Paramecium Calmodulin Mutants Defective in Ion Channel Regulation Associate with Melittin in the Absence of Calcium but Require It for Tertiary Collapse†

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Received October 3, 2000; Revised Manuscript Received November 16, 2000

ABSTRACT: Calmodulin (CaM) is a small acidic protein essential to calcium-mediated signal transduction. Conformational change driven by calcium binding controls its selective activation of myriad target proteins. In most well characterized cases, both homologous domains of CaM interact with a target protein. However, physiologically separable roles for the two domains were demonstrated by mutants of *Paramecium tetraurelia* [Kung, C. et al. (1992) *Cell Calcium 13*, 413], some of which have altered calcium affinities [Jaren, O. R. et al. (2000) *Biochemistry 39*, 6881]. To determine whether these mutants can associate with canonical targets in a calcium-dependent manner, their ability to bind melittin was assessed using analytical gel permeation chromatography, analytical ultracentrifugation, and fluorescence spectroscopy. The Stokes radius of wild-type PCaM and 11 of the mutants decreased dramatically upon binding melittin in the presence of calcium. Fluorescence spectra and sedimentation velocity studies showed that melittin bound to wild-type PCaM and mutants in a calcium-independent manner. However, there were domain-specific perturbations. Mutations in the N-domain of PCaM did not affect the spectrum of melittin (residue W19) under apo or calcium-saturated conditions, whereas most of the mutations in the C-domain did. These data are consistent with a calcium-dependent model of sequential target association whereby melittin (i) binds to the C-domain of PCaM in the absence of calcium, (ii) remains associated with the C-domain upon calcium binding to sites III and IV, and (iii) subsequently binds to the N-domain upon calcium binding to sites I and II of CaM, causing tertiary collapse.

Calmodulin (CaM)[1] is a small, acidic, calcium-binding protein that functions as the primary intracellular calcium sensor in eukaryotic cells. CaM is a member of the EF-hand family of proteins (*1*, *2*) with four helix−loop−helix calcium-binding sites (see Figure 1, panel A), two in each globular domain. Alignment of the four segments of *Paramecium* CaM (PCaM, see Figure 1, panel B) illustrates the high degree of sequence homology between the two domains (the N-terminal domain is composed of residues 1−75 and the C-terminal domain is residues 76−148) and among the four 12-residue calcium-binding sites.

Calmodulin associates with and activates over 30 different target proteins, many of which are essential for signal transduction, cell motility, growth, and morphogenesis, and calcium-activated ion channel regulation (*3*, *4*). In vivo studies by Kung et al. (*5*−*7*) have identified mutants in *Paramecium* CaM that cause deficits in ion channel regulation (both Na^+ and K^+ currents). These mutants were classified into two functional groups on the basis of their abnormal response to chemical stimuli. Mutants classified as *fast-2* were under-reactors to stimuli, a response associated with deficient Na^+ channel regulation (*5*). Mapping the underlying mutations revealed changes localized to the N-domain of PCaM, primarily between calcium-binding sites I and II (one mutation is within site II). The second group of mutants was classified as *pantophobiac*. They were over-reactors to stimuli, a response associated with deficient K^+ channel regulation. The underlying mutations were located in the C-domain of PCaM, primarily within calcium-binding sites III and IV (one is located in helix H in the hydrophobic cleft).

Biochemical characterization of 12 of these mutants [six in the N-domain and six in the C-domain (see Figure 1)] revealed that they can bind calcium and adopt conformational endstates equivalent to wild-type (WT) (*8*). The N-domain (under-reactive) mutations have no effect on calcium-binding to sites III and IV and appear to have minimal effects on sites I and II (W. S. Van Scyoc and M. A. Shea, unpublished). Most of the C-domain (over-reactive) mutations are in sites III and IV, and their intrinsic affinities and cooperativity are diminished. A consequence is that the sequential transition from apo to calcium-saturated PCaM is perturbed. Results from simulations, where the calcium affinities of sites III and IV were varied and calcium affinities of sites I and II were not altered by C-domain mutations (see Figure 7 in ref *8*), suggest that these mutations cause the affinities of the domains in PCaM to become more similar to each other than is observed in native PCaM (i.e., the separation of calcium affinities between the domains is smaller). The mutant M145V (see Figure 1) does not alter the calcium

† These studies were supported by a grant to M.A.S. from the National Institutes of Health (RO1 GM 57001).

