Neuronal Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) interacts with several prominent dendritic spine proteins, which have been termed CaMKII-associated proteins. The NR2B subunit of N-methyl-D-aspartate (NMDA)-type glutamate receptor, densin-180, and α-actinin bind comparably, approximately stoichiometric amounts of Thr\(^{286}\)-autophosphorylated CaMKII, forming a ternary complex (Robison, A. J., Bass, M. A., Jiao, Y., Macmillan, L. B., Carmody, L. C., Bartlett, R. K., and Colbran, R. J. (2005) J. Biol. Chem. 280, 35329–35336), but their impacts on CaMKII function are poorly understood. Here we show that these interactions are differentially regulated and exert distinct effects on CaMKII activity. Nonphosphorylated and Thr\(^{286}\)-autophosphorylated CaMKII bind to α-actinin with similar efficacy, but autophosphorylation at Thr\(^{286}\) or Ca\(^{2+}\)/calmodulin binding significantly reduce this binding. Moreover, α-actinin antagonizes CaMKII activation by Ca\(^{2+}\)/calmodulin, as assessed by autophosphorylation and phosphorylation of a peptide substrate. CaMKII binding to densin (1247–1542) is partially independent of Thr\(^{286}\) autophosphorylation and is unaffected by Ca\(^{2+}\)/calmodulin binding to CaMKII (7). However, mutagenesis studies indicate that Thr\(^{286}\) autophosphorylation stabilizes the synaptic targeting of green fluorescent protein-CaMKIIa localization in cultured hippocampal neurons suggest that transient translocation of soluble CaMKII to synapses in response to NMDA-type glutamate receptor (NMDAR) activation requires only Ca\(^{2+}\)/calmodulin binding to CaMKII (7). However, mutations studies indicate that Thr\(^{286}\) autophosphorylation stabilizes the synaptic targeting of green fluorescent protein-CaMKIIa, whereas Thr\(^{305}\)/Thr\(^{306}\) autophosphorylation promotes dissociation of synaptic green fluorescent protein-CaMKIIa (8). Consistent with these data, the transgenic mouse knock-in mutation of Thr\(^{305}\) in CaMKIIa to Asp shows reduced CaMKII association with the PSD and changes in long term potentiation and learning and memory (2, 9, 10). Interestingly, a mouse model of Angelman mental retardation syndrome that has deficits in long term potentiation and spatial learning also displays decreased PSD-associated CaMKII and increased autophosphorylation at Thr\(^{305}\)/Thr\(^{306}\) (11). Other studies have shown that PKC activation can also drive synaptic translocation of CaMKII by a mechanism that is dependent on the actin cytoskeleton (12). In combination, these studies suggest that multiple, complex cellular mechanisms control PSD targeting of CaMKII. However, the molecular basis for these dynamic interactions is poorly defined.

CaMKII binds several PSD-enriched CaMKII-associated proteins (CaMKAPs), including F-actin (13), cyclin-dependent protein kinase 5 (14), synGAP/β (15), α-actinin (14, 16), densin-180 (16, 17), and multiple NMDAR subunits (18–21). Our understanding of the regulation of CaMKII interactions with these PSD-associated CaMKAPs is incomplete. For example, some laboratories detect Ca\(^{2+}\)/calmodulin-depend
ent interactions of CaMKII with the NMDAR NR2B subunit that are enhanced by Thr286 autophosphorylation (21, 22), but others detect binding of only [P-T286]CaMKII to NR2B (18). Despite this discrepancy, it is clear that CaMKII activation is important for interactions with NR2B in intact cells (18, 21, 23, 24). In this respect, NR2B remains a strong candidate for mediating the activity-dependent synaptic translocation of CaMKII in neurons. However, although autophosphorylation at Thr305/306 promotes CaMKII dissociation from PSDs, it was reported to have only a modest effect on CaMKII binding to NR2B (22). In contrast, inactive CaMKIIe binds densin-180 in the absence of Ca2+/calmodulin; autophosphorylation at Thr286 potentiates this interaction (17), but the effects of Thr305/306 autophosphorylation are unknown. In addition, a minimal binding domain for [P-T286]CaMKII was recently identified in α-actinin, but the regulation of this interaction has not been explored (25). Thus, current knowledge of the dynamics of any single protein–protein interaction cannot account for PSD targeting in intact cells.

