Effects of Myosin Light Chain Kinase and Peptides on Ca\textsuperscript{2+} Exchange with the N- and C-terminal Ca\textsuperscript{2+} Binding Sites of Calmodulin*

J. David Johnson‡§, Christopher Snyder‡, Michael Walsh¶, and Maera Flynn†

From the Department of Medical Biochemistry, The Ohio University Medical Center, Columbus, Ohio 43210 and the Department of Medical Biochemistry, University of Calgary, Calgary, Alberta T2N 4N1, Canada

Myosin light chain kinase and peptides from the calmodulin (CaM) binding domains of myosin light chain kinase (RS-20, M-13), CaM kinase II, and the myristoylated alanine-rich protein kinase C substrate protein slowed Ca\textsuperscript{2+} dissociation from CaM's N-terminal sites from 405 ± 75/s to 1.8–2.9/s and from CaM's C-terminal sites from 2.4 ± 0.2/s to 0.1–0.4/s at 10°C. Since Ca\textsuperscript{2+} dissociates 5–29 times faster from the N-terminal in these CaM-peptide complexes and both lobes are required for activation, Ca\textsuperscript{2+} dissociation from the N-terminal could control target protein activation. Ca\textsuperscript{2+} binds 70 times faster to the N-terminal (1.6 × 10\textsuperscript{6} M\textsuperscript{-1} s\textsuperscript{-1}) than the C-terminal sites (2.3 × 10\textsuperscript{5} M\textsuperscript{-1} s\textsuperscript{-1}). In a 0.6-ms half-width Ca\textsuperscript{2+} transient, Ca\textsuperscript{2+} occupied >70% of the N-terminal but only 20% of the C-terminal sites. RS-20 produced a 9-fold and CaM kinase II a 6.3-fold increase in C-terminal Ca\textsuperscript{2+} affinity, suggesting that some target proteins may be bound to the C-terminal at resting [Ca\textsuperscript{2+}]. When this is the case, Ca\textsuperscript{2+} exchange with the faster N-terminal sites may regulate CaM's activation and inactivation of these target proteins during a Ca\textsuperscript{2+} transient.

CaM\textsuperscript{3} is a ubiquitous Ca\textsuperscript{2+} binding protein that consists of a N- and C-terminal lobe, each of which contains two EF hand Ca\textsuperscript{2+} binding sites (see Weinstein and Mehler (1994) and James et al. (1995) for reviews). The N- and C-terminal lobes are separated by an 8-turn \(\alpha\)-helix, and they undergo a Ca\textsuperscript{2+} dependent exposure of their hydrophobic binding pockets, which allows binding and activation of target protein (LaPorte et al., 1980; Tanaka and Hidaka, 1980). In the presence of Ca\textsuperscript{2+}, both the N- and C-terminal hydrophobic pockets of CaM bind an amphipathic \(\alpha\)-helical peptide domain within the target protein structure (O'Neil and DeGrado, 1990), and recently the high resolution x-ray and NMR structure of several Ca\textsuperscript{2+}-CaM-peptide complexes have been determined (Ikura et al., 1992; Meador et al., 1992, 1993). Ca\textsuperscript{2+}-CaM binding removes a pseudosubstrate inhibitory domain from many enzymes' active sites, and this results in the activation of smooth and skeletal muscle MLCK, CaM kinase II, calcineurin, plasmalogen Ca-ATPase, and cyclic nucleotide phosphodiesterase (see Kemp and Pearson (1991) and James et al. (1995) for review).

The CaM binding domains of many CaM target proteins have been identified, and peptides representing these domains from skeletal (M-13) and smooth (RS-20) muscle MLCK, CaM kinase II (C\textsubscript{2}K), and the MARCKS protein have been synthesized. These peptides bind CaM with nanomolar affinity as does the entire target protein (Blumenthal et al., 1985; Lucas et al., 1986; Payne et al., 1988; Verghese et al., 1994).

The binding of target proteins, their CaM binding domain peptides, and hydrophobic drugs all produce dramatic (7–40-fold) increases in CaM's affinity for Ca\textsuperscript{2+} (Keller et al., 1982; Olwin et al., 1984; Mills et al., 1985; Yagi and Yazawa, 1989; Kasturi et al., 1993). These increases in Ca\textsuperscript{2+} affinity might be reflected by decreases in the rate of Ca\textsuperscript{2+} dissociation from the lower affinity N- and higher affinity C-terminal EF hands of CaM. If target peptide and protein binding reduces the rate of Ca\textsuperscript{2+} dissociation from the CaM-protein (peptide) complex, then the rate of complex dissociation and inactivation could be delayed until long after the Ca\textsuperscript{2+} transient has subsided. In this paper, we determined the rates of Ca\textsuperscript{2+} exchange with the N- and C-terminal domains of CaM and examined the effects of MLCK and several CaM binding peptides on the rates of Ca\textsuperscript{2+} dissociation from the N- and C-terminal Ca\textsuperscript{2+} binding sites of CaM and on C-terminal Ca\textsuperscript{2+} affinity. We relate our results to the mechanism of CaM's activation and inactivation of its target proteins during a cellular Ca\textsuperscript{2+} transient.

