Activation of Myosin Light Chain Kinase and Nitric Oxide Synthase Activities by Engineered Calmodulins with Duplicated or Exchanged EF Hand Pairs†

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ABSTRACT: We have constructed three engineered calmodulins (CaMs) in which the two EF hand pairs have been substituted for one another or exchanged: CaMNN, the C-terminal EF hand pair (residues 82−148) has been replaced by a duplication of the N-terminal pair (residues 9−75); CaMCC, the N-terminal pair has been replaced by a duplication of the C-terminal pair; CaMCN, the two EF had pairs have been exchanged. Skeletal muscle myosin light chain kinase (skMLCK) activity is activated to 75% of the maximum level by CaMCC and to 45% of the maximum level by CaMCN and is not significantly activated by CaMNN; K_{act} or K_i values for the engineered CaMs are 2−3.5 nM. Smooth muscle myosin light chain kinase activity (gMLCK) is fully activated by CaMCN and is not significantly activated by either CaMNN or CaMCC; the K_{act} value for CaMCN is 2 nM and the K_i values for CaMNN and CaMCC are 10 and 40 nM, respectively. Cerebellar nitric oxide synthase activity (nNOS) is fully activated by CaMNN and CaMCN and is not significantly activated by CaMCC; the engineered CaMs have K_{act} or K_i values for this enzyme activity of 2−8 nM. These results indicate that the EF hand pairs contain distinct but overlapping sets of determinants for binding and activation of enzymes, with the greater degree of overlap in determinants for binding. Furthermore, while the structural changes associated with swapping the EF hand pairs do not affect activation of nNOS or gMLCK activities, they significantly reduce activation of skMLCK activity, indicating that this process requires specific determinants in CaM outside the EF hand pairs.

The ubiquitous Ca^{2+}-binding protein, calmodulin (CaM),[1] is responsible for the activation of numerous enzyme activities occurring when the intracellular Ca^{2+} ion concentration is elevated from a resting level less than 10^{-7} M to a level of $\sim 10^{-6}$ M (Manalan & Klee, 1984). Many of these enzyme activities are themselves at the top of regulatory cascades, such as CaM-dependent protein kinase II and nitric oxide synthase (Colbran & Soderling, 1990; Bredt & Snyder, 1990; Cho et al., 1992). CaM has considerable internal structural homology, its 148 residues consisting almost entirely of two pairs of EF hand Ca^{2+}-binding domains comprising residues 9−75 and 82−148 (Manalan & Klee, 1984; Babu et al., 1988). The main-chain coordinates of these regions are essentially superimposable (Babu et al., 1988). The crystal structure of CaM indicates a dumbbell-shaped molecule in which two globular lobes containing the EF hand pairs are joined by a shared central helix in which the central one-third is exposed to solvent on all sides (Babu et al., 1988). A reflection of CaMs internal homology is seen in its complexes with CaM-binding peptides, which exhibit an approximate 2-fold axis of symmetry relating the CaM lobes (Ikura et al., 1992; Meador et al., 1992). Several studies have suggested that the two CaM lobes share many determinants for binding and activation of target proteins (Newton et al., 1984; Guerini et al., 1984; Persechini et al., 1994). In spite of this, CaM has so far been observed to associated with model target peptides in only an antiparallel arrangement, where the C-terminal lobe of CaM is associated with the N-terminal region of the peptide and the N-terminal lobe of CaM is associated with the C-terminal region of the peptide (Ikura et al., 1991; Meador et al., 1992).

A difficulty with studies to date performed to investigate the roles of the CaM lobes is that they have utilized CaM tryptic fragments containing residues 1−75 or 78−148. To observe binding and/or enzyme activation, these fragments must be used at concentrations as much as 10^4-fold higher than required with intact CaM (Newton et al., 1984; Persechini et al., 1994). These CaM fragments also appear to compete with one another for binding to their respective sites on different targets, which necessitates complex kinetic analyses in order to extract information concerning the function of the lobes, particularly with respect to their functional interchangeability (Persechini et al., 1994). Furthermore, at high concentrations and, especially, without the constraint of the central helix, the fragments could bind to targets at exogenous sites or in relative orientations that are not possible with intact CaM. It is therefore important to confirm and extend our understanding of the functional properties of the CaM lobes using a more direct approach. This was undertaken using engineered proteins with high

