Sites on Calmodulin That Interact with the C-terminal Tail of Ca\textsubscript{v}1.2 Channel* [S]

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Two fragments of the C-terminal tail of the \(\alpha_1\) subunit (CT1, amino acids 1538–1692 and CT2, amino acids 1596–1692) of human cardiac L-type calcium channel (Ca\textsubscript{v}1.2) have been expressed, refolded, and purified. A single Ca\textsuperscript{2+}-calmodulin binds to each fragment, and this interaction with Ca\textsuperscript{2+}-calmodulin is required for proper folding of the fragment. Ca\textsuperscript{2+}-calmodulin, bound to these fragments, is in a more extended conformation than calmodulin bound to a synthetic peptide representing the IQ motif, suggesting that either the conformation of the IQ sequence is different in the context of the longer fragment, or other sequences within CT2 contribute to the binding of calmodulin. NMR amide chemical shift perturbation mapping shows the backbone conformation of the fragment. Ca\textsuperscript{2+} produces the greatest changes in the backbone amides of residues in the N-lobe relative to loops III and IV in the C-lobe. In conclusion, Ca\textsuperscript{2+}-calmodulin assumes a novel conformation when part of a complex with the C-terminal tail of the Ca\textsubscript{v}1.2 \(\alpha_1\) subunit that is not duplicated by synthetic peptides corresponding to the putative binding motifs.

Calmodulin (CaM), a ubiquitous Ca\textsuperscript{2+} sensor, directly or indirectly regulates excitation-contraction coupling and other important physiological functions in cardiac myocytes (3–7). Cardiac L-type Ca\textsuperscript{2+} channels (Ca\textsubscript{v}1.2) are modulated by the interaction of the channel \(\alpha_1\) subunit C-terminal tail with CaM (8), such that CaM binding to this region is required for both Ca\textsuperscript{2+}-dependent inactivation (CDI) and Ca\textsuperscript{2+}-dependent facilitation (CDF) of cardiac L-type Ca\textsuperscript{2+} channels (9–14). CDI is the process whereby the entry of Ca\textsuperscript{2+} enhances channel closing during a maintained depolarization (15, 16), whereas CDF is the process whereby increased basal Ca\textsuperscript{2+} or repeated transient depolarizations leads to increased channel opening (17). CDI of Ca\textsubscript{v}1.2 appears to be driven by Ca\textsuperscript{2+} binding to the C-lobe of CaM and is unaltered by the presence of intracellular Ca\textsuperscript{2+} buffers (18).

Although a number of sequences within the C-terminal tail of the \(\alpha_1\) subunit appear to be capable of binding CaM (1, 9, 19–21), it is unclear which actually contribute to CaM binding in the native channel. A sequence designated the IQ motif is required for both CDI and CDF, but the precise roles of other neighboring sequences remain to be elucidated. Peterson et al. (22) and De Leon et al. (23) identified critical determinants of CDI within the consensus Ca\textsuperscript{2+} binding motif (the EF hand) of the cardiac L-type Ca\textsuperscript{2+} channel. Specifically, Peterson et al. (22) found a four amino acid cluster (VVTTL) within the EF hand to be essential for CDI. However, Zhou et al. (24) and Qin et al. (13) maintained that CDI of Ca\textsubscript{v}1.2 did not require the EF hand motif. Zühlke and Reuter (21) suggested that CDI was a cooperative process involving three noncontiguous amino acid sequences: the EF hand motif, two hydrophilic residues (asparagine and glutamic acid, residues 1630 and 1631), and the IQ motif. To complicate matters further, two other sequences (amino acids 1609–1628, designated A motif; amino acids 1627–1652, designated C or CB motif) between the EF hand and the IQ motif have also been implicated in CaM binding (1, 9, 19–21).

Additional data to define the CaM binding site on the C-terminal tail of the \(\alpha_1\) subunit and to elucidate the mechanisms of CDI and CDF of Ca\textsubscript{v}1.2 are needed. The molecular details of the interactions and the stoichiometry of this interaction remain to be determined. In this report, we describe the expression of fragments of the \(\alpha_1\) subunit C-terminal domain and provide new details of its interaction with CaM.

EXPERIMENTAL PROCEDURES

Materials—\(^{15}\)N\textsubscript{H}\textsubscript{4}Cl and \(^{13}\)C-glucose were obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA). \(^{15}\)N-((2-iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid (1,5-IAEDANS) was purchased from Molecular Probes, Inc. (Eugene, OR). N-((4-Dimethylamino-3,5-dinitrophenyl)maleimide (DDPM) and other chemicals were purchased from Sigma. Peptides were synthesized in the core facility at Baylor College of Medicine under the direction of Dr. Richard Cook.

Expression and Purification of CT1, CT2, Wild-type Ca\textsubscript{M}, N-CaM, C-CaM, E12Q CaM, E34Q CaM, E1234Q CaM, and T34C CaM—We made two constructs of the C-terminal tail using the human Ca\textsubscript{v}1.2 \(\alpha_1\) subunit cDNA as a template. One construct codes for amino acids 1538–1692 (includes the EF hand regions and the A, C, and IQ motifs) and is designated CT1. A second construct codes for amino acids 1596–1692 (includes the A, C, and IQ motifs) and is designated CT2.
We subcloned the cDNA of these sequences into pET23a(+) and pET28a(+) vectors (Novagen, Madison, WI) between NdeI and HindIII sites and used these constructs for the expression of the fragments with and without a His tag. We transformed the subcloned vectors into BL21(DE3) host (Novagen) for expression and grew the cells in LB media containing the suitable antibiotics at 37°C. Cells were induced with 0.5 mM isopropyl-1-thio-D-galactopyranoside at an OD600 of 0.6 and incubated another 3 h at 37°C before harvesting. The cells were lysed with lysozyme and nuclease and soluble material was removed by sedimentation of the insoluble material. The insoluble pellet was washed with water and Triton X-100 to obtain an insoluble fraction enriched in inclusion body proteins. Wild-type CaM, N-CaM, and C-CaM were expressed and purified as described previously (25). The Ca2+ binding site mutants of CaM (E12Q CaM, E34Q CaM, and E1234Q CaM) were expressed and purified as described previously (26). Expression and purification of T34C/T110C CaM (cysteine substitution for threonine at positions 34 and 110 of CaM) is the same as that of wild-type CaM.

