Apocalmodulin and Ca^{2+}Calmodulin-Binding Sites on the $Ca_V1.2$ Channel

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ABSTRACT The cardiac L-type voltage-dependent calcium channel is responsible for initiating excitation-contraction coupling. Three sequences (amino acids 1609–1628, 1627–1652, and 1665–1685, designated *A*, *C*, and *IQ*, respectively) of its α_1 subunit contribute to calmodulin (CaM) binding and Ca^{2+}-dependent inactivation. Peptides matching the *A*, *C*, and *IQ* sequences all bind Ca^{2+}CaM. Longer peptides representing *A* plus *C* (*A-C*) or *C* plus *IQ* (*C-IQ*) bind only a single molecule of Ca^{2+}CaM. Apocalmodulin (ApoCaM) binds with low affinity to the *IQ* peptide and with higher affinity to the *C-IQ* peptide. Binding to the *IQ* and *C* peptides increases the Ca^{2+} affinity of the C-lobe of CaM, but only the *IQ* peptide alters the Ca^{2+} affinity of the N-lobe. Conversion of the isoleucine and glutamine residues of the *IQ* motif to alanines in the channel destroys inactivation (Zühlke et al., 2000). The double mutation in the peptide reduces the interaction with apoCaM. A mutant CaM unable to bind Ca^{2+} at sites 3 and 4 (which abolishes the ability of CaM to inactivate the channel) binds to the *IQ*, but not to the *C* or *A* peptide. Our data are consistent with a model in which apoCaM binding to the region around the *IQ* motif is necessary for the rapid binding of Ca^{2+} to the C-lobe of CaM. Upon Ca^{2+} binding, this lobe is likely to engage the *A-C* region.

INTRODUCTION

Calmodulin (CaM) is an important regulator of ion channels, serving as a Ca^{2+} sensor for a number of ligand- and voltage-gated ion channels (Saimi and Kung, 2002). The cardiac L-type voltage-dependent calcium channel ($Ca_v1.2$) is the cardiac isoform of the L-type voltage-dependent calcium channel, but is also found in a variety of other tissues. The binding of Ca^{2+} to CaM, bound to the carboxy-terminal tail of the α_1 subunit of the $Ca_V1.2$ channel, can enhance channel opening (*facilitation*) (Zühlke et al., 1999), accelerate channel closing (*inactivation*) (Peterson et al., 1999; Qin et al., 1999), and/or initiate changes in gene transcription by activating the neuronal MAPK pathway (Dolmetsch et al., 2001). The molecular details of the processes by which CaM performs such diverse functions are not clear. The amplitude and/or frequency of the Ca^{2+} signal itself and/or the functional state of the channel at the time of the change in the Ca^{2+} concentration may contribute to the functional outcome of Ca^{2+} binding to CaM bound to the channel.

In cardiac muscle, Ca^{2+}-dependent inactivation of the $Ca_V1.2$ channel occurs during maintained depolarizations (Catterall, 2000; McDonald et al., 1994). Both Ca^{2+} influx through the Ca^{2+} channel and CaM binding to a motif designated as *IQ* are required for this type of inactivation (Peterson et al., 1999). Other sequences within the channel also appear to contribute to the inactivation event. The I–II loop (Adams and Tanabe, 1997), the putative Ca^{2+} binding EF hand motif (Zühlke and Reuter, 1998), and several regions between the EF hand and the IQ motif (Pate et al., 2000; Peterson et al., 2000; Pitt et al., 2001; Soldatov et al., 1998) have been implicated in this process. Ca^{2+}-dependent inactivation is blocked by a mutant CaM that cannot bind Ca^{2+}, suggesting that the channel also binds apocalmodulin (ApoCaM or Ca^{2+} free CaM) (Peterson et al., 1999).

Channel facilitation is a phenomenon in which Ca^{2+} currents are enhanced after an increase in basal Ca^{2+} or repeated transient depolarizations (Anderson, 2001). CaM binding to the *IQ* motif is required for this process, since mutation of the isoleucine to a glutamate destroys CaM binding completely and abolishes both inactivation and facilitation (Zühlke et al., 1999). Mutation of the isoleucine (amino acid 1624) within this sequence to an alanine results in loss of Ca^{2+}-dependent inactivation and unmasks a strong facilitation by CaM (Zühlke et al., 1999, 2000). Mutation (*IQ* (*AA*)) of both the isoleucine and the glutamine to an alanine (I1624A/Q1625A) produces an even more pronounced facilitation and, again, abolishes inactivation (Zühlke et al., 2000).

Three different sequences in α_{1C} carboxyterminal tail of $Ca_v1.2$ have been suggested to contribute directly to CaM

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Abbreviations used: ApoCaM/apocalmodulin, Ca^{2+} free calmodulin; Ca^{2+}CaM, Ca^{2+} bound calmodulin; CaM, calmodulin; $Ca_v1.2$, cardiac L-type calcium channel; $Ca_v1.1$, skeletal L-type calcium channel; CHAPS, (3-[(3-chlolamidopropyl)-dimethylammonio]-1-propanesulfonate); DHPR, dihydropyridine receptor, another name for the L-type calcium channel; EF Hand, Ca^{2+} binding motif (Kretsinger, 1976); EGTA, ethylene glycol-bis (beta-aminoethyl ether)-*n*,*n*,*n*,*n*′-tetraacetic acid; E12Q CaM, CaM double mutant containing E31Q and E67Q that abolishes Ca^{2+} binding to the amino-terminal lobe of CaM; E34Q CaM, CaM double mutant containing E104Q and E140Q that abolishes Ca^{2+} binding to the carboxy-terminal lobe of CaM; F19W, CaM mutant in which the phenylalanine in position 19 was replaced with a tryptophan; F92W, CaM mutant in which the phenylalanine in position 92 was replaced with a tryptophan; IQ (AA), mutant in which the isoleucine and the first glutamine within the IQ peptide sequence were replaced with two alanines; IQW, mutant in which the first phenylalanine in the IQ peptide sequence was replaced with a tryptophan; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecylsulfate.

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binding (Pate et al., 2000; Pitt et al., 2001; Soldatov et al., 1998; Zühlke and Reuter, 1998). These sequences are amino acids 1609–1628 (Pitt et al., 2001; Soldatov et al., 1998), 1627–1652 (Pate et al., 2000; Pitt et al., 2001), and 1665–1685 (Pate et al., 2000; Soldatov et al., 1998; Zühlke and Reuter, 1998) (this numbering is that of the human cardiac sequence), designated *A*, *C*, and *IQ*, respectively. Synthetic peptides representing these sequences all bind Ca^{2+}CaM, but it is not clear how they together contribute to CaM binding to the channel. The *A* motif (amino acids 1565–1578 of the mouse sequence or 1609–1628 of the human sequence) represents a 1-8-14 CaM binding motif (Pitt et al., 2001). Pitt and co-workers proposed that the N-lobe of apoCaM binds to the A motif whereas the C-lobe binds at an unidentified site, providing a ''brake'' that slows inactivation. In this model, when Ca^{2+} enters via the channel, it binds to the C-lobe of CaM, allowing this lobe of CaM to bind to the *IQ* motif, producing inactivation. This model predicts an apoCaM binding site in the carboxyterminal tail with the *A* motif forming part of the binding site. An apoCaM site has not been identified. However, an expressed fragment of the carboxyterminal tail corresponding to amino acids 1551–1660 of the mouse sequence (human sequence 1599–1708) that includes the *A*, *C*, and *IQ* sequences appears to undergo a Ca^{2+}-dependent conformational change (Pitt et al., 2001). The binding of Ca^{2+} with nM affinity at some site within this sequence is thought to alter the conformation of this region of the carboxyterminal tail to allow apoCaM binding. Romanin et al. (2000) found that the mouse sequence 1571–1586 (human 1619–1634) represents a high affinity Ca^{2+} binding site. The Ca^{2+} dependence of the interaction of CaM with the carboxyterminal tail of the $Ca_V1.2$ channel, therefore, appears to arise from Ca^{2+} binding to both CaM and the channel itself. The affinity of the Ca^{2+} binding site on the channel is such that Ca^{2+} is likely to occupy this site at resting Ca^{2+} levels.

