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

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Ca\textsuperscript{2+}-dependent facilitation (CDF) of voltage-gated calcium current is a powerful mechanism for up-regulation of Ca\textsuperscript{2+} influx during repeated membrane depolarization. CDF of L-type Ca\textsuperscript{2+} channels (Ca\textsubscript{1.2}) contributes to the positive force–frequency effect in the heart and is believed to involve the activation of Ca\textsuperscript{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\textsubscript{1C} (Ca\textsubscript{α1.2}) is a CaMKII substrate and that CaMKII interaction with the COOH terminus of α\textsubscript{1C} is essential for CDF of L-type channels. Ca\textsuperscript{2+} influx triggers distinct features of CaMKII targeting and activity. After Ca\textsuperscript{2+}-induced targeting to α\textsubscript{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\textsuperscript{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\textsuperscript{2+}-dependent facilitation (CDF) of calcium channels serves to potentiate the Ca\textsuperscript{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\textsuperscript{2+} current through L-type channels (Ca\textsubscript{1.2}; 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\textsuperscript{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\textsuperscript{2+} channels displaying CDF (Dzhura et al., 2000). Further, immunocytochemical data suggest that the Ca\textsubscript{1.2} 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\textsuperscript{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
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\(^{285}\) 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 \(\sim 240\) kD, consistent with the molecular mass of α\(_{1C}\). The kinase activity could be attributed to CaMKII and not to another kinase coinmunoprecipitated with α\(_{1C}\) because inclusion of the CaMKII inhibitor autocomitide-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 (\(\sim 50\) kD) corresponding to the autophosphorylated form of the α subunit of CaMKII 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,1.2 function (Rotman et al., 1995; Bence-Hanulec et al., 2000; MeHugh et al., 2000). Similar to results with the intact channel (Fig. 1 A), we again noticed in multiple lanes an \(\sim 50\) kD \(^{32}\)P-labeled
CaMKII and Xpress-tagged experiments with HEK 293 cells coexpressing GFP-tagged the kinase to the pore-forming subunit was further evaluated in was consistent with the properties of ily detectable with a biotinylated calmodulin overlay, which

tial accessory subunits

to precipitate an (Fig. 3). The goal was to find out whether a di-

tion could be observed in vitro and whether differ-

ence activation states of CaMKII modulated binding. When

Figure 2. CaMKII coimmunoprecipitates and colocalizes with α1C. (A) Bioti-

ylated calmodulin overlay of rat cardiac sarcolemmal membranes after immu-

noprecipitation with an anti-α1C antibody. Purified α-CaMKII was run as a

control to demonstrate effectiveness of CaM overlay. An anti-α1C antibody

(tetra-coimmunoprecipitation of the GFP-CaMKII by the antibody to


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features of CaMKII that had been introduced for the kinase assay.

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kinase anchoring to the channel subunit as a whole.

CaMKII interacts specifically with α1C

We tested the possibility that CaMKII tethers to α1C in the rat heart by attempting to coimmunoprecipitate CaMKII with α1C (Fig. 2 A). An anti-α1C antibody (but not control IgG) coimmunoprecipitated a protein identified as the α iso-

form of CaMKII by biotinylated CaM overlay and apparent molecular mass. (B) Anti-GFP immunoblot after immunoprecipitation of GFP-CaMKII by con-

rol IgG (lane 4) or anti-α1C antibody (lane 5) from lysates of HEK 293 cells transiently transfected with GFP-CaMKII and α1C.

