Serine/threonine phosphorylation of calmodulin modulates its interaction with the binding domains of target enzymes

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The interaction of serine/threonine-phosphorylated calmodulin with synthetic peptides corresponding to the calmodulin-binding domains of six enzymes has been studied by fluorescence spectroscopy. For five peptides, the dissociation constant of the calmodulin–peptide complex (K_d) increased when calmodulin was phosphorylated. An increase of more than one order of magnitude was observed with peptides derived from smooth-muscle myosin light-chain kinase and cAMP phosphodiesterase. In contrast, only a slight increase in K_d was noted with two peptides derived from the plasma membrane Ca^{2+}-ATPase and for the peptide derived from nitric oxide synthase. No significant change in affinity was detected with the peptide derived from calcineurin. In contrast, a decrease in the dissociation constant was observed with the peptide derived from the Ca^{2+}-calmodulin dependent kinase II. Phosphorylation also affected the peptide–calmodulin binding stoichiometry: a decrease from two to one binding sites was observed with the peptides derived from myosin light-chain kinase and phosphodiesterase.

Key words: Ca^{2+} signalling, dansylation, peptide affinity, protein phosphorylation.

INTRODUCTION

The Ca^{2+}-activated form of calmodulin (CaM) activates numerous proteins and enzymes involved in different metabolic and signalling pathways such as glycogenolysis, nucleotide metabolism, heart muscle and smooth-muscle contraction, neuronal transmission and cell motility [1,2]. It has been suggested that a fraction of CaM is constitutively phosphorylated *in vivo*, e.g. in chicken fibroblasts [3], rat and chicken brain [4–6] and rat liver [7]. CaM is phosphorylated *in vitro* by two serine/threonine kinases: casein kinase II (CK II; EC 2.7.1.37), which phosphorylates Thr-79, Ser-81, Ser-101 and Thr-117 [6,8], and myosin light-chain kinase (MLCK; EC 2.7.1.37), which phosphorylates CaM on Thr-26 and Thr-29 [9]. Several tyrosine kinases, e.g. Src tyrosine kinase [3], rat spleen tyrosine kinase [10], epidermal growth factor tyrosine kinase [11] and the insulin receptor [12–14], have been shown to phosphorylate CaM on Tyr-99 and Tyr-138 as well.

The effect of the phosphorylation of CaM *in vitro* on the activation of CaM target enzymes has been studied in some detail [7,8,15–17]. Whereas serine/threonine phosphorylation only slightly diminished the ability of CaM to activate calcineurin (EC 3.1.3.36) [15,17], a more pronounced decrease was observed for cAMP phosphodiesterase (EC 3.1.4.17), MLCK, plasma membrane Ca^{2+}-ATPase (EC 3.6.1.38) and Ca^{2+}/CaM-dependent kinase II (EC 2.7.1.123) [7,15,17–19]. In general, serine/threonine phosphorylation of CaM seems to increase the activation constant (K_{act}, the concentration of CaM required for half-maximal activation) of target enzymes [7,15,17–19].

In this paper we describe the effect of the serine/threonine phosphorylation of CaM on its affinity for peptides corresponding to the CaM-binding domains of the plasma membrane Ca^{2+}-ATPase, MLCK, cAMP phosphodiesterase, Ca^{2+}/CaM-dependent kinase II, neuronal nitric oxide synthase (EC 1.14.13.39) and calcineurin. The CaM-binding domains have been synthesized and their dissociation constants (K_d) for CaM and phosphorylated calmodulin (PCaM) have been measured by fluorescence spectroscopy. Phosphorylation on serine/threonine residues (Thr-79, Ser-81, Ser-101 and Thr-117) modulates the interaction with the CaM-binding domains by either decreasing or increasing the dissociation constant (K_d). Whereas the binding stoichiometry of CaM with target proteins is known to be 1:1, the binding stoichiometry of two peptides (derived from MLCK and phosphodiesterase) has instead been found to be 2:1. In these cases phosphorylation decreased the number of binding sites from two to one.

