Regulation of Calcium/Calmodulin-dependent Protein Kinase II Docking to N-Methyl-d-aspartate Receptors by Calcium/Calmodulin and α-Actinin*

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Ca2⁺ influx through the N-methyl-d-aspartate (NMDA)-type glutamate receptor leads to activation and postsynaptic accumulation of Ca2⁺/calmodulin-dependent protein kinase II (CaMKII) and ultimately to long term potentiation, which is thought to be the physiological correlate of learning and memory. The NMDA receptor also serves as a CaMKII docking site in dendritic spines with high affinity binding sites located on its NR1 and NR2B subunits. We demonstrate that high affinity binding of CaMKII to NR1 requires autophosphorylation of Thr286. This autophosphorylation reduces the off rate to a level (t½ = ~23 min) that is similar to that observed for dissociation of the T286D mutant CaMKII (t½ = ~30 min) from spines after its glutamate-induced accumulation (Shen, K., Teruel, M. N., Connor, J. H., Shenolikar, S., and Meyer, T. (2000) Nat. Neurosci. 3, 881–886). CaMKII as well as the previously identified NR1 binding partners calmodulin and α-actinin bind to the short C-terminal portion of the C0 region of NR1. Like Ca2⁺/calmodulin, autophosphorylated CaMKII competes with α-actinin-2 for binding to NR1. We conclude that the NR1 C0 region is a key site for recruiting CaMKII to the postsynaptic site, where it may act in concert with calmodulin to modulate the stimulatory role of α-actinin interaction with the NMDA receptor.

Long term potentiation (LTP) in the hippocampus is a long-lasting increase in synaptic transmission, which likely constitutes a physiological correlate of learning and memory (1, 2). LTP usually requires Ca2⁺ influx through the NMDA receptor and subsequent activation of CaMKII by Ca2⁺/calmodulin (2–4). CaMKII is concentrated at postsynaptic densities (5, 6). This localization is strategically ideal for activation by Ca2⁺ influx through NMDA receptors and for phosphorylation of neighboring AMPA receptors, which contributes to LTP (3, 7, 8). Simultaneous binding of two or more Ca2⁺/calmodulin molecules to neighboring subunits within the same dodecameric CaMKII holoenzyme leads to intersubunit phosphorylation at Thr286 (4, 9). This mechanism enables CaMKII to decode Ca2⁺ spike frequency (10). Thr286 phosphorylation keeps the enzyme in a catalytically active conformation independent of the presence of Ca2⁺/calmodulin in the holoenzyme and thereby of cytosolic Ca2⁺ levels (4, 9). Coincident with its activation, CaMKII associates with the NMDA receptor subunits NR1 (11), NR2A (12), and NR2B (11, 13) and accumulates at postsynaptic spines (14, 15).

NMDA receptors in cortex and hippocampus mainly consist of one or two NR1 and two or three NR2A and 2B subunits (16–19). Specific interaction of CaMKII with NR2A (12, 20) is about an order of magnitude weaker than with NR1 or NR2B (13) and not easily detectable under several conditions (11, 21). CaMKII robustly binds to two independent sites on the 644-amino acid-long intracellular C terminus of NR2B. One site (NR2B/C) is formed by residues 1290–1309; the other site (NR2B/P) is localized within residues 839–1120 but has evaded further identification up to now (11, 13, 21, 22). Ca2⁺/calmodulin is sufficient to induce high affinity binding of CaMKII to NR2B/C (22). Ca2⁺/calmodulin binding and Thr286 phosphorylation remove the autoinhibitory segment of CaMKII to various degrees from its catalytic substrate-binding site (S site) and from the endogenous attachment site for Thr286, the T site (23). The NR2B1289–1310 sequence binds to the T site in the presence of Ca2⁺/calmodulin. The resulting interaction outlasts dissociation of Ca2⁺/calmodulin, thereby displacing Thr286 from the T site and the inhibitory segment from the catalytic S site for an extended period of time (22). The result is a lasting increase in the activity of NR2B/C-associated CaMKII independent of cytosolic Ca2⁺ or Thr286 phosphorylation.