Activity-dependent association of CaMKII with multiple cytoplasmic regions of α1C

To further define the interaction between CaMKII and α1C, we con-

structed a pull-down binding assay using various α1C-GST fusion proteins (Fig. 3). The goal was to find out whether a di-

rect interaction could be observed in vitro and whether different

activation states of CaMKII modulated binding. When

CaMKII was activated with Ca2+/CaM but not allowed to un-

dergo autophosphorylation (ATP not included), the kinase

bound to the NH2-terminal domain and the III-IV loop of α1C (Fig. 3, middle). Subsequent removal of Ca2+/CaM in the wash buffer reversed this binding (unpublished data). When CaMKII

was activated in the presence of Ca2+/CaM plus ATP on ice, conditions previously shown to produce predominantly Thr328

autophosphorylation (Lai et al., 1987; Lou and Schulman, 1989; Ikeda et al., 1991), CaMKII again bound to the NH2 ter-

minus 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 α1C requires activation of the kinase by Ca2+/CaM. A subsequent activation state, that produced by autophosphory-

lation, was necessary for binding to additional cytoplasmic regions of α1C.

To identify novel structural determinants of α1C that func-
tionally 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 autophosphory-

lated CaMKII (Fig. 3), a state of the kinase capable of supporting

fication of single channels (Dzhura et al., 2000); and it has been implicated in Ca2+-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 α1C, 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 autophosphory-

lated CaMKII that suggested that the kinase bound between

residues 1622 and 1669 of α1C. Because a weak interaction was also seen with a construct proximal to 1622, we generated a fusi-

on 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-
assessed 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 1622–1690) 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, TVGKFI), was identified as being nearly identical in α1C and α1A (Ca,2.1), 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 α1C fusion protein containing the amino acids EEDAAA in place of TVGKFI 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 α1C prevents CDF**

We then tested whether this site was critical for CDF by introducing the Mut6 mutation into α1C 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 (11654A; 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\(_{\text{Ca}}\) 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 α1C (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\(_{\text{Ca}}\) 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,1.2.

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 α1C (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\(_{\text{Ca}}\) 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,1.2.

The CaMKII binding site for the COOH terminus of α1C 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 α1C across a range of isoforms. The α, β, γ, δA, and δC 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 autophosphorylated, robust binding to the α1C COOH-terminal

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**Figure 4. Localization of the CaMKII binding site on the COOH terminus of α1C.** (A) Diagram of α1C fusion proteins used in GST pull-down assays with autophosphorylated α-CaMKII, exhibiting robust (+), partial (±), and no (−) binding. (B) Immunoblot with CB2 after GST pull down of 20 nM of purified autophosphorylated α-CaMKII, using α1C 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 CB2 of GST pull-down assays of purified autophosphorylated α-CaMKII, using α1C aa 1581–1690 (wild type [WT]), a \(1^{689-1589}VTKFY1^{699}\)→EDDAAA 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 Dunnet'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 CB2. (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.
tail was observed for each of these CaMKII isoforms, with the sole exception of \( \gamma_9 \)-CaMKII. Thus, the capability of interaction with Cav1.2 is a widespread property of the CaMKII family, including the \( \alpha_C \), \( \beta \), and \( \delta \) isoforms prevalent in brain and cardiac tissue.

Where is the binding site for \( \alpha_C \) on CaMKII? The conserved nature of the \( \alpha_C \) 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 \( \alpha_C \)-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 \( \alpha_C \) 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 \( \alpha_C \) 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 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 \( \alpha_C \) or to NR2B. The NR2B sequence that was

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**Figure 5.** CaMKII interaction with the COOH terminus of \( \alpha_C \) is essential for CDF. (A) \( I_{\text{Ba}} \) and scaled \( I_{\text{Ca}} \) traces during a train of 40 test pulses of \( V_h \) from \(-90 \text{ mV} \) to \(+20 \text{ mV} \) at 3.3 Hz recorded from oocytes expressing \( \alpha_C \) I1654A (I/A) or \( \alpha_C \) I1654A/1644TVGKFY1649 \( \rightarrow \) EEDAAA (I/A-Mut6). Bars, 500 nA and 25 ms. (B) Peak \( I_{\text{Ba}} \) and \( I_{\text{Ca}} \) 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 \( \pm \) SEM. (C) Changes in peak \( I_{\text{Ba}} \) and \( I_{\text{Ca}} \) conducted by \( \alpha_C \) I1654A (I/A) or \( \alpha_C \) I1654A/1644TVGKFY1649 \( \rightarrow \) EEDAAA (I/A-Mut6) at indicated stimulation frequencies \((n = 4–5)\). Values indicate means \( \pm \) 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 \( \alpha_C \) (Mut6) by CaMKII, performed as in Fig. 1 A. An anti-\( \alpha_C \) immunoblot of the samples used in the kinase reaction confirmed similar expression levels of the WT and mutant \( \alpha_C \) subunits. An anti-CaMKII immunoblot with CB2 confirmed the identity of the retained 50-kD \( ^3\text{P} \)-labeled protein as \( \alpha_C \)-CaMKII.