MATERIALS AND METHODS

Materials

Poly-(L-lysine) (average molecular mass 68 kDa) and dansyl chloride was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The protein assay reagent (bicinchoninic acid) was from Pierce (Oud-Beijerland, Netherlands). Trypsin (EC 3.4.21.4) was from Boehringer Mannheim (Germany). Human recombinant CaM was expressed in *Escherichia coli* and purified as described previously [20]. CK II was purified from bovine liver by the procedure of Meggio et al. [21]. The CaM-binding peptides

Abbreviations used: CaM, calmodulin; CK II, casein kinase II; dans-CaM, dansylated calmodulin; dans-PCaM, dansylated phosphorylated calmodulin; MALDI–TOF-MS, matrix-assisted laser desorption ionization–time-of-flight MS; MLCK, myosin light-chain kinase, PCaM, phosphorylated calmodulin; PDE, 3′,5′-cyclic nucleotide phosphodiesterase.

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Table 1 Amino acid sequence of the synthetic peptides derived from the CaM-binding domain of six enzymes

The peptides used were C20W and C21W of plasma-membrane Ca^{2+}-ATPase [66], NO30 of neuronal nitric oxide synthase [41], PDE16 of cAMP-phosphodiesterase [44], CaMKII24 of CaM-dependent kinase II [67], MLCK20 of smooth-muscle MLCK [23] and CN24 of the A subunit of calcineurin [68]. The hydrophobic anchor residues are indicated in bold type.

Name	Sequence	Residue numbers of the corresponding enzyme
C21W	**W**FRGLNRIQTQIKVVNA**F**RSS	1107–1127, Ca^{2+}-ATPase
C20W	LRRGQIL**W**FRGLNRIQTQIK	1100–1119, Ca^{2+}-ATPase
NO30	KRRAIG**F**KKLAEAVKFSAK**L**MGQAMAKRVK	725–754, nitric oxide synthase
CaMKII24	LKK**F**NARRKLKGAILTTM**L**ATRNF	290–313, CaM kinase II
PDE16	KM**W**QRLKGILRSLVKQ	25–40, phosphodiesterase
CN24	ARKEV**I**RNKIRAIGKMARV**F**SVLR	177–200, calcineurin
MLCK20	ARRK**W**QKTGHAVRAIGR**L**SSM	796–815, MLCK

were synthesized (Table 1) and dansylated as described previously [22]. The peptide PDE16 (in which PDE stands for 3′,5′-cyclic nucleotide phosphodiesterase) of sequence KMWQRLKGILR-**S**LVKQ (single-letter codes) was a substitution analogue of the original CaM-binding domain of sequence KMWQRLKGILR-**C**LVKQ. It contained a serine residue instead of cysteine at position 36 (shown in bold) to avoid dimerization. The MLCK20 peptide, RRKWQKTGHAVRAIGRLSSS, used in this study was synthesized in accordance with the sequence published by Lukas et al. [23] and has been studied previously [24,25], although the sequence of the full enzyme (AC p11799) in the database SWISS-PROT shows a methionine instead of a serine residue at position 513. All chemical reagents were of the highest purity available commercially.

Phosphorylation of CaM *in vitro*

Phosphorylation of CaM by CK II was performed by following the procedure described by Quadroni et al. [17]. In brief, 0.1 mg/ml CaM was incubated overnight at 37 °C in 50 mM Tris/HCl (pH 7.5)/10 mM $MgCl_2$/1 mM EGTA in the presence of 0.5 μM poly-(L-lysine) and 30–50 units of CK II. CaM and PCaM were separated after phenyl-Sepharose chromatography by PAGE [12 % (w/v) gel] under non-denaturing conditions in the presence of 1 mM EGTA.

Dansylation of CaM and PCaM

CaM and PCaM were incubated with 1 equiv. of dansyl chloride in 20 mM ammonium bicarbonate in the presence of 1 mM $CaCl_2$ for 2 h and then dialysed overnight against the same buffer [22]. The total concentrations of dansylated and non-dansylated CaM and PCaM in the stock solutions were determined by the bicinchoninic acid method, with native CaM as reference. In addition, the concentration of dansylated CaM (dans-CaM) was determined by UV absorption, with an ϵ_{320} value of 3400 $M^{-1}\cdot cm^{-1}$ [26]. The ratio of dansylation was calculated by dividing the concentration of the dans-CaM [or dansylated PCaM (dans-PCaM)] by the concentration of total CaM (dansylated and non-dansylated).

