Calmodulin interactions with IQ peptides from voltage-dependent calcium channels

D. J. Black, D. Brent Halling, David V. Mandich, Steen E. Pedersen, Ruth A. Altschuld, and Susan L. Hamilton

1Department of Molecular Physiology and Biophysics, Baylor College of Medicine, Houston, Texas; and 2Department of Molecular and Cellular Biochemistry, Ohio State University, Columbus, Ohio

Submitted 16 April 2004; accepted in final form 6 October 2004

Black, D. J., D. Brent Halling, David V. Mandich, Steen E. Pedersen, Ruth A. Altschuld, and Susan L. Hamilton. Calmodulin interactions with IQ peptides from voltage-dependent calcium channels. Am J Physiol Cell Physiol 288: C669–C676, 2005. First published October 20, 2004; doi:10.1152/ajpcell.00191.2004.—Calmodulin (CaM) functions as a Ca$^{2+}$ sensor for inactivation and, in some cases, facilitation of a variety of voltage-dependent Ca$^{2+}$ channels. A crucial determinant for CaM binding to these channels is the IQ motif in the COOH-terminal tail of the channel-forming subunit. The binding of CaM to IQ peptides from Lc-, P/Q-, and R-type, but not N-type, voltage-dependent Ca$^{2+}$ channels increases the Ca$^{2+}$ affinity of both lobes of CaM, producing similar N- and C-lobe Ca$^{2+}$ affinities. Ca$^{2+}$ associates with and dissociates from the N-lobe much more rapidly than the C-lobe when CaM is bound to the IQ peptide. Compared with the other IQ peptides, CaM-bound Lc-IQ has the highest Ca$^{2+}$ affinity and the most rapid rates of Ca$^{2+}$ association at both lobes, which is likely to make Ca$^{2+}$ binding to CaM, bound to this channel, less sensitive than other channels to intracellular Ca$^{2+}$ buffers. These kinetic differences in Ca$^{2+}$ binding to the lobes of CaM when bound to the different IQ motifs may explain both the ability of CaM to perform multiple functions in these channels and the differences in CaM regulation of the different voltage-dependent Ca$^{2+}$ channels.

Ca$^{2+}$-dependent inactivation; Ca$^{2+}$-dependent facilitation; apocalmodulin

The activities of several different voltage-dependent Ca$^{2+}$ channels are regulated by Ca$^{2+}$. Ca$^{2+}$-dependent inactivation (CDI) is the process whereby the entry of Ca$^{2+}$ enhances channel closing during a maintained depolarization (3, 9). In contrast, Ca$^{2+}$-dependent facilitation (CDF) is the process whereby increased basal Ca$^{2+}$ or repeated transient depolarizations leads to increased channel opening (1). Calmodulin (CaM) functions as a Ca$^{2+}$ sensor for both CDI of the voltage-dependent Ca$^{2+}$ channels (Cav), L-type (Cav1.2), P/Q (Cav2.1), N (Cav2.2), and R (Cav2.3), and CDF of the P/Q-type channel appears to be driven by Ca$^{2+}$ binding to the C-lobe of CaM (7, 8), whereas CDI of P/Q- (7), R-, and N-type channels (8) requires Ca$^{2+}$ binding to the N-lobe of CaM. The presence of intracellular Ca$^{2+}$ buffers does not alter L-type channel CDI or P/Q channel CDF (8). In contrast, CDF of the P/Q-type, N-type, and R-type channels is eliminated by strong Ca$^{2+}$ buffering (8).

Calmodulin binding to an “IQ-like” motif in the COOH-terminal region of the α1C-subunit of the Cav1.2 has been shown to be crucial for both CDI and CDF (14), raising questions as to how a single Ca$^{2+}$ sensor, binding at a single site, could produce opposite effects on channel activity. A mutant CaM (E1234Q) that cannot bind Ca$^{2+}$ at any of the four Ca$^{2+}$ binding sites (EF hands) competitively inhibits the interaction of Ca$^{2+}$-bound CaM with the cardiac L-type Ca$^{2+}$ channel (11), suggesting that both the Ca$^{2+}$-free and Ca$^{2+}$-bound forms of CaM bind to this channel. However, only the Ca$^{2+}$-bound form can produce inactivation (11). It has been proposed that CaM in the Ca$^{2+}$-free state (apoCaM) is anchored to the Ca$^{2+}$ channels in the region of the IQ motif (8). Our laboratory (12) has shown that a peptide representing amino acids 1627–1685 (human sequence) of the L-type Ca$^{2+}$ channel binds apoCaM.