An additional effect of CaMKAPs may be to modulate CaMKII activity in specific subcellular compartments. For example, NR2B can trap an autonomous form of CaMKII in the absence of Thr286 autophosphorylation in vitro (21). Recent studies in Drosophila have shown that Camguk is a CaMKAP that can modulate CaMKII activity and promote Thr305/306 autophosphorylation of CaMKII (26). However, the effects of other CaMKAPs on CaMKII activity are unknown.

Here, we compare the effects of autophosphorylation and of Ca2+/calmodulin binding on CaMKII interactions with NR2B, densin-180, and α-actinin, clarifying the mechanisms of CaMKII binding to NR2B and α-actinin. In addition, the effects of these CaMKAPs on CaMKII activity are described. The diversity of the interaction mechanisms and their distinct effects on CaMKII activity together suggest that CaMKAPs provide subtle and dynamic modulation of CaMKII activity in discrete subcellular compartments.

**MATERIALS AND METHODS**

**Proteins**—Purified glutathione S-transferase (GST) fusion proteins containing CaMKAP fragments were generated as described previously (25); GST-NR2B contains amino acids 1260–1339 of NR2B, with Ser1303 mutated to alanine (Fig. 1, 2, 4, and 6); GST-densin contains amino acids 1247–1542 of the D splice variant of densin-180, unless otherwise noted (Figs. 1 and 2); GST-actinin contains amino acids 819–894 of α-actinin-2 (Figs. 1, 2, 3A, and 4C). His6-tagged CaMKAP fragments were expressed from pRSET-A (Qiagen) and purified from isopropyl 1-thio-β-D-galactopyranoside-induced BL-21 (DE3) Escherichia coli using His-Select nickel affinity gel (Sigma) according to the manufacturer’s protocol. His6-NR2B contains amino acids 965–1482 (the entire cytosolic C terminus of the NR2B subunit), and His6-actinin contains amino acids 547–894 of α-actinin-2 (Fig. 3B and 4A). Murine CaMKIIe (wild-type and a double mutant of Thr286 and Thr306 autophosphorylation sites to Ala (T305A/T306A)) was purified from baculovirus-infected S9 cells, as described (27–29). Bovine brain calmodulin and recombinant chicken calmodulin expressed in E. coli were purified as described previously (30, 31). All purified proteins were quantified using Bradford (Bio-Rad) or bicinechonic acid (BCA, Pierce) assays.

**CaMKIIα Autophosphorylation**—CaMKIIα was autophosphorylated essentially as described (29). Briefly, selective Thr286 autophosphorylation was achieved by incubating CaMKIIα (5 μM subunit) on ice for 90 s with 50 mM HEPES, pH 7.5, 1 mg/ml bovine serum albumin, 1 mM dithiothreitol, 0.5 mM CaCl2, and 2.5 μM calmodulin. Cationic effects lacked GST fusion proteins. The reaction was initiated by the addition of [γ-32P]ATP (2500 cpm/pmol) and magnesium acetate to own μM and 10 μM, respectively, and incubations were performed at 4 °C. Aliquots (10

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Phosphorylation (as above, except using 500 μM ATP) was terminated using EDTA (4 μM final), and the Ca2+-independent reaction was continued for 2 min at 30 °C. For basal autophosphorylation at Thr305/306 and other sites, CaMKII (5 μM subunit) was incubated for 60 min at 30 °C with 50 mM HEPES, pH 7.5, 10 mM magnesium acetate, 1 mM EGTA, and 500 μM ATP, and the reaction was stopped with EDTA (12.5 mM, final).

