CaMKII tethers to L-type Ca^{2+} channels, establishing a local and dedicated integrator of Ca^{2+} signals for facilitation

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Ca^{2+}-dependent facilitation (CDF) of voltage-gated calcium current is a powerful mechanism for up-regulation of Ca^{2+} influx during repeated membrane depolarization. CDF of L-type Ca^{2+} channels ($Ca_v1.2$) contributes to the positive force–frequency effect in the heart and is believed to involve the activation of Ca^{2+}/calmodulin-dependent kinase II (CaMKII). How CaMKII is activated and what its substrates are have not yet been determined. We show that the pore-forming subunit α_{1C} ($Ca_v\alpha1.2$) is a CaMKII substrate and that CaMKII interaction with the COOH terminus of α_{1C} is essential for CDF of L-type channels. Ca^{2+} influx triggers distinct features of CaMKII targeting and activity. After Ca^{2+}-induced targeting to α_{1C}, CaMKII becomes tightly tethered to the channel, even after calcium returns to normal levels. In contrast, activity of the tethered CaMKII remains fully Ca^{2+}/CaM dependent, explaining its ability to operate as a calcium spike frequency detector. These findings clarify the molecular basis of CDF and demonstrate a novel enzymatic mechanism by which ion channel gating can be modulated by activity.

Introduction

Ca^{2+}-dependent facilitation (CDF) of calcium channels serves to potentiate the Ca^{2+} influx through the L-type channels during repeated activity. CDF is a feed-forward form of adaptive plasticity that is a critical regulatory feature of many excitable cells. In the heart, frequency-dependent potentiation of Ca^{2+} current through L-type channels ($Ca_v1.2$; Noble and Shimoni, 1981; Marban and Tsien, 1982; Lee, 1987; Schouten and Morad, 1989; Zygmunt and Maylie, 1990) contributes to the force–frequency relationship of cardiac contraction (Koch-Weser and Blinks, 1963). This increased contraction strength with faster heart rates contributes to the positive inotropic response during exercise (Ross et al., 1995) and is abnormal in heart failure (Feldman et al., 1988; Mulieri et al., 1992; Hasenfuss et al., 1994). In the brain, CDF of L-type channels may be important in relation to the privileged role of L-type channels in excitation–transcription coupling (Deisseroth et al., 2003). Despite these important physiological roles that are central to cardiac function and neuronal plasticity, there is little understanding of the molecular mechanism of CDF of L-type channels.

Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), a multifunctional Ser/Thr protein kinase, is a likely effector of CDF. Pharmacological inhibition of CaMKII abolishes CDF in the heart (Xiao et al., 1994; Yuan and Bers, 1994). Addition of activated CaMKII to the cytoplasmic face of cardiac myocyte membranes induces a high open-probability state of the channel that is consistent with the properties of Ca^{2+} channels displaying CDF (Dzhura et al., 2000). Further, immunocytochemical data suggest that the $Ca_v1.2$ and CaMKII are localized close to each other on the cardiomyocyte sarcolemmal membrane (Xiao et al., 1994), suggesting that the kinase has easy access to the channel.

CaMKII has structural and functional properties that make it an ideal candidate to sense the frequency of Ca^{2+} transients during neuronal firing or changes in cardiac rhythm and translate that frequency signal into activity-dependent alterations such as CDF. CaMKII is a multimeric holoenzyme composed of 12 subunits, with the subunit isoforms being derived from a family of four closely related genes (α, β, γ, and δ; Hudmon and Schulman, 2002b). In the brain, α-CaMKII has

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Abbreviations used in this paper: AIP-2, autocamtide-2–related inhibitory peptide; AKAP, A-kinase anchor protein; CaMKII, Ca^{2+}/calmodulin-dependent kinase II; CDF, Ca^{2+}-dependent facilitation; CDI, Ca^{2+}-dependent inactivation; HEK, human embryonic kidney; NMDAR, *N*-methyl-D-aspartate receptor; PKA, protein kinase A; P_o, open probability; PP1, protein phosphatase 1.

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been shown to play a key role in synaptic plasticity and learning/memory (Lisman et al., 2002). The γ and δ isoforms predominate in the heart and have been implicated in the regulation of gene expression as well as CDF (Zhang et al., 2002). In all of these isoforms, activation proceeds by Ca^{2+}/CaM binding to an autoregulatory region, which causes the removal of a pseudosubstrate domain from the catalytic site. After the initial stimulus, autophosphorylation of Thr^{286} or its equivalent (Thr^{287} in non–α isoforms) renders subsequent kinase activity independent (autonomous) of Ca^{2+} and CaM (Miller et al., 1988) and increases the kinase's affinity for CaM by >10,000-fold ("CaM trapping"; Meyer et al., 1992). These properties endow CaMKII with the ability to become persistently activated in a transition that is sharply dependent on the frequency of Ca^{2+} oscillations (De Koninck and Schulman, 1998; Eshete and Fields, 2001; Bayer et al., 2002; Bradshaw et al., 2003).

We now demonstrate for the first time that the subcellular localization of CaMKII is critical for its biological role as a frequency decoder of voltage-driven calcium spikes. We show that CaMKII phosphorylates α_{1C} and that tethering of CaMKII to the α_{1C} COOH terminus is an essential molecular feature of CDF. We present a molecular model for CDF in which a dedicated CaMKII holoenzyme acts as both a local sensor to monitor Ca^{2+} channel activity and as a resident kinase effector to regulate Ca^{2+} channel activity.

Results

The NH_2 and COOH termini of α_{1C} are substrates of CaMKII

Modulation of L-type channel gating by cytoplasmic delivery of constitutively active CaMKII is blocked by nonhydrolyzable analogues of ATP (Dzhura et al., 2000), suggesting that the kinase acts through phosphorylation of the channel or an associated regulatory protein. Because the kinase-induced increase in L-type Ca^{2+} current by both protein kinase A (PKA) and Src results from phosphorylation of α_{1C} (De Jongh et al., 1996; Bence-Hanulec et al., 2000), we first tested whether α_{1C} was also a substrate for activated CaMKII (Fig. 1 A). The addition of activated CaMKII to α_{1C} immunoprecipitated from lysates of L-type channel–expressing human embryonic kidney (HEK) cells resulted in the phosphorylation of protein migrating at ~240 kD, consistent with the molecular mass of α_{1C}. The kinase activity could be attributed to CaMKII and not to another kinase coimmunoprecipitated with α_{1C} because inclusion of the CaMKII inhibitor autocamtide-2–related inhibitory peptide (AIP-2) prevented phosphorylation; continued presence of the α_{1C} protein under this condition was confirmed by immunoblotting (Fig. 1 A, bottom). The immunoprecipitated and phosphorylated protein could be confidently identified as α_{1C} in light of the findings that no α_{1C} was immunoprecipitated and that ^{32}P was not incorporated when immunoprecipitation was performed with control IgG or with lysates of HEK cells in which α_{1C} had not been expressed. Interestingly, under conditions in which α_{1C} was phosphorylated by CaMKII (Fig. 1 A, lane 3), we noticed a ^{32}P-labeled protein (~50 kD) corresponding to the autophosphorylated form of the α subunit of CaMKII

