ABSTRACT The molecular basis of long-term potentiation (LTP), a long-lasting change in synaptic transmission, is of fundamental interest because of its implication in learning. Usually LTP depends on Ca\(^{2+}\) influx through postsynaptic N-methyl-d-aspartate (NMDA)-type glutamate receptors and subsequent activation of Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII). For a molecular understanding of LTP it is crucial to know how CaMKII is localized to its postsynaptic targets because protein kinases often are targeted to their substrates by adapter proteins. Here we show that CaMKII directly binds to the NMDA receptor subunits NR1 and NR2B. Moreover, activation of CaMKII by stimulation of NMDA receptors in forebrain slices increases this association. This interaction places CaMKII not only proximal to a major source of Ca\(^{2+}\) influx but also close to \(\alpha\)-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)-type glutamate receptors, which become phosphorylated upon stimulation of NMDA receptors in these forebrain slices. Identification of the postsynaptic adapter for CaMKII fills a critical gap in the understanding of LTP because CaMKII-mediated phosphorylation of AMPA receptors is an important step during LTP.

Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) mediates a variety of different cellular responses to Ca\(^{2+}\) influx (1, 2). An important source of Ca\(^{2+}\) influx into neurons is the N-methyl-D-aspartate (NMDA)-type glutamate receptor, which is activated by the excitatory neurotransmitter glutamate (2). NMDA- and \(\alpha\)-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)-type glutamate receptors are clustered at postsynaptic sites opposing presynaptic neurotransmitter release sites (3, 4). Brief trains of presynaptic high-frequency stimulation efficiently activate NMDA receptors (5), resulting in postsynaptic Ca\(^{2+}\) influx and long-term potentiation (LTP). LTP is a long-lasting increase in neurotransmission thought to represent the physiological correlate of learning and memory (5, 6). The induction of NMDA receptor-dependent LTP requires activation of CaMKII in the postsynaptic neuron (6, 7). CaMKII is enriched at postsynaptic densities (8, 9), where it is well placed for activation by Ca\(^{2+}\) influx through NMDA receptors and subsequent phosphorylation of neighboring AMPA receptors, an event contributing to LTP (7, 10). At least three CaMKII isoforms, usually called AKAPs, place cyclic AMP-dependent protein kinase (PKA) next to selected substrates such as AMPA receptors (11), and receptors for activated C kinase (RACKs) are important for subcellular localization of different protein kinase C (PKC) isozymes (12). Crucial information about the subcellular targeting of CaMKII is lacking. NMDA receptors would be ideal postsynaptic adapter sites for CaMKII, where it would have full access to Ca\(^{2+}\) influx through these receptors. Cortical NMDA receptors consist of one or two NR1 and two or three NR2A and NR2B subunits whose C termini are intracellular (13–16). We show that CaMKII is directly associated with NR1 and NR2B.

EXPERIMENTAL PROCEDURES

Materials. Tetrodotoxin, microcystin-LR, KN62, KN93, and GF109203X were purchased from Calbiochem, MK-801 and CPP were purchased from Research Biochemicals, and enhanced chemiluminescence detection kits were purchased from Amersham. [\(\gamma\)-32P]ATP (111 TBq/mmol) was obtained from New England Nuclear, protein A-Sepharose was from Sigma, recombinant CaMKII was from A. R. Means (Duke University, Durham, NC), and the AC3-I peptide as well as anti-CaMKII antibodies were from H. Schulman (Stanford University, CA).

Immunoprecipitation and Immunoblotting. Crude rat brain membranes were prepared as described (17) and extracted with deoxycholate (1%) at 0–4°C for solubilization of whole NMDA receptor complexes or with 1% SDS at 50°C, followed by dilution with Triton X-100, to obtain dissociated NMDA receptor subunits, (for details see refs. 17 and 18). Immunoprecipitations and immunoblots were performed with the NR1, NR2A, NR2B, GluR1, and GluR2/3-specific antibodies αNR1, αNR2A, αNR2B, αGluR1, and αGluR2/3, respectively, and with monoclonal mouse antibodies against CaMKIIα and β (anti-CaMKIIα and anti-CaMKIIβ, respectively) as described (17, 18). SDS treatment of either membrane fractions or immunoprecipitated glutamate receptor complexes at 50°C dissociates the receptor complexes and subsequently allows specific immunoprecipitation of individual subunits after neutralization of SDS by adding an excess of Triton X-100 (17, 18). The specificities of the antibodies have been carefully characterized (17–19). Chromatographically purified nonspecific mouse or rabbit IgG antibodies (Zymed) were used for control immunoprecipitations to test for non-specific antibody interactions.