How these three regions of the $Ca_V1.2$ channel allow CaM to function as a Ca^{2+} sensor for inactivation remains to be answered. We compared the ability of synthetic peptides representing the *A*, *C*, and *IQ* motifs to bind Ca^{2+}CaM, apoCaM, and Ca^{2+} binding site mutants of CaM and to alter the Ca^{2+} binding properties of CaM. We also examined the interaction of CaM with a peptide containing the *IQ* to *AA* substitution that abolishes Ca^{2+}-dependent inactivation of this channel.

MATERIALS AND METHODS

Materials

Bovine brain CaM (95% pure) was purchased from Sigma (St. Louis, MO) and dissolved in 10 mM MOPS (pH 7.4), 1 mM EGTA, and 0.02% NaN_3. CaMs with engineered mutations were constructed and isolated as described by Black et al. (2000). All peptides were synthesized at the protein lab facility at Baylor College of Medicine and were diluted into 200 mM MOPS (pH 7.4). Table 1 lists the peptides that are used in this study. All electrophoresis reagents were analytical grade from BioRad (Hercules, CA).

Methods

Electrophoretic mobility shift assays

The interactions of CaM with the peptides were evaluated by nondenaturing PAGE, under discontinuous conditions using a modified Laemmli technique (Laemmli, 1970). For analysis of interaction at low Ca^{2+} concentrations, the gels were prepared in triplicate with 1 mM EGTA in all solutions. CaM (2 μM) in either 200 μM free Ca^{2+} or 1 mM EGTA was incubated with the peptides in molar ratios of peptide:CaM of 0.1:1, 0.5:1, 1:1, 2:1, 3:1, 5:1, and 10:1. The extent of the interaction was quantified by densitometer analysis of Coomassie-stained gels of the uncomplexed CaM at each peptide:CaM molar ratio. Values were normalized to CaM in the absence of peptide. Mean normalized optical density values mean ± SE were plotted as a function of peptide:CaM ratio.

SDS PAGE

Polyacrylamide gel electrophoresis was performed as described by Laemmli (1970) or Schägger and von Jagow (1987). The latter gels are designated *Schägger gels* in this article.

Native to denatured two-dimensional gel electrophoreses

To determine the composition of the complexes detected by nondenaturing gel electrophoresis, lanes from the nondenaturing gel containing the CaM-peptide complex were excised. The gel strip was then loaded on top of a Schägger polyacrylamide gel for the second-dimension electrophoresis. The gap between the gel strip and the second-dimension resolving gel was sealed with melted agarose (1% agarose, 2% SDS, and 50 mM DTT) and the gels were electrophoresced for 1–2 h at 120–170V at 4°C. The two-dimensional gels were subsequently stained with Coomassie blue (R250).

Ca^{2+} affinity determination

Ca^{2+} affinity was determined using standardized Ca^{2+} solutions from Molecular Probes (Eugene, OR) composed of 30 mM MOPS (pH 7.2), 100

TABLE 1 Sequences of the $Ca_V1.2$ channel α_1 carboxy-terminal peptides

Peptide	Seq. no. Human	Amino acids
A	1609–1628	TLFALVRTALRIKTEGNLEQ
C	1627–1652	EQANEELRAIIKKIWKRTSMKLLDQV
IQ	1665–1685	KFYATFLIQEYFRKFKKRKEQ
A-C	1609–1653	TLFALVRTALRIKTEGNLEQANEELRAIIKKIWKRTSMKLLDQVV
C-IQ	1627–1685	EQANEELRAIIKKIWKRTSMKLLDQVVPPAGDDEVTVGKFYATFLIQEYFRKFKKRKEQ
IQW	1665–1685	KWYATFLIQEYFRKFKKRKEQ
IQ (*AA*)	1665–1685	KFYATFLAAEYFRKFKKRKEQ

mM KCl, 10 mM EGTA, and various Ca^{2+} concentrations. CaM (2.5 μM) and the specified channel peptide (10 μM) were incubated at room temperature. Tryptophan fluorescence was measured at 330 nm after 295-nm excitation. Each EC_{50} reported represents an average of 3–5 replicate titrations, mean ± SE.

CaM binding analysis by fluorescence

Assays of the interactions of CaM with the peptides by tryptophan fluorescence were performed in either a high Ca^{2+} buffer or a low Ca^{2+} buffer. The high Ca^{2+} buffer contained 1 mM EGTA, 300 mM NaCl, 50 mM MOPS at pH 7.4, 0.1% CHAPS, 100 μg/ml bovine serum albumin, 1.2 mM $CaCl_2$, and 0.02% NaN_3. Low Ca^{2+} buffer contains 1 mM EGTA, 300 mM NaCl, 50 mM MOPS pH 7.4, 0.1% CHAPS, 100 μg/ml bovine serum albumin, and 0.02% NaN_3. Peptides (10 μM) with or without CaM (2.5 μM) were incubated on an Orbit-P4 shaker in a 96 well Molecular Probes quartz plate at 25 rpm for 30 min before scanning. Fluorescence spectra were obtained on a SpectraMAX Gemini fluorometer (Molecular Devices, Sunnyvale, CA). We used four different excitation/emission protocols on all samples: 1) excitation at 295 nM and emission at 330 nm; 2) excitation at 275 nm and emission at 320 nm; 3) excitation at 295 nm and emission scanning from 310 nm to 400 nm; and 4) excitation at 275 nm and emission scanning from 310 nm to 400 nm. All experiments were done in triplicate and data are presented as a mean ± SE. Binding interactions produced blue shifts in the peak fluorescence accompanied by increases in fluorescence intensity. Ca^{2+} affinities determined by excitation at 295 nm were not statistically different from those determined by 275-nm excitation, despite the contribution of phenylalanine and tyrosine fluorescence with the latter protocol. Data shown in figures were obtained from the 295-nm excitation.

RESULTS

Peptides representing sequences within the α_1 carboxy-terminal tail differentially interact with Ca^{2+}CaM and apoCaM

Three different sequences have been implicated in Ca^{2+}CaM binding to the carboxyterminal tail of the $Ca_V1.2$ channel. We compared the ability of synthetic peptides matching these sequences to bind Ca^{2+}CaM. The peptides, listed in Table 1, fall into three categories: 1) peptides exactly matching the sequences within the carboxyterminal tail of the cardiac $Ca_V1.2$ channel (*A*, *C*, *IQ*, *A-C*, and *C-IQ*), 2) a peptide (*IQW*) prepared with a tryptophan in place of phenylalanine 1666 to allow us to monitor binding of CaM to the peptide by changes in the tryptophan fluorescence of the peptide, and 3) a peptide with amino acid substitutions corresponding to the mutations that destroyed Ca^{2+}-dependent inactivation (*IQ* (*AA*)) (Zühlke et al., 1999, 2000).

