Intermolecular tuning of calmodulin by target peptides and proteins: Differential effects on Ca^{2+} binding and implications for kinase activation

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Abstract

Ca^{2+} -activated calmodulin (CaM) regulates many target enzymes by docking to an amphiphilic target helix of variable sequence. This study compares the equilibrium Ca^{2+} binding and Ca^{2+} dissociation kinetics of CaM complexed to target peptides derived from five different CaM-regulated proteins: phosphorylase kinase, CaM-dependent protein kinase II, skeletal and smooth myosin light chain kinases, and the plasma membrane Ca^{2+} -ATPase. The results reveal that different target peptides can tune the Ca^{2+} binding affinities and kinetics of the two CaM domains over a wide range of Ca^{2+} concentrations and time scales. The five peptides increase the Ca^{2+} affinity of the N-terminal regulatory domain from 14- to 350-fold and slow its Ca^{2+} dissociation kinetics from 60- to 140-fold. Smaller effects are observed for the C-terminal domain, where peptides increase the apparent Ca^{2+} affinity 8- to 100-fold and slow dissociation kinetics 13- to 32-fold. In full-length skeletal myosin light chain kinase the inter-molecular tuning provided by the isolated target peptide is further modulated by other tuning interactions, resulting in a CaM–protein complex that has a 10-fold lower Ca^{2+} affinity than the analogous CaM–peptide complex. Unlike the CaM–peptide complexes, Ca^{2+} dissociation from the protein complex follows monoexponential kinetics in which all four Ca^{2+} ions dissociate at a rate comparable to the slow rate observed in the peptide complex. The two Ca^{2+} ions bound to the CaM N-terminal domain are substantially occluded in the CaM–protein complex. Overall, the results indicate that the cellular activation of myosin light chain kinase is likely to be triggered by the binding of free Ca_2^{2+} -CaM or Ca_4^{2+} -CaM after a Ca^{2+} signal has begun and that inactivation of the complex is initiated by a single rate-limiting event, which is proposed to be either the direct dissociation of Ca^{2+} ions from the bound C-terminal domain or the dissociation of Ca^{2+} loaded C-terminal domain from skMLCK. The observed target-induced variations in Ca^{2+} affinities and dissociation rates could serve to tune CaM activation and inactivation for different cellular pathways, and also must counterbalance the variable energetic costs of driving the activating conformational change in different target enzymes.

Keywords: calcium signaling; calmodulin; kinase regulation; protein–protein interactions

The ubiquitous protein calmodulin (CaM) plays a crucial role in Ca^{2+} signaling (Beckingham, 1995; Braun & Schulman, 1995; Clapham, 1995; Wolenski, 1995; Berridge, 1996), where it regulates a wide range of cellular processes by serving as an intracellular receptor for Ca^{2+} ions (Wheeler et al., 1995; Calakos & Scheller, 1996; Dawson & Dawson, 1996; Ghosh & Greenberg, 1996; Restrepo et al., 1996). CaM binds four Ca^{2+} ions and then undergoes a conformational change that greatly increases its affinity for various target proteins (Chazin, 1995a; Finn et al., 1995; Kuboniwa et al., 1995; Zhang et al., 1995; Ikura, 1996), leading to complex formation and activation or inactivation of numerous biochemical pathways (Crivici & Ikura, 1995; James et al., 1995). The

targets of such activation include cytoplasmic enzymes as diverse as protein kinases and phosphatases, adenylate cyclase, cytoskeletal proteins, ion channels, and Ca^{2+} pumps. There is increasing evidence that CaM is also involved in nuclear functions, including transcriptional control through interactions with specific DNA binding proteins (Bachs et al., 1994; Corneliussen et al., 1994; Baudier et al., 1995).

CaM is prototypical of a large family of ion binding proteins that utilize a helix-loop-helix motif known as the EF-hand to bind Ca^{2+} and Mg^{2+} (Kawasaki & Kretsinger, 1995; Kretsinger, 1996). A key functional requirement of the EF-hand motif is that the Ca^{2+} binding characteristics of a given site must be tailored, or “tuned,” to provide the optimal Ca^{2+} activation parameters required by a specific biochemical pathway (Chazin, 1995a,b; Falke et al., 1994; Linse & Forsén, 1995). In particular, the Ca^{2+} affinity, selectivity, and kinetics must be such that a given EF-hand protein is activated

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or inactivated at the appropriate Ca^{2+} concentration threshold and with the appropriate kinetics. Two broad classes of tuning mechanisms are used to achieve this goal; *intra-molecular* tuning and *inter-molecular* tuning. Intra-molecular tuning arises from the unique primary and tertiary structural features of different EF-hand proteins, which exhibit over 10^5 -fold ranges of Ca^{2+} binding affinities and kinetics. Such intra-molecular tuning is the result of a carefully orchestrated set of structural and electrostatic interactions in the EF-hand, particularly in the Ca^{2+} coordinating EF-loop itself (Falke et al., 1994; Linse & Forsén, 1995; Drake et al., 1996; Drake & Falke, 1996). While intra-molecular tuning is sufficient to tailor a given protein to a single biochemical role, multi-functional proteins such as CaM use additional inter-molecular tuning interactions to satisfy the diverse activation requirements of different signaling pathways.

Inter-molecular tuning relies on interactions between different proteins to modulate ion binding at an EF-hand site, providing an additional mechanism whereby ion binding characteristics can be optimized. CaM is one of the best characterized examples of inter-molecular EF-hand tuning because its Ca^{2+} affinity increases substantially in the presence of the activatable target enzymes to which it binds. Olwin and Storm (1985) showed that the macroscopic Ca^{2+} affinity of CaM increases from $\sim 14 \mu\text{M}$ in the absence of a target protein to 2.1, 1.1, and $0.6 \mu\text{M}$ in the presence of skeletal muscle troponin I, skeletal muscle myosin light chain kinase, and phosphodiesterase, respectively. In many target enzymes, CaM binding to its target helix results in the removal of an adjacent sequence from the enzyme active site, thereby triggering enzyme activation through relief of auto-inhibition (reviewed by James et al., 1995). Studies of CaM-dependent enzymes have revealed a consensus CaM-binding motif consisting of a ~ 20 residue long basic and amphiphilic α -helical sequence that exhibits structural similarity, but remarkably little sequence identity, among different target proteins (Cox et al., 1985; Erickson-Viitanen & DeGrado, 1987; O'Neil & DeGrado, 1990a; Strynadka & James, 1990; Afshar et al., 1994). Synthetic peptides corresponding to the CaM binding sequences of specific proteins serve as potent antagonists in Ca^{2+} /CaM-dependent activation assays and bind CaM with nanomolar affinities that are comparable to or tighter than those of the full-length proteins (Blumenthal et al., 1985; Lukas et al., 1986; Dasgupta et al., 1989; Anagli et al., 1995). Peptides can also increase the Ca^{2+} affinity and slow Ca^{2+} dissociation rates as compared to CaM in the absence of a target sequence (Yazawa et al., 1992; Stemmer & Klee, 1994; Bayley et al., 1996; Johnson et al., 1996; Persechini et al., 1996).

CaM has a dumbbell shaped structure in which two domains, termed the N and C domains, are linked by a flexible helix (Taylor et al., 1991; Finn et al., 1995; Kuboniwa et al., 1995; Zhang et al., 1995). Each domain binds two Ca^{2+} ions into a pair of EF-hand type sites. In the presence of a target peptide, the middle portion of the inter-domain helix melts and the two domains wrap around and engulf the target helix (Klevit et al., 1985; Ikura et al., 1992; Meador et al., 1992, 1993; Meador et al., 1995; Crivici & Ikura, 1995). The N domain of CaM interacts with the C-terminal end of the target peptide and vice versa. Due to variations in target sequences, the CaM-target interfaces are unique for each target helix, and different target helices can alter the conformation of a given CaM domain, as well as the relative orientation between the two domains (Meador et al., 1993; Crivici & Ikura, 1995). Moreover, different natural target sequences exhibit varying free energies of binding to Ca^{2+} -loaded CaM, and thus differ in their abilities to

drive the equilibrium toward the Ca^{2+} -loaded state (Crivici & Ikura, 1995). Together, these factors provide multiple sources of structural and thermodynamic variability that could be used to provide inter-molecular tuning of Ca^{2+} binding.

