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Regulation of the Ca^{2+}/CaM-Responsive Pool of CaMKII by Scaffold-Dependent Autophosphorylation

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Summary

CaMKII is critical for structural and functional plasticity. Here we show that Camguk (Cmg), the *Drosophila* homolog of CASK/Lin-2, associates in an ATP-regulated manner with CaMKII to catalyze formation of a pool of calcium-insensitive CaMKII. In the presence of Ca^{2+}/CaM, CaMKII complexed to Cmg can autophosphorylate at T287 and become constitutively active. In the absence of Ca^{2+}/CaM, ATP hydrolysis results in phosphorylation of T306 and inactivation of CaMKII. Cmg coexpression suppresses CaMKII activity in transfected cells, and the level of Cmg expression in *Drosophila* modulates postsynaptic T306 phosphorylation. These results suggest that Cmg, in the presence of Ca^{2+}/CaM, can provide a localized source of active kinase. When Ca^{2+}/CaM or synaptic activity is low, Cmg promotes inactivating autophosphorylation, producing CaMKII that requires phosphatase to reactivate. This interaction provides a mechanism by which the active postsynaptic pool of CaMKII can be controlled locally to differentiate active and inactive synapses.

Introduction

Refinement of synaptic circuitry by selective strengthening of some inputs at the expense of others is a basic mechanism of development and plasticity in the nervous system. The biochemical events that allow a neuron to decide if an input is to be retained or lost are incompletely understood. One molecule that has been implicated in both positive (Malinow et al., 1989) and negative (Mayford et al., 1995; Stevens et al., 1994) changes in synaptic strength and in activity-dependent modification of synaptic structure (Koh et al., 1999; Pratt et al., 2003; Wu and Cline, 1998) is calcium/calmodulin-dependent protein kinase II (CaMKII). For CaMKII, as for many signaling molecules, localization is a mechanism by which specificity of action is conferred (Bayer and Schulman, 2001). This type of local regulation of signaling is critical for initiating the synapse-specific plastic changes which are believed to underlie developmental and learning-related plasticity. Membrane associated guanylate kinases (MAGUKs) are a class of proteins that have been shown to be integral to the assembly of synaptic complexes. In this study, we present evidence that the MAGUK protein Camguk (Cmg) can bind to CaMKII. Interaction with Cmg localizes CaMKII and, in a Ca^{2+}/CaM-dependent manner, controls the amount of Ca^{2+}-responsive CaMKII in the postsynaptic cell, providing a mechanism by which CaMKII activity can be controlled based on the activity history of the synapse.

CaMKII is localized by a variety of protein partners. The NMDA receptor subunits NR2B (Bayer et al., 2001; Strack and Colbran, 1998) and NR2A (Gardoni et al., 2001), α-actinin and densin-180 (Strack et al., 2000; Walikonis et al., 2001), actin (Shen et al., 1998), and synapsin I (Benfenati et al., 1992) have all been shown to target the kinase to specific subcellular regions of mammalian neurons. To date, no direct interactions of CaMKII with MAGUK proteins have been demonstrated, although in *Drosophila*, CaMKII immunoprecipitates in a complex with Dlg, the fly PSD95 homolog (Koh et al., 1999). Localization of CaMKII to the postsynaptic density in neurons is dynamically regulated by the autophosphorylation state of the kinase (Elgersma et al., 2002; Shen and Meyer, 1999; Strack et al., 1997), neuronal activity (Shen and Meyer, 1999), and the activation of protein kinase C (Fong et al., 2002). Dynamic localization may be key to allowing CaMKII to participate in synapse-specific events.

Localizing interactions are also important for regulating CaMKII activity. The interaction of CaMKII with NR2B requires Ca^{2+}/CaM to be bound to the kinase to expose residues in the catalytic region of the enzyme that normally interact with its autoinhibitory domain. Once this region is exposed, a sequence in NR2B that resembles the kinase autoinhibitory domain is able to tether CaMKII to the channel. This Ca^{2+}/CaM-dependent interaction of CaMKII with NR2B locks the kinase in an active conformation even after Ca^{2+}/CaM has dissociated (Bayer et al., 2001). These results suggest that the interaction surfaces used for intramolecular interactions between the catalytic and regulatory regions of CaMKII can be used to create new, activity-regulating interactions with other proteins.

Cmg is the *Drosophila* homolog of the mammalian CASK protein (Hata et al., 1996) and *C. elegans* Lin-2 (Hoskins et al., 1996). Cmg, CASK, and Lin-2 form a MAGUK subfamily which is defined by an N-terminal CaMKII-like domain in addition to the SH3, PDZ, and guanylate kinase domains typical of the MAGUK family (Dimitratos et al., 1997). In mammalian neurons, CASK has been shown to be localized to synapses both pre- and postsynaptically where it associates with cell surface molecules like neurexins (Hata et al., 1996), calcium channels (Maximov et al., 1999), and syndecans (Hsueh et al., 1998), the cytoskeletal protein 4.1 (Biederer and Sudhof, 2001), PSD95 (Chetkovich et al., 2002), and other adaptor molecules (Butz et al., 1998). CASK may also participate in signaling to the nucleus by directly interacting with a transcription factor (Hsueh et al., 2000).

In this study, we present evidence that binding of Cmg to the CaMKII regulatory domain is stimulated by trinucleotide in the CaMKII ATP binding site. Association

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involves the N terminus of the CaMKII autoregulatory domain, while release from the complex is accomplished via autophosphorylation of the C-terminal CaM binding domain. This suggests a model in which high intracellular Ca^{2+} can protect the complex by blocking autophosphorylation of the CaM binding domain and keep synaptic Cmg-bound CaMKII active. When Ca^{2+} is low and Ca^{2+}/CaM dissociates from CaMKII, Cmg promotes inactivating autophosphorylation. This interaction provides a mechanism for local regulation of the Ca^{2+}/CaM-responsive pool of CaMKII. In vivo, Cmg levels and synaptic activity regulate postsynaptic CaMKII T306 autophosphorylation, and Cmg null animals have defects in plastic behaviors, indicating that this interaction is an important regulator of synaptic plasticity in the intact animal.

Results

Camguk and CaMKII Localize to Synaptic Regions of the *Drosophila* Brain and Neuromuscular Junction and Coimmunoprecipitate

In the wild-type *Drosophila* larval CNS, Cmg is expressed in synaptic regions of the ventral ganglion and brain lobes (Figure 1A, panel 1). Cmg is also expressed at the neuromuscular junction (NMJ). Weak presynaptic terminal staining can be seen (Figure 1A, panel 3, arrowhead), with stronger postsynaptic body wall muscle expression. Animals that are transheterozygous for two deletions that remove the *cmg* gene (Martin and Ollo, 1996) show no staining with anti-Cmg antibodies (Figure 1A, panels 2 and 4). To determine if Cmg colocalized with CaMKII, we expressed Cmg and GFP-CaMKII in the presynaptic compartment using *C164-GAL4* (Packard et al., 2002), detecting Cmg using an antibody. Figure 1B (panels 1–3) shows that these two proteins have completely overlapping distribution. To assess postsynaptic colocalization, we expressed Cmg in muscle using *24B-GAL4* (Brand and Perrimon, 1993) and detected Cmg and endogenous CaMKII using an antibody. Panels 4–6 show that CaMKII is present at high levels both pre- and postsynaptically and that postsynaptic Cmg colocalizes with CaMKII to the subsynaptic reticulum surrounding bouton contact sites.

The colocalization and homology of CaMKII and Cmg and the multimeric nature of CaMKII suggested that they might directly interact. In tsA201 cells, a derivative of HEK 293, transfected with both Cmg and HA-tagged CaMKII, immunoprecipitation of CaMKII brought down Cmg (Figure 1C, right lane). In untransfected cells or cells transfected with Cmg only, antibody to tagged CaMKII did not precipitate Cmg (Figure 1C, left and middle lanes). CaMKII and Cmg also interacted in *Drosophila* heads. Complete immunoprecipitation of all of the Cmg from an extract of fly heads (Figure 1D, top left panel) is able to precipitate a substantial portion of the CaMKII (Figure 1D, bottom left panel) while preimmune serum brought down neither (Figure 1D, right panel). These data demonstrate that CaMKII and Cmg are colocalized in *Drosophila* and can be reciprocally coimmunoprecipitated. It is also clear from the native tissue coimmunoprecipitation that the amount of CaMKII in *Drosophila* heads outstrips the binding ability of endogenous Cmg.

Association of Camguk and CaMKII Is Stimulated by Trinucleotide but Not CaM or Autophosphorylation

To investigate the interaction mechanism, we developed an in vitro association assay using GST-Cmg fusion protein coupled to glutathione-sepharose. During the development of this assay, we noted that the presence of nucleotides affected the recovery of CaMKII. In Figure 2A, Cmg-coupled beads were incubated with CaMKII and nucleotide as indicated. Ca^{2+}/CaM was present during loading of the nucleotide but not during binding to Cmg, and it is not required for the interaction (data not shown). Cmg-bound kinase was eluted by boiling in SDS after washes in 0.8 M NaCl and 3 mM EGTA and detected by immunoblotting with anti-CaMKII antibody. CaMKII was able to bind to Cmg in the absence of nucleotides (data not shown), but binding was enhanced by addition of the nonhydrolyzable ATP analog AMP-PNP. ADP did not enhance binding relative to the basal level, and addition of ATP led to a significant decrease in the amount of kinase associated with Cmg when compared to all other conditions.