The initial Ca2⁺-induced translocation of CaMKII to postsynaptic sites requires Ca2⁺/calmodulin binding to CaMKII but not Thr286 phosphorylation (14, 15). These studies did not identify the postsynaptic attachment sites for CaMKII, but our earlier evidence indicates that the initial Ca2⁺-induced postsynaptic accumulation is at least in part due to Ca2⁺/calmodulin-dependent association of CaMKII with the NR2B/C site (22). However, the time course of persistent CaMKII trans-
location to the postsynaptic site does depend on Thr286 phosphorylation; prolonging autophosphorylation by inhibiting phosphatase 1 and mimicking Thr286 phosphorylation by introducing a T286D mutation resulted in a much reduced dissociation rate of CaMKII from postsynaptic sites (14, 15). Auto-phosphorylation of CaMKII increases its affinity for NR2B/C (13, 21, 22). This auto-phosphorylation-induced increase in NR2B/C binding is comparable with that caused by Ca2+/calmodulin (22) (see Fig. 1C). This auto-phosphorylation-dependent interaction could therefore contribute to a more enduring association of CaMKII with the postsynaptic site. However, serine 1303 in NR2B/C is the major CaMKII phosphorylation site on NR2B (24), and phosphorylation of this serine inhibits CaMKII binding to this site (21), thereby antagonizing an otherwise potentially lasting interaction between NR2B/C and CaMKII. We now show that auto-phosphorylation of Thr286 decreases the off rate for CaMKII release from NR1 by a factor of 10 and brings it into a time range similar to that observed for dissociation of Thr286-phosphorylated CaMKII from synaptic sites in neuronal cultures. Together with previous work our results argue for a complex regulatory mechanism of the CaMKII-NMDA receptor interaction.

Previous work indicates that calmodulin itself and also α-actinin-2 can directly bind to the C terminus of NR1 (25–27), thereby displacing α-actinin (26, 28). The physiological significance of these findings is highlighted by earlier results demonstrating that an intact actin cytoskeleton and interaction with α-actinin support NMDA receptor activity (28, 29). Using peptide libraries provided by Drs. F. Zheng and P. J. Conn, Emory University, Atlanta, and inserted into pGEX-6P-3; accession number D13211 (31); kindly supported NMDA receptor activity (28, 29). Using peptide libraries provided by Drs. F. Zheng and P. J. Conn, Emory University, Atlanta, and inserted into pGEX-6P-3; accession number D13211 (31); kindly

Materials—GST-NMDA Receptor Fusion Proteins—Recombinant CaMKIIa (1–2 μg) was preincubated for 5 min at 0 °C (to promote Thr286 auto-phosphorylation in selected samples) in basic phosphatase buffer (50 mM HEPES, pH 7.4, 10 mM MgCl2, 1 mM CaCl2, 1 mM EDTA) and centrifuged at 10,000 × g for 20 min, and the pellet was resuspended in buffer A (50 mM HEPES, pH 7.4, 50 mM NaCl, 3 mM MgCl2, 1 mM CaCl2, 1 mM EDTA). The suspension was added to a 5-ml calmodulin-Sepharose (Sigma) column, equilibrated with buffer A, and incubated for 1 h under tilting. The column was washed with 1 column volume of buffer A, followed by a high salt wash (buffer A plus 950 mM NaCl). The CaMKII was eluted by chelating Ca2+ via incubating the column for 1 h in 1 column volume of 50 mM HEPES, pH 7.4, 50 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1 mM EDTA, 1 mM DTT. Additional fractions were eluted with several more column volumes of the same buffer. The protein concentration was determined by A280 reading and with the bicinchoninic acid protein assay (Pierce). The various fractions were analyzed by SDS-PAGE, followed by Coomasie staining to control for purity and potential degradation and by immunoblotting to identify CaMKII. CaMKII was dialyzed against 10 mM HEPES, pH 7.5, 5 mM DTT, 1 mM EDTA and stored at −20 °C in the dialysis buffer with 50% glycerol added to it.

CaMKII and α-Actinin Binding and Competition Assays with NMDA Receptor Fusion Proteins—Recombinant CaMKIIa (1–2 μg) was preincubated for 5 min at 0 °C (to promote Thr286 auto-phosphorylation in selected samples) in basic phosphatase buffer (50 mM HEPES-NaOH, pH 7.4, 10 mM MgCl2) containing, if indicated, 500 μM CaCl2, 1 μM calmodulin, and 500 μM ATP. In some samples EGTA was added, which completely removed calcium/calmodulin from the kinase (22). To induce Thr286 Thr286 phosphorylation in selected samples (Fig. 1 only), the incubation was continued for another 15 min at 30 °C. The kinase mixture was then added to 20 μl of glutathione-Sepharose resin samples loaded with about 1.0 μg of GST fusion proteins or GST alone (as estimated by SDS-PAGE and subsequent staining with Coomassie Brilliant Blue in addition to a quantitative bicinchoninic acid-protein assay (Pierce) (11)). After incubation for 2–3 h at 0–4 °C in and washing of the samples with TBS (10 mM Tris-Cl, pH 7.4, 150 mM NaCl) containing 0.1% Triton X-100, the proteins were separated by SDS-PAGE and transferred onto nitrocellulose for immunoblotting and quantification by ImageQuant (Molecular Dynamics) analysis. Competition by α-actinin was tested by the addition of up to 10 μg α-actinin to the resin samples during the CaMKII-binding step. Binding of α-actinin to GST-NR1 was measured in the same way in the absence and presence of Ca2+ and increasing calmodulin concentrations.