To determine the apparent affinity of the peptides for CaM, the peptides were incubated with CaM at several different peptide:CaM ratios and electrophoresced on nondenaturing gels. Fig. 1 *A* shows the nondenaturing gels of Ca^{2+}CaM in the presence of increasing concentrations of each of these peptides. The summarized data from the densitometer analyses of replicate gels for all the peptides are shown in Fig. 1 *C*. In this gel system the peptide alone does not enter the gel due to its positive charge. In the high Ca^{2+} gels the complex of the peptide and CaM can be seen above the free CaM band. All of the peptides tested bind Ca^{2+}CaM. Both *IQ* (*AA*) and *IQW* (data not shown) bind Ca^{2+}CaM with the same apparent affinity as the *IQ* peptide.

The ability of a Ca^{2+} binding site mutant of CaM to block Ca^{2+}-dependent inactivation (Peterson et al., 1999) suggests that the $Ca_V1.2$ Ca^{2+} channel binds both apoCaM and Ca^{2+}CaM. Although apoCaM binding to the carboxyl tail has been suggested by Peterson et al. (1999) and Pitt et al. (2001), an actual apoCaM binding site has not been demonstrated. In the nondenaturing gel system, apoCaM binding was detected to the *IQ*, *IQW* (data not shown), and *C-IQ* peptides (Fig. 1 *B*). The summarized data from the densitometer analyses of replicate gels for all the peptides are shown in Fig. 1 *D* (low Ca^{2+}). In the low Ca^{2+} gels, the CaM-peptide complex does not migrate as a discrete band, consistent with previous observations (Pate et al., 2000; Rodney et al., 2001). In low Ca^{2+}, the extent of interaction was assessed by the disappearance of the free CaM band and was confirmed by fluorescence (see below). The peptide *C-IQ* has the highest apparent affinity for apoCaM but the *IQ* peptide shows significant binding. No binding of apoCaM to *IQ* (*AA*), *C*, or *A* was detected. The *IQ* to *AA* mutations in the $Ca_V1.2$ channel destroy Ca^{2+}-dependent inactivation (Zühlke et al., 1999, 2000) and these changes in the peptide destroy its ability to bind apoCaM.

The longer peptides have two motifs which, as separate peptides, both bind CaM. These findings raise the question of how many CaMs bind to the longer peptides. In Fig. 1 we examined the effects of increasing the peptide:CaM ratio on complex formation. These conditions of peptide in molar excess would not tend to favor the formation of a complex with two CaMs bound to one peptide, even if a peptide were capable of binding two CaMs. If, however, both lobes of CaM are able to bind to sequences within a peptide, the high concentrations of peptide might lead to a ternary complex with two peptides bound to a single molecule of CaM (one peptide at each lobe). We see no evidence for a complex of two peptides and one CaM for the *A*, *C*, *AC*, or *IQ* peptides. However, at high *C-IQ*:CaM ratios, there is a loss in the CaM-peptide complex (Fig. 1 *A*, *lane 8*), suggesting that the two lobes of CaM may each be able to bind a peptide at this high concentration of peptide. Alternatively, this loss of the complex from the gels could reflect a decrease in the solubility of the peptide at these high concentrations. A more important question is whether more than one CaM can bind to the longer peptides that have two CaM binding motifs. To address this question we developed a two-dimensional gel system in which the first dimension was a nondenaturing gel and the second dimension was an SDS gel. Typical two-dimensional gels (using peptides *IQ* and *C-IQ*) are shown in Fig. 2, *A* and *B*. To determine the amount of CaM and peptide in the two-dimensional gel, an SDS gel, identical to that used in the second dimension, was created with known amounts of CaM and peptide (Fig. 2, *C* and *D*). Using densitometric analysis we calculated the molar ratio of CaM