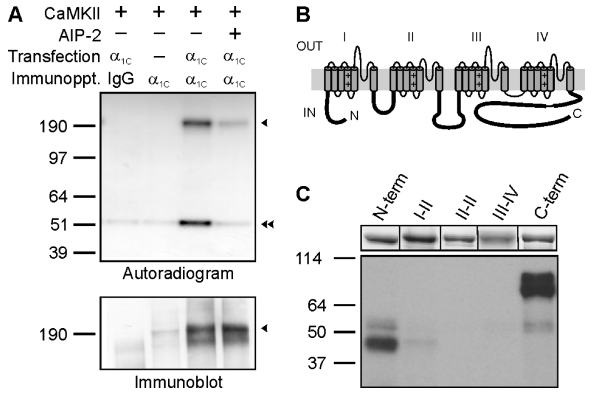

Figure 1. **Phosphorylation of the α_{1C} subunit by CaMKII.** (A) Autoradiogram showing phosphorylation of α_{1C} by CaMKII. Lysates from HEK cells transfected with α_{1C} and β2b (lanes 2–4) or nontransfected cells (lane 1) were immunoprecipitated with an anti-α_{1C} antibody (lanes 1, 3, and 4) or control IgG (lane 2) and then incubated with purified α-CaMKII in the presence of Ca^{2+}/CaM and Mg^{2+}/ATP^{32} as described in Materials and methods. 200 nM of the CaMKII inhibitor peptide AIP-2 (Calbiochem) was included (lane 4) to demonstrate kinase specificity. Phosphorylated α_{1C} is indicated by an arrowhead; autophosphorylated CaMKII, retained after the kinase reaction despite extensive washing of the immunoprecipitate, is indicated with a double arrowhead. An anti-α_{1C} immunoblot of the samples used in the kinase reaction is shown below the autoradiogram. (B) Schematic of α_{1C}. Thick black lines highlight regions used to generate GST fusion proteins. (C) GST fusion proteins enriched from bacterial lysates using glutathione–sepharose were incubated with purified α-CaMKII in the presence of Ca^{2+}/CaM and Mg^{2+}/ATP^{32} as described in Materials and methods. After extensive washes, proteins were eluted using SDS-PAGE sample buffer. Autoradiogram of fusion proteins separated by SDS-PAGE after phosphorylation by CaMKII. C-term refers to the more distal COOH-terminal fusion protein, containing aa 1669–2171. Above the autoradiogram is the Coomassie blue–stained band for each fusion protein, indicating nearly equal loading of substrate for all fusion proteins.

that had been introduced for the kinase assay. Immunoblots with an anti-CaMKII antibody confirmed its identity (not depicted, but see Fig. 5 E). The retention of CaMKII, despite extensive washing of the immobilized α_{1C}, suggested that α_{1C} may serve as an anchoring protein as well as a substrate for the kinase. The near absence of retention when AIP-2 was added to the reaction gave an early indication about the mechanism of anchoring (see Fig. 6 B).

Having demonstrated that α_{1C} was a CaMKII substrate, we next ran tests to determine which of the intracellular domains of α_{1C} were phosphorylated by CaMKII. GST fusion proteins were generated for the entire sequence of each of the intracellular domains of the α_{1C} subunit except the large cytoplasmic tail, which was represented by two complementary fragments (aa 1507–1622 and 1669–2171; Fig. 1 B). When the fusion proteins were tested in an in vitro kinase assay, significant incorporation of ^{32}P was only observed for the NH_2-terminal construct and the COOH-terminal fusion protein containing aa 1669–2171 (Fig. 1 C) and not the fusion protein containing aa 1507–1622 (not depicted). The finding that CaMKII can phosphorylate NH_2- and COOH-terminal regions of α_{1C} is provocative in light of previous data suggesting that these regions may be targets of kinase action for modulation of $Ca_v1.2$ function (Rotman et al., 1995; Bence-Hanulec et al., 2000; McHugh et al., 2000). Similar to results with the intact channel (Fig. 1 A), we again noticed in multiple lanes an ~50-kD ^{32}P-labeled

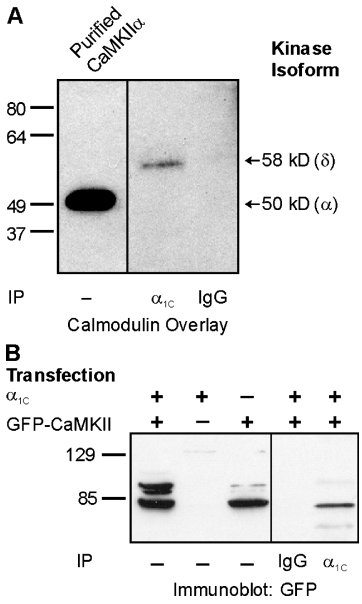

Figure 2. **CaMKII coimmunoprecipitates and colocalizes with α_{1C}.** (A) Biotinylated calmodulin overlay of rat cardiac sarcolemmal membranes after immunoprecipitation with an anti-α_{1C} antibody. Purified α-CaMKII was run as a control to demonstrate effectiveness of CaM overlay. An anti-α_{1C} antibody (but not control IgG) coimmunoprecipitated a protein identified as the δ isoform of CaMKII by biotinylated CaM overlay and apparent molecular mass. (B) Anti-GFP immunoblot after immunoprecipitation of GFP-CaMKII by control IgG (lane 4) or anti-α_{1C} antibody (lane 5) from lysates of HEK 293 cells transiently transfected with GFP-CaMKII and α_{1C}.

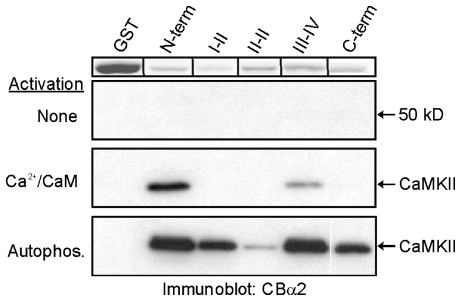

Figure 3. **Activity-dependent interaction of CaMKII with the cytoplasmic determinants of α_{1C}.** Immunoblots using an mAb (CBα2) for CaMKII after a GST pull-down assay with 20 nM of native (top), Ca^{2+}/CaM-activated (middle), or Ca^{2+}/CaM/autophosphorylated α-CaMKII (bottom). GST fusion proteins contained various cytoplasmic regions of α_{1C} just as in Fig. 1 C. Panel above the immunoblots shows a representative Ponceau stain of each fusion protein. Although only one Ponceau staining profile is shown, all blots were run in parallel, and equal loading of all fusions proteins was independently verified.

protein corresponding to the autophosphorylated form of the α subunit of CaMKII that had been introduced for the kinase assay. The finding that α-CaMKII could be retained by individual domains of α_{1C} suggested that these domains might contribute to the kinase anchoring to the channel subunit as a whole.

CaMKII interacts specifically with α_{1C}

We tested the possibility that CaMKII tethers to α_{1C} in the rat heart by attempting to coimmunoprecipitate CaMKII with α_{1C} (Fig. 2 A). An anti-α_{1C} antibody (but not control IgG) coimmunoprecipitated an ~58-kD protein from a rat heart that was easily detectable with a biotinylated calmodulin overlay, which was consistent with the properties of δ-CaMKII. Tethering of the kinase to the pore-forming subunit was further evaluated in experiments with HEK 293 cells coexpressing GFP-tagged CaMKII and Xpress-tagged α_{1C}, along with the calcium channel accessory subunits $\alpha_2\delta$ and β_2 (Fig. 2 B). We observed coimmunoprecipitation of the GFP-CaMKII by the antibody to epitope-tagged α_{1C} (Fig. 2 B, lane 5), but not by a control IgG (lane 4).