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[1] Abbreviations: PCR, polymerase chain reaction; nNOS, rat cerebellar nitric oxide synthase; skMLCK, rabbit skeletal muscle myosin light chain kinase; gMLCK, chicken gizzard myosin light chain kinase; CaM, calmodulin; CaMCN, engineered protein in which CaM residues 9−75 and 82−148 have been exchanged; CaMCC, engineered protein in which CaM residues 9−75 have been replaced by the sequence of CaM residues 82−148; CaMNN, engineered protein in which CaM residues 82−148 have been replaced by the sequence of CaM residues 9−75; central helix linker, CaM residues 76−81; N-terminal leader, CaM residues 1−8.

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enzyme-binding affinities in which the two EF hand pairs (residues 9−75 and 82−148) have been exchanged or substituted for one another.

MATERIALS AND METHODS

Expression vectors for CaMNN, CaMCN, and CaMCC were assembled as follows. Our initial vector construction consisting of the CaM-encoding sequence cloned in M13mp18 that we have described previously (Persechini et al., 1989), except for the presence of a *Pvu*II endonuclease site whose DNA sequence, CAGCTG, encodes Q3 and L4 in the CaM amino acid sequence. This silent alteration in the CaM-encoding DNA sequence was introduced using primer-extension mutagenesis as described by Zoller and Smith (1984), with modifications suggested by Kunkel (1985). This CaM-encoding sequence was excised from M13mp18 via *Nco*I and *Pst*I endonuclease sites and inserted into the multiple cloning site of a pMEX7 vector with expression under control of a *trp-lac* hybrid promoter (United States Biochemicals). Substitution and exchange of DNA sequences to generate DNA encoding the desired engineered proteins were achieved using two rounds of PCR. In the first round, DNA segments encoding residues 9−75 or 82−148 in CaM were generated using primers with additional flanking sequences allowing the PCR products to be be combined, by virtue of a common DNA sequence encoding residues 76−81 in the CaM central helix, and amplified in a second round of PCR to give the DNA sequences encoding the desired engineered proteins. The final PCR products were cleaved with *Pvu*II and *Pst*I, purified by gel electrophoresis, and inserted into the original pMEX7 clone in place of the native CaM-encoding sequence. The identities of all engineered CaM-encoding DNAs were confirmed by performing cycle sequencing using fluorescently labeled dideoxynucleotides and *Taq* polymerase supplied in kit form by Applied Biosystems International and used according to the manufacturer's instructions. Native and engineered CaMs were expressed in *Escherichia coli* and purified as described by Persechini et al. (1989). Quantitative amino acid analysis and optical density data were used to calculate extinction coefficients for the engineered proteins which are as follows: CaMNN, $E_{257}^{0.1\%} = 0.13$; CaMCC, $E_{274}^{0.1\%} = 0.46$; and CaMCN, $E_{276}^{0.1\%} = 0.25$. Optical densities at the indicated wavelengths were determined after extensive dialysis of the purified proteins against buffer containing 10 mM ammonium bicarbonate and 50 μM $CaCl_2$.

skMLCK was expressed in Sf9 cells using a recombinant baculovirus and purified as described by Fitzsimmons et al. (1992). nNOS was expressed in stably transfected HEK A293 cells and purified as described by McMillan et al. (1992). gMLCK purified as described by Herring (1991) and the recombinant baculovirus used for expression of skMLCK were gifts from the laboratory of James T. Stull. Enzyme activation and competitive inhibition experiments were performed as described elsewhere (Persechini et al., 1994). All enzyme activities are expressed relative to the maximal enzyme activities measured in the presence of 100 nM CaM, which are ~300 nmol min^{-1} (mg of protein)$^{-1}$ for nNos and ~20 μmol min^{-1} (mg of protein)$^{-1}$ for skMLCK or gMLCK. Enzyme activation data were fit to an equation of the form