**Refolding of CT1 (Amino Acids 1538–1692) and CT2 (Amino Acids 1596–1692).—**The inclusion bodies were solubilized with Inclusion Body solubilization reagent (Pierce) and refolded by dialyzing against 90 mM Tris-HCl, pH 7.4, 6 mM urea, 6 mM calcium in the presence of CaM (wild-type CaM or CaM mutants). Water was added gradually to the dialysis buffer to the urea concentration to 2 M. The remaining urea was removed by dialysis against 30 mM Tris-HCl, pH 7.4 and 2 mM calcium. Soluble and insoluble proteins were then separated by centrifugation at 48,000 × g for 1 h at 4°C. The tags were removed using a Thrombin Cleavage Capture kit (Novagen). The refolding of the presence of CaM was also examined at three different Ca2+ concentrations.

**Preparation of CT1 for Analysis of CaM Binding by Nondenaturing Gel Electrophoresis—**The untagged, solubilized CT1 was further purified using chelating Sepharose (Amersham Biosciences). To avoid CT1 precipitation, all buffers (equilibration, binding, wash, and elution buffer) included a 50% solution of the Inclusion Body solubilization reagent. CT1 was eluted with 10 mM imidazole. The purified CT1 was refolded by dialysis against 90 mM Tris-HCl, pH 7.4, 6 mM urea, 3% Triton X-100, gradually adding water into the dialysis buffer until 2 M urea was reached. At this stage CT1 was dialyzed against 50 mM MOPS, pH 7.4, 1% Triton X-100. Increasing amounts of refolded CT1 were incubated with CaM (2 μg in 20 μL of buffer) per sample) for 30 min at room temperature in 50 mM MOPS (pH 7.4), 1% Triton X-100, and 2 mM CaCl2 or 2 mM EGTA. Nondenaturing PAGE (15%) was performed to assess complex formation of CT1 and CaM. Protein concentrations were determined by the method of Lowry (27) using bovine serum albumin as a standard.

**Preparation of CT1, CT2, and Donor/Acceptor-labeled CaM for Fluorescence Resonance Energy Transfer (FRET)—**The purified complex of CT1 or CT2-CaM was obtained by refolding CT1 or CT2 in the presence of CaM. His tags were cleaved with thrombin. The samples were dialyzed against 30 mM Tris-HCl buffer, pH 7.4, with EGTA to precipitate CT1 or CT2. The pellet was washed with 30 mM Tris-HCl buffer, pH 7.4, to obtain purified CT1 or CT2 (insoluble). Samples were solubilized in the Inclusion Body solubilization reagent. Donor/acceptor-labeled CaM (D/A CaM) was obtained by labeling T34C/T110C CaM with 1.5-IAEDANS (the donor) and DDPM (the acceptor). To obtain T34C/T110C CaM that was only labeled with the donor, T34C/T110C CaM was labeled with 0.4 mol of donor/mol of protein in 20 mM Tris-HCl, pH 7.5, 100 mM KCl for 2 h at 20°C in the dark. Free donor was removed by a desalting column packed with size exclusion medium, Bio-Gel P-6DG (Bio-Rad). The product had 0.31 mol of the donor per mol of protein. A portion of this partially labeled protein was saved as the donor-alone protein, while the rest was labeled with excess acceptor to give D/A CaM saturated by acceptor at the same reaction conditions. Free acceptor was removed by the same desalting column as above.

**FRET—**D/A CaM (0.5 μM) was incubated with increasing concentrations of CT1, CT2, A-peptide, C-peptide, or IQ-peptide for 5 min at 20°C in 20 mM MOPS pH 7.5, 100 mM KCl, 2 mM CaCl2, or 2 mM EGTA. Fluorescence data were collected on a PTI QuantaMaster spectrofluorometer. The excitation wavelength was set at 334 nm and emission spectra were scanned from 400 nm to 600 nm with a 5-nm slit width for excitation and a 10-nm slit width for emission. Spectra were processed by subtracting the background of buffer and/or additives where appropriate and by averaging three sets of scans. To ensure that the fluorescence changes were due to the changes of distance between donor and acceptor and not to the interaction of these fragments with the donor, parallel experiments were performed with donor-alone-labeled CaM. Data were compared with the FRET obtained with a CaM kinase II peptide (FNARRKLGAILTMLATRN, designated FNA peptide) that was known to bring the two lobes of CaM in close proximity (28).

To study the apparent affinity of CT2 and the synthetic peptides for CaM, we titrated D/A CaM in 20 mM MOPS pH 7.5, 100 mM KCl, 2 mM CaCl2 with increasing CT1-, CT2-, IQ-, A-, or C-peptide. Fluorescence emission data at 490 nm were read on an ISS PC1 Photon Counting Spectrofluorometer (ISS, Champaign, Illinois), with a 1-mm slit for excitation and 2-mm slit for emission, at a 334 nm excitation wavelength. Data were processed by subtracting the background of buffer and/or additives where appropriate. The data were fit with a four-parameter Hill equation in SigmaPlot.

**Preparation of CT1 or CT229,N13C-labeled CaM Complex for Nuclear Magnetic Resonance Spectroscopy (NMR)—**29,N13C-labeled CaM was created by growing cells in M9 minimal medium using an expression plasmid coding for D/A CaM. 34NH4Cl and 6-[U-13C]glucose were the only nitrogen and carbon sources for the cells. Other steps involved in expression and purification of 29,N13C-labeled CaM were the same as for wild-type CaM. The complex of 29,N13C-labeled CaM/CT1 or CT2 for NMR was obtained by refolding the solubilized CT1 or CT2 in the presence of 29,N13C-labeled CaM. The His tag was cleaved with biotinylated thrombin, and the thrombin was removed by binding to streptavidin agarose (Novagen). The cleaved His tag was removed from the complex solution during dialysis against the final buffer (10 mM imidazole pH 6.4, 2 mM CaCl2) with a 10,000 MWCO cassette (Pierce).

