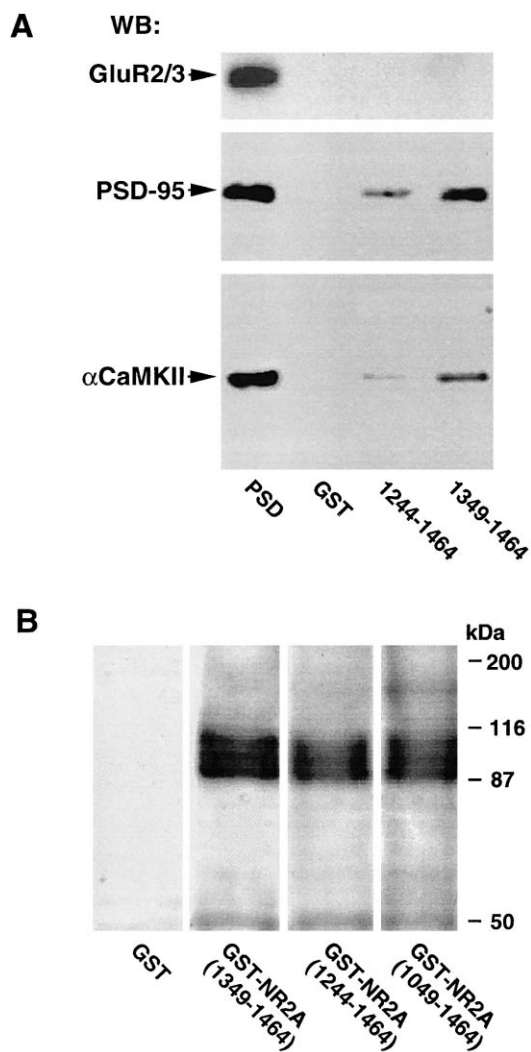


Fig. 1. A: 'Pull-out' of  $\alpha$ CaMKII, PSD-95 and GluR2/3 from hippocampal PSD by GST, GST-NR2A(1244-1464) and GST-NR2A(1349-1464) purified fusion proteins. Data are representatives of three independent experiments performed on different PSD preparations and replicated four times in each PSD preparation. B: GST-NR2A(1349-1464), GST-NR2A(1244-1464) and GST-NR2A(1049-1464) fusion proteins bind in overlay experiments on the PSD-95 family and  $\alpha$ CaMKII. Native PSD proteins were separated in SDS-PAGE (running gel: acrylamide 6%) and electroblotted. Blocked nitrocellulose was incubated with eluted GST, GST-NR2A(1349-1464), GST-NR2A(1244-1464) and GST-NR2A(1049-1464). The nitrocellulose sheet is revealed by western blotting (WB) using anti-GST polyclonal antibody. Data are representatives of three independent experiments performed on different PSD preparations and replicated four times in each PSD preparation.

fragments bind the PSD-95 family of proteins, as expected and previously reported by others [20,22].

### 3.2. CaMKII-dependent phosphorylation of GST-NR2A C-terminal fusion proteins

Since NR2A subunits of the NMDA receptor complex have been described to be substrates for CaMKII, we have investigated whether the binding of CaMKII to NR2A occurs in a region where Ser/Thr phosphate acceptor sites for CaMKII are present.

Sequence comparison between NR2A and NR2B shows that the region between 1255–1298 shows a modest homology.

Within this region, a domain (I-1284–D-1291) containing Ser-1289, a putative phosphate acceptor site for CaMKII, is present. GST-NR2A fusion proteins were incubated in the presence of  $\alpha$ CaMKII(1-315), a region containing the catalytic and the regulatory domain, in a buffer containing  $\text{Ca}^{2+}$ /CaM in the presence of ATP as phosphate donor. Fig. 2 shows a representative autoradiograph of GST-NR2A fusion fragments L-1244–V-1464, S-1349–V-1464 phosphorylation. The radioactive band at 50 kDa corresponding to NR2A(1244-1464) GST fusion protein is clearly visible (right-most lane). The S-1349–V-1464 fragment did not show any phosphorylation, thus demonstrating the presence of the CaMKII phosphosite in the region comprised between 1244 and 1348, where the Ser-1289 is located. The phosphorylation signal shown by fragment (1244–1464) is specific since GST alone did not show any phospho-band (left-most lane, Fig. 2).

