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Communication

Translocation of Autophosphorylated Calcium/Calmodulin-dependent Protein Kinase II to the Postsynaptic Density*

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Stefan Strack, Sukwoo Choi, David M. Lovinger, and Roger J. Colbran‡

From the Department of Molecular Physiology and Biophysics, Vanderbilt University Medical Center, Nashville, Tennessee 37232-0615

Calcium/calmodulin-dependent protein kinase II (CaMKII) undergoes calcium-dependent autophosphorylation, generating a calcium-independent form that may serve as a molecular substrate for memory. Here we show that calcium-independent CaMKII specifically binds to isolated postsynaptic densities (PSDs), leading to enhanced phosphorylation of many PSD proteins including the α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA)-type glutamate receptor. Furthermore, binding to PSDs changes CaMKII from a substrate for protein phosphatase 2A to a protein phosphatase 1 substrate. Translocation of CaMKII to PSDs occurs in hippocampal slices following treatments that induce CaMKII autophosphorylation and a form of long term potentiation. Thus, synaptic activation leads to accumulation of autophosphorylated, activated CaMKII in the PSD. This increases substrate phosphorylation and affects regulation of the kinase by protein phosphatases, which may contribute to enhancement of synaptic strength.

CaMKII[1] isoforms comprise a family of broad specificity, calcium-activated kinases (1, 2). The α and β isoforms are abundantly expressed in the brain, with α making up as much as 2% of total protein in certain brain regions (3). CaMKII is particularly enriched in PSDs (4, 5), cytoskeletal specializations apposed to the postsynaptic membrane of excitatory synapses that are thought to be scaffolds for neurotransmitter receptors, ion channels, and their postsynaptic modulators and effectors (reviewed in Refs. 6 and 7). Earlier reports suggested that CaMKIIα constitutes as much as 50% of total PSD protein (8–10), but PSDs prepared from rapidly homogenized brains are only 2–3-fold enriched in CaMKIIα compared with whole forebrain extracts (3, 11). CaMKIIα knockout mice show impaired hippocampal long term potentiation, a cellular model for learning and memory (12). Conversely, introduction of CaMKIIα into neurons augments postsynaptic responses and occludes further electrically induced long term potentiation (13, 14).

CaMKIIα undergoes calcium/calmodulin-dependent autophosphorylation on Thr^{286} in its regulatory domain, rendering the kinase partially calcium-independent (1, 2). This reaction has been proposed as a "molecular switch," translating transient calcium elevation into prolonged kinase activity (15, 16), which becomes subject to regulation by protein phosphatases. In addition, Thr^{286} autophosphorylation promotes binding of CaMKIIα to a 190-kDa PSD protein by gel overlay (17). The present results extend these findings, demonstrating that Thr^{286} autophosphorylation controls subcellular targeting of CaMKII in neurons with important functional consequences.

EXPERIMENTAL PROCEDURES

Purification and Labeling of Recombinant CaMKIIα—CaMKIIα was expressed in Sf9 cells and purified (17). [^{35}S]CaMKIIα (≈1200 cpm/pmol) was purified from cells metabolically labeled with 35 μCi/ml [^{35}S]methionine for 58 h prior to harvesting. Kinase was autophosphorylated in the presence (Thr^{286}) or the absence (Thr^{305}/Thr^{306}) of calcium/calmodulin (0.2–0.6 and 0.6–1.2 mol ^{32}P/mol kinase subunit, respectively) (17). Appropriate autophosphorylation of [^{35}S]CaMKIIα with unlabeled ATP was confirmed by assaying calcium-dependent and -independent kinase activities (18) using specific CaMKII substrate autocamtide-2 (10 μM) (19).