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**Figure 6.** The binding site for the COOH terminus of \( \alpha_C \) 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 \( \alpha_C \) [aa 1509–1905] on lysates of HEK 293 cells transiently transfected with the CaMKII isoforms \((\alpha_C, \beta, \delta, \gamma_C, \), and \( \gamma_2 \) arrows) after thioauto phosphorylation. In lanes 6 and 7, lysates of untransfected cells were run with [+] and without [−] purified thiophosphorylated \( \alpha_C \)-CaMKII added to the lysate. (B) Immunoblot using an mAb (CB2) for CaMKII after GST pull downs, using a fusion protein from the COOH terminus of \( \alpha_C \) (aa 1509–1905) and 20 mM of purified autophosphorylated \( \alpha_C \)-CaMKII. In addition, 20 \( \mu \text{M} \) of the indicated peptide was added to each binding reaction. (C) Sequence alignment of CaMKII binding sites from the COOH termini of NR2B and \( \alpha_C \) with the autoregulatory domain from \( \alpha_C \)-CaMKII.
measurements of CaMKII recovered after treatment with recombinant PP1 indicate means

found to support interaction with CaMKII closely resembles the autoregulatory domain of CaMKII surrounding Thr286 (Fig. 6 C; Bayer et al., 2001). In turn, both of these stretches of amino acids show significant resemblance to the region of α1C 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 α1C-CaMKII interaction. Although the corresponding regions of α1C 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 α1C produces a dedicated Ca2+ sensor

The functional nature of the channel–kinase interaction could follow one of several possible scenarios. During recurrent rises and falls in Ca2+, the enzyme might cycle on and off the channel. Alternatively, CaMKII might remain anchored to α1C with its activity persistently switched on, like CaMKII associated with the NMDAR (Bayer et al., 2001). Finally, CaMKII might stay tethered to the α1C subunit, like PKA associated with Ca.1.2 through A-kinase anchor protein (AKAP; Tavalin et al., 1999), but with kinase activity modulated by local changes in Ca2+/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 Ca2+ elevation or the kinase activation that initially drove the interaction.

When the Ca2+ chelator EGTA was added immediately after the preautophosphorylation reaction, the binding of CaMKII to the α1C COOH-terminal tail was inhibited (Fig. 7 A). In contrast, once autophosphorylated CaMKII had bound to the α1C 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 α1C 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 Thr286 had been completely dephosphorylated by PP1 treatment after the initial kinase binding (Fig. 7 B). Thus, although Ca2+/CaM and autophosphorylation were necessary for CaMKII to bind to the α1C COOH terminus, the same conditions were no longer required to sustain the interaction.

Tethered CaMKII retains its dependence on Ca2+/CaM for activity

Because the CaMKII binding for both α1C and NR2B appears to localize to the catalytic domain of the kinase, we asked whether α1C 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 Ca2+/CaM and autophosphorylation (Bayer et al., 2001). To determine how CaMKII is regulated when it is stably bound to the α1C COOH terminus, we examined the Ca2+/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 α1C-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 α1C COOH terminus but depended strictly on CaMKII autophosphorylation. After PP1 treatment, tethered CaMKII could be reactivated by Ca2+/CaM. In these respects, CaMKII binding to α1C or to NR2B had very different effects on the activity of the kinase. As mentioned in Discussion, the association of CaMKII to the α1C 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 Ca2+ entry through Ca2+ 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