The variable lobe dependence and differential response to added Ca$^{2+}$ buffers among the voltage-dependent Ca$^{2+}$ channels (8) imply that the N and C lobes of CaM are likely to have very different Ca$^{2+}$ binding properties when bound to the different channels. We have examined the Ca$^{2+}$ binding properties of CaM bound to peptides matching the sequences of the IQ motifs of the Lc (cardiac), P/Q, R, and N-type voltage-dependent Ca$^{2+}$ channels.

MATERIALS AND METHODS

Materials

Bovine brain CaM (95% pure) was purchased from Sigma (St. Louis, MO), solubilized in 10 mM MOPS (pH 7.4), 1 mM EGTA, 0.02% NaN$_3$, and quantified by absorption from 320 to 277 nm to obtain stock solutions of 300 μM (2). All peptides were synthesized at the protein laboratory facility at Baylor College of Medicine and were diluted in 200 mM MOPS (pH 7.4) for assays. F19W and F92W were purified as described by Black et al. (2).

Methods

Determination of affinity of F19W and F92W for LcIQ peptide. LcIQ peptide has a predicted isoelectric point $>$10, requiring the need to silanize the negatively charged quartz cuvettes and glassware to prevent loss of peptide. The peptides and glassware were dipped in a solution of 1% N-trimethoxysilylpropyl-$N,N,N$-triethyiammonium (United Chemical Technologies, Bristol, PA) in methanol, rinsed in 100% methanol, then baked at 115°C for several hours. A range of LcIQ concentrations (from 0 to 1,500 nM) were prepared in solutions containing 200 mM CaM (F19W, F92W, or control), 30 mM MOPS, 100 mM KCl, 1 mM EGTA, and 2 mM CaCl$_2$. The buffer was titrated to pH 7.2 after all the ingredients were added. After 45 min of incubation, the fluorescence spectra were collected on a fluorometer (model PC-1; ISS, Urbana-Champaign, IL) by tryptophan excitation

* D. J. Black and D. B. Halling contributed equally to this study.

Address for reprint requests and other correspondence: S. L. Hamilton, Dept. of Molecular Physiology and Biophysics, BCM 335, Baylor College of Medicine, 1 Baylor Plaza, Houston, TX 77030 (E-mail: susanl@bcm.tmc.edu).

http://www.ajpcell.org 0363-6143/05 $8.00  Copyright © 2005 the American Physiological Society C669

First published October 20, 2004; doi:10.1152/ajpcell.00191.2004.
at 295 nm using a 0.5-mm slit (4 nm band pass) and collecting the emission spectrum from 310 to 400 nm. A solar blind excitation filter and a 295-nm cut-on emission filter (Oriel, Stamford, CT) were used to reduce interference.

Because peptide binding shifts both the peak emission and the intensity of emission from F19W CaM and F92W CaM, the extent of binding is not simply proportional to intensity changes at any particular wavelength or to the shift of peak emission. To determine the extent of peptide binding from the spectral data, spectra at various peptide concentrations were assumed to be linear combinations of the spectra for unbound and fully bound CaM. Matrix methods were then used to extract the relative contribution of each end point spectrum to the spectra at intermediate concentrations as follows: \( S = B \cdot W \), where \( S \) is the matrix of the spectral data at all concentrations of peptide; \( B \) is a basis-set matrix of the two end point spectra (zero and maximal peptide concentration); and \( W \) is the matrix containing the relative contributions of the two end point spectra to the spectra at all peptide concentrations. The values of \( W \) essentially represent the extent of titration; here, \( W \) contains the titration data of interest. To solve for \( W \), we used the following equation: \( W = B^{-1} \cdot S \). Because the \( B \) matrix is generally rectangular and cannot be inverted, the matrix pseudo-inverse was applied to determine \( B^{-1} \) and then calculate \( W \), as implemented in MatLab (version 13; MathWorks, Natick, MA). The values in \( W \) were then plotted against peptide concentration to determine the titration curves.

The concentration range of binding was similar to the concentration of CaM; therefore, a significant amount of peptide was bound at the lower ends of the titration curves. To plot the data in terms of free peptide concentration, the free peptide concentration was calculated from the total concentration added and corrected for the bound peptide. Bound peptide was determined from the extent of the titration, given by the values in the matrix \( W \), and the concentration of CaM and assuming 1:1 stoichiometry of binding: [Bound peptide] = \( w \times [\text{CaM}] \), where \( w \) represents the weight for the corresponding peptide concentration taken from \( W \). Then [free peptide] = [added peptide] – [bound peptide].

