Ca\textsuperscript{2+}, Caldesmon, and Myosin Light Chain Kinase Exchange with Calmodulin*

Rama Kasturi, Charles Vasulka, and J. David Johnson

From the Ohio State University Medical Center, Department of Medical Biochemistry, Columbus, Ohio 43210

(Received for publication, October 19, 1992)

Wheat calmodulin (CaM) was labeled at Cys-27 with the sulphydryl-specific fluorescent probe 2-(4'-mal-elimidoanilino)naphthalene-6-sulfonic acid (MIANS), to form MIANS-CaM. In the presence of Ca\textsuperscript{2+}, MIANS-CaM undergoes a large fluorescence increase when it binds myosin light chain kinase (MLCK) and caldesmon (CaD), but little fluorescence change when it binds CaM antagonists or Ca\textsuperscript{2+}. MLCK associates with MIANS-CaM at a rate of 2.8 \times 10^7 \text{ M}^{-1} \text{s}^{-1} and dissociates at 0.031 s\textsuperscript{-1} (K_d = 1.1 nM). Protein kinase A phosphorylation of MLCK (P-MLCK) produces a 3.5-fold decrease in its association rate with CaM and a 6-fold increase in its dissociation rate (K_d = 23 nM). CaD associates with MIANS-CaM with a rate of 5.3 \times 10^6 \text{ M}^{-1} \text{s}^{-1} and dissociates at 57 s\textsuperscript{-1} (K_d = 108 nM).

EGTA disrupts the CaM-MLCK, CaM-P-MLCK, and the CaM-CaD complexes at rates of 3.5 s\textsuperscript{-1}, 6.5 s\textsuperscript{-1}, and 13.5 s\textsuperscript{-1}, respectively. MLCK, therefore, dissociates from CaM more quickly by Ca\textsuperscript{2+} removal while the lower affinity CaD is dissociated more quickly by competition from higher affinity CaM target proteins than by Ca\textsuperscript{2+} removal. MLCK binding to CaD slowed Ca\textsuperscript{2+} dissociation from CaM's C-terminal Ca\textsuperscript{2+}-binding sites from 30 s\textsuperscript{-1} to 6 s\textsuperscript{-1} while CaD had little effect on Ca\textsuperscript{2+} dissociation from these sites. During a Ca\textsuperscript{2+} transient, CaM could exchange with MLCK and CaD rapidly enough for these proteins to be directly involved in the contraction/relaxation cycle of smooth muscle.

Calmodulin (CaM) is a ubiquitous Ca\textsuperscript{2+}-binding protein which senses a rise in cytosolic Ca\textsuperscript{2+} and activates numerous cellular proteins and enzymes to modulate cyclic nucleotide levels, Ca\textsuperscript{2+} homeostasis, and protein phosphorylation-dephosphorylation (see Chueh (1980), Klee and Newton (1985), Johnson and Mills (1986), and Means et al. (1991)). As cytosolic Ca\textsuperscript{2+} falls, CaM dissociates from most of its target proteins and they inactivate. In this manner, CaM regulates many cellular processes including smooth muscle contraction, neurosecretion, glandular secretion, cell division, cell proliferation, and motility.

CaM binds Ca\textsuperscript{2+} rapidly (t_\text{on} < 2 ms) (Johnson et al., 1981; Tsutara and Sano, 1990), but little is known concerning the rate at which it binds its various target proteins as cytosolic Ca\textsuperscript{2+} rises or the rate at which it dissociates from these proteins as the Ca\textsuperscript{2+} transient subsides. CaM-target protein complexes dissociate through several mechanisms. 1) A reduction in cytosolic Ca\textsuperscript{2+}. 2) Competition for Ca\textsuperscript{2+}. CaM by higher affinity target proteins. 3) Phosphorylation of target proteins (i.e. MARCKS, neomodulin, and MLCK) which reduce their affinity for CaM.

CaM plays a major role in the generation and maintenance of force in smooth muscle by binding its target contractile proteins, myosin light chain kinase (MLCK) and caldesmon (CaD) (see Hartshorne (1987), for review). CaM activation of MLCK and the subsequent phosphorylation of the 20-kDa myosin light chain is essential for smooth muscle contraction (Perry and Grand, 1979; Adelstein and Klee, 1981; Sparrow et al., 1981; Kamm and Stull, 1985). CaM binding to CaD on actin is thought to reverse CaD's inhibition of actomyosin ATPase activity (Soube et al., 1988; Pritchard and Marston, 1989).

The rise in smooth muscle intracellular free [Ca\textsuperscript{2+}] is separated from tension generation by a 200–400-ms delay generally ascribed to the regulatory events that precede actomyosin cross-bridge formation (Somlyo and Somlyo, 1989). During this delay, CaM must associate with MLCK to facilitate myosin light chain phosphorylation and may associate with CaD to relieve its inhibition of actomyosin ATPase. Presently, little is known about the rate at which CaM interacts with MLCK and CaD or the rate at which CaM dissociates from these proteins as the Ca\textsuperscript{2+} signal is attenuated.

High affinity target proteins including MLCK (Olwin et al., 1984), troponin I (Keller et al., 1982), and phosphodiesterase (Chu et al., 1982) and high affinity peptides/drugs, including melittin (Maulet and Cox, 1983), mastoparan (Yasawa et al., 1987), the CaM-binding region of MLCK (Yagi et al., 1988), and calmidazolium (Mills et al. 1988) produce dramatic increases (7–40-fold) in Ca\textsuperscript{2+} binding to CaM. This suggests that CaM in the presence of high affinity target proteins should exhibit increased sensitivity to Ca\textsuperscript{2+} and that these high affinity CaM-target protein complexes should dissociate more slowly as the Ca\textsuperscript{2+} transient subsides due to a slower rate of Ca\textsuperscript{2+} dissociation.