EXPERIMENTAL PROCEDURES

Materials—Quin 2 and Mg-Fura-2 were purchased from Molecular Probes; phenyl-Sepharose CL-4B, TNS, calmidazolium, and EGTA were purchased from Research Organics (Cleveland, OH); and hydroxyapatite was from Bio-Rad. All other chemicals were of analytical grade.

C\textsubscript{2}K peptide was purchased from L.C. Laboratories (Woburn, MD), RS-20 peptide was from American Peptide Co. (Sunnyvale, CA), M-13 peptide was purchased from Peptide Technologies (Gaithersburg, MD), and MARCKS peptide was the generous gift of Dr. Perry Blackshear (Duke University, Durham, NC). CaM41/75 and CaM85/112 were generously provided by Dr. Zenon Grabarek (Boston Biomedical Institute, Boston, MA).

Protein Purification—CaM was purified from bovine brain as described previously by Kasturi et al. (1993). MLCK was purified to electrophoretic homogeneity from chicken gizzard smooth muscle as described by Nagai et al. (1984).
Ca\(^{2+}\) Exchange with Calmodulin

**Methods**—All static fluorescence measurements were performed on a Perkin-Elmer LS5 Spectrofluorimeter at 10 °C. Free [Ca\(^{2+}\)] was calculated as described by Robertson and Potter (1984). Kinetic measurements were performed by mixing an equal volume (50 \(\mu\)l) of each reagent together in an Applied Photophysics Ltd. (Leatherhead, UK) model SF-17 MV stopped flow instrument. This instrument had a dead time of 1.6 ms and flow rate of 17 \(\mu\)l/s. The samples were excited using a 150-watt Xenon arc source at the specified wavelength. Fluorescence emission was monitored through the specified interference filters. The curve fitting program (by P. J. King, Applied Photophysics Ltd.) uses the non-linear Levenberg-Marquardt algorithm.

The changes in Quin fluorescence as it dissociates Ca\(^{2+}\) from CaM were converted to moles of Ca\(^{2+}\) dissociating from CaM by mixing increasing concentrations of Ca\(^{2+}\) (0, 10, 20, 30, 40, 50, and 60 \(\mu\)M) with Quin 2. Quin 2 fluorescence increased linearly as a function of increasing [Ca\(^{2+}\)], allowing us to directly relate a change in Quin 2 fluorescence to the number of moles of Ca\(^{2+}\) dissociating per mole of CaM. Calibration curves were performed at the end of each experiment using the same Quin 2 solutions and experimental conditions as used in the experiments. The amplitude of the change in Quin 2 fluorescence was extrapolated from an exponential fit of the data in Fig. 1A, inset.

**Computer Modeling**—The computer simulations were performed using KSIM version 1.1 (N. C. Millar, UCLA School of Medicine, Los Angeles). The \(k_{on}\) of EGTA for Ca\(^{2+}\) at 10 °C and low ionic strength (pH 7.0) was calculated to be 4.23 \(\times\) 10\(^{-7}\) M\(^{-1}\) s\(^{-1}\) from the program of Robertson and Potter (1984). The rate of Ca\(^{2+}\) dissociation from EGTA (\(k_{off}\)) was determined by stopped flow experiments where EGTA (10 \(\mu\)M) and Ca\(^{2+}\) (20 \(\mu\)M) were mixed with Quin 2 (150 \(\mu\)M) in the same buffer (20 mM Hepes, pH 7.0), at 10 °C. The Quin 2 fluorescence increased at the \(k_{off}\) of EGTA, 0.55 \(\pm\) 0.03/s. Our calculated \(k_{on}\) of 1.3 \(\times\) 10\(^{6}\) M\(^{-1}\) s\(^{-1}\) (from \(k_{on} = k_{on}/k_{off}\)) was in agreement with the value of 9.2 \(\times\) 10\(^{5}\) M\(^{-1}\) s\(^{-1}\) determined by Smith et al. (1984) at 16 °C in 0.1 M ionic strength (pH 6.8). Modeling of the Ca\(^{2+}\) transient and the Ca\(^{2+}\) occupancy of the N- and C-terminal sites of CaM assumed the following initial concentrations: N- and C-terminal Ca\(^{2+}\) binding sites (4 \(\mu\)M), Ca\(^{2+}\) (100 \(\mu\)M), and EGTA (1 mM), which represent the reaction conditions in the stopped flow experiments of Fig. 5 after mixing. The kinetic parameters used for Ca\(^{2+}\) exchange with the N- and C-terminal sites of CaM were those determined as described under “Results” and as cited in Table I.