Activity-dependent association of CaMKII with multiple cytoplasmic regions of α_{1C}

To further define the interaction between CaMKII and α_{1C}, we constructed a pull-down binding assay using various α_{1C}-GST fusion proteins (Fig. 3). The goal was to find out whether a direct interaction could be observed in vitro and whether different activation states of CaMKII modulated binding. When CaMKII was activated with Ca^{2+}/CaM but not allowed to undergo autophosphorylation (ATP not included), the kinase bound to the NH_2-terminal domain and the III-IV loop of α_{1C} (Fig. 3, middle). Subsequent removal of Ca^{2+}/CaM in the wash buffer reversed this binding (unpublished data). When CaMKII was activated in the presence of Ca^{2+}/CaM plus ATP on ice, conditions previously shown to produce predominantly Thr^{286} autophosphorylation (Lai et al., 1987; Lou and Schulman, 1989; Ikeda et al., 1991), CaMKII again bound to the NH_2 terminus and III-IV loop, but additionally bound to the I-II loop and the COOH terminus (Fig. 3, bottom). In contrast, CaMKII did not bind to any of the cytoplasmic region–containing GST fusion proteins in the absence of activating stimuli (Fig. 3, top). We concluded that the initiation of a direct interaction between CaMKII and α_{1C} requires activation of the kinase by Ca^{2+}/CaM. A subsequent activation state, that produced by autophosphorylation, was necessary for binding to additional cytoplasmic regions of α_{1C}.

To identify novel structural determinants of α_{1C} that functionally affect CDF, we initially focused on the COOH terminus. This region displays an appropriate combination of attributes for CaMKII-mediated CDF: it is a target for phosphorylation by CaMKII (Fig. 1 C); it binds preferentially to autophosphorylated CaMKII (Fig. 3), a state of the kinase capable of supporting facilitation of single channels (Dzhura et al., 2000); and it has been implicated in Ca^{2+}-dependent modulation of channel function (Hell et al., 1995; Zühlke et al., 1999; Gao et al., 2001). To delimit the locus of CaMKII binding within the COOH-terminal tail of α_{1C}, we used a series of GST fusion proteins corresponding to different portions of this region (Fig. 4 A). We found a pattern of interactions with autophosphorylated CaMKII that suggested that the kinase bound between residues 1622 and 1669 of α_{1C}. Because a weak interaction was also seen with a construct proximal to 1622, we generated a fusion protein spanning aa 1581–1690 for additional testing and found a clear interaction (Fig. 4 A). To further narrow down the CaMKII interaction site within this 110-aa region, we probed its interaction with autophosphorylated CaMKII and as-

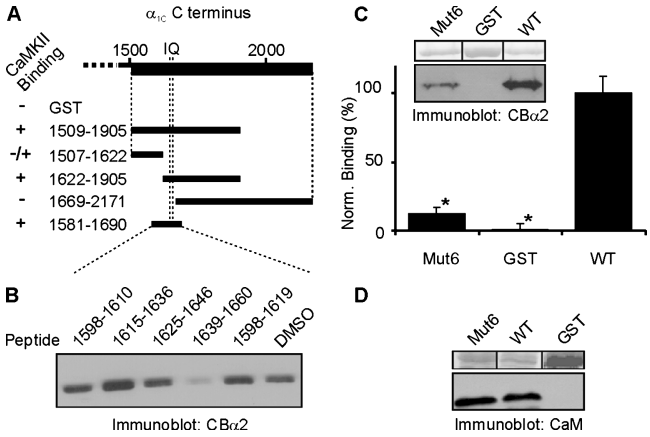

Figure 4. **Localization of the CaMKII binding site on the COOH terminus of α_{1C}.** (A) Diagram of α_{1C} fusion proteins used in GST pull-down assays with autophosphorylated α-CaMKII, exhibiting robust (+), partial (±), and no (−) binding. (B) Immunoblot with CBα2 after GST pull down of 20 nM of purified autophosphorylated α-CaMKII, using α_{1C} aa 1581–1690 fused to GST. Pull-down assay performed in the presence of 40 μM of the indicated peptide or the peptide diluent DMSO. (C) Quantification after immunoblot with CBα2 of GST pull-down assays of purified autophosphorylated α-CaMKII, using α_{1C} aa 1581–1690 (wild type [WT]), a ^{1644}TVGKFY^{1649} → EEDAAA mutant (Mut6), or GST alone shows that Mut6 blocks CaMKII binding. Panel above the immunoblots shows a representative Ponceau stain of each fusion protein. *, $P < 0.001$ for a one-way analysis of variance followed by Dunnett's test to identify specific pair-wise differences between the means for Mut6 versus WT and GST versus WT (n = 4–8). Inset shows an exemplar immunoblot with CBα2. (D) An exemplar immunoblot with an anti-CaM antibody, showing that CaM binding is not affected by the Mut6 mutation. Panel above the immunoblots shows a representative Ponceau stain of each fusion protein.

sessed interference by a series of ∼22 overlapping aa peptides (Fig. 4 B; Pitt et al., 2001). A peptide generated from residues 1639–1660 dramatically reduced the interaction of the kinase with the 1581–1690 fusion protein. In contrast, the CaMKII interaction was not inhibited by two peptides (1589–1610 and 1615–1636) that corresponded to sites important for tethering of apoCaM (Pitt et al., 2001; Kim et al., 2004). One stretch of six residues within the 1639–1660 peptide, TVGKF($^{Y}/_{I}$)A, was identified as being nearly identical in α_{1C} and α_{1A} ($Ca_v2.1$), the pore-forming subunit of P/Q-type Ca^{2+} channels, which display their own form of CDF (Lee et al., 1999; DeMaria et al., 2001). Accordingly, we constructed an α_{1C} fusion protein containing the amino acids EEDAAA in place of TVGKFY within an otherwise wild-type sequence of residues 1581–1690 (Mut6). CaMKII binding to the Mut6 fusion protein was reduced by 87.3 ± 4.5% relative to binding to wild-type 1581–1690 fusion protein (Fig. 4 C). In contrast, the same amino acid substitution left CaM binding to this mutant fusion protein unaffected (Fig. 4 D).

Disruption of CaMKII binding to the COOH terminus of α_{1C} prevents CDF

We then tested whether this site was critical for CDF by introducing the Mut6 mutation into α_{1C} subunits of L-type channels expressed in *Xenopus laevis* oocytes. Because L-type channels also display a strong Ca^{2+}-dependent inactivation (CDI) process that could diminish our ability to detect facilitation, we sought conditions under which CDF could be observed without the counteraction of CDI. Fortunately, robust CDF during trains of depolarizing pulses can be obtained by means of a point mutation within the IQ motif (I1654A; Zühlke et al., 1999, 2000) that eliminates CDI. In this setting, the Mut6 modification of the CaMKII interaction site completely abolished CDF (Fig. 5, A–C). There was no potentiation of I_{Ca} at any point during the train of 40 successive depolarizations within the entire range of frequencies tested (0.5–3.3 Hz). Abolition of the Ca^{2+}-dependent facilitatory process was also observed in experiments using a two-pulse protocol and finely graded changes in interpulse interval. Ca^{2+} currents evoked by the second pulse averaged 110% of those elicited by the first pulse at a time interval when the peak Ba^{2+} current had only recovered to ∼95% (Fig. 5 D). A comparable difference between recovery of Ca^{2+} and Ba^{2+} currents was seen in wild-type α_{1C} (Zühlke et al., 1999, 2000) but was likewise abolished by the Mut6 modification (unpublished data). Thus, in both of the approaches used to assess facilitation—potentiation of I_{Ca} during trains of depolarizations and recovery from the aftereffects of a single pulse—CDF was abolished by mutation of the COOH-terminal CaMKII interaction site on $Ca_v1.2$.