To determine if the ATP effect was due to phosphorylation by CaMKII, we repeated the experiment using K43M CaMKII, a point mutant that can bind but not hydrolyze ATP (Hanson et al., 1994, and data not shown). For this mutant, both ATP and AMP-PNP supported association, while ADP did not (Figure 2A, bottom panel). These data imply that binding of trinucleotide facilitates interaction, not phosphorylation of CaMKII or Cmg in the binding reaction. The ability of ATP to decrease recovery of wt but not K43M CaMKII suggested that hydrolysis of the bound ATP may either block association or actively dissociate the complex.

Both CaMKII and Cmg have nucleotide binding motifs. CaMKII has a catalytic domain ATP binding site, while Cmg has a guanine nucleotide binding motif in its GUK domain and potential ATP binding sites in its CK and GUK domains. To determine if Cmg was able to bind ATP, we incubated GST-Cmg with 5 μM radioactively α labeled ATP and crosslinked with UV irradiation. CaMKII was used as a positive control. Specificity of binding was demonstrated by competition with a 40-fold excess of cold ATP or ADP. Only CaMKII was able to bind radioactive ATP, and this binding was completely blocked by both cold ADP and ATP (Figure 2B). These results imply that ATP promotes association by binding to CaMKII, not Cmg.

Dissociation of the Camguk/CaMKII Complex Requires ATP Hydrolysis

Although binding of trinucleotide stimulated association with Cmg, incubation of CaMKII and Cmg with ATP under conditions which allow ATP hydrolysis decreased Cmg binding (Figure 2A). To determine if this decrease was due to reduced association or if it was due to an active dissociation process, we made preassembled CaMKII:Cmg complex on beads using AMP-PNP. Nucleotide in this complex can be exchanged by washing the beads to remove AMP-PNP and Ca^{2+}/CaM and incubating with an excess of another nucleotide. Nucleotide can also be completely stripped from the complex by addition of EDTA. Figure 3A shows the results of exchange and stripping on CaMKII recovery. In the top

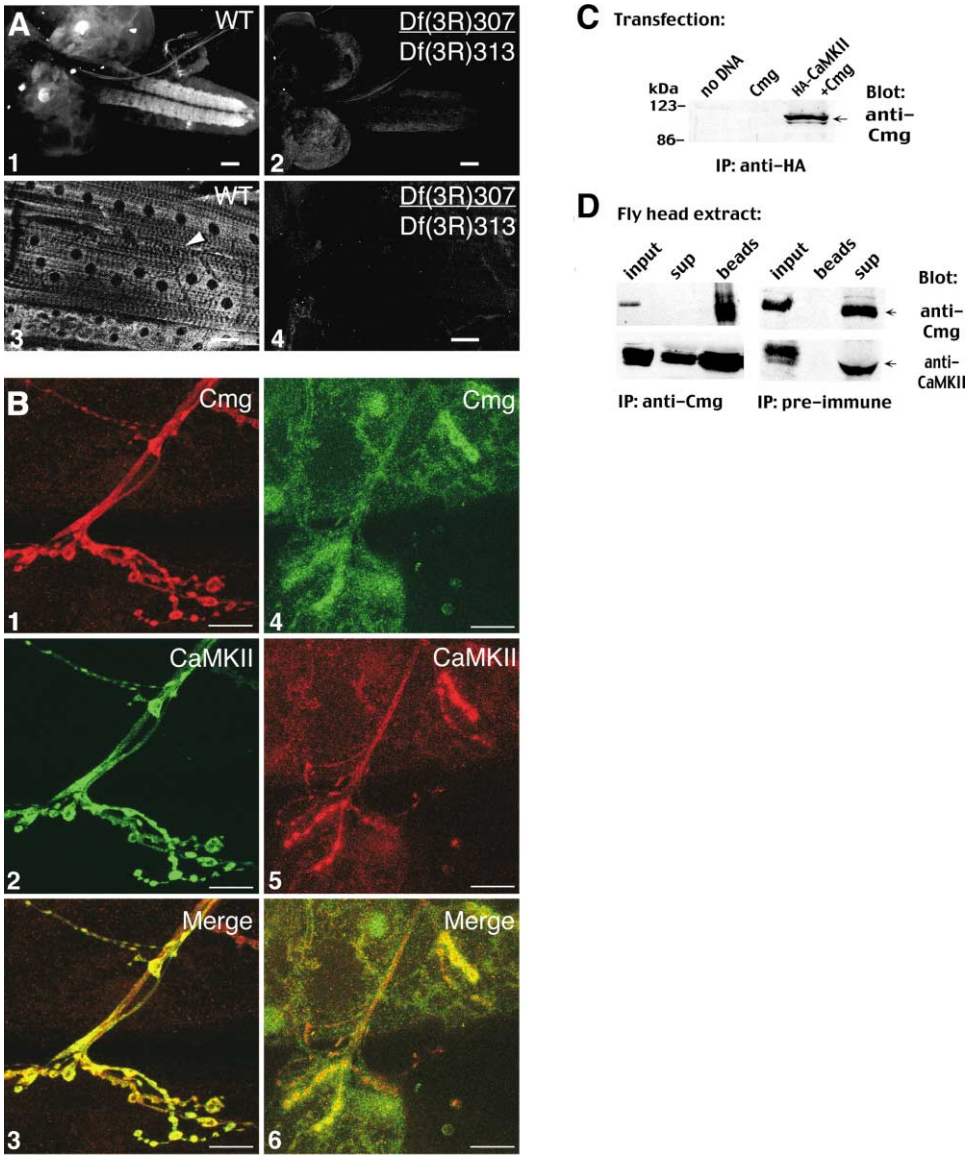

Figure 1. CaMKII and Camguk Interaction In Vitro and In Vivo

(A) Cmg is expressed in the CNS neuropil and body wall muscle of *Drosophila* third instar larvae. Wild-type and Cmg null *Df(3)x307/Df(3)x313* larvae were dissected and stained in parallel. Images were acquired on the same day at identical settings to allow direct comparison. Panels 1 and 2 show a wild-type brain and a Cmg null brain stained with affinity-purified rabbit anti-Cmg (1:40) visualized with FITC-conjugated anti-rabbit Ig (1:180). Panels 3 and 4 show muscles 12 and 13 of wild-type and Cmg null third instar larvae stained with preadsorbed 1:700 guinea pig anti-Cmg. Staining is visualized with FITC-conjugated anti-guinea pig Ig (1:180). Scale bars, 40 μ.

(B) Cmg and CaMKII colocalize presynaptically and postsynaptically at the larval NMJ. To visualize presynaptic colocalization, a motorneuron-specific GAL4 line, *C164-GAL4* (Packard et al., 2002), was used to express *UAS-Cmg* and *UAS-GFP-CaMKII*. Tissue was stained with rabbit anti-Cmg (1:700) and staining visualized using Cy5-conjugated anti-rabbit Ig (1:180). To visualize postsynaptic colocalization, a muscle-specific GAL4 line, *24B-GAL4* (Brand and Perrimon, 1993), was used to express *UAS-Cmg* in larval body wall muscle. Tissue was stained with preadsorbed guinea pig anti-Cmg (1:700) visualized with FITC-conjugated anti-guinea pig Ig (1:180) and with rabbit anti-CaMKII (1:3000) visualized with Cy5-conjugated anti-rabbit Ig (1:180). Scale bars, 16 μ.

(C) Cmg binds to CaMKII in transfected tsA201 cells. Cmg was transfected alone or with HA-tagged CaMKII into tsA201 cells. Cell lysates were immunoprecipitated with anti-HA antibody and analyzed by immunoblotting with anti-Cmg (1:1000).

(D) Cmg binds to CaMKII in native tissue. Fly head homogenates from adult Canton-S wild-type flies were immunoprecipitated with anti-Cmg (left panel) or preimmune serum (right panel) and protein A/G sepharose beads. 10% of input homogenate along with supernatant (sup), and coimmunoprecipitates (beads) were analyzed by immunoblotting using anti-Cmg and anti-CaMKII (1:1000) antibodies.

panel, wt CaMKII was bound in the presence of AMP-PNP (left lane) then incubated in the absence of Ca^{2+}/CaM at 30°C for either 2 or 20 min with ATP, ADP, or EDTA. Exchange of AMP-PNP for ATP resulted in a fast dissociation of the complex. Treatment of the K43M:Cmg complex with ATP did not cause release of kinase (Figure 3A, lower panel). Stripping of nucleotide by EDTA treatment or replacement of AMP-PNP with ADP did not

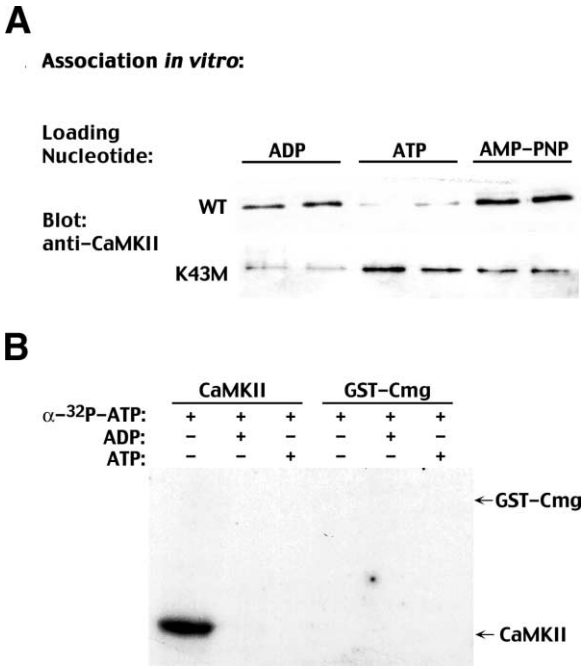

Figure 2. Nucleotides Regulate CaMKII:Cmg Complex Formation

(A) Trinucleotide binding and hydrolysis regulate CaMKII/Cmg interaction in vitro. ADP, ATP or AMP-PNP (100 μM), a nonhydrolyzable ATP analog, was incubated with purified CaMKII, either wild-type (wt, top panel) or "kinase dead" K43M (bottom panel) at 30°C for 2 min and bound to GST-Cmg beads at 4°C for 1 hr in the association assay. Bound proteins were resolved by 10% SDS-PAGE and probed with anti-CaMKII antibody. Representative blots of duplicate reactions for each condition from six independent experiments are shown.