**NMR Methodology—**NMR experiments were collected on a DRX 600 MHz spectrometer instrument using a 5-mm TXI probe or cryoprobe at 47°C. Complexes of CaM29–13C-labeled CaM bound to CT1 or CT2 were prepared as described above and then concentrated to 0.5–1 mM. Peptide-CaM complexes were prepared by adding the appropriate peptide directly to the Ca2+-CaM NMR sample. Final NMR samples contained 10 mM imidazole, and 2 mM CaCl2 in 95% H2O, 5% D2O at pH 6.4. Amide chemical shifts for free Ca2+-CaM in the absence of salt were assigned by comparison to reported chemical shifts for free Ca2+-CaM in 100 mM KCl (29) and confirmed by the following triple resonance experiments: 1H, 15N,13C-One-bond HSQC, 15N,13C-COSY, and 15Nedited NOESY-HSQC. CaM complexes were assigned using the above mentioned experiments and in addition three-dimensional HNCO and HN(CA)CO experiments were also collected. All data were processed using Felix 2002 software from Accelrys.

**RESULTS**

**Expression of Fragments of the C-terminal Tail of the CaV1.2 α1c Subunit and Analysis of Their Ability to Bind CaM—**To elucidate the determinants of the molecular interactions of CaM with the C-terminal tail of the CaV1.2 α1c subunit, we expressed or synthesized fragments of this region containing all or part of the putative binding motifs (Fig. 1). For these studies we used three synthetic peptides designated A (amino acids 1609–1628), C (amino acids 1627–1652), and IQ (amino acids 1665–1685). We expressed two fragments of the C-termi-
Calcium Channels, Calmodulin, and Ca$^{2+}$

Fig. 2. Calcium-dependent complex formation of CT1 with CaM in 15% polyacrylamide nondenaturing gel. CT1 was incubated with 2 μg of CaM (in 20 μl, final 6 μM) were incubated for 30 min at room temperature in 50 mM MOPS, pH 7.4, 1% Triton X-100 with 2 mM Ca$^{2+}$ (A) or 2 mM EGTA (B) before electrophoresis. Lane 1, 2 μg CT1 alone; lanes 2–9, the molar ratio of CT1:CaM is 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0, respectively. I/I$_{0}$ designates the ratio of the intensity of the CaM band in the presence of CT1 versus that of CaM alone (C), and intensities were obtained by densitometric analysis of Coomassie Blue-stained CaM bands from three independent nondenaturing gels. The curve with filled squares is for the low Ca$^{2+}$ condition and the one with filled circles is for the high Ca$^{2+}$ condition.

The molecule of soluble CT1 or CT2 can be obtained by extraction of the inclusion bodies and a subsequent refolding step (see “Experimental Procedures”). The interaction of refolded CT1 with CaM as assessed by nondenaturing gel electrophoresis is shown in Fig. 2. CT1 is highly positively charged (isoelectric point 10.5) and does not enter the nondenaturing gel at either high or low Ca$^{2+}$ concentrations (lane 1 of Fig. 2, A and B, respectively). The interaction of CT1 with CaM was assessed by the appearance of a more slowly migrating complex CT1/CaM or by a decrease in the intensity of CaM band. A stable CT1-CaM complex was formed at high Ca$^{2+}$ concentrations (Fig. 2A). The densitometric analysis of the disappearance of the CaM band at different fragment concentrations is shown in Fig. 2C. A very limited and low affinity interaction of this fragment was detected with apoCaM (assessed by the disappearance of the apoCaM band on the gel, Fig. 2, B and C).

Although only a small amount of soluble CT1 or CT2 can be obtained as described above, the presence of CaM during the refolding step greatly enhances the amount of CT1 and CT2 that can be isolated in a soluble form. As shown in the SDS-polyacrylamide gel of the soluble fraction in Fig. 3, the amount of soluble CT1 is directly proportional to the CaM added (lane 2 of Fig. 3A). Other proteins and excess CT1 are found in the insoluble pellet (lane 3 of Fig. 3A). The CT1-CaM complex remains soluble even when concentrated to 100 mg of protein per ml. The complex of CT1 and CaM showed evidence of 2 complexes in nondenaturing gels (Fig. 3B, lane 2). Using N-terminal sequencing we found that some proteolysis after amino acid 1595 had occurred during the isolation. This observation led us to create CT2.

CT2 also required the presence of CaM in the refolding step (lane 4 of Fig. 3A), and the amount of soluble protein obtained was proportional to the amount of CaM added (lane 5 of Fig. 3A). The presence of free CaM in nondenaturing gels of CT1 but not CT2 (lane 2 of Fig. 3B) suggests that only one CaM is binding to CT1 and CT2. The free CaM in the refolding step (lane 4 of Fig. 3B) could be detected at all Ca$^{2+}$ concentrations in the presence of CaM (lanes 1–5), but was maximal at concentrations above 100 μM. Without CaM, no facilitated refolding was detected at any Ca$^{2+}$ concentration for either CT1 and CT2 (lanes 6–10). The molecular equivalents of CaM required to refold CT1 and CT2 suggests that only one CaM is binding to CT1 and CT2.

Ca$^{2+}$ Binding to Both Lobes of CaM Is Required for Maximal CaM-assisted Refolding—To evaluate the effects of Ca$^{2+}$ binding to the N- and C-lobes of CaM on the facilitated refolding we examined the ability of Ca$^{2+}$ binding site mutants of CaM to promote refolding at high Ca$^{2+}$ concentrations. For these experiments we used: 1) N-CaM, a CaM composed of amino acids...
approximately equimolar quantities; CaM alone; extraction and refolding of CT2 in the presence of CaM. CaM and CT1 are present in approximately equimolar quantities. Lane 3, soluble fraction after CaM assisted refolding of CT1; lane 4, soluble fractions obtained from extraction and refolding of CT2 in the presence of CaM; CaM and CT2 are present in approximately equimolar quantities; lane 5, insoluble fraction after CaM assisted refolding of CT2. B, non-denaturing gel electrophoresis: lane 1, CaM alone; lane 2, soluble fractions obtained from extraction and refolding of CT1 in the presence of CaM; lane 3, soluble fractions obtained from extraction and refolding of CT2 in the presence of CaM.