CaMKII Assays. Depoxidate-extracted NMDA receptor complexes, SDS-dissociated NMDA receptor subunits, or Triton X-100-solubilized AMPA receptor complexes were immunoprecipitated and incubated for 30 min at 32°C in 50 μl phosphorylation buffer (50 mM Hepes-NaOH, pH 7.4/10 mM MgCl\(_2\)) containing 1 μM microcystin-LR, 50 μM unlabeled ATP, and 0.2 μM [\(\gamma\)-32P]ATP (111 TBq/mmol; DuPont/NEN) (for more details see refs. 17 and 20). To activate CaMKII, 1 mM calmodulin and 0.5 mM CaCl\(_2\) were included and, in some experiments, exogenous recombinant CaMKIIα.

This paper was submitted directly (Track II) to the Proceedings office. Abbreviations: AMPA, \(\alpha\)-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; CaMKII, Ca\(^{2+}\)/calmodulin-dependent protein kinase II; CPP, \((\pm\)-3-(2-carboxypropionylamine-4-yl))-1-phosphonic acid; GST, glutathione S-transferase; LTP, long-term potentiation; NMDA, N-methyl-D-aspartate; PKA, cyclic AMP-dependent protein kinase; PKC, protein kinase C.

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RESULTS AND DISCUSSION

NMDA receptor complexes were solubilized with deoxycholate, immunoprecipitated from rat forebrain, and incubated with \( [\gamma^{32}P]ATP \). Two prominent phosphorylated polypeptides were detectable under conditions that activate CaMKII (Fig. 1, lane 1). The endogenous kinase activity required \( Ca^{2+} \) and calmodulin (Fig. 1, lanes 4–8) and was blocked by the CaMKII-selective inhibitor KN93 (Fig. 1, lane 10) but not by a PKA-specific inhibitory PKI peptide (26) or the bisindolylmaleimide GF109203X, which inhibits multiple PKC isoforms (Fig. 1, lanes 11–13). The efficacy of the two inhibitors for PKA and PKC was confirmed in parallel experiments (M. A. Davare and J.W.H., unpublished results) using the \( Ca^{2+} \) channel \( \alpha_{1C} \) subunit as a substrate for purified PKA and PKC, respectively (20). These data indicate that CaMKII is part of the NMDA receptor complex.

The specificity of the immunoprecipitation of the CaMKII activity with NMDA receptors was confirmed with control antibodies (Fig. 1, lane 2). The negative results obtained with the control precipitations exclude the possibility that the phosphorylated polypeptides bound nonspecifically to the immunoprecipitating antibodies or the resin. Because CaMKII is a highly abundant neuronal protein, it is possible that the observed interaction with NMDA receptors may be a result of nonspecific binding of CaMKII to this receptor. Therefore, we carefully evaluated whether CaMKII would also associate with the AMPA receptor complex under our conditions. Like NMDA receptors, AMPA receptors are hetero-

![Figure 1](image_url)
Accordingly, CaMKII does not binding to any membrane protein in an indiscriminate fashion.

To evaluate whether the larger $^{32}$P-labeled polypeptide was identical to NR2A or 2B, which migrate with an apparent $M_r$ of about 200 kDa in our SDS/PAGE system (17), NMDA receptors were phosphorylated by the endogenous kinase and dissociated with SDS before NR2A and 2B were separately precipitated (17). $^{32}$P-labeled polypeptides of about 200 kDa were detectable in NR2A and 2B precipitations (Fig. 1, lanes 14–16), demonstrating that both NR2A and NR2B were phosphorylated by the endogenous kinase.

CaMKII is a multimeric complex formed by homologous $\alpha$ and $\beta$ subunits ($M_r = 50$ and 60 kDa, respectively) (1). Both subunits undergo rapid autophosphorylation in the presence of Ca$^{2+}$ and calmodulin, allowing their detection by autoradiography (1). Accordingly, the signal at 55 kDa probably is autophosphorylated CaMKII$\alpha$. A phosphorylated polypeptide of 65 kDa also was observed often, suggesting that the $\beta$ subunit is present in the NMDA receptor complex as well (data not shown). However, the 65-kDa signal was very weak, probably because in the forebrain the $\beta$ subunit is much less prevalent than the $\alpha$ subunit (1).

