Molecular Basis of Calmodulin Tethering and Ca^{2+}-dependent Inactivation of L-type Ca^{2+} Channels*

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Ca^{2+}-dependent inactivation (CDI) of L-type Ca^{2+} channels plays a critical role in controlling Ca^{2+} entry and downstream signal transduction in excitable cells. Ca^{2+}-insensitive forms of calmodulin (CaM) act as dominant negatives to prevent CDI, suggesting that CaM acts as a resident Ca^{2+} sensor. However, it is not known how the Ca^{2+} sensor is constitutively tethered. We have found that the tethering of Ca^{2+}-insensitive CaM was localized to the C-terminal tail of α_{1C}, close to the CDI effector motif, and that it depended on nanomolar Ca^{2+} concentrations, likely attained in quiescent cells. Two stretches of amino acids were found to support the tethering and to contain putative CaM-binding sequences close to or overlapping residues previously shown to affect CDI and Ca^{2+}-independent inactivation. Synthetic peptides containing these sequences displayed differences in CaM-binding properties, both in affinity and Ca^{2+} dependence, leading us to propose a novel mechanism for CDI. In contrast to a traditional disinhibitory scenario, we suggest that apoCaM is tethered at two sites and signals actively to slow inactivation. When the C-terminal lobe of CaM binds to the nearby CaM effector sequence (IQ motif), the braking effect is relieved, and CDI is accelerated.

The voltage-gated L-type Ca^{2+} channel is unique among ion channels in displaying two gating properties that are regulated by the ion that permeates the channel, calcium-dependent inactivation (CDI) and calcium-dependent facilitation (CDF). These feedback mechanisms are of critical importance for regulation of the electromechanical activity of the heart and other essential physiological processes. CDI helps determine the length of the cardiac action potential plateau (1) and CDF contributes to the positive force-frequency relationship of the cardiac contraction (2).

Several lines of evidence from recent work suggest that the calcium sensor mediating both of these processes may be the calcium-binding protein calmodulin (CaM). We (3) and others (4, 5) have shown that there is a Ca^{2+}-dependent CaM-binding sequence (“IQ motif”) in the cytoplasmic C-terminal tail of the pore-forming α_{1C} subunit of the channel, within a region previously shown to confer Ca^{2+} sensitivity (6). We have also shown that those mutations within the IQ motif that render the channel subunit unable to bind CaM also disrupt CDI (3, 7), suggesting that the IQ motif serves as the effector region for CDI. Furthermore, we (3) and others (4) have shown CDI can be blocked in a dominant negative fashion by those CaM mutants that lack Ca^{2+} binding in their C-terminal EF-hand domains.

Several important questions remain unanswered about how Ca^{2+} and CaM might regulate L-type Ca^{2+} channel inactivation. The first question concerns how CaM may be tethered to the L-type channel (8). There are multiple reasons for thinking that there must be a binding site that tethers the Ca^{2+} sensor in the channel’s resting state, keeping it poised for signaling as soon as Ca^{2+} entry begins. Without tethering it would be difficult to explain the rapid development of CDI, beginning within milliseconds after L-type channel opening is initiated by depolarization (5). Tethering would also explain the dominant negative inhibitory action of mutant CaM molecules inasmuch as their binding to the tethering site would preclude binding of wild-type CaM (3, 4, 7). Recent studies have identified sequences in a cytoplasmic domain of the α_{1C} subunit that display significant affinity for CaM even at low Ca^{2+} concentrations (9–11), but these do not appear to bind to dominant negative CaM mutants, as would be expected for the putative tethering site. Thus, the molecular basis for CaM tethering remains unclear.

A second question concerns the nature of the interaction between CaM and the IQ motif. How does CaM interact with the IQ motif so that CaM performs its effector functions? Is this interaction similar to CaM interactions with well known partners such as Ca^{2+}/CaM-dependent protein kinase II (CaMKII) or myosin light chain kinase?

A third question concerns puzzling alterations in Ca^{2+}-independent inactivation, produced by modifying amino acids within the IQ motif or in its general vicinity. Such modifications produce prominent changes in the kinetics of Ba^{2+} currents through L-type channels (3, 7, 12). How can one account for these experimental observations if the regulation of inactivation were strictly dependent on formation of a Ca^{2+}-CaM complex?

In addressing each of these questions, our experiments indi-
icated that CaM acts through a switch-like mechanism significantly different than its classical disinhibitory mode of action.