To determine dissociation rates, unphosphorylated (no ATP present during preincubation and binding) and auto-phosphorylated recombinant CaMKIIa (1–2 μg) was incubated with immobilized CaMKIIa obtained in the previous paragraph for 1.5 h at 4 °C (initial experiments revealed that binding equilibrium was reached within less than 1 h; 24 h equilibration time did not result in more CaMKII binding). Matrix-bound NR1-CaMKII complexes were obtained by centrifugation, followed by three quick washing steps, which took less than 3 min. The resin was then resuspended in either 1 or 10 ml of buffer A (32) and centrifuged at 160,000 g for 10 min. Centrifugation was repeated if necessary. Matrix-bound CaMKIIa was obtained by centrifugation at the indicated time points, followed by two quick washing steps taking less than 60 s, extraction with SDS sample buffer, immunoblotting, and quantification as described. The koff value was obtained from single exponential best fit curves of experimental
Peptide Synthesis—Fmoc peptide synthesis was performed manually in a solid phase approach as described by the manufacturer (Chiron Mimotopes). In brief, the peptides were synthesized on derivatized polypropylene pins arranged in a 96-well plate format. Each condensation reaction began with the removal of the Fmoc protecting group with trifluoroacetic acid (50% in DMF, 1–2% ethanol or anisole) followed trifluoroacetic acid treatment and successive washes with 10% triethylenamine in DMF, methanol, triethylamine, methanol, and DMF completed the neutralization sequence.

Peptide Binding Assays—Pins with the peptides were utilized for binding assays in 96-well plates by an ELISA procedure with recombinant CaMKIIα, poly-his-α-actinin-2, or brain calmodulin, followed by antibodies against CaMKIIα, the poly-his tag, or calmodulin, respectively, and ultimately by horseradish peroxidase-coupled goat antimouse IgG antibodies for all three proteins. All binding assays and antibody incubations were done in 20 mM phosphate buffer, pH 7.4, containing 150 mM NaCl, 2% bovine serum albumin, and 0.1% Tween 20. To detect the horseradish peroxidase-labeled antibodies, 150 μl of the 1-step slow TMB-ELISA solution (Pierce) was added to each well. A500 readings were taken every 5 min for up to 30 min to follow the reaction. Finally 100 μl of 1 M H2SO4 was added to stop the reaction for A500 readings, which often showed reduced background compared with the readings at A500 taken without H2SO4. Because the peptides were synthesized on a noncleavable peptide block, repeated binding assays could be performed after regenerating the block by ultrasound treatment in a bath sonicator in 62.5 mM Tris-Cl, pH 6.8, 1% SDS, 20 mM dithiothreitol, for 30 min at 55°C (power setting 6). This procedure removed bound target and antibody from the peptide without breaking the bond between the peptide and the pin. Subsequent control ELISA tests with primary and secondary antibodies only confirmed that no antigen remained on the pins. After sonication, the pins were washed five times with water and five times with TBS and stored in TBS containing 0.02% azide. All of the noncleavable peptide blocks contained positive control peptides, including AC3-I (KKALHRQETVID) (11) and NR2B1256–1306 (RNKLRRQHSYD) (21, 22).

RESULTS

Regulation of CaMKII Binding to NR1 and NR2B by Calmodulin and Autophosphorylation—The NMDA receptor appears to be a major docking site for CaMKII at postsynaptic sites (11–13, 20–22). The initial accumulation of CaMKII at postsynaptic sites requires Ca2+/calmodulin binding to CaMKII, but persistent CaMKII clustering depends on Thr286 phosphorylation (14, 15). To evaluate the binding partners and molecular mechanisms of CaMKII recruitment and retention at postsynaptic sites, we determined the effect of Ca2+/calmodulin and the effect of autophosphorylation of CaMKII on its binding to the three high affinity interaction sites NR1, NR2B/P, and NR2B/C (Fig. 1).

The GST fusion proteins NR1, NR2B/P, and NR2B/C were immobilized on glutathione-Sepharose and incubated with recombinant CaMKII in the absence and presence of Ca2+/calmodulin and ATP before washing and detection of CaMKII by immunoblotting. CaMKII bound to all three fusion proteins even in the absence of Ca2+/calmodulin and ATP. The resulting interactions were rather weak, explaining why they have not been observed in other studies (13, 21). These Ca2+/calmodulin- and autophosphorylation-independent interactions were nevertheless specific because negative controls with plain GST performed in parallel did not yield signals for CaMKII (data not shown; but see Ref. 11). Ca2+ by itself had no effect, but together with calmodulin it increased association of CaMKII with NR2B/C (Fig. 1C). Ca2+/calmodulin was not sufficient to stimulate CaMKII binding to NR2B/P or NR1 (Fig. 1, A and B).