Fig. 3. CaM-mediated refolding of CT1 and CT2. CT1 and CT2 were expressed in E. coli and were found almost exclusively in inclusion bodies. The inclusion bodies were solubilized with Pierce Inclusion Body solubilization reagent. Fragments were refolded by dialyzing against 30 mM Tris-HCl, pH 7.4, 2 mM calcium in the presence of CaM. Soluble and insoluble fractions were analyzed by either SDS denaturing gel electrophoresis (A) or non-denaturing gel electrophoresis (B). A, lane 1, CaM alone; lane 2, soluble fractions obtained from extraction and refolding of CT1 in the presence of CaM. CaM and CT1 are present in approximately equimolar quantities. Lane 3, soluble fraction after CaM assisted refolding of CT1; lane 4, soluble fractions obtained from extraction and refolding of CT2 in the presence of CaM; CaM and CT2 are present in approximately equimolar quantities; lane 5, insoluble fraction after CaM assisted refolding of CT2. B, non-denaturing gel electrophoresis: lane 1, CaM alone; lane 2, soluble fractions obtained from extraction and refolding of CT1 in the presence of CaM; lane 3, soluble fractions obtained from extraction and refolding of CT2 in the presence of CaM.

Fig. 4. The effect of Ca2+ concentration on the facilitated refolding. CT1 and CT2, were refolded as described in Fig. 3 at different free calcium concentrations, in the presence or absence of CaM. A shows the soluble CT1, and B shows the soluble CT2, obtained after refolding in the presence of CaM at <10 μM, 100 μM, 10 mM, and 100 mM free Ca2+ (lanes 1–5, respectively) or in the absence of CaM at <10 μM, 100 μM, 10 mM, and 2 mM free Ca2+ (lanes 6–10, respectively) as assessed by SDS-PAGE.

Fig. 5. Effect of mutation of Ca2+ binding sites on CaM on the ability of CaM to facilitate refolding of CT1 and CT2. CT1 and CT2 inclusion bodies were solubilized with Inclusion Body solubilization reagent, refolded by dialyzing against 30 mM Tris-HCl pH 7.4, 2 mM calcium in the presence of various CaM mutants with the same molar concentration. Supernatants were used for SDS 15% PAGE. CT1 and CT2 refolded in the presence of CaM mutants are shown in Fig. 4, A and B, respectively. Lane 1, CaM standard; lane 2, CT1 or CT2 refolded with CaM; lane 3, N-CaM (amino acids 1–75 of CaM) standard; lane 4, CT1 or CT2 refolded with E12Q; lane 5, C-CaM (amino acids 76–148 of CaM) standard; lane 6, CT1 or CT2 refolded with C-CaM; lane 7, E12Q standard (no Ca2+ binding to the N-lobe of CaM); lane 8, CT1 or CT2 refolded with E34Q; lane 9, E34Q standard (no Ca2+ binding to the C-lobe of CaM); lane 10, CT1 or CT2 refolded with E12Q and E34Q; lane 11, E12Q and E34Q standard (no Ca2+ binding to any of the 4 sites of CaM); lane 12, CT1 or CT2 refolded with E1234Q; lane 13, CT1 or CT2 refolded without any of CaM mutants.

A-peptide (Fig. 6C) all decreased the fluorescence of D/A CaM. In contrast, the C-peptide (Fig. 6D) increased the fluorescence of D/A CaM. These findings suggest that the lobes of CaM are closer together when bound to CT2, the I/Q-peptide, and the A-peptide compared with the C-peptide. The longer CT1 fragment produces similar FRET changes to those determined with
CT2 (not shown). As a control we examined the fluorescence at 490 nm with CaM that was labeled only with the donor compound (D/CaM) and found that the fluorescence did not change upon addition of the peptides or fragments (data not shown).

With CT1, CT2, and IQ, saturation of the FRET changes was obtained at 1:1 molar ratios of fragment/CaM (Fig. 6, A and B). A higher molar ratio for saturation of the FRET changes was required for both the A- and C-peptides (Fig. 6, C and D), consistent with our findings that the IQ-peptide has a higher affinity for CaM than either the A- or C-peptide (1, 19). Analysis of the concentration dependence of the FRET changes (Fig. 6E) was used to calculate apparent affinities for CaM of 37 ± 3

![Diagram of FRET changes](image.png)

**Fig. 6. Effects of the fragment CT2 and synthetic peptides on FRET of D/A CaM.** Fluorescence donor and acceptor-labeled CaM (D/A CaM, 0.5 μM) and increasing CT2 (A), IQ-peptide (B), A-peptide (C), and C-peptide (D) were incubated for 5 min at 20 °C in 20 mM MOPS pH 7.5, 100 mM KCl, and 2 mM CaCl₂ before fluorescence data were collected. The number assigned to the curve indicates the molar ratio of CT2 (A), IQ-peptide (B), A-peptide (C), or C-peptide (D) to D/A CaM, respectively. Curve FNA shows the maximum fluorescence decrease of D/A CaM induced by FNA-peptide that makes the two lobes of CaM close each other. E shows FRET data obtained using increasing concentration of CT2, IQ, A, or C-peptide with a single concentration of D/A CaM. CT2 (●), Hill coefficient = 1.3 and EC₅₀ = 37 ± 3 nM; IQ (■), Hill coefficient = 1.1 and EC₅₀ = 45 ± 4 nM; A-peptide (▲), Hill coefficient = 1.1 and EC₅₀ = 76 ± 5 nM; and C-peptide (△).
nm for CT2 (n = 3), 45 ± 4 nm for IQ (n = 3), and 76 ± 5 nm (n = 3) for A-peptide). The titration curve of CT1 is essentially identical to that of CT2 (data not shown). Our measured $K_d$ for CaM binding to CT2 is comparable to the value ($K_d = 163$ nM) reported by Erickson et al. (30) for CaM binding to full-length $\alpha_1$C. The maximal fluorescence decrease of the D/A CaM in the presence of either the IQ-peptide or A-peptide is similar to that obtained with the control FNA-peptide (Fig. 6, B and C), which is known to bring the two lobes of CaM into close proximity (28). In contrast, the fragments CT1 and CT2 produced an intermediate decrease of D/A CaM fluorescence, suggesting that the conformation of CaM bound to the fragment is different than when bound to either A- or IQ-peptides. This could reflect a difference in conformation of these sequences (IQ or A) within the larger fragment or a contribution of the C sequence to the CaM interaction. At low Ca$^{2+}$ concentrations, the fragments and peptides did not produce FRET of D/A CaM (data not shown), indicating that the either conformational change in CaM or its interaction with the fragments and peptides are Ca$^{2+}$-dependent.