**EXPERIMENTAL PROCEDURES**

cDNA Construction and Site-directed Mutagenesis—For the gel shift experiment in Fig. 1, the coding sequences of CaM and CaM<sub>234</sub> (provided by J. Adelman, Oregon Health Sciences University) were amplified by PCR and cloned into pGEX-4T-1 (Amersham Pharmacia Biotech). Constructs for the GST fusion proteins containing amino acids 1477–1592 and 1592–1875 of the α<sub>1C</sub> cytoplasmic tail were the generous gift of M. Hosey (Northwestern University) and have been described previously (13), although the numbering of the amino acids in this report reflects that of the cDNA clone 77wt that has been described previously (3). The construct for the GST fusion protein containing amino acids 1551–1660 was generated by PCR amplification using primers 5′-CCGAATTCACGAGGAGTTTCTCAAG-3′ and 5′-GGCTCGAGGTCGGCAGGCACTCAG-3′ and cloning of the restriction enzyme-digested product into the EcoRI and Xhol sites of pGEX-4T-1. The construct for the GST fusion protein containing amino acids 1512–1563 was generated by PCR amplification using primers 5′-CCGAATTCACGAGGAGTTTCTCAAG-3′ and 5′-GGCTCGAGGTCGGCAGGCACTCAG-3′ and cloning of the restriction enzyme-digested product into the EcoRI and Xhol sites of pGEX-4T-1. The construct for the GST fusion protein containing amino acids 1551–1660 was generated by PCR amplification using primers 5′-CCGAATTCACGAGGAGTTTCTCAAG-3′ and 5′-GGCTCGAGGTCGGCAGGCACTCAG-3′ and cloning of the restriction enzyme-digested product into the EcoRI and Xhol sites of pGEX-4T-1. The construct for the GST fusion protein containing amino acids 1512–1563 was generated by PCR amplification using primers 5′-CCGAATTCACGAGGAGTTTCTCAAG-3′ and 5′-GGCTCGAGGTCGGCAGGCACTCAG-3′ and cloning of the restriction enzyme-digested product into the EcoRI and Xhol sites of pGEX-4T-1.

Full-length 77wt to form the deletion mutant 77d<sub>13</sub>, and the deletion PpuI and SphI XhoI and SpeI restriction sites (underlined sequences). Fragment A and B were subsequently digested with the restriction enzymes PpuI and SphI for 30 min, aliquots of supernatant were snap-frozen and stored at −80 °C. For CaM and CaM<sub>234</sub> used in Fig. 2, protein was allowed to bind to glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) in 150 mM NaCl, 50 mM Tris, pH 7.4, 0.1% Triton for 30 min at 4 °C, washed extensively, and then cleaved from GST with thrombin without added Ca<sup>2+</sup>. For binding experiments between fusion proteins and CaM or CaM<sub>234</sub>, CaM or CaM<sub>234</sub> was then added and allowed to interact with the fusion protein for 4 h at 4 °C. The beads were then washed extensively with binding buffer containing the indicated amounts of free Ca<sup>2+</sup> and the retained protein eluted with SDS sample buffer. The protein was then subjected to SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and detected by immunoblot with a polyclonal antibody against CaM (Zymed Laboratories Inc.). The ability of this antibody to detect CaM<sub>234</sub> equally as well as CaM was confirmed (data not shown).

**RESULTS**

**Interactions between CaM and the IQ Motif as the Basis for Tethering**—A constitutive interaction between CaM and the channel has been postulated to keep CaM strategically positioned to sense Ca<sup>2+</sup> entry and to induce inactivation rapidly, but the molecular basis for the tethering is unclear. Because of its homology...
to Ca<sup>2+</sup>-independent CaM-binding sequences found in unconventional myosins and neuromodulin (17), we initially considered the possibility that the IQ motif in the C-terminal tail could serve as the tethering site for CaM at basal levels of [Ca<sup>2+</sup>]<sub>i</sub>. Although our previous results (3) showed that the C-terminal tail could not bind CaM in the absence of Ca<sup>2+</sup><sup>+</sup>, we considered that tethering to the IQ motif might require Ca<sup>2+</sup> concentrations of 20–100 nM, like those in resting cells, and might be missed in zero Ca<sup>2+</sup>.

To test this possibility, we chose to examine interactions between the IQ motif and CaM<sub>234</sub>, one of the Ca<sup>2+</sup>-insensitive CaM molecules that act as dominant negatives to prevent CDI. Because CaM<sub>234</sub> clearly lacks the ability to act as a Ca<sup>2+</sup> effector (3, 7), any binding that it displayed with the IQ motif could be regarded as an unambiguous indication of a tethering interaction. Accordingly, we generated CaM<sub>234</sub> and wild-type CaM by bacterial expression and tested the purified proteins for interaction with the IQ motif in a gel shift assay (Fig. 1). In the absence of added peptide, the CaM<sub>234</sub> protein migrated for interaction with the IQ motif in a gel shift assay (Fig. 1). In the presence of added peptide, the CaM<sub>234</sub> protein migrated faster than wild-type CaM protein, confirming that this CaM mutant is unable to bind Ca<sup>2+</sup> and undergo the characteristic Ca<sup>2+</sup>-induced shift in mobility. The addition of the IQ motif peptide did not induce a shift in the mobility of CaM<sub>234</sub>, regardless of whether in the presence of 1 mM Ca<sup>2+</sup> (Fig. 1) or in 2 mM EGTA (data not shown). This indicated that CaM<sub>234</sub> cannot bind to the IQ motif and that the basis for its dominant negative effect must be sought elsewhere.