Previous studies have characterized the effects of individual target peptides on Ca^{2+} binding to CaM (Vorherr et al., 1990; Yazawa et al., 1992; Bayley et al., 1996) or have compared the effects of multiple target sequences on Ca^{2+} dissociation kinetics (Johnson et al., 1996; Persechini et al., 1996). The present work extends these studies by systematically comparing both the equilibrium and kinetic aspects of Ca^{2+} binding to nine CaM-peptide complexes and one CaM-protein complex. This comparative analysis utilizes target peptides derived from five CaM-regulated enzymes: CaM-dependent protein kinase II α (MKII), phosphorylase kinase (PhK5), smooth and skeletal myosin light chain kinases (smMLCK and skMLCK), and the plasma membrane Ca^{2+} pump (CaATPase). Direct detection of Ca^{2+} binding by flow dialysis experiments reveals that each of the target peptides substantially increases the Ca^{2+} affinity of CaM, but the range of these affinity enhancements is significantly broader than previously determined using intact target proteins. Stopped-flow kinetic studies indicate that the affinity enhancements are largely accounted for by slowed Ca^{2+} dissociation from the Ca^{2+} -CaM-peptide complexes, while the calculated Ca^{2+} association rates remain relatively constant. Although all five peptides yield similar ratios of Ca^{2+} affinities and dissociation rates for the two domains, experiments using shorter peptides confirm that it is possible to tune Ca^{2+} binding to the two domains independently of each other, and the magnitude of tuning increases with the length of the peptide. Finally, a direct comparison of Ca^{2+} binding and kinetic data obtained using the full-length skMLCK protein and its corresponding CaM-binding peptide reveal the presence of important additional tuning interactions in the intact protein. Thus, the overall Ca^{2+} concentration threshold of CaM-dependent activation is controlled both by the docking of CaM to the target helix and other features specific to a given target protein. The implications of these findings for different mechanisms of Ca^{2+} /CaM-dependent kinase activation are discussed.

Results

Selection of target peptides

The goal of this inter-molecular tuning study was to directly compare the effects of different target peptides and proteins on the equilibrium and kinetics of Ca^{2+} binding to calmodulin using a representative set of well-defined target sequences. The use of identical laboratory conditions is crucial to determining the range of tuning effects because differences in ionic strength, temperature, and pH can have profound effects on Ca^{2+} binding parameters. A total of nine peptides corresponding to full or partial CaM binding motifs from five different enzymes were examined, as summarized in Table 1. The five full-length peptides bind CaM with high affinities in the presence of Ca^{2+} , having dissociation constants in the low nanomolar range as listed in Table 1.

Three peptides (MKII, smMLCK, and skMLCK) correspond to the full CaM binding motifs of regulatory kinases where structures of the CaM-peptide complexes have been determined by solution NMR (Ikura et al., 1992) and X-ray crystallography methods (Meador et al., 1992, 1993). The fourth full-length target peptide is from the plasma membrane Ca^{2+} ATPase, and the fifth peptide, PhK5, corresponds to one of two CaM binding sequences found in the

Table 1. Sequences of full-length and partial CaM target peptides

Peptide	Sequence ^a	Affinity
PhK5	•L R R L I D A Y A F R I Y G H W V K K G Q Q Q N R•	<20 nM ^b
CaATPase	R G Q I L W F R G L N R I Q T Q I K V V N A F S	~1 nM ^c
smMLCK	R R K W Q K T G H A V R A I G R L S S	1–2 nM ^d
skMLCK	K R R W K K N F I A V S A A N R F K K I S S S	≤1 nM ^e
MKII	F N A R R K L K G A I L T T M L A T R N F S	<1 nM ^f
sk-N11	•K R R W K K N F I A V•	0.4 μM ^g
sk-C10	•A N R F K K I S S S•	ND
CaATP-N18	R G Q I L W F R G L N R I Q T Q I K	ND
CaATP-C17	R G L N R I Q T Q I K V V N A F S	ND

^aUnderline indicates regions of smMLCK, skMLCK, and MKII peptides established to be an ordered α -helix in the known structures of the CaM–peptide complexes. Bold indicates residues making the primary hydrophobic interactions with CaM in these complexes, putative anchor residues for other peptides are indicated in italics, • denotes peptide termini that were blocked by acetylation or amidation, and ND = affinity not determined.

^bDasgupta et al. (1989). ^cVorherr et al. (1990). ^dEnyedi et al. (1989). ^eLukas et al. (1986) Malencik et al. (1982). ^fFitzsimons et al. (1992). ^gBlumenthal et al. (1985). ^hFindlay et al. (1995). ⁱHanley et al. (1987). ^gDetermined by tryptophan fluorescence (see Methods).

catalytic γ subunit of phosphorylase kinase (PhK), a multi-subunit enzyme that tightly binds CaM as its δ -subunit. Four shorter peptides, representing the N- or C-terminal portions of the skMLCK and CaATPase helices, were generated to examine the effects of partial peptides on Ca^{2+} binding to CaM (Table 1). In each case, these shorter peptides contain only one of the two hydrophobic anchors proposed to dominate the binding interactions with the corresponding hydrophobic patches found on each domain of CaM.

Peptides were added in 3- to 30-fold molar excess to ensure that CaM was fully saturated with peptide during the Ca^{2+} binding experiments. Control experiments confirmed that, as expected given the nanomolar affinity of each full-length peptide for CaM, varying concentrations of peptide ranging from 3–30 μM yielded the same Ca^{2+} affinity, within error (data not shown). For technical reasons (see Methods), the equilibrium binding experiments included 0.5 mM Mg^{2+} , which significantly reduced the scatter in the flow-dialysis data, presumably by blocking non-specific divalent ion binding sites on the dialysis membrane. At this concentration, Mg^{2+} interacts quite weakly with CaM and the CaM–target complexes, and is largely a spectator ion. Parallel Ca^{2+} binding experiments carried out in the absence and presence of Mg^{2+} for some of the CaM–peptide complexes showed that the inclusion of Mg^{2+} resulted in less than a twofold change in the observed Ca^{2+} dissociation constants, consistent with the nearly Mg^{2+} -independent binding of Ca^{2+} previously noted for the CaM–skMLCK protein complex (Johnson et al., 1981). Addition of Mg^{2+} at concentrations as high as 10 mM was unable to promote formation of CaM–peptide complexes, as judged by peptide tryptophan fluorescence (data not shown).

Measurements of Ca^{2+} binding to CaM–peptide complexes

Equilibrium Ca^{2+} binding to CaM and its target peptide complexes was measured using flow dialysis, a technique that enables rapid determination of a complete binding curve for high-affinity ligand interactions (Colowick & Womack, 1969; Porumb, 1994). Figure 1 directly compares the Ca^{2+} binding profiles of CaM alone and in the presence of the five full-length peptides. The Ca^{2+} concentrations yielding half-saturation ($[\text{Ca}^{2+}]_{1/2}$), summarized in Table 2, reveal that target peptides increase the macroscopic Ca^{2+} affinity

of CaM by as much as two orders of magnitude, as previously shown in similar peptide studies using a variety of experimental conditions (Yazawa et al., 1992; Stemmer & Klee, 1994; Bayley et al., 1996). Here, the controlled conditions enable direct comparison of intermolecular tuning by the five different peptides. The MKII and skMLCK peptides generate the greatest enhancement of macroscopic Ca^{2+} affinity (240- and 220-fold, respectively), while the PhK5 peptide provides the smallest enhancement (13-fold). Additionally, the steepness of the Ca^{2+} binding profile varies slightly between different CaM–peptide pairs, suggesting that selected targets can differentially adjust either the cooperativity of Ca^{2+} binding or the relative Ca^{2+} affinities of the two CaM domains. Together,

Fig. 1. Ca^{2+} binding curves for calmodulin in the presence of synthetic peptides corresponding to CaM target sequences of five different enzymes. Shown are data for peptides derived from phosphorylase kinase (PhK5), erythrocyte Ca^{2+} ATPase (CaATPase), smooth and skeletal myosin light chain kinases (smMLCK and skMLCK), and the CaM-dependent multi-functional kinase II α from bovine brain (MKII). Binding data were obtained by flow dialysis in 100 mM KCl, 0.5 mM MgCl_2 , 20 mM MOPS, pH 7.4 at 25 °C. Each curve represents the calculated best fit to a pair of independent domains that each bind two Ca^{2+} ions in a cooperative manner, yielding the parameters listed in Table 2. Data from three or four different determinations were combined to give the range of points shown for each CaM–peptide complex.

Table 2. Effects of target peptides on the equilibrium and kinetics of Ca^{2+} binding to calmodulin

Peptide	[Ca] _{1/2} ^a (μM)	Equilibrium Ca^{2+} binding to the two CaM domains ^b				Ca^{2+} dissociation ^c	
		K_{D1} (μM)	K_{D2} (μM)	H_1	H_2	k_{offA} (s^{-1})	k_{offB} (s^{-1})
None	7.5	2.0 ± 0.1	13.0 ± 0.6	1.4 ± 0.2	1.7 ± 0.3	11.8 ± 0.1	$\geq 850 \pm 70$
PhK5	0.60	0.24 ± 0.08	0.9 ± 0.2	1.1 ± 0.3	1.7 ± 0.4	0.90 ± 0.01	13.2 ± 0.2
CaATPase	0.12	0.09 ± 0.01	0.2 ± 0.1	2.0 ± 0.3	1.4 ± 0.2	0.69 ± 0.03	12.1 ± 0.5
smMLCK	0.17	0.10 ± 0.06	0.2 ± 0.1	1.5 ± 0.7	1.7 ± 0.7	0.70 ± 0.02	12.5 ± 0.6
skMLCK	0.034	0.02 ± 0.01	0.08 ± 0.06	2.3 ± 1.8	0.9 ± 0.6	0.36 ± 0.01	6.4 ± 0.6
MKII	0.031	0.03 ± 0.01	0.04 ± 0.01	0.9 ± 0.3	2.0 ± 0.8	0.49 ± 0.03	6.0 ± 0.4
sk-N11		0.28 ± 0.01	6.0 ± 0.2	1.7 ± 0.1	1.8 ± 0.1	0.73 ± 0.01	450 ± 10
sk-C10		3.4 ± 0.8	4.0 ± 1.0	1.4 ± 0.4	1.2 ± 0.4	6.8 ± 0.1	650 ± 50
sk-N11 + sk-C10		0.32 ± 0.01	4.7 ± 0.2	1.3 ± 0.1	2.0 ± 0.1	ND	ND
CaATP-N18		0.12 ± 0.01	3.9 ± 0.2	1.6 ± 0.3	1.7 ± 0.1	0.63 ± 0.01	260 ± 10
CaATP-C17		0.66 ± 0.13	2.4 ± 0.2	1.2 ± 0.1	1.8 ± 0.3	1.72 ± 0.04	15.2 ± 0.4

^aMacroscopic Ca^{2+} affinity determined by the midpoint of the Ca^{2+} saturation curve determined at 25 °C in 100 mM KCl, 0.5 mM MgCl_2 , 20 mM MOPS, pH 7.4.