Isolation of PSDs—PSDs were prepared from adult rat forebrains flash frozen within 45 s of euthanasia by detergent lysis of synaptosomes (20) except that 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 10 μg/ml leupeptin were included in all buffers. Synaptosomes were lysed in 1% (v/v) Triton X-100 and 150 mM KCl, and a second subsequent sucrose gradient was omitted because it yielded no further purification. PSDs displayed typical "donut" morphology by video-enhanced differential-interference contrast microscopy (21). PSDs prepared in the absence or the presence of the protein phosphatase inhibitor microcystin-LR (1 μM) contained similar amounts of CaMKII protein (5–10% by quantitative immunoblotting (17)) and calcium/calmodulin-dependent CaMKII activity (100–150 nmol/min/mg with 10 μM autocamtide-2). Calcium-independent kinase activity was <2% and ≈10% of calcium/calmodulin-dependent kinase activity when PSDs were isolated in the absence or the presence of microcystin-LR, respectively. Therefore, CaMKII (Thr^{286}) was almost completely dephosphorylated during normal PSD isolation (minus microcystin-LR).

In Vitro Binding of CaMKIIα—PSDs and ^{35}S- or ^{32}P-labeled CaMKIIα were incubated at the indicated concentrations, temperatures, and times in Buffer A (20 mM HEPES, pH 7.5, 10 mM dithiothreitol, 0.1% (v/v) Triton X-100, 1 mM benzamidine, 20 μg/ml leupeptin) containing 1 mg/ml bovine serum albumin, 0.1 M NaCl, 1 μM microcystin-LR, and EDTA and EGTA at 2.5-fold molar excess over Mg^{2+} and Ca^{2+}, respectively, carried over from the autophosphorylation. Binding was terminated by centrifugation of 90 μl through 0.5 ml of HEPES-buffered 0.5 M sucrose cushion into 20 μl of 10% glutaraldehyde in 0.6 M sucrose in a horizontal rotor (5000 × *g*, 45–60 s). The sucrose cushions were aspirated (excess ligand), and the tube bottoms (PSD pellets) were cut off and counted.

Protein Phosphatase Assays—Soluble [T286-^{32}P]CaMKIIα (0.1–0.2 μM subunit) was incubated with 25–50 μg/ml isolated PSDs, whereas resuspended PSD·[T286-^{32}P]CaMKIIα complexes (2–5 μg kinase/mg PSD) were incubated at a final concentration of 70 μg/ml PSD protein. Incubations were conducted for 30 min at 30 °C in Buffer A containing 1 mM EGTA, 1 mg/ml bovine serum albumin, and 0.1 M NaCl, plus specific inhibitors or activators indicated below. Phosphatase activity

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‡ To whom correspondence should be addressed: Dept. of Molecular Physiology and Biophysics, Vanderbilt University Medical Center, Nashville, TN 37232-0615. Tel.: 615-936-1630; Fax: 615-322-7236; E-mail: Roger.Colbran@mcmail.vanderbilt.edu.

[1] The abbreviations used are: CaMKII, calcium/calmodulin-dependent protein kinase II; CaMKIIα or β, α or β isoform of CaMKII; [T286-P]CaMKIIα, CaMKIIα autophosphorylated on threonine 286; [T306-P]CaMKIIα, CaMKIIα autophosphorylated on threonine 305 and/or threonine 306; PSD, postsynaptic density; PP, protein serine/threonine phosphatase; TEA, tetraethylammonium; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid.