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Figure 7. CaMKII interaction with the COOH terminus of α1C is not reversed by dephosphorylation or CaM dissociation, and tethered CaMKII requires autophosphorylation or Ca2+/CaM for activity. (A and B) Immunoblots with CBx2 or a phosphospecific CaMKII mAb after GST pull-down assays, using α1C aa 1509–1905 and 20 nM of autophosphorylated α1C-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 α1C aa 1509–1905. Ca2+/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.
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 \(\alpha_{1C}\) subunit of L-type channels in the heart as indicated by coimmunoprecipitation. Second, specific regions of the \(\alpha_{1C}\) subunit have the capability to directly anchor activated CaMKII. Third, CaMKII can phosphorylate \(\alpha_{1C}\) in regions previously implicated in regulating channel function. Fourth, a mutation in the COOH terminus of \(\alpha_{1C}\) 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 thio-phosphorylated (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 \(\alpha_{1C}\) 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 \(\alpha_{1C}\). 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 \(\alpha_{1C}\), and if the depolarization frequency suffices to produce CaMKII autophosphorylation onThr\(^{286}\), the resulting displacement of the kinase’s autoregulatory domain exposes a potent...
anchoring site for the $\alpha_{1C}$ 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 $\alpha_{1C}$. 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. 7D), 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 $\alpha_{1C}$ (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 $\alpha_{1C}$ 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).

**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 $\alpha_{1C}$ as targets for CaMKII binding. First, completely inactive CaMKII will not initiate binding to any of these subunits. Second, in both NR2B and $\alpha_{1C}$, 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. 6B; 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 $\alpha_{1C}$ COOH-terminal tail (Fig. 6B). Third, in both NR1 and $\alpha_{1C}$, 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 $\alpha_{1C}$ 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; Zühkle 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).

**Comparisons with L-type channel modulation by other kinases**

The tethering of CaMKII to $\alpha_{1C}$ 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 $\alpha_{1C}$. 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$_2$C$_2$ 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 $\beta$-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.
There are also critical functional differences between α1C and NR2B in their interaction with CaMKII. Although the COOH-terminal tails of α1C 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 α1C 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 α1C; our experiments show that interaction with the α1C 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 α1C 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 α1C 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 α1C subunit was 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 α1C, the auxiliary Ca$^{2+}$ channel subunits β1 and α2δ, were performed as previously described (Zühkle et al., 2000). Before recording whole cell I_o 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_Ca and I_o recordings were performed essentially as described previously (Zühkle 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 3122 A/D interface (Axon Instruments, Inc.) to a personal computer. I_Ca and I_o were recorded in the same ionic. Oocyte current filters were set 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 α1C (available from Genbank/EMBL/DDB) under accession no. X15539) NH2 terminus (aa 1–154), II intracellular loop (aa 435–554), III/IV intracellular loop (aa 784–931), III/IV intracellular loop (aa 1197–1250), and two COOH-terminal fragments (aa 1581–1690 and 1669–1717) were cloned into pGEX-4T-1, and GST fusion proteins were generated. The plasmids encoding the COOH-terminal fragments CTS (aa 1507–1622), CT12 (aa 1509–1905), and CT23 (aa 1622–1905) were provided by M. Hasey (Northwestern University, Evanston, IL).

Peptides

Peptides spanning α1C residues 1581–1690 have previously been described (Pitt et al., 1999). The N-methyl-aspartate-sensitive 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 anti-α1C (Alomone Laboratories) 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 α1C, α2δ, β2, 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-α1C antibody (Alomone). After SDS-PAGE, immunoblotting was performed with an anti-GFP antibody (Covance).

Expression and purification of CaMKII

αCaMKII was expressed and purified essentially as described previously (Bradshaw et al., 2002). Additional CaMKII isoforms were generated by transient expression in HEK 293 cells (Stratagene). 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 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 mM 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). Prewautophosphorylation 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 Thr286 (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 densitometric measurement of band intensity using ImageJ 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 α1C

Purified α1C-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 His6-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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