$$v = \frac{F_{act}[\text{activator}]}{K_{act} + [\text{activator}]} \quad (1)$$

where F_{act} is the maximal activation expressed as a fraction of the enzyme activity measured in the presence of 100 nM native CaM, v is the observed enzyme activity, and K_{act} is the concentration of activator giving half-maximal activation. Data for enzyme inhibition were fit to an equation of the form

$$v = \frac{\frac{[\text{CaM}]}{K_{CaM}} + F_i\frac{[\text{inhibitor}]}{K_i}}{1 + \frac{[\text{CaM}]}{K_{CaM}} + \frac{[\text{inhibitor}]}{K_i}} \quad (2)$$

where K_{CaM} is the CaM concentration giving half-maximal activation, K_i is the apparent dissociation constant of the inhibitor, and F_i is the fractional activation by the inhibitor. Nonlinear least-squares fits of these equations to the experimental data were performed using the Prism software package (GraphPad, Inc.). The predicted apparent dissociation constants (K_{calc}) for the complexes between CaMNN or CaMCC and nNOS, skMLCK, or gMLCK were calculated from the relation $K_{calc} = K_{CaM}R$. For CaMCC, R is the apparent dissociation constant for the complex between CaM fragment 78−148 and the enzyme site normally occupied by the N-terminal CaM lobe expressed as a fraction of the apparent dissociation constant for binding of CaM fragment 1−75 to this site. For CaMNN, R is the apparent dissociation constant for the complex between CaM fragment 1−75 and the enzyme site normally occupied by the C-terminal CaM lobe expressed as a fraction of the apparent dissociation constant for binding of CaM fragment 78−148 for this site. Values for the different apparent affinities of the CaM fragments and for K_{CaM} were taken from Persechini et al. (1994).

RESULTS

CaMCN, in which residues 82−148 and 9−75 have been exchanged, appears to bind and activate nNOS and gMLCK activities in a manner that is not significantly different from what is seen with CaM (Figure 1; Table 1). In contrast, CaMCN activates skMLCK activity to only 45% of the maximum level seen with a saturing CaM concentration, although it also appears to bind this enzyme as well as does the native protein (Figure 1; Table 1). Hence, the structural changes associated with swapping the EF hand pairs have no effect on binding or activation of nNOS and gMLCK but significantly reduce activation of skMLCK activity. CaMCC, in which residues 82−148 replace residues 9−75, activates skMLCK activity to 75% of the maximum with a K_{act} value of 3.5 nM (Figure 2; Table 1). CaMCC activates nNOS and gMLCK to levels less than 10% of the maximum. Data for competitive inhibition of CaM-dependent nNOS and gMLCK activities by CaMCC indicate K_i values for these proteins of 2.5 and 40 nM, respectively (Figure 3; Table 1). CaMNN activates both skMLCK and gMLCK to levels less than 15% of the maximum but fully activates nNOS activity with a K_{act} value of 8 nM (Figure 2; Table 1). Competitive inhibition of CaM-dependent skMLCK and gMLCK activities indicates K_i values for CaMNN of 2 and 10 nM, respectively (Figure 3; Table 1).

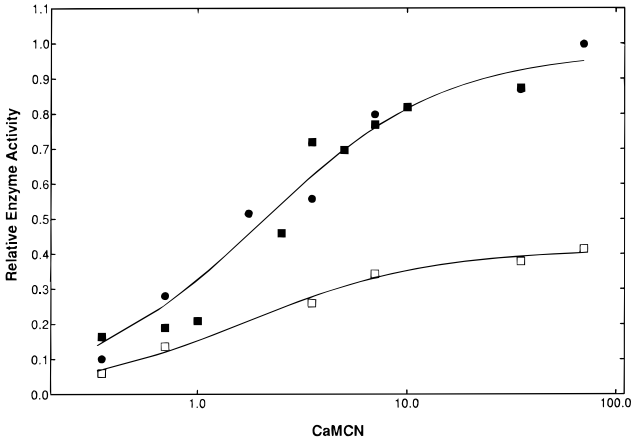

FIGURE 1: Activation of enzyme activity by CaMCN. Data for activation of skMLCK (□), gMLCK (●), and nNOS (■) activites are presented. Enzyme concentrations in all assays were 0.5 nM with CaMCN added to the indicated final concentrations. Curves in the figures were generated according to eq 1 using K_{act} values of 2 nM for all three sets of enzyme activation data. An F_{act} value of 1 was used for nNOS and gMLCK; a value of 0.45 was used for skMLCK.