We have previously labeled spinach CaM with a fluorescent sulphydryl selective probe MIANS to produce a biologically active fluorescent CaM which undergoes a fluorescence increase exclusively upon target protein binding (Mills et al., 1988). In the present study, we use an analogous MIANS-labeled wheat CaM (MIANS-CaM) and fluorescence stopped-flow techniques to determine the association and dissociation rate constants of MIANS-CaM with MLCK, PKA-phos-

* This work was supported by National Institutes of Health Grant RO1 DK33727 (to J. D. J.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom all correspondence should be addressed: Medical Biochemistry, 333 Hamilton Hall, 1645 Neil Ave., Columbus, OH 43210. Tel: 614-292-0104; Fax: 614-292-4118.

The abbreviations used are: CaM, calmodulin; CaD, caldesmon; MIANS-CaM, wheat CaM covalently labeled with MIANS; MLCK, myosin light chain kinase; PKA, cyclic AMP-dependent protein kinase; P-MLCK, MLCK phosphorylated by PKA; Quin 2, 2-(2-bis(carboxyethyl)aminomethyl-6-methoxy-8-bis(carboxyethyl)-aminoninoline; MOPS, 3-(N-morpholino)propanesulfonic acid.
phorylated MLCK (P-MLCK), and CaD. We relate the rate of EGTA-induced CaM-target protein complex disruption to the rates of Ca\(^{2+}\) dissociation from the C-terminal high affinity Ca\(^{2+}\)-binding sites of CaM, CaM-MLCK, and CaM-CaD. These studies should further our understanding of how high affinity target proteins increase Ca\(^{2+}\) affinity and slow Ca\(^{2+}\) dissociation from CaM and allow us to better understand the temporal nature of CaM’s interaction with its smooth muscle target proteins during a Ca\(^{2+}\) transient.

**EXPERIMENTAL PROCEDURES**

**Materials**—[\(\gamma\)-\(^{32}\)P]ATP was purchased from Amersham; 2-(4'-maleimidobenzoylethyl)-naphthalene-6-sulfonic acid (MIANS) from Molecular Probes (Eugene, OR); phenyl-Sepharose CL-4B, Quin 2, and EGTA from Sigma; and hydroxyapatite from Bio-Rad. All other chemicals used were of analytical grade unless specified otherwise.

**Methods**—Plant CaM was purified from wheat germ according to the method of Strasburg et al. (1988) with the modifications of Mills et al. (1988). Difficulties with achieving stoichiometric labeling of wheat CaM with sulphydryl selective probes have been reported (Zot et al., 1990). Purified wheat CaM sometimes runs as a monomer (17 kDa) and a dimer (34-35 kDa) on 12% polyacrylamide gels, suggesting the possibility of an intermolecular disulfide bond. This could explain the decreased sulphydryl reactivity and decreased stoichiometry of labeling of wheat CaM with sulphydryl selective probes observed by Zot et al. (1990). To overcome this problem, purified wheat CaM (2 mg/ml) was incubated with 6 mM guanidine HCl and dithiothreitol (1 mM) for 24 h at 4 °C, then exhaustively dialyzed against 10 mM MOPS buffer, pH 7.0, 90 mM KCl, 2 mM EGTA to remove the guanidine HCl and dithiothreitol. This wheat CaM was incubated with a 10-fold molar excess of MIANS for 24-30 h at 4 °C. The sample was exhaustively dialyzed against 10 mM MOPS buffer, pH 7.0, 90 mM KCl, to remove free label. The incorporation under these conditions was 0.8-1.1 mol of MIANS/mol of CaM (assuming \(E_{200} = 20,000\) M\(^{-1}\) cm\(^{-1}\) for MIANS). This fluorescently labeled wheat CaM (CaM-MIANS) ran as a single fluorescent band on 12% polyacrylamide gels and as a single peak on C\(_18\) reverse phase chromatography, suggesting that each molecule of CaM was labeled with one molecule of MIANS.

CaM was purified from bovine brain according to the method of Gopalakrishna et al. (1982) followed by hydroxyapatite column chromatography. MLCK and CaD were purified from chicken gizzard according to the method of Adelstein and Klee (1981) and Lynch and Bretscher (1986), respectively. Smooth muscle myosin light chain was a generous gift of Dr. Primal Delanerolle (University of Illinois).

MLCK activity was assayed as described by Adelstein and Klee (1981). Phosphorylation of MLCK (250 nM) was carried out by the addition of 10 μM PKA (2.5 mg/ml) to 1 ml of 10 mM MOPS buffer, pH 7.0, 90 mM MgCl\(_2\), 100 μM ATP, 200 μM EGTA at 22 °C for 20 min. The stoichiometry of MLCK phosphorylation (1.5 mol of P/mol of kinase) was determined by phosphorylating MLCK as above except in the presence of [\(\gamma\)-\(^{32}\)P]ATP (0.01 μCi/μl). MIANS-CaM was excited at 320 nm and its fluorescence monitored at 440 nm. All static fluorescence measurements were carried out in a Perkin-Elmer LS5 Spectrofluorometer at 22 °C. Kinetic measurements were carried out in a stopped-flow instrument (Applied Photophysics Ltd., United Kingdom, model SF.17 MV) with a dead time of 1.6 ms at 22 °C. The sample was excited at 320 nm (for MIANS-CaM) and 335 nm (for Quin 2) using a 150-W xenon arc source. Fluorescence emission was detected through narrow band-pass (10 nm) interference filters (440 nm for MIANS-CaM, 510 nm for Quin 2).