Determination of the dansylation sites on CaM and PCaM

Dans-CaM and dans-PCaM (15 μg) were digested in two steps: first with CNBr and then with trypsin as described by Quadroni et al. [7]. As control, non-dansylated CaM and PCaM were digested under identical conditions. The masses of the digested peptides were determined by using matrix-assisted laser desorption ionization–time-of-flight MS (MALDI–TOF-MS). The digest peptides were separated by HPLC and the peptide containing the dansyl group (UV absorption at 330 nm) was isolated.

Fluorescence measurements

The fluorescence experiments were performed on a Spex fluorolog 1680 double-wavelength spectrophotometer connected to a SpectrAcq Control Module and using Datamax version 1.03 software (Jobin Yvon/Spex). Quartz cuvettes with a path length of 10 mm and a volume of 3 ml were used. All measurements were made at room temperature. The fluorescence emission spectra of the dansylated proteins or peptides were recorded between 400 and 550 nm (bandwidth 4 nm) after excitation of the dansyl moiety at 340 nm (bandwidth 6 nm). The experiments were performed in 20 mM Hepes buffer (pH 7.2)/130 mM KCl/1 mM $CaCl_2$. The concentrations of the dansylated peptides (dans-C20W and dans-C21W) were determined with an ϵ_{320} of 3400 $M^{-1}\cdot cm^{-1}$ [26]. The concentrations of the other peptides were determined by using their intrinsic UV absorption, taking ϵ_{280} as 5600 $M^{-1}\cdot cm^{-1}$ or ϵ_{254} as 200 $M^{-1}\cdot cm^{-1}$ for tryptophan or phenylalanine residues respectively, or by amino acid analysis.

Titration of CaM and PCaM with peptides dans-C20W and dans-C21W

Peptide dans-C20W or dans-C21W (150 nM) was titrated with increasing amounts of CaM or PCaM. The titration was performed until saturation. Sample dilution as a result of the titration was less than 5 %.

The dissociation constants (K_d) for the complexes dans-C20W·CaM (or C21W·CaM) and dans-C20W·PCaM or (C21W·PCaM) were calculated by using a non-linear regression procedure:

$$F = F_0 + (F_\infty - F_0)\{([E_t] + [S_0] + K_d) - [([E_t] + [S_0] + K_d)^2 - (4[S_0][E_t])]^{\frac{1}{2}}\}/(2[S_0]) \quad (1)$$

where K_d = [dans-C20W][CaM]/[dans-C20W·CaM], F_0, F_∞ and F are the relative fluorescence intensities of dans-C20W in the absence of CaM, at a saturating concentration of CaM and at an intermediate concentration of CaM respectively, $[S_0]$ is the total concentration of dansylated peptide and $[E_t]$ is the total concentration of CaM (or PCaM) present after each addition.

Titration of dans-CaM and dans-PCaM with CaM target peptides (Table 1)

The experiments were performed as described above with 53 nM dans-CaM or 35 nM dans-PCaM, except for the titrations with the peptide CaMKII24, in which 10 nM dans-CaM or dans-PCaM was used. The data were fitted with:

$$F = F_0 + (F_\infty - F_0)\{([S_t] + [E_0] + K_d) - [([S_t] + [E_0] + K_d)^2 - (4[E_0][S_t])]^{\frac{1}{2}}\}/2[E_0] \quad (2)$$

where K_d = [dans-CaM][peptide]/[dans-CaM·peptide], F_0, F_∞ and F are the relative fluorescence intensities of dans-CaM or dans-PCaM in the absence of peptide, at a saturating concentration of peptide and at an intermediate concentration of peptide respectively, $[E_0]$ is the total concentration of dans-CaM and $[S_t]$ is the total concentration of peptide present in the cuvette after each addition.

For all calculations it was assumed that the fractional change in fluorescence was directly proportional to the fraction of dans-peptide/CaM or dans-CaM/peptide complexes formed as reported previously in titration experiments with dans-CaM [27–30].

The fitting of the data was performed with a non-linear regression that allowed the estimation of the parameters $[S_0]$ or $[E_0]$, K_d, F_0 and $(F_\infty - F_0)$, indicating the reliability of the fit.

RESULTS

Phosphorylation of CaM

The extent of phosphorylation was determined by MALDI–TOF-MS and was comparable with that previously reported: 70% of the species was triphosphorylated, 15–25% was monophosphorylated and diphosphorylated and 5–15% was tetraphosphorylated [17]. Non-phosphorylated CaM was not detected in the sample of PCaM eluted from the gel. CaM phosphorylation could not be performed to completion, as mentioned previously by others [8,16].