Data were fit to a four-parameter Hill equation using SigmaPlot (SSPS, Chicago, IL),

\[
F = \frac{y_{\text{max}} + (A \cdot [\text{Free IQ}]^3)/K_d}{[\text{Free IQ}]^3} + \frac{[\text{Free IQ}]^3}{K_d}
\]

where \( K_d \) is the apparent dissociation constant for the Lc-IQ peptide binding to CaM, \( y_{\text{max}} \) is the minimum fluorescence, \( h \) is the Hill coefficient, and \( A \) is the difference in magnitude in the fluorescence between the peptide-free and peptide-saturated CaM. \( A \) was determined by averaging the data obtained at the upper plateau and subtracting the average value for peptide free CaM.

**Determination of Ca\(^{2+}\) affinity of F19W and F92W: 1 \( \mu \text{M} \) F19W or F92W.** CaM was incubated with 5 \( \mu \text{M} \) peptide for \( \geq 1 \) h at room temperature in standardized Ca\(^{2+}\) buffers prepared from Ca\(^{2+}\) calibration kits available from Molecular Probes (Eugene, OR). Fluorescence data were collected on an ISS PC-1 fluorometer using 1 mm slits (8 nm bandwidth) and a 295-nm cut-on emission filter (Oriel). A 295-nm excitation wavelength was used, and the emission was monitored at 330 nm. Data were plotted and analyzed using SigmaPlot (SSPS) software as described above.

**Ca\(^{2+}\) dissociation kinetics.** As previously described by Black et al. (2), an Applied Photophysics (model SX.18MV; Leatherhead, UK) stopped-flow instrument was used to measure rates of Ca\(^{2+}\) dissociation (\( k_{\text{off}} \)) at 22°C. The apparatus has a dead time of 1.35 ms. A 150-W Xenon arc source was used for excitation. The dissociation rates were extracted using the nonlinear Levenberg-Marquardt algorithm implemented in software provided by P. J. King of Applied Photophysics. Each observed rate represents the concerted release of two Ca\(^{2+}\) from either the NH\(_2\) terminal or the COOH terminal Ca\(^{2+}\)-binding sites. Each trace represents an average of 5–8 individual traces fit with either a single or double exponential as needed (variance \( < 4 \times 10^{-5} \)). All fits of the kinetic traces occurred after premixing was complete. Tryptophan fluorescence was measured after rapid mixing of 50 \( \mu \text{M} \) of F19W or F92W CaM (4 \( \mu \text{M} \), peptide (20 \( \mu \text{M} \), and Ca\(^{2+}\) (200 \( \mu \text{M} \) in 10 mM MOPS, 90 mM KCl, pH 7.0, with an equal volume of EGTA (10 mM). A 320-nm cut-on filter (Oriel) was used to minimize contamination from changes in the intrinsic tyrosine fluorescence of CaM and peptides. Each tryptophan fluorescence trace was fit with the following single exponential equation: \( A e^{-kt} + C \), where \( A \) is the amplitude of the fluorescence change, and \( k \) is the rate at which the change is occurring.

Ca\(^{2+}\) dissociation rates were verified by the rapid mixing of CaM (5 \( \mu \text{M} \), IQ peptide (40 \( \mu \text{M} \), Ca\(^{2+}\) (15 \( \mu \text{M} \) in 10 mM MOPS, and 90 mM KCl, pH 7.0, with an equal volume of the fluorescent Ca\(^{2+}\) chelator Quin-2 (150 \( \mu \text{M} \)). A 510-nm broad band-pass filter (Oriel) monitored the emission due to excitation at 330 nm. Each Quin-2 fluorescence trace was fit with the double exponential equation \( A_1 e^{-k_{1t}} + A_2 e^{-k_{2t}} + C \), where \( A_1 \) and \( A_2 \) are the individual amplitudes of each component of the fluorescence change, and \( k_1 \) and \( k_2 \) are the corresponding rates of change. A double exponential was required to fit the Quin-2 signal reporting both the NH\(_2\) terminal (fast) and COOH terminal (slow) Ca\(^{2+}\) dissociation rates. The changes in Quin-2 fluorescence were converted into moles of Ca\(^{2+}\) dissociating from CaM, as previously described by Johnson et al. (4). Briefly, monitoring the increase in Quin-2 fluorescence with increasing concentrations of Ca\(^{2+}\) (10, 20, 40, and 80 \( \mu \text{M} \) allowed for the conversion of observed Quin-2 fluorescence to molar [Ca\(^{2+}\)]. Quin-2 fluorescence increased linearly as a function of total [Ca\(^{2+}\)]; allowing the Quin-2 fluorescence increase to be used to calculate the total number of moles of Ca\(^{2+}\) that dissociate from each lobe of CaM.