The curve fitting program (software by A. J. King, Applied Photophysics Ltd.) uses the nonlinear Levenberg-Marquardt algorithm (30). Free [Ca\(^{2+}\)] was calculated using the following logarithmic association constants for metals and \(H^+\) to EGTA: \(H^+\) to EGTA\(^{-}\), 9.46; \(H^+\) to EGTA\(^{-}\), 8.85; \(H^+\) to EGTA\(^{2-}\), 2.68; \(H^+\) to EGTA\(^{3-}\), 2.96; \(Ca^{2+}\) to EGTA\(^{-}\), 11.0; \(Ca^{2+}\) to EGTA\(^{2-}\), 5.32; \(Mg^{2+}\) to EGTA\(^{-}\), 5.21; and \(Mg^{2+}\) to EGTA\(^{2-}\), 3.37.

Protein concentrations were determined by the Bradford (1976) method using \(\gamma\)-globulin as the standard or by UV absorption using the following extinction coefficients: vertebrate CaM \(E_{280nm} = 1.9\) (Klee, 1977); CaD \(E_{280nm} = 3.3\) (Grazetta et al., 1988), and MLCK \(E_{280nm} = 11.4\) (Adelstein and Klee, 1981).

**RESULTS**

**Brain CaM, Wheat CaM, and MIANS-CaM Activation of MLCK**—Brain CaM, wheat CaM, and MIANS-CaM activate MLCK (100 nM) with a similar dose dependence and maximal MLCK activity is observed near stoichiometric concentrations (100 nM) of each CaM (Fig. 1A). MIANS-CaM stimulates MLCK’s activity to ~70% of that observed with brain CaM and unlabeled wheat CaM. Brain CaM, wheat CaM, and MIANS-CaM all activate MLCK half-maximally at \(pCa = 6.55\) in a cooperative manner (Hill coefficient = 4.0) (Fig. 1B). Thus, all three CaMs produce a similar Ca\(^{2+}\)-dependent activation of MLCK although MIANS-CaM exhibits a slightly lower stimulation of MLCK activity.

**Interaction of MLCK, P-MLCK, and CaD with MIANS-CaM**—The increase in the fluorescence intensity of MIANS-CaM (50 nM) is shown as a function of increasing concentrations of MLCK, P-MLCK, and CaD in Fig. 2. MLCK binds to MIANS-CaM stoichiometrically with an \(E_d\) of 14 nM (\(F/F_0 = 3.2\)). Titrations of lower concentrations of MIANS-CaM (10 nM) with MLCK were also stoichiometric suggesting a high affinity (\(K_c \leq 1\) nM) of MLCK for MIANS-CaM. P-MLCK shows a reduced affinity for MIANS-CaM with an
**Ca²⁺ and Protein Exchange with Calmodulin**

The % increase of MIANS-CaM's (50 nM) fluorescence is shown as a function of total added MLCK (A), P-MLCK (B), or CaD (C). Titrations were conducted in 1 ml of 10 mM MOPS, pH 7.0, 90 mM KCl, 100 μM CaCl₂. MLCK was phosphorylated (1.5 mol/mol) as described under "Experimental Procedures" except that 10 μl of PKA (2.5 mg/ml) were added to 75 μl of MLCK (9.6 μM) and incubated at 22 °C for 1 h. 100% fluorescence increase corresponded to a 3.2- (±MLCK), 2.6- (±P-MLCK), and 1.8-fold (+CaD) enhancement, respectively. Each point is the mean of 3 titrations.

**Fig. 2.** The % increase of MIANS-CaM's (50 nM) fluorescence is shown as a function of total added MLCK (A), P-MLCK (B), or CaD (C). Titrations were conducted in 1 ml of 10 mM MOPS, pH 7.0, 90 mM KCl, 100 μM CaCl₂. MLCK was phosphorylated (1.5 mol/mol) as described under "Experimental Procedures" except that 10 μl of PKA (2.5 mg/ml) were added to 75 μl of MLCK (9.6 μM) and incubated at 22 °C for 1 h. 100% fluorescence increase corresponded to a 3.2- (±MLCK), 2.6- (±P-MLCK), and 1.8-fold (+CaD) enhancement, respectively. Each point is the mean of 3 titrations.

**Fig. 3.** Kinetic traces of the rates of MLCK (62.5 nM) association with 0.2, 0.4, and 0.7 μM MIANS-CaM. MLCK and MIANS-CaM in 10 mM MOPS buffer, pH 7.0, 90 mM KCl, 0.2 mM EGTA, 0.4 mM CaCl₂, 10 mM MgCl₂, 100 mM ATP at 22 °C, were rapidly mixed with a 1:1 ratio in the stopped-flow instrument and the fluorescence change recorded as a function of time as described under "Experimental Procedures." The inset shows a plot of the observed rate (kobs) of MIANS-CaM-MLCK complex formation versus [MIANS-CaM] (62.5 nM) associated with MLCK. The kobs for each concentration of MIANS-CaM was determined from the rate of fluorescence increase that occurs when MLCK (62.5 nM) complexes with MIANS-CaM (0.19–0.69 μM) in the above buffer. Control experiments (MIANS-CaM + Ca²⁺ versus Ca⁺⁺) indicated that we observed kinetically >90% of the fluorescence increase that occurs at the highest [MIANS-CaM] used. The data of 6-8 traces at each [MIANS-CaM] were well fit as single exponential processes (variance < 1 x 10⁻⁵).