Our previous demonstration that a peptide containing the IQ motif could induce a shift in mobility of wild-type CaM even in the presence of 100 nM added Ca<sup>2+</sup> (3) does not in itself establish a tethering role for the IQ motif. Micromolar concentrations of CaM are required in this assay in order to visualize CaM, thus requiring micromolar concentrations of peptide to achieve appropriate stoichiometry. The concentration of peptide used in such assays is therefore more than 10-fold greater than the K<sub>d</sub> value of 50 nM for interactions between IQ motif-containing peptide and CaM that we subsequently determined (7). Thus, the law of mass action would favor the detection of significant binding, even if the IQ motif interacted relatively weakly with CaM at 100 nM Ca<sup>2+</sup>.

**Consideration of the I-II Loop as a Possible Tethering Site**—To expand our search for a tethering domain, we scanned the putative intracellular portions of the α<sub>1C</sub> subunit for sequences with homology to known CaM-binding domains, using the criteria as defined by Rhoads and Friedberg (17). We found three sites in the I-II intracellular loop (amino acids 438–451, 520–532, and 542–555, Fig. 2A), one site in the cytoplasmic tail (amino acids 1565–1578, see below), but none in the N-terminal domain. Ivanina et al. (18) reported that CaM binds to a fusion protein containing the N-terminal domain; presumably CaM binds to a site that lacks homology to consensus sequences. The 520–532 sequence conforms to a consensus 1-5-10 CaM-binding motif, and the sequences 438–451 and 542–555 correspond to a consensus 1-8-14 type B CaM-binding domain. The 438–451 sequence contains amino acids 520–532, FNCAGRALHRISKFSKRWR, monitored as an increase in dCaM fluorescence at λ = 480 nm. Data are fit to a binding curve with K<sub>d</sub> = 90 nM and a Hill coefficient of 1.0. C, immunoblot for CaM showing interaction of CaM with GST fusion proteins containing I-II loop (amino acids 453–555) and the IQ motif (amino acids 1592–1875). GST was used as a control. The 7th lane is blank, and the 8th lane contains purified CaM as a control for the antibody. [Ca<sup>2+</sup>] = 1.0 mM, [EGTA] = 2.5 mM. D, current traces showing lack of effect on CDI of a 77α<sub>1C</sub> mutant in which amino acids 520–532 were deleted (77d13). Currents were recorded during a 400-ms depolarizing step to +20 mV from a holding potential of −90 mV. I<sub>B</sub><sub>a</sub> and I<sub>c</sub><sub>a</sub> were obtained from the same oocytes, and traces were scaled to equalize peak currents.

Accordingly, we synthesized peptides that contained these sequences and looked for interactions with 5-dimethylaminonaphthalene-1-sulfonyl-CaM (dCaM). In the presence of Ca<sup>2+</sup>, the 520–532 peptide increased the fluorescence of dCaM, with a concentration dependence corresponding to a binding affinity of ~80 nM (Fig. 2B). Significant interaction was detected in the presence of Ca<sup>2+</sup>. No significant interaction, either in the presence or absence of Ca<sup>2+</sup>, was detected with the 438–451 and the 542–555 peptides. To confirm the CaM-binding properties of the 520–532 peptide, we generated GST fusion proteins that contained the I-II loop and, for comparison, additional GST fusion proteins incorporating a segment of the C-terminal tail that included the IQ motif (Fig. 2C). Potential interactions between the fusion proteins and CaM were examined in the presence or absence of Ca<sup>2+</sup> and detected by an immunoblot for CaM. As shown in Fig. 2C, the I-II loop fusion protein was able to bind CaM only in the presence of Ca<sup>2+</sup> but not in its absence, confirming the results obtained with fluorescently labeled CaM. This suggested that CaM was not bound constitutively to the I-II loop in zero Ca<sup>2+</sup> and was therefore unlikely to serve as the CaM-tethering site.

As a further test of the possible involvement of the I-II loop if not in tethering, as a possible effector site, we generated an α<sub>1C</sub> subunit that lacked the amino acids 520–532. This mutant was co-injected with α<sub>B</sub> and β<sub>i</sub> into Xenopus oocytes and L-type Ca<sup>2+</sup> currents were recorded. As shown in Fig. 2D, Ca<sup>2+</sup>-dependent inactivation and the dominant negative inhibition of CDI by CaM<sub>1234</sub> were both unaffected by the deletion in α<sub>1C</sub>. We took this as strong evidence that the deleted se-
sequence does not play a significant role in supporting either the tethering or effectors aspects of Ca\(^{2+}\) sensing in CDI.