To further test whether the endogenous kinase is CaMKII, NR2A and NR2B were phosphorylated either by endogenous kinase or after dissociation with SDS by recombinant exogenous CaMKII$\alpha$ and analyzed by two-dimensional phosphopeptide mapping. The pattern of the major phosphopeptides derived from NR2A (A.S.L. and J.W.H., data not shown) or NR2B (Fig. 1B) were very similar upon phosphorylation by endogenous or exogenous kinase. These results provide additional evidence for the hypothesis that the endogenous kinase is CaMKII.

CaMKII complexes were immunoprecipitated from forebrain deoxycholate extracts with anti-CaMKII$\alpha$ and anti-CaMKII$\beta$ antibodies, which were mixed for higher efficiency. Subsequent immunoblotting with antibodies against NR1, 2A, and 2B subunits demonstrated their presence in CaMKII precipitates but not in control precipitates (Fig. 2A, lanes 1–3), corroborating that CaMKII is associated with NMDA receptor complexes.

Deoxycholate-solubilized NMDA receptor complexes contain not only various receptor subunits but also structural proteins of the PSD-95 family (18). To investigate whether CaMKII directly interacts with receptor subunits, NMDA receptor complexes were treated with SDS under conditions that completely separate the NMDA receptor subunits from each other and from members of the PSD-95 family (refs. 17 and 18; A.S.L. and J.W.H., unpublished results). Subsequently, NR1, 2A, and 2B subunits were immunoprecipitated individually. Recombinant CaMKII$\alpha$ was preincubated in $[\gamma-^{32}$P]ATP-containing phosphorylation buffer for radioactive labeling by autophosphorylation before the immunocomplexes were added to this mixture. CaMKII$\alpha$ stably bound to NR1 and NR2B, but not NR2A (Fig. 2A, lanes 2, 4, 5). In similar experiments, CaMKII$\alpha$ did not coprecipitate with AMPA receptor GluR1 subunits (A.S.L. and J.W.H., data not shown). The finding that CaMKII$\alpha$ did not form stable complexes with NR2A or GluR1, which possess high degrees of sequence similarities with NR1 and 2B, demonstrates the specificity of the binding of CaMKII$\alpha$ to NR1 and 2B. Using increasing amounts of CaMKII$\alpha$, we observed that binding of CaMKII$\alpha$ to NR1 as well as to NR2B was saturable (Fig. 3B). NR2A and 2B, but not NR1 (Fig. 3A, lanes 4, 5, and 2, respectively; see also Fig. 1), are phosphorylated under these conditions; however, $^{32}$P-labeled NR2A and 2B were absent in NR1 precipitates, further indicating that NR1 and NR2 subunits were efficiently dissociated during the SDS extraction and that they did not reassociate during the immunoprecipitation. When no recombinant CaMKII was added during these experiments, no phosphorylated polypeptide was detectable, arguing that the endogenous CaMKII had been removed completely during dissociation with SDS (Fig. 3A, lanes 8–10).

The synthetic peptide AC3-I is derived from the autoinhibitory domain spanning residues 278–290 of CaMKII$\alpha$ and inhibits CaMKII by competitively binding to the catalytic
CaMKIIα binding to NR1 and NR2B. (A and B) Crude membrane fractions were extracted and NMDA receptor subunits were dissociated with SDS before immunoprecipitation of individual subunits as indicated on the bottom (A) or top (B). Control immunoprecipitations were performed with nonspecific murine (A, lane 1) or rabbit IgG (A, lanes 7 and 10). Recombinant CaMKIIα was preincubated under phosphorylation conditions in the presence of \( \gamma \)-\( ^{32} \)P-ATP for labeling by autophosphorylation, and immunocomplexes were incubated with the whole phosphorylation mixture. The signal at 55 kDa (A, lanes 2, 3, 5, and 6) reflects binding of recombinant and autophosphorylated CaMKIIα rather than the presence of the endogenous kinase because no phosphorylation was detectable if recombinant CaMKIIα was omitted (A, lanes 8–10). Phosphorylation of NR2B (A, lane 6) but not association of CaMKIIα with NR1 (A, lane 3) or NR2B (A, lane 6) was inhibited if the competitive catalytic site inhibitor AC3-I (20 μM) was added after autophosphorylation of CaMKIIα (27). Similar results were obtained in two other experiments. Binding of CaMKIIα to NR1 and NR2B was saturable (B; the amounts of CaMKIIα associated with NR1 or NR2B were quantified after SDS/PAGE by using PhosphorImager analysis; values are means ± SEM; n = 3). (C) CaMKIIα was preincubated under phosphorylation conditions with \( \gamma \)-\( ^{32} \)P-ATP for labeling by autophosphorylation. Twenty microliters of glutathione Sepharose loaded with approximately 2 μg (as confirmed by staining with Coomassie brilliant blue) of GST or GST fusion proteins (as indicated at the bottom) then was added (together with 20 μM AC3-I when indicated) before samples were washed and analyzed by SDS/PAGE and autoradiography. GST-NR2B839–1482, 839–1346, and 1120–1482, but not 839–1120 or GST alone, were phosphorylated by CaMKII in an AC3-I-sensitive manner (Upper). Autophosphorylated CaMKIIα bound equally well to all four GST-NR2B fusion proteins but not to GST alone or to nonrelevant GST fusion proteins with the Src or Abl SH3 domain or cyclin G2 (Lower).