**Effect of CT1 and CT2 on Backbone Amide Chemical Shifts of Ca$^{2+}$-CaM**—We used amide chemical shift perturbation mapping to compare the interactions of CaM with the expressed fragments of the C-terminal tail of the Ca$_1.2$ $\alpha_1$ subunit (CT1 and CT2) versus the synthetic peptides (C and IQ) corresponding to the putative CaM binding sites in this sequence. Our study was performed in the presence of both CT1 and CT2 are insoluble in the absence of Ca$^{2+}$-CaM.

Fig. 7A compares $^1$H,$^1$N HSQC spectra for free (black) and CT2-bound (red) Ca$^{2+}$-CaM. The absence of heterogeneity is consistent with a dominant conformation of Ca$^{2+}$-CaM bound to CT2 in a 1:1 complex. The boxed region in Fig. 7A is enlarged in Fig. 7, B–D to compare the effects of CT2 and CT1 on chemical shifts for selected amino acids in the N- and C-terminal lobes of CaM. Chemical shifts for the 11 amino acids are identical for Ca$^{2+}$-CaM bound to CT2 or CT1 (Fig. 7, C and D). In fact, the cross-peaks for only 5 residues (Ser$^{17}$, Glu$^{54}$, Met$^{77}$, Asp$^{78}$, and Thr$^{78}$) do not directly overlap in the spectra for CaM bound to CT2 versus CT1. These data indicate that sequences N-terminal to Met$^{1596}$ in the cytoplasmic tail of Ca$_1.2$ have minor, if any, effect on the conformation of Ca$^{2+}$-CaM.

The histogram in Fig. 8A summarizes the weighted chemical shift difference per residue for free CaM versus CaM bound to CT2. Also indicated are the locations of helices A–H and the four Ca$^{2+}$ binding loops. The N-domain and C-domain include residues 1–76 and 80–148, respectively. Residues 77–79 form a flexible linker between domains (31). The dashed line at 0.46 ppm corresponds to the mean plus the S.D. of chemical shift differences for all residues, and is used as reference for very significant changes. Bars denoted with closed circles correspond to hydrophobic residues with chemical shift changes ≥0.46 ppm.

CT2 causes large changes in the backbone amide chemical shifts for residues 77 and 78 located in the flexible tether that links helices D and E. Changes in this region, which functions as a hinge to allow the N- and C-domains of CaM to bind to small peptides (32, 33), are consistent with Fig. 6, showing that CT2 causes CaM to assume a more compact conformation. Large chemical shift changes in the N-domain are seen for hydrophobic amino acids Phe$^{19}$, Val$^{55}$, Leu$^{69}$, Met$^{71}$, and Met$^{72}$. In contrast, only hydrophobic residue Phe$^{92}$ in the C-lobe has a chemical shift perturbation ≥0.46 ppm. The majority of C-lobe amides with chemical shifts perturbation of ≥0.35 are associated with hydrophilic residues.

Fig. 9 shows chemical shift differences induced by CT2 mapped on the surface of Ca$^{2+}$-CaM bound to a peptide from smooth muscle myosin light chain kinase (34). We selected a peptide-bonded model because binding targets to Ca$^{2+}$-CaM stabilizes the intrinsically flexible N- and C-domains in more open conformations, as defined by interhelical angles (35). Ca$^{2+}$-CaM was bound to the myosin light chain kinase peptide was chosen since it is a well studied complex, and since the major difference in backbone tertiary structure of CaM bound to different target peptides is localized to the flexible linker between the N- and C-domains while the structures of the respective domains, especially the C-domain, is similar in the different complexes (36, 37). White indicates $\Delta \delta$ of <0.04 ppm, shades of red indicate increasing $\Delta \delta$ between 0.04 and 0.46 ppm, and red dark indicates $\Delta \delta$ ≥ 0.46 ppm. The two views of each domain are rotated roughly 180° degrees to contrast effects on surfaces that include the Ca$^{2+}$ binding loops and the hydrophobic pockets. The yellow contours encompass contiguous hydrophobic surfaces in the N- and C-lobes.

As anticipated from Fig. 8A, the N-domain hydrophobic pocket shown in the upper panel of Fig. 9 has a well defined red surface because of large chemical shift perturbations of hydrophobic residues. The hydrophobic pocket of the C-domain does not show a well grouped cluster of residues that are greatly affected by CT2. Instead, dark red surfaces in the C-domain of Fig. 9 are associated primarily with polar and charged residues that border the hydrophobic pocket. Similar patterns were seen when using structures of Ca$^{2+}$-CaM bound to peptides from the Ca$^{2+}$-ATPase, CaM-dependent protein kinase II, and anthraces edema factor (see Supplemental Information).