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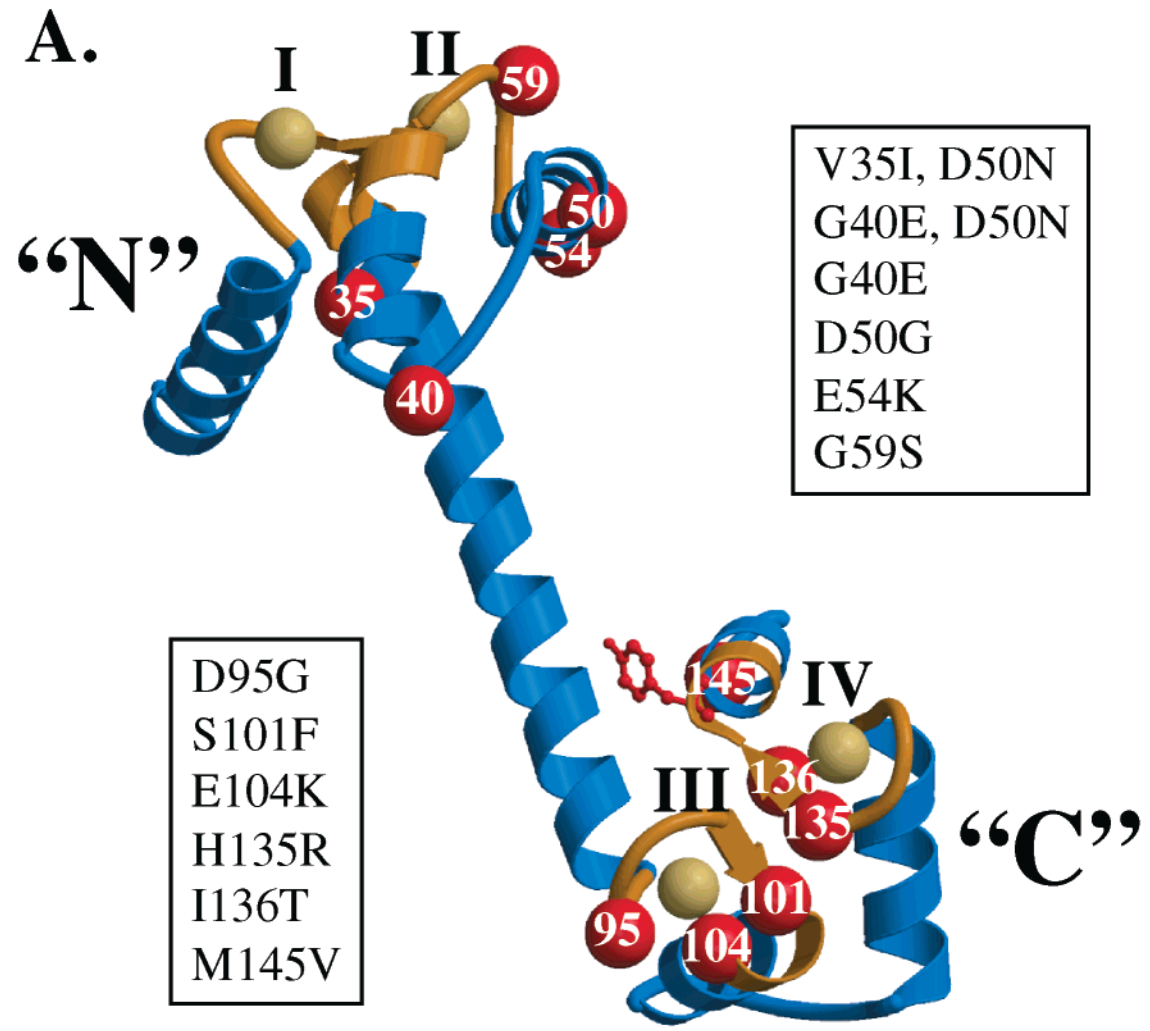
[1] Abbreviations: PCaM, *Paramecium* calmodulin (1−148); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, *N*-(2-hydroxy-ethyl)piperazine-*N*′-2-ethanesulfonic acid; λ_{max}-wavelength at maximum fluorescence intensity; I_{max}maximum fluorescence intensity; R_s, Stokes radius; WT, wild-type.