To examine the mechanism by which the mutation prevented CDF, we tested whether the Mut6 channel was still a substrate for CaMKII using an in vitro assay like that in Fig. 1, in which the availability of kinase for phosphorylation was not limited and not dependent on tethering to the COOH terminus. Disruption of the CaMKII binding site on the COOH terminus by the Mut6 substitution did not reduce ^{32}P incorporation into α_{1C} (Fig. 5 E), suggesting that the α_{1C} retained its ability to undergo phosphorylation. Together, these data support the hypothesis that CDF depends on tethering of CaMKII to this COOH-terminal site. Like the wild-type α_{1C}, the Mut6 α_{1C} displayed an interaction with autophosphorylated (^{32}P-labeled) CaMKII. The retention of kinase binding was not surprising in light of the multiple sites on α_{1C} for CaMKII interaction that we had previously identified (Fig. 3).

The CaMKII binding site for the COOH terminus of α_{1C} is conserved among multiple CaMKII isoforms and localizes to the catalytic domain

Although we had used α-CaMKII, the predominantly brain-enriched isoform studied in the preceding in vitro experiments, there are several other CaMKII isoforms that differ in their cellular and subcellular distributions (Hudmon and Schulman, 2002a). The δ isoform, the major CaMKII isoform in the heart (Edman and Schulman, 1994), was of particular interest (Fig. 2 A). Accordingly, we examined the generality of CaMKII interactions with the COOH-terminal tail of α_{1C} across a range of isoforms. The α, β, γ_B, δ_A, and δ_C isoforms were transiently expressed in HEK 293 cells for use as source material in pull-down assays and detected by the sensitive calmodulin overlay technique (Glenney and Weber, 1983; Fig. 6 A). In the absence of autophosphorylation, no binding was ever observed for any of the isoforms tested (unpublished data). However, once autothiophosphorylated, robust binding to the α_{1C} COOH-terminal

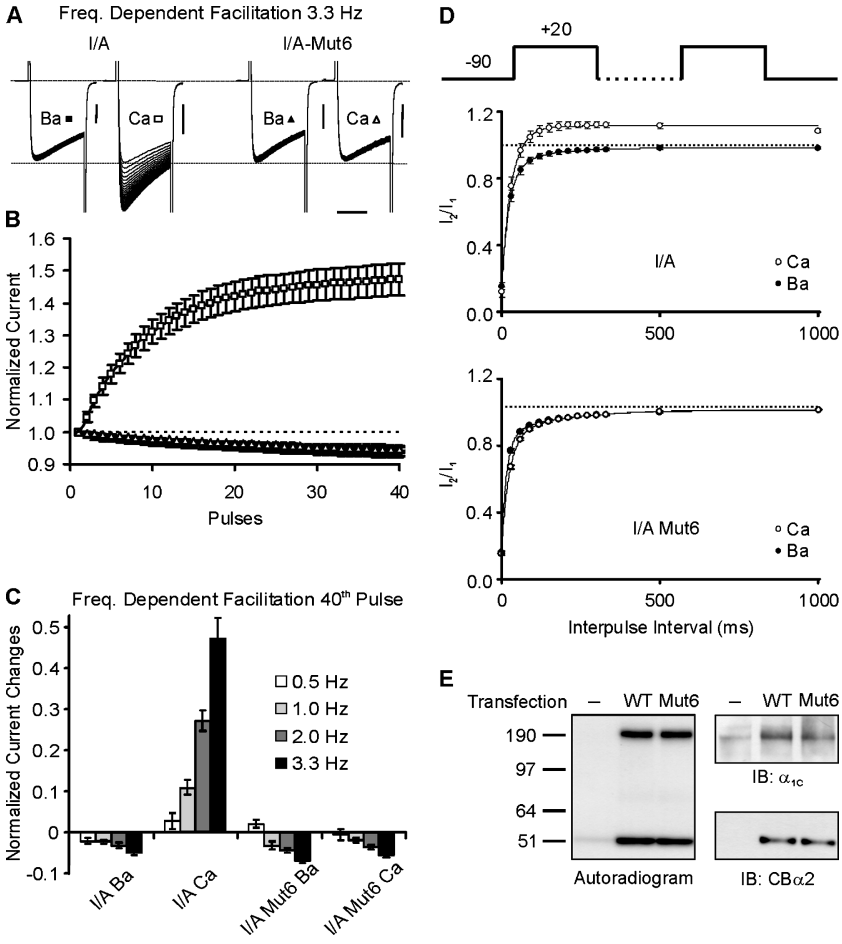

Figure 5. **CaMKII interaction with the COOH terminus of α_{1C} is essential for CDF.** (A) I_{Ba} and scaled I_{Ca} traces during a train of 40 test pulses of V_h from –90 mV to +20 mV at 3.3 Hz recorded from oocytes expressing α_{1C} I1654A (I/A) or α_{1C} I1654A/^{1644}TVGKFY^{1649} → EEDAAA (I/A-Mut6). Bars, 500 nA and 25 ms. (B) Peak I_{Ba} and I_{Ca} during trains of 40 repetitive test pulses at 3.3 Hz, normalized to the current amplitude at the beginning of each train (n = 4–5). Values indicate means ± SEM. (C) Changes in peak I_{Ba} and I_{Ca} conducted by α_{1C} I1654A (I/A) or α_{1C} I1654A/^{1644}TVGKFY^{1649} → EEDAAA (I/A-Mut6) at indicated stimulation frequencies (n = 4–5) Values indicate means ± SEM. (D) Summary of the recovery from inactivation after a two-step protocol for I/A and I/A-Mut6. The length of the prepulse was individually determined for each oocyte to produce ~75–90% inactivation. (E) Autoradiograph showing phosphorylation of wild type (WT) or mutant α_{1C} (Mut6) by CaMKII, performed as in Fig. 1 A. An anti-α_{1C} immunoblot of the samples used in the kinase reaction confirmed similar expression levels of the WT and mutant α_{1C} subunits. An anti-CaMKII immunoblot with CBα2 confirmed the identity of the retained 50-kD ^{32}P-labeled protein as α-CaMKII.

tail was observed for each of these CaMKII isoforms, with the sole exception of γ_B-CaMKII. Thus, the capability of interaction with $Ca_v1.2$ is a widespread property of the CaMKII family, including the α/β and δ isoforms prevalent in brain and cardiac tissue.