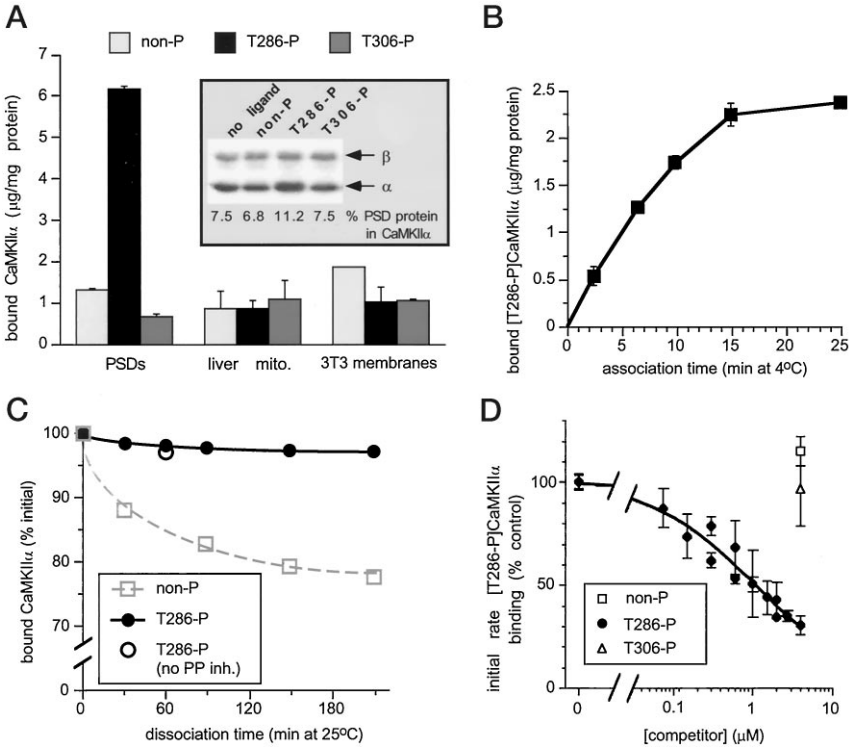

FIG. 1. **Characterization of CaMKIIα binding to PSDs *in vitro*.** *A*, comparison of CaMKIIα binding to different cellular structures. Nonphosphorylated or Thr^{286}- or Thr^{306}-autophosphorylated (*non-P*, *T286-P*, and *T306-P*, respectively) [^{35}S]CaMKII (1 μM each) was incubated with PSDs, liver mitochondria (*mito.*), or NIH 3T3 fibroblast membranes (0.25 mg/ml each) for 10 min at 25 °C. *Inset*, PSDs were incubated with each form of CaMKIIα (2.5 μM), washed twice, and analyzed by quantitative CaMKII immunoblot. The amounts of endogenous CaMKIIβ does not change following binding. Data (mean ± S.D., $n = 2$) representative of two (mitochondria and membranes) or four (PSDs) experiments are shown. *B*, time course of binding. Incubations at 4 °C containing 50 nM [T286-^{32}P]CaMKIIα and 0.1 mg/ml PSDs were stopped at the indicated times. *C*, time course of dissociation. PSDs (0.25 mg/ml) were incubated (25 °C, 15 min) with [T286-P] (●, ○) or nonphosphorylated (□) [^{35}S]CaMKIIα (1 μM) and unbound kinase was removed by centrifugation. PSD·CaMKIIα complexes (16.8 μg [T286-P]CaMKIIα per mg, 1 μg/mg nonphosphorylated) were resuspended in fresh buffer with (●, □) or without (○) microcystin-LR (1 μM), and [^{35}S]CaMKII released into the supernatant was assayed at the indicated times. Data (means ± S.D., $n = 2$) representative of three experiments are shown in *B* and *C*. *D*, determination of binding affinity. Initial rates of binding to PSDs were determined by incubating PSDs (0.1 mg/ml) with [T286-^{32}P]CaMKIIα (50 nM) for 5 min at 4 °C in the absence or the presence of nonradioactive CaMKIIα in different autophosphorylation states. Mean ± S.D. ($n = 2$) data from two experiments are plotted. The apparent IC_{50} for [T286-P]CaMKIIα was 1.06 μM (95% confidence interval: 0.87–1.31 μM).

was quantitated as trichloroacetic acid-soluble (20%, w/v) [^{32}P]phosphate by scintillation counting. Blanks, with no PSDs in soluble substrate assays or 2.5 μM microcystin-LR in "PSD complex" substrate assays, were subtracted from all assays to control for [^{32}P]phosphate present at the beginning of the incubation. Microcystin-LR completely blocked dephosphorylation in both assays. PP1 activity was calculated by subtraction of dephosphorylation measured in the presence of 0.2 μM Inhibitor-2 (Upstate Biotechnology, Inc.) from total "constitutive" dephosphorylation. PP2A activity was defined as activity inhibited by 2.5 nM okadaic acid (LC Laboratories); activity measured in the presence of 2.5 μM okadaic acid was assigned to unknown okadaic acid-resistant phosphatase(s); PP2C activity was defined as that stimulated by 10 mM magnesium acetate in the presence of 2.5 μM okadaic acid. Each protein phosphatase activity was expressed as a percentage of constitutive activity. Activities do not add up to 100% because inhibitor concentrations were chosen for optimum selectivity, not maximal efficacy.