Separation of the different phosphorylated species (monophosphorylated, diphosphorylated, triphosphorylated and tetraphosphorylated) of CaM was not possible with non-denaturing gel electrophoresis, probably owing to the particular migration properties of CaM on a gel. As already reported, whereas PCaM should have run faster on the native gel, it ran more slowly [17]. However, CaM is known to have an abnormal migration even on an SDS gel, in which the migration rate depends on the presence of Ca^{2+} [1].

Site of dansylation of dans-CaM and dans-PCaM

After digestion by CNBr and trypsin, analysis of the digested peptides derived from dans-CaM and dans-PCaM showed the presence of an additional peptide of mass 1781 (Figures 1 and 2). This peptide was isolated by HPLC and showed a UV absorption at 330 nm, indicating the presence of the dansyl group. The mass of the dansylated peptide (1781) could be correlated only with a dansylation at Lys-115, corresponding to the peptide T^{110}NLGE-dnsK-LTDEEVDEhms^{124}, in which dns and hms stand for dansyl and homoserine respectively. Dansylation was found to occur at this residue in both CaM and PCaM. Lys-115 is usually trimethylated in CaM originating from animal tissue.

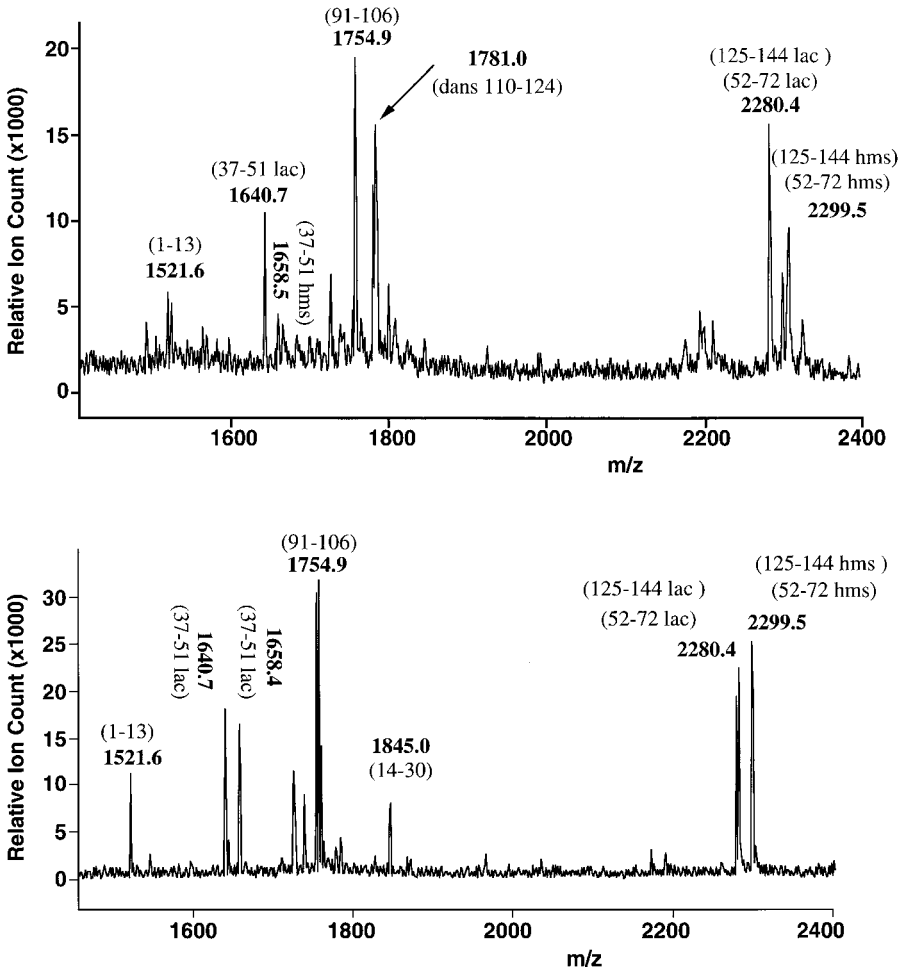

Figure 1 MALDI–TOF-MS spectra of CNBr/tryptic digests of human recombinant dans-CaM (upper panel) and human recombinant CaM (lower panel)

The peak indicated with an arrow corresponds to the dansylated peptide of mass 1781 (residues T^{110}NLGE*KLTDEEVDEhms^{124}). Abbreviations: dns, dansyl(*); hms, homoserine; lac, homoserine lactone.