^bDissociation constants (K_{D1} , K_{D2}) and Hill coefficients (H_1 , H_2) obtained by fitting the data as two independent binding events, each of which cooperatively binds a pair of Ca^{2+} ions. Domain assignments are discussed in the text (K_{D1} , H_1 , k_{offA} represent the C-terminal domain; K_{D2} , H_2 , k_{offB} the N-terminal domain).

^c Ca^{2+} dissociation rates determined by fitting the stopped-flow Quin-2 fluorescence signal to a double exponential decay curve. The indicated values are averages of rates determined over a range of peptide concentrations (10–15 μM , see Results). Data were obtained at 25 °C in 100 mM KCl, 20 mM MOPS, pH 7.4.

these results provide direct, model-independent evidence for intermolecular tuning of Ca^{2+} binding to calmodulin.

Quantitative analysis of the Ca^{2+} binding curves was carried out by fitting the data to a model of two independent domains, each of which bind a pair of Ca^{2+} ions in a single cooperative event (Porumb, 1994). This approach is justified by observations that the Ca^{2+} binding profile of free CaM can be reconstructed from those of its two isolated domains, by evidence for domain-independence in the binding and dissociation of Ca^{2+} in CaM–target complexes, and by the observation that individual Ca^{2+} site mutations perturb mainly the two sites in the same domain (Linse et al., 1991; Starovasiluk et al., 1992; Finn et al., 1995; Bayley et al., 1996; Johnson et al., 1996). Table 2 presents the resulting equilibrium dissociation constants and Hill coefficients of the high-affinity (K_{D1} , H_1) and low affinity (K_{D2} , H_2) CaM domains. In the absence of target peptide, the two domains bind Ca^{2+} with dissociation constants of 2 and 13 μM and Hill coefficients of 1.4 and 1.7, respectively. These data agree with previously published values from other laboratories, which have assigned the high-affinity Ca^{2+} binding component as the C-terminal domain and the low-affinity component as the N-terminal domain (Haiech et al., 1981; Linse et al., 1991). Upon addition of peptides, the Ca^{2+} dissociation constants of the high- and low-affinity components decrease up to 100-fold and 320-fold, respectively. With the exception of PhK5, peptide binding decreases the ratio of the two affinities (K_{D2}/K_{D1}) from 6.5 to about 2, indicating that the Ca^{2+} affinities of the two CaM domains become more equal upon association with a target peptide. Such equalization of the two binding events accounts, in part, for the increased steepness observed for Ca^{2+} binding to the four highest affinity complexes (Fig. 1). Enhanced Ca^{2+} binding cooperativity within one or both CaM domains may also contribute to the greater steepness, but the precision of the measured Hill coefficients is insufficient to ascertain the importance of such effects.

Ca^{2+} dissociation kinetics

The rates of Ca^{2+} dissociation from CaM were determined at 25 °C by stopped-flow fluorescence measurements using Quin-2, a fluorescent chelator that exhibits rapid, high-affinity Ca^{2+} binding, resulting in a fivefold reduction in its fluorescence (Martin et al., 1985). Solutions were made in the same buffer used for the flow-dialysis experiments but without the 0.5 mM MgCl_2 used to increase binding curve precision because Mg^{2+} binds to Quin-2 and would interfere with the kinetic assay. Because Mg^{2+} is largely a spectator ion in the equilibrium binding experiments (see above), its omission does not obviate direct comparisons of the equilibrium and kinetic data, such as calculation of Ca^{2+} association rates.

In the absence of a target sequence, free CaM exhibited bi-exponential Ca^{2+} dissociation kinetics yielding time constants of $\geq 850 \text{ s}^{-1}$ and 11.8 s^{-1} (Fig. 2), where each dissociation event can be attributed to the essentially simultaneous dissociation of two Ca^{2+} ions from one domain of CaM (Suko et al., 1986; Martin et al., 1992; Johnson et al., 1996; Persechini et al., 1996). In the presence of the target peptides these events are slowed dramatically, with the highest Ca^{2+} affinity CaM–peptide complexes yielding the slowest Ca^{2+} dissociation rates. The best-fit apparent rate constants for the slow (k_{offA}) and fast (k_{offB}) dissociation events are listed in Table 2. The rate of the slow dissociation event decreases as much as 33-fold, from 12 s^{-1} in the absence of peptide to 0.36 s^{-1} in the presence of the skMLCK peptide. Even more drastic changes are observed for the fast dissociation event, which slows at least 140-fold, decreasing from $\geq 850 \text{ s}^{-1}$ to 6 s^{-1} . As was observed for the Ca^{2+} affinities of the two domains, all five target peptides bring the relative Ca^{2+} dissociation rates of the two domains closer together. In the absence of a target peptide the ratio $k_{\text{offB}}/k_{\text{offA}}$ is ~ 70 , but this value decreases to ~ 15 in the presence of target peptide (Table 3).

Fig. 2. Stopped-flow measurements of Ca^{2+} dissociation from CaM-peptide complexes. Shown are Quin-2 fluorescence data for CaM and CaM-PhK5 and CaM-smMLCK complexes. Data were obtained at 25 °C by mixing solutions of 5 μM CaM and 10 μM peptide in 60 μM CaCl_2 with 150 μM Quin-2, both in 100 mM KCl, 20 mM MOPS, pH 7.4. Inset shows first 35 ms of the dissociation curves and Table 2 lists the k_{off} values determined by fitting to double exponential decay curves after masking the first 1.0 ms to account for the dead time of the Applied Photophysics model 17MV stopped flow apparatus.

Domain assignments of equilibrium and kinetic parameters

It is important to assign the pairs of equilibrium dissociation constants (K_{D1} , K_{D2}) and dissociation rate constants (k_{offA} , k_{offB}) to the N and C domains of CaM. For free CaM, it is well established that the N domain provides the low affinity, rapidly dissociating component, while the C domain is responsible for the high affinity, slowly dissociating component (Martin et al., 1985, 1992; Linse et al., 1991; Johnson et al., 1996). We have confirmed this assignment in our *Drosophila* CaM by stopped-flow absorbance measurements at 276 nm, where the Ca^{2+} -dependent absorbance of Tyr-138 provides a specific probe of Ca^{2+} binding to EF-hand site IV in the C domain (Maune et al., 1992). Tyr absorbance was found to change exponentially at a rate of 12 s^{-1} when Ca^{2+} -saturated CaM was mixed with EDTA (data not shown), which is in excellent agreement with the slow Quin-2 detected rate of

11.8 s^{-1} and shows that the slowly dissociating component can be attributed to the C domain. However, in the presence of target peptide this method of domain assignment is compromised by the background absorbance of aromatic side chains on the peptides.

Evidence provided by several other approaches argues that the same domain assignments made in the absence of peptide continue to hold in the Ca^{2+} binding reactions of all five CaM-target peptide complexes. First, the fast dissociating component (k_{offB}) observed for the various CaM-peptide complexes is generally comparable to the slow C component (k_{offA}) observed in CaM alone. Because peptide binding greatly increases the Ca^{2+} affinities of both domains, it is unlikely that the k_{off} value for one domain would be relatively unaffected while that of the other domain undergoes a 3000-fold decrease (from a k_{offB} of $\geq 850 \text{ s}^{-1}$ to a k_{offA} of 0.26 s^{-1}). Second, if one assumes that the low- and high-affinity equilibrium constants, K_{D2} and K_{D1} , also continue to represent binding to the N and C domains in the CaM-peptide complexes, then the apparent on-rate constants of the two domains can be calculated as $k_{\text{onN}} = k_{\text{offB}}/K_{D2}$ and $k_{\text{onC}} = k_{\text{offA}}/K_{D1}$. As shown in Table 3, these calculations show that the k_{onN} of the N domain remains generally constant at $\sim 6 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ for free CaM and its peptide complexes, while the k_{onC} of the C domain is constant at $\sim 1 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$. These relationships indicate that the N and C domains of CaM retain their respective identities as the (i) low-affinity, rapidly dissociating, and (ii) high-affinity, slowly dissociating components of the Ca^{2+} binding reactions in the CaM-peptide complexes. Furthermore, these data show that the affinity increases observed for both domains arise almost purely from decreases in the Ca^{2+} dissociation rates. Finally, the same domain assignments have also been obtained in equilibrium and kinetic studies of CaM complexed with MLCK and several other target sequences. In their recent kinetic studies, Johnson et al. (1996) used disulfide locked CaM domains and Ca^{2+} transients to show that the N domain both binds and releases Ca^{2+} faster than does the C domain in four CaM-peptide complexes, and Persechini et al. (1996) used N and C domain fragments of CaM to assign the slower Ca^{2+} dissociation events to the C domain in MLCK and nitric oxide synthase target peptide complexes. Combined with the results presented here, these data suggest that most, if not all, CaM-peptide complexes retain a conserved order of Ca^{2+} affinity and release rates from the two CaM domains.