Preincubation of CaMKII with Ca2+/calmodulin and ATP under conditions that result in Thr286 phosphorylation was necessary to induce high affinity binding of CaMKII to NR1 and NR2B/P (Fig. 1, A and B). These results indicate high affinity interactions of NR2B/C with the Ca2+/calmodulin-bound form of CaMKII and of NR1 and NR2B/P with Thr286-phosphorylated CaMKII.

Focusing on NR1, we tested whether Thr286 phosphorylation is not only necessary but also sufficient for high affinity interaction of CaMKII with NR1. GST-NR1 and, as negative control, GST alone were immobilized on anti-GST antibody-coated 96-well plates. The samples were incubated with wt and T286A- and T286D-mutated CaMKII before washing and immunoblotting. This procedure is less sensitive in our hands than the pull-down experiments with fusion proteins on glutathione-Sepharose and only permits detection of the high affinity interaction between CaMKII and NR1. When indicated, CaMKII had been pretreated with Ca2+/calmodulin and ATP under conditions that result in Thr286 phosphorylation in the wild type form. As before, wt CaMKII bound with high affinity to GST-NR1 only after Thr286 phosphorylation (Fig. 2, lanes 1 and 2). CaMKII did not show an interaction with NR1 when ATP was omitted during the procedure to prevent Thr286 phosphorylation, whether Ca2+/calmodulin was present or not (lanes 3 and 4). The addition of EGTA after pretreatment completely removes Ca2+/calmodulin from CaMKII (22) but did not result in a reduction of Thr286-phosphorylated CaMKII binding to NR1 (compare lanes 1 and 2). These results show that high

FIG. 1. Ca2+/calmodulin and Thr286 phosphorylation differentially regulates CaMKIIα association with its NMDA receptor-binding sites NR1, NR2B/P, and NR2B/C. GST fusion proteins NR1 (residues 725–936, which include the C0, C1, and C2 region), NR2B/P (839–1120), and NR2B/C (1120–1482) were immobilized on glutathione-Sepharose and incubated with recombinant wt CaMKIIα that had been pretreated without or with Ca2+, calmodulin, and ATP as indicated. Some samples (lane 5) were further preincubated for 15 min at 30°C with EGTA added after initial prephosphorylation of Thr286 in the presence of Ca2+/calmodulin and ATP; the latter procedure results in additional phosphorylation of Thr285 and Thr286. After washing, the amount of bound CaMKII was determined by immunoblotting (lower panels) and subsequent densitometry (n = 3; error bars, S.D.). Probing of the blots with anti-GST antibodies indicated that comparable amounts of the GST fusion proteins were present in all samples (not shown; see Ref. 11).
CaMKII Docking on NMDA Receptors

**Identification of the Binding Site for CaMKII, Calmodulin, and α-Actinin on NR1**—The precise CaMKII attachment site has been defined for NR2B/C (21) that may play a crucial role in the initial 5-min preincubation of CaMKII with Ca\(^{2+}\)/calmodulin and ATP. Dissociation of the resulting CaMKII-NR1 complexes were measured after 50-fold dilution by determining how much CaMKII was left on the beads at various time points. Phosphorylation increased the half-life of the NR1-CaMKII complex by a factor of 10 from 2.4 to 23 min (Fig. 3). After dilution, matrix bound CaMKII was obtained by two quick washing steps taking less than 1 min at the indicated time points and quantified by immunoblotting and densitometry. No electrophoretic mobility shifts of CaMKII were visible in any sample, thus indicating that no hyperphosphorylation had occurred under any of these conditions. The \(k_{\text{off}}\) values were obtained from single exponential best fit curves of experimental data. All of the data fits were performed with PRISM software (version 3.02, GraphPad Software, Inc. San Diego, CA) (n = 3 ± S.D.).

**Regulation of CaMKII Dissociation from NR1 by Autophosphorylation**—We hypothesize that the increase in affinity of the CaMKII interaction upon Thr\(^{286}\) phosphorylation could at least in part be responsible for the very slow redistribution rate of the T286D mutant of CaMKII after postsynaptic accumulation. CaMKII was incubated with GST-NR1 adsorbed onto glutathione-Sepharose in the presence and absence of Ca\(^{2+}\)/calmodulin and ATP. Dissociation of the resulting CaMKII-NR1 complexes were measured after 50-fold dilution by determining how much CaMKII was left on the beads at various time points. Phosphorylation increased the half-life of the NR1-CaMKII complex by a factor of 10 from 2.4 to 23 min (Fig. 3). There was no significant difference in these off rates when dissociation was initiated by a 500-fold instead of a 50-fold dilution, indicating that the observed off rates were true off rates and not contaminated by substantial rebinding or other factors. These results link Thr\(^{286}\) phosphorylation and the resulting decrease in the dissociation rate of an NR1-CaMKII complex to a strongly reduced relocation rate of synaptically clustered CaMKII. The relocation rate of wt CaMKII is much faster than that of the T286D mutant and depends at least in part on phosphatase activity (14, 15). Our results suggest that phosphorylation of Thr\(^{286}\) reduces dissociation rates for CaMKII from NR1 and thereby from the postsynaptic site. Accordingly, dephosphorylation of Thr\(^{286}\) should increase NR1 dissociation and, thereby, increase the relocation rate.