**GST Cosegregation Assays**—Purified GST fusion proteins or GST alone (~250 nM full-length protein) were incubated with CaMKIIα (~250 nM in the indicated autophosphorylation state) and glutathionagarose beads (Sigma; 25 μl of packed resin) in pull-down buffer (50 mM Tris-HCl pH 7.5, 200 mM NaCl, 0.5% Triton X-100) to a final volume of 500 μl for 2 h at 4 °C. Free Ca2+ concentrations were calculated using an internet-based tool (32) or were set using a Ca2+/EGTA buffering system (33). Where indicated, various nucleotides (100 μM) were added to pull-down buffer during binding only. Beads were sedimented on the gel and washed in 500 μl of pull-down buffer 6 times for 5 min each. After transfer of beads to new microcentrifuge tubes, proteins were eluted with SDS-PAGE sample buffer, resolved by SDS-PAGE, and transferred to nitrocellulose membranes. Proteins on the membrane were visualized by staining with Ponceau S and quantified from digitally scanned images using ImageJ (rsb.info.nih.gov/ij). Pilot studies established that pixel densities of individual protein bands are linearly related to the amount of protein loaded on the gel in the range of 0.04–2.5 μg/lane for both GST and CaMKIIα (0.7–4 pmol). Amounts of CaMKIIα sedimented on the beads were normalized to the recovered GST fusion protein, and background binding to GST alone was subtracted. To confirm the identity of the Ponceau-stained proteins, membranes were immunoblotted for CaMKII using affinity-purified polyclonal goat anti-CaMKII primary antibodies (29) and alkaline-phosphatase-conjugated secondary antibodies (Jackson ImmunoResearch).

**Kinase Assays**—Kinase assays were performed essentially as previously reported (28). Briefly, nonphosphorylated CaMKIIα or [P-T286]CaMKII were preincubated for 15–30 min on ice with the indicated concentrations of CaMKAPs (as GST- or His6-tagged proteins) in the presence of 1 mM calcium (Ca2+-dependent) or 1 mM EGTA (Ca2+-independent), respectively. Phosphorylation of the model peptide substrate, syntide-2 (20 μM), was then initiated by the addition of [γ-32P]ATP (0.2–4 μM final).

**Kinetic Analyses of Inhibition by NR2B**—Ca2+/calmodulin-dependent activity of non-phosphorylated CaMKIIα was assayed in the presence of the indicated concentrations of NR2B (1290–1309: S1303A) peptide (AQQKNRKNKLRRQHYDFTFDV, Macromolecular Resources, Fort Collins, Colorado). Two model peptide substrates were used: syntide-2 is based on a phosphorylation site in glycogen synthase, whereas autocamtide-2 is based on the sequence surrounding the Thr286 autophosphorylation site in CaMKII. Assays were performed using either fixed [γ-32P]ATP concentrations (0.4 mM) and variable peptide substrate concentrations or fixed peptide substrate concentrations (0.2 mM syntide-2 or 0.1 mM autocamtide-2) and variable [γ-32P]ATP concentrations. Raw data were fit to Michaelis-Menten kinetics and inhibitory mechanisms were investigated using double-reciprocal (Lineweaver-Burk) plots (Prism 4.0, GraphPad).

**Initial rates of CaMKII Autophosphorylation**—CaMKIIα (1.25 μM) was preincubated with GST, GST-NR2B, or GST-actinin (2.5 μM each) in 50 mM HEPES, pH 7.5, 1 mg/ml bovine serum albumin, 1 mM dithiothreitol, 0.5 mM CaCl2, and 2.5 mM calmodulin. Control reactions lacked GST fusion proteins. The reaction was initiated by the addition of [γ-32P]ATP (2500 cpm/pmol) and magnesium acetate to 20 μM and 10 μM, respectively, and incubations were performed at 4 °C. Aliquots (10

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µl of each reaction were spotted on P81 phosphocellulose papers after 10, 20, 30, and 40 s to determine the initial reaction rates (mol/mol/s), which were calculated from the slope of a line fitted to the data. Additional 10-µl aliquots were quenched by the addition of SDS after 50 s and analyzed by autoradiography of SDS-polyacrylamide gels.

RESULTS

Effects of Autophosphorylation on Interactions—Previous studies have shown that CaMKII associates with NR2B, densin-180, and α-actinin in brain (see the Introduction). GST-NR2B, GST-densin, and GST-actinin contain the CaMKII binding domains from the parent proteins and bind similar, approximately stoichiometric amounts of [P-T²⁸⁶]CaMKII in glutathione-agarose cosedimentation assays (Fig. 1) (25). However, non-phosphorylated CaMKII bound equally well to GST-actinin and at a somewhat reduced level to GST-densin (33% of that detected using [P-T²⁸⁶]CaMKII) but failed to bind significantly to GST-NR2B under these conditions (but see Fig. 6) (Fig. 1, A and B, compare white and black bars in B). Thus, Thr²⁸⁶ autophosphorylation alone was required for CaMKII binding to NR2B and potentiated CaMKII binding to densin by about 3-fold but had no effect on CaMKII binding to actinin.