10.1021/bi0023091 CCC: $20.00
Published on Web 01/03/2001



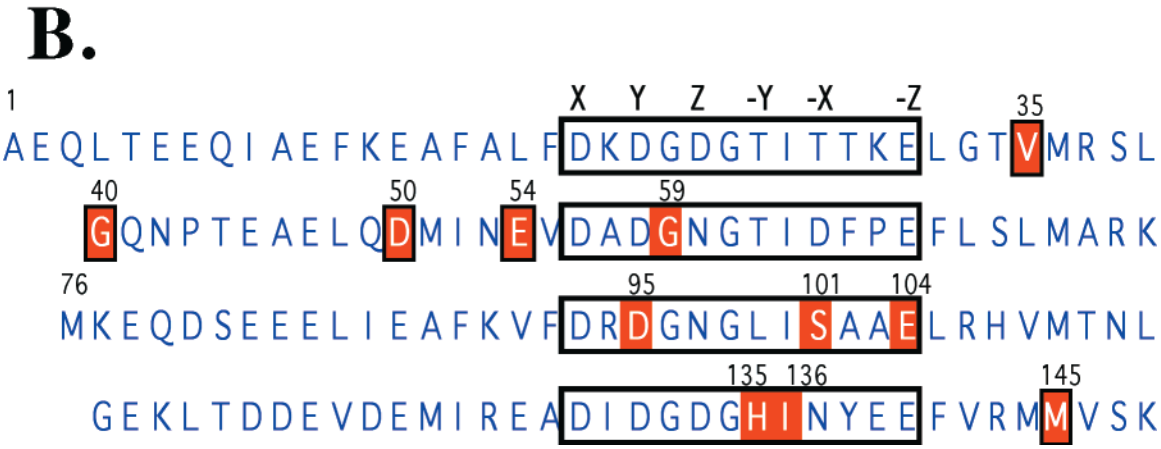


FIGURE 1: (A) Ribbon diagram of the α-carbon backbone of $(Ca^{2+})_4$−PCaM created using MOLSCRIPT (*38*) and RASTER3D (*39*). Coordinates were taken from the RCSB Protein Data Bank file 1osa.pdb (ref *40*; http://www.rcsb.org/pdb/). The four calcium-binding sites are numbered I, II, III, and IV and calcium ions are colored yellow. Locations of mutations of PCaM isolated on the basis of abnormal swimming behavior (*5*) and included in this study are indicated by red spheres. Substitutions are listed in boxes next to each domain. (B) Amino acid sequence of wild-type PCaM aligned to illustrate sequence similarity among the four calcium-binding sites enclosed in boxes. Positions within each site involved in chelating calcium ion are indicated as X, Y, Z, −Y, −X and −Z (*41*, *42*). Mutations in PCaM identified by Kung et al. (*5*) are highlighted in red.

affinity of sites III and IV. This implies that its main defect is in target association or conformational change required for activation.

The mutants may be subdivided according to whether they (a) bind calcium normally, but do not bind target(s), (b) do not bind calcium normally but are still able to bind target [e.g., similar to some *Drosophila* CaM mutants (*9*)], (c) bind both calcium and some targets in a normal fashion, or (d) bind neither calcium nor target. Calcium titrations monitored by fluorescence showed that over-reactive mutants with

sequence differences in sites III and IV have a lower intrinsic affinity for calcium and diminished cooperativity between sites III and IV (*8*) suggesting they may be in class "b". However, the calcium-binding properties of sites III and IV in six under-reactive N-domain mutants and the mutant altered in helix H (M145V) are equivalent to wild-type. Thus, unless sites I and II are dramatically affected in these, it appears that some of these mutants may be defective only in the calcium-dependence of their target interactions (class "a" mutants).

CaM can associate with and activate a variety of different targets. There are examples of CaM functioning as an intrinsic subunit [e.g., phosphorylase kinase (*10*)] and as a transient activator of targets through association-mediated release of inhibition of catalytic sites or protein−protein interaction sites (*3*). CaM may also be inhibited by association with various pharmacological agents [e.g., trifluoperazine (*11*)]. The flexibility of the tether connecting the two domains of CaM allows CaM to adjust its tertiary structure to accommodate a multitude of targets. It can also bind small peptides such as melittin and a portion of smMLCK even if they are synthesized with all D-amino acids (*12*).

Many physiological targets are too large for direct structural characterization of a co-complex with CaM. To study the interactions between CaM and its targets, an accepted strategy is to examine CaM interactions with a small peptide representing the binding domain of the target. Most of the identified domains (*3*) are 14−26 residue peptides that have a propensity to form basic amphipathic helices (*13*). Studies of the interactions between CaM and these small peptides have demonstrated that the orientation of target interaction with CaM can be antiparallel [$N\text{-}_{CaM}$: $C\text{-}_{target}$; e.g., myosin light chain kinases (*14*−*16*)] or parallel [$N\text{-}_{CaM}$: $N\text{-}_{target}$; e.g., CaMKK (*17*) and melittin (*18*, *19*)].

Melittin is a well-accepted model for studying CaM−target interactions because it is small (26 amino acids), binds to $(Ca^{2+})_4$−CaM with high affinity (*20*, *21*), and has a spectroscopic reporter (W19) that changes upon binding to CaM (*22*, *23*). Previous biophysical studies of the interaction between CaM and melittin indicate that a low affinity complex [K_d of 10 μM (*21*)] forms between CaM and melittin under apo conditions and a high affinity complex [K_d of 3 nM (*20*) to 0.11 μM (*24*)] forms in the presence of saturating calcium. Small-angle X-ray scattering (*25*), fluorescence energy transfer (*24*), and gel permeation chromatography studies (*23*, *26*) suggest that the interaction between $(Ca^{2+})_4$−CaM and melittin is similar to the high-resolution structure of a peptide of CaMKK bound to $(Ca^{2+})_4$−CaM (i.e., target binding causes $(Ca^{2+})_4$−CaM to collapse with both domains interacting with target). Therefore, melittin is an appropriate model for studying the effect of the mutations in the domains of PCaM on target interaction.

In this study, analytical gel permeation chromatography, analytical sedimentation velocity, and tryptophan fluorescence were used to monitor the association of PCaM with melittin. With these approaches, mutants from both the under-reactive and the over-reactive classes were compared to wild-type PCaM to test whether they could associate with melittin in a calcium-dependent manner and whether the complexes formed were structurally similar to that adopted by wild-type PCaM.