Where is the binding site for α_{1C} on CaMKII? The conserved nature of the α_{1C} binding site between brain and cardiac CaMKII isoforms favored a binding site that is conserved among the different kinase isoforms. We examined the conserved catalytic domain of α-CaMKII, based on a recent report describing its interaction with the COOH terminus of the NR2B subunit of the neuronal *N*-methyl-D-aspartate receptor (NMDAR; Bayer et al., 2001). Indeed, binding of the COOH-terminal tail of α_{1C} to autophosphorylated CaMKII was blocked by a peptide modeled after the CaMKII binding site of the NR2B subunit (NR2B peptide; Fig. 6 B). Further, binding of α_{1C} to CaMKII was potently blocked by peptides designed around Thr^{286} and the autoregulatory domain of CaMKII, including the peptide substrate AC-2 and the peptide inhibitor AC-3i (Fig. 6 B), as well as AIP-2 (Fig. 1 A). As expected, the control peptide AC-3c had no effect on binding. Both sets of observations resemble previous findings using peptide inhibition to study binding of CaMKII to NR2B (Strack et al., 2000; Bayer et al., 2001). A logical conclusion is that similar or identical molecular determinants on CaMKII are responsible for binding either to α_{1C} or to NR2B. The NR2B sequence that was

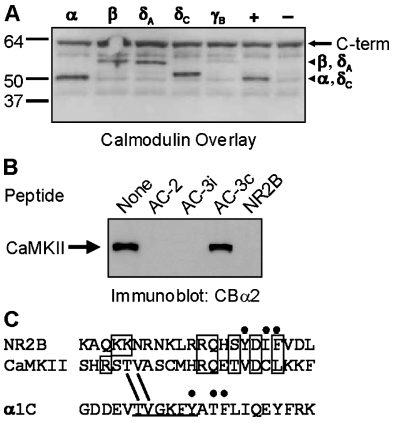

Figure 6. **The binding site for the COOH terminus of α_{1C} on CaMKII is localized near the catalytic domain.** (A) Biotinylated CaM overlay of GST pull downs, using a fusion protein from the COOH terminus of α_{1C} (aa 1509–1905) on lysates of HEK 293 cells transiently transfected with the CaMKII isoforms (α, β, δ_A, δC, and γ_B; arrows) after thioautophosphorylation. In lanes 6 and 7, lysates of untransfected cells were run with (+) and without (–) purified thiophosphorylated α-CaMKII added to the lysate. (B) Immunoblot using an mAb (CBα2) for CaMKII after GST pull downs, using a fusion protein from the COOH terminus of α_{1c} (aa 1509–1905) and 20 nM of purified autophosphorylated α-CaMKII. In addition, 20 μM of the indicated peptide was added to each binding reaction. (C) Sequence alignment of CaMKII binding sites from the COOH termini of NR2B and α_{1C} with the autoregulatory domain from α-CaMKII.

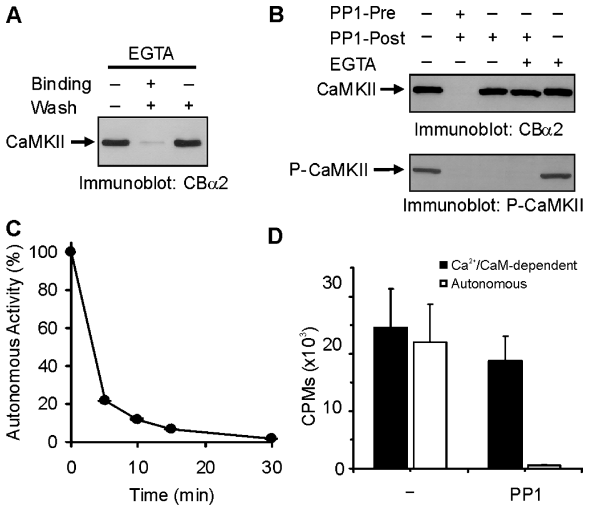

Figure 7. **CaMKII interaction with the COOH terminus of α_{1C} is not reversed by dephosphorylation or CaM dissociation, and tethered CaMKII requires autophosphorylation or Ca^{2+}/CaM for activity.** (A and B) Immunoblots with CBα2 or a phosphospecific CaMKII mAb after GST pull-down assays, using α_{1C} aa 1509–1905 and 20 nM of autophosphorylated α-CaMKII. (A) 5 mM EGTA was present in the binding reaction and/or in the wash. (B) Purified recombinant PP1 was added before (PP1-Pre) or after (PP1-Post) the binding reaction in the presence or absence of 5 mM EGTA, as indicated. (C) Time course of reversal of CaMKII autonomous activity after PP1 treatment in solution ($n = 4$). (D) Activity measurements, using peptide AC-2 as a substrate, of CaMKII recovered in GST pull-down assays, using α_{1C} aa 1509–1905. Ca^{2+}/CaM-dependent and autonomous activity measurements of CaMKII recovered after treatment with recombinant PP1 for 30 min (PP1) or no treatment (−) in the binding assay ($n = 4$) Values indicate means ± SD.

found to support interaction with CaMKII closely resembles the autoregulatory domain of CaMKII surrounding Thr^{286} (Fig. 6 C; Bayer et al., 2001). In turn, both of these stretches of amino acids show significant resemblance to the region of α_{1C} that we identified as critical for CaMKII interaction by peptide competition (Fig. 4 B), and that includes the TVGKFY sequence that was altered to the detriment of the α_{1C}–CaMKII interaction. Although the corresponding regions of α_{1C} and NR2B display points of sequence similarity (Fig. 6 C, dots and dashes), the overall degree of homology is limited.

CaMKII binding to the COOH terminus of α_{1C} produces a dedicated Ca^{2+} sensor

The functional nature of the channel–kinase interaction could follow one of several possible scenarios. During recurrent rises and falls in Ca^{2+}, the enzyme might cycle on and off the channel. Alternatively, CaMKII might remain anchored to α_{1C} with its activity persistently switched on, like CaMKII associated with the NMDAR (Bayer et al., 2001). Finally, CaMKII might stay tethered to the α_{1C} subunit, like PKA associated with $Ca_v1.2$ through A-kinase anchor protein (AKAP; Tavalin et al., 1999), but with kinase activity modulated by local changes in Ca^{2+}/CaM, similar to the way that PKA is regulated by cAMP for β-adrenergic modulation (Gao et al., 1997). To explore these possibilities, we tested whether CaMKII dissociated from the COOH-terminal tail on reversal of the Ca^{2+} elevation or the kinase activation that initially drove the interaction.

When the Ca^{2+} chelator EGTA was added immediately after the preautophosphorylation reaction, the binding of CaMKII to the α_{1C} COOH-terminal tail was inhibited (Fig. 7 A). In contrast, once autophosphorylated CaMKII had bound to the α_{1C} COOH-terminal tail, EGTA in the wash buffer (two or three rounds of washing, each lasting ~5 min) failed to dissociate the kinase (Fig. 7 A). Dephosphorylation of autophosphorylated CaMKII with protein phosphatase 1 (PP1) before presenting the kinase to the α_{1C} COOH-terminal fusion protein prevented binding (Fig. 7 B). However, dephosphorylation of CaMKII after binding did not. Even the combination of post-binding dephosphorylation and EGTA application failed to reverse binding (Fig. 7 B). In control experiments, immunoblotting with the phosphospecific antibody indicated that Thr^{286} had been completely dephosphorylated by PP1 treatment after the initial kinase binding (Fig. 7 B). Thus, although Ca^{2+}/CaM and autophosphorylation were necessary for CaMKII to bind to the α_{1C} COOH terminus, the same conditions were no longer required to sustain the interaction.

Tethered CaMKII retains its dependence on Ca^{2+}/CaM for activity

Because the CaMKII binding for both α_{1C} and NR2B appears to localize to the catalytic domain of the kinase, we asked whether α_{1C} binding to CaMKII regulates its kinase activity, as in the case of NR2B. When bound to NR2B, CaMKII remains active in phosphorylating substrates even in the absence of Ca^{2+}/CaM and autophosphorylation (Bayer et al., 2001). To determine how CaMKII is regulated when it is stably bound to the α_{1C} COOH terminus, we examined the Ca^{2+}/CaM-dependent and -independent (autonomous) activity after PP1 treatment. Dephosphorylation by PP1, assessed by tracking the loss of autonomous activity for soluble kinase, was complete within 30 min (Fig. 7 C). Under similar conditions, we observed that treatment of α_{1C}-bound kinase with PP1 completely eliminated autonomous activity (remaining activity was 1.2 ± 0.6% of that without PP1 treatment; Fig. 7 D). Thus, autonomous activity of bound CaMKII was not maintained merely by interaction of the kinase with the α_{1C} COOH terminus but depended strictly on CaMKII autophosphorylation. After PP1 treatment, tethered CaMKII could be reactivated by Ca^{2+}/CaM. In these respects, CaMKII binding to α_{1C} or to NR2B had very different effects on the activity of the kinase. As mentioned in Discussion, the association of CaMKII to the α_{1C} COOH terminus is well suited to localize the kinase in close proximity to its regulatory target but not to keep the kinase constitutively active.