Ca²⁺-independent autophosphorylation also selectively regulated these interactions. Basal Ca²⁺-independent autophosphorylation alone failed to support CaMKII binding to GST-NR2B and had no significant effect on binding to GST-densin (Fig. 1A and B, compare light gray and white bars in B). Moreover, Ca²⁺-independent autophosphorylation after initial Thr²⁸⁶ autophosphorylation had no significant effect on CaMKII binding to GST-NR2B or GST-densin (Fig. 1, A and B, compare dark gray and black bars in B). However, CaMKII binding to GST-actinin was substantially reduced (70–90%) by Ca²⁺-independent autophosphorylation regardless of whether Thr²⁸⁶ was initially autophosphorylated (Fig. 1, A and B). Thr³⁰⁵ and Thr³⁰⁶ have been identified as major sites of Ca²⁺-independent autophosphorylation, although additional sites have been identified (e.g. Ser³¹³) (22, 25). Mutation of both Thr³⁰⁵ and Thr³⁰⁶ to Ala abrogated the effect of Ca²⁺-independent autophosphorylation on CaMKII binding to GST-actinin. However, the double-mutated kinase bound normally to GST-NR2B (i.e. in a Thr²⁸⁶ autophosphorylation-dependent manner) (Fig. 1C). Thus, Ca²⁺-independent autophosphorylation at Thr³⁰⁵ and/or Thr³⁰⁶ abrogates CaMKII binding to α-actinin but has no effect on CaMKII binding to GST-NR2B or GST-densin.

Regulation of Interactions by Ca²⁺/Calmodulin—Because Thr³⁰⁵/³⁰⁶ autophosphorylation prevents binding of CaMKII to both Ca²⁺/calmodulin (28) and α-actinin (see Fig. 1), we compared the effects of Ca²⁺/calmodulin on binding of non-phosphorylated CaMKII and [P-T²⁸⁶]CaMKII to these CaMKAPs. These assays were performed in the presence of either CaCl₂ (5 mM) or chelator (1.2 mM EDTA) because excess calmodulin (2.5 µM) and chelators (1.5 mM EDTA) were always present as a carryover from the kinase preincubation. The addition of Ca²⁺ had no significant effect on the binding of non-phosphorylated CaMKII to α-actinin, NR2B, or densin-180 (Fig. 2, A and B, compare the white and dark gray bars in B). However, Ca²⁺ reduced binding of [P-T²⁸⁶]CaMKII to α-actinin by >90% without affecting binding to NR2B or densin-180 (Fig. 2, A and B, compare the black and light gray bars in B). The effect of Ca²⁺ on binding of [P-T²⁸⁶]CaMKII to α-actinin was dose-dependent, with an estimated EC₅₀ of 11 µM free Ca²⁺ (Fig. 3A). The affinity of CaMKII for calmodulin is increased ~1000-fold by Thr²⁸⁶ autophosphorylation (34), presumably sufficient to allow Ca²⁺/calmodulin to displace α-actinin from [P-T²⁸⁶]CaMKII but not from nonphosphorylated CaMKII under these conditions.

To determine whether calmodulin and α-actinin also compete for nonphosphorylated CaMKII, we examined the effect of α-actinin on calmodulin-dependent CaMKII activation. His₅₅-α-actinin (1 µM) increased the apparent kₐ for calmodulin from ~150 nM to ~1.5 µM (Fig. 3B, inset); double-reciprocal (Lineweaver-Burk) analysis of these data yielded straight lines intersecting on the y axis (Fig. 3B), indicative of competitive inhibition of CaMKII by α-actinin with respect to Ca²⁺/calmodulin.
CaM. In addition, \( /H9251/-\)actinin inhibited \( /H11001/-\)calmodulin-dependent substrate (autocamtide-2) phosphorylation (Fig. 4A, E) \( /H11015\) and autophosphorylation (Fig. 4C) but had no effect on the \( /H11001/-\)independent activity of \( /H9262/-\)CaMKII (Fig. 4A). In combination, these data show that \( /H9251/-\)actinin and calmodulin compete for binding to both \( /H9262/-\)CaMKII and non-phosphorylated CaMKII.