Table 1: Steady-State Kinetic Parameters for Binding and Activation of Enzymes by CaMNN, CaMCN, and CaMCC[a]

	K_{act}	K_i	K_{calc}	F_{act}
		nNOS		
CaM	1			1
CaMNN	8		10	1.1
CaMCN	2			1
CaMCC		2.5	10	<0.1
		skMLCK		
CaM	1			1
CaMNN		2	40	<0.15
CaMCN	2			0.45
CaMCC	3.5		7	0.75
		gMLCK		
CaM	1			1
CaMNN		10	80	<0.15
CaMCN	2			1
CaMCC		40	25	<0.1

[a] K_{act} is the concentration (nM) of CaM or engineered CaM required for half-maximal activation of enzyme activity. K_i is the apparent dissociation constant (nM) based on competitive inhibition of CaM-dependent enzyme activity. F_{act} is the maximal enzyme activity expressed relative to the level of activity achieved with 100 nM CaM. K_{calc} is a predicted apparent dissociation constant (nM) calculated as described under Materials and Methods, except for the K_{calc} for CaMCC binding to nNOS, which is taken to be equivalent to the apparent dissociation constant for binding of CaM fragment 78−148 (Persechini et al., 1994).

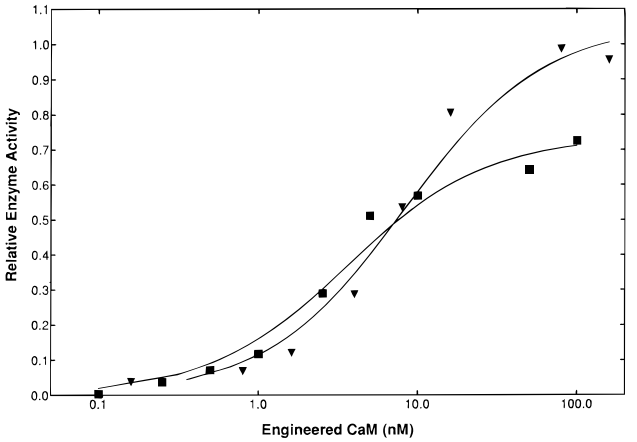

FIGURE 2: Activation of enzyme activity by CaMNN and CaMCC. Assay conditions were as described in the legend to Figure 1. Data for activation of skMLCK by CaMCC (■), and nNOS activity by CaMNN (▼) are presented. Data for enzyme activation giving F_{act} values less than 0.15 are not shown (Table 1). Curves in the figure were generated according to eq 1 using K_{act} values of 8 (nNOS) and 3.5 nM (skMLCK) and F_{act} values of 1 (nNOS) and 0.75 (skMLCK).

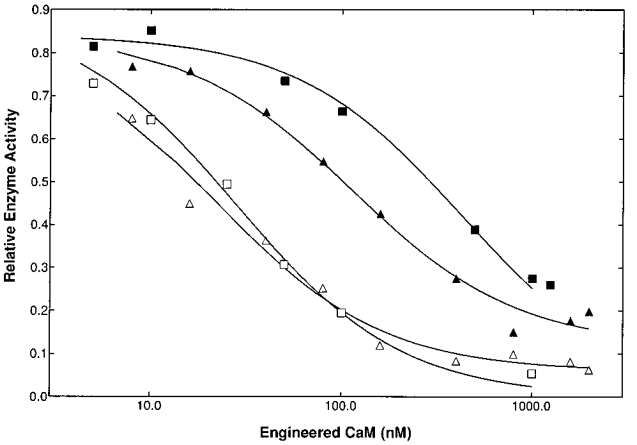

FIGURE 3: Inhibition of enzyme activity by CaMNN and CaMCC. Enzymes were at final concentrations of 1 nM with 10 nM added CaM. Data for inhibition of nNOS (□) and gMLCK (■) activites by CaMCC and of skMLCK (△) and gMLCK (▲) activities by CaMNN are presented. Curves in the figure were generated according to eq 2 using K_i values for CaMCC of 2.5 (nNOS) and 40 nM (gMLCK) and for CaMNN of 2 (skMLCK) and 10 nM (gMLCK). For CaMCC inhibition data a value for F_i of 0 was used. For CaMNN inhibition data F_i values of 0.06 (skMLCK) and 0.12 (gMLCK) were used.