**Autophosphorylation of Thr\(^{286}\)**—Induced by glutamate receptor-mediated Ca\(^{2+}\) influx (15). The hyperphosphorylation clearly reduced the affinity of CaMKII for NR1, NR2B/P, and NR2B/C, although the affinity was in each case still more than 2-fold higher than the affinity of CaMKII for NR1 and thereby from the postsynaptic site. About 10 \(\mu\)g of GST-NR1C0-C1-C2 (residues 725–938) was immobilized on 96-well plates coated with antibodies against GST and incubated with pretreated CaMKII. The samples were diluted 50-fold (in some experiments 500-fold; not shown) with buffers and cofactors that were identical to those used during the phosphorylation and binding step. After dilution, matrix bound CaMKII was obtained by two quick washing steps taking less than 1 min at the indicated time points and quantified by immunoblotting and densitometry. CaMKII docking on NMDA receptors can be induced by glutamate receptor-mediated Ca\(^{2+}\) influx (15). The latter finding suggests that Thr\(^{286}\) phosphorylation contributes to acceleration in relocation of postsynaptically clustered CaMKII. Thr\(^{286}\) phosphorylation should decrease affinity for the NMDA receptor if the NMDA receptor is to a substantial degree responsible for CaMKII anchoring at postsynaptic sites. We induced Thr\(^{286}\) phosphorylation by adding EGTA after the initial 5-min preincubation of CaMKII with Ca\(^{2+}\)/calmodulin and ATP to the phosphorylation reaction mixture and by continuing the preincubation for 15 min at increased temperature (30 °C). The effectiveness of this established protocol in causing hyperphosphorylation is demonstrated by the expected decrease in the electrophoretic mobility of CaMKII (Fig. 1, right lanes). The hyperphosphorylation clearly reduced the affinity of CaMKII for NR1, NR2B/P, and NR2B/C, although the affinity was in each case still more than 2-fold higher than the weak basal binding (Fig. 1). Accordingly, Thr\(^{286}\) phosphorylation may contribute to a reduction in the affinity of CaMKII for the NMDA receptor and may foster release of CaMKII from the postsynaptic site (15). We note that the addition of EGTA alone to remove calcium/calmodulin from CaMKII without raising the temperature did, as expected, not result in hyperphosphorylation because the electrophoretic mobility did not shift without the treatment at elevated temperature (Fig. 2, lane 2).

**Identification of the Binding Site for CaMKII, Calmodulin, and α-Actinin on NR1**—The precise CaMKII attachment site has been defined for NR2B/C (21) that may play a crucial role in the initial Ca\(^{2+}\)/calmodulin-induced CaMKII binding (22) but not for NR1, which may be critical for sustained CaMKII...
The C0 region of NR1 exists in four splice variants. All CaMKII activity (29). To define the precise interaction sites for CaMKII, the channel depends on an intact actin cytoskeleton for optimal anchoring upon Thr286 phosphorylation (see above). The intra-cellular C terminus of NR1 contains a CaMKII docking site and neighboring residues that help to support the observed interactions. For example, specific CaMKII binding starts with peptide 12 and ends with peptide 17 as designated in Fig. 5. The stretch of residues that is common to these two peptides (and to peptides 13–16; LAFAAVNV) is therefore necessary for CaMKII binding and constitutes the minimal or core sequence. However, peptides 12 and 17 show only a relatively low level of CaMKII binding compared with peptides 13–16, indicating that residues next to the core sequence also contribute to binding (i.e., QMQ N-terminal and WRK C-terminal to the core sequence). The optimal binding peptide covered NR1 (847–858) for CaMKII and for Ca2+/calmodulin and α-actinin-2 NR1 (850–862). The overlap in the binding segment for Ca2+/calmodulin and α-actinin-2 explains their competition for C0 association.

**Competition between CaMKII and α Actinin for Association with NR1**—The overlap of the association segments for CaMKII, Ca2+/calmodulin, and α-actinin-2 suggests that these three proteins might compete for C0 binding. As demonstrated earlier (26), we found that Ca2+/calmodulin can displace α-actinin-2 (Fig. 6). The important point of this experiment is that at least 0.5–1 μM calmodulin is required for substantial displacement of α-actinin-2; 100 nM calmodulin had little, if any, effect. This observation allowed us to preincubate CaMKII with Ca2+ and ATP in the presence of 100 nM calmodulin to stimulate Thr286 phosphorylation without subsequent removal of calmodulin before the binding competition assays with α-actinin-2. Increasing amounts of α-actinin-2 prevented more and more CaMKII binding (Fig. 7). These results demonstrate a competitive relationship between these two proteins for C0 association.