**RESULTS**

**Ca\(^{2+}\) Dissociation from the N- and C-terminal Ca\(^{2+}\) Binding Sites**

Sites of CaM—Bayley et al. (1984) have previously used Quin 2 fluorescence to measure the rates of Ca\(^{2+}\) dissociation from CaM. Fig. 1A shows Ca\(^{2+}\) dissociation from CaM’s N- and C-terminal Ca\(^{2+}\) binding sites as measured by the increase in Quin 2 fluorescence, which occurs upon Ca\(^{2+}\) binding. Ca\(^{2+}\) dissociated from CaM as a biphasic process with 1.2 \(\pm\) 0.2 mol of Ca\(^{2+}\) dissociating at 405 ± 75 s (Fig. 1A, inset) and 1.6 \(\pm\) 0.2 mol of Ca\(^{2+}\) dissociating nearly 170 times more slowly at 2.4 \(\pm\) 0.2 s. Thus, under the conditions used in our studies, we clearly observe Ca\(^{2+}\) dissociation from both the N- and C-terminal Ca\(^{2+}\) binding sites of CaM.

**Effect of MLCK and CaM Binding Peptides on Ca\(^{2+}\) Dissociation from CaM**—The effects of MLCK and several CaM binding peptides on Ca\(^{2+}\) dissociation from CaM were determined using Quin 2 fluorescence. Fig. 1B shows the effect of smooth muscle MLCK, its CaM binding peptide (RS-20), and the MARCKS peptide on Ca\(^{2+}\) dissociation from CaM. In the presence of protein or peptide, the rapid phase of Ca\(^{2+}\) dissociation (405 s) was eliminated. Instead, biphasic Ca\(^{2+}\) dissociation rates of 2.28 ± 0.20 s\(^{-1}\) and 0.39 ± 0.15 s\(^{-1}\) were observed for MLCK, RS-20, and MARCKS peptide, respectively. M-13 peptide, from skeletal muscle MLCK, was similar to RS-20 with biphasic Ca\(^{2+}\) dissociation rates of 2.86 ± 0.06 s\(^{-1}\) and 1.14 ± 0.02 s\(^{-1}\). Ca\(^{2+}\) dissociation was similar to the MARCKS peptide with biphasic Ca\(^{2+}\) dissociation rates of 2.9 ± 0.3 s\(^{-1}\) and 0.29 ± 0.03 s\(^{-1}\). Thus, MLCK and all of these high affinity peptides abolished the rapid rate of Ca\(^{2+}\) dissociation from the N-terminal of CaM and result in ~2 mol of Ca\(^{2+}\) dissociating at 1.8–2.9 s\(^{-1}\) and ~2 mol of Ca\(^{2+}\) dissociating at 0.1–0.4 s\(^{-1}\).

**Effect of Peptide on Ca\(^{2+}\) Dissociation from the C-terminal Ca\(^{2+}\) Binding Sites**—CaM’s two tyrosine residues (Tyr-99 and Tyr-138) are in the C-terminal half of the molecule and undergo a large fluorescence increase when Ca\(^{2+}\) binds to the C-terminal sites of CaM (Dedman et al., 1977; George et al., 1993). Fig. 2 shows the time course of the EGTA-induced decrease in Ca\(^{2+}\)-CaM tyrosine fluorescence. These data agree with our Quin 2 studies and verify that Ca\(^{2+}\) dissociates from the C-terminal sites of CaM at a rate of 2.1 ± 0.1 s\(^{-1}\) at 10 °C. The addition of MARCKS peptide, which has no Tyr or Trp residues, reduces the rate of Ca\(^{2+}\) dissociation from these C-terminal sites to 0.32 ± 0.02 s\(^{-1}\).