(B) ATP binds to CaMKII but not Cmg. Purified recombinant wild-type CaMKII and GST-Cmg were incubated with 5 μM [α-^{32}P]-ATP (40 Ci/mmol) in the presence or absence (indicated by "+" or "−" at the top of the figure) of a 40-fold excess of ADP or ATP at 4°C. The mixture was exposed to UV to crosslink nucleotide to proteins, resolved by 7.5% SDS-PAGE, and visualized by autoradiography.

cause dissociation of either wt or K43M CaMKII. The continued presence of trinucleotide in the catalytic domain binding site is therefore not required to maintain the interaction between the two proteins, suggesting that either interaction with Cmg locks the kinase in the ATP-bound conformation or that the intial trinucleotide-dependent interaction may be supplemented by a secondary interaction with another part of the protein. These results also indicate that, for the wt kinase, ATP hydrolysis actively disrupts association with Cmg.

Dissociated CaMKII Does Not Bind CaM

In the experiment shown in Figure 3A, the recovery of CaMKII remaining bound to Cmg-sepharose beads was assessed by immunoblotting using anti-CaMKII. In a separate set of experiments we used biotinylated CaM to detect CaMKII and did not find CaM binding activity in the fraction that had undergone ATP-dependent dissociation from the CaMKII:Cmg complex. Figure 3B (left panel) shows that CaMKII immunoreactivity is present in the input, the Cmg-bound fraction, and the ATP-eluted dissociated fraction. The same samples assayed by CaM overlay show that the dissociated kinase no longer binds CaM. Loss of CaM binding and kinase activation have been shown to occur with autophosphorylation of residues within the CaM binding domain (Colbran and Soderling, 1990; Patton et al., 1990; Wang et al., 1998). These results suggested that, during dissociation from Cmg, CaMKII may become phosphorylated at T306 and/or T307 within the CaM binding domain.

Dissociation Is Mediated by Autophosphorylation of the CaMKII CaM Binding Domain

To test the possibility that autophosphorylation of CaMKII at T306/7 in its CaM binding domain is integral to the mechanism of dissociation, we examined the effects of activation of CaMKII with Ca^{2+}/CaM and of T306/7 point mutants in our dissociation assay. Preformed CaMKII:Cmg complex was made using AMP-PNP, and excess AMP-PNP and CaM were washed out. Note that under these conditions no autophosphorylation can occur during formation of the complex. CaM washout was verified by immunoblotting with an anti-CaM antibody (data not shown). ATP or ADP was exchanged into the complex and dissociation monitored by immunoblotting using anti-CaMKII antiserum. For wt CaMKII, ATP should support dissociation, while ADP would preserve the complex.

The first condition we tested was dissociation in the presence of Ca^{2+}/CaM. This condition would activate the kinase but would also block access of the catalytic site to T306/7. Addition of Ca^{2+}/CaM with either ADP or ATP protected the complex from dissociation (Figure 3C). This result suggested that activation of the kinase, while required (since K43M CaMKII fails to dissociate, Figure 3A), is insufficient to cause dissociation in the continued presence of Ca^{2+}/CaM. This experiment supports the idea that autophosphorylation of the CaM binding domain is required for release and disrupts the Cmg:CaMKII interaction.

To more directly test the idea that this region was critical to the interaction, we examined the ability of kinases with mutations there to associate with Cmg. Figure 3D shows that T306DT307D, T306ST307S, and T306AT307A CaMKII all fail to associate with Cmg even in the presence of AMP-PNP. The ability of T306DT307D to bind nucleotides was verified by UV crosslinking; T306AT307A and T306ST307S are catalytically active, and T306ST307S is able to autophosphorylate its CaM binding domain, suggesting that these mutants are structurally intact (data not shown). Thus, T306 and/or T307 are critical residues for Cmg binding, consistent with phosphorylation causing dissolution of the complex.

In solution, phosphorylation of residues in the CaM binding domain of CaMKII occurs at a very low rate in the absence of Ca^{2+}/CaM (Colbran, 1993; Hanson and Schulman, 1992) and at a robust rate when the enzyme has been made Ca^{2+} independent by previous autophosphorylation at T286 (Lou and Schulman, 1989) or T287 (Wang et al., 1998). In the case of release from the Cmg complex, T287 phosphorylation is not required for T306/7 phosphorylation. Since the CaMKII:Cmg complex is formed in the presence of AMP-PNP, no T287 autophosphorylation can occur, as this nucleotide does not transfer phosphate at a significant rate. Once CaM

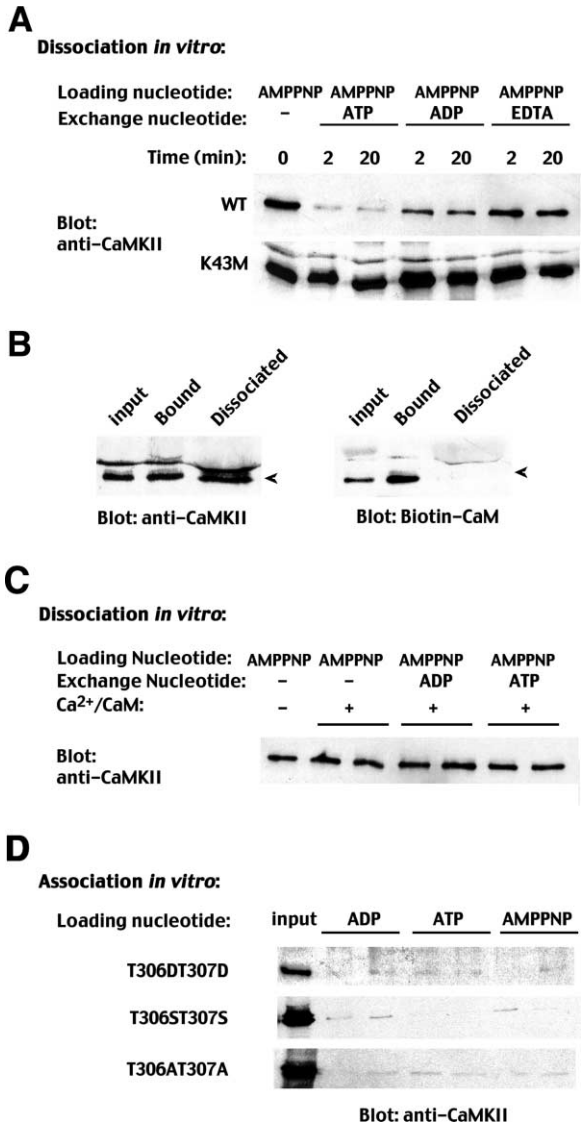

Figure 3. Autophosphorylation of the CaM Binding Domain of CaMKII Dissociates CaMKII:Cmg Complex

(A) ATP hydrolysis dissociates the CaMKII/Cmg complex. Complexes were preassembled on GST-Cmg beads with wild-type (wt) CaMKII (top panel) or catalytically inactive K43M (bottom panel) using AMP-PNP and washed to remove Ca^{2+}/CaM and excess AMP-PNP. Dissociation was initiated by exchanging in 1 mM ADP, ATP, or EDTA to the protein complex for 2 or 20 min at 30°C. Cmg-bound CaMKII was recovered from beads and detected by immunoblotting. Figure shows a representative result from six experiments.

(B) CaMKII dissociated from the CaMKII:Cmg complex cannot bind CaM. Equivalent amounts of soluble CaMKII (5% input), CaMKII on GST-Cmg beads (bound), and CaMKII from supernatant of the dissociation reaction (dissociated) were separated by SDS-PAGE. A membrane probed with anti-CaMKII is shown in the left panel, and a membrane probed with biotin-CaM is shown on the right. Arrowheads show the position of CaMKII. Figure shows a representative result from three experiments.