Discussion

CDF is a powerful positive feedback mechanism that allows excitable cells such as myocytes and neurons to modulate Ca^{2+} entry through Ca^{2+} channels according to the previous pattern of repetitive activity. The functional consequences are clearest in the heart, where CDF of L-type channels is required for sinoatrial pacemaker activity (Vinogradova et al., 2000) and contributes to the myocardial force–frequency relationship (Koch-Weser and Blinks, 1963). However, CDF or related

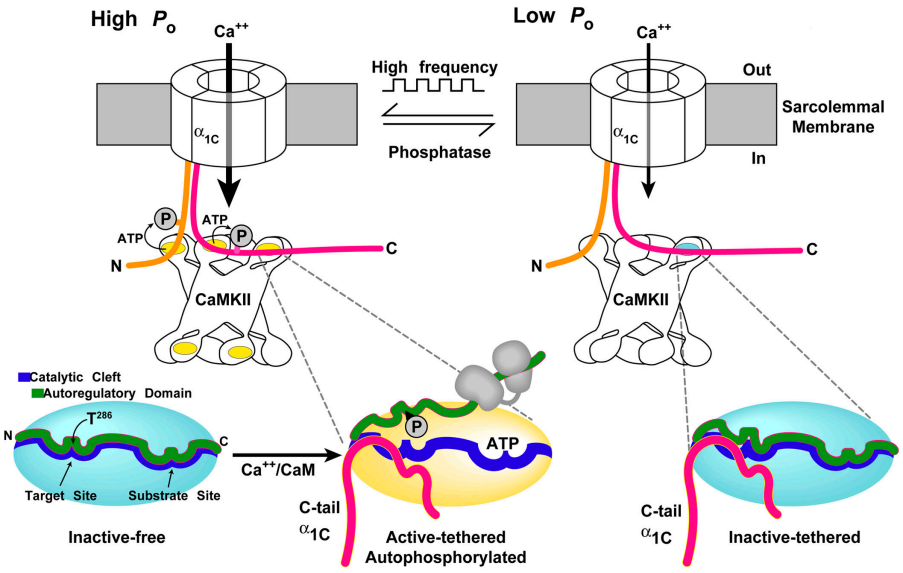

Figure 8. **Proposed mechanism of CaMKII binding to α_{1C} to form a local and dedicated Ca^{2+} spike integrator for CDF.** A catalytic core and autoregulatory domain for a prototypical CaMKII inactive subunit is shown on the bottom left (inactive is indicated by green). Ca^{2+}/CaM activation and Thr^{286} autophosphorylation displace the CaMKII autoregulatory domain within the catalytic lobe to activate the subunit (yellow) and to expose an α_{1C} tethering site. The CaMKII holoenzyme remains bound to the α_{1C} COOH terminus even after removal of the Ca^{2+}/CaM stimulus, and CaMKII dephosphorylation produces an inactive subunit. CaMKII may remain tethered to other cytoplasmic domains of α_{1C} as well. High depolarization frequencies would produce a threshold level of activated/autophosphorylated CaMKII subunits that increase the P_o of the channel via phosphorylation of the NH_2 and/or COOH termini (top left). At low depolarization frequencies and under the influence of phosphatase action, CaMKII activation would not be produced, favoring a low P_o for α_{1C} (top right).

phenomena have also been described for voltage-gated Ca^{2+} channels in neurons (Cuttle et al., 1998), smooth muscle cells (McCarron et al., 1992), and adrenal glomerulosa cells (Wolfe et al., 2002). Although not described in neurons, CDF of L-type channels could play a major role in supporting their privileged status in mediating excitation–transcription coupling and long-term synaptic plasticity (Bradley and Finkbeiner, 2002; West et al., 2002; Deisseroth et al., 2003).

We have presented several new findings that advance our understanding of CDF of L-type channels. First, CaMKII associates with the pore-forming α_{1C} subunit of L-type channels in the heart as indicated by coimmunoprecipitation. Second, specific regions of the α_{1C} subunit have the capability to directly anchor activated CaMKII. Third, CaMKII can phosphorylate α_{1C} in regions previously implicated in regulating channel function. Fourth, a mutation in the COOH terminus of α_{1C} that disrupted CaMKII binding to that region completely abolished CDF. Fifth, once tethered to the COOH terminus, CaMKII can be completely dephosphorylated and deactivated, even though it persists in its association and retains its dependence on Ca^{2+}/CaM. Thus, we conclude that the localization and targeting of CaMKII to the COOH terminus of the L-type channel is critical for CDF. Our experiments suggest that individual L-type channels can take advantage of CaMKII as a frequency detector for the activity-dependent regulation of their Ca^{2+} influx. The tethered kinase provides a local and specific integrator of preceding channel activity that controls future channel function through feed-forward autoregulation.

A working model for unifying disparate observations on CDF

Our findings provide a biochemical and molecular explanation of earlier findings that suggested that CDF was mediated by CaMKII. Ca^{2+} buffer experiments revealed that CDF depended on a calcium signal near the channel (Hryshko and Bers, 1990). Pharmacological inhibition of CaMKII abolished CDF (Anderson et al., 1994; Xiao et al., 1994; Yuan and Bers, 1994). Immunostaining showed that autophosphorylated CaMKII was concentrated near the surface membrane of cardiomyocytes (Xiao et al., 1994; Vinogradova et al., 2000). More recently, Dzhura et al. (2000) found that direct application of thiophosphorylated (constitutively activated) CaMKII to the cytoplasmic face of cardiac myocyte membranes induced a high open probability (P_o) mode of L-type channel activity, thereby accounting for CDF; the modulatory effect could be prevented by nonhydrolyzable ATP analogues or CaM kinase blockers, further implicating the importance of phosphorylation by CaMKII.

Our results not only uncover key molecular underpinnings of those earlier studies but also resolve several unanswered questions. How can a ubiquitous CaMKII fulfill the requirement for a local Ca^{2+} signal in CDF (Hryshko and Bers, 1990; Vinogradova et al., 2000)? Is autophosphorylated CaMKII concentrated near the cell surface (Xiao et al., 1994; Vinogradova et al., 2000) simply because Ca^{2+} is highest near sites of influx (Hryshko and Bers, 1990)? Is a membrane localization of CaMKII achieved by tethering to L-type channels, and is such targeting necessary for CDF? Does CaMKII mediate CDF by directly phosphorylating the pore-forming α_{1C} subunit or an auxiliary protein (Anderson et al., 1994)?