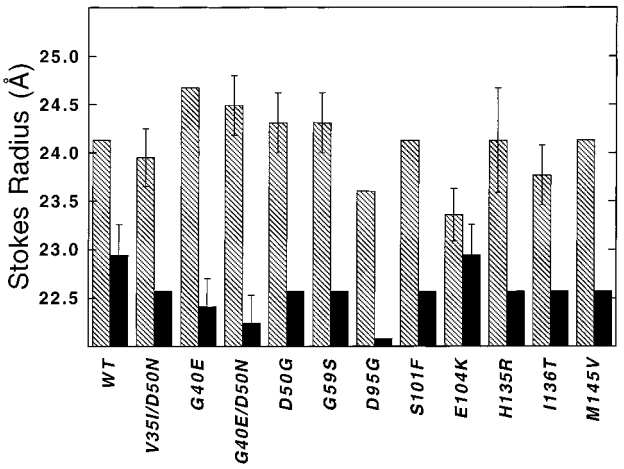


FIGURE 2: Stokes radii for $(Ca^{2+})_4$−PCaM (wild-type and mutants) in the absence (hatched bar) and presence (black bar) of equimolar levels of melittin. Three trials were performed for each protein. The absence of an error bar indicates that replicate measurements of R_s were identical to within 0.01 Å.

MATERIALS AND METHODS

Analytical gel permeation chromatography studies were performed on a Superdex-75 column (Pharmacia) using an FPLC (model LCC-500 Plus; Pharmacia). Samples of wild-type (WT) or mutant PCaM were diluted to 50 μM using either the apo buffer (50 mM HEPES, 100 mM KCl, 50 μM EGTA; pH 7.42 ± 0.01 at 23 °C) or calcium-saturated buffer (apo buffer, 3 mM $CaCl_2$) in the absence or presence of 50 μM melittin. Elution profiles of injected volumes of 100 μL protein samples were monitored at 254 nm with a 0.4 mL/min flow rate and 0.2 cm/mL chart speed. All chromatographic separations were performed at room temperature (20−23 °C). Stokes radii were calculated based on the elution profiles of standard proteins [bovine serum albumin (BSA), chymotrypsin, RNase A, and ovalbumin] used at a final concentration of 4 mg/mL each as described previously (*27*). The average Stokes radius from three determinations is reported in Figure 2. The absence of an error bar indicates that R_s values were identical to within 0.01 Å in all trials.

Analytical Ultracentrifugation. Sedimentation velocity studies of WT PCaM with or without melittin under apo and calcium-saturated conditions were conducted at 22 ± 1 °C on a Beckman XL-I analytical ultracentrifuge at 55 000 rpm. Apo conditions were established by dilution of PCaM (± 40 μM melittin) to 40 μM with apo buffer (50 mM HEPES, 100 mM KCl, 50 μM EGTA; pH 7.42 ± 0.01) or calcium-saturated buffer (apo buffer + 3 mM $CaCl_2$). Sedimentation was monitored at 280 nm every 90 s for 3.5 h. Data were analyzed using the approach developed by Walter Stafford (*28*) and the software program *DCDT+* developed by John Philo (http://www.jphilo.mailway.com). Sedimentation coefficients were resolved by fitting the resulting distributions (see Figure 3) to a single Gaussian or the sum of two Gaussians using the software *Kaleidagraph 3.08* (Synergy Software, Reading, PA).

The sedimentation coefficient (*s*) is inversely related to the Stokes radius (R_s) as shown in the following equation (*29*),

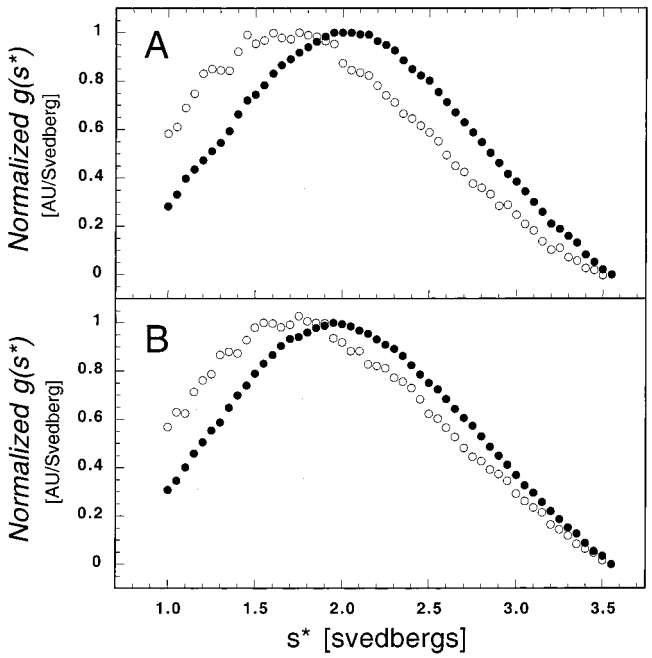


FIGURE 3: Sedimentation velocity study of wild-type PCaM in the absence (open circles) or presence (filled circles) of melittin in a 1:1 molar ratio under (A) apo and (B) calcium-saturated conditions. Sedimentation of PCaM was monitored by absorbance at 280 nm using a Beckman XL-I analytical ultracentrifuge. Data analysis is described in Materials and Methods.

$$s = \frac{M(1 - \nu\rho)}{N_o 6\Pi\eta R_s} \quad (1)$$

where M is molecular weight (16.7 kDa for PCaM, 19.5 kDa for PCaM/melittin), ν is partial specific volume, ρ is solvent density, N_o is Avogadro's number, and η is the viscosity of water at 20 °C. This relationship can be used to predict the change in Stokes radii caused by ligand binding when the corresponding change in sedimentation coefficients is determined.