Fig. 3. CaMKIIα binding to NR1 and NR2B. (A and B) Crude membrane fractions were extracted and NMDA receptor subunits were dissociated with SDS before immunoprecipitation of individual subunits as indicated on the bottom (A) or top (B). Control immunoprecipitations were performed with nonspecific murine (A, lane 1) or rabbit IgG (A, lanes 7 and 10). Recombinant CaMKIIα was preincubated under phosphorylation conditions in the presence of \( \gamma \)-\( ^{32} \)P-ATP for labeling by autophosphorylation, and immunocomplexes were incubated with the whole phosphorylation mixture. The signal at 55 kDa (A, lanes 2, 3, 5, and 6) reflects binding of recombinant and autophosphorylated CaMKIIα rather than the presence of the endogenous kinase because no phosphorylation was detectable if recombinant CaMKIIα was omitted (A, lanes 8–10). Phosphorylation of NR2B (A, lane 6) but not association of CaMKIIα with NR1 (A, lane 3) or NR2B (A, lane 6) was inhibited if the competitive catalytic site inhibitor AC3-I (20 μM) was added after autophosphorylation of CaMKIIα (27). Similar results were obtained in two other experiments. Binding of CaMKIIα to NR1 and NR2B was saturable (B; the amounts of CaMKIIα associated with NR1 or NR2B were quantified after SDS/PAGE by using PhosphorImager analysis; values are means ± SEM; n = 3). (C) CaMKIIα was preincubated under phosphorylation conditions with \( \gamma \)-\( ^{32} \)P-ATP for labeling by autophosphorylation. Twenty microliters of glutathione Sepharose loaded with approximately 2 μg (as confirmed by staining with Coomassie brilliant blue) of GST or GST fusion proteins (as indicated at the bottom) then was added (together with 20 μM AC3-I when indicated) before samples were washed and analyzed by SDS/PAGE and autoradiography. GST-NR2B839–1482, 839–1346, and 1120–1482, but not 839–1120 or GST alone, were phosphorylated by CaMKII in an AC3-I-sensitive manner (Upper). Autophosphorylated CaMKIIα bound equally well to all four GST-NR2B fusion proteins but not to GST alone or to nonrelevant GST fusion proteins with the Src or Abl SH3 domain or cyclin G2 (Lower).
Autophosphorylation of CaMKII makes the kinase Ca$^{2+}$-independent (1) and is important for its binding to residues 1260–1309 (28). To investigate whether binding to 839–1120 can occur independently of autophosphorylation of CaMKII, we incubated GST-NR2B839–1120 with recombinant CaMKII by immunoblotting with anti-CaMKII (A.S.L. and J.W.H., unpublished data). Therefore, association with our binding site does not require autophosphorylation of CaMKII. Accordingly, nonphosphorylated CaMKII can also bind to GST-NR2B839–1482 (A.S.L. and J.W.H., unpublished data). However, because CaMKII has to be autophosphorylated for association with 1260–1309 (28), autophosphorylation increases CaMKII binding to GST-NR2B839–1482 (A.S.L. and J.W.H., unpublished data) and also to a 190-kDa protein in the postsynaptic density, which is most likely NR2B (30).