Calculation of Ca\(^{2+}\) association rate constant. Ca\(^{2+}\) association rates were estimated using the relationship \( k_{\text{on}} = k_{\text{off}}/K_d \), assuming that \( k_{\text{off}} \) and \( K_d \) represent the concerted release or binding events of Ca\(^{2+}\) ions from the Ca\(^{2+}\)-binding sites of the mutant CaM, as described by Wang et al. (13). \( k_{\text{off}} \) represents the dissociation of both Ca\(^{2+}\) ions from each lobe of CaM due to the inability to distinguish the dissociation events of the individual ions via Quin-2 and/or tryptophan fluorescence. Apparent \( K_d \) values represent the concerted binding of both Ca\(^{2+}\) ions assuming that both Ca\(^{2+}\) ions bind indistinguishably to the Ca\(^{2+}\) binding sites of the CaM mutants.

### RESULTS

**Affinity of F19W and F92W for IQ Peptides**

To determine the effects of the different IQ peptides on Ca\(^{2+}\) binding properties of the N- and C-lobes of CaM, we used CaM mutants with phenylalanine 19 or 92 mutated to tryptophan. Tryptophan substitutions within the paired EF hands of each domain of CaM are sensitive to local Ca\(^{2+}\)-dependent conformational changes (2, 5). F19W and F92W CaM are similar to earlier CaM mutants used to assess the site-specific order of Ca\(^{2+}\) binding to CaM and the effect of the chelating residues within the Ca\(^{2+}\) binding loops on Ca\(^{2+}\) affinity (2, 5). The IQ peptides used in this study are listed in Table 1.

**Table 1. Peptides used for this study**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Channel</th>
<th>Sequence Number</th>
<th>Amino Acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lc-IQ</td>
<td>Ca,1.2</td>
<td>1665–1685</td>
<td>KFYATPLIQEYFKRPKRRKQ</td>
</tr>
<tr>
<td>P/Q-IQ</td>
<td>Ca,1.2</td>
<td>1955–1975</td>
<td>KYIAAMYEMYESGSKKKLQ</td>
</tr>
<tr>
<td>N-IQ</td>
<td>Ca,2.2</td>
<td>1853–1873</td>
<td>KYIAAMLFDFYKYKKNTTRDQ</td>
</tr>
<tr>
<td>R-IQ</td>
<td>Ca,2.3</td>
<td>1866–1886</td>
<td>KYIAAMIDYYQSKVKKQR</td>
</tr>
</tbody>
</table>

IQ, motif for binding calmodulin; Lc, P/Q, N, and R, channel types; Ca\(^{2+}\)-dependent Ca\(^{2+}\) channel. Alignment of the IQ binding peptides from \( \alpha_o \) subunits of the indicated voltage-activated Ca\(^{2+}\) channels. Sequence numbers correspond to the human sequence.
To demonstrate that the tryptophan substitutions in CaM do not alter its affinity for the peptides, we examined the tryptophan fluorescence of Ca\(^{2+}\)-saturated F19W and F92W with increasing concentrations of the Lc-IQ peptide (Fig. 1). Figure 1, A and B, shows the change in the emission spectra of F19W and F92W in high Ca\(^{2+}\) upon addition of saturating concentrations of Lc-IQ. Figure 1C shows the formation of the Lc-IQ-mutant CaM complexes as a function of peptide concentration. The apparent \(K_d\) for Lc-IQ binding to F19W was 50 nM with a Hill coefficient of 1.5. The apparent \(K_d\) for Lc-IQ binding to F92W was 66 nM with a Hill coefficient of 1.6. The \(K_d\)s for Lc-IQ binding to these mutant CaM were similar to the values obtained by Zuhlke et al. (15) using dansylated CaM. The Hill coefficient is, however, somewhat higher than the value of 1 obtained by Zuhlke et al. (15). This is likely due to the overestimation of the free concentration of the peptide at low concentrations arising from the loss of peptide due to sticking to the walls of the cuvette (data not shown). This problem is alleviated by silanizing the cuvette. However, all subsequent studies are performed at high peptide concentrations where this is not a problem. To assess the Ca\(^{2+}\) affinity of F19W or F92W CaM bound to the different IQ peptides, we chose a 5:1 ratio of the IQ peptides to CaM. Our previous studies (10, 12) demonstrated that this ratio saturates the CaM and each peptide forms a 1:1 complex with CaM. Although longer peptides appear to bind apoCaM, the IQ peptides alone showed little or no binding of apoCaM (10, 12).

**Ca\(^{2+}\) Affinity of CaM Bound to IQ Peptides Derived from Different Voltage-Activated Ca\(^{2+}\) Channels**

Fluorescence emission (330 nm) of F19W and F92W alone and in the presence of different IQ peptides as a function of Ca\(^{2+}\) concentration are shown in Figs. 2 and 3, respectively. Data for all of the peptides are summarized in Table 2. All of the IQ peptides, except P/Q-IQ, bind to CaM with a high affinity, as evidenced by the high \(K_d\) values. The P/Q-IQ peptide has a lower affinity for CaM, as shown by the lower \(K_d\) value. The Hill coefficients for the binding of IQ peptides to CaM are all close to 1, indicating that the binding is cooperative.