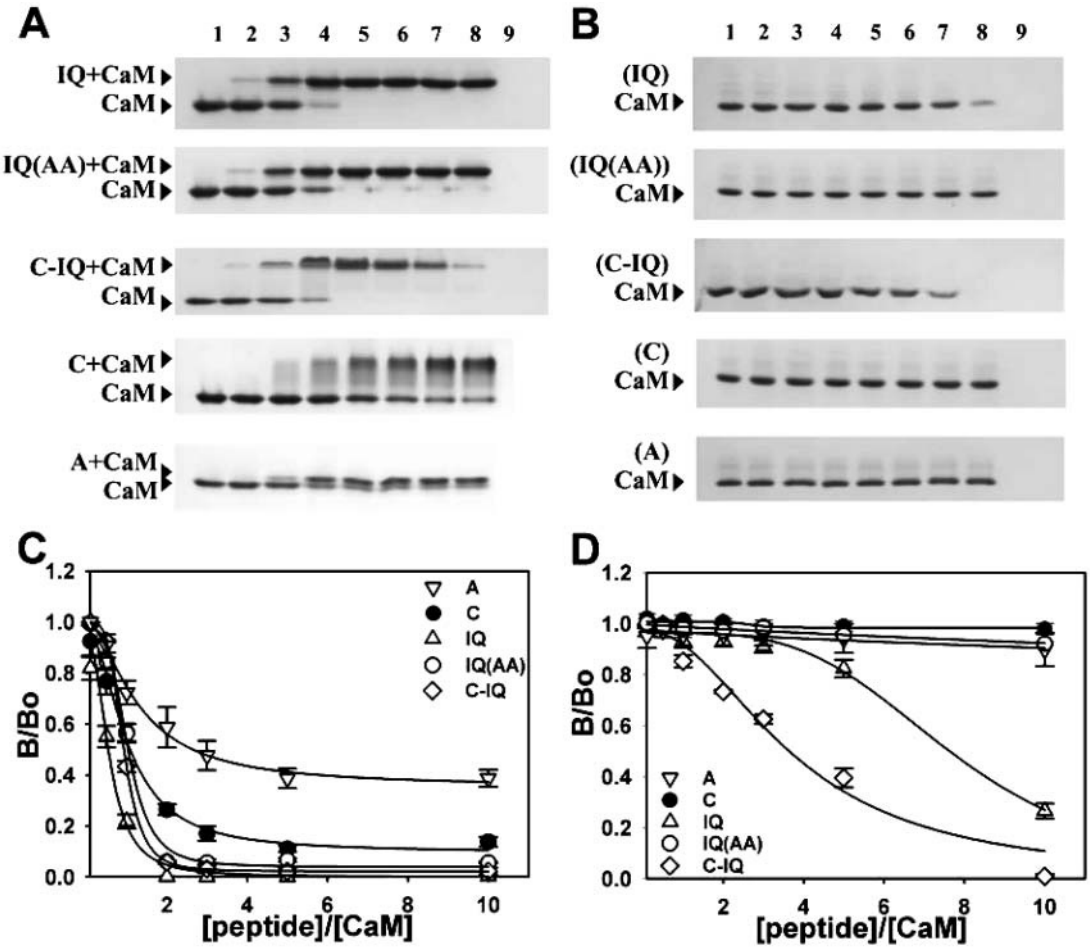

FIGURE 1 The interaction of the $Ca_V1.2$ channel α_1 carboxy-terminal peptides with Ca^{2+}CaM and apoCaM. CaM (2 μM) was incubated with peptide in increasing peptide:CaM molar ratios before electrophoresis on 20% nondenaturing gels in the presence of either 200 μM Ca^{2+} (*A*), or 1 mM EGTA (*B*). Shown are representative Coomassie-blue stained 20% nondenaturing gels of samples containing CaM and increasing molar ratios of *IQ*, *IQ* (*AA*), *C-IQ*, *C*, and *A*. The first lane in each gel contains CaM only. Peptide:CaM ratios used were 0:1, 0.1:1, 0.5:1, 1:1, 2:1, 3:1, 5:1, 10:1, and 1:0. (*C* and *D*) The relative amount of CaM on the gel at each peptide concentration (*B*) was determined by densitometry and was normalized to CaM in the absence of peptide (*B0*). The data are plotted as the mean *B*/*B0* ± SE ($n = 3$) vs. peptide:CaM ratio. *C* summarizes the results from experiments performed in 200 μM Ca^{2+} and *D* summarizes the results from experiments performed in 1 mM EGTA. The graph shows CaM with increasing peptide concentration described by the following symbols: ●, *C*; ◇, *C-IQ*; ▽, *A*; △, *IQ*; and ○, *IQ* (*AA*).

to peptide in each of the CaM-peptide complexes (Fig. 2 *C*). All peptides were tested initially at a CaM:peptide ratio of 1:5, identical to that used in Fig. 1. The longer peptides were also analyzed under conditions of excess CaM (CaM:peptide of 5:1). Experiments were also performed with equimolar ratios of CaM:peptide and gave similar results (data not shown). The composition of each of the complexes (moles of CaM per mole peptide) is summarized in Fig. 2 *E*. At all CaM:peptide ratios, the complexes were composed of one molecule of CaM for each molecule of peptide. These findings suggest that the different sequences contribute to a single CaM binding site. In support of this, we recently determined that the skeletal muscle $Ca_v1.1$ channel, which differs from the cardiac sequence only at a few amino acids in these regions, binds a single CaM per channel (unpublished observation).

Ca^{2+} concentration dependence of the interaction of α_1 carboxy-terminal peptides with CaM

A number of CaM binding peptides have been shown to increase the affinity of CaM for Ca^{2+} (Johnson et al., 1996; Kasturi et al., 1993; Keller et al., 1982; Kilhoffer et al., 1992; Olwin et al., 1984; Peterson et al., 1999). Peptide *C* has a tryptophan that allows its interaction with CaM to be analyzed by fluorescence. To measure the affinity of CaM for Ca^{2+} when bound to the *IQ* region we generated a mutant peptide (*IQW*) in which the first phenylalanine in the peptide sequence was mutated to a tryptophan. This mutant *IQ* peptide binds Ca^{2+}CaM and apoCaM with an apparent affinity similar to that of the wild-type *IQ* peptide. The differences in the fluorescence of these peptides can be used to analyze the Ca^{2+}-concentration dependence of the