(C) Ca^{2+}/CaM protects the CaMKII:Cmg complex from dissociation. CaMKII:Cmg complex was preassembled in the presence of AMP-PNP. Dissociation was initiated by adding Ca^{2+}/CaM alone or exchanging in ADP or ATP in the presence of Ca^{2+}/CaM to the protein complex. Duplicate reactions are shown for each condition. Figure shows a representative result from four experiments.

(D) T306/T307 mutants do not bind to Cmg. ADP, ATP, or AMP-PNP (100 μM), the nonhydrolyzable ATP analog, was incubated with purified CaMKII mutants T306DT307D, T306ST307S, or T306AT307A at 30°C for 2 min and bound with immobilized GST-Cmg beads at 4°C for 1 hr in the association assay. Bound proteins were resolved by SDS-PAGE and probed with anti-CaMKII antibody. 5% soluble input and duplicate binding reactions are shown for each condition. Figure shows a representative result from seven experiments.

is stripped from the complex, addition of ATP caused almost complete dissociation within 2 min (Figure 3A). These results suggest that within the CaMKII:Cmg complex, phosphorylation of T306/7 is fast and independent of T287 phosphorylation.

CaMKII Autoinhibitory Domain Peptides Disrupt Interaction with Camguk

The stimulation of binding by ATP and the ability of Cmg and CaMKII to initially associate in the presence of Ca^{2+}/CaM, which blocks access to T306/7 in the C-terminal region of the autoinhibitory domain, suggested that the interaction of these two proteins may occur via multiple regions of CaMKII. Based on work by many groups on the activation mechanism of CaMKII, we reasoned that ATP binding to CaMKII might expose autoinhibitory domain residues; one of the roles of the autoinhibitory domain of CaMKII is to gate ATP binding to the kinase catalytic domain. The K_m for ATP of the fully autoinhibited kinase (kinase with no bound Ca^{2+}/CaM) is approximately 10-fold higher than for the Ca^{2+}/CaM activated kinase, and the N terminus of the autoinhibitory domain has been shown to be important for this ATP site regulation (Brickey et al., 1994). To test the hypothesis that interactions with both the N and C termini of the autoinhibitory domain of CaMKII were important for Cmg binding, we assessed the ability of peptide analogs of this region (Figure 4A) to inhibit complex formation. AC3I, a small substrate peptide analog, and a scrambled control peptide did not inhibit binding to Cmg, indicating that interactions with the substrate binding site were not important for complex formation (Figure 4B). A longer peptide (281–302, T286A) that contains the N-terminal sequences thought to mediate ATP site gating, but not those that bind CaM, is able to inhibit binding by 50% with an IC50 of 37 μM. This peptide has been shown to inhibit the kinase by acting competitively with ATP and will protect the ATP site from chemical modification (Colbran et al., 1989). Complete inhibition of CaMKII binding, with an IC50 of 13 μM, is seen with a longer peptide (275–310) which contains both the N-terminal and CaM binding sequences of CaMKII. A peptide that binds to CaM but is unrelated to the CaMKII autoregulatory domain in sequence (VIP) does not block binding. These results suggest that both N- and C-terminal residues in the CaMKII autoinhibitory domain have a role in its interaction with Cmg and that full interaction requires both ends of the autoinihibitory domain.

Association with Camguk Modulates CaMKII Activity In Vitro

Phosphorylation of CaMKII in its CaM binding domain has been shown to prevent activation of the enzyme by Ca^{2+}/CaM (Colbran and Soderling, 1990; Lou and

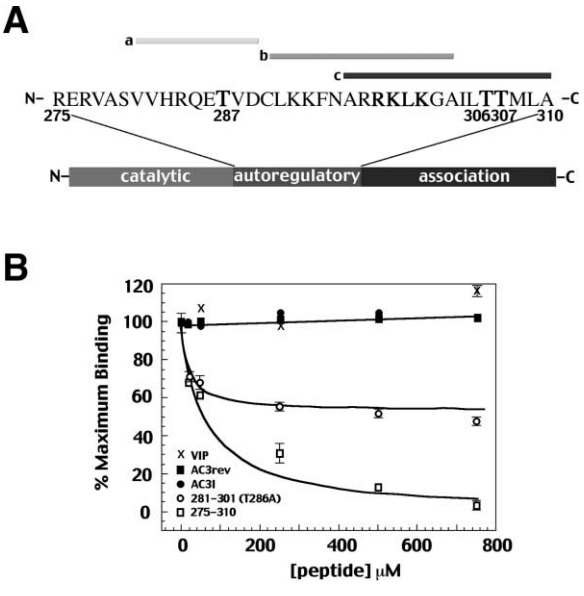

Figure 4. Mapping Interaction of the Autoinhibitory Domain

CaMKII autoregulatory domain peptides block Cmg binding. (A) Schematic diagram of CaMKII shows key autophosphorylated threonine residues, the pseudosubstrate region (in bold), and the regions of the autoregulatory domain that were demonstrated to competitively block (Aa) ATP binding site interaction, (Ab) exogenous substrate binding sites in the catalytic domain, and (Ac) CaM binding (Colbran et al., 1989; Payne et al., 1988; Smith et al., 1992; Yang and Schulman, 1999). (B) A substrate analog peptide (KKALHRQEAVDAL; AC3I), an N-terminal autoinhibitory domain peptide (aa 281–301, T286A; ala peptide), and a peptide encompassing the entire autoregulatory domain (aa 275–310) were used as competitors in the association assay done in the absence of Ca^{2+}/CaM. Vasoactive intestinal peptide (VIP), which binds CaM but is unrelated in sequence to CaMKII, and AC3I rev (an AC3I reverse sequence peptide) were included as controls. Bound CaMKII was measured by immunoblotting and quantified by densitometry. Data are presented as mean ± SEM, n ≥ 3.

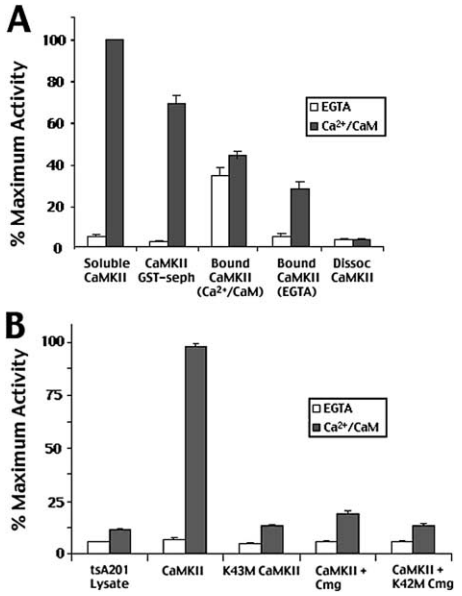

Figure 5. Cmg Modulates CaMKII Activity

(A) In vitro interaction with Cmg modulates CaMKII activity. Phosphorylation of the AC3 peptide substrate catalyzed by equivalent amounts (determined by immunoblotting) of CaMKII was measured in the presence of EGTA (white bars) or Ca^{2+}/CaM (gray bars). Activity was normalized to the maximum stimulated activity of soluble CaMKII. Data are presented as mean ± SEM, n ≥ 4.

(B) Cmg cotransfection inhibits CaMKII activity. Phosphorylation of AC3 peptide catalyzed by equivalent amounts (determined by immunoblotting) of CaMKII in extracts of transfected tsA201 cells was measured in the presence of EGTA (white bars) or Ca^{2+}/CaM (gray bars). Transfection conditions are indicated below each pair of bars. Activity was normalized to the maximum stimulated activity of extracts transfected with CaMKII alone. Data are presented as mean ± SEM, n ≥ 4.

Schulman, 1989; Patton et al., 1990). This suggests that one of the functions of the CaMKII:Cmg interaction may be to regulate the activity of CaMKII. We measured the ability of CaMKII to phosphorylate a peptide substrate, autocamtide-3 (AC3), when associated with or after dissociation from Cmg. As a baseline, we measured the activities of soluble CaMKII and soluble CaMKII mixed with GST-sepharose beads. The activity of the kinase under these conditions is almost completely Ca^{2+}/CaM dependent (Figure 5A). All activity results are reported normalized for the amount of immunoreactive kinase in the fraction assayed.

We first examined the activity of CaMKII in complex with Cmg. The CaMKII:Cmg complex was formed in the presence of Ca^{2+}/CaM or EGTA and washed to remove binding buffer before assay. Figure 5A shows that CaMKII, when complexed with Cmg-sepharose under conditions that allow T287 phosphorylation (+Ca^{2+}/CaM), has a significantly increased amount of constitutive activity ($p < 0.05$, ANOVA with Fisher's PLSD test). Constitutive activity was associated with phosphorylation of T287 as measured by immunoblotting with a phosphospecific antibody (data not shown). When the complex is formed in the presence of EGTA, there is no constitutive activity and no T287 phosphorylation. The maximum calcium-stimulated activity of CaMKII is not affected by the assembly conditions ($p > 0.05$), although the kinase in complex with Cmg sepharose has significantly less activity than free kinase ($p < 0.05$). These data show that autophosphorylation of CaMKII in complex with Cmg can generate constitutive kinase activity.