**Effects of CaMKAPs on CaMKII Activity**—As controls for inhibition experiments described above, GST and GST-densin (1402–1452) were shown to have no effect on CaMKII activity (not shown). In contrast to a previous report that NR2B can trap an autonomously active form of CaMKII in the absence of Thr286 autophosphorylation (21), GST-NR2B potently inhibited both \( /H11001/-\)calmodulin-dependent exogenous peptide substrate phosphorylation and \( /H11001/-\)calmodulin-dependent autophosphorylation under our conditions (Fig. 4, B and C). In addition, GST-NR2B inhibited the \( /H11001/-\)independent activity of \( /H9262/-\)CaMKII with similar potency (EC50s 10–20 nM) (Fig. 4B). In contrast, \( /H9251/-\)actinin inhibited only \( /H11001/-\)calmodulin-dependent activity of non-phosphorylated CaMKII (see Fig. 4A). Moreover, CaMKII activity was inhibited with similar potency by a synthetic peptide corresponding to the minimal CaMKII binding domain in NR2B (residues 1290–1309) as well as...
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by the entire C-terminal tail of NR2B (His8−NR2B (965−1482)) (data not shown).

Kinetics of CaMKII Inhibition by NR2B—To gain a better understanding of the interaction between NR2B and CaMKII, we determined the mechanism for inhibition of Ca2+/calmodulin-dependent CaMKII activity by NR2B. Inhibition kinetics were determined with respect to two model peptide substrates with different modes of interaction at the catalytic site (syntide-2 and autocamtide-2) (35) as well as with respect to ATP. At fixed (saturating) ATP concentrations, inhibition by NR2B was competitive with respect to syntide-2 (Fig. 5A, lines intersect on the x axis) and competitive with respect to autocamtide-2 (Fig. 5C, lines intersect on the y axis). Surprisingly, at fixed saturating concentrations of either peptide substrate the inhibition was uncompetitive with respect to variable ATP concentrations (Fig. 5, B and D, lines essentially parallel).

Effect of Nucleotides on NR2B-CaMKII Interaction—Uncompetitive inhibition of CaMKII with respect to ATP indicates that nucleotide binding is required for the interaction of non-phosphorylated kinase with NR2B. Therefore, we examined the effect of an adenine nucleotide on CaMKII binding to GST-NR2B under a variety of conditions. ADP (100 μM) was used to avoid potential confounding effects of ongoing phosphorylation during incubation. Binding of GST-NR2B to [P-T286]CaMKII was unaffected by the addition of ADP (although it should be noted that 4 μM adenine nucleotide was carried into the [P-T286]CaMKII incubations from the autophosphorylation reaction). However, the addition of ADP revealed a Ca2+-dependent interaction of non-phosphorylated CaMKII with NR2B (Fig. 6) that was not seen in the absence of ADP (cf. Fig. 2). This Ca2+/calmodulin-dependent interaction was also supported by AMP and by a non-hydrolyzable analog of ATP (AMP-PNP) (data not shown). Thus, the combined effect of binding both nucleotide and Ca2+/calmodulin induces conformational changes in nonphosphorylated CaMKII that facilitate interaction with NR2B.

FIGURE 6. Nucleotides stabilize Ca2+-dependent binding of CaMKII to NR2B. GST-NR2B was incubated with [P-T286]CaMKII (left) or nonphosphorylated CaMKII (right) in the presence of varying free Ca2+ concentrations (∼10 μM L−1, 4 μM H−1) with or without 100 μM ADP, as indicated. Complexes were analyzed as in Fig. 1. Data are representative of three separate experiments.

FIGURE 5. Kinetics of CaMKII Inhibition by NR2B. A and B, Ca2+/CaM-dependent CaMKII activity was assayed in the presence of 0 (●), 100 (▲), or 200 (▼) nM NR2B peptide using varying syntide-2 concentrations and constant ATP (▲) or varying ATP concentrations and constant syntide-2 (●). The main graphs show Lineweaver-Burk (double-reciprocal) plots of the data, with the inset showing raw data (v = μmol/min/mg in the insets). C and D, Ca2+/CaM-dependent CaMKII activity was assayed in the presence of 0 (●), 1 (▲), or 2 (▼) μM NR2B peptide as in panels A and B, except using autocamtide-2 (AC-2) as a substrate. All data are representative of three–six separate experiments.