CaM41/75 is a mutant CaM in which cysteine residues have been introduced at positions 41 and 75 by site-directed mutagenesis.
Effect of MARCKS peptide on Ca\(^{2+}\) dissociation from CaM and CaM41/75 C-terminal Ca\(^{2+}\) binding sites. The upper traces show the increase in Quin 2 fluorescence associated with Ca\(^{2+}\) dissociation from the C-terminal sites of CaM41/75 with and without MARCKS peptide. CaM41/75 (4 \(\mu\)M) + Ca\(^{2+}\) (60 \(\mu\)M) ± MARCKS peptide (10 \(\mu\)M) in 20 mM Hepes, pH 7.0, was rapidly mixed with an equal volume of Quin 2 (150 \(\mu\)M) in the same buffer at 10°C. Quin 2 fluorescence was monitored as described in the legend to Fig. 1. The lower traces show the decrease in CaM tyrosine fluorescence when CaM ± MARCKS peptide was rapidly mixed with EGTA. CaM (4 \(\mu\)M) + Ca\(^{2+}\) (100 \(\mu\)M) ± MARCKS peptide (10 \(\mu\)M) in 20 mM Hepes, pH 7.0, was rapidly mixed with an equal volume of EGTA (5 mM) in the same buffer at 10°C. Control experiments where CaM + Ca\(^{2+}\) were reacted with buffer + Ca\(^{2+}\) were flat lines, which began at the starting amplitude of the EGTA trace. CaM tyrosine fluorescence was measured through a UV-transmitting black glass filter (UG1 from Oriel (Standford, CT)) with excitation at 275 nm. Each trace is an average of five traces fit with a single exponential (variance = 2.2 × 10\(^{-4}\)).

2. Z. Grabarek, personal communication.


Fig. 3. Effects of RS-20, MARCKS, and C-K peptide on Ca\(^{2+}\) binding to the C-terminal sites of CaM or CaM41/75. The Ca\(^{2+}\) dependence of the increase in tryptophan fluorescence for CaM (○), CaM41/75 (●), CaM + C-K peptide (△), or CaM + MARCKS peptide (□) and the Ca\(^{2+}\) dependence of the increase in RS-20 tryptophan fluorescence in the presence of CaM41/75 (□) are shown as a function of pCa.

Fig. 4 (Ca trace). The free [Ca\(^{2+}\)] decays at ~1200/s to an equilibrium value of pCa 7.4, producing a 0.6-ms half-width Ca\(^{2+}\) transient. This Ca\(^{2+}\) transient can be produced and visualized in the stopped flow by following Mg-Fura-2 fluorescence when 200 μM Ca\(^{2+}\) is mixed with Mg-Fura-2 in the presence of 2 mM EGTA (Fig. 4, MF2 trace). Ca\(^{2+}\) bound to Mg-Fura-2 during the dead time and then dissociated at ~1300/s, as EGTA chelated Ca\(^{2+}\). Thus, the simulation and Mg-Fura-2 fluorescence precisely define the time course of this Ca\(^{2+}\) transient.

Fig. 4 also simulates the time course of occupancy of the N- and C-terminal Ca\(^{2+}\) binding sites of CaM (using the on- and off-rates shown in Table I) during this Ca\(^{2+}\) transient. This simulation suggests that the faster N-terminal sites would be 96% occupied at 0.4 ms and that they would lose this Ca\(^{2+}\) to EGTA at ~240/s. The slower C-terminal Ca\(^{2+}\) binding sites would only be 18% occupied, and they would then lose this Ca\(^{2+}\) to EGTA at 2.4/s.

Ca\(^{2+}\) binding to both the N- and C-terminal lobes of CaM exposes hydrophobic sites that bind TNS and cause a large increase in TNS fluorescence. Fig. 5 (trace A) shows the time course of the decrease in TNS fluorescence when the Ca\(^{2+}\)-CaM-TNS complex is mixed with EGTA. Similar to Suko et al. (1985), we observed a biphasic process in which 54% of the TNS fluorescence decrease occurs at 405 ± 20/s (Fig. 5, trace A).

### Table I

| Peptide or protein | k\(_{\text{off}}\) | k\(_{\text{on}}\) | k\(_{\text{on}}\) |
|-------------------|---------------|---------------|---------------|
|                   | s\(^{-1}\)     | M             | M\(^{-1}\) s\(^{-1}\) |
| ---               | ---           | ---           | ---           |
| None              | 405           | 2.6 × 10\(^{-6}\) | 1.6 × 10\(^{8}\) |
| C2K               | 2.9           | ---           | ---           |
| MARCKS            | 2.6           | ---           | ---           |
| MLCK              | 2.3           | ---           | ---           |
| RS20              | 1.8           | ---           | ---           |

---

Ca\(^{2+}\) Exchange with Calmodulin
that occur in the transient occupancy experiments (Fig. 5, trace B) are compared to the amplitudes of the decreases in the EGTA experiments (Fig. 5, trace A), we find that 70% of the N-terminal and 20% of the C-terminal Ca$^{2+}$ binding sites are occupied during the transient occupancy experiments. These data are consistent with the simulation of Fig. 4, where 96% of the N-terminal sites (with a Ca$^{2+}$ on-rate of $1.2 \times 10^8$ M$^{-1}$ s$^{-1}$) and 18% of the C-terminal sites (with a Ca$^{2+}$ on-rate of $1.2 \times 10^7$ M$^{-1}$ s$^{-1}$) would be occupied during this Ca$^{2+}$ transient. Increasing the [Ca$^{2+}$] produced greater occupancy of both the N- and C-terminal hydrophobic pockets by TNS. Thus, Ca$^{2+}$ binding is the rate-limiting step and not the rate of opening of the hydrophobic pocket or TNS binding. These data confirm the Ca$^{2+}$ on-rates we calculated in Table I and the simulation using these on-rates (Fig. 4), which shows that the N-terminal Ca$^{2+}$ binding sites are almost fully occupied during a rapid Ca$^{2+}$ transient, while the C-terminal sites, with a 70-fold slower Ca$^{2+}$ on-rate, are not.