Table 3. Calculated peptide-induced effects on Ca^{2+} binding to the two CaM domains

Peptide	Ca affinity ^a enhancement	K_{D2}/K_{D1}	$k_{\text{offB}}/k_{\text{offA}}$ ^b	$k_{\text{onN}}^{\text{N}}$ (calc) ($\times 10^6 \text{ M}^{-1} \text{ s}^{-1}$) ^c	$k_{\text{onC}}^{\text{C}}$ (calc) ($\times 10^6 \text{ M}^{-1} \text{ s}^{-1}$) ^c
None	1×	6.5 ± 0.4	$\geq 72 \pm 6$	$\geq 65 \pm 6$	5.9 ± 0.3
PhK5	12×	3.8 ± 1.5	14.7 ± 0.1	15 ± 2	3.8 ± 0.9
CaATPase	62×	1.9 ± 1.2	17.5 ± 0.1	60 ± 20	7.7 ± 0.6
smMLCK	44×	2.0 ± 1.6	18.0 ± 0.3	60 ± 20	7 ± 3
skMLCK	220×	4.0 ± 3.4	15.9 ± 0.9	80 ± 40	18 ± 5
MKII	240×	1.3 ± 0.6	11.8 ± 0.6	150 ± 30	16 ± 4

^aCalculated as the ratio $\{[\text{Ca}]_{1/2} \text{ of CaM}\}/\{[\text{Ca}]_{1/2} \text{ of CaM-peptide}\}$ based on values found in Table 2.

^bAverage ratio determined over a range of peptide concentrations (10–15 μM) approaching saturation of the observed k_{off} values (see Results).

^cFor the N-terminal domain, $k_{\text{onN}}^{\text{N}} = k_{\text{offB}}/K_{D2}$; for the C-terminal domain, $k_{\text{onC}}^{\text{C}} = k_{\text{offA}}/K_{D1}$ (see Results and Table 2). Note that the indicated apparent rates include small systematic errors due to (1) the presence of Mg^{2+} in the K_D determinations and (2) the peptide concentration dependence of the k_{off} measurements (see Results).

Effects of partial peptides on Ca^{2+} binding and dissociation

To further investigate the inter-molecular tuning of the two CaM domains, four partial peptides were designed to interact more strongly with a specific domain of CaM. First, the 23-residue skMLCK peptide was split into an N-terminal 11-residue peptide, sk-N11, and a C-terminal 10-residue peptide, sk-C10. The Ser-Ala residues in the middle of the full-length peptide were omitted to minimize steric clash between the two partial peptides, making it theoretically possible for both peptides to bind CaM simultaneously. The termini were blocked to prevent adverse interactions between a charged peptide terminus and hydrophobic groups on the CaM interface. Based on the structure of the CaM-skMLCK peptide complex (Ikura et al., 1992), sk-N11 is predicted to interact only with the C domain of CaM, while sk-C10 peptide would interact only with the N domain. Recent NMR studies have confirmed that a shorter N-terminal peptide selectively binds the C domain of CaM and shifts its Ca^{2+} binding parameters (Findlay et al., 1995; Bayley et al., 1996). Second, a pair of longer partial peptides were made based on the CaATPase peptide sequence. Designated CaATP-N18 and CaATP-C17, these two peptides are N- and C-terminal fragments that contain all the residues up to, but not including, the putative second hydrophobic anchor sequence, i.e., the Trp-Phe residues in the N-terminal half and the two valines in the C-terminal half of the peptide (Table 1). Previous NMR (Vorherr et al., 1990) and equilibrium Ca^{2+} binding studies (Yazawa et al., 1992) have shown that a CaATP-N18 peptide with a Leu-Arg N-terminal extension binds selectively to the C-terminal domain of CaM and elicits a biphasic Ca^{2+} binding curve. Here, we seek to directly compare the effects of this N-terminal peptide with a similarly constructed C-terminal peptide starting immediately after the N-terminal hydrophobic anchor residues.

Figure 3 summarizes the CaM Ca^{2+} binding curves obtained in the presence of these peptides, and the corresponding equilibrium and kinetic constants are listed in Table 2. Data from the N-terminal peptides, sk-N11 and CaATP-N18, show that these peptides can greatly enhance the Ca^{2+} affinity of one domain over the other, resulting in a clearly biphasic binding curve. Qualitatively, the data are in good agreement with earlier Ca^{2+} binding data from similar peptides (Yazawa et al., 1992; Bayley et al., 1996), and both the observed Ca^{2+} dissociation rates and binding constants confirm that these N-terminal peptides mainly affect the higher affinity, slower dissociating C domain. The sk-N11 peptide enhances the Ca^{2+} affinity of the C domain sevenfold and decreases its Ca^{2+} dissociation rate 16-fold and CaATP-N18 increases the Ca^{2+} affinity of the C domain 17-fold and decreases its Ca^{2+} dissociation rate 19-fold. The effects on the N domain are only two- to threefold for both N-terminal peptides.

Domain specific tuning is also observed for the two C-terminal peptides, sk-C10 and CaATP-C17, which selectively perturb the Ca^{2+} binding parameters of the N domain to a greater degree than those of the C domain. CaATP-C17 decreases the fast Ca^{2+} dissociation rate from the N domain 56-fold while having only a sevenfold effect on the C domain, and the respective effects on equilibrium Ca^{2+} binding are five- and threefold. Smaller effects are seen for sk-C10, which selectively increases the Ca^{2+} affinity of the N domain only threefold even at peptide concentrations as high as 500 μM (data not shown). Finally, an equimolar mixture of the sk-N11 and sk-C10 peptides have approximately the same effects on Ca^{2+} binding to the two CaM domains as do the individual peptides (Fig. 3A). These data indicate that the two peptides

are not able to act in concert to increase the Ca^{2+} affinity of the complex to the level seen for the full-length skMLCK peptide and argue against the existence of strong inter-domain tuning effects whereby the binding of Ca^{2+} and/or peptide to one CaM domain significantly alters the binding equilibria of the other.

Three conclusions can be drawn from the partial peptide results. First, N-terminal peptides as short as 11 residues are capable of selectively tuning the Ca^{2+} affinity and kinetics of the C domain, with only minor effects on the N domain, as recently observed by Bayley et al. (1996). Increasing the peptide length further enhances the Ca^{2+} binding properties of both CaM domains, but the Ca^{2+} binding curve remains clearly biphasic, indicating that the preferential tuning of the C domain is retained. Second, C-terminal peptides appear to interact selectively with the N domain, but they do not elicit biphasic binding curves even if their length is sufficient to demonstrate clear effects on the Ca^{2+} affinities of both domains. The lack of a biphasic curve is not unexpected because there is an

Fig. 3. A: Ca^{2+} binding curves for calmodulin in the presence of the two half-length peptides sk-N11 and sk-C10. Heavy lines show data obtained for sk-N11 (diamonds) and sk-C10 (open circles) at 17 μM , and filled circles delineate a mixture of both peptides present at 15 μM each. Essentially identical results were obtained with higher peptide concentrations (up to 500 μM each). The Ca^{2+} binding curves of CaM alone (— — —) and in the presence of the full-length skMLCK peptide (—) are shown for comparison. Other conditions were as in the Figure 1 legend. B: Analogous data obtained using the CaATP-N18 (diamonds) and CaATP-C17 (open circles) partial peptides present at 30 μM each. Data for CaM alone (— — —) and in the presence of the full-length CaATPase peptide (—) are shown for comparison. Best-fit Ca^{2+} dissociation constants are listed in Table 2.

inherent sixfold difference in the Ca^{2+} affinities of the two domains that must be overcome by C-terminal peptides, but only enhanced by N-terminal peptides, in order to see a biphasic Ca^{2+} binding curve. Third, both hydrophobic anchor residues are required to elicit the full enhancement of Ca^{2+} affinity seen with the full-length peptides, and these residues must be presented on the same peptide.