**Fig. 1.** Determination of the affinity of Ca\(^{2+}\)-saturated calmodulin (CaM) mutant, in which phenylalanine in positions 19 and 92 have been replaced with tryptophan (F19W and F92W) for motif binding for Lc CaM (Lc-IQ). F19W or F92W was incubated with LcIQ peptide, as described in MATERIALS AND METHODS. Spectra were collected for each LcIQ concentration: A: F19W spectra with no LcIQ and 1.300 nM free LcIQ. B: the same data shown for F92W spectra. The end point spectra in A or B are used to calculate their relative contribution to each spectrum at the intermediate concentrations of LcIQ, as described in MATERIALS AND METHODS. The relative contribution, or spectral weight, of the end point spectrum is plotted vs. LcIQ peptide. C: spectral weights of F19W and F92W as a function of increasing LcIQ. The curves shown in this figure are to the averaged data points; the values shown for Hill slope and EC\(_{50}\) are averages of fits from three different trials. Average correlation coefficient (\(R^2\)) values for fits of at least three individual data sets are 0.989 ± 0.002 for F19W and 0.983 ± 0.002 for F92W. RFU, relative fluorescence units.

**Fig. 2.** Changes in tryptophan fluorescence of F19W in the presence of the IQ peptides as a function of Ca\(^{2+}\) concentration. The IQ peptides (5 \(\mu\)M) were incubated with F19W (1 \(\mu\)M) for 1 h at room temperature. A: F19W alone, F19W with Lc-IQ, and F19W with P/Q-IQ. B: F19W alone, F19W with R-IQ, and F19W with N-IQ. Each data point represents an average of at least 3 titrations ± SD fit with a modified Hill equation. \(R^2\) values for all the fits are as follows: 0.987 ± 0.008 for F19W alone, 0.989 ± 0.007 with Lc-IQ, 0.994 ± 0.002 with P/Q-IQ, 0.993 ± 0.008 with R-IQ, and 0.991 ± 0.004 with N-IQ.
except for N-IQ, increased the maximal fluorescence yield of F19W (see Table 2). All of the peptides, except for Lc-IQ, increased the maximal fluorescence yield for F92W. When not bound to a peptide, the C-lobe of CaM has a higher affinity for Ca\(^{2+}\) than the N-lobe, but when bound to any of the IQ peptides, the affinity of the two lobes for Ca\(^{2+}\) is similar. The Lc-IQ peptide produces the greatest increase in the Ca\(^{2+}\) affinities of both lobes of CaM (~100× increase in apparent Ca\(^{2+}\) affinity of the N-lobe and ~20× increase in apparent Ca\(^{2+}\) affinity of the C-lobe). The N-IQ peptide produces a small effect on the Ca\(^{2+}\) affinity of the N-lobe of CaM (~2× increase in apparent Ca\(^{2+}\) affinity), but does not alter the Ca\(^{2+}\) affinity of the C-lobe.

The fluorescence of F92W in the presence of Lc-IQ first increases with increasing Ca\(^{2+}\) concentrations, then, before reaching a maximal fluorescence, decreases and finally plateaus. These findings suggest that an intermediate state exists for the CaM peptide complex with fewer than four Ca\(^{2+}\) ions bound. One explanation of this phenomenon is that the N-lobe, upon binding Ca\(^{2+}\), comes close enough to the tryptophan in the C-lobe to cause some quenching. This would suggest that lobe-lobe interactions are occurring in the 4 Ca\(^{2+}\) bound state that are not present when only the C-lobe is Ca\(^{2+}\) bound. To test this possibility, we created an N-lobe Ca\(^{2+}\) binding site mutant of F92W CaM by mutating the glutamic acid residues in the ε positions of the N-lobe EF hands to glutamines, abolishing Ca\(^{2+}\) binding. As shown in Fig. 4, the fluorescence obtained at the low Ca\(^{2+}\) concentrations in the presence of Lc-IQ is similar for F92W and the mutant F92W that cannot bind Ca\(^{2+}\) at the N-lobe. However, at higher Ca\(^{2+}\) concentrations, the fluorescence of the mutant F92W that cannot bind Ca\(^{2+}\) at the N-lobe continues to increase. This mutant may provide a more accurate value of C-lobe Ca\(^{2+}\) affinity for CaM complexed to Lc-IQ. The apparent Ca\(^{2+}\) affinity of the C-lobe of the mutant CaM that cannot bind Ca\(^{2+}\) at the N-lobe complexed to the LC-IQ peptide is 0.05 ± 0.01 μM (n = 3). When CaM is complexed to Lc-IQ, the Ca\(^{2+}\) affinity of the N-lobe (EC\(_{50} = 0.13 ± 0.01 \mu\)M) is likely to be slightly higher than that of the C-lobe (EC\(_{50} = 0.07 ± 0.01\)). The maximal fluorescence of the F92W mutant that cannot bind Ca\(^{2+}\) at the N-lobe is similar to F92W in the presence of the other IQ peptides (data not shown). Also, the maximal fluorescence obtained with F19W was similar when bound to all of the peptides, and, therefore, it seems unlikely that the C-lobe binding is quenching the N-lobe fluorescence of F19W. The high Ca\(^{2+}\) affinities of CaM bound to Lc-IQ supports the finding that the presence of intracellular Ca\(^{2+}\) buffers does not alter L-type channel Ca\(^{2+}\)-dependent inactivation.