**ED₅₀ of 27 nM (F/F₀ = 2.6).** CaD binds to MIANS-CaM with much lower affinity than MLCK and P-MLCK with an ED₅₀ of 60 nM (F/F₀ = 1.8). None of these target proteins increase MIANS-CaM's fluorescence in the absence of Ca²⁺ (+2 mM EGTA) and Ca⁺⁺ binding to MIANS-CaM alone produces only a small decrease (F/F₀ = 0.8) in its fluorescence intensity. These results are consistent with Ca⁺⁺-dependent target protein binding to MIANS-CaM, with the order of affinity being MLCK > P-MLCK > CaD.

**Kinetics of MLCK Binding to MIANS-CaM.** The second order rate constant for the association of MLCK with increasing [MIANS-CaM] was determined by approximating pseudo-first order reaction conditions. Fig. 3 shows the rates of MLCK (62.5 nM) association with MIANS-CaM at 0.2 (3.8 ± 0.03 s⁻¹), 0.4 (10.1 ± 0.06 s⁻¹), and 0.7 (17.7 ± 0.139 s⁻¹) μM MIANS-CaM. A plot of kobs versus [MIANS-CaM] shows that the rate of complex formation increases with increasing [MIANS-CaM], yielding a second order rate constant (k₅₀) of 2.8 x 10⁷ ± 0.18 x 10⁷ M⁻¹s⁻¹ (Fig. 3, inset). The rates of complex formation were identical when MLCK + Ca²⁺ was rapidly mixed with MIANS-CaM + Ca²⁺ as when MLCK + MIANS-CaM + EGTA was rapidly mixed with excess Ca²⁺. Thus, the association of MLCK with MIANS-CaM is not rate-limited by Ca²⁺ binding to CaM (data not shown).

**Kinetics of MLCK Dissociation from MIANS-CaM.** The dissociation rate constant (kₜ₅₀) of the MIANS-CaM-MLCK complex was determined by displacing MLCK from the complex with a 20-fold molar excess of brain CaM over MIANS-CaM (Fig. 4). Complex dissociation occurs very slowly, with a rate of 0.031 ± 0.010 s⁻¹. The kₜ₅₀ of MLCK for MIANS-CaM determined from kₜ₅₀ = k₅₀/kₐ₅₀ is 1.1 nM. In the presence of Ca²⁺, the high affinity CaM-antagonist peptide melittin binds to MIANS-CaM without increasing its fluorescence and prevents MLCK and CaD from binding and increasing MIANS-CaM's fluorescence. Melittin (1–3 μM) and a peptide (RS-20) corresponding to the CaM-binding region of MLCK disrupted the MIANS-CaM-MLCK complex with a rate of 0.031 s⁻¹ (data not shown).

**Kinetics of P-MLCK Exchange with MIANS-CaM.** To determine the effect of PKA phosphorylation of MLCK on its rate of association with and dissociation from MIANS-CaM, MLCK was phosphorylated to 1.5 mol of P/mol of MLCK. Fig. 4 compares the rates of MLCK and P-MLCK dissociation from MIANS-CaM with a 20-fold excess of brain CaM over MIANS-CaM. P-MLCK dissociates from MIANS-CaM six times more rapidly than MLCK (0.18 s⁻¹ compared to 0.031 s⁻¹), consistent with a reduced affinity of P-MLCK for CaM. The second order rate constant for the association of P-MLCK with MIANS-CaM (using MIANS-CaM in 9-fold molar excess over P-MLCK (125 nM)) was 8.0 x 10⁶ ± 0.2 x 10⁶ M⁻¹s⁻¹ (data not shown). Thus, PKA phosphorylation of MLCK results in an ~3.5-fold decrease in its rate of association with MIANS-CaM and a 6-fold increase in its rate of dissociation from MIANS-CaM. This results in a significant decrease in the fluorescence intensity of MIANS-CaM.

**Fig. 4.** Displacement of MLCK and P-MLCK from MIANS-CaM by excess unlabeled brain CaM. MIANS-CaM (200 nM) and MLCK (250 nM) or P-MLCK (250 nM) were rapidly mixed with CaM (4 μM) and the corresponding fluorescence decrease was monitored as a function of time. The buffer used was as described in the legend to Fig. 3. MLCK was phosphorylated (1.5 mol/mol) by PKA as described under "Experimental Procedures." Control experiments with MIANS-CaM + MLCK or MIANS-CaM + P-MLCK was rapidly mixed with buffer without excess unlabeled brain CaM exhibited only a small <3% linear decrease in fluorescence (over 200 s) due to a slight photobleaching of MIANS-CaM. The data shown for the dissociation of MLCK from MIANS-CaM is the mean of 4 traces with control traces (no unlabeled CaM) subtracted. This data was fit with a single exponential with a rate of 0.031 s⁻¹ with normalized equation y = 1.8 x 10⁻⁶. The dissociation of P-MLCK from MIANS-CaM (mean of 4 traces with 4 control traces subtracted) was fit with a single exponential with a rate of 0.185 s⁻¹ (variance = 1.9 x 10⁻⁶).
an ~20-fold decrease in MLCK's affinity for MIANS-CaM ($K_d = 22.5 \text{ nM}$) upon phosphorylation.