Fluorescence Spectroscopy of Melittin. Fluorescence emission spectra of PCaM, melittin, and mixtures of PCaM and melittin under apo and calcium-saturated conditions were collected at 22 ± 1 °C using a Spectra CQ Fluorolog-3 SPEX, model FL3-21 (Instruments SA, Edison, NJ) with an excitation wavelength of 289 nm, a step size of 0.1 nm, a time constant of 0.5 s, 2.5 nm slit widths, and a constant voltage of 950 mV. Apo conditions were established by dilution of melittin and PCaM with apo buffer (50 mM HEPES, 100 mM KCl, 50 μM EGTA; pH 7.42 ± 0.01) to final concentrations of 3.0 μM melittin and/or 4.5 μM PCaM (i.e., excess PCaM). Calcium-saturated conditions were established by adding 1 M $CaCl_2$ to the identical apo samples to establish a final $CaCl_2$ concentration of 3 mM. Emission spectra presented for melittin in solution with PCaM (Figure 4, panels A−D) were corrected for background emission of PCaM in the absence of melittin. To allow for direct comparison of the spectrum for melittin alone with its spectrum in the presence of PCaM (WT or one of the 11 mutants), all spectra were normalized to the fluorescence intensity at 355 nm. Spectra shown in Figure 4 are representative of three to six emission scans measured for each PCaM sample.

RESULTS

Analytical Gel Permeation Chromatography. Comparison of the Stokes radii of WT PCaM with and without melittin shows that melittin binding to calcium-saturated PCaM resulted in a 1.19 Å decrease in Stokes radius (see Figure 2 and Table 1). Comparable results were observed for all mutants (except E104K) under these conditions where the Stokes radius decreased between 0.42 and 2.26 Å upon binding melittin. Very little change in Stokes radius was observed for WT PCaM and mutants with and without melittin in the absence of calcium (data not shown).

Analytical Sedimentation Velocity. The properties of WT PCaM in the absence and presence of melittin under apo and calcium-saturating conditions were compared. In the absence of calcium, the resolved sedimentation coefficient (s*) of PCaM increased by 0.34 S in the presence of melittin (Figure 3, panel A). In the presence of saturating levels of calcium, the s* of PCaM increased by 0.38 S in the presence of melittin (Figure 3, panel B). The standard error in the individual measurements of s* was 0.07−0.10 S, significantly smaller than the calculated values of Δs*. This indicated that melittin binds to PCaM in a calcium-independent manner (i.e., it binds to both apo and calcium-saturated PCaM).

Using the inverse relationship between s and Stokes radius (see eq 1), the Stokes radius of $(Ca^{2+})_4$−CaM was predicted to decrease by 5.2%; the observed decrease for WT PCaM was in very close agreement with this prediction (4.93%). Apo PCaM was predicted to decrease by 2.7% upon binding melittin; however, no significant change in Stokes radius was observed.

Trp Fluorescence of Melittin. The emission spectrum of melittin changed upon addition of WT PCaM. Shifts in the wavelength at maximum intensity of tryptophan fluorescence (λ_{max}) indicated a change in the environment of W19 of melittin under both apo and calcium-saturating conditions (Figure 4, panels A−D). In the absence of PCaM, λ_{max} was 355.3 nm under apo conditions and was almost identical (356.3 nm) in the presence of calcium (see data in Supporting Information). Upon addition of WT PCaM, the values of λ_{max} shifted by ∼10 Å (to 345.7 nm in the absence of calcium and 346.4 in the presence of saturating levels of calcium).

The tryptophan fluorescence of melittin was unaffected by mutations in the N-terminal domain of PCaM in both the absence (Figure 4, panel A; average λ_{max} of 344.8 ± 0.0.5 nm) and presence of calcium (Figure 4, panel C; average λ_{max} of 346.3 ± 0.8 nm). This suggests that the environment surrounding W19 in melittin is not affected by either these mutations or by calcium binding to sites I and II in this domain. In contrast, the effects of mutations in the C-domain of CaM were more variable (i.e., larger standard deviation). Shifts in λ_{max} under both apo (Figure 4, panel B; average $\lambda_{max}$was 345.8 ± 4.2 nm) and calcium-saturating (Figure 4, panel D; average λ_{max} was 344.2 ± 2.5 nm) conditions were observed. The largest variations were observed for M145V under apo conditions and E104K under calcium-saturating conditions. This indicates that the environment of the tryptophan in melittin when in the hydrophobic cleft of the C-domain is altered by these mutations regardless of the degree of calcium occupancy of sites III and IV.