Comparison of intermolecular tuning by a full-length protein and its isolated target peptide

Figure 4 compares equilibrium Ca^{2+} binding curves obtained from CaM in the presence of the full-length skMLCK protein (generously provided by Dr. Peter Kennelly, Virginia Tech, Blacksburg, VA). The temperature of these experiments was lowered to 20 °C to maintain maximal stability of the Ca^{2+} activated CaM-skMLCK complex during the experiment (Kennelly et al., 1990). It is readily apparent that the intact protein increases the Ca^{2+} affinity of CaM to a significantly smaller extent than does the isolated target peptide, which enhances the macroscopic Ca^{2+} affinity 220-fold (Table 2). The protein increases the macroscopic Ca^{2+} affinity only 13-fold to a value of 560 nM, in excellent agreement with the value of 700 nM measured by Crouch et al. (1981). The new flow-dialysis data for the CaM-protein complex further enable resolution of the Ca^{2+} affinities of the two CaM domains, yielding values of 380 and 800 nM (Table 5). These affinities result in only partial Ca^{2+} occupancy of the CaM-skMLCK complex at resting cellular Ca^{2+} levels (~100 nM), as required for effective Ca^{2+} -CaM mediated activation in vivo. In contrast, the much higher affinities observed for the skMLCK peptide complex, 20 and 80 nM, would predict significant Ca^{2+} occupancy and activation even in the absence of a Ca^{2+} signal.

At 20 °C the Ca^{2+} dissociation timecourse of free CaM showed ~2 Ca^{2+} dissociating from the N domain at a rate of 730 s^{-1} and ~2 Ca^{2+} dissociating from the C domain at a rate of 8.4 s^{-1} (Table 5). When skMLCK was added, the Ca^{2+} dissociation time course became a single exponential process exhibiting a rate of 0.52 s^{-1} and a stoichiometry of 3.4 Ca^{2+} per CaM (Fig. 5,

Fig. 4. Comparison of Ca^{2+} binding to CaM in the presence of the skMLCK peptide and the complete skMLCK protein. Experiments were carried out using 1 μM CaM and 4 μM skMLCK protein or peptide at 20 °C in 100 mM KCl, 0.5 mM MgCl_2 , 20 mM MOPS, pH 7.4. Best-fit Ca^{2+} binding constants are listed in Table 5.

Fig. 5. Kinetics of Ca^{2+} release from the CaM-skMLCK protein complex. The fitted curve corresponds to a single exponential dissociation curve having a rate of 0.52 s^{-1} and an observed amplitude 3.4 Ca^{2+} per CaM, as summarized in Table 5. The inset shows an expanded view of the first 85 ms, which were sampled with a dwell time of 25 μs ; note the absence of a significant rapid component in the protein complex. The experiment was carried out at 20 °C by stopped-flow mixing a solution of 150 μM Quin-2 with an equal volume of 1 μM CaM and 2 μM skMLCK in 100 μM CaCl_2 , where both solutions were buffered in 20 mM MOPS, 100 mM KCl, 1 mM DTT, pH 7.4. At the post-mixing concentrations of Quin-2 and CaCl_2 , the Ca^{2+} occupancy of the CaM-skMLCK complex is expected to be ~17%, resulting in ~0.7 Ca^{2+} ions per CaM being retained by the complex. Thus, the observed amplitude of the kinetic trace corresponds to the maximum expected stoichiometry for the dissociation of four bound Ca^{2+} ions (see Results).

Table 5). This rate was independent of skMLCK concentration when skMLCK was present at 1.5- to 2.5-fold excess over CaM. Although the observed amplitude of this dissociation event is 3.4 Ca^{2+} per CaM, it most likely represents the complete release of all four Ca^{2+} ions from the CaM-skMLCK complex for two reasons. First, at the end of the stopped flow experiment the free Ca^{2+} concentration will be about 0.2 μM based on the post-mixing concentrations of Quin-2 (75 μM) and Ca^{2+} (50 μM) and a K_D of ~0.1 μM for the interaction between Ca^{2+} and Quin-2. This final free Ca^{2+} concentration is effectively being buffered by the Quin-2 and is sufficient to maintain partial Ca^{2+} saturation of the 1 μM CaM-skMLCK complex. Based on the K_D values reported in Table 5, the complex will be 17% saturated and have a stoichiometry of ~0.7 Ca^{2+} ions per complex, thereby fully accounting for the “missing” Ca^{2+} ions in the dissociation time course. Second, the single exponential behavior of the dissociation time course argues against the existence of another dissociation event—attempts to add a second exponential component to the curve fit are not statistically justified based on the resulting χ^2 values and parameter errors. It follows that the complete dissociation of all four Ca^{2+} ions occurs in response to a single rate-limiting event that triggers skMLCK inactivation.

An alternative interpretation that three Ca^{2+} ions dissociate at the observed rate while a fourth ion dissociates too rapidly to observe at a rate of >1000 s^{-1} (Persechini et al., 1996) is ruled out by several factors. First, this interpretation does not account for the Ca^{2+} still bound to the protein complex at the end of the dissociation time course, as defined by the final free Ca^{2+} concentration due to buffering by the Ca^{2+} chelator (see above). Second, the binding of CaM to skMLCK is expected to slow, rather than increase, the rates of Ca^{2+} dissociation from CaM because the Ca^{2+} affinity of the CaM-

skMLCK complex is 13-fold higher than that of free CaM. Third, the biphasic time course observed for free CaM clearly becomes monophasic for the CaM-skMLCK complex.

Discussion

CaM tuning by isolated target peptides

Overall, the results indicate that isolated peptides representing five different natural CaM binding sequences can increase the macroscopic Ca^{2+} affinity and slow Ca^{2+} dissociation by over two orders of magnitude relative to in the absence of peptide. These tuning effects are extremely peptide specific, with different CaM target sequences eliciting a 20-fold range of Ca^{2+} affinity enhancements and a threefold range of Ca^{2+} dissociation rates. Deconvolution of the data into two independent equilibrium constants and dissociation rates, each representing a cooperative 2Ca^{2+} process occurring at one of the two CaM domains, reveals that the two domains are differentially tuned by target peptides. Inter-molecular tuning of both Ca^{2+} affinity and dissociation kinetics is three- to fivefold stronger for the N domain than for the C domain, thereby making the Ca^{2+} binding parameters of the two domains more similar than in the absence of peptide. This domain specific tuning may have important functional implications, because it is thought that the N domain plays a central role in activation within cells while the C domain may be largely Ca^{2+} occupied even in the absence of a Ca^{2+} signal (Bayley et al., 1996; Johnson et al., 1996).

It is not yet clear what structural and electrostatic features of isolated target peptides are responsible for their different effects on Ca^{2+} affinity. The range of peptide-induced Ca^{2+} affinities, which corresponds to a free energy range of $\sim 6 \text{ kcal mol}^{-1}$ per four Ca^{2+} ions (Table 4), is unlikely to stem from the varying helix-formation propensities of aqueous target peptides for two reasons. First, the Ca^{2+} binding experiments were conducted at peptide concentrations sufficient to saturate the peptide binding equilibrium (see

Methods). Under such conditions, assuming that CaM selectively binds a helical conformer, the peptide association reaction is dominated by the binding of pre-formed target helices, which are expected to account for $\sim 20\%$ of the total solution population. Second, the free energy of the helix-coil transition for a typical 20–25 residue peptide is on the order of 1 kcal mol^{-1} (O'Neil & DeGrado, 1990b; Scholtz & Baldwin, 1992; Chakrabarty et al., 1994), which is small compared to the 6 kcal mol^{-1} range of Ca^{2+} binding energy triggered by the different target peptides (Table 4).

Instead, the results suggest that two distinct types of inter-molecular contacts may dominate the tuning of Ca^{2+} binding to CaM–target complexes: domain-specific contacts and dual-domain contacts. Domain-specific tuning stems from direct interactions between a given CaM domain and the region of the target peptide that is docked to that domain. Such tuning is illustrated by partial peptides from two different target sequences. N-terminal partial peptides (sk-N11 and CaATP-N18) selectively tighten Ca^{2+} binding to the high-affinity C domain of CaM, and C-terminal peptides (sk-C10 and CaATP-C17) have dominant effects on Ca^{2+} binding to the lower affinity N domain. It is clear that partial peptides from both ends of the target sequence retain the same domain specificity of tuning that they exhibit in the full-length peptide. However, the dual-domain contacts provided by the full-length skMLCK and CaATPase peptides are able to enhance the Ca^{2+} affinity of both CaM domains by a significantly larger factor than do their corresponding partial peptides; for the skMLCK peptide there is an additional 14-fold affinity enhancement beyond that attributable to the domain-specific interaction of sk-N11. This extra enhancement is not observed when the sk-N11 and sk-C10 peptides are added to CaM simultaneously, demonstrating that the full effect requires a covalent linkage between the N- and C-terminal regions of the skMLCK target sequence. Such covalent continuity is likely to increase the stability of the Ca_4^{2+} –CaM–target complex, thereby favoring the fully Ca^{2+} -occupied form of CaM. The Ca^{2+} binding curves obtained in the presence of the CaATP-N18 and CaATP-C17 peptides further support this model. The increased lengths of

Table 4. Estimated free energies of Ca^{2+} binding to CaM–target complexes

Target peptide	ΔG_1^a (kcal mol $^{-1}$ per Ca^{2+})	ΔG_2^a (kcal mol $^{-1}$ per Ca^{2+})	$2\Delta\Delta G_1 + 2\Delta\Delta G_2^b$ (kcal mol $^{-1}$ per 4 Ca^{2+})
None	–7.8	–6.7	—
PhK5	–9.0	–8.2	–5.7
CaATPase	–9.6	–9.1	–8.6
smMLCK	–9.5	–9.1	–8.5
skMLCK	–10	–9.7	–12
MKII	–10	–10	–12
skMLCK Protein (20 °C)	–8.8	–8.3	–5.3

^aCalculated at $\Delta G = -RT \ln K_D$ based on the K_{D1} and K_{D2} values determined by fitting the equilibrium binding data to the two cooperative event model (Table 2). Using the domain assignments described in Results, ΔG_1 and ΔG_2 correspond to the average free energy of binding a single Ca^{2+} ion to the C and N domain, respectively.