**A Broader Search for a Tethering Site in the C-terminal Cytoplasmic Tail**—Lacking evidence for involvement of the I-II loop, we proceeded to focus on the fourth CaM-binding site identified in our scan, in the C-terminal tail. Our previous experiments had weighed against possible tethering sites in regions of the C-terminal tail other than the IQ motif, because a Ile\(^{1624}\) → Glu substitution within the IQ motif (I/E mutation) appeared to render the tail unable to bind CaM in 1 mM Ca\(^{2+}\) or in EGTA (3). To check this result, we carried out further experiments, taking care to allow longer incubations of agarose-CaM with the in vitro translated \[^{35}S\]methionine-labeled \(\alpha_{1C}\) fragment (Fig. 3). The new data showed a clear Ca\(^{2+}\)-dependence of interaction between CaM and the C-terminal protein harboring the I/E mutation, although at reduced levels compared with wild-type protein. It is unlikely that this interaction represents residual binding to the mutated IQ site, since the concentration of \[^{35}S\]labeled peptide in the reaction was about 10\(^{-17}\) M, far below the \(K_d\) value we have calculated for a IQ motif peptide containing the I/E mutation (>5 \(\mu\)M). A further hint that the cytoplasmatic tail could support CaM binding outside of the IQ motif is provided by comparison of results from Qin et al. (5) with our own findings (7). Extreme disruption of the IQ motif (IQEYFRKAAEYAAAA) spared CaM binding to a fusion protein containing amino acids 1542–1688 (numbering as in 77wt) (5), even though a less severe modification (IQEYFRKAAEYAAAA) decreased the affinity of CaM for the IQ motif peptide by -100-fold (7).

Analysis of amino acids 1565–1578 showed that it conformed to a 1-8-14, type A motif (17). Interestingly, this site contains the short sequence \[^{1572}IKTEG\]\[^{1576}\], previously identified as essential for CDI by analysis of deletions or domain swaps (6, 12, 20, 21). Further consideration of the entire region between \(~1565\) and \(~1650\) as a locus of possible tethering sites was suggested by recent studies documenting the binding of CaM to sequences containing the \[^{1600}LLDQV\]\[^{1604}\] motif (9–11), another short sequence identified as important for CDI (6, 12, 20, 21). Accordingly, we tested various GST fusion proteins containing these sequences for possible interactions, not only with CaM but also with the dominant negative CaM\(_{1234}\) as a definitive test for tethering (Fig. 4).

Our first attempts were made with a GST fusion protein incorporating amino acids 1477–1592, within which the \[^{1572}IKTEG\]\[^{1576}\] sequence resides (Fig. 4A, gray segment). A second GST fusion protein consisted of the amino acids 1592–1875, which includes the sequence \[^{1600}LLDQV\]\[^{1604}\] and the IQ motif (Fig. 4A, black segment). The binding of CaM or CaM\(_{1234}\) to these fusion proteins was examined in the presence of 100 nM Ca\(^{2+}\), to approximate basal [Ca\(^{2+}\)]\(_{i}\) in quiescent cells. As shown in Fig. 4B (lanes 1 and 2), the 1477–1592 fusion protein failed to bind CaM (WT) or CaM\(_{1234}\) (4-), suggesting that the corresponding stretch of \(\alpha_{1C}\) is not sufficient to serve a tethering function. In a somewhat different pattern, the 1592–1875 fusion protein was found to bind CaM but not CaM\(_{1234}\). Because this fusion protein included the IQ motif, the simplest interpretation was that this motif was responsible for binding Ca\(^{2+}\)-CaM, in a reaction driven by the high local concentration of IQ motif in this assay, despite the presence of only 100 nM Ca\(^{2+}\). In any case, it was clear that aa 1592–1875 of \(\alpha_{1C}\) were also not sufficient to serve a tethering function, since CaM\(_{1234}\) failed to bind (Fig. 4B, lane 4).

Tests with the 1477–1592 and 1592–1875 fusion proteins did not exclude a remaining possibility that binding of the mutant CaM might depend jointly on essential sequences within both of the corresponding regions of \(\alpha_{1C}\). This idea was tested with an additional fusion protein containing the amino acids 1551–1660 (Fig. 4A), depicted with both gray and black shading to indicate that it straddled the junction between the first two fusion proteins. In this case, both CaM and CaM\(_{1234}\) bound equally well (Fig. 4B), conforming to the pattern expected for a genuine tethering region.