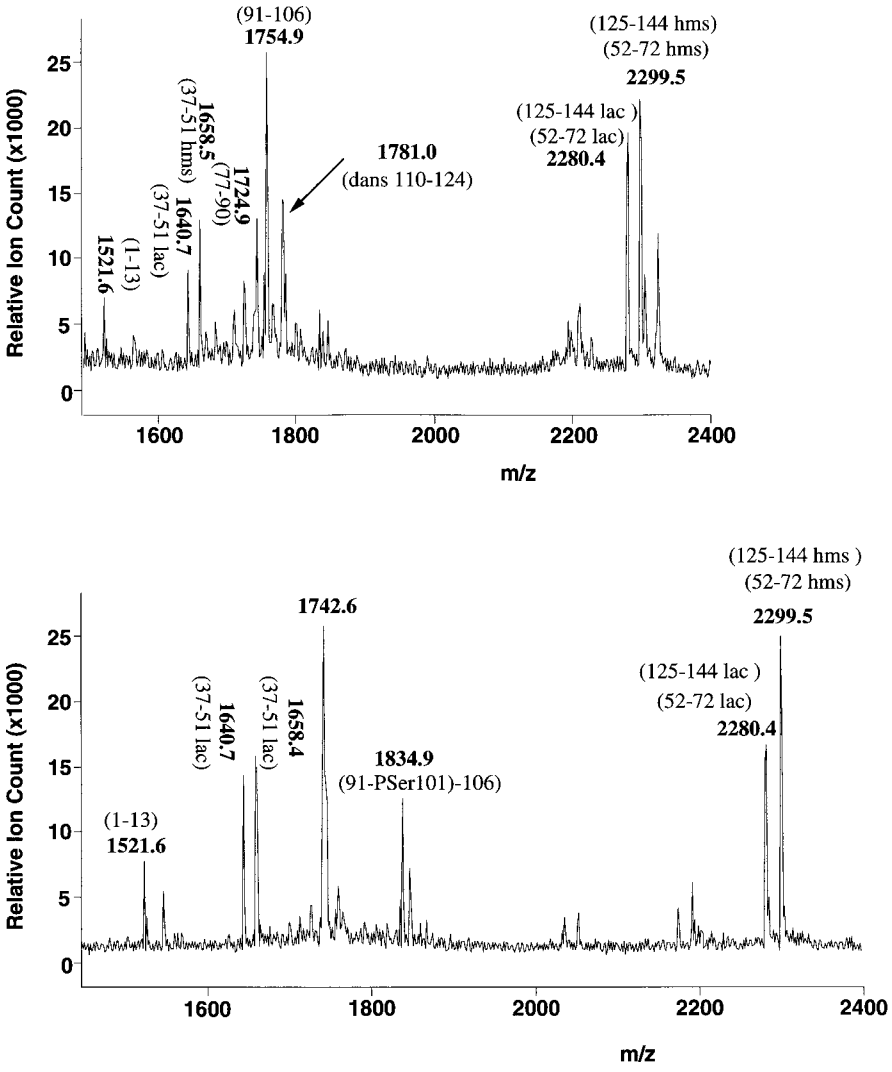

Figure 2 MALDI–TOF-MS spectra of CNBr/tryptic digests of dansylated human recombinant PCaM (upper panel) and human recombinant PCaM (lower panel)

The peak at *m/z* 1742.6 could not be identified. The peak indicated with an arrow corresponds to the dansylated peptide of mass 1781 (residues T^{110}NLGE*KLTDEEVDEhms^{124}). Abbreviations: dns, dansyl (*); hms, homoserine; lac, homoserine lactone.

Binding of CaM and PCaM to the peptides derived from the Ca^{2+}-ATPase

The binding of CaM and PCaM to the peptides derived from the Ca^{2+}-ATPase were studied by following the change in the fluorescence properties of the dansyl group N-terminally attached to the peptides C20W and C21W.