NMDA receptor-mediated Ca$^{2+}$ influx and induction of LTP stimulates autophosphorylation of CaMKII (10, 31, 32). Therefore, Ca$^{2+}$ influx may help to recruit CaMKII to the postsynaptic site by increasing its association with the NMDA receptor. Incubation of rat brain slices with 200 μM of NMDA in the presence of the Na$^{+}$ channel blocker tetrodotoxin induces NMDA receptor-mediated Ca$^{2+}$ influx and activation of Ca$^{2+}$-dependent events at postsynaptic sites without damaging the physiological integrity of the slices (33). Following this protocol, cortical slices were incubated with and without NMDA and crude membrane fractions were prepared and solubilized with deoxycholate. After immunoprecipitation with a mixture of antibodies against CaMKII and β, NR1 and 2B were detected by immunoblotting. A strong increase in NR1 and 2B immunoreactivity present in the immunoprecipitated CaMKII complexes is obvious upon stimulation with NMDA (Fig. 2 A, lanes 4 and 5, and B). Pretreatment with the NMDA receptor antagonist MK801 or the CaMKII inhibitor KN62 blocked this NMDA-induced effect (Fig. 2, lanes 7 and 8). Thus, NMDA receptor-mediated Ca$^{2+}$ influx and subsequent autophosphorylation of CaMKII, which can be inhibited with KN62, strongly increased the association of CaMKII with the NMDA receptor in intact neurons.

Phosphorylation of the AMPA receptor GluR1 subunit by CaMKII plays a central role in LTP that is dependent on Ca$^{2+}$ influx through NMDA receptors (7, 10). As demonstrated in the previous paragraph, NMDA receptor-mediated Ca$^{2+}$ influx, which causes autophosphorylation and thereby activation of CaMKII, also results in a strong increase of CaMKII recruitment to postsynaptic sites by its association with NMDA receptors. Because NMDA receptors and AMPA receptors are colocalized, this association may coordinate phosphorylation of AMPA receptors by CaMKII. Therefore, we investigated whether NMDA receptor activation in cortical slices causes CaMKII-dependent phosphorylation of AMPA receptors. All cortical slices were treated in the presence of the bisindolylmaleimide GF109203X to block PKC because serine-831 in the main phosphorylation site for CaMKII on the GluR1 subunit, also can be phosphorylated by PKC (34, 35).

After solubilization, AMPA receptor complexes were immunoprecipitated and analyzed by immunoblotting or back-phosphorylation (22, 36) with recombinant CaMKII. Immunoblotting with an antibody against GluR1 and with an antibody that reacts with GluR2 and GluR3 revealed that similar amounts of the respective subunits were present after immunoprecipitation from slices treated either under control conditions or with NMDA (Fig. 4A). After back-phosphorylation, immunoprecipitated AMPA receptor complexes were dissociated with SDS, and GluR1 or a combination of GluR2/3 separately was reprecipitated (18). Incubation with NMDA reduced back-phosphorylation of GluR1 but not GluR2/3 (Fig. 4 B and C). These results show that stimulation with NMDA induces phosphorylation of GluR1 at phosphorylation sites for CaMKII in intact neurons. The effect on back-phosphorylation was inhibited by the NMDA receptor blocker CPP and MK801 and by the CaMKII inhibitor KN93 (Fig. 4C). These findings indicate that NMDA-induced phosphorylation of GluR1 was mediated by NMDA receptor stimulation and activation of CaMKII. CaMKII-mediated phosphorylation of serine-831 in the GluR1 subunit of the AMPA receptor increases its activity (35). Our results indicate that Ca$^{2+}$ influx through the NMDA receptor can potentiate AMPA receptors by stimulating phosphorylation of GluR1 by CaMKII.

In summary, our findings demonstrate that CaMKII directly interacts with NR1 and NR2B. Upon NMDA receptor-mediated Ca$^{2+}$ influx and subsequent autophosphorylation, binding of CaMKII to NMDA receptors and, thereby, recruitment of CaMKII to the postsynaptic site are increased. This interaction places CaMKII at an ideal location for stimulation by Ca$^{2+}$ influx through NMDA receptors. It also brings CaMKII in close proximity to AMPA receptors that are phosphorylated and subsequently up-regulated in their activity by CaMKII upon NMDA receptor-mediated Ca$^{2+}$ influx, especially during LTP (7, 10). Accordingly, NMDA receptors not only are sources for Ca$^{2+}$ but also are part of the postsynaptic adapter sites for CaMKII. The recruitment of CaMKII by the NMDA receptor is likely to be crucial for the induction of CaMKII-dependent LTP. Our experiments identify the first synapse-specific anchoring protein for CaMKII. It is now possible to identify the exact sequences of the NMDA receptor and CaMKII that mediate the association of CaMKII with NMDA receptor subunits. It then will be feasible to test the physiological relevance of the interaction between CaMKII.
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