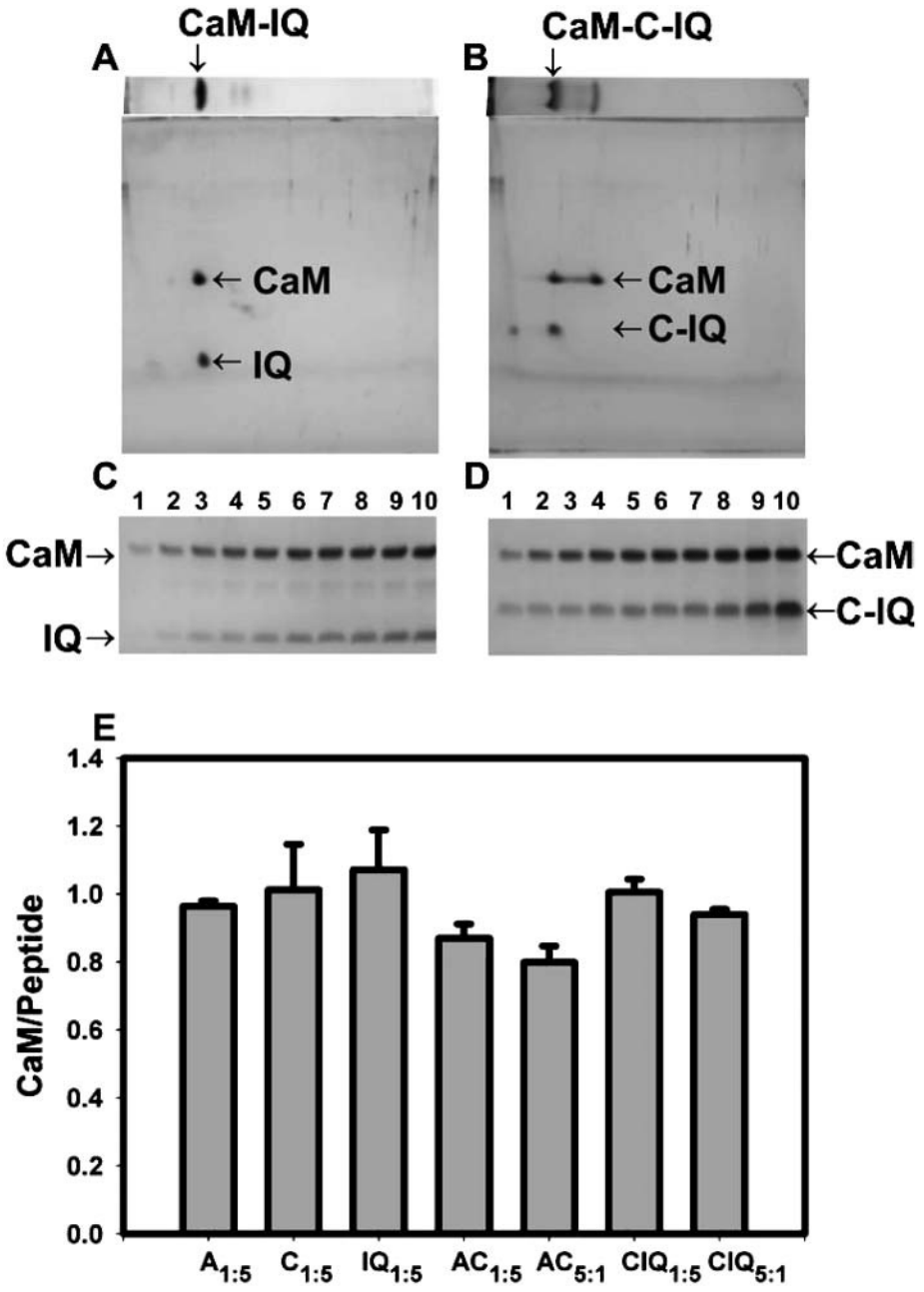

FIGURE 2 Each peptide binds one molecule of CaM. CaM (2.5 μM or 50 μM) was incubated with peptide (10 μM) and the complex was electrophoresed in the first dimension in the nondenaturing gels and in the second dimension on an SDS Schägger gel as described in Methods. The gel was stained with Coomassie blue and the optical densities of the CaM and peptide spots were determined by densitometry. In parallel, a Schägger gel was electrophoresced with known quantities of CaM and peptide to use as standard curves for determination of the amount of peptide and CaM on the two-dimensional gels. (*A*) Coomassie-blue stained two-dimensional gel of CaM + peptide *IQ* obtained with a 1:5 CaM:peptide ratio. (*B*) Coomassie-blue stained two-dimensional gel of CaM + peptide *C-IQ* obtained with a 1:5 CaM:peptide ratio. (*C*) Protein standard gel: Coomassie-blue stained Schägger gel of known amounts of CaM (20–200 pmoles) and peptide *IQ* (20–200 pmoles). (*D*) Protein standard gel: Coomassie-blue stained Schägger gel of increasing known amounts of CaM (20–200 pmoles) and peptide *C-IQ* (20–200 pmoles). (*E*) Summary of densitometric analysis of the two-dimensional gels of the CaM-peptide complexes. The subscripts on the peptide name on the *x*-axis represent the CaM:peptide ratios used in the incubation before the first-dimension gel. The *y*-axis CaM:peptide ratio represents the composition of the CaM-peptide complexed as assessed on the second-dimension gel. The data shown represent data obtained from 3–4 different two-dimensional gels.

interaction of the peptides with CaM. The binding of CaM to each of the peptides examined in our study produces both a Ca^{2+}-dependent blue shift and an enhancement in the tryptophan emission spectrum. The interaction of these peptides with CaM occurs at a lower Ca^{2+}concentration than is required to saturate the lobes of unbound CaM (see below) indicating that these peptides increase the affinity of CaM for Ca^{2+} (Fig. 3). Table 2 summarizes the relative affinities of the Ca^{2+}-dependent interaction of these peptides with CaM. These values were identical using two different excitation/emission protocols: 1) excitation at 275 nm and emission at 320 nm, and 2) excitation at 295 nm and emission at 330 nm. Using an excitation of 275 nm and an emission of 320 nm we can also obtain an estimate of the Ca^{2+} affinity of CaM alone using tyrosine fluorescence. Under our conditions the EC_{50} for Ca^{2+} binding to CaM as determined by the change in the fluorescence of the tyrosine and phenylalanines of CaM with increasing Ca^{2+} concentrations is 1.2 $\pm$ 0.3 μM ($n = 4$).

Tryptophan fluorescence analysis of Ca^{2+} binding to CaM that is bound to *C*, *IQ*, or *IQ* (*AA*)

The above peptide fluorescence studies are limited by the need for a tryptophan in the peptide and cannot be used to evaluate the binding of Ca^{2+} to the N- and C-lobes of CaM. One approach to assessing the apparent affinity of the N- and

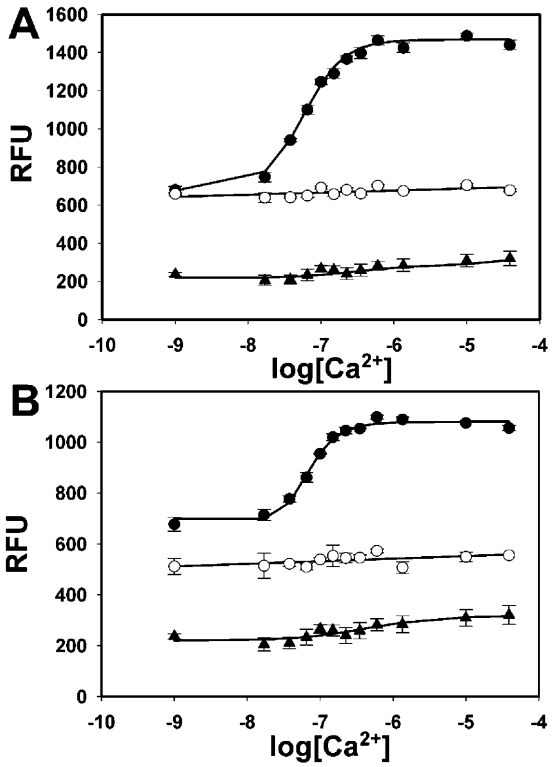

FIGURE 3 Detection of Ca^{2+}-dependent interaction of CaM with the peptides by tryptophan fluorescence. Using Ca^{2+} standards from Molecular Probes, the affinity of CaM for Ca^{2+} in the presence of peptide was determined by measuring an increase in fluorescence emission at 330 nm after the tryptophan was excited at 295 nm. 2.5 μM CaM was incubated with 10 μM peptide for one half-hour before the fluorescent studies. The calculations for affinity are described in Methods. Data are plotted as relative fluorescence units (*RFU*) vs. log [Ca^{2+}], (*A*) ●, Peptide *C* + CaM; ○, Peptide *C* alone; and ▲, CaM alone. (*B*) ●, Peptide *IQW* + CaM; ○, *IQW* alone; and ▲, CaM alone.

C-lobes of CaM for Ca^{2+} involves using mutant CaMs in which a tryptophan has been substituted for a phenylalanine at either residue 19 (F19W) or residue 92 (F92W). Tryptophan residues at these positions are extremely sensitive to changes in the conformation of CaM that occur when Ca^{2+} binds to that lobe (Black et al., unpublished data) and are similar to earlier isofunctional CaMs used to assess the order of Ca^{2+} binding to CaM (Kilhoffer et al., 1992; Romanin et al., 2000). We have used F19W and F92W CaM to assess the effects of the peptides on the apparent affinity of the N- and C-terminal lobes of CaM for Ca^{2+}.

TABLE 2 EC50 of CaM for Ca^{2+} in the presence of the $Ca_V1.2$ channel peptides

Peptide + CaM	EC_{50} for Ca^{2+}
IQW + CaM	0.07 ± 0.01 μM ($n = 3$)
C + CaM	0.05 ± 0.01 μM ($n = 8$)
C-IQ + CaM	0.14 ± 0.06 μM ($n = 3$)
A-C + CaM	0.05 ± 0.01 μM ($n = 3$)
CaM	1.2 ± 0.3 μM ($n = 4$)

To examine the change in tryptophan fluorescence of F19W and F92W, we excited at 295 nm and measured fluorescence at 330 nm. Data comparing *IQ* to *IQ* (*AA*) with F19W and F92W are shown in Fig. 4, *A* and *B*, respectively. The EC_{50}s are listed in Table 3. The *IQ* and *IQ* (*AA*) peptides increase the Ca^{2+} affinity of both lobes of CaM as detected by the leftward shift in the Ca^{2+} dependence of the change in tryptophan fluorescence for both F19W and F92W. These findings suggest that both lobes of CaM are interacting with both the *IQ* and *IQ* (*AA*) peptides. There are no significant differences in the EC_{50}s for Ca^{2+} binding to either mutant CaM bound to the *IQ* and *IQ* (*AA*) peptides. There is, however, a significant increase in fluorescence enhancement of F92W complexed to *IQ* (*AA*) as compared to *IQ*, suggesting that the interactions of the C-lobe of F92W with the *IQ* (*AA*) peptide are somewhat different than the interactions of this lobe with the *IQ* peptide.