DISCUSSION

The proteins we have constructed allow us to confirm and extend our understanding of the contributions made by the CaM lobes and the central helix linker region to activation of different enzyme activities. Furthermore, the engineered proteins allow us to investigate the extent to which specific functional determinants in the CaM lobes reside within the EF hand pairs.

We address this latter point primarily through CaMCN, in which residues 9−75 and 82−148 have been exchanged. CaMCN appears to bind and activate nNOS or gMLCK activities in a manner that is not significantly different from native CaM (Table I). This indicates that CaMCN forms complexes with these enzymes such that the exchanged EF hand pair amino acid sequences are bound at the locations they occupy in the corresponding native CaM−enzyme complexes. This is to be expected on the basis of the CaM−peptide structures that have been determined, which indicate little or no involvement of the linker or leader sequences in CaM (Ikura et al., 1992; Meador et al., 1992). In order for CaMCN to bind in the expected orientation, the leader and linker residues in the CaMCN−target complex must adopt positions in relation to the CaM-binding domain quite different from those seen in the native CaM−peptide complex (Figure 4). This suggests that the specific CaM determinants required for activation of nNOS and gMLCK activities are contained within the EF hand pairs.

In contrast, the results obtained with CaMCN and skMLCK suggest at least a requirement for specific contacts

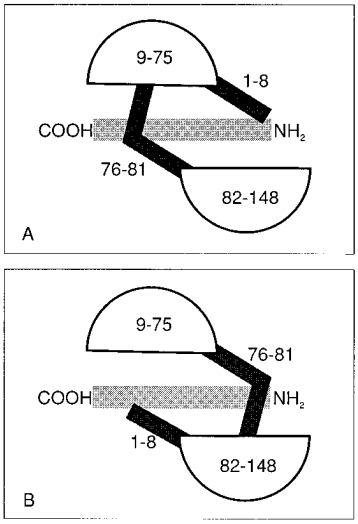

FIGURE 4: Schematic representations of CaM−peptide and CaMCN−peptide complexes. (A) Schematic for the CaM−peptide complex based on the structure for the complex between CaM and the CaM-binding domain in skMLCK (Ikura et al., 1991). The positions of the central helix linker (residues 76−81) and the N-terminal leader (residues 1−8) are indicated as are the positions of the N- and C-terminal EF hand pairs (residues 9−75 and 82−148). The N- and C-terminal ends on the bound peptide are indicated. (B) Schematic for the CaMCN−peptide complex assuming that the positions of residues 9−75 and 82−148 in relation to the bound peptide are the same as in the CaM−peptide complex. The sequence designations used for the N- and C-terminal EF hand pairs in CaMCN reflect their origins in native CaM and are used for clarity.

between this enzyme and residues in the N-terminal leader sequence of CaM that cannot form in a CaMCN−skMLCK complex of the type suggested in Figure 4 (Persechini et al., 1994). Consistent with this, we have reported similar levels of skMLCK activation by CaM fragment 78−148, which lacks residues 1−8 (Persechini et al., 1994). It is reasonably well established that amino acid residues in the central helix linker region play little or no specific role in the activation of skMLCK activity, although the tethering function of this region is necessary for high-affinity target binding (Putkey et al., 1988; Persechini & Kretsinger, 1988; Persechini et al., 1989, 1994). Nevertheless, a specific involvement of this region in activation of skMLCK activity cannot be ruled out.

It is evident that activation of skMLCK activity by CaM involves determinants outside the CaM EF hand pairs. Of course, this is also a possibility with the CaM−nNOS and CaM−gMLCK complexes, although our results suggest that any involvement of the leader or linker sequences of CaM in activation of these enzyme activities must either be rather nonspecific or independent of the spatial relationship between these regions and the EF hand pairs.