The histogram in Fig. 8A, and the ribbon and surface representations on the right hand side of Fig. 9 (which highlights the Ca$^{2+}$ binding loops) show more extensive chemical shift perturbations for residues in Ca$^{2+}$ binding loops I and II in the N-domain relative to loops III and IV of the C-domain. This contrast is especially striking for residues 56–67 in loop II and residues 129–140 in loop IV, which have average chemical shift changes of 0.21 and 0.07 ppm, respectively. Loop IV is also distinguished by a relatively small chemical shift perturbation for Ile$^{128}$ (0.17 ppm), which immediately precedes the loop. The corresponding residue preceding loops I, II, and III (Phe$^{15}$, Val$^{55}$, and Phe$^{92}$, respectively) have large chemical shift changes (0.51–1.1 ppm).

CaM-target interactions are known to modulate the Ca$^{2+}$ binding properties of CaM (38–43). CaM-mediated facilitation and inactivation of channel activity in response to Ca$^{2+}$ levels and frequency of oscillation may be coupled to channel-induced modulation of Ca$^{2+}$ binding to CaM. The data in Fig. 8A suggest that this modulation is targeted to sites I-III.

**Comparison of CT2 and IQ-Peptide on Chemical Shifts of Ca$^{2+}$-CaM**—Titration of Ca$^{2+}$-CaM with the IQ-peptide caused changes in backbone amide chemical shifts that were characteristic of slow exchange on the NMR time scale. Thus, backbone assignments for $^{15}$N,$^{13}$C-labeled Ca$^{2+}$-CaM bound to the IQ-peptide were made using triple resonance experiments described under “Experimental Procedures.” Fig. 8B compares backbone amide chemical shifts for free CaM versus the CaM-IQ peptide complexes. Patterns of chemical shift perturbations induced by the peptide are similar to those caused by CT2. The greatest magnitude of change is seen for residues 55–94, which span helices D and E. Hydrophobic residues in the N-domain of CaM are affected to a greater extent than those in the C-domain. Residues in loops I and II are affected to a greater extent than those in loops III and IV. However, as shown in Fig. 8C, 112 out of 148 backbone amide shifts differ by greater than the precision of the measurement when comparing CaM bound to CT2 versus the IQ-peptide. The average chemical shift difference for these residues (0.16 ppm) is 4-fold
FIG. 7. Effect of CT proteins on the 1H-15N HSQC spectra of Ca2+-CaM. A shows overlaid spectra of free Ca2+-CaM (black contours) with Ca2+-CaM bound to CT2 (red contours). The boxed region in A is expanded in B for free Ca2+-CaM and includes sequence-specific assignments. C and D, compare this region in the spectra for free Ca2+-CaM (black) with those of CaM bound to CT2 and CT1 (red), respectively. Red arrows in C and D indicate directions of chemical shift perturbation for several CaM backbone amides when complexed to CT proteins.
greater than the error for the calculation. The greatest differences are found in the helices A–D of the N-lobe. Helix E is also different. A comparison of the relative chemical shift differences between free CaM and CaM bound to either CT2 or the IQ-peptide suggests that the IQ-peptide alters the conformation of the N-lobe to a greater extent than CT2.

**Comparison of Effects of IQ- and C-Peptides**—Both the IQ and C-peptides were soluble at the concentrations required for NMR analysis, but the A-peptide was not and was, therefore, not studied further. Peptide C showed relatively weak binding to CaM with fast exchange characteristics on the NMR time scale. This allowed us to assign 118 backbone amides by following the change in chemical shifts during titration of Ca²⁺/CaM with the C-peptide. Fig. 8 shows that chemical shift changes induced by the C-peptide are lesser in magnitude than for CT2 or the IQ-peptide, because of weaker binding affinity, but are comparable to the effect of the IQ protein PEP-19 reported previously (44). Several features of Fig. 8 are notable. First, there is a lack of effect of the C-peptide on residues in the central hinge region of CaM. This is particularly evident for residues 77 and 78, which are greatly affected by CT2 and the IQ-peptide, but show little change upon association of the C-peptide. This is consistent with Fig. 6 showing that, in contrast to CT2 and the IQ-peptide, the C-peptide does not induce CaM to adopt a compact conformation. Second, relative to CT2 and the IQ-peptide, the C-peptide causes relatively large changes in chemical shifts for residues in Ca²⁺ binding loop IV. Third, chemical shift changes are observed in both N- and C-lobes of CaM. Since FRET experiments in Fig. 6 show CaM to be extended when bound to the C-peptide, single peptides may interact with both the N- and C-lobes, or one molecule of the C-peptide may bind to each lobe forming a ternary complex. Such a ternary complex may only be apparent at the high concentrations of CaM and peptide used for NMR experiments.

**DISCUSSION**

We have expressed and refolded two different fragments (CT1 and CT2) of the C-terminal tail of the α₁ subunit of the Caᵥ₁.2 channel. Refolding is greatly facilitated by the presence of CaM. Both CT1 and CT2 contain all three motifs (A, C, and IQ), which have been suggested to contribute to CaM binding (1, 9, 19–21). Despite the fact that synthetic peptides representing these motifs can each bind CaM, the expressed fragments (CT1 and CT2) bind only a single molecule of CaM, suggesting that either two of these sequences do not bind CaM when part of the larger protein or that the three sequences contribute to a single CaM binding site. A stoichiometry of one CaM binding to each Caᵥ₁.2 subunit is consistent with the findings of Mori et al. (45).

Peterson et al. (22) found that a four amino acid cluster (VVTTL) within the F helix of the EF hand motif of the C-terminal tail of the α₁ subunit of the Caᵥ₁.2 channel is essential for CDI and suggested that the EF hand motif of α₁ was involved in the transduction of Ca²⁺ binding to CaM into channel inactivation. A conformational coupling between Ca²⁺–CaM binding and the region of the EF hand (or the VVTTL sequence identified by Peterson et al., Ref. 22) seems unlikely since the conformation of CaM, as indicated by amide chemical shifts, is essentially identical when bound to CT1, which has the EF hand and the VVTTL sequence, and CT2, which does not. We
conclude that the sequences in the cytoplasmic tail N-terminal to Met\textsuperscript{1596}, including the EF motif, do not participate in binding to Ca\textsuperscript{2+}-CaM. A role of this region in association with apoCaM has yet to be determined.