We also examined the effects of peptide *C* on the Ca^{2+} affinity of F19W and F92W. These data are shown in Fig. 4, *C* and *D*, respectively. To analyze peptide *C*'s interactions with the fluorescent CaMs, we had to consider the tryptophan in the peptide *C* sequence, which complicates the fluorescent signal. To evaluate the contribution of this tryptophan to the total fluorescence change, we compared the fluorescence of peptide *C* obtained with F19W and F92W with that obtained with wild-type CaM (no tryptophan). The Ca^{2+}-dependent changes in tryptophan fluorescence of F92W complexed to peptide *C* occur at approximately the same Ca^{2+} concentration range as seen with the peptide bound to wild-type CaM (Fig. 3 *D*), suggesting that the interaction is primarily controlled by the C-lobe of CaM. The titration curve obtained with F19W and peptide *C* (Fig. 4 *C*) is, however, distinctly biphasic. The first phase corresponds to the change in fluorescence of the peptide upon interaction with the C-lobe of CaM, and the second phase is similar to the Ca^{2+} dependence for the change in fluorescence of the N-terminal lobe of F19W in the absence of peptide *C*. A similar biphasic curve was obtained with peptide *A-C* (summarized in Table 3). Together these data suggest that either only the C-lobe of Ca^{2+}CaM binds to the *C* and *A-C* peptides, or that the interaction of the N-lobe with these sequences does not alter the Ca^{2+} affinity of this lobe.

In contrast to the findings with peptides *C*, *A-C*, and *IQ*, under the conditions of this assay, peptide *A* does not greatly alter the affinity of either F19W or F92W for Ca^{2+}. Data for peptide *A* with F19W and F92W are shown in Fig. 4, *E* and *F*, respectively. We do not detect a change in fluorescence in either F19W or F92W until the Ca^{2+}concentration reaches a level where the CaMs would bind Ca^{2+} in the absence of peptide. Upon Ca^{2+} saturation, peptide *A* binds both F19W and F92W, producing both an enhancement and a blue shift in the isofunctional CaM's tryptophan fluorescence. Since Ca^{2+} increases the affinity of CaM for the peptide, the peptide must, therefore, increase the affinity of CaM for Ca^{2+}. This was not detected, suggesting that peptide *A* must

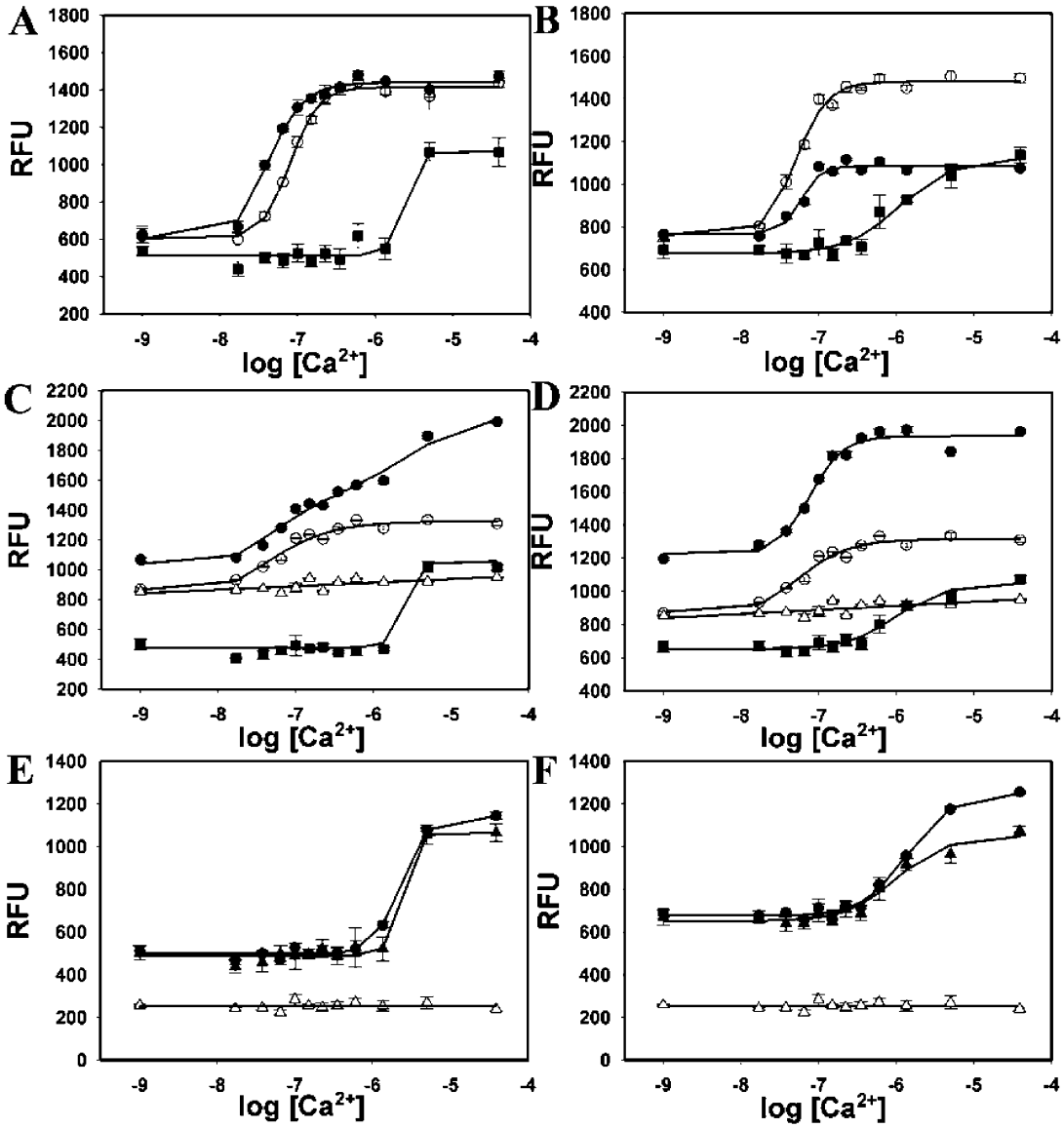

FIGURE 4 Effect of Ca^{2+} on the interaction of peptides with F19WCaM and F92WCaM. F19W and F92W are CaM mutants that contain an F-to-W mutation at amino acids 19 and 92, respectively. At different Ca^{2+} concentrations, the tryptophan was excited at 295 nm and the emission in relative fluorescence (*RFU*) was detected at 330 nm. Data plotted are the mean of three trials ± SE. (*A*) *IQ* and *IQ* (*AA*) with F19W. ●, F19W + *IQ*; ○, F19W + *IQ* (*AA*); and ■, F19W alone. (*B*) F92W with *IQ* and *IQ* (*AA*). ●, F92W + *IQ*; ○, F92W + *IQ* (*AA*); and ■, F92W alone. (*C*) Peptide *C* with F19W. ●, F19W + *C*; ○, CaM + *C*; △, *C* alone; and ■, F19W alone. (*D*) Peptide *C* with F92W. ●, F92W + *C*; ○, CaM + *C*; △, *C* alone; and ■, F92W alone. (*E*) Peptide *A* with F19W. ●, F19W + *A*; ▲, F19W alone; and △, *A* alone. (*F*) Peptide *A* with F92W. ●, F92W + *A*; ▲, F92W alone; and △, *A* alone.

have a very low affinity for apoCaM and much higher concentrations of peptide would be required to detect peptide *A* effects on the Ca^{2+} affinity of CaM.