We next examined activity of kinase that had been released from the Cmg complex by incubation with ATP. Complex was formed in the presence of AMP-PNP, ATP was exchanged in, Cmg-sepharose beads were spun out, and released CaMKII was collected in the supernatant. Figure 5A shows that this fraction has significantly less Ca^{2+}/CaM-dependent activity than free or complexed CaMKII ($p < 0.05$ compared to all other conditions). This result is consistent with the loss of CaM binding and the predicted effects of T306/7 autophosphorylation. These data suggest that when the kinase is in complex with Cmg, it can become constitutively active due to Ca^{2+}/CaM-dependent autophosphorylation. In the absence of Ca^{2+}/CaM, the kinase can be autophosphorylated in its CaM binding domain and released in an inactive form.

Camguk Regulates CaMKII in Transfected Cells

The data obtained using purified proteins in vitro suggest that Cmg may have a profound inhibitory effect on

CaMKII activity in vivo if the level of Ca^{2+}/CaM is low. To test this, we assayed CaMKII activity in extracts of tsA201 cells, a nonexcitable human kidney cell line, that had been transfected with CaMKII and/or Cmg. The amount of DNA used for transfections was adjusted to produce approximately equimolar amounts of the two proteins. Figure 5B shows that in these extracts, cotransfection with Cmg brought CaMKII activity down to a level of activity not significantly different from untransfected extracts or extracts of cells transfected with catalytically inactive K43M CaMKII ($p > 0.0001$, ANOVA with Fisher's PLSD test). Mutation of the ATP binding motif in Cmg did not prevent this effect, showing that Cmg catalytic activity is not required for regulation of CaMKII.

Camguk and PP2A Levels Modulate CaMKII Regulatory Domain Autophosphorylation In Vivo in Adult *Drosophila* Heads

To determine if Cmg can affect CaMKII autophosphorylation in native tissue, we made head extracts from flies genetically modified to have different levels of Cmg and assessed the amount of T306 autophosphorylation by immunoblotting using a rabbit antiserum made against a peptide with the sequence CRRKLKGAIL-pT-TMLATRN. This antiserum recognizes rat αCaMKII only when it is phosphorylated at T305 (Elgersma et al., 2002) and *Drosophila* CaMKII only when it is autophosphorylated in conditions that promote T306/7 autophosphorylation (Figure 6A). This antibody does not recognize CaMKII that has a T306A mutation. A comparison of the level of T306 phosphorylation between animals null for Cmg expression (*Df(3R)x313/Df(3R)x307*), animals expressing normal levels of Cmg (Canton-S wild-type), and animals with elevated levels of neuronal Cmg (*C155;UAS-Cmg*) shows that pT306 autophosphorylation in vivo is promoted by Cmg (Figure 6B, left panel). The magnitude of changes seen with manipulation of Cmg levels suggests that interaction with Cmg is the major regulator of T306 phosphorylation in *Drosophila*. In vitro both protein phosphatases 1 and 2A (PP1 and PP2A) have been shown to dephosphorylate threonines in the CaM binding domain (Patton et al., 1990). In *Drosophila*, genetic reduction of PP2A profoundly increases T306 phosphorylation in adult heads, while reduction of PP1 activity has no effect (Figure 6B, right panel), suggesting that in vivo PP2A is the relevant phosphatase for these sites. Modulation of Cmg or phosphatase levels does not affect the amount of CaMKII protein (Figure 6B). These data support the idea that Cmg can act in neurons to regulate the size of the Ca^{2+}/CaM-activatable pool of CaMKII and that phosphorylation of this site is dynamically regulated by the ratio of kinase to phosphatase activity.

Camguk and PP2A Modulate Postsynaptic CaMKII Regulatory Domain Autophosphorylation

We assessed the ability of Cmg to modulate synaptic CaMKII autophosphorylation by immunocytochemical staining of pT306 in the third instar larval CNS and NMJ. The NMJ experiments also allowed us to ask whether regulation of pT306 phosphorylation was occurring presynaptically or postsynaptically. To allow direct comparison of pT306 levels, tissues were dissected, stained, and viewed in parallel and confocal images taken at identical settings. Larval brains from wt and Cmg null animals show significantly different levels of pT306 in both neuronal cell bodies and neuropil regions (Figure 6C, panel 1). At the NMJ, synaptic staining is also reduced in Cmg null animals (compare panels 2 and 3). To test whether the CaMKII that was responsive to Cmg levels was predominantly pre- or postsynaptic, we used selective overexpression of a Cmg transgene. When overexpressed in the presynaptic neuron (panel 4), there was little increase in pT306 staining. Postsynaptic overexpression of Cmg, however, greatly increased both the synaptic and muscle levels of pT306 (panel 5). The increased bouton staining reflects pT306 in the subsynaptic reticulum of the postsynaptic cell, as evidenced by its peripheral distribution. The pattern and level of enhancement seen with postsynaptic Cmg expression was mimicked by genetic reduction in the levels of PP2A (panel 6). These data suggest that the interaction of Cmg and CaMKII produces a modification of postsynaptic CaMKII. Presynaptic pT306 is relatively unaffected by alteration of Cmg levels, even though there is CaMKII present in the presynaptic terminal (Figure 1B, panel 5). These data imply that the pre- and postsynaptic complexes that contain CaMKII and Cmg are qualitatively different and that presynaptic CaMKII may be "protected" from interaction with Cmg by another binding partner.

Synaptic Activity Modulates T306 Phosphorylation

Ca^{2+}/CaM-inhibited dissociation implies that synaptic activity, by changing postsynaptic calcium levels, could regulate the level of CaMKII inactivation produced by interaction with Cmg. To test this, we quantitatively assessed the level of postsynaptic pT306 immunoreactivity in animals that had either increased or decreased activity at the NMJ. Increased activity was produced by mutation of the *eag* potassium channel gene (Wu et al., 1983). This mutant has spontaneous action potentials in the motor nerves and increased postsynaptic calcium influx. Decreased postsynaptic activity was produced by expressing a hyperpolarizing potassium channel, dORKΔC (Nitabach et al., 2002), both pre- and postsynaptically at muscle 12. The dORKΔC transgene suppresses presynaptic activity and prevents the muscle membrane from depolarizing, decreasing voltage-gated calcium channel-mediated calcium influx into the postsynaptic cell. Since the Cmg-responsive pool of CaMKII is postsynaptic, the intensity of bouton staining was used as a measure of the effect of activity manipulations on CaMKII associated with the subsynaptic reticulum. The intensity of anti-pT306 staining was significantly ($p < 0.0001$, Student's t test) decreased by synaptic hyperactivity and significantly increased ($p < 0.0001$, Student's t test) by synaptic silencing (Figure 6D).

Camguk Is Required for Courtship Habituation in *Drosophila*

Cmg null animals were known to have impaired locomotion (Martin and Ollo, 1996, and J.M., see Experimental Procedures), but their ability to learn had not been tested. Modification of courtship behavior in *Drosophila* has been used to test both associative and nonassocia-

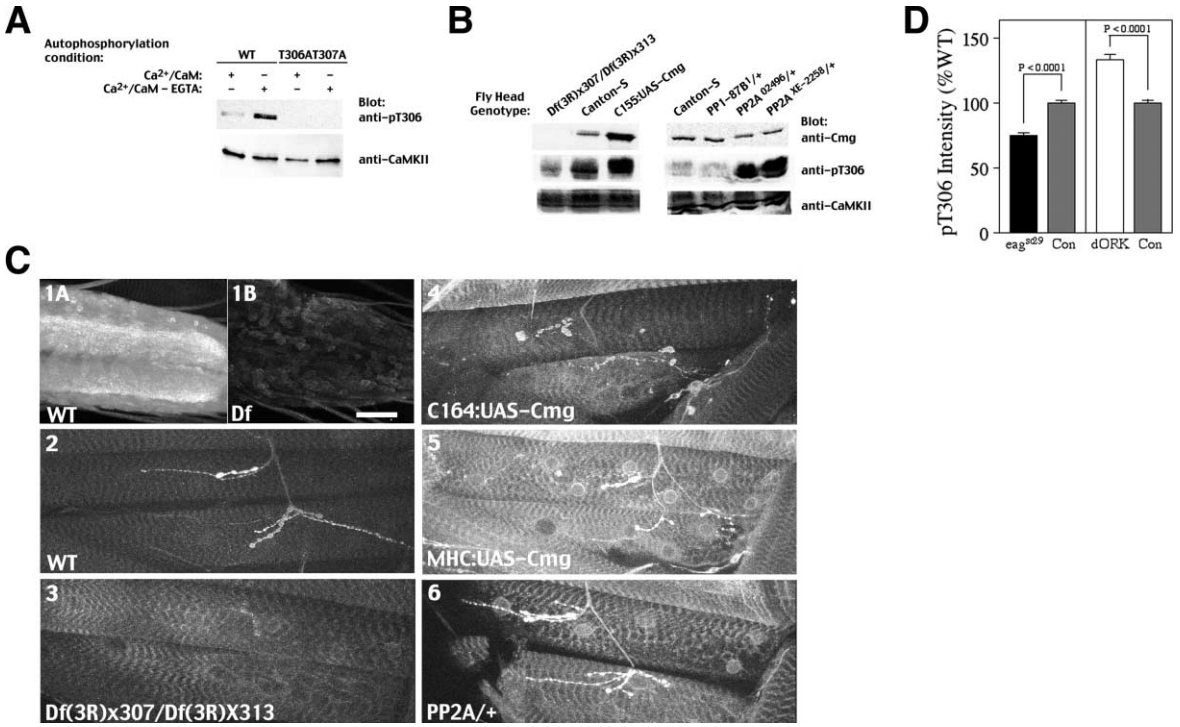

Figure 6. Cmg Levels Modulate T306 Phosphorylation In Vivo

(A) Specificity of the anti-pT306 antibody. Purified wild-type CaMKII (wt) or T306AT307A mutant was autophosphorylated for 15 s in the presence of Ca^{2+}/CaM or for 15 s in the presence of Ca^{2+}/CaM followed by 45 s in the presence of EGTA to induce bursts of inhibitory autophosphorylation at T306/7. Kinase (20 ng) after reaction was separated by SDS-PAGE and probed with anti-pT306 (1:2000, top panel) or anti-CaMKII (1:1000, bottom panel).