**Kinetics of CaD Binding to MIANS-CaM**—The second order rate constant for the association of CaD with increasing [MIANS-CaM] was determined by approximating pseudo-first order reaction conditions. Fig. 5 shows that CaD (200 nM) binds to 0.2 \( \mu \text{M} \) MIANS-CaM at 157 \( \pm \) 5 s\(^{-1}\) and to 1 \( \mu \text{M} \) MIANS-CaM at 582 \( \pm \) 41 s\(^{-1}\). A plot of $k_{obs}$ versus [MIANS-CaM] shows that the rate of complex formation ($k_{obs}$) increases with increasing [MIANS-CaM], yielding a second order rate constant of 5.3 \( \text{M}^{-1} \text{s}^{-1}\) (Fig. 5, inset). The rates of complex formation were identical when CaD + Ca\(^{2+}\) was rapidly mixed with MIANS-CaM + Ca\(^{2+}\) as when CaD + MIANS-CaM + EGTA was rapidly mixed with excess Ca\(^{2+}\). Thus, the rate of association of CaD with MIANS-CaM is not rate-limited by the binding of Ca\(^{2+}\) to CaM (data not shown).

**Kinetics of CaD Dissociation from MIANS-CaM**—The dissociation rate constant ($k_{off}$) of the MIANS-CaM-CaD complex was determined by displacing CaD from the complex with a 20-fold molar excess of brain CaM over MIANS-CaM (Fig. 5). Complex dissociation occurred rapidly with a rate of 5.7 \( \pm \) 4 s\(^{-1}\). The $K_0$ of CaD for MIANS-CaM determined from these on and off rates is 108 nM. Thus, the lower affinity protein, CaD, dissociates much more rapidly (57 s\(^{-1}\)) from MIANS-CaM than the higher affinity MLCK (0.031 s\(^{-1}\)) or P-MLCK (0.18 s\(^{-1}\)). Melittin (0.25-1 \( \mu \text{M} \)) disruption of the MIANS-CaM-CaD complex occurred with a similar rate of 42 \( \pm \) 2 s\(^{-1}\) (data not shown).

**EGTA Disruption of MIANS-CaM-Target Protein Complexes**—MLCK, P-MLCK, and CaD have very different affinities for CaM (1.1, 22.5, and 108 nM, respectively). We wished to determine if these differences in affinity for CaM would affect the rate at which EGTA disrupts each CaM-target protein complex. EGTA disrupts the MIANS-CaM-MLCK complex at 3.5 \( \pm \) 0.03 s\(^{-1}\), the MIANS-CaM-P-MLCK complex at 6.5 \( \pm \) 0.05 s\(^{-1}\), and the MIANS-CaM-CaD complex at 13.5 \( \pm \) 0.15 s\(^{-1}\) (Fig. 7). Thus, for these target proteins, EGTA disruption of higher affinity MIANS-CaM-target protein complexes occurs more slowly than EGTA disruption of lower affinity MIANS-CaM-target protein complexes.

**Calcium Dissociation from CaM and CaM-Target Protein Complexes**—When Quin 2 (a fluorescent Ca\(^{2+}\) chelator) is rapidly mixed with a Ca\(^{2+}\)-calcium-binding protein complex, its fluorescence increases at a rate which is equal to the rate of Ca\(^{2+}\) dissociation from that Ca\(^{2+}\) binding protein. Free (unbound) Ca\(^{2+}\) binds to Quin 2 very rapidly ($k_{off} = 7.5 \times 10^8 \text{ M}^{-1} \text{s}^{-1}$) and is not rate-limiting (Bayley et al., 1984). To determine the effect of target protein binding on CaM's affinity for Ca\(^{2+}\), we monitored Ca\(^{2+}\) dissociation from wheat CaM in the absence and presence of target proteins, using Quin 2 fluorescence. Ca\(^{2+}\) (10 \( \mu \text{M} \)) dissociates from wheat CaM (0.5 \( \mu \text{M} \)) as a single exponential process with a rate of 30 \( \pm \) 0.7 s\(^{-1}\) (Fig. 8). MLCK produced a dramatic decrease in the rate of Ca\(^{2+}\) dissociation from CaM (6 \( \pm \) 0.1 s\(^{-1}\)), while CaD had little effect on the rate of Ca\(^{2+}\) dissociation from CaM (28 \( \pm \) 0.4 s\(^{-1}\)) (Fig. 8). Neither CaD nor MLCK produced any observable change in Quin 2 fluorescence in the absence of CaM. The amplitude of the Quin 2-Ca\(^{2+}\) signal observed in the presence of MLCK or CaD was twice that observed with wheat CaM alone. However, only the higher affinity target
protein MLCK produced a dramatic 5-fold decrease (30 s⁻¹ to 0 s⁻¹) in the observed rate of Ca²⁺ dissociation from CaM.