Ca^{2+} binding site mutants of CaM as tools to analyze the interaction of α_1 carboxy-terminal peptides with CaM

The above data suggest that the lobes of CaM have multiple possible binding sites on the $Ca_v1.2$ channel and, therefore, may move upon binding Ca^{2+}. We have used CaM mutants that cannot bind Ca^{2+} at either the N- or C-lobe to determine the Ca^{2+} dependence of the interactions with the peptides. Each CaM mutant has glutamine substitutions in two of the E-F hands to inactivate Ca^{2+} binding to that lobe of CaM. These Ca^{2+} binding mutants are E12Q (mutations at both E31 and E67) and E34Q (mutations at both E104 and E140), respectively. We tested the interactions of these CaM mutants with *IQ*, *IQ* (*AA*), *A*, and *C* peptides at high Ca^{2+} using gel mobility shift assays as shown in Fig. 5. E12Q binds to peptides *C*, *IQ*, and *IQ* (*AA*), but not to the *A* peptide, suggesting that Ca^{2+} binding to the N-lobe of CaM is

TABLE 3 EC50 of F19WCaM and F92WCaM for Ca^{2+} in the presence of the $Ca_V1.2$ channel peptides

Peptide + CaM	EC_{50} for Ca^{2+} (μM)
A + F19W	3.7 ± 0.8 ($n = 5$)
A + F92W	2.1 ± 0.2 ($n = 5$)
C + F19W	0.05 ± 0.03
	8 ± 5 ($n = 3$)
C + F92W	0.06 ± 0.04 ($n = 3$)
IQ + F19W	0.03 ± 0.01 ($n = 8$)
IQ + F92W	0.05 ± 0.01 ($n = 9$)
A-C + F19W	0.1
	2.3 ($n = 2$)
A-C + F92W	0.13 ± 0.04 ($n = 3$)
C-IQ + F19W	0.08 (n =1)
C-IQ + F92W	0.08 ($n = 1$)
IQ (*AA*) + F19W	0.05 ± 0.01 ($n = 5$)
IQ (*AA*) + F92W	0.05 ± 0.01 ($n = 6$)
F19W	2.1 ± 0.4 μM ($n = 6$)
F92W	0.8 ± 0.1 μM ($n = 6$)

All peptides were tested at a peptide:CaM ratio of 5:1.

necessary for the interaction of CaM with the *A* peptide. E34Q interacts with both *IQ* and *IQ* (*AA*) but has a decreased affinity for *A*, *C*, and *A-C*. Together these findings suggest that the *C* peptide interaction with CaM is enhanced by Ca^{2+} binding to the C-lobe and the *A* peptide interaction is enhanced by Ca^{2+} binding to both lobes of CaM. Several aspects of the *IQ* interactions are surprising. Since the *IQ* peptide appears to bind both lobes of Ca^{2+}CaM (as evidenced by the increase in Ca^{2+}affinity of both lobes when bound to *IQ*), we anticipated a loss of affinity with CaM mutated in either the N-lobe or C-lobe Ca^{2+} binding sites. Instead, we found that both E12Q and E34Q bind to both the *IQ* and *IQ* (*AA*) peptides with apparent affinities similar to wild-type CaM.

DISCUSSION

Three different sequences in the carboxyterminal tail of the $Ca_V1.2$ channel have been implicated in CaM binding and in Ca^{2+}-dependent inactivation (Peterson et al., 1999; Pate et al., 2000; Zühlke et al., 2000; Pitt et al., 2001). We have compared the ability of peptides matching these sequences in the $Ca_V1.2$ channel to bind Ca^{2+}CaM, apoCaM, and Ca^{2+} binding site mutants of CaM and to alter the Ca^{2+}affinity of CaM.

We confirm that all three sequences (*A*, *C*, and *IQ*) bind Ca^{2+}CaM. Peterson et al. (1999) showed that a mutant CaM that does not bind Ca^{2+} at any of its four sites blocks the ability of Ca^{2+}CaM to produce inactivation, suggesting that the channel binds apoCaM and that Ca^{2+} binding to CaM is required for Ca^{2+}-dependent inactivation. More specifically, this group showed that Ca^{2+} binding to sites 3 and 4 at the C-lobe of CaM is required for Ca^{2+}-dependent inactivation (Peterson et al., 1999; Alseikhan et al., 2002). Our comparison of the ability of the different peptides to bind apoCaM, E12Q, and E34Q offers some possible explanations for these observations. The *IQ* peptide alone binds apoCaM whereas the *A* and *C* peptides do not. The *C-IQ* peptide, however, binds apoCaM with higher apparent affinity than *IQ* alone. We propose that the *C-IQ* region on the carboxyl terminal tail of the α_1 subunit of the $Ca_v1.2$ channel is a candidate for the apoCaM binding site on the channel.

CaM mutated at Ca^{2+} binding sites 1 and 2 in the N-lobe of CaM (E12Q) binds to the *IQ* and *C* peptides, but not to the *A* peptide. CaM mutated at Ca^{2+} binding sites 3 and 4 in the C-lobe of CaM binds to the *IQ* peptide, but not to the *C* or *A* peptides. The mutations in CaM that abolish inactivation, therefore, appear to correlate with the changes in its interactions with the *A* and *C* peptides.

Our findings suggest that the *IQ* peptide can interact with CaM in a variety of states: Ca^{2+} free, Ca^{2+} bound only at the N-lobe, Ca^{2+} bound only at the C-lobe, and fully Ca^{2+} bound. In contrast, the *C* peptide can bind CaM with its N-lobe Ca^{2+} free and its C-lobe Ca^{2+} bound or with both lobes Ca^{2+} bound. The *A* peptide requires Ca^{2+} binding to both lobes of CaM for interaction. These findings are consistent with the effects of the peptides on the Ca^{2+} affinity of the lobes of CaM. The *IQ* peptide increases the affinity of both the N- and C-lobes of CaM for Ca^{2+}, suggesting an interaction with both lobes. In contrast, the *C* peptide increases the Ca^{2+}affinity of only the C-lobe of CaM. The *A* peptide, however, appears to have a low affinity for apoCaM and a higher affinity for Ca^{2+}CaM. The low affinity for apoCaM suggests that higher concentrations of peptide *A* would be required to see an affect on Ca^{2+} affinity of CaM. One possible explanation of the ability of CaM mutated at Ca^{2+} binding sites 3 and 4 to block Ca^{2+}-dependent inactivation (Peterson et al., 1999; Alseikhan et al., 2002) is that these mutations in CaM alter its interactions with the *A-C* sequence.