Phosphorylation of PSD Proteins and Immunoprecipitation—After incubation without or with 1 μM [T286-P]CaMKIIα, PSDs (0.3 mg/ml) were washed by pelleting twice and suspended in Buffer A containing 10 mM magnesium acetate, 0.5 mg/ml bovine serum albumin, 1 μM microcystin-LR, and either 0.5 mM $CaCl_2$, 6 μM calmodulin, or 1 mM EGTA. Phosphorylations were started by adding 0.5 μM [γ-^{32}P]ATP (100 cpm/nmol) and stopped with 25 mM EDTA after 1 min on ice. GluR1 was immunoprecipitated (22) using antibody from Chemicon. No phosphorylated band co-migrating with GluR1 was detected in control precipitations with normal serum (not shown).

Translocation of CaMKII in Hippocampal Slices—Preparation and electrophysiological recordings from hippocampal slice were described previously (23). Four to six slices (400 μm, 3–6 week old rats) were divided into control/experimental hemislices and maintained in oxygenated, artificial cerebrospinal fluid for 60 min at 32 °C. After treatment with or without calyculin A (1 μM for 60 min) or tetraethylammonium (TEA) (25 mM for 10 min, 15-min wash) slices were flash frozen in liquid N_2. Slices were homogenized in 50 mM Tris, pH 7.5, 1% (v/v) Triton X-100, 0.1 M KCl, 20 mM sodium pyrophosphate, 50 mM NaF, 50 mM sodium β-glycerophosphate, 1 μM microcystin-LR, 1 mM benzamidine, 20 μg/ml leupeptin, and the PSD-enriched particulate fraction was isolated by centrifugation (30 min at 14,000 × *g*). Crude PSD fractions were immunoblotted in triplicate for the indicated proteins using mixed alkaline phosphatase- and ^{125}I-labeled secondary antibodies. Gamma counts for CaMKIIα/β and GluR1 in each lane were normalized to neurofilament heavy subunit (NF-H) immunoreactivity. Normalized values for experimental slices were expressed as a percentage of control slice values. Quantifications were performed in the linear range of the assay, which was determined separately for each protein by probing serial extract dilutions.

RESULTS AND DISCUSSION

Thr^{286} autophosphorylated CaMKIIα has been shown previously to bind a 190-kDa PSD protein on gel overlays (17). One criticism of gel overlays is that denatured proteins are partially renatured prior to binding, potentially exposing binding domain(s) that are cryptic in native protein or PSDs. To examine possible interactions of CaMKII under native conditions, we investigated binding of [^{35}S]CaMKIIα to isolated PSDs (Fig. 1*A*). Calcium/calmodulin-dependent autophosphorylation at Thr^{306}, generating the calcium-independent form of the kinase ([T286-P]CaMKII), enhanced *in vitro* binding about 5-fold. Calcium/calmodulin-independent autophosphorylation at Thr^{305}/Thr^{306}, inactivating the enzyme ([T306-P]CaMKII), reduced binding somewhat below nonphosphorylated kinase. Binding of [T286-P]CaMKII by PSDs was independent of calcium/calmodulin or magnesium (not shown). The apparent binding was not

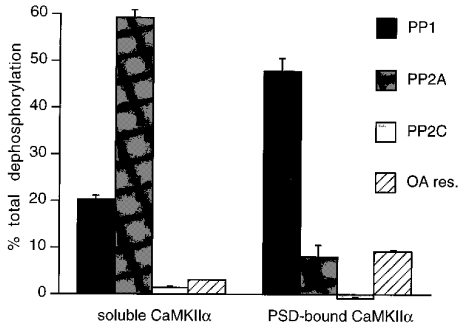

FIG. 2. **Dephosphorylation of [T286-P]CaMKIIα.** Protein phosphatases endogenous to isolated PSDs were allowed to dephosphorylate soluble and [T286-^{32}P]CaMKIIα previously bound to PSDs. Specific inhibitors and activators were used to define contributions of PP1, PP2A, PP2C, and an okadaic acid-resistant activity (see "Experimental Procedures"). Binding of CaMKIIα to PSDs had no effect on the dephosphorylation of glycogen phosphorylase *a*, a selective PP1 substrate, or casein, a substrate for PP2A and PP2C (not shown), demonstrating that CaMKIIα binding to PSDs does not activate PP1 or inactivate PP2A. Similar data were obtained in four or five independent experiments.