The observation that the C-terminal sites were only 20% occupied by Ca$^{2+}$ during this rapid Ca$^{2+}$ transient was verified using CaM-tyrosine fluorescence. When CaM (4 mM) and Ca$^{2+}$ (200 mM) were mixed with 2 mM EGTA, the C-terminal tyrosine fluorescence decreased at a rate of $2.1 \times 10^6$ s$^{-1}$ as Ca$^{2+}$ dissociated from the C-terminal sites (Fig. 6, CaM + Ca versus EGTA trace). When a rapid Ca$^{2+}$ transient was produced by mixing Ca$^{2+}$ (200 mM) with CaM (4 mM) in the presence of 2 mM EGTA, the tyrosine fluorescence rose to only 23% of the intensity seen with Ca$^{2+}$ saturated CaM and then decayed back at 2.1/s (Fig. 6, CaM + EGTA versus Ca trace). When the [Ca$^{2+}$] was increased to 400 $\mu$M, the tyrosine fluorescence increased to 42% of the intensity seen with Ca$^{2+}$ saturated CaM. This suggests that Ca$^{2+}$ binding to the C-terminal is the rate-limiting step.

Our studies show that Ca$^{2+}$ dissociates from the N-terminal Ca$^{2+}$ bonding sites of CaM at 405/s and from the higher affinity C-terminal sites at 2.4/s at 10°C. These results agree with the work of Bayley et al. (1984) who have shown that Quin 2 chelates Ca$^{2+}$ from the N- and C-terminal sites of CaM at 293 ± 93/s and 2.1 ± 0.4/s, respectively, at 11°C. Martin et al. (1992) have also shown that the N-terminal Ca$^{2+}$ binding sites lose Ca$^{2+}$ rapidly (389 ± 64/s) while the higher affinity C-terminal Ca$^{2+}$ binding sites lose Ca$^{2+}$ more slowly (11 ± 2.4/s) at 18°C and low ionic strength.

Peptide and MLCK binding to CaM reduced the rate of Ca$^{2+}$ dissociation from the N-terminal sites from 405/s to 1.8-2.9/s (a 140-225-fold decrease) and from the C-terminal Ca$^{2+}$ binding sites from 2.4/s to 0.1-0.4/s (a 6-24-fold decrease).

Martin et al. (1985) have shown that the CaM antagonist trifluoperazine slows Ca$^{2+}$ dissociation from the CaM N-terminal sites from 310/s to 15/s and from the CaM C-terminal sites from 3.6/s to 0.5/s at 13.4°C. Further, calmidazolium slowed Ca$^{2+}$ dissociation to 4/s and 0.2/s for the N- and C-terminal sites, respectively (data not shown). Thus, these CaM antagonist drugs, which bind to both the N- and C-terminal hydrophobic pockets, produce similar decreases in Ca$^{2+}$ off-rate as peptide.

The high resolution structure of CaM complexed with RS-20 (Meador et al., 1992), C$_K$ peptide (Meador et al., 1993), and M-13 (Ikura et al., 1992) suggest that peptide binding dramatically alters CaM structure. The central helix of CaM unwind, allowing the hydrophobic pockets on the N- and C-terminal lobes to engulf these peptides. The amphipathic nature of these peptides allows them to shield the N- and C-terminal hydrophobic pockets of CaM from solvent (O’Neil and Degrado, 1990) and to stabilize the Ca$^{2+}$ bound state of CaM. This results in
peptide-induced increases in Ca\(^{2+}\) affinity, which we see expressed as large decreases in the rate of Ca\(^{2+}\) dissociation from both halves of CaM.

While all of the peptides produced similar dramatic decreases in the rate of Ca\(^{2+}\) dissociation from the N-terminal of CaM, both RS-20 and M-13 slowed Ca\(^{2+}\) dissociation from the C-terminal 3-fold more than either C\(_{2}\)K or MARCKS. Comparison of the high resolution CaM-peptide structures indicates that there are ~40% less contacts between CaM-C\(_{2}\)K compared to CaM-RS-20 (Meador et al., 1993). Furthermore, both RS-20 and M-13 have an aromatic Trp residue in their N-terminal domain, which makes extensive contacts with the C-terminal of CaM (Ikura et al., 1992; Meador et al., 1992). This could explain why RS-20 and M-13 reduce Ca\(^{2+}\) dissociation from the C-terminal to ~0.1/s while C\(_{2}\)K and MARCKS peptide reduce Ca\(^{2+}\) dissociation to only ~0.3/s. While every residue of RS-20, M-13, and C\(_{2}\)K forms contacts with CaM, the Trp residue in the N-terminal of RS-20 and M-13 may further stabilize the Ca\(^{2+}\) bound state, increase Ca\(^{2+}\) affinity, and decrease the rate of Ca\(^{2+}\) dissociation from the C-terminal.