Ca^{2+}-dependent inactivation of the channel can also be abolished by the mutation of the isoleucine and glutamine residues of the *IQ* motif in the channel to alanines (Zühlke et al., 2000). These mutations in the synthetic *IQ* peptide do not alter the apparent affinity of the peptide for Ca^{2+}CaM, the apparent affinity of the CaM for Ca^{2+}, or the apparent affinity for interactions of the peptide with either E12Q or E34Q. The latter finding is somewhat surprising if both lobes of CaM interact with *IQ*. There are several possible explanations of the lack of a major effect of the Ca^{2+} binding site mutations on the interaction with *IQ*: 1), when one lobe binds *IQ* in the Ca^{2+} bound state the other lobe can still bind in the Ca^{2+} free state and any apparent affinity differences are small; 2), there is only a single site on the *IQ* motif for interaction with a lobe of CaM and this site can engage either the N- or the C-lobe of Ca^{2+}CaM with equal affinity; 3) the affinity for a Ca^{2+} bound lobe is greater in the absence of Ca^{2+} binding to the second lobe; or 4) the *IQ* peptide is interacting with a region on CaM that is exposed by Ca^{2+} binding to either the N- or C-lobes. The first

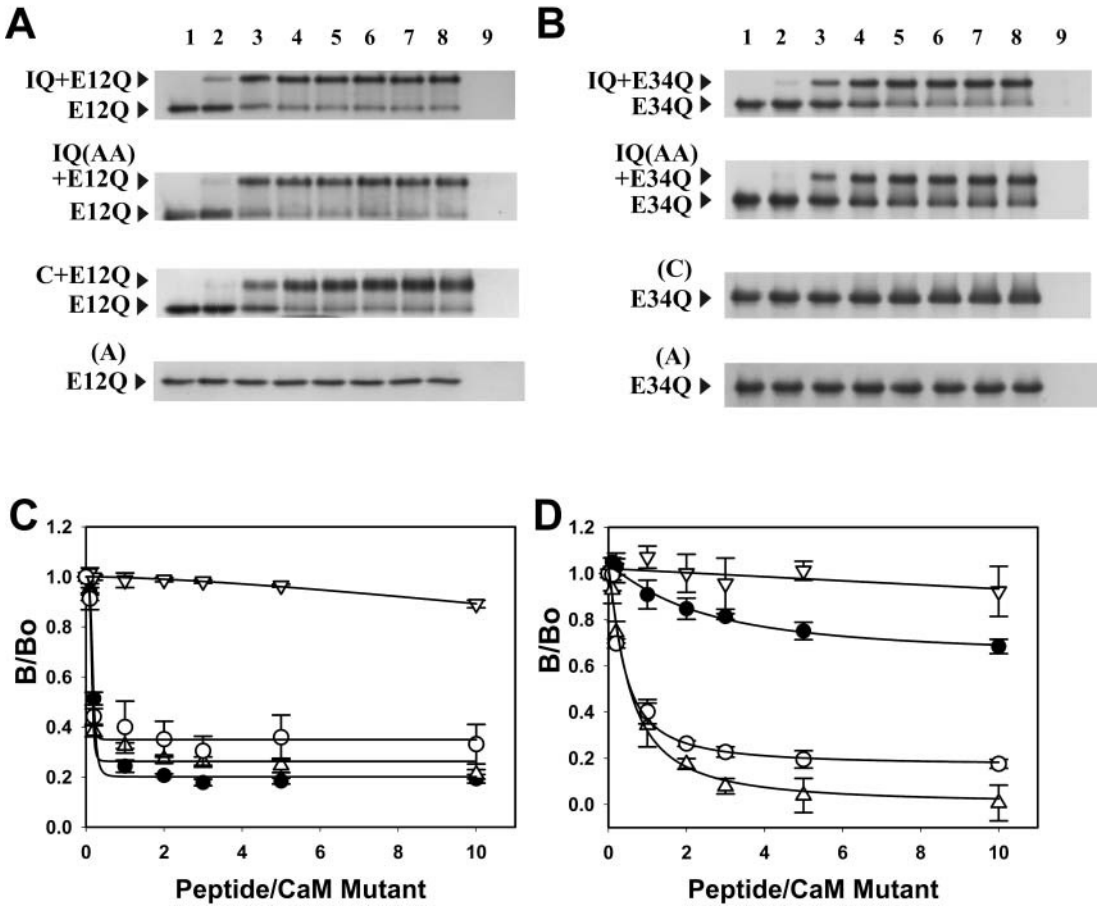

FIGURE 5 Interaction of E12Q and E34Q CaMs with the cardiac $Ca_V1.2$ channel peptides *A*, *C*, *IQ*, and *IQ* (*AA*). CaM (3 μM) was incubated with peptide in increasing peptide:CaM molar ratios before electrophoresis on 20% nondenaturing gels (added 200 μM Ca^{2+}). (*A* and *B*) The representative Coomassie-blue stained 20% nondenaturing gels of samples containing E12Q CaM (*A*) or E34Q CaM (*B*) and increasing molar ratios of *IQ*, *IQ* (*AA*), *C*, and *A* are evaluated. Ratios of peptide:CaM (beginning in *lane 2*) are 0:1, 0.1:1, 0.5:1, 1:1, 2:1, 3:1, 5:1, 10:1, and 1:0. (*C* and *D*) The optical density of the CaM band on the gel in the presence of increasing peptide (*B*) was determined by densitometry and was normalized to the optical density of the CaM band in the absence of peptide (*B0*). The data are plotted as the mean *B*/*B0* ± SE ($n > 3$) vs. peptide:CaM ratio. (*C*) Summary of data with E12Q. ●, *C* + E12Q; △, *IQ* + E12Q; ○, *IQ* (*AA*) + E12Q; and ▽, *A* + E12Q. (*D*) Summary of data with E34Q. ●, *C* + E34Q; △, *IQ* + E34Q; ○, *IQ* (*AA*) + E34Q; and ▽, *A* + E34Q.

possibility seems unlikely since the *IQ* (*AA*) mutation greatly decreases apoCaM binding, but shows normal binding of E12Q and E34Q under conditions where one lobe is Ca^{2+} bound. We think the most likely explanation is that only one lobe of Ca^{2+}CaM is binding to the *IQ* peptide and this can be either Ca^{2+}-bound lobe. This could be a phenomenon that does not occur in the native channel but is rather found only with using isolated peptides and CaM at high concentrations. Alternatively, this type of lobe equivalence for CaM interaction with the *IQ* motif interaction in the native channel could contribute to the complexity of the modulation of this channel by CaM.

Another issue addressed in this manuscript is how many CaMs bind to a peptide that has more than one of these binding motifs. We find that the *A-C* and *C-IQ* peptides bind only a single molecule of CaM, suggesting that the three sequences are likely to contribute to a single CaM binding site and that the lobes of CaM can move within the site.

In summary we have shown that, 1), the *C-IQ* peptide binds apoCaM; 2), the *IQ* peptide increases the Ca^{2+} affinity of both the N- and C-lobes of CaM, but can bind CaM with only one lobe (either one) Ca^{2+} bound; 3), the *C* peptide increases the Ca^{2+} affinity of only the C-lobe of CaM and has decreased affinity for a CaM that cannot bind Ca^{2+} at its C-lobe; and 4), the *IQ* to *AA* mutation that, in the channel, abolishes Ca^{2+}-dependent inactivation, primarily alters the ability of the *IQ* peptide to bind apoCaM. The ability of the Ca^{2+} binding state of CaM to regulate its interactions with the different sequences may suggest mechanisms whereby this molecule can function in different regulatory roles on the channel. A model consistent with our peptide data is that apoCaM binds to the *C-IQ* region of the channel and this interaction is required for Ca^{2+}-dependent inactivation. Upon an increase in cytoplasmic Ca^{2+} concentration, Ca^{2+} binding to the C-lobe of CaM may favor its movement to the *A-C* region, leading to Ca^{2+}-dependent inactivation.

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