**Ca\(^{2+}\)-Dependence of Tethering to a C-terminal Region**—Initially, we attempted to confirm our results by looking for interactions between apocalmodulin and the candidate-tethering region by carrying out experiments in the absence of Ca\(^{2+}\) (EGTA). This failed (Fig. 3C), suggesting that interactions between CaM and the 1551–1660 fusion protein might be critically different in zero Ca\(^{2+}\) versus 100 nM Ca\(^{2+}\). This would be consistent with our previous finding that in the absence of Ca\(^{2+}\), agaro-CaM bound poorly to \[^{35}S\]methionine-labeled in vitro translated protein corresponding to the whole cytoskeletal tail (Fig. 3). Further experiments were carried out to look systematically for such a Ca\(^{2+}\)-dependence (Fig. 4D). Here we used CaM\(_{1234}\), which lacks any ability to interact with submucronar Ca\(^{2+}\), to focus on possible Ca\(^{2+}\)-dependence arising from the channel sequence itself. Consistent with the previous result, there was little interaction of CaM\(_{1234}\) and the 1551–1660 fusion protein in the absence of Ca\(^{2+}\). When Ca\(^{2+}\) was added to the reaction, the degree of interaction was fully saturated at Ca\(^{2+}\) concentrations of 100 nM or higher and was half-maximal at ~10 nM Ca\(^{2+}\) (Fig. 4E). Thus, while dependent on Ca\(^{2+}\), the putative tethering interaction would be effective at typical cytoplasmic Ca\(^{2+}\) concentrations in cells at rest.

**Non-contiguous Peptides Involved in CaM Tethering**—To take a more systematic approach to the structural determinants of the CaM interactions in the region between amino acids 1551–1660, we examined a series of overlapping peptides that covered most of this region in a staggered fashion (Fig. 5A). These were designed as follows: A-(1558–1579), B-(1568–1589), C-(1585–1606), D-(1595–1616), E-(1609–1630), and F-(1619–1638). Peptide-CaM binding was tested in fluorescence assays using dCaM (23). The vertical axis in Fig. 5B denotes the fluorescence increase at 480 nm, a measure of the
extent of the interaction. For the purposes of an initial survey, the assays were performed in the presence of 1 μM Ca\(^{2+}\) (3) since peptide F-(1619–1638) contained the IQ motif and provided a convenient point of reference. As shown in Fig. 5B, dCaM displayed significant interactions with peptide A-(1558–1579), which contains the IKTEG sequence and the consensus 1-8-14, type A CaM-binding motif, peptide C-(1585–1606), which contains the LLDQV sequence, and reference peptide F-(1619–1638), which contains the IQ motif. In contrast, dCaM failed to show interactions with the peptides B-(1568–1589), D-(1595–1616), and E-(1609–1630).

The experiments with the peptide series were of considerable interest from more than one point of view. First, finding significant CaM interactions with multiple nonoverlapping peptides suggested that the determinants of CaM tethering might reside in noncontinuous stretches of amino acids within the αC C terminus. Second, peptide A contains the signature sequence IKTEG, and peptide C includes the signature sequence LLDQV, both of demonstrated importance for CDI (12, 24). Third, the positions of the interacting peptides A and C may help explain the earlier pattern of results with GST fusion proteins (Fig. 4B); significant interactions with CaM and CaM\(_{1234}\) were found for the fusion protein 1551–1660, which subsumes both peptides A-(1558–1579) and C-(1585–1606), but no interaction was detected with fusion protein 1477–1592, which incorporates A but not C. The interaction of CaM with fusion protein 1592–1875, which contains F, part of C, but not A, confirms our previous observation of a Ca\(^{2+}\)-dependent, high affinity interaction of CaM with the IQ peptide (contained in F) and demonstrates that tethering at resting Ca\(^{2+}\) levels requires at least all of A and C.

**CaM Interactions with Various Peptides Show Differing Patterns of Ca\(^{2+}\) Dependence**—If peptides A and C contribute to the tethering site, one might expect the peptides to be able to bind CaM at ambient Ca\(^{2+}\) levels in resting cells. To test this assumption, we determined the Ca\(^{2+}\)-dependence of CaM binding to both of these peptides and peptide F, which contains the IQ motif. The spectrum for dCaM at a given concentration of free Ca\(^{2+}\) was subtracted from the spectrum of the same dCaM sample after addition of a saturating concentration of added peptide. This yielded accurate estimates of the peptide-dependent increase in dCaM fluorescence (Fig. 5C, top). The data enabled us to determine the Ca\(^{2+}\)-dependence of CaM bound to peptide A at zero Ca\(^{2+}\) and remained fairly constant up to 1 μM free Ca\(^{2+}\), consistent with a site that serves a tethering function. At higher levels of free Ca\(^{2+}\), the dCaM signal in the presence of peptide showed a further increase, suggesting either additional dCaM binding or changes in the nature of the dCaM-peptide A interaction and in the environment of the fluorophore. Peptide C showed an intermediate Ca\(^{2+}\)-dependence (Fig. 5C, middle).
Little dCaM bound in zero Ca\(^{2+}\), but binding increased with rising concentrations of free Ca\(^{2+}\) (K\(_{0.5}\) ~100 nM), suggesting that a significant portion of dCaM bound to peptide C at resting levels of Ca\(^{2+}\), and that peptide C may serve a tethering function. In contrast, peptide F showed little or no interaction at levels of Ca\(^{2+}\) found in resting cells, and increasingly strong effects on the fluorescent signal as Ca\(^{2+}\) was elevated, with a K\(_{0.5}\) of ~400 nM (Fig. 5C, bottom). This buttressed the idea that Ca\(^{2+}\) drives the CaM-IQ motif interaction, thereby accelerating inactivation once the channel has opened and Ca\(^{2+}\) has risen.