^bThe net effect of target peptide binding to CaM on the binding free energy of four Ca^{2+} , relative to the binding free energy of four Ca^{2+} in the absence of target peptide. For each domain, the effect of target peptide on the binding of a single Ca^{2+} ($\Delta\Delta G_n$) is given by

$$\Delta\Delta G_n = \Delta G_n^{\text{CaM} \cdot \text{target}} - \Delta G_n^{\text{CaM}}$$

where n specifies the domain and $\Delta G_n^{\text{CaM} \cdot \text{target}}$ and ΔG_n^{CaM} are the appropriate values from elsewhere in this table.

these peptides result in Ca^{2+} affinities which are closer, but not equal, to those seen in the full-length CaATPase peptide complex. The CaATPase partial peptides reiterate the seemingly strict requirement for hydrophobic anchor residues at both ends of the peptide in order to elicit the full enhancement of Ca^{2+} binding, as suggested by the deep hydrophobic pockets formed on each domain of CaM upon Ca^{2+} binding (reviewed by Ikura, 1996).

Ca^{2+} affinity tuning: Target peptides vs. target proteins

The target sequences that generate the highest Ca^{2+} affinities, MKII and skMLCK, actually shift the macroscopic K_D for Ca^{2+} binding to ~ 30 nM, which is below the resting level of free Ca^{2+} in a typical eukaryotic cell, estimated to be ~ 100 nM (Clapham, 1995). Even the K_D of the regulatory N domain is observed to shift below the resting Ca^{2+} level (Table 2), which could lead to constitutive CaM-dependent activation in vivo. It follows that intact target proteins must have additional tuning interactions that weaken the macroscopic Ca^{2+} affinity of their CaM complexes to ensure that the affinity of the N domain, in particular, is above the resting intracellular Ca^{2+} level. The native skMLCK protein, for example, has been shown to generate a macroscopic Ca^{2+} affinity of ~ 700 nM under conditions comparable to those of the present study (Crouch et al., 1981) and similar results have been observed for the smooth muscle isoform (Fitzsimons et al., 1992). The onset of Ca^{2+} -CaM-dependent activation of skMLCK has also been observed to require significantly higher Ca^{2+} concentrations than those needed to saturate direct Ca^{2+} binding to CaM in the presence of the skMLCK peptide (Yagi et al., 1989), further suggesting that the interactions between CaM and its targets are weaker for full-length proteins than for their individual target helices. Using our experimental conditions, we have confirmed that the Ca^{2+}

affinity of the CaM-skMLCK protein complex is at least an order of magnitude weaker than that of the corresponding CaM-skMLCK peptide complex. For the protein complex the K_D values of the N and C domains are 800 nM and 380 nM, respectively, as compared to 80 nM and 20 nM for two domains in the peptide complex.

The lower Ca^{2+} affinity observed for the CaM-skMLCK protein complex has important implications for the activation of its CaM-regulated pathway in a cellular environment. One proposed mechanism for activation, based on the higher Ca^{2+} affinity observed for the C domain of a CaM-peptide complex, begins with CaM pre-bound to the target protein via its C-terminal domain at resting levels of intracellular Ca^{2+} . Enzyme activation then only requires the binding of Ca^{2+} to the lower affinity N domain during a Ca^{2+} signal (Bayley et al., 1996; Johnson et al., 1996). Such a mechanism is quite plausible and is likely to describe the activation of a number of CaM target proteins. However, the present results for the CaM-skMLCK protein complex do not predict significant Ca^{2+} occupancy ($<10\%$) of the C domain at resting levels of intracellular Ca^{2+} . Rather, the higher Ca^{2+} threshold required for saturation of the CaM-protein complex as compared to the CaM-peptide complex suggests that, within cells, skMLCK activation is initiated some time after the Ca^{2+} signal has begun, either by the binding of Ca_2^{2+} -CaM via its Ca^{2+} loaded C domain or by the essentially simultaneous binding of both domains of Ca_4^{2+} -CaM to the target enzyme. Clearly, both the Ca^{2+} threshold and kinetics will be quite different for mechanisms beginning with pre-bound versus freely diffusing CaM. Because different cell types vary greatly in the fraction of CaM that is pre-bound, and because certain target complexes are known to use CaM as a bound subunit (Klee & Vanaman, 1982; Means, 1988; Luby-Phelps et al., 1995), it appears likely that both extremes of activation mechanisms are physiologically important, providing a wide range of thresholds and timecourses for Ca^{2+} /CaM activation.

Table 5. Ca^{2+} binding to calmodulin-skMLCK peptide and protein complexes

	CaM	CaM-skMLCK peptide	CaM-skMLCK protein ^a
Equilibrium Ca^{2+} binding ^b			
K_{D1} (μM)	2.0 ± 0.1	0.02 ± 0.01	0.38 ± 0.05
K_{D2} (μM)	13.0 ± 0.6	0.08 ± 0.06	0.80 ± 0.06
H_1	1.4 ± 0.2	2.3 ± 1.8	1.6 ± 0.2
H_2	1.7 ± 0.3	0.9 ± 0.6	1.6 ± 0.5
Ca^{2+} dissociation ^c			
k_{offA} (s^{-1})	11.8 ± 0.1	8.4 ± 0.2^a	0.36 ± 0.01
n_A ($\text{Ca}^{2+}/\text{CaM}$)		2.1 ± 0.1^a	3.4 ± 0.1
k_{offB} (s^{-1})	$\geq 850 \pm 70$	730 ± 60^a	—
n_B ($\text{Ca}^{2+}/\text{CaM}$)		2.4 ± 0.2^a	ND ^d
Calculated Ca^{2+} k_{on} ^e			
N domain ($\times 10^6 \text{ M}^{-1} \text{ s}^{-1}$)	$\geq 65 \pm 6$	80 ± 40	0.65 ± 0.04
C domain ($\times 10^6 \text{ M}^{-1} \text{ s}^{-1}$)	5.9 ± 0.3	18 ± 5	1.4 ± 0.1

^aData obtained at 20°C to maximize CaM-skMLCK complex stability (see Results).

^bTwo cooperative site binding model (see Methods and Table 2). Data obtained in 20 mM MOPS, 100 mM KCl, 0.5 mM MgCl_2 , pH 7.4 at 25°C.

^cSame conditions as above except MgCl_2 was omitted. n indicates observed dissociation stoichiometry.

^dNot determined since the high Ca^{2+} affinities of the CaM-peptide complexes interfere with stoichiometry determination (see Results and Methods).

^eApparent k_{on} value as described in Table 3.

Ca^{2+} release tuning: Target peptides vs. target proteins

The kinetics of Ca^{2+} release from the CaM-skMLCK protein complex differ substantially from those of all the CaM-peptide complexes in that Ca^{2+} dissociation from the N domain is slowed to the same time scale as C domain dissociation, yielding mono-exponential kinetics in which all four Ca^{2+} ions dissociate at a single observed rate of 0.52 s^{-1} . Ca^{2+} dissociation from the CaM N domain is 10-fold slower in the protein complex than in the peptide complex, while dissociation from the C domain is essentially unchanged. The observation that Ca^{2+} dissociation from the N domain is substantially slower in the protein complex than in the peptide complex, despite having a lower Ca^{2+} affinity, indicates that the N domain Ca^{2+} sites are somehow occluded in the complex with the full-length protein. Such occlusion may stem from steric or electrostatic barriers blocking a dissociation pathway to the solvent or could represent the inhibition of a structural rearrangement required for Ca^{2+} release from the coordination site. Functionally, the occlusion serves to extend the lifetime of the activated protein complex.

The monophasic Ca^{2+} dissociation curve observed for the release of all four Ca^{2+} ions from the activated CaM-skMLCK protein complex implies that there is a single rate-limiting event resulting

in complete enzyme inactivation. The specific nature of this trigger event is not known, but several lines of evidence suggest that it may be driven by the dissociation of the CaM C domain from the N-terminal end of the target sequence. A similar model has been proposed by Bayley et al. (1996), who have argued that dissociation of a C domain- Ca^{2+} -target complex is an ordered two-step process initiated by the target peptide dissociating from Ca^{2+} -loaded CaM and followed by the rapid loss of Ca^{2+} from the resulting Ca^{2+} -C domain complex. As predicted by their model, the observed Ca^{2+} dissociation rate of $\sim 0.52 \text{ s}^{-1}$ from the CaM-skMLCK protein complex (Table 5) correlates well with the $\sim 1 \text{ s}^{-1}$ rate of skMLCK inactivation when triggered by addition of Ca^{2+} chelators (Persechini et al. 1996; Stull et al., 1986) and the $\sim 2 \text{ s}^{-1}$ rate determined for CaM-skMLCK complex dissociation (Johnson et al., 1981).