**DISCUSSION**

Previous studies indicate that the NR1, NR2A, and NR2B subunits of the NMDA receptor complex serve as postsynaptic anchor sites for CaMKII and that these interactions are directly regulated by the autophosphorylation state of the kinase (11–13, 21, 34). Binding to the NR2B/C site has received the most attention up to now and was further evaluated in this study with respect to dependence on calmodulin and autophosphorylation on Thr286 as well as on Thr305/306. Similar to earlier publications (13, 21, 22), our results indicate that CaMKII binds with high affinity to NR2B/C when autophosphorylated on Thr286 independent of the presence of Ca2+ and calmodulin (Fig. 1). A comparable level of binding was observed without autophosphorylation as long as Ca2+ and calmodulin were present; binding in the absence of Ca2+/calmodulin and Thr286 phosphorylation was weak. These results indicate that displacement of the autoinhibitory loop of CaMKII by either Ca2+/calmodulin binding or Thr286 phosphorylation induces high affinity binding to NR2B/C (see also Ref. 22). Once CaMKII is bound to NR2B/C, Ca2+/calmodulin can be removed from the nonphosphorylated kinase without displacing NR2B/C. NR2B/C association keeps CaMKII at a high activity level even after calmodulin is removed, whether or not the kinase underwent Thr286 phosphorylation. This mechanism constitutes a second way to generate a lasting, autonomous activity of CaMKII beyond the initial binding of calmodulin as occurring during a rise in intracellular Ca2+, which is usually transient (22).

Redistribution of CaMKII after its Ca2+-induced accumulation at postsynaptic sites is quite fast (t1/2 is ~1 min) (14, 15), raising the question of which mechanisms contribute to a re-
versal of the CaMKII-NMDA receptor interaction. Ser\textsuperscript{1303} is part of the NR2B/C site, which comprises residues 1290–1309 (13, 21). Ser\textsuperscript{1303} is the major CaMKII phosphorylation site in the presence of 0.5 mM CaCl\textsubscript{2}, 0.1 mM CaM, and 0.5 mM ATP and then added to the incubation mixture, which was then allowed to reach a second equilibrium for another 1 h. After washing of the complex, we detected a strong specific signal for CaMKII by immunoblotting with anti-CaMKII in the absence of α-actinin (lower panel, lane 1). CaMKII binding to NR1-GST was progressively more antagonized by increasing amounts of α-actinin with half-maximal inhibition occurring between 0.5 and 1.0 μM α-actinin (lanes 3 and 4).

The difference between the more modest effect of hyperphosphorylation on NR2B/C binding in our hands as compared with Strack and Colbran (13) may be due to the higher level of Thr\textsuperscript{286} phosphorylation in our experiments (we allowed near stoichiometric Thr\textsuperscript{286} phosphorylation at low temperatures before inducing Thr\textsuperscript{286} phosphorylation by adding EGTA and raising the temperature for 15 min to 30 °C). Thr\textsuperscript{286} phosphorylation prevents Ca\textsuperscript{2+}/calmodulin from associating with CaMKII, and Ca\textsuperscript{2+}/calmodulin from itself can stimulate CaMKII interaction with NR2B/C. Therefore, it is possible that the reduction of CaMKII binding to NR2B/C upon Thr\textsuperscript{286} phosphorylation is in part due to the irreversible displacement of calmodulin from the kinase. In any case, collectively the study by Strack and Colbran (13) and our results argue that Thr\textsuperscript{286} phosphorylation results in reduced CaMKII affinity for NR2B/C. This effect, together with Ser\textsuperscript{1303}

![Normalized Binding Signal Intensity](image)

**Fig. 5.** Identification of the CaMKIIα, calmodulin, and α-actinin binding site on NR1. A library of 12-residue-long peptides was synthesized on polystyrene lanterns via a noncleavable seryl-glycyl linker at their C termini. The first peptide corresponded to the membrane-proximal 12 residues of the C0 region (residues 834–845 of NR1). Subsequent peptide sequences were shifted by one position toward the C terminus and covered the C0, C1, C2, and C2’ regions and all of the possible connections between these regions (not all peptides are shown). After deprotection of the peptides, which remained covalently linked to the pins, successive binding assays with recombinant CaMKII (preincubated with Ca\textsuperscript{2+}, 100 mM calmodulin and ATP to induce Thr\textsuperscript{286} phosphorylation for high affinity binding), Ca\textsuperscript{2+}/calmodulin, and α-actinin were performed in a modified ELISA fashion with respective primary and secondary antibodies. After each assay, the bound proteins were removed by sonication in 1% SDS; mock assays with antibodies alone confirmed that the corresponding proteins had completely been removed by the sonication. Shown are consecutive peptides covering the C0 region, which specifically binds CaMKII, calmodulin, and α-actinin. Peptides further downstream only exhibited background levels of binding similar to the first nine peptides of the panel (not shown).