These results with peptides A and C differ somewhat from those obtained with the fusion proteins in Fig. 4. No CaM interaction was detected with fusion protein 1477–1592, which contained peptide A, even if Ca\(^{2+}\) was present; in the absence of Ca\(^{2+}\), no CaM interaction was detected with fusion protein 1551–1660, although it contained peptides A and C. The dCaM assay may reveal weaker interactions at single contact sites, whereas detection in the GST-pulldown assay may require higher affinity binding like that afforded by multiple points of interaction. Furthermore, in the absence of Ca\(^{2+}\), a fusion protein may attain a conformation that is not permissive for CaM binding.

The N- and C-terminal Domains of CaM and Interactions with Peptides A, C, and F—The finding that multiple, non-contiguous sequences in the C-terminal region interact with CaM, and do so with different Ca\(^{2+}\) requirements, prompted us to perform new tests to determine which of these sequences might accelerate inactivation by binding CaM and which might tether CaM in readiness for such action. A clear criterion for the accelerator site was established by previous experiments with CaM mutants in which various Ca\(^{2+}\)-binding EF hands were disabled (4). CDI was spared following expression of CaM\(_{44A}\), a CaM mutant in which the two N-terminal EF hands were mutated to Ca\(^{2+}\)-insensitive forms. In contrast CaM\(_{44A}\), a CaM mutant in which the two C-terminal EF hands are disabled, ablated CDI in a dominant negative manner.

These results predicted that the accelerator site should interact preferentially with the C-terminal lobe of CaM, the region where wild-type Ca\(^{2+}\) binding is critical, rather than the N-terminal lobe, where loss of Ca\(^{2+}\) binding can be tolerated. On the other hand, the findings with dominant negative CaMs made no specific prediction about which domain(s) of CaM should interact with the tethering site, so long as this binding occurs at resting Ca\(^{2+}\) levels (4). Accordingly, we set up competition experiments with CaM and its N- or C-terminal domains, generated by limited proteolysis (25). We examined the ability of CaM or its domains to interfere with the interaction between fluorescent CaM and peptides A, C, and F (Fig. 6). Since CaM and both the N-terminal and C-terminal lobes of CaM are Ca\(^{2+}\)-binding proteins, their addition at the concentrations used in this competition assay would affect any attempts to buffer Ca\(^{2+}\) at low levels and therefore affect the dCaM signal, independent of their ability to compete with dCaM. Accordingly, to be fully certain that changes in Ca\(^{2+}\) concentration would not affect interpretation of the results, we used solutions containing 1 mM Ca\(^{2+}\) to study CaM interactions with peptides A, C, and F. In all three cases, raising the concentration of unlabeled CaM progressively diminished the fluorescence signal generated by interaction of peptide with dCaM. In the case of peptide C (Fig. 6B), the N- and C-terminal domains of CaM were equally effective in reducing the fluorescent signal. In contrast, the hemi-CaMs competed differently for binding of dCaM to peptide F, which contains the IQ motif (Fig. 6C). The C-terminal lobe competed effectively, whereas the N-terminal lobe did not compete well (K\(_{I}\) for C-terminal lobe ~46-fold lower than K\(_{I}\) for N-terminal lobe). In agreeing with earlier experiments with CaM\(_{44A}\) (4), this preference supported the prevailing hypothesis that speeding of inactivation by CaM involves CaM binding to the IQ motif. The employment of the C-terminal domain of CaM as the Ca\(^{2+}\) signal transducer finds precedent in the control of the Ca\(^{2+}\)-activated K\(^{+}\) current in Paramecium (26).

Peptide A also demonstrated a preference for interaction with the N-lobe of CaM over the other (Fig. 6A), but in this case, it was the N-terminal lobe of CaM rather than the C-terminal lobe that competed more effectively with dCaM for binding to peptide A. The K\(_{I}\) for the N-terminal lobe was ~10-fold lower than the K\(_{I}\) for the C-terminal lobe, the opposite of what was found with peptide F.