**DISCUSSION**

Brain and wheat CaMs are 90% homologous (Watterson et al., 1980; Toda et al., 1985) and exhibit nearly identical activation of cyclic nucleotide phosphodiesterase (Mann and Vanaman, 1989) and erythrocyte Ca²⁺/Mg²⁺ATPase (Strasburg et al. 1988; Mann and Vanaman, 1989). While all three CaMs activate MLCK with a second order rate constant (kₗaff) of 2.8 × 10⁸ M⁻¹ s⁻¹ and dissociates with a rate of 0.031 s⁻¹. Bowman et al. (1992) have recently reported that MIANS-labeled spinach CaM associates with skeletal muscle MLCK with a rate of 4.6 × 10⁷ M⁻¹ s⁻¹ resulting in a nearly parallel activation of MLCK. This value is in good agreement with the rate we determined for CaM association with smooth muscle MLCK. The rate of CaM-binding skeletal MLCK was close to the limiting value for a diffusion controlled association (5 × 10⁷ M⁻¹ s⁻¹) of these two proteins (Bowman et al., 1992). Bowman et al. (1992) also reported a slower (2 s⁻¹) isomerization process that occurred after binding similar to the rate (6 s⁻¹) that we had previously observed with CaM binding skeletal muscle MLCK (Johnson et al., 1981). We do not see this slower process with MIANS-CaM binding smooth muscle MLCK.

While the rate of dissociation of the CaM-MLCK complex has not been previously measured, Chau et al. (1982) have reported CaM dissociation from the higher affinity (Kᵩ = 0.1 nM) CaM-PDE complex at a rate of 0.008 s⁻¹. Since MLCK's affinity for CaM is approximately 10-fold lower than PDE's affinity, its dissociation rate might be expected to be 10-fold faster, as our data indicates. Melittin, RS-20, and excess unlabeled CaM disrupted the MIANS-CaM. MLCK complex (see Table I). MIANS-CaM with a second order rate constant (kₗoff) of 2.8 × 10⁸ M⁻¹ s⁻¹ and dissociates with a rate of 0.031 s⁻¹.

Use of MIANS-CaM allowed us to fully characterize the bimolecular association and dissociation rates of CaM-MLCK, CaM-P-MLCK, and CaM-CaD complexes (see Table I). MIANS-CaM with a second order rate constant (kₗaff) of 2.8 × 10⁸ M⁻¹ s⁻¹ and dissociates with a rate of 0.031 s⁻¹. Bowman et al. (1992) have recently reported that MIANS-labeled spinach CaM associates with skeletal muscle MLCK with a rate of 4.6 × 10⁷ M⁻¹ s⁻¹ resulting in a nearly parallel activation of MLCK. This value is in good agreement with the rate we determined for CaM association with smooth muscle MLCK. The rate of CaM-binding skeletal MLCK was close to the limiting value for a diffusion controlled association (5 × 10⁷ M⁻¹ s⁻¹) of these two proteins (Bowman et al., 1992). Bowman et al. (1992) also reported a slower (2 s⁻¹) isomerization process that occurred after binding similar to the rate (6 s⁻¹) that we had previously observed with CaM binding skeletal muscle MLCK (Johnson et al., 1981). We do not see this slower process with MIANS-CaM binding smooth muscle MLCK.

While the rate of dissociation of the CaM-MLCK complex has not been previously measured, Chau et al. (1982) have reported CaM dissociation from the higher affinity (Kᵩ = 0.1 nM) CaM-PDE complex at a rate of 0.008 s⁻¹. Since MLCK's affinity for CaM is approximately 10-fold lower than PDE's affinity, its dissociation rate might be expected to be 10-fold faster, as our data indicates. Melittin, RS-20, and excess unlabeled CaM disrupted the MIANS-CaM-MLCK complex with essentially identical rates. The dissociation rate constant (kₗoff) of 0.031 s⁻¹ for the MIANS-CaM-MLCK complex is

![Graph](image)

**FIG. 8.** Kinetic traces of the increase in Quin 2 fluorescence produced by its chelation of Ca²⁺ from CaM and CaM-target protein complexes. Wheat CaM (0.5 μM), wheat CaM + MLCK (0.6 μM), or wheat CaM + SM-MLCK (1.5 μM) in 10 mM MOPS buffer, pH 7.0, 90 mM KCl, 10 μM CaCl₂, 0.1 mM dithiothreitol was rapidly mixed with Quin 2 (80 μM) in the same buffer (with no added CaCl₂). Control traces (Ca²⁺ versus Quin 2) were essentially flat and were subtracted from each data set (n = 6–8 traces). Each curve was well fit to a single exponential (variance < 1 × 10⁻³). Control experiments where each target protein + Ca²⁺ (in the absence of CaM) was rapidly mixed with Quin yielded no time-dependent fluorescence change.
consistently with a $K_d = (k_{d off}/k_{d on})$ of MLCK for CaM of 1.1 nM. This $K_d$ value of MLCK for MINS-CaM is in reasonable agreement with previously reported $K_d$ values of 0.5–3 nM for the CaM-smooth muscle MLCK interaction (Conti and Adelstein, 1981; Kamm and Stull, 1985; Ikebe and Reardon, 1990).

PKA phosphorylation of MLCK produced a ~3.5-fold decrease in its rate of association and a ~6-fold increase in its rate of dissociation from MINS-CaM. Thus phosphorylation of MLCK reduces its affinity for CaM from $K_d = 1.1$ to 22.5 nM. Our kinetic results are consistent with Conti and Adelstein’s (1981) and Kamm and Stull’s (1985) reports of a shift in the $K_d$ of MLCK for CaM from 1–3 to 10–50 nM upon PKA phosphorylation. Our results show that phosphorylation of MLCK produces dramatic increases in the rate at which it dissociates from CaM and presumably inactivates. While PKA phosphorylation of MLCK could decrease the amount of CaM-MLCK complex and thereby decrease smooth muscle contraction (Conti and Adelstein, 1981), the physiological relevance of this process has been questioned (see Kamm and Stull (1985) and Hatashorne (1987)).