Experiments with CaMCC and CaMNN provide important confirmation of our earlier analysis of the roles played by the two CaM lobes based on studies with CaM tryptic fragments 1−75 and 78−148 (Persechini et al., 1994). Except for the complexes between CaMNN and skMLCK or gMLCK, there is excellent agreement between the observed K_i or K_{act} values for the CaMNN− and CaMCC−enzyme complexes examined and the values calculated on the basis of the apparent affinities of the CaM tryptic fragments (Table 1). Thus, the relative contributions of the EF hand pairs to the affinities of the different CaM−enzyme complexes examined can be accurately determined using CaM tryptic fragments 1−75 and 78−148. In particular, this result indicates that the central helix linker contributes to the affinity of different CaM−enzyme complexes only through a generalized tethering function; it does not appear to contribute to the specificity or avidity of the interactions formed between these enzymes and each of the CaM lobes. This conclusion is also supported by the observed high-affinity enzyme binding by CaMCN, in which the position of the central helix linker in relation to the two EF hand pairs has been dramatically altered.

The patterns of enzyme activation observed when a single CaM fragment occupies the sites for both CaM lobes on enzymes are remarkably similar to what we have observed with CaMNN and CaMCC (Table 1). In analogy with CaMCC, fragment 78−148 can activate skMLCK activity to 65% of the maximum level but does not signficantly activate either gMLCK or nNOS activities; in analogy with CaMNN, fragment 1−75 can activate nNOS activity to a level of 50% but activates skMLCK and gMLCK activities to ≤20% of their maximum levels (Persechini et al., 1994). This agreement between the behavior of CaMCC or CaMNN, which are effective at nanomolar concentrations, and CaM fragments, which are effective only at concentrations thousands of times higher, is significant because it demonstrates that the experimental results obtained with CaM fragments are not due to artifacts associated with the high fragment concentrations used (Persechini et al., 1994).

It is not clear why CaMNN binds skMLCK and gMLCK with K_i values 10−20-fold greater than expected on the basis of studies of CaM fragment 1−75. One possibility is that when it is not flanked by residues 1−8, the N-terminal EF hand pair binds more avidly to the site normally occupied by the C-terminal pair. If this proves to be true, it may also help to explain the relatively low level of activation of skMLCK activity by CaMCN, since it could result in a significant fraction of CaMCN−skMLCK complexes containing CaMCN bound in the polarity opposite that modeled in Figure 4. Investigations of possible effects of the N-terminal leader on the target-binding affinity of the N-terminal EF hand pair and on establishment of the polarity of bound CaM are ongoing in this laboratory.

The results we present here suggest that the EF hand pairs contain the specific determinants for binding and activation of gMLCK and nNOS activities, while activation of skMLCK activity appears to require specific determinants in at least the N-terminal leader sequence. In addition, our results indicate that the EF hand pairs contain distinct, but overlapping sets of determinants for binding and activation of enzymes, with the greatest degree of overlap in those determinants for enzyme binding. Hence, by intramolecular substitution of the EF hand pairs, engineered CaMs that cause little or no activation of gMLCK, skMLCK, and nNOS enzyme activities can be generated that exhibit K_i or K_{act} values for these enzymes only 2−10-fold greater than the corresponding K_{act} values for native CaM. VanBerkum and Means (1991) have similarly reported an engineered CaM containing three amino acid substitutions that binds gMLCK with an apparent dissociation constant 20−40-fold greater than that observed with native CaM and is a poor activator of this enzyme activity. The behavior of CaMNN with gMLCK and skMLCK is particularly striking in that the K_i values for competitive inhibition of these enzyme activities

by CaMNN are not significantly different from the K_{act} values observed with native CaM. This indicates that both lobes of CaMNN are interacting with the CaM-binding domains in skMLCK and gMLCK in analogy with bound CaM. Thus, it would appear that occupancy of the CaM-binding domains in these enzymes is necessary but not sufficient for activation of their enzyme activities. We cannot draw a similar conclusion with respect to the lack of activation of nNOS activity by CaMCC, since it is possible that only one lobe in CaMCC binds the CaM-binding domain in nNOS. So full occupancy of the CaM-binding domain may be both necessary and sufficient for activation of this enzyme activity.

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