![CaMKII Docking on NMDA Receptors](image)

**Fig. 6.** Competition of calmodulin and α-actinin for NR1 binding. NR1\textsubscript{G}GST (encoding the C0-C2’ regions) or GST alone were incubated with purified His\textsubscript{6}-tagged α-actinin-2 fusion protein (residues 344–894) in the absence or presence of Ca\textsuperscript{2+} and various amounts of calmodulin as given at the bottom. After washing of the resin, α-actinin-2 was visualized by immunoblotting with the anti-His\textsubscript{6} antibody. α-actinin binding to NR1 is Ca\textsuperscript{2+}-independent (upper panel, lanes 1–3) but inhibited by calmodulin at and above concentrations of 0.5 μM (upper panel, lanes 4–7). Stripping the blots with SDS and dithiothreitol (42, 43) and probing with the antibody against GST demonstrates that similar amounts of NR1-GST and higher amounts of GST had been loaded (lower panel)
phosphorylation of NR2B, promotes dissociation of CaMKII from this site after increased Ca\(^{2+}\) concentrations will return to basal levels inside the neuron. Phosphorylation of Ser\(^{370}\) in the NR2B subunit and of Thr\(^{305}\) and Thr\(^{306}\) in the CaMKII autoinhibitory loop may therefore promote redistribution of CaMKII after its Ca\(^{2+}\)-induced accumulation at postsynaptic sites.

We previously identified two additional high affinity binding sites for CaMKII, NR2B/P, which is upstream of NR2B/C, and the C terminus of NR1 (11). In contrast to NR2B/C binding, Ca\(^{2+}\)/calmodulin is neither sufficient nor necessary to stimulate association of CaMKII with NR1 or NR2B/P (Figs. 1 and 2). Rather, autophosphorylation of Thr\(^{306}\) or its mutation to aspartate induces high affinity interaction with NR1 and NR2B/P, whether Ca\(^{2+}\)/calmodulin is present or not (Figs. 1 and 2; see also Ref. 22). We demonstrated that Thr\(^{306}\) autophosphorylation reduced the dissociation rate of CaMKII from the NR1 site from t\(_{1/2}\) of 2.4 to 23 min (Fig. 3). Compared with wt, the T286D mutant of CaMKII exhibits a prolonged accumulation at the postsynaptic site following Ca\(^{2+}\) influx in intact neurons (t\(_{1/2}\) is \(-1\) min for wt and \(-30\) min for T286D) (14, 15). Furthermore, the T286A mutant of CaMKII, which can only bind to NR2B/C but not to NR2B/P or NR1, has a very fast redistribution rate after postsynaptic accumulation (t\(_{1/2}\) is \(<10\) s) (15). Our results indicate that Thr\(^{306}\) phosphorylation-induced high affinity binding to NR1 and NR2B/P may act by prolonging the postsynaptic residence time of CaMKII.

The data described in the preceding paragraph collectively also suggest that dephosphorylation of Thr\(^{306}\) in wt CaMKII is important to reverse CaMKII clustering on a time scale in the range of 1 min. In fact, the phosphatase inhibitor calcineurin A strongly decreases the redistribution rate (t\(_{1/2}\) for wt CaMKII is \(-17\) min in the presence of calcineurin A) (15). A second mechanism that contributes to this redistribution is phosphorylation of Thr\(^{305}\) and Thr\(^{306}\) of CaMKII; when phosphorylation of these two residues is prevented by mutating both residues to alanine, t\(_{1/2}\) is increased to \(-9\) min (15). We found that high affinity binding to all three sites (i.e. NR1, NR2B/P, and NR2B/C) was decreased, although clearly not abolished, when CaMKII was pretreated under conditions that promoted Thr\(^{305,306}\) autophosphorylation, presumably due to the presence of Ca\(^{2+}\)/calmodulin and the proteasome inhibitor MG132 (34). A preferential dephosphorylation of Thr\(^{306}\) over Thr\(^{305,306}\) on CaMKII bound to the NMDA receptor would reduce binding affinity and promote the release of CaMKII from postsynaptic sites.

Using GST fusion proteins and different peptide libraries, which spanned the C terminus of NR1 and also the region of NR2B that contained the NR2B/P site, we were able to precisely define the CaMKII binding site in NR1 (Figs. 4 and 5) but not NR2B/P. \(^3\)CaMKII binds to the C-terminal portion of the C0 region of NR1 (residues 845–862). The same region also interacts with \(\alpha\)-actinin-2 and Ca\(^{2+}\)/calmodulin (Fig. 5). These data are in agreement with earlier findings that calmodulin and \(\alpha\)-actinin-2 bind to the C0 region (25–27) and also identify the exact binding site for those two proteins within this region. The overlap of \(\alpha\)-actinin and Ca\(^{2+}\)/calmodulin-binding sites offers an explanation for the mechanism of their competitive interaction. These proteins may require at least overlapping if not identical sets of binding target residues for interaction and simultaneous binding may therefore not be possible.