Recently, protein kinase C phosphorylation of CaM-binding proteins including, neuromodulin and MARCKS protein, has been shown to dramatically reduce their affinity for CaM (see Liu and Storm (1990) and McIlroy et al. (1991)). We have shown that phosphorylation of MARCKS protein results in its rapid dissociation from CaM allowing CaM to bind to and activate other target proteins (McIlroy et al., 1991). Thus, phosphorylation of CaM target proteins which results in a decreased affinity and a more rapid dissociation of CaM, may be a common mechanism for altering CaM-target protein interactions (see also, Malencik and Anderson (1982)).

CaD associates with MINS-CaM with a second order rate constant ($k_{d on}$) of $5.3 \times 10^5$ M$^{-1}$ s$^{-1}$ and dissociates with a rate of 57 s$^{-1}$. Both melittin and excess unlabeled CaM disrupt this complex with very similar rates. The $K_d$ for CaD for CaM determined from these studies (108 nM) is in reasonable agreement with the $K_d$ of 75 nM reported by Malencik et al. (1989).

EGTA disrupts the MINS-CaM target protein complexes producing a complete reversal of the fluorescence increases that occur upon their binding MINS-CaM. EGTA disruption of the CaM-MLCK, CaM-P-MLCK, and CaM-CaD complexes occur with rates of 3.5 s$^{-1}$, 6.5 s$^{-1}$, and 13.5 s$^{-1}$, respectively. Our rate for EGTA disruption of the MINS-CaM-MLCK complex (3.5 s$^{-1}$) agrees well with the rate obtained by Johnson et al. (1981) for EGTA disruption of the CaM-skeletal muscle MLCK complex (2 s$^{-1}$) and with the rate of skeletal muscle MLCK inactivation (1 s$^{-1}$) upon Ca$^{2+}$ chelation (Stull et al., 1986). For these three target proteins, the higher affinity proteins dissociate from CaM more slowly than the lower affinity proteins as Ca$^{2+}$ is removed. This suggests that higher affinity target proteins may increase Ca$^{2+}$ affinity and slow Ca$^{2+}$ dissociation from CaM more than lower affinity proteins.

The effect of target protein binding on Ca$^{2+}$ dissociation from CaM was determined using Quin 2 fluorescence. In the absence of target protein and at 10–40 μM added Ca$^{2+}$, calcium dissociates from wheat CaM as a single exponential process with a rate of 30 s$^{-1}$. Martin et al. (1985) and Bayley et al. (1984) have used Quin 2 and anilinonaphthalenesulfonate fluorescence to measure fast (240–600 s$^{-1}$) and slow (10–24 s$^{-1}$) Ca$^{2+}$ dissociation rates from the lower affinity (N-terminal) and the higher affinity (C-terminal) sites of brain CaM, respectively. We did not observe the fast (240–600 s$^{-1}$) Ca$^{2+}$ dissociation from the low affinity N-terminal Ca$^{2+}$-binding sites because Ca$^{2+}$ dissociation from these sites cannot be observed at physiological ionic strength and temperatures (Martin et al., 1992).

When CaM has a single Tyr-138 residue in its C-terminal lobe (Toda et al., 1985), EGTA induced a ~2-fold decrease in wheat CaM's tyrosine fluorescence with a rate of $34 \pm 3$ s$^{-1}$ (data not shown). This agrees with our rate for Ca$^{2+}$ dissociation from wheat CaM using Quin 2 fluorescence (30 s$^{-1}$), and suggests that Quin 2 is reporting Ca$^{2+}$ dissociation from the high affinity C-terminal Ca$^{2+}$-binding sites of CaM.

The higher affinity MLCK produced a 5-fold decrease in the rate of Ca$^{2+}$ dissociation from CaM (30 s$^{-1}$ to 6 s$^{-1}$), while the lower affinity CaD produced only a slight decrease in the rate of Ca$^{2+}$ dissociation from CaM (30 s$^{-1}$ to 28 s$^{-1}$). The high affinity CaM-binding peptide melittin produced a 5-fold decrease in the rate of Ca$^{2+}$ dissociation from CaM (from 30 s$^{-1}$ to 6 s$^{-1}$), similar to MLCK. The amplitude of the Quin 2 fluorescence signal seen upon its chelation of Ca$^{2+}$ from the CaM-MLCK, CaM-CaD, and CaM-melittin complexes was ~2-fold greater than the signal observed upon Quin chelation of Ca$^{2+}$ from wheat CaM alone. This suggests that target protein (or peptide) binding to CaM increases Ca$^{2+}$ binding to CaM (at 10 μM added Ca$^{2+}$) and that CaM might not saturate with Ca$^{2+}$ during a Ca$^{2+}$ transient in the absence of high affinity target proteins. Alternatively, the rate of Ca$^{2+}$ dissociation from the fast exchanging low affinity N-terminal Ca$^{2+}$-binding sites could be sufficiently reduced by target protein (or peptide) binding, so that dissociation of Ca$^{2+}$ from these sites might now contribute to the amplitude of the observed Quin 2 fluorescence signal. Consistent with this, we have found that melittin and RS-20 dramatically slow Ca$^{2+}$ dissociation from the low affinity N-terminal and the high affinity C-terminal Ca$^{2+}$-binding sites on CaM under conditions (low ionic strength, 10 °C) where Ca$^{2+}$ dissociation from both classes of sites can be observed. This is consistent with the recent X-ray crystallographic studies of Meador et al. (1992) which show a tight association of the MLCK peptide with both the N- and C-terminal lobes of CaM. Thus, high affinity target protein binding to CaM produces dramatic (5-fold) reductions in the rate of Ca$^{2+}$ dissociation from the high affinity C-terminal sites of CaM and presumably also slows Ca$^{2+}$ dissociation from the low affinity N-terminal Ca$^{2+}$-binding sites.