The effector and tethering functions performed by the various peptides depend not only on the Ca\(^{2+}\) dependence of the interaction (Fig. 5C) but also on the affinity of the peptides for...
CaM. All three peptides were found capable of interacting with CaM at the high Ca\(^{2+}\) levels that allow CaM to perform its effector function. However, one would expect that at maximum, only two out of the three would be able to interact simultaneously with CaM, presumably one peptide sequence per N- or C-terminal lobe of CaM. This prompted us to characterize the concentration dependence of binding of peptides A or C to dCaM, with the goal of determining which peptide(s) would bind CaM most favorably in high Ca\(^{2+}\). We had previously shown that peptide F, which contains the IQ motif, interacts with high affinity with CaM (K\(_{0.5} \approx 50\) nM peptide) (7) (broken line in Fig. 7). In these new experiments, we determined that the interaction of peptide A with CaM conformed to a 1:1 binding curve, with a much weaker affinity (K\(_{0.5} \approx 1\) µM peptide, right curve, Fig. 7). In contrast, the interaction of peptide C and CaM followed a steeper binding curve, with intermediate affinity (middle curve).

**DISCUSSION**

Our findings provided clear answers to fundamental but unanswered questions about CaM action as follows: how CaM is tethered to the L-type channel before performing its accelerator function, how the CaM interacts with the effector IQ domain, and how the CaM sensor is able to act so quickly to initiate Ca\(^{2+}\)-dependent inactivation once Ca\(^{2+}\) entry has begun.

A Structural Basis for CaM Tethering—Knowing how CaM is tethered to resting L-type channels is critical to understanding how CDI occurs so quickly and how mutant CaM molecules exert their dominant negative effects. The C-terminal cytoplasmic domain was initially deemed unpromising as a locus for anchoring CaM because it failed to bind CaM in the absence of Ca\(^{2+}\) (4). We re-examined the involvement of this region by focusing on peptide interactions with CaM\(_{1234}\), arguably the most stringent test for a genuine locus of CaM tethering (4). Because CaM\(_{1234}\) completely lacks high affinity Ca\(^{2+}\) interactions, we were able to run tests for interaction in the presence of nonzero Ca\(^{2+}\) concentrations. The results demonstrated clearly that a portion of the C-terminal domain was able to bind CaM\(_{1234}\) or CaM, but only at 10–100 nM Ca\(^{2+}\), levels normally maintained in cells at rest. Thus, tethering does occur in the C-terminal cytoplasmic region but is complicated by a Ca\(^{2+}\) dependence intrinsic to the channel itself.

We found that the C-terminal tethering does not require the IQ motif but relies instead on two noncontiguous stretches of amino acids, roughly 51 and 24 aa to the N-terminal side of the IQ motif. The involvement in tethering of the more N-terminal group of residues (peptide A) is a novel finding, whereas our conclusions about the other set of amino acids (peptide C) are similar to those put forward in recent papers from the groups of Soldatov (10), Hamilton (9), and Maulet (11). The definitive test for tethering, binding of a dominant negative CaM molecule, is also a new result that we present here. Uncovering multiple sites for tethering relatively close to the IQ motif has important functional implications because the proximity of loci of tethering and acceleration would support a very fast development of CDI. It is also noteworthy that the sequences in peptides A and C are present in s\(_{1c}\), subunits but not in s\(_{1a}, \) s\(_{1b}\), or s\(_{1c}\); consistent with the fact that CDI is much faster and more prominent in L-type channels than in (P/Q), N-, or R-type channels.

Structural Basis of CDI Acceleration, Binding of C-terminal Lobe of CaM to IQ Motif—To identify the part of CaM that interacts with the IQ motif, we examined the competition between CaM fragments and fluorescent CaM for binding to IQ peptide. The finding that the IQ domain interaction selectively involves the C-terminal domain of CaM, not the N-terminal domain (Fig. 6C), provides a satisfying explanation of two important findings from previous biophysical experiments. First,
CDI remains intact in the presence of an excess of CaM_{12} but not CaM_{34} (4). Second, the cooperativity of CDI is much less than that of the transition from apocalmodulin to fully Ca^{2+}-saturated CaM (27).

A Model for CDI Based on Rapid Switching between CaM Configurations—Here we present a working hypothesis for Ca^{2+}-dependent inactivation of L-type channels that takes into account recent findings on how the N- and C-terminal lobes of CaM help tether CaM and support its effector actions (Fig. 8). Regions of the C-terminal cytoplasmic region of α_{1C}, are labeled A, C, and IQ, in accordance with the peptide segments used in our earlier binding studies. At resting Ca^{2+} levels (left panel), calmodulin is depicted as bound to sequences A and C. This is based on the finding that CaM shows significant interactions with the corresponding peptides even at very low Ca^{2+} (Fig. 5C). The CaM is shown as oriented with its N-terminal lobe associated with A, as suggested by Fig. 6A, whereas peptide C is depicted as lying between the two CaM lobes, reflecting its ability to interact with either lobe (Fig. 6B). This arrangement is subject to the caveat that the orientation of CaM lobes was examined in the presence of saturating Ca^{2+} concentrations, but we believe that the results extend to basal Ca^{2+} levels in resting cells since the binding of CaM_{1234} to the tethering regions remained constant over a wide range of Ca^{2+} concentrations (Fig. 4).