Our results show that the C-terminal of CaM has a 2.6-fold greater affinity for Ca\(^{2+}\) than the N-terminal of CaM. This agrees with the results of Minowa and Yagi (1984) who have shown a 3.4-fold higher Ca\(^{2+}\) affinity at the C-terminal of the molecule. Since the N-terminal of CaM has a 170-fold faster Ca\(^{2+}\) off-rate than the C-terminal but only a 2–3-fold lower Ca\(^{2+}\) affinity, it follows that the N-terminal of CaM must have a much faster Ca\(^{2+}\) on-rate than the C-terminal. Utilizing the K\(_{a}\) and Ca\(^{2+}\) on-rates for the N- and C-terminal Ca\(^{2+}\) binding sites of CaM, we calculated their Ca\(^{2+}\) on-rates to be 1.6 x 10\(^8\) M\(^{-1}\) s\(^{-1}\) and 2.3 x 10\(^8\) M\(^{-1}\) s\(^{-1}\), respectively.

Estimates of Ca\(^{2+}\) on-rate from K\(_{off}/K_{on}\) could be complicated by the cooperativity that exists between the two Ca\(^{2+}\) binding sites in each lobe of CaM. Therefore, it was necessary to verify the faster Ca\(^{2+}\) on-rate to the N-terminal Ca\(^{2+}\) sites by an additional method. Currently, there is no way to directly measure the Ca\(^{2+}\) on-rate to the N-terminal Ca\(^{2+}\) binding sites in a stopped flow apparatus. Our computer simulations (Fig. 4) were conducted assuming the Ca\(^{2+}\) off-rates determined by our stopped flow studies and the calculated Ca\(^{2+}\) on-rates cited above and in Table I. They suggest that during a rapid Ca\(^{2+}\) transient (half-width 0.6 ms), Ca\(^{2+}\) would occupy 96% of the N-terminal sites and 18% of the C-terminal sites. We have produced this rapid Ca\(^{2+}\) transient in the stopped flow and verified (using CaM and TNS fluorescence) that the N-terminal sites are >70% saturated by this Ca\(^{2+}\) transient, while the slower C-terminal sites are only 20% occupied. Thus, our calculated Ca\(^{2+}\) on-rates for the N- and C-terminal sites of CaM are verified by modeling (Fig. 4) and by the Ca\(^{2+}\) transient occupancy experiments using CaM-TNS (Fig. 5).

Ca\(^{2+}\) not only binds quickly to the N-terminal Ca\(^{2+}\) sites, but it also rapidly exposes the N-terminal hydrophobic pocket to accommodate TNS and presumably protein binding. Thus, only the N-terminal Ca\(^{2+}\) binding sites on CaM can respond to a very rapid rise and fall in Ca\(^{2+}\) by opening and closing their hydrophobic binding pocket. The Ca\(^{2+}\) transient (0.6 ms half-width) produced in our stopped flow experiments is much faster than physiological Ca\(^{2+}\) transients, which exhibit half-widths from milliseconds to minutes. Clearly, during these longer Ca\(^{2+}\) transients, both the N- and C-terminal Ca\(^{2+}\) binding sites would be occupied. The rapid “artificial” Ca\(^{2+}\) transient produced in our stopped flow experiments was designed and used to verify the faster Ca\(^{2+}\) on-rate to the N-terminal sites of CaM relative to the C-terminal sites.

The N-terminal Ca\(^{2+}\) binding sites of CaM have a ~70-fold faster Ca\(^{2+}\) on-rate and a ~170-fold faster Ca\(^{2+}\) off-rate than the higher affinity C-terminal Ca\(^{2+}\) binding sites. Therefore, the N-terminal sites of CaM resemble the N-terminal sites of TnC, which have a fast Ca\(^{2+}\) on-rate (~2 x 10\(^9\) M\(^{-1}\) s\(^{-1}\)) and off-rate (400/s) (Johson et al., 1979, 1994) compared to the higher affinity C-terminal Ca\(^{2+}\) binding sites.