Tentative answers to these questions can be put forward in the context of a working hypothesis that emerges from our findings on L-type channel–CaMKII interactions (Fig. 8). In a quiescent excitable cell, CaMKII is free in the cytoplasm (Fig. 8, bottom left) inasmuch as the inactive form of the kinase did not significantly interact with any of the cytoplasmic regions of α_{1C}. After an initial Ca^{2+} entry, recruitment to the channel takes place in an activity-dependent manner. CaM binding to soluble CaMKII targets the kinase to certain intracellular domains of α_{1C}, and if the depolarization frequency suffices to produce CaMKII autophosphorylation on Thr^{286}, the resulting displacement of the kinase's autoregulatory domain exposes a potent

anchoring site for the α_{1C} COOH terminus (Fig. 8, bottom middle). Observations that autophosphorylated CaMKII is concentrated at the myocyte sarcolemma (Xiao et al., 1994; Vinogradova et al., 2000) can be explained at least in part by a direct interaction of the kinase with α_{1C}. Moreover, the requirement for a local Ca^{2+} signal to trigger CDF (Hryshko and Bers, 1990; Vinogradova et al., 2000) would arise if the necessary phosphorylation could only be achieved by a tethered kinase that is modulated by CaM molecules in the immediate vicinity of the channel-anchored CaMKII.

Once established, this interaction may persist even after Ca^{2+} is lowered and the kinase is completely dephosphorylated (Fig. 7 D), so that CaMKII remains tightly tethered to the channel as long as the cell is intermittently active (Fig. 8, bottom right). This scenario capitalizes on the dodecameric structure of the CaMKII holoenzyme (Kolodziej et al., 2000) by using one or more kinase subunits for the purpose of subcellular localization (Fig. 8, top left). The existence of multiple CaMKII interaction sites on α_{1C} (Fig. 3) may serve to couple the channel and the kinase more tightly and/or orient the large, dodecameric kinase for efficient phosphorylation. The securing of CaMKII in close proximity to key substrate sites on intracellular loops of the channel protein produces a high rate of channel phosphorylation and promotes a pattern of gating with high P_o (mode 2; Dzhura et al., 2000). Lowering of the frequency of Ca^{2+} influx reduces kinase activation and allows phosphatases to prevail in dephosphorylating both the channel and its associated CaMKII, driving the channel into a low P_o gating mode (Fig. 8, top right). Because the resident CaMKII can be fully dephosphorylated while remaining associated with the channel, its modulatory activity can be graded over the widest possible working range. By virtue of its position, the anchored kinase has a tremendous kinetic advantage over cytosolic CaMKII molecules and essentially monopolizes the modulatory function. Accordingly, a mutation in α_{1C} that rendered the cytoplasmic tail unable to bind CaMKII completely abolished CDF (Fig. 5). Thus, tethering of CaMKII to the COOH terminus of the channel is critical for making it competent for CDF. The combined channel–kinase complex represents a dedicated frequency detector that responds specifically to local Ca^{2+} signaling.

Looking beyond Ca^{2+} channels in surface membranes, Ca^{2+} sequestration into intracellular Ca^{2+} stores undergoes a frequency-dependent acceleration in myocardial cells, which is also critically dependent on CaMKII (DeSantiago et al., 2002). It remains unclear whether this action of CaMKII depends on activity-dependent targeting and whether frequency-dependent modulation is a common feature of Ca^{2+} signaling proteins (Maier and Bers, 2002).

Comparisons with L-type channel modulation by other kinases

The tethering of CaMKII to α_{1C} adds some unique elements to the repertoire of mechanisms used by signaling molecules to link stimulus to cellular response. The L-type channel–CaMKII interaction takes advantage of the multimeric CaMKII holoenzyme, using one or a limited number of its 12 catalytic subunits for anchoring and therefore circumventing the use of auxiliary proteins such as AKAPs or receptors for activated protein kinase C, which tether PKA or PKC, respectively (Bunemann et al., 1999; Tavalin et al., 1999; Schechtman and Mochly-Rosen, 2001; Dorn and Mochly-Rosen, 2002). Another distinction lies in the persistent tethering of CaMKII and its catalytic domains to α_{1C}. The spatial zone of catalytic activity is delimited by the distance from site of anchored subunit to most distant subunit of that holoenzyme. Dissociation of the PKA R_2C_2 complex from AKAPs leads to the immediate loss of catalytic localization once the C subunits are liberated and thereby activated over a much larger spatial volume. This mechanism is ideal for enabling catalytic subunits to diffuse from the site of activation to the nucleus (Harootunian et al., 1993) and is acceptable if β-adrenergic potentiation of L-type Ca^{2+} currents (Gao et al., 1997; Hulme et al., 2002) requires rapid responsiveness but only on infrequent occasions. The persistent tethering of the CaMKII holoenzyme might be better suited for continuous operation as an integrator of L-type Ca^{2+} channel activity, endowed with briskly reversible Ca^{2+} responsiveness and dedicated to a limited number of channels.

Similarities and contrasts with CaMKII–NMDAR interactions

Like L-type channels, NMDARs are predominant Ca^{2+} entry pathways in neurons for triggering synaptic plasticity and signaling to the nucleus, and CaMKII is tethered to the NR1 and NR2B subunits of the NMDAR, so our experiments provide interesting points of comparison with previous work showing the direct binding of CaMKII to the NR2B and NR1 subunits of NMDARs (Strack and Colbran, 1998; Leonard et al., 1999, 2002; Strack et al., 2000; Bayer et al., 2001). There are telling similarities between NMDAR subunits and α_{1C} as targets for CaMKII binding. First, completely inactive CaMKII will not initiate binding to any of these subunits. Second, in both NR2B and α_{1C}, a COOH-terminal domain of the membrane protein competes with the autoregulatory domain of CaMKII for binding to the kinase, as shown by peptide competition (Fig. 6 B; Strack et al., 2000). This similarity was highlighted by the finding that a peptide based on the CaMKII binding site on NR2B prevented the kinase from interacting with the α_{1C} COOH-terminal tail (Fig. 6 B). Third, in both NR1 and α_{1C}, the site of CaMKII binding lies close to a site for CaM binding. In the C0 domain of NR1, the amino acids most critical for CaMKII binding lie three residues NH_2-terminal to those most important for CaM binding (Leonard et al., 2002). Likewise, the α_{1C} sequence implicated in the CaMKII interaction (Mut6) lies between stretches of amino acids, among them the IQ motif, that are critical for CaM tethering and effector action (Peterson et al., 1999; Zühlke et al., 1999, 2000; Pate et al., 2000; Romanin et al., 2000; Pitt et al., 2001; Erickson et al., 2003; Kim et al., 2004). Further studies will be needed to understand how the activity of the anchored CaMKII may be integrated with the Ca^{2+}-sensing properties of the CaM–IQ domain complex for regulation of L-type channel gating and for downstream signaling to nuclear cAMP response element–binding protein (Dolmetsch et al., 2001).

There are also critical functional differences between α_{1C} and NR2B in their interaction with CaMKII. Although the COOH-terminal tails of α_{1C} and NR2B use overlapping sites on CaMKII for binding, the two channels exhibit significant differences in kinase activation state requirements and in consequences of tethering. The NR2B COOH terminus displays a high-affinity interaction with CaMKII that merely requires Ca^{2+}/CaM activation of CaMKII, not autophosphorylation (Bayer et al., 2001). In contrast, the COOH terminus of α_{1C} only binds to autophosphorylated CaMKII (Fig. 3). Binding of CaMKII to NR2B alters kinase function, causing maintained kinase activity even in the absence of Ca^{2+}/CaM or autophosphorylation. This is not the case for CaMKII binding to α_{1C}; our experiments show that interaction with the α_{1C} COOH terminus does not circumvent the autoinhibitory function of the bound kinase. The contrasting properties might arise from substantial differences in the respective COOH-terminal sequences of α_{1C} and NR2B (Fig. 6 C) and might offer specific advantages appropriate to the different roles of the two channels. Establishment of sustained CaMKII activity after transient NMDAR signaling seems perfectly appropriate as a means of supporting enduring effects, e.g., long-term potentiation and long-term depression (Lisman et al., 2002). On the other hand, CDF of L-type channels would suffer a significant loss of dynamic range if the α_{1C} COOH-terminal interaction with CaMKII were to cause constitutive kinase activity. The retention of dependence on Ca^{2+}/CaM for enzymatic activity is well suited for the operation of CaMKII as a built-in integrator of the frequency of prior Ca^{2+} signaling (Hudmon and Schulman, 2002a; Maier and Bers, 2002).