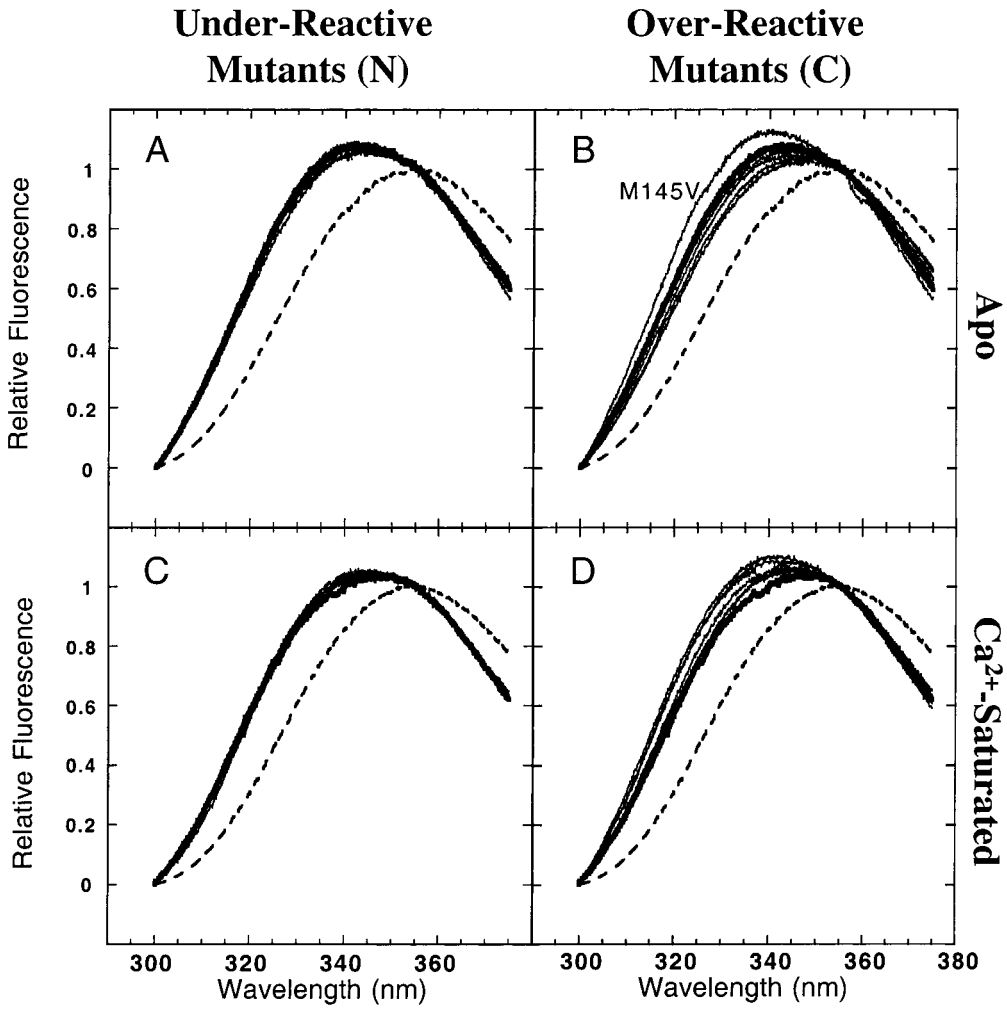


FIGURE 4: Fluorescence emission spectra of melittin in the absence (dashed line) or presence (solid lines) of PCaM WT (bold) and mutants under apo (top panels, A and B) and calcium-saturated (bottom panels, C and D) conditions. Panels A and C compare tryptophan fluorescence emission spectra of melittin in the presence of PCaM mutants altered in the N-domain. Panels B and D show the effect on melittin of association with PCaM mutants altered in the C-domain. All spectra were normalized to 355 nm.

Table 1: Effect of Calcium and Melittin on Stokes Radii of PCaM Mutants

protein	Ca^{2+}-saturated R_s[a] (Å)	Ca^{2+}-saturated R_s[a] (Å) + melittin	ΔR_s[b]
WT PCaM	24.13 ± 0.00	22.94 ± 0.32	1.19
V35I/D50N	23.95 ± 0.30	22.57 ± 0.00	1.38
G40E	24.67 ± 0.00	22.41 ± 0.29	2.26
G40E/D50N	24.49 ± 0.31	22.24 ± 0.29	2.25
D50G	24.31 ± 0.31	22.57 ± 0.00	1.74
G59S	24.31 ± 0.31	22.57 ± 0.00	1.74
D95G	23.60 ± 0.00	22.08 ± 0.00	1.52
S101F	24.13 ± 0.00	22.57 ± 0.00	1.56
E104K	23.36 ± 0.27	22.94 ± 0.32	0.42
H135R	24.13 ± 0.54	22.57 ± 0.00	1.56
I136T	23.77 ± 0.31	22.57 ± 0.00	1.20
M145V	24.13 ± 0.00	22.57 ± 0.00	1.56

[a] Stokes radii were calculated as described previously (*27*). [b] Difference between Stokes radii (ΔR_s) in the absence and presence of melittin.

DISCUSSION

Domain-Specific Effects of Mutations on PCaM. Two classes of *Paramecium* CaM mutants were identified by Kung et al. (*5*−*7*) and shown to affect ion channel regulation. These mutants were the first reported example of domain-specific target activation by calmodulin. *Paramecia* with mutations in the N-domain exhibited deficient calcium-dependent sodium channel regulation, while mutations in the C-domain of PCaM exhibited deficient calcium-dependent potassium channel regulation (*7*). Biochemical characterization of the calcium-binding properties of 12 of these mutants revealed that they were able to bind calcium and undergo conformational changes equivalent to wild-type PCaM. Therefore, a logical conclusion is that these mutants are defective in target association. In this study, analytical gel permeation chromatography, analytical sedimentation velocity studies, and fluorescence spectroscopy were used to determine whether PCaM mutants defective in ion channel regulation retain the ability to bind target proteins. Comparisons were made using melittin as a model target peptide because it was expected to associate with the wild-type and N-domain (under-reactive) mutants that have wild-type C-domains. This was inferred from a wide array of biophysical studies that have spanned nearly two decades.