### 3.3. Effect of CaMKII autophosphorylation on the NR2A kinase association

To test whether CaMKII autophosphorylation might influence NR2A association, PSDs purified from rat hippocampus were first phosphorylated under conditions known to activate CaMKII, thereby promoting kinase autophosphorylation, and offered to GST-NR2A(1349-1464) immobilised on agarose beads in a 'pull-out' assay. The binding of CaMKII in the PSD fraction to GST-NR2A(1349-1464) was detected by immunoblot analysis using a monoclonal anti- $\alpha$ CaMKII antibody in the bound material, as described above.

Fig. 3A shows results of a representative pull-out experiment in which the same amount of GST-NR2A(1349-1464), as judged by Western blotting with an anti-GST antibody and by ponceau staining of the nitrocellulose membrane after blotting, was incubated with phospho- or dephospho-PSDs. The endogenous phosphorylation of PSDs increased the binding of  $\alpha$ CaMKII to NR2A(1349-1464) by  $+316.2 \pm 18.4\%$  ( $P < 0.01$ ).

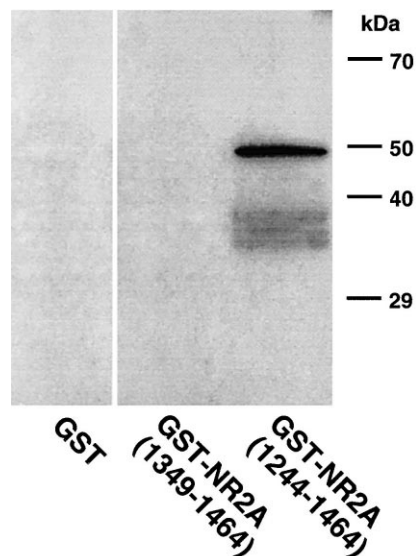


Fig. 2. In vitro phosphorylation of GST-NR2A fusion proteins. GST, GST-NR2A(1244-1464) and GST-NR2A(1349-1464) purified fusion proteins were incubated with 50 U of  $\alpha$ CaMKII(1-315) (New England BioLabs) for 30 min at 37°C. Proteins were separated by SDS-PAGE (running gel: acrylamide 11%) and phosphoproteins were revealed by autoradiography.

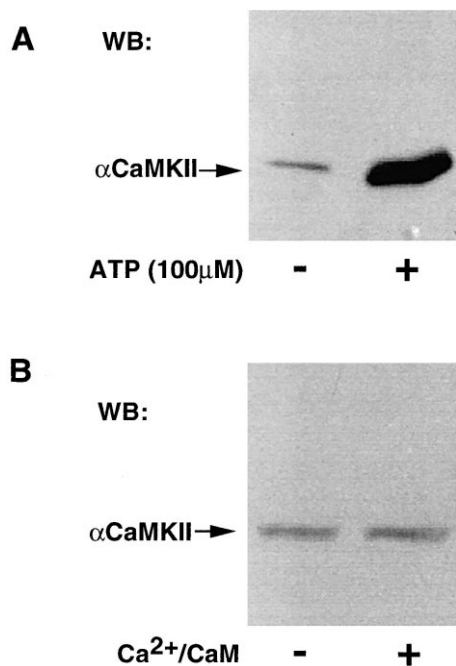


Fig. 3. Autophosphorylation of CaMKII affects binding to NR2A. A: PSDs purified from rat hippocampus were incubated in presence or absence of ATP (100 μM) under conditions known to activate CaMKII and were offered to GST-NR2A(1349-1464) immobilised on agarose beads. αCaMKII bound to fusion protein was recognised by an immunoblot with specific monoclonal antibody. B: Pull-out experiments performed in absence or presence of Ca<sup>2+</sup>/CaM.

Moreover, the binding of αCaMKII to NR2A(1349-1464) is strictly related to the autophosphorylation of the kinase since addition of calcium/calmodulin alone does not influence the αCaMKII association to NR2A (Fig. 3B).

We then tested which domain of αCaMKII could be responsible for the NR2A C-tail binding. To answer this question, in a first set of experiments, GST-NR2A(1349-1464) fusion proteins immobilised on agarose beads were incubated in the presence of 100 ng of αCaMKII(1-315), before incubation in a batch with PSD proteins. Using these experimental conditions, the amount of native αCaMKII bound to NR2A(1349-1464) was significantly lower ( $-72.2 \pm 5.4\%$ ;  $P < 0.01$ ), thus indicating that the exogenous added αCaMKII(1-315) competed with the native kinase for NR2A binding (Fig. 4). In a second set of experiments, a fusion protein between GST and the αCaMKII association domain (amino acids 315–478) was obtained, immobilised on agarose beads and incubated for pull-out and overlay experiments with native PSD. No signal for association of this kinase domain with the native NMDA receptor subunits was found (data not shown).