Significantly, the observed Ca^{2+} dissociation rate of the protein complex (0.52 s^{-1}) is similar to the rate of Ca^{2+} dissociation from the C domain in both the CaM-skMLCK peptide complex (0.36 s^{-1}) and the CaM-sk-N11 partial peptide complex (0.73 s^{-1}), supporting the hypothesis that a dissociation event involving the C domain is the rate-limiting step in the breakdown of the CaM-protein complex. Dissociation of two Ca^{2+} ions from the bound C domain, or dissociation of the fully Ca^{2+} -occupied C domain, would trigger dissociation of the CaM-protein complex and the

Fig. 6. Model for the Ca^{2+} -CaM triggered activation and inactivation of skeletal muscle myosin light-chain kinase (skMLCK). Shown are the most common sequence of events and intermediates during activation (steps 1–3) and inactivation (steps 4–6) of skMLCK under cellular conditions. At resting Ca^{2+} levels (10^{-7} M) CaM (red) exists primarily as the free apo protein while the target sequence (blue) is docked in or near to the kinase active site, where it effectively blocks kinase activity. When a Ca^{2+} signal, or influx, occurs the peak Ca^{2+} levels (10^{-5} M) drive Ca^{2+} binding to CaM, first to the high-affinity C domain (step 1) and subsequently to the lower affinity N domain (step 3). These steps simultaneously trigger the binding of the two domains to the target helix in the ordered manner shown (steps 2 and 3), thereby activating the kinase and occluding the two N domain Ca^{2+} ions (outlines). Finally, as the Ca^{2+} concentration falls back to its resting level during Ca^{2+} efflux the C domain typically dissociates first from the target, yielding a transient intermediate where the N domain remains bound with its occluded Ca^{2+} ions (step 4). Immediately thereafter the bound CaM and its remaining Ca^{2+} ions are released, returning the system to the inactive state (steps 5 and 6). Overall, the predominant equilibrium species are a) the auto-inhibited kinase and free apo CaM (at resting Ca^{2+} levels; left box), and b) the active kinase in its complex with Ca_4^{2+} -CaM (at peak Ca^{2+} levels; right box).

rapid release of the remaining Ca^{2+} from the N and C domains of CaM at rates comparable to those observed in free CaM. Such a model is attractive because it implies that the rate of target enzyme inactivation is dominated by interactions between the CaM C domain and the N-terminal half of the target helix, with little additional tuning provided by the rest of the protein, and as such can be predicted by studying the behavior of CaM-peptide complexes. It should be pointed out, however, that the kinetic data presented here do in no way exclude the CaM N domain from playing a role in triggering the deactivation of the CaM-skMLCK protein complex, especially considering the striking occlusion of Ca^{2+} dissociation from the N domain sites, which may lie at a critical docking interface. Figure 6 presents a schematic model summarizing the predominant molecular events during the activation and inactivation of skMLCK *in vivo*.

Origins of the different tuning observed for target peptides and proteins

One molecular explanation for the different tuning effects observed when CaM is complexed with isolated peptides and full-length proteins can be derived from the mechanism of CaM-dependent enzyme regulation. Many CaM-regulated kinases, including skMLCK, smMLCK, and MKII, are activated by the removal of an autoinhibitory sequence from the active site of the enzyme, an event that can be triggered either by the binding of CaM to a nearby sequence or by phosphorylation of key residues (Zurini et al., 1984; Edelman et al., 1985; Pearson et al., 1988; Olson et al., 1990; Gallagher et al., 1993; Brickey et al., 1994; Krueger et al., 1995; Goldberg et al., 1996). For these proteins, the smaller Ca^{2+} affinity enhancement observed for the full-size enzyme relative to the isolated target peptide is likely to stem, at least in part, from the energetic cost of removing autoinhibition. The full-length Ca^{2+} ATPase molecule provides an analogous energetic cost to CaM binding that is absent in the corresponding target peptide, because CaM binding is 40-fold tighter for the isolated peptide than to the intact protein (Enyedi et al., 1989). Such considerations imply that target peptides from enzymes exhibiting the strongest autoinhibition will yield the highest Ca^{2+} affinity CaM-peptide complexes.

Superimposed on the need to counterbalance the energetic cost of auto-inhibition is the independent requirement that each CaM-target enzyme complex must exhibit Ca^{2+} concentration threshold and kinetic parameters that are optimized for its specific physiological role. As a result, the interactions between CaM and the target sequence must be carefully adjusted to account not only for the unique features of the target molecule, but also for the requirements of the pathway that the target molecule regulates. It should be emphasized that while the binding of CaM to its target sequence provides an important component of the overall tuning, the bound CaM appears to interact with additional surfaces of the target molecule, thereby increasing the number of contacts that could contribute to intermolecular tuning. An example of such additional tuning effects is the 10-fold reduction in the observed rate of Ca^{2+} dissociation from the N domain in the CaM-skMLCK protein complex as compared to the analogous peptide complex. Such kinetic tuning may serve a critical physiological role by optimizing the rate of kinase inactivation for skeletal muscle function.

In closing, the present study further defines the nature of the intermolecular tuning generated by the binding of CaM to isolated target helices. The data show that target peptides can tune the Ca^{2+} binding characteristics of CaM over a much broader range than

previously observed for intact target proteins. Although the isolated target sequence clearly provides a major component of intermolecular tuning, additional interactions occur in the full-length CaM-protein complex that bring the threshold for Ca^{2+} activation into the physiologically appropriate range and further tune the kinetics of Ca^{2+} dissociation. It is clear that a molecular understanding of the interaction between CaM and its targets will require further dissection of the target sequences to identify the key structural and electrostatic motifs that regulate Ca^{2+} binding to and release from this important mediator of Ca^{2+} signaling.

Methods

Calmodulin and peptides

The Ca^{2+} binding experiments were carried out using *Drosophila* calmodulin that was overexpressed with the pJFM39 vector (Maune et al., 1992) in *E. coli* strain AR68 (A. Shatzman, SmithKline Beecham Inc., King of Prussia, PA) using temperature induction of the phage λ P₁ promoter. *Drosophila* CaM differs from vertebrate CaM at only three positions (Tyr 99 → Phe, Gln 143 → Thr, Ala 147 → Ser) (Smith et al., 1987) and activates target enzymes with an efficiency comparable to that of the vertebrate form (Gao et al., 1993). The protein was purified using phenyl-sepharose chromatography followed by a G-25 gel filtration column to remove EGTA (Putkey et al., 1985), exchanged into 100 mM KCl, 20 mM MOPS, 5 mM CaCl_2 , pH 7.4 and concentrated to ~150 μM using an Amicon YM10 membrane prior to being snap frozen in for storage at -70°C . The final protein preparation exhibited its characteristic Ca^{2+} -dependent mobility shift on SDS-polyacrylamide gels (Klee et al., 1979) and was >99% pure as judged by Coomassie and silver staining. Liquid chromatography electrospray mass-spectrometry (LC/ESI-MS) showed that in the presence of Ca^{2+} the protein eluted from a Poros C₄ reverse phase column in two peaks; a less hydrophobic species with a molecular weight of 16,679 Da and a more hydrophobic 16,838 Da species that is consistent with the binding of four Ca^{2+} ions.

Peptides corresponding to the CaM binding domains of CaM-regulated enzymes were synthesized and purified to >90% homogeneity by Macromolecular Resources (Colorado State University, Fort Collins, CO) or by Dr. Spencer Anthony-Cahill (Somatogen Inc., Boulder, CO), and the expected molecular masses were confirmed by mass spectrometry. Stock solutions of 0.9–1.5 mM peptide were prepared in the buffer used for flow dialysis experiments (see below), where peptide concentrations were determined by absorbance based on Trp ($\epsilon_{280} = 5600 \text{ M}^{-1} \text{ cm}^{-1}$) or Phe ($\epsilon_{258} = 200 \text{ M}^{-1} \text{ cm}^{-1}$) residues. The affinity of the sk-N11 peptide for Ca^{2+} loaded CaM was determined by observing peptide Trp fluorescence during direct titration with CaM, resulting in a 0.4 μM K_D . *In vivo* total concentrations of CaM and its target proteins have generally been determined to be in the 1 μM range, although the concentration of freely diffusible CaM is lower (Klee & Vanaman, 1982; Luby-Phelps et al., 1995), and experimental CaM and target concentrations similar to these were used. A CaM concentration of ~0.9 μM was used in experiments involving the skMLCK, smMLCK, Ca^{2+} ATPase, and MKII peptides, where the Ca^{2+} affinities of the complexes were 0.2 μM or less. Experiments involving PhK5 and the four partial peptides were done at 1–7 μM CaM to more accurately detect the weaker Ca^{2+} binding of these complexes, and free CaM experiments were conducted at 10–25 μM protein. At high stock peptide concentrations the

Ca^{2+} ATPase peptide exhibited solubility problems, as previously noted by Vorherr et al. (1990). It did, however, become soluble in the standard flow dialysis buffer upon addition of formic acid to 1%. To compensate for the increased acidity, the MOPS concentration of the flow dialysis buffer was increased from 20 to 50 mM and the pH was adjusted to 7.6 prior to adding the peptide stock solution, resulting in a final pH of 7.4.