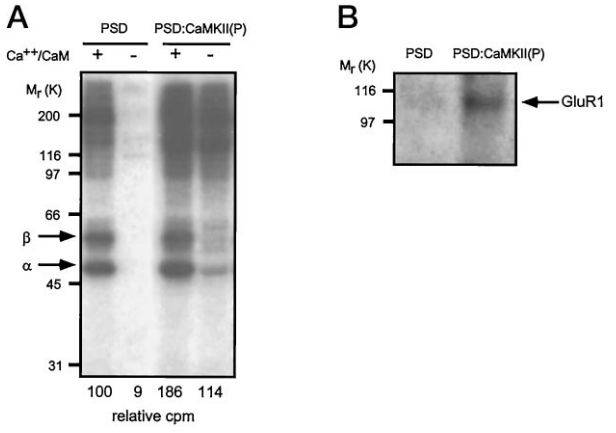

FIG. 3. **Activity of CaMKIIα bound to PSDs.** *A*, phosphorylation of PSD proteins. PSDs or PSDs with bound [T286-P]CaMKIIα (*PSD-:CaMKII(P)*) phosphorylated in the absence (−) or the presence (+) of calcium/calmodulin were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. The relative radioactivities of each lane (duplicate determinations by scintillation counting) are indicated. *Arrows* mark autophosphorylated α and β subunits of endogenous CaMKII. *B*, identification of the glutamate receptor GluR1 subunit as a substrate. GluR1 immunoprecipitations were carried out from samples of PSD and PSD·CaMKII(P) phosphorylated in the absence of calcium/calmodulin (*lanes 2* and *4* of *panel A*). Similar data were obtained in four (*A*) and two (*B*) independent experiments.

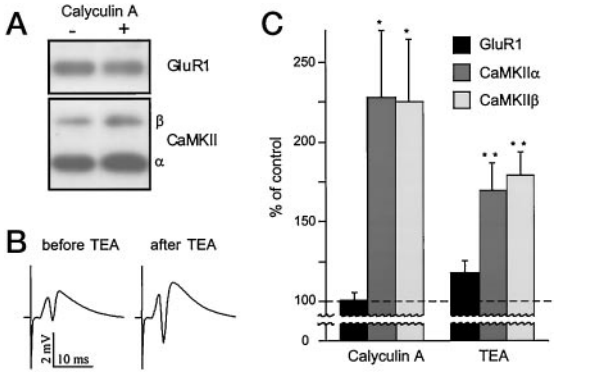

FIG. 4. **Translocation of CaMKII in hippocampal slices.** *A*, effect of calyculin A. Representative immunoblot of PSD-enriched fraction from hippocampal slices of the same rat treated with or without 1 μM calyculin A for 60 min. *B*, TEA potentiates synaptic transmission. Slices were treated with 25 mM TEA for 10 min while recording field potentials in the CA1 region in response to test stimuli applied to the Schaffer collateral. Averages of 10 traces obtained before treatment and 15 min after treatment are shown. *C*, summary of translocation experiments. Calyculin A (1 μM for 60 min) and TEA (25 mM for 10 min, 15 min wash) treatment resulted in a significant increase of both α and β isoforms of CaMKII (* $p < 0.05$; ** $p < 0.01$; $n = 6$) in PSD-enriched fractions, whereas GluR1 ($n = 3$) remained unaffected.

due to exchange of [^{35}S]CaMKII for endogenous enzyme but represented true accumulation of CaMKIIα in the PSD (Fig. 1*A*, *inset*). [T286-P]CaMKIIα binding to PSDs was specific and may be functionally relevant, because the low levels of binding to mitochondria and membranes were unaffected by autophosphorylation (Fig. 1*A*).