**Fig. 3.** Changes in tryptophan fluorescence of F92W in the presence of the IQ peptides as a function of Ca\(^{2+}\) concentration. The IQ peptides (5 μM) were incubated with F92W (1μM) for 1 h at room temperature. A: F92W alone, F92W with Lc-IQ, and F92W with PQ-IQ. B: F92W alone, F92W with R-IQ, and F92W with N-IQ. Each data point represents an average of at least 3 titrations ± SD fit with a modified Hill equation. R\(^2\) values for all the fits are as follows: 0.993 ± 0.003 for F92W alone, 0.936 ± 0.014 with Lc-IQ, 0.988 ± 0.001 with PQ-IQ, 0.995 ± 0.004 with R-IQ, and 0.995 ± 0.006 with N-IQ.

**Table 2. Effect of IQ peptides on Ca\(^{2+}\) affinity of CaM: equilibrium binding parameters**

<table>
<thead>
<tr>
<th>IQ Peptide</th>
<th>F19W K(_d) μM</th>
<th>ΔRFU</th>
<th>Apparent Hill No.</th>
<th>F92W K(_d) μM</th>
<th>ΔRFU</th>
<th>Apparent Hill No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lc-IQ</td>
<td>0.05±0.01</td>
<td>1.62±0.02</td>
<td>2.2±0.2</td>
<td>0.07±0.01</td>
<td>0.54±0.02</td>
<td>4.3±0.8</td>
</tr>
<tr>
<td>PQ-IQ</td>
<td>0.23±0.06</td>
<td>1.74±0.02</td>
<td>2.7±0.2</td>
<td>0.26±0.02</td>
<td>1.03±0.02</td>
<td>1.5±0.3</td>
</tr>
<tr>
<td>R-IQ</td>
<td>0.53±0.03</td>
<td>1.80±0.05</td>
<td>2.5±0.4</td>
<td>0.41±0.01</td>
<td>1.15±0.02</td>
<td>2.2±0.3</td>
</tr>
<tr>
<td>N-IQ</td>
<td>2.58±0.24</td>
<td>1.12±0.02</td>
<td>2.8±0.2</td>
<td>1.47±0.09</td>
<td>1.18±0.01</td>
<td>1.5±0.1</td>
</tr>
<tr>
<td>No peptide</td>
<td>4.83±0.68</td>
<td>1.32±0.06</td>
<td>2.3±0.3</td>
<td>1.40±0.09</td>
<td>0.93±0.02</td>
<td>1.7±0.1</td>
</tr>
</tbody>
</table>

Values are means ± SD. CaM, calmodulin; F19W, CaM mutant in which phenylalanine in position 19 has been replaced with tryptophan; ΔRFU, increase in maximal fluorescence yield; F92W, CaM mutant; K\(_d\), rate of dissociation.
Effects of Peptides on Dissociation of Ca\textsuperscript{2+} Measured by Changes in Quin-2 Fluorescence

To determine whether the F19W or F92W mutations in CaM are altering the Ca\textsuperscript{2+} binding properties of CaM and to measure Ca\textsuperscript{2+} dissociation from the lobes of CaM by an alternative method, we used stopped flow measurements of changes in the Quin-2 fluorescence upon binding Ca\textsuperscript{2+} released from the CaM-IQ complexes (Fig. 7). For these experiments, we used wild-type CaM, F19W, and F92W complexed to the peptides. As expected, two kinetic components were detected in the Quin-2 experiments (dissociation from both the N and C lobes). The dissociation values obtained are summarized in Table 4. The values for the faster component detected with Quin-2 were similar to the dissociation rates calculated from the change in tryptophan fluorescence of F19W for each peptide while the values for the slower component were similar to values obtained from the changes in tryptophan fluorescence of F92W (compare Tables 3 and 4). In addition, the two rates of Ca\textsuperscript{2+} dissociation from wild-type CaM bound to each of the peptides were similar to that of F19W and F92W. These findings suggest that the tryptophan substitutions themselves did not greatly alter the Ca\textsuperscript{2+} binding properties of CaM when complexed to the IQ peptides. The close correlation between the 2 kinetic components detected by Quin-2 fluorescence (Table 4) and the rates of decrease in the tryptophan fluo-