The IQ motif is pictured as not contributing to the CaM tethering, based on three pieces of evidence. First, dominant negative CaMs do not interact with the IQ motif (Fig. 1). Second, mutation of the IQ motif spares a significant degree of CaM binding to the cytoplasmic tail (Fig. 3). Third, CaM binding to a smaller fusion protein that contains the tethering sites is unaffected by disruption of the IQ motif (5).

Upon elevation of cytosolic Ca^{2+} to micromolar levels (right panel), the IQ motif is pictured as shifting to the C-terminal lobe of CaM, whereas the N-terminal lobe remains anchored on peptide A and both lobes continue to harbor peptide C. In support of this are data showing that the C-terminal lobe of Ca^{2+}/CaM interacts directly with the IQ motif (Fig. 6C). Our model favors the rapid development of inactivation, since it requires only minor conformational changes, namely IQ peptide association with the C-terminal lobe of CaM driven by the elevation of Ca^{2+}. Restoration of resting levels of cytosolic Ca^{2+} would favor a return to the original CaM configuration (left) and a prompt removal of inactivation, leaving the CaM tethered for another round of inactivation.

Implications of Multiple, Discrete Sequences for Tethering and Effector Action—This model deviates from the classical scenario for CaM action that originated from studies of CaMKII and myosin light chain kinase (28, 29). In this scenario, both lobes of Ca^{2+}/CaM wrap around a short target peptide and bind close to each other, thereby removing the repressive effect of the peptide and nearby residues on the catalytic site of the enzyme. A similar mechanism has been proposed for CDI (4).

Our hypothesis is most closely aligned with current thinking about gating of the SK potassium channel, another ion channel regulated by Ca^{2+} through constitutively bound CaM (16, 32). Recently, Schumacher and colleagues (33) provided an elegant crystal structural analysis of CaM bound to CaM binding domain (CBD) from the C-terminal cytoplasmic tail of the SK channel. In the CaM_{CBD} complex, both lobes of CaM engage in multiple interactions with distinct α-helical stretches of the two CBDs. The C-terminal lobe of a CaM lacks bound Ca^{2+} but interacts closely with separate helical segments from one CBD; the N-terminal lobe of the CaM holds two Ca^{2+} ions and engages the other CBD. Thus, in striking correspondence with our inferences for the Ca^{2+} channel, CaM tethering in the SK channel involves two α-helical segments and the effector interaction takes place by recruitment of a third α-helical segment. In both cases, binding of Ca^{2+} to only one of the CaM lobes is sufficient to trigger gating, but in the L-type Ca^{2+} channel this is the C-terminal lobe and not the N-terminal lobe as in the SK channel.

In the present case, these distinct and specific interactions may confer functional advantages for control of channel gating. Employing several points of attachment for a single CaM molecule may allow the Ca^{2+} sensor to satisfy the disparate requirements of the CDI mechanism. On one hand, the C-terminal lobe must rapidly engage the IQ motif, of known importance for CDI; on the other hand, the sensor must stay securely attached, to remain immediately ready for repeated rounds of inactivation. The model we present would ensure the retention of a CaM molecule, allowing it to function over and over again, as implied by the persistent expression of CDI in planar bilayers bathed in solutions devoid of CaM (34).

Another Function for Peptides A and C, Control of Ca^{2+}-independent Inactivation?—Our biochemical observations fit well with earlier biophysical studies of CDI (12, 24) that pointed to importance of groups of amino acids, IKTEG and LLDQV, that fall within peptides A and C. Swapping either of these five-residue segments leads to significant changes in inactivation properties, not only a reduction in the Ca^{2+} dependence but also a speeding of inactivation with Ba^{2+} as a charge carrier. In combination, the domain swaps completely abolish all Ca^{2+} dependence and greatly hasten inactivation of I_{Ba}. Altered inactivation kinetics in the absence of Ca^{2+} entry would not be predicted simply because of the removal of a tethered Ca^{2+} sensor. The effects on I_{Ba} provided a clue that one or both of these amino acid sequences may exert an effect beyond that expected for a passive tether.

Therefore, our model attributes yet another function to the CaM tethering and effector sites. We hypothesize that sequences in peptides A and C not only tether CaM, but in binding apoCaM also help create a deceleratory signal to retard inactivation when cytosolic Ca^{2+} levels are low. Modifying either sequence would alter the local conformation of the CaM-tethering site and disrupt the braking effect, as manifested by the observed speeding of I_{Ba} inactivation (12, 24). When cytosolic Ca^{2+} suddenly rises, the strongly favored interaction between CaM and the IQ motif will change the local configuration...
CaM Tethering and CDI of L-type Ca^{2+} Channels

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