#### 4. Discussion

The present study shows that the C-terminal region of the NR2A subunit of the NMDA receptor complex interacts with native αCaMKII from rat hippocampal PSDs and that this interaction occurs with unphosphorylated CaMKII, but is modulated by phosphorylation. In addition, we show that CaMKII is able to phosphorylate NR2A subunit in the region 1244–1349, most likely on Ser-1289.

Targeting of CaMKII to NMDA receptor subunits has previously been described. However, all these studies [25,30] focused their attention primarily on NR2B subunits, which were reported to be one of the major substrates for CaMKII in PSD [3]. Nevertheless, given the high concentration of NR2A subunits in hippocampal native glutamatergic synapses and the importance of these subunits in the NMDA receptor complex in the modulation of synaptic efficacy, it was of great relevance to demonstrate the interaction of NR2A and CaMKII.

NR2A is by far the most abundant subunit assembled with NR1 in the mature glutamatergic synapse [27] and it is likely to play a major role in synaptic plasticity modulating LTP and LTD. In fact, Sprengel et al. [31] demonstrated that LTP is defective in animals with a C-terminal truncation of post-natally expressed NR1/NR2A heteromeric receptors, but with an intact NR1/NR2B complex. Therefore, given the fundamental role played by NR2A in modulating the synaptic efficacy and plasticity, the association of CaMKII to NR2A appears to be particularly relevant and it may represent a molecular strategy used by the cell to finally tune the NMDA receptor function and subsequent cellular response.

Strack and Colbran [25] recently demonstrated that recombinant CaMKII is targeted to the NR2B C-terminal region upon autophosphorylation. Using an artificial cell system and recombinant in vitro phosphorylated CaMKII, they showed an association between P-Thr-286-αCaMKII and NR2B(1260-1309). More recently, Leonard et al. [30] described an interaction of the kinase with NR2B(839-1120) and the NR1 C-tail. They reported that in this new site on NR2B subunits, the association of CaMKII occurs also in absence of kinase autophosphorylation. Despite their apparent discrepancies, the studies of Strack and Colbran [25] and Leonard et al. [30] agree in identifying NMDA receptor subunits as a target for CaMKII in PSD, thus underlying the importance of such an association in modulating the synaptic strength in the excitatory synapses. Of relevance to this complex protein-protein interaction, the results presented here show that the 1349–1464 C-terminal region of NR2A is capable of interacting with both αCaMKII and PSD-95.

αCaMKII is able to bind NR2A(1349-1464) also in a unphosphorylated form (Fig. 2A). In fact, a low but significant amount of CaMKII is bound to NR2A(1349-1464) when native PSDs are offered to the beads. One might speculate that in these conditions, the endogenous quiescent kinase binds to NR2A(1349-1464) [32]. In addition, the fact that the recombinant catalytic domain is able to displace the binding to native

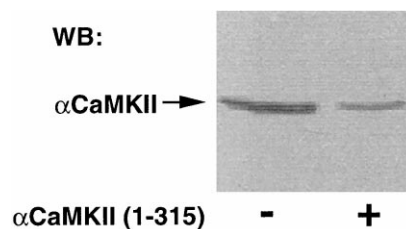


Fig. 4. The association of αCaMKII to NR2A is via the catalytic domain. PSDs incubated in absence or presence of 100 ng of αCaMKII(1-315) were incubated in a pull-out experiment with GST-NR2A(1349-1464). The amount of αCaMKII bound to the fusion protein was monitored by immunoblot analysis with anti-αCaMKII.

PSD (Fig. 3A) further strengthens this hypothesis. Consistent with this observation, Shen and Meyer [33] reported that NMDA receptor activation in hippocampal neurons in culture reversibly translocates green fluorescent protein-tagged CaMKII from F-actin to PSD but that the kinase dead mutant (Lys-42-Arg-42) as well as autophosphorylation deficient mutant still translocate to the PSD domain, thus suggesting that kinase autophosphorylation is not a needed prerequisite. Nevertheless, they reported that kinase autophosphorylation appears to prolong the association of the kinase with the PSD domain. Our data, showing that when PSD is phosphorylated under conditions known to activate endogenous CaMKII and leading to kinase autophosphorylation, the association of CaMKII to NR2A(1349-1464) strongly increases, are in line with this observation and with data reported by others [25], indicating that autophosphorylated kinase is more prone to association.

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