[T286-P]CaMKIIα binding to PSDs was rapid, reaching saturation after 15 min at 4 °C (Fig. 1*B*) or 5 min at 25 °C (not shown). Whereas nonphosphorylated kinase bound reversibly (≈25% dissociated in 3 h), binding of [T286-P]CaMKIIα appeared essentially irreversible under these conditions (<3% dissociated in 3 h) (Fig. 1*C*). Interestingly, although binding to PSDs was enhanced by autophosphorylation, continued phosphorylation was not required to maintain the interaction because dissociation was not accelerated when dephosphorylation of bound kinase by endogenous protein phosphatases was allowed (calcium-independent CaMKII activity 17 *versus* 10% ± microcystin-LR at 60 min) (Fig. 1*C*, *open circle*). This may explain why during PSD isolation CaMKII remains PSD-associated even though it is mostly dephosphorylated (see "Experimental Procedures"). In fact, [T286-P]CaMKIIα dissociated from PSDs as slowly as endogenous CaMKIIα measured by immunoblotting (not shown), suggesting that *in vitro* and *in vivo* association of CaMKII with the PSD are mechanistically similar. The reason for this very slow reversibility is not known, but mechanisms such as proteolysis (24, 25) may be required to dissociate CaMKII from the PSD *in vivo*.

To assess the affinity of binding to PSDs, initial rates of [T286-^{32}P]CaMKIIα binding were measured in the absence or the presence of nonradioactive kinase. Whereas non-P CaMKIIα and [T306-P]CaMKIIα were poor competitors, [T286-P]CaMKIIα was an effective competitor (apparent IC_{50} ≈1.1 μM) (Fig. 1*D*). Because this IC_{50} is approximately 10 times lower than the average concentration of CaMKII in forebrain (3, 17), sufficient CaMKII exists for binding to be regulated *in vivo*. Significantly, this IC_{50} is very similar to the affinity of [T286-P]CaMKIIα for p190 estimated by gel overlay (17); p190 is therefore a candidate for targeting the calcium-independent form of CaMKII to PSDs.

It seemed important to determine which protein phosphatases (PPs) influence the lifetime of calcium-independent CaMKII in the PSD. CaMKII can be dephosphorylated by purified PP1 (26), PP2A (27), and PP2C (28), but not by PP2B. Each of these major phosphatases is present in PSDs, although only PP1 is enriched in this fraction (29). Their activities can be distinguished based on requirements for divalent cations and sensitivity to specific inhibitors (30). With soluble exogenous [T286-^{32}P]CaMKIIα as the substrate, the PP2A:PP1 activity ratio was 3:1 (Fig. 2), in agreement with previous data (29). However, under these conditions, 5–10% of the substrate binds to PSDs. When assays were repeated using [T286-^{32}P]-CaMKIIα previously bound to the PSD as a substrate, PP1 appeared to be mostly responsible for dephosphorylation (PP2A:PP1 activity ratio, 1:6; Fig. 2).[2] This is consistent with

[2] Total activities in soluble and PSD-bound CaMKII phosphatase assays were 7.8 and 0.6 pmol/min/mg, respectively. It is difficult to interpret this difference because both substrate and enzyme are immobilized on the PSD in the latter assay. The apparent decrease in phosphatase activity after binding may reflect a preferential loss of the PP2A component or may simply be due to restricted mobility of PSD-bound CaMKII.

previous reports that dephosphorylation of CaMKII endogenous to PSDs is catalyzed by PP1 (29, 31, 32); however, CaMKII endogenous to isolated PSDs (*i.e. in vivo* translocated CaMKII) may represent a modified form of the kinase (46), possibly due to post-mortem ischemic conditions (11, 47). The present data demonstrate that interaction of the calcium-independent form of CaMKII with PSDs directly regulates its inactivation, in that binding to the PSD *in vitro* converts CaMKII from a PP2A substrate to a substrate for PSD-bound PP1. The mechanism for this change in protein phosphatases responsible for dephosphorylating Thr^{286} is unknown. CaMKII may undergo a conformational change after binding to PSDs that favors dephosphorylation by PP1. Alternatively, dephosphorylation by PP1 may be enhanced by physical proximity in the PSD.