The fluorescence emission spectrum of peptide dans-C20W exhibited a maximum at 535 nm, which was blue-shifted in the presence of CaM (maximum at 512 nm) or PCaM (maximum at 508 nm) (Figure 3, upper panel). The quantum yield of the dansyl group was increased 8-fold in both cases. These results are in good agreement with previous studies [27] and indicate that the dansyl group is in a more hydrophobic environment when bound to CaM and PCaM.

The fluorescence emission spectra of dans-C21W in the presence and absence of CaM are shown in Figure 3 (lower panel). The maximum of the fluorescence emission was again shifted from 535 to 508 nm in the presence of CaM; the same shift was observed in the presence of PCaM. The quantum yield of the dansyl group was increased 5.5-fold and 5-fold in the presence of CaM and PCaM respectively.

Peptide dans-C20W bound CaM and PCaM with a K_d of 21.5 ± 3.7 (mean $\pm$ S.D.) and 28.1 ± 4 nM for CaM and PCaM respectively (Figure 4 and Table 2). Peptide dans-C21W bound CaM and PCaM with a higher affinity than did peptide dans-C20W. The K_d values were 7.9 ± 5.7 and 19.1 ± 6.6 nM for CaM and PCaM respectively (Table 2). The change in affinity was probably due to the presence of two aromatic residues (Trp-1107 and Phe-1124) in the peptide C21W instead of one (Trp-1107) in C20W (Table 1). These aromatic residues are known to bind to the hydrophobic pockets of CaM and are responsible for the specific interaction of the CaM-binding domains with CaM [31–34].

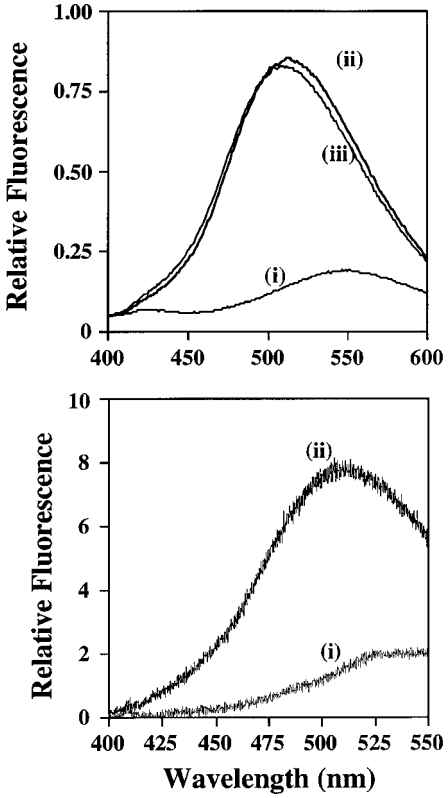

Figure 3 Fluorescence emission spectra of calmodulin complexed with dansylated peptides

Upper panel: fluorescence emission spectra of dans-C20W (150 nM) (trace i) incubated with CaM (200 nM) (trace ii) or PCaM (200 nM) (trace iii). Lower panel: fluorescence emission spectra of dans-C21W (150 nM) (trace i) incubated with CaM (200 nM) (trace ii). Experimental conditions: 20 mM Hepes (pH 7.2)/130 mM KCl/1 mM $CaCl_2$. The relative fluorescence intensity is shown in arbitrary units. Excitation was at 340 nm and the spectra were recorded from 400 to 550 nm.