Although Ca\textsuperscript{2+} binding to the EF hand does not appear to play a role in regulating the conformation of CaM bound to these fragments, Pitt \textit{et al.} (9) suggested that there was a site within the C-terminal tail of the a\textsubscript{1} subunit outside of the putative EF hand between amino acids 1551–1660 of the mouse sequence (corresponding to amino acids 1599–1708 of the human sequence) that binds Ca\textsuperscript{2+} with high affinity, allowing the binding of apoCaM. Romanin \textit{et al.} (46) also found that Ca\textsuperscript{2+} binding to a C-terminal sequence (amino acids 1571–1585 of the mouse sequence, corresponding to amino acids 1619–1633 of the human sequence) was required for apoCaM binding. These sequences are contained within both CT1 and CT2 and may play an important role in the interaction with apoCaM as proposed by these authors. However, a Ca\textsuperscript{2+} binding site mutant of CaM (E1234Q), even at Ca\textsuperscript{2+} concentrations expected to saturate these putative Ca\textsuperscript{2+} binding sites on CT1 and CT2, did not produce facilitated refolding of the expressed fragments, suggesting that this is a property of Ca\textsuperscript{2+}-CaM. CaM mutants unable to bind Ca\textsuperscript{2+} at either the N or C-lobes could partially support refolding, but binding to both lobes of CaM is required for maximal facilitated refolding of CT1 and CT2.

Previous studies from our laboratory have demonstrated that the IQ-peptide binds Ca\textsuperscript{2+}-CaM with higher affinity than the A- or C-peptides (19). We have also shown that the C- and IQ-peptides, but not the A-peptide, increase the affinity of the C-terminal lobe of CaM for Ca\textsuperscript{2+}. Only the IQ-peptide increases the affinity of the N-terminal lobe of CaM for Ca\textsuperscript{2+}. The differences in Ca\textsuperscript{2+} affinity of the lobes of CaM when bound to the different sequences and the differences in affinity for these CaM binding motifs for the lobes of CaM may allow CaM to assume different conformations within the binding site. Our FRET data suggest that CaM is in a more extended conformation when bound to CT2 than to the IQ-peptide and is in a very extended conformation when bound to the C-peptide. Amide chemical shift perturbation mapping experiments support the FRET data in that amides in the central linker regions of CaM are greatly affected upon binding to CT2 and IQ, but not to the C-peptide. The differences between CT2 and IQ-peptide bound to CaM could arise, in part, from competition with other sequences within CT2 for binding to the N-lobe of CaM. For example, the IQ motif in CT2 could anchor Ca\textsuperscript{2+}-CaM via its C-terminal lobe, allowing the N-terminal lobe to interact with other sequences (A and/or C). The N-terminal lobe could be either dominated by interaction with the A or C region or might be in fast exchange between the IQ regions and A and/or C regions, thereby promoting a slightly more extended conformation of CaM bound to CT2 versus IQ. This is consistent with the model of Pitt \textit{et al.} (9). The relative competitive binding advantage between regions in CT2 for binding to domains of CaM could change significantly depending on the binding of Ca\textsuperscript{2+} to CaM and/or the functional state of the channel itself.

Fig. 8 shows that differences between the conformation of CaM bound to CT2 and IQ extend beyond central linker region and include backbone differences throughout the N- and C-domains. This is in striking contrast to the results of Kranz \textit{et al.} (47) who used backbone amide chemical shift perturbation mapping to conclude that the backbone conformation of CaM is virtually identical when bound to either intact CaM kinase I (CKI) or the CaM binding peptide from CKI. Contributions from multiple sequences in CT2 to the CaM binding site may be a general feature of IQ motif proteins that is not shared by proteins such as CKI, which are regulated by autoinhibition involving a restricted CaM binding region.

Amide chemical shifts are extremely sensitive to conformational changes, and can result from allosteric effects, but Figs. 8 and 9 are consistent with interactions between CT2 and regions in the N- and C-lobes of Ca\textsuperscript{2+}-CaM that include hydrophobic surfaces. This is consistent with the structural paradigm established from NMR and crystal structures of CaM-target complexes that hydrophobic surfaces in Ca\textsuperscript{2+}-CaM.
interact with a variety of ligands (28, 32, 37, 48, 49). However, Figs. 8 and 9 reveal an interesting pattern of effects on hydrophobic and hydrophilic residues that suggest a greater contribution of hydrophobic interactions for binding CT2 to the N-domain of CaM, while binding to the C-lobe may rely more on electrostatic interactions with residues that border the hydrophobic pocket. This is consistent with previous studies demonstrating a role for electrostatic interactions in binding the IQ motif containing protein RC3 and its IQ-peptide to apo and Ca\(^{2+}\)CaM (50–53). A different chemical basis for interaction of CT2 with the N- and C-lobes of CaM could be important for the lobe movement on CT2 in response to changes in Ca\(^{2+}\) levels.

A final observation of our NMR studies is that CT fragments and the IQ-peptide have a greater effect on amide chemical shifts of residues located Ca\(^{2+}\) binding loops I and II in the N-lobe, especially loop II, relative to loops III and IV in the C-lobe. This may reflect important differential modulation of the Ca\(^{2+}\) binding properties of the N- and C-domains of CaM. For example, these data are consistent with our previous study (19) showing that the IQ-peptide produces approximately a 70-fold increase in the Ca\(^{2+}\) affinity of the N-lobe of CaM, but only a 16-fold increase in the Ca\(^{2+}\) affinity of the C-lobe. Since the IQ-peptide has a greater overall magnitude of effect than CT2 on the N-lobe of CaM, we predict modulation of Ca\(^{2+}\) to the N-lobe by these ligands.

In summary, these studies suggest that CaM interacts in a unique fashion with the C-terminal tail of the CaV\(_{1.2}\) \(\alpha_1\) subunit: 1) the C-tail of CaV\(_{1.2}\) binds a single CaM; 2) the conformation or initial folding of this region may be dependent on the presence of CaM; 3) the binding site cannot be adequately mimicked by synthetic peptides, suggesting multiple sites of interaction or a conformation not obtainable in a short peptide; 4) the N-lobe of CaM interacts primarily via hydrophobic interaction while the C-lobe of CaM involves significantly more electrostatic interactions; and 5) the 3rd and 4th EF hand are not greatly affected by the binding of CaM at the binding site.