Calcium Dependence of Association. Small-angle X-ray scattering studies of the calcium dependence of CaM binding to melittin indicate that the shape of apo CaM does not change in the presence of melittin, whereas $(Ca^{2+})_4$−CaM undergoes a shape transition described as dumbell to more globular (*25*). Fluorescence energy transfer studies have shown that the distance between the Cys 27 in site I and

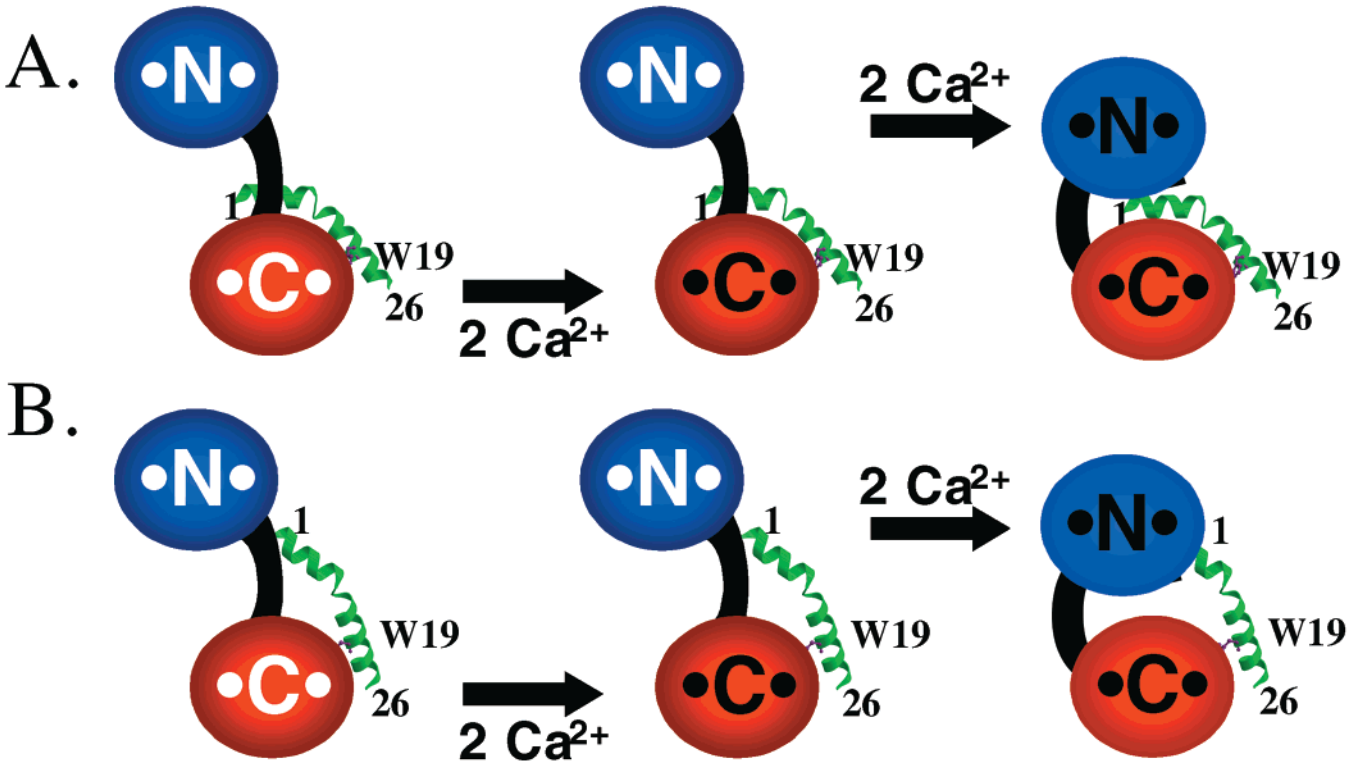


FIGURE 5: Models for the calcium-dependent interaction between melittin and PCaM. In the absence of calcium (far left), PCaM is extended and the peptide melittin (green; its W19 is purple) interacts with either (A) the C-domain (red sphere) alone or (B) with both the N- and C-domains of PCaM. As the C-domain sites III and IV (small circles) become filled with calcium (small black circles), the structure remains extended. CaM collapses when calcium binds to the N-domain sites I and II.

Tyr 139 in site IV of wheat $(Ca^{2+})_4$−CaM decreases by ~3 Å (from 22.4 to 19.5 Å) upon melittin complex formation, which is comparable to the change induced by other autoinhibitory model peptides (*24*).

Analytical gel permeation chromatography studies conducted in the absence of calcium showed no significant change in Stokes radius of PCaM (wild-type or mutants) in the presence of melittin (data not shown). These results are in agreement with previous studies of CaM and melittin using the same technique (*23*, *26*) and offer evidence for a weak association in the absence of calcium. However, in the presence of saturating levels of calcium, a significant decrease in Stokes radius was observed for all of the PCaM mutants in the presence of melittin (see Figure 2 and Table 1) indicating that these mutations have not abolished the melittin binding site in CaM.