Fig. 4. Changes in tryptophan fluorescence of mutant F92W (no N-lobe Ca\textsuperscript{2+} binding) in the presence of the IQ peptides as a function of Ca\textsuperscript{2+} concentration. The Lc-IQ peptide (5 \mu M) was incubated with F92W (no N-lobe Ca\textsuperscript{2+} binding) (1 \mu M) for 1 h at room temperature. F92W with Lc-IQ (same data as in Fig. 2). Each data point represents an average of at least 3 titrations ± SD fit with a modified Hill equation.

Fig. 5. Ca\textsuperscript{2+} dissociation rates from F19W in the presence of IQ peptides derived from the voltage-gated Ca\textsuperscript{2+} channels. Trytrophan fluorescence was measured after rapid mixing of equal volumes (50 l) of F19W (4 \mu M), peptide (20 \mu M), and Ca\textsuperscript{2+} (200 \mu M) in 10 mM MOPS, 90 mM KCl, pH 7.0 and EGTA (10 mM). This figure shows the decrease in tryptophan fluorescence associated with Ca\textsuperscript{2+} dissociation from the NH\textsubscript{2} terminal sites of F19W and in the presence and absence of the IQ peptides (Lc-IQ in A, all others in B). Rates were obtained as described in MATERIALS AND METHODS. Each trace is an average of 8 oversampled traces fit with a single or double exponential equation as required (variance <4.0 \times 10^{-5}) and is representative of 3–5 independent determinations.
rescence as Ca\(^{2+}\)/H\(11001\) dissociates from the F19W and F92W complexes (Table 3) suggest that these tryptophan mutants accurately report Ca\(^{2+}\)/H\(11001\) binding events in the N-lobe and C-lobe of CaM. The N-IQ peptide produced the smallest effects on Ca\(^{2+}\)/H\(11001\) dissociation from the N-lobe.

Figure 8 shows a comparison of the EGTA-induced alteration in tryptophan fluorescence and Quin-2 fluorescence traces obtained when F92W is complexed with Lc-IQ. An exponential fit of the increase in fluorescence reveals a rate similar to the fast component (N-lobe) dissociation from the Quin-2 studies. The rates of change are 6.8 s\(^{-1}\) for the initial increase in tryptophan fluorescence and 0.74/s for the following decrease of the F92W-Lc-IQ complex as Ca\(^{2+}\)/H\(11001\) is released. The Quin-2 change in fluorescence is biphasic with rates of 6.6 s\(^{-1}\) and 0.75 s\(^{-1}\) for Ca\(^{2+}\)/H\(11001\) dissociating from the F92W-Lc-IQ complex directly (Fig. 8, Tables 3 and 4). Thus the early increase in fluorescence correlates with the rate of dissociation of Ca\(^{2+}\)/H\(11001\) from the N-lobe of CaM, suggesting that dissociation of Ca\(^{2+}\)/H\(11001\) from the N-lobe is altering the environment of the tryptophan at position 92 in the C-lobe.

Calculation of Rates of Ca\(^{2+}\) Association

While there is a good correlation between the decrease in the rates of Ca\(^{2+}\)/H\(11001\) dissociation and the observed increases in Ca\(^{2+}\)/H\(11001\) affinities, the decreases observed in the rates of Ca\(^{2+}\)/H\(11001\) dissociation are not alone large enough to account for the observed changes in Ca\(^{2+}\)/H\(11001\) affinities. Alterations in the Ca\(^{2+}\)/H\(11001\) association rates \(k_{\text{on}}\) are also likely to be occurring. We estimated the Ca\(^{2+}\)/H\(11001\) association rates for the N and C-lobes of F19W and F92W, respectively, in the presence and absence of the IQ peptides (Table 5) using the relationship:

\[
\frac{k_{\text{on}}}{K_{\text{d,final}}} = \frac{1}{K_{\text{d,final}}} + \frac{1}{K_{\text{d,initial}}}.
\]

In all cases the calculated Ca\(^{2+}\)/H\(11001\) association rate to the N-lobe was decreased when F19W was complexed with an IQ peptide. The binding of CaM to the peptides, therefore, apparently inhibits...
Table 4. Effect of IQ peptides on $K_d$ of Ca$^{2+}$ from CaM as measured by changes Quin-2 fluorescence