(B) Cmg and PP2A modulate pT306 levels in vivo. Extracts of fly heads from strains expressing different levels of Cmg (left panel) or reduced levels of PP1 or PP2A (right panel) were assessed for T306 phosphorylation. *Df(3R)x307/Df(3R)x313* has overlapping deletions that eliminate the *cmg* gene, Canton-S is wild-type, and *C155; UAS-Cmg* overexpresses Cmg in the nervous system. Heterozygous PP2A deficiencies express 70%–80% normal activity (Snaith et al., 1996). An equal amount of protein from each extract was separated by SDS-PAGE and probed with affinity purified anti-Cmg (1:100, top panel), anti-pT306 (middle panel), or anti-CaMKII (1:1000, bottom panel).

(C) Cmg modulates postsynaptic CaMKII autophosphorylation. Third instar larvae were dissected and stained with anti-pT306 in parallel and imaged at identical settings. Tissues and genotypes are (C1A) wild-type brain; (C1B) *Df(3)x307/Df(3)x313)* brain; (C2) wild-type NMJ (muscles 12 and 13 are shown for all genotypes); (C3) *Df(3)x307/Df(3)x313* NMJ; (C4) Cmg motor neuron overexpresser *C164-GAL4; UAS-Cmg* NMJ; (C5) Cmg muscle overexpresser *MHC-GAL4; UAS-Cmg* NMJ; (C6) phosphatase mutant heterozygote $PP2A^{02496}/+$ NMJ. Scale bar, 40 μ.

(D) Synaptic activity regulates pT306 levels. Animals with increased synaptic activity (eag^{sc29}) or with decreased synaptic activity (*BG487-GAL4/+;UAS-dORKΔC/+*) were stained with anti-pT306 (1:1000) and anti-HRP (1:500). Muscle 12 intensity values for each channel were collected blind to genotype and the ratio of pT306/HRP calculated and normalized to 100% for the control genotype.

tive memory formation; CaMKII is required for both (Griffith et al., 1993). Exposure of a male to a mated female leads to decreased courtship of a subsequently presented virgin (Siegel and Hall, 1979). This suppression reflects formation of an associative memory (Tompkins et al., 1983) that requires mushroom body and central complex circuits (Joiner and Griffith, 1999). Courtship suppression also occurs during the training period via a non-mushroom body central circuit (Joiner and Griffith, 1999). Habituation to a courtship stimulating cue can be demonstrated after training with immature males (Gailey et al., 1982). In these behavioral assays, the locomotor defects of the Cmg null flies could only cause an underestimate of behavioral defects since "failure" in the assay is signified by an increase in courtship activity. Wild-type, Cmg null, and *cmg* deficiency heterozygotes were tested for mated female conditioning. All genotypes were normal for associative memory formation when compared to males that had been sham conditioned in an empty chamber (Figure 7A, right panel). Behavior during the training period, however, was abnormal; Cmg null males failed to decrease courtship during training (Figure 7A, left panel, $p < 0.0003$). Deficiency heterozygotes were intermediate; *Df(3)x313/+* was significantly different from Cmg null ($p < 0.0003$), and *Df(3)x307/+* was significantly different from wild-type ($p < 0.0003$). To determine if the Cmg null had a habituation defect, we examined the response to immature males, who emit courtship promoting pheromones distinct from the female type. Cmg null animals failed to habituate (Figure 7B, $p < 0.0002$). Courting Cmg null males had normal initial courtship levels and ability to discriminate between virgin and mated females, indicating that peripheral sensory pathways were intact (see Experimental Procedures).

Discussion

Camguk Regulates a Pool of Ca^{2+}/CaM Unresponsive CaMKII

Cmg, a MAGUK protein with an N-terminal CaMKII-like domain, can bind to CaMKII and regulate its activity.

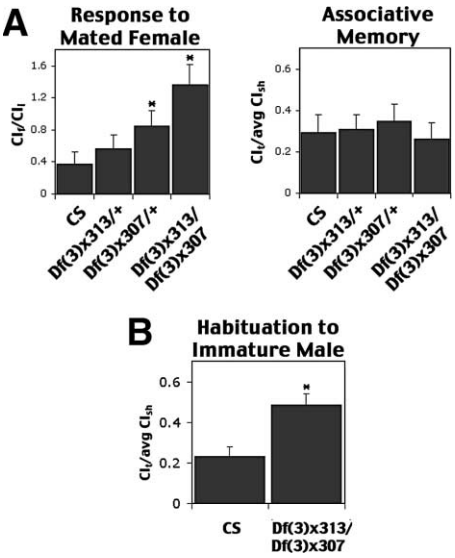

Figure 7. Camguk-Deficient Flies Do Not Habituate Normally

(A) Courtship conditioning using a mated female trainer and an anesthetized virgin female tester. The response to the mated female during training is measured as the Courtship Index (CI) of the last 10 min of training (CI_f), divided by the CI of the initial 10 min (CI_i) of the 1 hr training period. Values can range from 0 to 1, with higher values indicating poorer performance. For wt males, a value of $\leq$0.5 is normal. wt is significantly different from *Df(3)x307/+* and *Df(3)x313/Df(3)x307* (indicated by *), and *Df(3)x313/+* is significantly different from *Df(3)x313/Df(3)x307* (indicated by *) by one-way ANOVA [$F_{(3,80)} = 6.9127$, $p < 0.0003$; Tukey-Kramer, $\alpha = 0.05$]. Associative memory is measured as the ratio of CI during the test period (CI_t) to average sham CI (CI_{sh}) [one-way ANOVA, $F_{(3,65)} = 0.2568$, $p > 0.85$]. Higher values indicate poorer performance. For wt males, a value of $\leq$0.5 is normal.

(B) Habituation test, following 30 min training with an immature male. Habituation was measured as the ratio of CI_t to average CI_{sham} and was significantly disrupted in Cmg nulls [t Test, $F_{(1,54)} = 15.5475$, $p < 0.0002$].

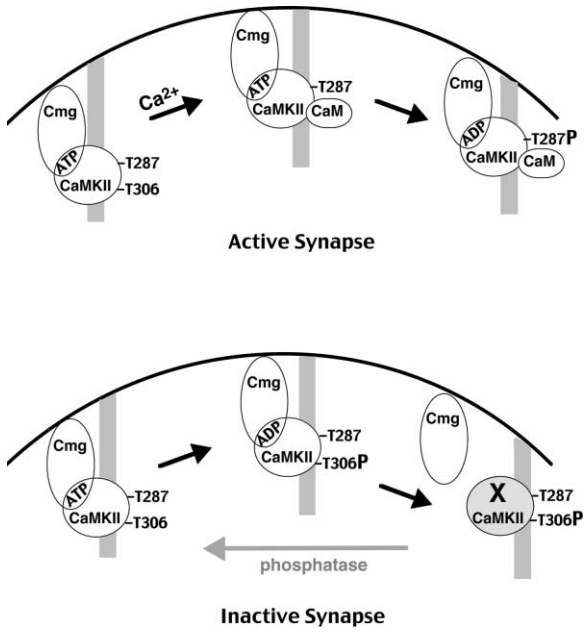

Figure 8. Model for Regulation of CaMKII Activity by Cmg

In the postsynaptic cell, basal ATP levels, which are in the millimolar range, allow CaMKII to bind to Cmg. The gray bar represents synaptic binding partners of CaMKII that have Cmg-independent interactions with the kinase. (A) At synapses that are active and have available Ca^{2+}/CaM, CaMKII complexed to Cmg is active and can undergo autophosphorylation at T287. T306 remains unphosphorylated because it is protected by bound CaM. Once the kinase is autophosphorylated at T287, CaM is trapped and remains associated with the complex even after Ca^{2+} levels have gone down. (B) At inactive synapses, CaMKII complexed to Cmg undergoes autophosphorylation at T306. This releases CaMKII from Cmg in a form which cannot be activated by Ca^{2+}/CaM until it is dephosphorylated.

Association of Cmg with CaMKII is stimulated by ATP bound to the CaMKII catalytic domain. At physiological levels of ATP, Cmg and CaMKII are likely to be associated. ATP hydrolysis in the presence of Ca^{2+}/CaM leads to autophosphorylation of CaMKII on T287 and production of a Cmg-localized constitutively active kinase. Cmg can also promote ATP hydrolysis by CaMKII in the absence of Ca^{2+}/CaM. Under these conditions and without prior T287 autophosphorylation, CaMKII preferentially autophosphorylates its own CaM binding domain. This reaction leads to the fast release of the CaMKII in a form that cannot be activated by Ca^{2+}/CaM. The ability of genetic manipulation of Cmg, PP2A, and synaptic activity levels in vivo to alter the amount of postsynaptic CaMKII that is in the inactivated state argues strongly that these reactions are physiologically relevant and define a new mechanism for the regulation of CaMKII activity.