DISCUSSION

PSD targeting of CaMKII appears to involve differentially regulated CaMKII interactions with multiple PSD-enriched proteins (see the Introduction). In addition to targeting CaMKII to specific subcellular locations and substrates, CaMKAPs may directly modulate CaMKII activity. For example, Camguk localizes a pool of Drosophila CaMKII that can be readily activated and Thr286-autophosphorylated but in the absence of Ca2+. Camguk promotes Thr505/506 autophosphorylation, creating an inactive pool of kinase (36). However, similar regulation of mammalian CaMKII has not been reported. The present work suggests that NR2B, densin-180, and α-actinin may make different contributions to the dynamic subcellular targeting of CaMKII and differentially modulate CaMKII activity.

CaMKII Interaction with NR2B—Much attention has focused on NR2B because of the potential involvement in activity-dependent synaptic translocation of CaMKII (see the Introduction). There are two distinct CaMKII binding sites within the C-terminal intracellular region of NR2B. Thr286 autophosphorylation is required for binding to a poorly characterized membrane-proximal CaMKII binding site (within residues 839−1120) (19). Autophosphorylation at Thr286 is also necessary for interaction with a high affinity binding site in NR2B corresponding to residues 1290−1309 (Fig. 2), as initially reported (18). We sometimes detect a weak Ca2+/calmodulin-stimulated CaMKII interaction with GST-NR2B (barely detected in Fig. 2A), but on average this is not sig-
significantly greater than nonspecific interactions with GST (Fig. 2B). However, we show here that adenine nucleotides stabilize a strong Ca\(^{2+}\)/calmodulin-dependent interaction between CaMKII and NR2B (Fig. 6). Others have previously reported that Ca\(^{2+}\)/calmodulin binding to CaMKII is sufficient to promote interactions with residues 1290–1309 of NR2B, but it is unclear whether these incubations contained a source of nucleotides (19, 21, 22).

Preincubation of CaMKII and NR2B with Ca\(^{2+}\)/calmodulin traps an autonomously active form of CaMKII in subsequent kinase assays without autophosphorylation at Thr\(^{305/306}\) (21). Although we confirmed this observation (data not shown), we found that NR2B potently inhibits Ca\(^{2+}\)/calmodulin-stimulated and autonomous CaMKII activity when added directly to kinase assays (Fig. 4). Importantly, inhibition was not a function of the specific GST fusion protein constructs used initially, because similar inhibition was detected using a synthetic peptide analog of amino acid residues 1290–1309 and using a His-tagged protein corresponding to the entire cytosolic C terminus of NR2B (not shown). The apparent inhibitory mechanism depends on the peptide substrate used; inhibition is competitive with autocamtide-2 but non-competitive with syntide-2 (Fig. 5). This difference in NR2B inhibitory mechanisms is likely explained by a previous report that these peptide substrates can bind in different ways at the active site (35). Autocamtide-2 is based on the sequence surrounding Thr\(^{286}\) in the CaMKII autoinhibitory domain, which is similar to the sequence surrounding Ser\(^{1303}\) in NR2B, whereas syntide-2 is based on sequences surrounding a phosphorylation site in glycogen synthase (37).

The mechanisms of CaMKII inhibition by NR2B with respect to peptide substrates are reminiscent of CaMKII inhibition by its autoinhibitory domain peptides (35, 38, 39), perhaps not surprising considering the sequence similarity between NR2B and the autoinhibitory domain. These prior studies suggested a model in which the N-terminal portion of the autoinhibitory domain (surrounding Thr\(^{286}\)) occupies a binding pocket on the C-terminal lobe of the catalytic domain termed the T-site, whereas the C-terminal portion acts as a pseudosubstrate, occluding the substrate binding (S) site (39). Interaction of autoinhibitory domain peptides at the T site appears to induce long range conformational changes in the ATP binding site of CaMKII, resulting in apparent competitive inhibition of CaMKII with respect to ATP. Thus, occupation of the T site and the nucleotide binding site are mutually exclusive (35, 38, 39). However, inhibition by NR2B (1290–1309) is uncompetitive with respect to ATP irrespective of the peptide substrate used (Fig. 5, B and D), suggesting that NR2B inhibition requires prior nucleotide binding to CaMKII. This is further demonstrated by the fact that nucleotides support a Ca\(^{2+}\)/calmodulin-dependent interaction with NR2B (Fig. 6). Thus, it is apparent that NR2B and the autoinhibitory domain are differentially coupled to nucleotide binding even though the nucleotide binding site is on the N-terminal lobe of the catalytic domain, and the T site is on the C-terminal lobe. More studies will be required to elucidate mechanisms for this differential communication.