Determination of Ca^{2+} binding curves and dissociation constants

Ca^{2+} binding measurements were carried out by flow dialysis (Porumb, 1994; Colowick & Womack, 1969) at 25 °C using a home-built, temperature-controlled three-cell flow dialyzer machined from Teflon. The flow dialyzer and all plasticware were decalcified by washing in 33% nitric acid, followed by a 20 mM EDTA bath and extensive rinsing with distilled water. The flow dialysis buffer, containing 100 mM KCl, 0.5 mM MgCl_2 , 20 mM MOPS at pH 7.4, was decalcified by passage over a Chelex-100 column (BioRad) prior to adding MgCl_2 . The presence of this second divalent ion significantly shortened the response time of the flow-dialysis apparatus and reduced scatter in the data points, presumably because Mg^{2+} acts to block non-specific divalent ion binding sites on the dialysis membrane. The enhanced precision resulting from the inclusion of Mg^{2+} was most striking for the highest Ca^{2+} affinity complexes (i.e., those experiments extending to very low free Ca^{2+} concentrations), where reliable binding curves could not be obtained in the absence of Mg^{2+} .

Freshly decalcified CaM was prepared for each Ca^{2+} binding experiment using a small Chelex-100 column whose effectiveness was ascertained by atomic absorption spectroscopy. The upper sample chamber of the dialyzer held a 1–1.7 mL sample and the lower pumped chamber had a total volume of 70 μL , which followed a serpentine path in contact with the dialysis membrane. Spectra-Por 7 metal-free membranes with a 1000 MWCO were used after extensive washing. The lower chamber was pumped at a rate of $\sim 300 \mu\text{L}/\text{min}$ using a Pharmacia P-3 peristaltic pump while collecting five 0.9-min fractions for each Ca^{2+} addition to the upper sample chamber. All Ca^{2+} additions were made using a 100 μL Hamilton syringe attached to a 50-step repeating injector and fitted with narrow plastic protein-gel loading tips. Ca^{2+} solutions were made using 99.99% pure CaCl_2 (Aldrich) and all buffer and protein solution volumes were determined by weight after correcting for buffer density.

Ca^{2+} binding curves were generated from ^{45}Ca scintillation counting data and were corrected for the loss of both radioactive ^{45}Ca and non-radioactive ^{40}Ca during the experiment as previously described (Maune et al., 1992; Yazawa et al., 1992; Stemmer & Klee, 1994). Typically, only 5–10% of the ^{45}Ca initially added to the sample cell diffused across the membrane during the experiment. Finally, the 2σ confidence interval reported by the scintillation counter was propagated by standard methods to produce errors in both the free Ca^{2+} concentration and the $\text{Ca}^{2+}/\text{CaM}$ stoichiometry at each point in the titration curve. Ca^{2+} binding constants were determined by fitting the data to the following equation describing the binding of four ions as two cooperative binding events (Porumb, 1994), where X is the free Ca^{2+} concentration, the factor c absorbs errors in the stoichiometry determination, and the K_D and H terms are the macroscopic dissociation constants and Hill coefficients of the two binding events.

$$\text{Ca}^{2+}/\text{CaM} = c \left[\frac{2}{1 + (K_{D1}/X)^{H1}} + \frac{2}{1 + (K_{D2}/X)^{H2}} \right].$$

The data were fit using KaleidaGraph 3.0 software for the Macintosh (Synergy Software, Reading, PA), which employs a Levenberg-Marquardt algorithm to determine the non-linear least squares best fit. For each point in the titration curve, the root mean square of the relative errors in both the $[\text{Ca}^{2+}]_{\text{Free}}$ and $\text{Ca}^{2+}/\text{CaM}$ dimensions (RMS error = $[(\Delta x/x)^2 + (\Delta y/y)^2]^{1/2}$) was designated as a data weighting factor during curve fitting. Data points determined to higher precision were thus given more emphasis during the fitting process. Accounting for error in both dimensions significantly improved the quality of the curve fits by balancing the dominant error in the $[\text{Ca}^{2+}]_{\text{Free}}$ early in the titration with the dominant error in the $\text{Ca}^{2+}/\text{CaM}$ stoichiometry found late in the titration.

Stopped flow dissociation kinetics

The time courses of Ca^{2+} dissociation from CaM and its peptide complexes were determined by fluorescence using the chelator Quin-2 (Molecular Probes, Eugene, OR) in an Applied Photophysics model 17MV stopped flow apparatus with a minimum dwell time of 20 μs and a dual time-base signal acquisition mode that allows one to accurately digitize both fast and slow events in a single experiment. The dead time of the stopped-flow apparatus was determined using the reduction of 2,6-dichlorophenolindophenol by ascorbic acid as described by Tonomura et al. (1978), revealing a dead time of 1.0 ms and a linear response up to an apparent reaction rate of $\sim 750 \text{ s}^{-1}$. Consequently, the first 1.5 ms of the observed dissociation time courses were omitted when curve fitting the data and rates in excess of 750 s^{-1} are designated as lower limits.

For the CaM-peptide complex studies, solutions containing 4–6 μM CaM, a range of 0–15 μM peptide, and 60 μM CaCl_2 in Mg^{2+} -free flow dialysis buffer were stopped-flow mixed with a solution of 200 μM Quin-2 in the same buffer. Quin-2 fluorescence was observed through a 490 nm bandpass filter ($\lambda_{\text{ex}} = 332 \text{ nm}$). A total of eight transients were acquired at each peptide concentration. The stopped flow apparatus was used in absorbance mode to observe changes in Tyr 138 absorbance at 276 nm as CaM solutions were mixed with 5 mM EDTA.

Although the major effect on the Ca^{2+} dissociation rate was established at equimolar concentrations of CaM and peptide, the observed dissociation rate decreased further when increasing concentrations of peptide were added. As recently pointed out by Bayley et al. (1996), this effect can be attributed to the rebinding of Ca^{2+} to CaM rather than Quin-2 after an initial dissociation event; such back-flow of Ca^{2+} reduces the observed dissociation rate and the effect becomes greater as the CaM-target equilibrium is pushed further toward formation of the high Ca^{2+} affinity CaM-target complex by addition of excess peptide. Consequently, the Ca^{2+} dissociation rates for all five peptides were determined over a range of peptide concentrations, and the reported rates (Table 2) are averages representing the asymptotic values achieved at saturating peptide concentrations (10–15 μM peptide and 3–5 μM CaM).

The Ca^{2+} dissociation rate of the CaM-skMLCK protein complex was determined at 20 °C using 150 μM Quin-2 in one syringe and 100 μM CaCl_2 with 1 μM CaM and 0–2.5 μM skMLCK in the other. Dithiothreitol was added to 1 mM. A skMLCK titration was used to confirm that the skMLCK to CaM ratio, based on

Bradford and BCA protein concentration assays, was sufficient to saturate the interaction between the two proteins. Separate experiments using a series of CaCl_2 solutions with concentrations ranging from 73 to 112 μM showed that the Quin-2 fluorescence response was linear over this range with a slope of 15.0 mV/ μM Ca^{2+} added, and this slope was used to convert the amplitudes of the observed dissociation rates into Ca^{2+} stoichiometries. Note that the high Ca^{2+} affinities of the CaM-peptide complexes interfere with the amplitude-based stoichiometry determination due to significant residual binding of Ca^{2+} at the end of the kinetic time-course (see Results). Consequently, only data from free CaM and the CaM-skMLCK protein complex were analyzed in terms of the number of Ca^{2+} ions dissociating per CaM molecule.

Calculated association constants

It should be noted that the calculated k_{on} ($= k_{off}/K_D$) values listed in Table 3 are best considered as *apparent* constants for two reasons. First, the flow dialysis binding experiments required the presence of 0.5 mM Mg^{2+} to get reliable Ca^{2+} K_D values for the high affinity CaM-target complexes, but the k_{off} values were determined in the absence of Mg^{2+} as it would interfere with Ca^{2+} binding to Quin-2. Although Mg^{2+} is largely a spectator ion in this system, the 0.5 mM concentration of Mg^{2+} may increase the Ca^{2+} K_D values up to a maximum of twofold due to competitive binding to EF-hand sites (see below). Second, the observed k_{off} values for the CaM-target complexes are themselves apparent rate constants that are slightly dependent on the target peptide concentration (Bayley et al., 1996; see Methods). Overall, these two effects will have minimal impact on the comparisons among the different CaM-peptide complexes because the binding experiments were all carried out under identical conditions and the reported k_{off} rates represent the plateau values observed at saturating amounts of peptide.

Comparison of our k_{on} values with those obtained by other laboratories in the absence of Mg^{2+} can be made by considering the effect of competing Mg^{2+} ions on the observed Ca^{2+} K_D value, which is described by the relationship

$$K_D^{obs} = K_D(1 + [\text{Mg}]/K_{D_{\text{Mg}^{2+}}}).$$

Tsai et al. (1987) investigated the binding of Mg^{2+} to CaM by NMR methods and determined that Mg^{2+} binds the CaM N and C domains with dissociation constants of ~ 0.5 mM and 3.3 mM, respectively, in the absence monovalent ions. Applying this correction to our binding data determined in the presence of 0.5 mM Mg^{2+} indicates that the added Mg^{2+} results in a small twofold increase in the N domain K_D and a negligible ~ 1.15 -fold effect on the C domain K_D .

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