Analytical sedimentation velocity studies showed an increase in the sedimentation coefficient for WT PCaM in the presence of melittin under both apo and calcium-saturated conditions (Figure 3). These data provide unequivocal support for the conclusion that an interaction between melittin and PCaM exists in the absence of calcium. This conclusion is supported by tryptophan fluorescence, gel disk electrophoresis and microcalorimetry which detected a low affinity [K_d of 10 μM (*21*)] complex in absence of calcium (*20*, *21*, *23*, *26*) and a 1:1 complex [K_d ranging from 0.11 μM (*24*) to 3 nM (*20*)] in the presence of saturating calcium (*18*, *20*, *21*, *23*, *26*, *30*).

Comparative analysis of the observed and predicted change in Stokes radii upon binding melittin (see eq 1 and Results) indicates that the similar Stokes radii of the apo PCaMs observed in the gel permeation chromatography study in the absence and presence of melittin is likely due to an interaction that is too weak for detection using this technique. Because melittin is smaller and has a larger partition coefficient than CaM, when it dissociates it may be retained on the column as CaM progresses through the gel bed. Thus, we have concluded that the small zone chromatographic method we used is inadequate to analyze an interaction as weak as that of apo CaM with melittin.

Fluorescence spectroscopy studies have taken advantage of the differences in amino acid composition between CaM and melittin. *Paramecium* CaM contains one tyrosine (Y138, see Figure 1) and no tryptophan residues, while melittin contains one tryptophan (W19). Using fluorescence spectroscopy to study localized conformational changes in CaM has been well documented (*8*, *31*−*36*). In this study, the environment of W19 in melittin was altered by WT PCaM binding in both the absence and presence of calcium [see Figure 4, panels A−D), consistent with prior findings with bovine CaM (*21*, *23*)]. The average value of λ_{max} for the N-domain mutants of PCaM was similar to that for WT PCaM. Significant variability in the shift of λ_{max} for W19 fluorescence of melittin was observed upon addition of the C-domain mutants of PCaM under apo and calcium-saturating conditions. This indicated that the environment of W19 when in the cleft of the C-domain is altered by the mutations regardless of the occupancy of sites III and IV by calcium (i.e., calcium binding does not "rescue" conformational heterogeneity).

Alternative Models for Binding and Collapse. On the basis of these data, the models shown in Figure 5 were formulated

to depict the calcium-dependent, domain-specific interactions of PCaM and melittin. In Figure 5, panel A, the structure of CaM is shown extended in the absence of calcium. Under these conditions, melittin associates with PCaM. The fluorescence spectra indicate that interaction occurs between the C-domain of PCaM and the C-terminal end of melittin where W19 is located. The lack of change in the λ_{max} and the maximum fluorescence intensity (I_{max}) for the N-domain mutants of PCaM suggest there is no direct interaction with melittin or none that affects W19. In this model, we assume that the interaction between PCaM and melittin under apo conditions is not disrupted by calcium-binding to sites III and IV and that the presence of melittin has no effect on the order of calcium binding or association of melittin to sites I and II in the N-domain. The structure of this intermediate species would be comparable to that reported for the complex between CaM and the peptide from the plasma membrane Ca^{2+}-pump [1CFF.pdb (*37*); http://www.rcsb.org/pdb/]. A significant shift in λ_{max} for the C-domain mutants of PCaM under both apo and calcium-saturating conditions suggests that melittin binds to the C-domain of PCaM at all levels of calcium, consistent with results obtained from analytical ultracentrifugation studies. The dramatic decrease in Stokes radius seen only under calcium-saturating conditions suggests that a collapse occurs only when calcium occupies all four sites (i.e., after it binds to sites I and II in the N-domain).

An alternative model for the calcium-dependent interaction between melittin and PCaM is shown in Figure 5, panel B, where both the N- and C-terminal domains of PCaM interact with melittin in the absence of calcium. However, the interaction with the N-domain is spectroscopically silent and does not change the Stokes radius. When calcium binds to sites I and II in the N-domain, this interaction is altered and mediates tertiary collapse of the structure. It should be noted that many other models of propagated effects are also consistent with these data. We favor these two on the basis of comparison with the literature on CaM−melittin interactions.

NMR studies of the interaction between $(Ca^{2+})_4$−CaM and melittin indicate binding causes perturbation in both the N- and C-domains of CaM and the orientation of the interaction is parallel, rather than the more common antiparallel orientation (*18*, *19*). However, if melittin associates in a parallel orientation, W19 would interact directly with the C-domain of PCaM and report primarily on local environmental differences. It would be unlikely to report on melittin interaction with the N-domain of PCaM. Therefore, it is not possible to distinguish between these two models using tryptophan fluorescence alone. This will require studies in which residue-specific probes of the N-domain are used to determine whether melittin can associate with a fragment containing those residues.

ACKNOWLEDGMENT

We thank Walter Stafford for his advice on analysis of analytical sedimentation velocity data and John Philo for his recommendation of software for analysis of these data on a Power Macintosh. We thank the University of Iowa Molecular Analysis Facility for amino acid analysis used for determination of protein concentrations.

SUPPORTING INFORMATION AVAILABLE

Fluorescence wavelength and intensity maxima of melittin associated with PCaM mutants (Table S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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BI0023091