Both the N- and C-terminal lobes of CaM must bind Ca\(^{2+}\) and expose their hydrophobic pockets to activate target proteins (Perschini and Kretsginger, 1988). Our Ca\(^{2+}\) titrations (Fig. 3) indicate that RS-20 and C\(_{2}\)K increase Ca\(^{2+}\) affinity at CaM’s C-terminal Ca\(^{2+}\) binding sites so dramatically that these sites may be 50–80% occupied even at the resting levels of Ca\(^{2+}\) found in smooth muscle cells (~pCa 6.8, Cornwell and Lincoln 1989). Therefore, the higher affinity C-terminal lobe of CaM may be bound to some target proteins at resting Ca\(^{2+}\) levels. While this would not result in enzyme activation, the subsequent rapid binding of Ca\(^{2+}\) to the N-terminal sites of CaM would allow it to bind also, resulting in target protein activation. A similar mechanism exists in skeletal muscle troponin C, where the higher affinity C-terminal Ca\(^{2+}\)-Mg\(^{2+}\) sites stabilize the troponin complex at resting levels of Ca\(^{2+}\), and the rapid exchange of Ca\(^{2+}\) with the faster N-terminal sites regulates contraction and relaxation (Potter and Johnson, 1981).

Ca\(^{2+}\) dissociates from CaM after cellular free [Ca\(^{2+}\)] falls in a Ca\(^{2+}\) transient. This results in the disruption of most CaM-target complexes and target protein inactivation. Since both halves of CaM are required for activation, the removal of Ca\(^{2+}\) from either the N- or C-terminal Ca\(^{2+}\) binding site would result in enzyme inactivation. Our data (Table I) indicate that in the presence of MLCK and peptides, Ca\(^{2+}\) dissociates from the N-terminal sites of CaM 5–29 times faster (1.8–2.9/s) than it dissociates from the C-terminal half of the molecule (0.1–0.4/s). This suggests that as the [Ca\(^{2+}\)] falls, the N-terminal half of CaM would dissociate first, resulting in enzyme inactivation at a rate slower than 2–3/s (at 10 °C and low ionic strength). At 20 °C, in the presence of RS-20 and KCl, Ca\(^{2+}\) dissociates from the N-terminal sites of CaM at 4.5/s and from the C-terminal sites of CaM at 0.18/s. If Ca\(^{2+}\) dissociation from the N-terminal of CaM controls the disruption and inactivation of the CaM-MLCK complex, then these events must occur slower than 4.5/s at 20 °C. Consistent with this, we have previously shown that EGTA disrupts the CaM-skeletal muscle MLCK complex at 2/s (Johson et al., 1981) and the CaM-smooth muscle MLCK complex at 3.2/s (Kasturi et al., 1993) at 20 °C. Stull et al. (1986) have also demonstrated that EGTA can inactivate CaM-activated MLCK at a rate of ~1/s. Thus, Ca\(^{2+}\) dissociation from the N-terminal sites of CaM in the CaM-MLCK complex is necessary for control disruption of these complexes and the inactivation of MLCK. Ca\(^{2+}\) dissociation from the C-terminal sites at 0.18/s is too slow to be responsible for the disruption of this complex and inactivation of MLCK. Thus, Ca\(^{2+}\) dissociation from the N-terminal sites of CaM, in the CaM-MLCK complex, appears to control the rate of enzyme inactivation as the [Ca\(^{2+}\)] falls.

Ca\(^{2+}\) dissociates from the N-terminal sites of CaM hundreds of times more slowly when a target protein is bound (1.8–2.9/s). This allows a rapid Ca\(^{2+}\) transient to result in the binding and presumable activation of a CaM target protein for over 300–400 ms. Thus, the fast rate of Ca\(^{2+}\) binding to the N-terminal of CaM allows it to rapidly “sense” the Ca\(^{2+}\) transient and bind target proteins. The dramatic increases in Ca\(^{2+}\) affinity and the reductions in the rate of Ca\(^{2+}\) dissociation observed in the CaM-target protein complex ensures that CaM-activated enzymes can remain active long after a rapid Ca\(^{2+}\) transient has subsided.

Acknowledgments—We gratefully acknowledge Cindy Sutherland for purification of MLCK, Dr. Perry Blackshear (Duke University) for the
gift of MARCKS peptide, and Dr. Zenon Grabarek (Boston Biomedical Research Institute) for helpful comments on the manuscript and for the gift of CaM 41/75 and CaM 85/112.