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REFERENCES

1. Pate, P., Mochca-Morales, J., Wu, Y., Zhang, J. Z., Rodney, G. G., Serysheva, I. I., Williams, B. Y., Anderson, M. E., and Hamilton, S. L. (2000) J. Biol. Chem. 275, 39786–39792
2. Kretsinger, R. H. (1976) Annu. Rev. Biochem. 45, 239–266
3. Yang, D., Song, L. S., Zhu, W. Z., Chakir, K., Wang, W., Wu, C., Wang, Y., Xiao, R. F., Chen, S. R., and Cheng, H. (2003) Circ. Res. 92, 659–667
4. Walsh, K. B., and Cheng, Q. (2004) Am. J. Physiol. Heart Circ. Physiol. 286, H186–H194
5. Wu, Y., Kimbrough, J. T., Perez-Reyes, E., Wei, X., Soong, T. W., Smutch, T. P., and Yue, D. T. (1995) Science 270, 1553–1554
6. Tang, W., Halling, D. B., Black, D. J., Pate, P., Zhang, J. Z., Pedersen, S., Altschuld, R. A., and Hamilton, S. L. (2003) Biophys. J. 85, 1538–1547
7. Wang, Y., Zhang, J. Z., Pedro, S., Zhang, J. Z., Pedersen, S., Altschuld, R. A., and Hamilton, S. L. (2003) Biophys. J. 85, 1538–1547
8. Zühlke, R. D., DeMaria, C. D., Erickson, M. G., Mori, M. X., Alekseiak, B. A., and Yue, D. T. (2003) Neuro 39, 951–960
9. Tang, W., Halling, D. B., Black, D. J., Pate, P., Zhang, J. Z., Pedersen, S., Altschuld, R. A., and Hamilton, S. L. (2003) Biophys. J. 85, 1538–1547
10. McDonald, T. Y., Pelzer, S., Tratzwein, W., and Pelzer, D. J. (1994) Physiol. Rev. 74, 365–507
11. Catterall, W. A. (2000) Annu. Rev. Cell Dev. Biol. 16, 521–555
12. Anderson, M. E. (2001) J. Mol. Cell. Cardiol. 33, 639–650
13. De Leon, M., Wang, Y., Jones, L., Perez-Reyes, E., Wei, X., Soong, T. W., Smutch, T. P., and Yue, D. T. (1995) Science 270, 1592–1596
14. Putkey, J. A., Kleerekoper, Q., Gaertner, T. R., and Waxham, M. N. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 12430–12435
15. Crivici, A., and Ikura, M. (2003) Cell 112, 159–171
16. Johnson, J. D., Snyder, C., Walsh, M., and Flynn, M. (1996) J. Biol. Chem. 271, 761–767
17. Kasturi, R., Vasulka, C., and Johnson, J. D. (1993) J. Biol. Chem. 268, 7958–7964
18. Kranz, J. K., Lee, E. K., Nairn, A. C., and Wand, A. J. (2002) J. Biol. Chem. 277, 10949–10955
19. Peersen, O. B., Madsen, T. S., and Falke, J. J. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 2301–2305
20. Ricci, G. M., Liang, H., Mori, M. X., and Yue, D. T. (2000) J. Biol. Chem. 275, 39786–39792
21. Zühlke, R. D., DeMaria, C. D., Erickson, M. G., Mori, M. X., Alekseiak, B. A., and Yue, D. T. (1999) Neuron 22, 549–558
22. Peterson, B. Z., DeMaria, C. D., Adelman, J. P., and Yue, D. T. (1999) Neuron 22, 549–558
23. Qin, N., Oleese, R., Bransley, M., Lin, T., and Birnbauer, L. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 23435–23438
24. Soldatov, N. M., Zühlke, R. D., Bouron, A., and Reuter, H. (1997) J. Biol. Chem. 272, 3560–3566
25. McDonald, T. Y., Pelzer, S., Tratzwein, W., and Pelzer, D. J. (1994) Physiol. Rev. 74, 365–507
26. Cathcart, J. W., and Crivici, A. (1999) J. Mol. Biol. 286, 8099–8106
27. Zühlke, R. D., and Resh, M. D. (1998) J. Biol. Chem. 273, 951–960
28. Peterson, B. Z., Lee, J. S., Mullen, J. G., Wang, Y., de Leon, M., and Yue, D. T. (2000) Biophys. J. 78, 1906–1920
29. De Leon, M., Wang, Y., Jones, L., Perez-Reyes, E., Wei, X., Soong, T. W., Smutch, T. P., and Yue, D. T. (1995) Science 270, 1592–1596
30. Zhou, J., Oleese, R., Qin, N., Noveti, F., Birnbauer, L., and Stefani, E. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 2301–2305
31. Xiong, L. W., Newman, R. A., Rodney, G. G., Thomas, O., Zhang, J. Z., Pereschini, A., Shea, M. A., and Hamilton, S. L. (2002) J. Biol. Chem. 277, 40862–40870
32. Rodney, G. G., Krol, J., Williams, B., Beckingham, K., and Hamilton, S. L. (2001) Biochemistry 40, 12430–12435
33. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
34. Kurokawa, H., Osawa, M., Kurihara, H., Katayama, N., Tokumitsu, H., Swindells, M. B., Kainosho, M., and Ikura, M. (2001) J. Mol. Biol. 312, 59–68
35. Hoflich, K. P., and Ikura, M. (2002) Cell 108, 739–742
36. Erickson, M. G., Liang, H., Mori, M. X., and Yue, D. T. (2003) Neuron 39, 97–107
37. Barbat, G., Ikura, M., Kay, L. E., Pastor, R. W., and, and Bax, A. (1992) Biochemistry 31, 5269–5278