AKAP79 is a protein that is thought to anchor inactive forms of protein kinase A, protein kinase C, and PP2B to PSDs (33, 34). In contrast, CaMKII bound to PSDs remains active, because binding of [T286-P]CaMKIIα to PSDs increased calcium-independent activity toward autocamtide-2 peptide substrate by an amount corresponding to binding of [T286-^{32}P]CaMKIIα quantified in parallel reactions (not shown). More importantly, binding of [T286-P]CaMKIIα ($\approx$10 μg/mg PSD protein) led to a $\approx$2-fold increase of calcium-dependent phosphorylation and a $\approx$13-fold increase of calcium-independent phosphorylation of many proteins in PSDs (Fig. 3*A*).

In the presence of calcium/calmodulin, CaMKII phosphorylates GluR1 subunits of AMPA-type glutamate receptors in PSDs, increasing channel permeability and postsynaptic responses (22). GluR1 immunoprecipitation experiments were carried out with PSDs phosphorylated in the absence of calcium, with or without bound [T286-P]CaMKIIα. Phosphorylated GluR1 was immunoprecipitated only from PSDs to which [T286-P]CaMKIIα had been bound (Fig. 3*B*), identifying GluR1 as a substrate for the PSD-bound, autophosphorylated, calcium-independent form of CaMKII.

Brain injuries involving increased intracellular calcium such as ischemia/hypoxia (11, 35), hypoglycemia (36), and excitotoxic insults (37) cause CaMKII translocation to the cytoskeleton, including PSDs. Ischemia also transiently increases calcium-independent CaMKII activity (38). We therefore tested the hypothesis that Thr^{286} autophosphorylation is sufficient for translocation of CaMKII to occur in neurons. Calyculin A, a cell-permeant protein phosphatase inhibitor, was shown previously to increase Thr^{286} phosphorylation in hippocampal slices (39) without affecting general excitability or viability (40). Here, calyculin A increased calcium-independent CaMKII activity in hippocampal slice extracts from 25.6 $\pm$ 1.2% to 43.4 $\pm$ 3.7% ($n = 6$, $p < 0.01$), accompanied by a >2-fold increase in CaMKII associated with PSD-enriched fractions (Fig. 4, *A* and *C*). We next induced a form of long term potentiation with the K^+-channel blocker TEA (41). There was a 83.6 $\pm$ 21.7% ($n = 4$, $p < 0.05$) enhancement of transmission at CA3-CA1 synapses (Fig. 4*B*) 15 min after TEA removal, accompanied by increased calcium-independent CaMKII activity (20.3 $\pm$ 1.9% to 26.7 $\pm$ 4.2%, $n = 5$, $p = 0.07$) and a 70–80% increase of CaMKII protein in the PSD-enriched fraction (Fig. 4*C*). Thus, CaMKII translocation occurs in intact neurons in response to treatments that induce CaMKII autophosphorylation and potentiate synaptic transmission.

In conclusion, the present data suggest a molecular mechanism for association of CaMKII with the PSD that is likely to be important in physiological and pathological states. Stimulation of CaMKII autophosphorylation by calcium influx into dendritic spines may increase the amount of active CaMKII in the PSD resulting in enhanced phosphorylation of GluR1 and other key substrates, in turn leading to an enhanced postsynaptic response. In addition, translocation may sequester CaMKII away from the cytosolic phosphatase activity of PP2A, making it available for dephosphorylation by PSD-bound PP1 only. Interestingly, PP1 itself is highly regulated by phosphorylation and association with targeting and inhibitory subunits (42) and is also involved in synaptic plasticity (43). Finally, because CaMKII has been proposed to play a structural role in the PSD (5), translocation of substantial amounts of cytosolic CaMKII to the PSD in response to calcium signals may result in long term changes in synapse morphology (44, 45).

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