REFERENCES

Bayley, P., Ahlstrom, P., Martin, S. R., and Forsen, S. (1984) Biochem. Biophys. Res. Commun. 120, 185–191
Blumenthal, D. K., Takio, K., Edelman, A. M., Charlbonneau, H., Titani, K., Walsh, K. A., and Krebs, E. G. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 3187–3191
Cornwell, T. L., and Lincoln, T. M. (1989) J. Biol. Chem. 264, 146–155
Dedman, J. R., Potter, J. D., Jackson, R. L., Johnson, J. D., and Means, A. R. (1977) J. Biol. Chem. 252, 8415–8422
George, S. E., Su, Z., Fan, D., and Means, A. R. (1993) J. Biol. Chem. 268, 2521–2522
Grabarek, Z., Fan, R. Y., and Gergely, J. (1991) Biophys. J. 59, 23 (abstr.)
Ikura, M., Clore, G. M., Gronenborn, A. M., Zhu, G., Klee, C. B., and Bax, A. (1992) Science 256, 632–638
James, P., Vorherr, T., and Carafoli, E. (1995) Trends Biochem. Sci. 20, 38–42
Johnson, J. D., Charlton, S. C., and Potter, J. D. (1979) J. Biol. Chem. 254, 3497–3502
Johnson, J. D., Hofroyde, M. J., Crouch, T. H., Solaro, R. J., and Potter, J. D. (1981) J. Biol. Chem. 256, 12194–12198
Johnson, J. D., Wittenaue, L. A., Thulin, E., Forsen, S., and Vogel, H. J. (1986) Biochemistry 25, 2226–2231
Johnson, J. D., Nakamura, R. J., Vasulka, C., and Smillie, L. B. (1994) J. Biol. Chem. 269, 8919–8923
Kasturi, R., Vasulka, C., and Johnson, J. D. (1993) J. Biol. Chem. 268, 7958–7964
Keller, C. H., Oligow, B. B., LaPorte, D. C., and Storm, D. R. (1982) Biochemistry 21, 156–162
Kemp, B. E., and Pearson, R. B. (1991) Biochim. Biophys. Acta 1094, 67–76
LaPorte, D. C., Wierman, B. M., and Storm, D. R. (1980) Biochemistry 19, 3814–3819
Lukas, T. J., Burgess, W. H., Prendergast, F. G., Lau, W., and Watterson, D. M. (1986) Biochemistry 25, 1458–1464
Martin, S. R., Anderson, Telem, A., Bayley, P. M., Drakenberg, T., and Forsen, S. (1985) Eur. J. Biochem. 151, 543–550
Martin, S. R., Maune, J. F., Beckingham, K., and Bayley, P. (1992) Eur. J. Biochem. 205, 1107–1114
Meador, W. E., Means, A. R., and Quiocho, F. A. (1992) Science 257, 1251–1256
Meador, W. E., Means, A. R., and Quiocho, F. A. (1993) Science 262, 1718–1721
Mills, J. S., Bailey, B. L., and Johnson, J. D. (1985) Biochemistry 24, 4987–4993
Minowa, O., and Yagi, K. (1984) J. Biochem. (Tokyo) 56, 1175–1182
Ngai, P. K., Carruthers, C. A., and Walsh, M. P. (1984) Biochem. J. 218, 863–870
Olwin, B. B., Edelman, A. M., Krebs, E. G., and Storm, D. R. (1984) J. Biol. Chem. 259, 10949–10955
O'Neill, K. T., and DeGrado, W. F. (1990) Trends Biochem. Sci. 15, 59–64
Payne, M. E., Fong, Y. L., Ono, T., Cobran, R. J., Kemp, B. E., Soderling, T. R., and Means, A. R. (1988) J. Biol. Chem. 263, 7190–7195
Persechini, A., and Krebs, R. H. (1988) J. Biol. Chem. 263, 12175–12178
Potter, J. D. and Johnson, J. D. (1981) in Calcium and Cell Function (Cheung, W. Y., ed) Vol. II, pp. 145–173, Academic Press, New York
Robertson, S., and Potter, J. D. (1984) Methods Pharmacol. 5, 63–75
Smith, P. D., Liesegang, G. W., Berger, R. L., Czerlinski, G., and Podolsky, R. J. (1984) Anal. Biochem. 143, 188–195
Stull, J. T., Nunnally, M. H., and Midhoff, C. H. (1986) in The Enzymes (Krebs, E. G., and Boyer, P. D., eds) pp. 113–166, Academic Press, Orlando, FL
Suko, J. P., Pidlich, J., and Bertel, O. (1985) Eur. J. Biochem. 153, 451–457
Tanaka, T., and Hidaka, H. (1980) J. Biol. Chem. 255, 11078–11080
Verghese, G. M., Johnson, J. D., Vasulka, C., Haupt, D. M., Stumpo, D. J., and Blackshear, P. J. (1994) J. Biol. Chem. 269, 9361–9367
Yagi, K., and Yazawa, M. (1989) in Calcium Protein Signaling (Hidaka, H., Carafoli, E., Means, A. R., and Tanaka, T., eds) pp. 147–154, Plenum Publishing Co, New York
Weinstein, H., and Meffer, E. L. (1994) Annu. Rev. Physiol. 56, 213–236