Differences in the interaction of NR2B and the autoinhibitory domain of CaMKII catalytic domains can also be inferred from the fact that CaMKII efficiently phosphorylates Ser\(^{1303}\) in NR2B within the complex (40), whereas Thr\(^{286}\) in peptide analogs of the autoinhibitory domain is a poor CaMKII substrate unless Ca\(^{2+}\)/calmodulin is bound to the peptide, abrogating its inhibitory interaction at the T site (38). Moreover, Thr\(^{286}\) in the autoinhibitory domain is a poor substrate for its cognate catalytic domain even after binding of Ca\(^{2+}\)/calmodulin and is phosphorylated by the catalytic domain of an adjacent subunit in the activated holoenzyme (41–43). Differences in the interaction mechanisms are further supported by the fact that mutation of Ile\(^{305}\) in the catalytic domain to Lys severely compromises binding to NR2B (21) but only marginally disrupts the autoinhibitory interaction (39). In combination, these data strongly suggest that NR2B does not directly mimic the autoinhibitory domain in binding to the T site in the catalytic domain.

CaMKII is routinely exposed to millimolar adenine nucleotide concentrations in intact cells. Removal of the autoinhibitory domain from the T site in response to Ca\(^{2+}\)/calmodulin binding facilitates nucleotide binding, which may be sufficient to induce CaMKII binding to NR2B in the absence of Thr\(^{286}\) autophosphorylation. This may explain activity-dependent synaptic translocation of T286A-mutated CaMKIIa in intact cells (7). However, ATP is the predominant nucleotide under physiological conditions. Thus, it seems likely that wild type CaMKII will autophosphorylate at Thr\(^{286}\) before it can diffuse to and bind NR2B. Autophosphorylation at Thr\(^{286}\) may stabilize the interaction. Our data suggest that CaMKII subunits interacting with NR2B will be inhibited, but it is possible that NR2B can trap autonomous kinase activity once Thr\(^{286}\) gets dephosphorylated. Whichever effect dominates in situ, steric constraints suggest that only a fraction of the subunits in a CaMKII holoenzyme are capable of simultaneously interacting with NR2B(s) anchored in the membrane. Thus, interaction of a few CaMKII subunits from the holoenzyme with NR2B may localize additional CaMKII subunits to the NMDAR and PSDs that do not directly interact with NR2B. Subunits that are not bound to NR2B presumably remain active to phosphorylate nearby proteins, although it is possible that intersubunit cooperativity allows NR2B to affect the activity of the entire holoenzyme. Consistent with this model, interaction of CaMKII with the high affinity binding site in NR2B was recently shown to enhance phosphorylation of a model substrate engineered onto the same membrane-targeted polypeptide (24). However, dexamethasone treatment of rats decreased the amount of PSD-associated NR2B and increased the amount of PSD-associated CaMKII activity (44). Moreover, although levels of Thr\(^{286}\) autophosphorylation are elevated for 60 min after long term potentiation induction, Ca\(^{2+}\)/calmodulin-independent CaMKII activity is elevated for only 5 min (45). These data are consistent with the idea that NR2B can also inhibit CaMKII activity in vivo.

Despite evidence supporting an important role for CaMKII interaction with NR2B in cells, this interaction cannot fully account for CaMKII targeting. For example, autophosphorylation at Thr\(^{205/306}\) promotes CaMKII dissociation from PSDs (8, 9) but does not significantly affect binding to NR2B (Fig. 1).