When the Ca\(^{2+}\) concentration is low, i.e. under resting conditions, \(\alpha\)-actinin interacts with the NMDA receptor and keeps its activity level high; upon Ca\(^{2+}\) influx, Ca\(^{2+}\)/calmodulin can displace \(\alpha\)-actinin and decrease the receptor activity (25–28) (Fig. 6). If the Ca\(^{2+}\)/calmodulin-mediated reduction in NMDA receptor activity is mainly due to the displacement of \(\alpha\)-actinin, the competition between CaMKII and \(\alpha\)-actinin for NR1 interaction (Fig. 7) would predict that CaMKII binding down-regulates the receptor activity by a similar mechanism, i.e. displacement of \(\alpha\)-actinin. However, CaMKII binding per se could change the NMDA receptor conformation in a way that is at variance with a conformational change induced by Ca\(^{2+}\)/calmodulin. Accordingly, it is possible that CaMKII and Ca\(^{2+}\)/calmodulin affect the NMDA receptor activity differently. In any case, the observation that CaMKII associates with the same segment of NR1 as Ca\(^{2+}\)/calmodulin and \(\alpha\)-actinin will stimulate a careful analysis of the effect of CaMKII on NMDA receptor activity. We have started to perform electrophysiologically experiments along these lines.

CaMKII binding to the NMDA receptor may be critical for several forms of synaptic plasticity, especially LTP. Ca\(^{2+}\) influx through the NMDA receptor and subsequent activation of CaMKII are critical steps in the induction of LTP (2–4). Docking of CaMKII on NMDA receptors puts CaMKII at a location that is strategically ideal for activation by Ca\(^{2+}\) influx through NMDA receptors and subsequent phosphorylation of neighboring AMPA receptors, thereby contributing to LTP (3, 7, 8). We started to evaluate the relevance of CaMKII association with the NMDA receptor for the phosphorylation of AMPA receptors and for the induction of LTP. In addition to up-regulation of AMPA receptor activity by CaMKII-mediated phosphorylation, an increased number of AMPA receptors accumulate during LTP at the postsynaptic site (36). A similar accumulation was induced by overexpression of a constitutively active form of CaMKII (37). It is currently unclear whether this CaMKII-induced AMPA receptor accumulation depends on anchoring of CaMKII by the NMDA receptor as recently suggested by Lisman et al. (3, 38). Defining the exact binding sites for CaMKII on the NMDA receptor provides the molecular basis for future studies, which will test the hypothesis that CaMKII anchoring at the NMDA receptor helps to control the number of AMPA receptors by specifically disturbing the CaMKII-NMDA receptor interactions.

Ca\(^{2+}\) influx through NMDA receptors plays a crucial role not only in LTP but also in stroke-related neuronal damage. Uncontrolled release of glutamate under anoxic conditions leads to overstimulation of the NMDA receptor and ultimately neuronal damage (39, 40). In rodents, administration of NMDA receptor antagonists prevents neuronal loss to a large extent even when given more than 1 h after the anoxic conditions occurred. Unfortunately, these drugs did not alleviate neuronal damage in clinical trials, perhaps because humans, unlike rodents, may not tolerate a strong block of NMDA receptor activity (39). CaMKII has also been implicated in stroke-induced neuronal damage (41). Disrupting the interaction between CaMKII and the NMDA receptor may specifically inhibit CaMKII signaling downstream of the NMDA receptor-activated Ca\(^{2+}\) influx as occurring during hypoxia and thereby prevent neuronal dysfunction following stroke without affecting other vital signaling pathways involving CaMKII or the NMDA receptor.

In summary, our results reveal several layers of complexity for CaMKII binding to the NMDA receptor. It involves multiple

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\(^3\) M. A. Merrill and J. W. Hell, unpublished data.
interactions that are likely to form simultaneously between the NMDA receptor and CaMKII complexes, thereby stabilizing their association. We also identified multiple regulatory mechanisms of this interaction. Defining the precise interaction sites and how these interactions are regulated provides the basis for future studies that will delineate the role of CaMKII binding to the NMDA receptors under physiological conditions such as synaptic plasticity and pathological conditions such as stroke. Because excessive Ca\textsuperscript{2+} influx through the NMDA receptor as well as subsequent activation of CaMKII have been implicated in stroke-related neuronal damage, unraveling the molecular basis for these interactions will provide the necessary basis for developing drugs that can disrupt these interactions and thereby inhibit the NMDA receptor-CaMKII signaling pathway with high specificity. Such drugs may be able to effectively alleviate neuronal damage following stroke.

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