<table>
<thead>
<tr>
<th>IQ Peptide</th>
<th>CaM $k_{-1}$ (s$^{-1}$)</th>
<th>CaM $k_{-2}$ (s$^{-1}$)</th>
<th>F19W $k_{-1}$ (s$^{-1}$)</th>
<th>F19W $k_{-2}$ (s$^{-1}$)</th>
<th>F92W $k_{-1}$ (s$^{-1}$)</th>
<th>F92W $k_{-2}$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lc-IQ</td>
<td>5.6±0.1</td>
<td>0.9±0.1</td>
<td>5.6±0.3</td>
<td>0.8±0.1</td>
<td>6.4±0.1</td>
<td>0.8±0.1</td>
</tr>
<tr>
<td>P/Q-IQ</td>
<td>13±3</td>
<td>2.0±0.1</td>
<td>4.3±0.5</td>
<td>1.8±0.2</td>
<td>13±1</td>
<td>1.4±0.1</td>
</tr>
<tr>
<td>R-IQ</td>
<td>7.4±0.7</td>
<td>1.6±0.1</td>
<td>8.9±0.2</td>
<td>2.0±0.1</td>
<td>17±1</td>
<td>1.1±0.1</td>
</tr>
<tr>
<td>N-IQ</td>
<td>25±2</td>
<td>1.9±0.1</td>
<td>57±6</td>
<td>1.9±0.1</td>
<td>21±2</td>
<td>1.8±0.1</td>
</tr>
<tr>
<td>No peptide</td>
<td>&gt;1,000</td>
<td>7.5±0.2</td>
<td>670±50</td>
<td>7.5±0.2</td>
<td>&gt;1,000</td>
<td>4.9±0.2</td>
</tr>
</tbody>
</table>

Fig. 8. Comparison dissociation rates from F92W tryptophan fluorescence with Quin-2 data. This figure shows changes in tryptophan fluorescence associated with Ca$^{2+}$ dissociation from F92W complexed to Lc-IQ with the changes in Quin-2 fluorescence. Rates were obtained as stated in MATERIALS AND METHODS. Each trace is an average of 8 oversampled traces fit with a single or double exponential equation as required (variance <4.0 × 10$^{-3}$) and is representative of 3–5 independent determinations.

Table 5. Estimated rates of association of Ca$^{2+}$ to N-lobe of F19W and C-lobe of F92W

<table>
<thead>
<tr>
<th>IQ</th>
<th>F19W $k_{+1}$ 10$^6$ (M$^{-1}$s$^{-1}$)</th>
<th>F92W $k_{+1}$ 10$^6$ (M$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lc</td>
<td>112</td>
<td>6.5*</td>
</tr>
<tr>
<td>P/Q</td>
<td>18.7</td>
<td>5.2</td>
</tr>
<tr>
<td>R</td>
<td>16.8</td>
<td>2.6</td>
</tr>
<tr>
<td>N</td>
<td>22.1</td>
<td>1.2</td>
</tr>
<tr>
<td>No peptide</td>
<td>139</td>
<td>2.8</td>
</tr>
</tbody>
</table>

*The value for $k_{+1}$ in the presence of Lc-IQ was calculated using the $K_d$ determined with the mutant F92W that did not bind Ca$^{2+}$ at the N-lobe.
Ca\textsuperscript{2+}-dependent facilitation and inactivation is related to its ability when in complex with the IQ motifs to assume multiple conformational states controlled by differential binding of Ca\textsuperscript{2+} at its N- and C-lobes in response to different types of Ca\textsuperscript{2+} signals.

ACKNOWLEDGMENTS

We thank Dr. Irina Serysheva, Dr. Brett Mitchell, Dr. William Durham, Dr. Cristina Danila, Charles Gilman, and Mariah Baker for comments on the manuscript.

GRANTS

This study was supported by grants from the Muscular Dystrophy Association and National Institutes of Health Grants AR-44802, AR-41802, and AR-44864 (all to S. L. Hamilton), DK-33727 and HL-48835 (to R. A. Altschuld), and NS-35212 (to S. Pedersen).

REFERENCES

1. Anderson ME. Ca\textsuperscript{2+}-dependent regulation of cardiac L-type Ca\textsuperscript{2+} channels: is a unifying mechanism at hand? J Mol Cell Cardiol 33: 639–650, 2001.
2. Black DJ, Tikunova SB, Johnson JD, and Davis JP. Acid pairs increase the N-terminal Ca\textsuperscript{2+} affinity of CaM by increasing the rate of Ca\textsuperscript{2+} association. Biochemistry 39: 13831–13837, 2000.