**CaMKII Interaction with Densin-180**—Densin-180 is a PSD-enriched, transmembrane glycoprotein (46) that was recently shown to play a key role in regulating neuronal morphology and branching (47). Initial studies showed that CaMKII interacts with a C-terminal domain in densin-180 that is distinct from CaMKII binding domains in other CaMKAPs and that fragments of densin-180 constitutively target CaMKII in transfected HEK293 cells (17). Here we confirm that CaMKII binding to densin-180 is partially autonomous, although the amount of activation-independent binding appears to depend on the assay conditions (Fig. 1) (16, 17). We have shown for the first time that CaMKII binding to densin is unaffected by Ca\(^{2+}\)/calmodulin binding or by Ca\(^{2+}\)-independent autophosphorylation at Thr\(^{205/306}\) and other sites (Figs. 1 and 2) and does not modulate CaMKII activity (not shown). These observations are not surprising since densin-180 interacts with the C-terminal association domain of CaMKII (16, 25). Thus, densin-180 may function to anchor both inactive and active CaMKII holoenzymes in intact cells.

**CaMKII Interaction with α-Actinin**—CaMKII interacts with α-actinin, a ubiquitous actin-bundling protein (16). A C-terminal 75- amino
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acid domain of α-actinin is necessary and sufficient for CaMKII binding (25). Here we show that α-actinin binding in complex with CaMKII with similar efficacy but that CaMKII transients allow calmodulin (purple) to bind nonphosphorylated CaMKII (CaMKII/CaM, black circles) facilitates binding to NR2B and inhibition of individual subunits. After CaMKII levels decrease and CaMKII/CaM dissociates (P-T305, NR2B may remain bound to and inhibit α-actinin. Additional autophosphorylation at T305/306, α-actinin, may prevent actinin binding.

FIGURE 7. Working model of CaMKII complexes with CaMKAPs. Under basal conditions (Non-P), nonphosphorylated CaMKII catalytic domains are inactive (red), and the holoenzyme (shown as a hexamer for clarity) may bind to densin-180 and α-actinin. In the absence of CaMKII, the kinase may undergo basal autophosphorylation (P-T305/306, white circles), preventing interaction with α-actinin. CaMKII autophosphorylation strongly abrogated the NR1 and NR2B subunits under basal conditions (48), possibly localizing this interaction may target an inactive pool of nonphosphorylated regulatory domain of CaMKII (25). Here we show that α-actinin binding may interact near the calmodulin binding domain. Indeed, we have shown that α-actinin binds to the C-terminal-truncated monomeric catalytic/ regulatory domain of CaMKII (25). However, synthetic peptide analogs of the calmodulin binding domain cannot effectively compete with α-actinin for binding to CaMKII (data not shown). Further studies will be required to precisely identify the α-actinin binding site in CaMKII.

Implications for CaMKII Signaling Complexes—The present studies suggest provocative models for tight control of localized CaMKII activity (Fig. 7). Constitutive interaction of the C-terminal association domain with densin-180 may serve to prevent CaMKII holoenzymes from escaping the PSD, leaving catalytic and regulatory domains available for dynamically modulated interactions with α-actinin or NR2B. Antagonism of CaMKII/calmodulin-dependent CaMKII activation by α-actinin may serve to prevent inappropriate kinase activation at low levels of CaMKII influx. Binding to NR2B could inhibit some subunits in the kinase holoenzyme, whereas others remain active or bound to other CaMKAPs. Indeed, we recently showed that [P-T305]CaMKIIα can simultaneously interact with densin, α-actinin, and NR2B to form a putative PSD-anchored signalosome (25). Additional interactions with NMDAR NR1 or NR2A subunits or other CaMKAPs (see the Introduction) may provide more nuances to the assembly and function of these complexes. Moreover, some CaMKAPs interact with each other independently of CaMKII (16, 48) creating additional possibilities that are not illustrated in Fig. 7. For example, α-actinin associates with NMDAR NR1 and NR2B subunits under basal conditions (48), possibly localizing inactive CaMKII to functional NMDARs. Local NMDAR-mediated CaMKII influx might promote sequential CaMKII dissociation from α-actinin, activation, and binding to NR2B. Such events may play a role in the NR2 subunit-selective modulation of NMDAR desensitization (49). Additional experiments must be designed to test various aspects of these models.

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