Equilibrium Ca$^{2+}$ binding data have shown that skeletal muscle MLCK (Olwin et al., 1984), melittin (Mauel and Cox, 1983), troponin I (Keller et al., 1982), and cyclic nucleotide phosphodiesterase (Huang et al., 1981) binding to CaM induce a 7–35-fold increase in CaM's Ca$^{2+}$ affinity. Our studies are consistent with the finding that high affinity target proteins (MLCK) dramatically increase CaM's affinity for Ca$^{2+}$ and assume a part of this increase in Ca$^{2+}$ affinity results from a reduced rate of Ca$^{2+}$ dissociation from CaM's C-terminal Ca$^{2+}$-binding sites.

For the proteins we studied, the higher affinity target proteins were dissociated from CaM more slowly by EGTA and they decreased the rate of Ca$^{2+}$ dissociation from the C-terminal Ca$^{2+}$-binding sites of CaM more dramatically than the lower affinity target proteins. Ca$^{2+}$ dissociation from the C-terminal sites of each CaM-target protein complex occurs ~2-fold faster than the rate of EGTA-induced complex disruption. Either a slower EGTA-induced conformational change is required for complex dissociation or Ca$^{2+}$ dissociation from the N-terminal sites could be involved in complex disruption.

MLCK exchanges with CaM a hundred times more slowly (0.031 s$^{-1}$) than the rate at which this complex is disrupted

---

1 J. D. Johnson and C. Vasulka, unpublished observations.
upon Ca\(^{2+}\) chelation (3.5 s\(^{-1}\)). Thus, high affinity target proteins, like MLCK, may allow CaM to sense Ca\(^{2+}\) in the physiological range and to bind and activate these proteins until the Ca\(^{2+}\) transient subsides. Lower affinity target proteins, like CaD, exchange with CaM 4 times more rapidly (57 s\(^{-1}\)) than the rate at which the complex is disrupted by Ca\(^{2+}\) chelation (13.5 s\(^{-1}\)). This could allow a rapid exchange of CaM with low affinity proteins like CaD, even in the presence of Ca\(^{2+}\). Phosphorylation of target proteins (like MLCK) which reduce their affinity for CaM can dramatically increase their rate of exchange with CaM.

Kamm and Stull (1986) have shown that Ca\(^{2+}\)-CaM can bind and activate MLCK resulting in a phosphorylation of myosin light chain with a rate of 1.1 s\(^{-1}\). Our on-rates of CaM to MLCK indicate that even at 1 \(\mu\)M of each protein, this complex could form at rates in excess of 150 s\(^{-1}\). Thus, CaM binding to MLCK should not be rate-limiting for myosin light chain phosphorylation.

Ca\(^{2+}\)-CaM is also thought to bind CaD and reverse its inhibition of the actin-myosin interaction (see Hartshorne (1987), Sobue et al. (1988) and Pritchard and Marston (1989)). Since the on-rate of CaM for CaD (5.3 \(\times\) 10\(^{5}\) M\(^{-1}\) s\(^{-1}\)) and the exchange rate of CaM with CaD (57 s\(^{-1}\)) are so rapid, CaM could first associate with CaD and then dissociate and bind MLCK rapidly enough to facilitate smooth muscle contraction. Presently it is not known if such a transfer occurs, but our kinetic studies indicate that it would be feasible.

In conclusion, the association rate constants for the CaM-CaD and CaM-MLCK complexes determined from our studies suggest that following a transient rise in intracellular [Ca\(^{2+}\)], CaM could bind CaD and MLCK within the ~200-400-ms interval that precedes the generation of force (Somlyo and Hippenms, 1989). A decrease in intracellular [Ca\(^{2+}\)] could dissociate CaM from MLCK, P-MLCK, and CaD, with rates that are sufficiently rapid to account for their participation in the process of smooth muscle relaxation. High affinity CaM target proteins dramatically increase CaM's affinity for Ca\(^{2+}\) (Olwin et al., 1984; Huang et al., 1981) and dramatically decrease the rate of Ca\(^{2+}\) dissociation from CaM. This would allow CaM to be saturated by lower concentrations of [Ca\(^{2+}\)] (low micromolar range) in the presence of high affinity target proteins and would facilitate a prolonged activation of these high affinity target proteins as the Ca\(^{2+}\) transient subsides. These kinetic studies should help to further our understanding of the temporal nature of the Ca\(^{2+}\)-dependent events that modulate the contraction-relaxation cycle of smooth muscle during a Ca\(^{2+}\) transient.

REFERENCES

Kose-Kosicka, D., Bzdega, T., and Johnson, J. D. (1990) Biochemistry 29, 1673-1679

Ca\(^{2+}\) and Protein Exchange with Calmodulin