What is the function of this interaction in vivo? One possible role is to provide a basis for maintaining differences between active and inactive synapses (Figure 8). The effect of *cmg* on courtship conditioning, a behavior known to require CaMKII, is suggestive of a role for this interaction in behavioral plasticity. Differences in synaptic strength can fine tune networks and are crucial to most memory models (Young, 1979). Local CaMKII inactivation by Cmg could serve to decrease the gain on CaMKII-mediated calcium signaling at synapses that have been inactive. At low Ca^{2+}, T306 autophosphorylation would occur, and inactive kinase would be released from Cmg. This provides a "use it or lose it" mechanism for preserving synapse-specific strength differentials, since CaMKII tethered to Cmg at synapses that see higher Ca^{2+} levels would be protected from inactivating autophosphorylation by bound CaM. This complexed kinase can undergo T287 autophosphorylation, resulting in constitutive kinase activity and a slowed CaM dissociation rate (Meyer et al., 1992) that would further prolong the lifetime of the complex. Whether the Cmg-mediated inactivation of CaMKII operates locally or globally remains to be determined and likely depends on the ability of the inactivated kinase to escape from complexes with its other binding partners in the postsynaptic density.

Establishment of a pool of autophosphorylated CaMKII that cannot be directly stimulated by Ca^{2+}/CaM also provides a mechanism for phosphatase activity to regulate CaMKII. In vitro, both PP1 and PP2A are able to dephosphorylate the CaM binding domain of CaMKII (Patton et al., 1991). In *Drosophila*, we show that PP2A is the relevant in vivo phosphatase for postsynaptic pT306. The opposing actions of Cmg and PP2A provide machinery for dynamic regulation of the maximum level of calcium-activatable CaMKII at synapses.

In mouse, knockin of a T305D mutation (Elgersma et al., 2002) blocked LTP and learning and decreased postsynaptic localization of the kinase, suggesting that this mutant was unable to interact with one or more of its normal synaptic binding partners. Inability to undergo inactivating autophosphorylation (T305VT306A knockin) was associated with a lower threshold for LTP and loss of behavioral fine tuning. These data argue that, in mammals, autophosphorylation of the CaM binding domain of CaMKII is also an important regulatory mechanism for both function and localization of CaMKII. Whether in mammals CASK (the Cmg homolog) is the critical synaptic binding partner and regulator of synaptic T305 autophosphorylation is unknown.

A Novel CaMKII Tethering Mechanism

In this study we also demonstrate a novel (to our knowledge) binding mechanism for CaMKII on a MAGUK scaffolding protein. We propose that this interaction consists of two discrete steps. First, binding of ATP to the catalytic domain of CaMKII reveals an interaction surface on the kinase that forms an initial bond to the Cmg protein. This initial interaction can occur either in the presence or absence of Ca^{2+}/CaM, implying that it does not involve the C-terminal CaM binding sequences of the autoinhibitory domain. The ability of a peptide corresponding to the N terminus of the autoinhibitory domain to partially block complex formation suggests that this region, which is known to be involved in regulation of ATP binding (Brickey et al., 1994; Colbran et al., 1989) or the catalytic domain surface that the N terminus of the autoinhibitory domain binds to, is the site of this initial interaction.

Once an initial interaction is established, bound ATP is no longer required. This could be due to a stabilization of the ATP-bound conformation by Cmg binding. Stabilization of the open form of the kinase autoregulatory domain is postulated to occur after Ca^{2+}/CaM-dependent NR2B binding, since this interaction is insensitive to CaM stripping (Bayer et al., 2001). Another possibility for the loss of ATP requirement is that a secondary interaction occurs that abrogates the need for the initial interaction. We have shown that such a secondary interaction does occur, but this does not rule out a concurrent stabilization of the ATP-bound conformation.

The secondary interaction that forms after ATP-dependent binding of Cmg involves the C-terminal region of the autoinhibitory domain, including the CaM binding sequences. Three lines of evidence suggest that this secondary interaction provides the most stable form of the complex. First, N-terminal peptides only partially block complex formation, while peptides that contain the CaM binding domain completely block complex formation. Second, CaMKIIs with mutations of T306/7 do not bind to Cmg at all. These mutants are fully capable of binding ATP, but the exposure of the initial binding domain is insufficient to maintain a salt-stable interaction in the absence of a normal CaM binding domain. The failure of the relatively subtle threonine to serine mutant to bind suggests a direct interaction of Cmg with T306/7. Third, the natural release mechanism of this complex is via autophosphorylation of the CaM binding domain. Protection of this region by Ca^{2+}/CaM can prevent both the autophosphorylation and dissociation of CaMKII.

The two-step association mechanism and the autophosphorylation-regulated dissociation of CaMKII from Cmg provide a new way in which CaMKII can be localized and regulated using its autoinhibitory domain. As defined for catalytic activation, the regulatory region of CaMKII is a small but complicated protein interaction domain. It is becoming clear that this region can support multiple intra- and intermolecular interactions that have profound effects on local CaMKII activity and synaptic plasticity.

Experimental Procedures

Antibodies

Anti-Cmg rabbit sera were generated at Foster animal facility (Brandeis) using a GST-Cmg (amino acids 152–897) as antigen. Serum was purified on a GST-Cmg coupled Affigel-15 column (Bio-Rad) and preabsorbed against *cmg* null fly embryo extract. Specificity was determined by immunoblot of Canton-S wild-type and *cmg* null flies. Anti-CaMKII rabbit polyclonal antibodies were generated as described (Koh et al., 1999; Long and Griffith, 2000). Anti-CaMKII guinea pig antiserum was made at Alpha Diagnostic International using GST-catalytic domain (amino acids 1–265) as antigen. Specificity was determined by immunoblot of purified proteins and tissue extracts. Guinea pig anti-Cmg was a gift from Peter Bryant (UC Irvine). Anti-CaMKII T306/7 phosphospecific antiserum was provided by Alcino Silva (UCLA) and David Sweatt (Baylor College of Medicine). Other reagents include anti-HA mouse monoclonal antibody 12ECA5 (Roche Applied Science), anti-CaM mouse monoclonal (Upstate Biotechnology), Cy5-anti-rabbit (Jackson Immunolabs), FITC- anti-rabbit (Jackson Immunolabs), HRP-conjugated secondary antibodies (Sigma, anti-guinea pig, Amersham-Pharmacia Biotech, anti-rabbit and anti-mouse), streptavidin-HRP (Jackson ImmunoResearch Laboratories), and biotinylated CaM.

Plasmids and Plasmid Construction

For cell line expression, CaMKII and Cmg were cloned into pSRα or pcDNA3. Site-directed mutagenesis was done using QuickChange (Stratagene). For insect cells, mutant sequences were cloned into Bac-to-Bac pFastBac (Invitrogen) containing wt CaMKII (a gift of Neal Waxham, UT Houston Medical School). pGEX4T-2 plasmids were provided by Gisela Wilson (University of Michigan).

Coimmunoprecipitation from tsA201 Cells

tsA201 cells were grown in DME (Life Technologies) with 10% Cosmic calf serum (HyClone), 100 units/ml penicillin, and 100 μg/ml streptomycin. Transfection (15 μg of Cmg and/or 5.0–8.0 μg of HA-tagged CaMKII DNA) was done using calcium phosphate (Sambrook et al., 1989). Sixteen hours later, medium was replaced and dishes transferred to 25°C for 48 hr. Cells were scraped off the dishes into ice-cold RIPA (1X PBS pH 7.4, 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, fresh protease inhibitor cocktail [Roche], 1 mM DTT, 0.1 mg/ml PMSF, 1 mM Na_3VO_4, 50 mM NaF, 1 μM okadaic acid, and Ser/Thr phosphatase inhibitor cocktail I [Sigma]) for 30 min. Supernatants were collected at 14,000 $\times$ *g* at 4°C for 15 min.

For immunoprecipitations, 200 μg of supernatant was precleared with 20 μl of 50% protein A/G agarose slurry (Santa Cruz). Supernatants were incubated with 2 μg anti-HA antibody for 2 hr at 4°C followed by 20 μl of 50% protein A/G agarose overnight. Protein A/G beads were spun at 1000 $\times$ *g* for 5 min and washed five times in high-salt RIPA (0.8 M NaCl). Immunoprecipitates were eluted by boiling in SDS-PAGE loading buffer (3% SDS, 8.3% glycerol, 6.2 mM Tris [pH 8.9]) and analyzed by SDS-PAGE followed by electrophoretic transfer onto Hybond-C nitrocellulose membrane (Amersham-Pharmacia Biotech) and immunoblotting with anti-Cmg. Bands were visualized with HRP-conjugated secondary antibodies and chemiluminescence (ECL, Amersham-Pharmacia Biotech).