Materials and methods

Oocyte recordings

The plasmid encoding the rabbit cardiac α_{1C} subunit used for expression in *X. laevis* oocytes, pCARDHE, was a gift of W. Sather (University of Colorado, Denver, CO). In vitro transcription and microinjection into *X. laevis* oocytes (provided by J. Riley and S. Siegelbaum, Columbia University, New York, NY) of α_{1C}, the auxiliary Ca^{2+} channel subunits β_1 and $\alpha_2\delta$, were performed as previously described (Zühlke et al., 2000). Before recording whole cell I_{Ba} or I_{Ca}, oocytes were injected with 25–50 nl of 100 mM BAPTA solution, pH 7.4, to minimize contaminating Ca^{2+}-activated Cl^- currents. I_{Ba} and I_{Ca} recordings were performed essentially as described previously (Zühlke et al., 2000) with a standard two-electrode voltage clamp configuration using an oocyte clamp amplifier (OC-725C; Warner Instrument Corp.) connected through a Digidata 3122A A/D interface (Axon Instruments, Inc.) to a personal computer. I_{Ba} and I_{Ca} were recorded in the same oocyte. Ionic currents were filtered at 1 kHz by an integral 4-pole Bessel filter, sampled at 10 kHz, and analyzed with Clampfit 8.1.

GST fusion proteins

PCR fragments corresponding to the a1C (available from Genbank/EMBL/DDBJ under accession no. X15539) NH2 terminus (aa 1–154), I-II intracellular loop (aa 435–554), II-III intracellular loop (aa 784–931), III-IV intracellular loop (aa 1197–1250), and two COOH-terminal fragments (aa 1581–1690 and 1669–2171) were cloned into pGEX-4T-1, and GST fusion proteins were generated. The plasmids encoding the COOH-terminal fragments CT5 (aa 1507–1622), CT12 (aa 1509–1905), and CT23 (aa 1622–1905) were provided by M. Hosey (Northwestern University, Evanston, IL).

Peptides

Peptides spanning α_{1C} residues 1581–1690 have previously been described (Pitt et al., 2001). The *N*-methyl-D-aspartate-L peptide (Bayer et al., 2001) and peptides AC-2 (Hanson et al., 1989), AC-3i (Braun and Schulman, 1995), and AC-3c (Braun and Schulman, 1995) have been described elsewhere.

Immunoprecipitation

Rat cardiac sarcolemmal membranes were provided by S.O. Marx (Columbia University, New York, NY). Immunoprecipitation was performed with either an anti-α_{1C} (Alomone) or control IgG in 150 mM NaCl, 50 mM Tris, pH 8.0, 1% Triton, and Complete protease inhibitor cocktail (Roche). After SDS-PAGE, calmodulin overlay was performed with biotin-conjugated calmodulin (STI Signal Transduction) and detected with Vectastain ABC kit (Vector Laboratories). HEK 293 cells were transfected with α_{1C}, $\alpha_2\delta$, $\beta 2$, and GFP-CaMKII using Lipofectamine 2000 (Invitrogen) as instructed by the manufacturer. After 48 h, they were washed in ice-cold PBS and then lysed in 150 mM NaCl, 50 mM Tris, pH 8.0, 1% Triton, and Complete protease inhibitor cocktail, and immunoprecipitation was performed with the anti-α_{1C} antibody (Alamone). After SDS-PAGE, immunoblotting was performed with an anti-GFP antibody (Covance).

Expression and purification of CaMKII

α-CaMKII was expressed and purified essentially as described previously (Bradshaw et al., 2002). Additional CaMKII isoforms were generated by transient expression in HEK 293 cells (Srα plasmid containing the α, β, δ_A, δ_C, or γ_B isoforms). After 72 h, cells were lysed in 10 mM Tris/5% Betaine/150 mM sodium perchlorate, pH 7.5, by brief sonication. Cell lysates were centrifuged for 30 min at 14,000 *g* at 4°C, and the supernatants were aliquoted, snap frozen, and stored at –80°C.

GST binding assay

The binding reactions were accomplished in Tris-binding buffer (50 mM Tris, 150 mM NaCl, 0.1% T-20, pH 7.4, and 0.1% BSA) containing 20 nM purified CaMKII. The total protein from the HEK 293 cell lysates added to each binding reaction ranged from 9 to 22 μg, as determined by normalizing for the amount of CaMKII activity (Singla et al., 2001). Preautophosphorylayion of CaMKII (purified and lysate) was performed on ice for 5 min in Tris-binding buffer plus 1 mM $CaCl_2$, 5 μM CaM, 1 mM ATP, and 5 mM $MgCl_2$ to restrict the sites of autophosphorylation to primarily Thr^{286} (Lai et al., 1987; Lou and Schulman, 1989; Ikeda et al., 1991). Final concentration of these components in the binding reaction (1:40) was 0.025 mM $CaCl_2$, 0.125 μM CaM, 0.025 mM ATP, and 0.125 mM $MgCl_2$. The binding reaction was rocked for 1 h at 4°C, and the beads were extensively washed in Tris-binding buffer (2–3 times for 5 min each). CaMKII binding was quantified using densiotometric measurement of band intensity using 1D Image Analysis Software (Eastman Kodak Co.). Multiple exposure times, as well as a standard curve generated by dilution analysis, ensured linearity in the chemiluminescence intensity. One-way analysis of variance was performed, and Dunnett's test was used to identify specific pair-wise differences between the means. Comparison analyses were conducted using SPSS Version 10.1.3 (SPSS, Inc.).

Calmodulin binding assay

The bound GST proteins–sepharose complex was prepared as described in the previous section. Purified CaM (Singla et al., 2001) was applied in the presence of 1 mM $CaCl_2$ for 1 h before multiple washes of Tris-binding buffer plus 1 mM $CaCl_2$. Immunoblotting was performed as described previously (Pitt et al., 2001).

CaMKII phosphorylation of α_{1C}

Purified α-CaMKII was incubated with bound GST fusion proteins or immunoprecipitated material bound to PKA in the presence of Ca^{2+}/CaM (2 mM/10 μM) and Mg^{2+}/ATP (5 mM/50 μM ATP) plus 10–50 μCi ATP^{32} for 15 min at RT. For the GST proteins, CaMKII was activated before exposure to the substrate reaction on ice (as described in GST binding assay) to produce an autophosphorylated enzyme. After the phosphorylation, the beads were washed extensively in PBS (plus 5 mM EDTA) and 2× SDS-PAGE sample buffer was added and SDS-PAGE was performed. The gels were Coomassie stained and exhaustively destained. The gels were dried down, and P^{32}-labeled proteins were detected using autoradiography.

CaMKII dephosphorylation using PP1

CaMKII was dephosphorylated using a Hisx6-tagged PP1 catalytic subunit construct (provided by A. Nairn, Yale University, New Haven, CT) purified by Ni-NTA affinity chromatography (provided by M. Bradshaw, Stanford University, Stanford, CA).

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