Fly Strains

Flies were grown on standard medium at 25°C. The *cmg* deficiency lines *Df(3R)x307* and *Df(3R)x313* (Martin and Ollo, 1996) were obtained from Gisela Wilson at University of Michigan. Null *cmg* flies came from crosses between these strains. Panneural Cmg overexpresser lines were made by crossing the *10MI UAS-Cmg* transgenic (a gift of Peter Bryant) with the *C155-GAL4* driver (Lin et al., 1994). The phosphatase mutants *PP1-87B^{1}/CyO*, *PP2A^{02496}/CyO*, and *PP2A$^{XE\text{-}2258}$/CyO* and the muscle-specific *24B-GAL4* were from the Bloomington Stock Center. *MHC-GAL4* was a gift of Graeme Davis (UCSF); *C164-GAL4* (motorneuron specific) and *BG487-GAL4* (Budnik, 1996) were provided by Vivian Budnik (U Mass, Amherst). *UAS-dORKΔC* was a gift of Todd Holmes (NYU). *BG487-GAL4* has primarily postsynaptic expression at muscles 6 and 7 and both pre- and postsynaptic expression at muscle 12 (J.J.L.H., unpublished data).

Fly Head Extracts

Adult flies were collected, frozen in liquid nitrogen, and decapitated by vortexing. Heads were collected by sieving. An equal number of frozen heads from each strain was homogenized and solubilized in ice-cold RIPA buffer for 30 min. Homogenates were spun at 500 × *g* and then 14,000 × *g* for 15 min at 4°C to remove debris. For immunoprecipitation, 150 μg protein was immunoprecipitated with 15 μg of anti-Cmg antibody, separated by SDS-PAGE, and analyzed by immunoblot.

Expression and Purification of GST Fusion Proteins

Fusion protein constructs in pGEX (Amersham-Pharmacia Biotech) were transformed into ProTet BL21 (Clontech), grown at 37°C in LB with 100 μg/ml carbenicillin, and induced with 0.4 mM IPTG at 30°C for 6 hr. GSH-sepharose purification was done according to the manufacturer's protocol (Amersham Pharmacia Biotech) except that the elution buffer was pH 8.8 and contained 50 mM GSH. Eluates were pooled and dialyzed.

Expression and Purification of Fly CaMKII

Sf21 cells (Life Technologies) were maintained in EX-Cell 400 media with 50 u/ml penicillin and 50 μg/ml streptomycin at 27°C at a density between 4 × 10^5 and 2 × 10^6. Cells were grown overnight to a density 1.3–2.0 × 10^6. CaMKII in pFastBac (InVitrogen) was used to infect cells at 0.25 ml virus/50 ml media and cells grown for 72 hr before harvest. Protein was purified using the protocol described in Putkey and Waxham, 1996. CaMKII was stored in 10 mM HEPES, pH 7.5, 0.1 mM EGTA, 100 mM KCl, and 50% glycerol at −80°C.

In Vitro Association Assay

CaMKII (1 μg) was incubated with 100 μM of either ADP, ATP, or AMP-PNP in binding buffer (20 mM sodium phosphate buffer [pH 7.2], 0.2% Triton X-100, 0.6 M NaCl, 50 mM PIPES, 15 mM $MgCl_2$, 0.1% BSA) plus 1 mM $CaCl_2$ and 3 μM CaM at 30°C for 2 min. Ice-cold EGTA (3 mM) was added at the end of 2 min incubation to unload Ca^{2+}/CaM from CaMKII, as Ca^{2+}/CaM was used initially to facilitate nucleotide loading but was not required. Immobilized GST-fusion protein beads (10 μg), prepared by crosslinking GST-fusion proteins to GSH beads covalently by DMP (Pierce), were added and incubated at 4°C for 1 hr with CaMKII. Five washes in binding buffer plus 0.8 M NaCl and 3 mM EGTA were followed by centrifugation at 500 × *g* for 5 min. Bound CaMKII was eluted by boiling in SDS-PAGE loading buffer and analyzed by SDS-PAGE and immunoblotting.

In Vitro Dissociation Assay

CaMKII was loaded with AMP-PNP, bound to Cmg beads, and washed as described above to make "preassembled" complexes. ATP/Mg^{2+}, ADP/Mg^{2+} (1 mM/15 mM), or EDTA (1 mM) was added to the CaMKII bound beads in the presence or absence of Ca^{2+}/CaM at 4°C for 10 min to exchange out AMP-PNP, and beads were transferred to 30°C for 2 or 20 min. Dissociated kinase was separated from beads by spinning at 500 × *g* for 5 min followed by SDS-PAGE and analysis using anti-CaMKII antibodies or biotin-CaM overlay.

Nucleotide Binding Assay

Purified CaMKII or GST-Cmg (5 μg) was incubated with 10 μCi of [α-^{32}P]-ATP (Perkin Elmer Life Sciences) in 50 μl binding buffer plus 1 mM DTT, 5 mM $MgCl_2$, and 5 μM ATP. For nucleotide competition, 200 μM of cold ADP and ATP were included in the incubation. After binding at 4°C for 10 min, samples were exposed to UV light at 254 nm with an energy dose of 0.9999 J/cm^2 in a Stratagene UV crosslinker and resolved by SDS-PAGE. Gels were vacuum dried, and binding of ATP to CaMKII or Cmg was visualized by autoradiography.

Peptide Competition

CaMKII autoregulatory domain peptides (amino acids 279–291 [AC3I, T287A], 281–301 [Ala, T287A], 275–310) and control peptides (AC3 rev and vasoactive intestinal peptide [VIP]) were preincubated with CaMKII in binding buffer plus 1 mM EGTA for 10 min at 4°C. GST-Cmg immobilized beads were added and incubated with CaMKII and peptides at 4°C for 1 hr. Bound kinase was eluted by boiling in 1X loading buffer and analyzed by SDS-PAGE and immunoblotting.

CaMKII Assay

Cell lysates or protein samples with normalized CaMKII immunoreactivity that was equivalent to 50 ng of purified CaMKII were assayed as described (Wang et al., 1998). Each 50 μl assay reaction contained 50 μM 1 Ci/mmol [γ-^{32}P]-ATP, 50 mM PIPES [pH 7.0], 15 mM $MgCl_2$, 50 μM AC3 peptide, 0.1 mg/ml BSA, 4.5 μM CaM, and 1 mM $CaCl_2$ or EGTA and was assayed at 30°C for 1 min.

Immunocytochemistry

Third instar larval brains and NMJs were dissected and stained as described (Park et al., 2002). All preparations within each experiment were processed in parallel and images acquired with identical setting using the 20× (for larval brain) or 40× (for muscle 12 NMJ) objective of a Leica TCS SP2 confocal scanning microscope.

For quantitation of anti-pT306 staining, preps were counterstained with goat anti-HRP conjugated to FITC (Cappel, 1:500). The fluorescence intensity of the synaptic pT306 signal was measured at individual boutons on muscle 12 (n = 64 boutons from four independent NMJs). pT306 intensity values were normalized to the HRP-FITC (Cappel) signal of the same bouton. Values from *eag^{sc29}* animals were compared to a Canton S wild-type control, and values from *BG487-GAL4/+; UAS-dORKΔC /+* were compared to *BG487-GAL4/+* controls. The mean of the control intensity was set at 100% and all values normalized to this standard. Data were collected blind to genotype and are presented as mean ± SEM.

Behavioral Assay

Behavior was observed under red lights, at 25°C, 70% relative humidity in plexiglass mating chambers (8 mm diameter, 3 mm high) with wet filter paper. For courtship conditioning, a 4- to 6-day-old male was placed with a 3- to 5-day-old Canton-S female that was mated the day before. After 1 hr of training, the male was transferred to a new chamber and observed for a 10 min test period with a CO_2-anesthetized virgin female collected that day. Habituation assays used immature (0–6 hr old) Canton-S males as trainers and testers and a training period of 30 min. Sham controls were done for all tests by training a male alone in a chamber, then testing as usual. Locomotion was measured by counting the number of times a male crossed the chamber in 3 min. Female discrimination was determined by comparing the amount of courtship of 3- to 5-day-old, CO_2-anesthetized virgins to mated females during a 10 min test period.

Courtship index (CI) is the amount of time courting divided by the time observed. Behavior during training is measured by dividing the CI for the final 10 min period of the training period (CI_f) by the CI for the initial 10 min (CI_i). Memory is calculated by taking the CI of the 10 min test period (CI_t) as a percentage of the average sham CI (CI_{sham}). Test males who copulate during training or who have a CI_i of less than 0.1 are not analyzed.

Data were subjected to a square root transformation to approximate normality and tested with one-way ANOVA with genotype as the main effect using JMP (version 3.1.5 for Macintosh). Locomotor data were normal and tested with ANOVA with genotype as the main effect using Statview (version 4.5 for Macintosh). The locomotor

indices of Canton-S (85 ± 5), *Df(3)x307/+* (78 ± 4), and *Df(3)x313/+* (81 ± 3) were significantly higher than that of *Df(3)x307/Df(3)x307* (58 ± 3) with $p \geq 0.0004$ for all comparisons [$F_{(3,92)} = 9.170$; Bonferroni-Dunn pairwise comparisons]. Female discrimination data were transformed and tested with ANOVA with genotype as the main effect ($p > 0.13$ for both virgin and mated female courtship, indicating that pheromone-sensing pathways are intact).

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