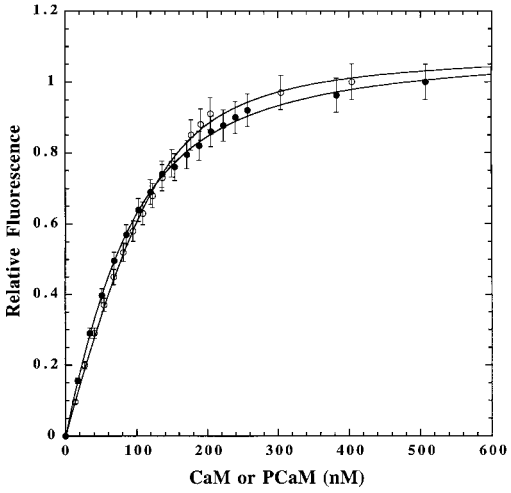

Figure 4 Fluorescence titration of dansylated peptide dans-C20W with CaM or PCaM

Peptide dans-C20W (150 nM) was treated with CaM (○) or PCaM (●). The averages of three experiments are plotted against the concentration of total CaM or PCaM. The continuous line shows the fit of the titration. The fit of the parameter $[S_0]$ gave values of 133 ± 5 and 166 ± 10 nM (means $\pm$ S.D.) for the titrations with CaM and PCaM respectively. The error bars represent S.D. Experimental conditions: 20 mM Hepes (pH 7.2)/130 mM KCl/1 mM $CaCl_2$. Excitation was at 340 nm and the fluorescence intensity was recorded at the maximum emission.

Binding of dans-CaM and dans-PCaM to the CaM-binding domains of other CaM targets

The affinities of the peptides CaMKII24, NO30, PDE16, CN24 and MLCK20 for CaM or PCaM were determined by titration with dans-CaM or dans-PCaM. Samples of CaM and PCaM were dansylated under identical conditions and in parallel. The degrees of dansylation were 70 % and 66 % for CaM and PCaM respectively. The Ca^{2+}-dependent changes in the fluorescence properties of the dansyl group (intensity and maximum wavelength position) of dans-PCaM were similar to those of dans-CaM.

The titrations of dans-CaM and dans-PCaM with the peptides are presented in Figure 5 and Table 2. All except one titration could be fitted to eqn. (2), corresponding to a one-binding-site model. Peptide NO30 bound to PCaM with lower affinity (K_d 5.5 ± 1.1 nM) than to CaM (K_d 1.9 ± 1.3 nM) without a change in the stoichiometry (Figure 5a).

In contrast, peptide CaMKII24 was had a higher affinity for dans-PCaM (K_d 0.6 ± 0.3 nM) than for dans-CaM (K_d 1.9 ± 0.8 nM) (Figure 5b); binding occurred with a 1:1 stoichiometry for CaM and PCaM. However, fluorescence quenching was observed for a peptide-to-CaM stoichiometry of more than one, suggesting the presence of a second binding site with lower affinity.

Table 2 Binding and kinetic parameters of CaM-binding proteins in the presence of CaM or PCaM

Each K_d is an average $\pm$ S.D. from three to five experiments. The catalytic parameters V_{max} and K_{act} were measured with the full enzymes in the presence of CaM or PCaM treated in the same way as in the binding experiments [17]. For experimental conditions, see the Materials and methods section.

Enzyme	Peptide	$K^{PCaM}_{act}/K^{CaM}_{act}$	$V^{PCaM}_{max}/V^{CaM}_{max}$	K^{CaM}_{d} (nM)	K^{PCaM}_{d} (nM)
Ca^{2+}-ATPase	C21W	3	0.85	21.5 ± 3.7	28.1 ± 4.0
Ca^{2+}-ATPase	C20W	–	–	7.9 ± 5.7	19.1 ± 6.6
Nitric oxide synthase	NO30	2	2.6	1.9 ± 1.3	5.5 ± 1.1
CaM kinase II	CaMKII24	2.8	0.2	1.9 ± 0.8	0.6 ± 0.3
Phosphodiesterase	PDE16	5	1	0.5 ± 0.3	10.6 ± 4.7
Calcineurin	CN24	5.8	1	20.2 ± 8.2	17.6 ± 8.7
MLCK	MLCK20	3.8	1	< 1	6.2 ± 2.4

Titrations of dans-CaM with peptide PDE16 suggested the presence of two binding sites (Figure 5c). Eqn. (2) was used to fit the data under the assumptions of two independent binding sites of similar affinities and a similar change in fluorescence on binding of the peptide to each site. K_d was estimated as 0.5 ± 0.3 nM for each site. Phosphorylation decreased the binding stoichiometry from 2 to 1 with a significant decrease in affinity (K_d 10.6 ± 4 .7 nM) (Figure 5c).

Peptide CN-24 bound dans-CaM (K_d 20.2 ± 8.2) with a similar affinity to that for dans-PCaM (K_d 17.6 ± 8.7) without a change in the stoichiometry (Figure 5d).

The titration of dans-CaM with peptide MLCK20 was surprisingly sigmoidal, reaching saturation with two bound peptides per dans-CaM molecule (Figure 5e). The same titration

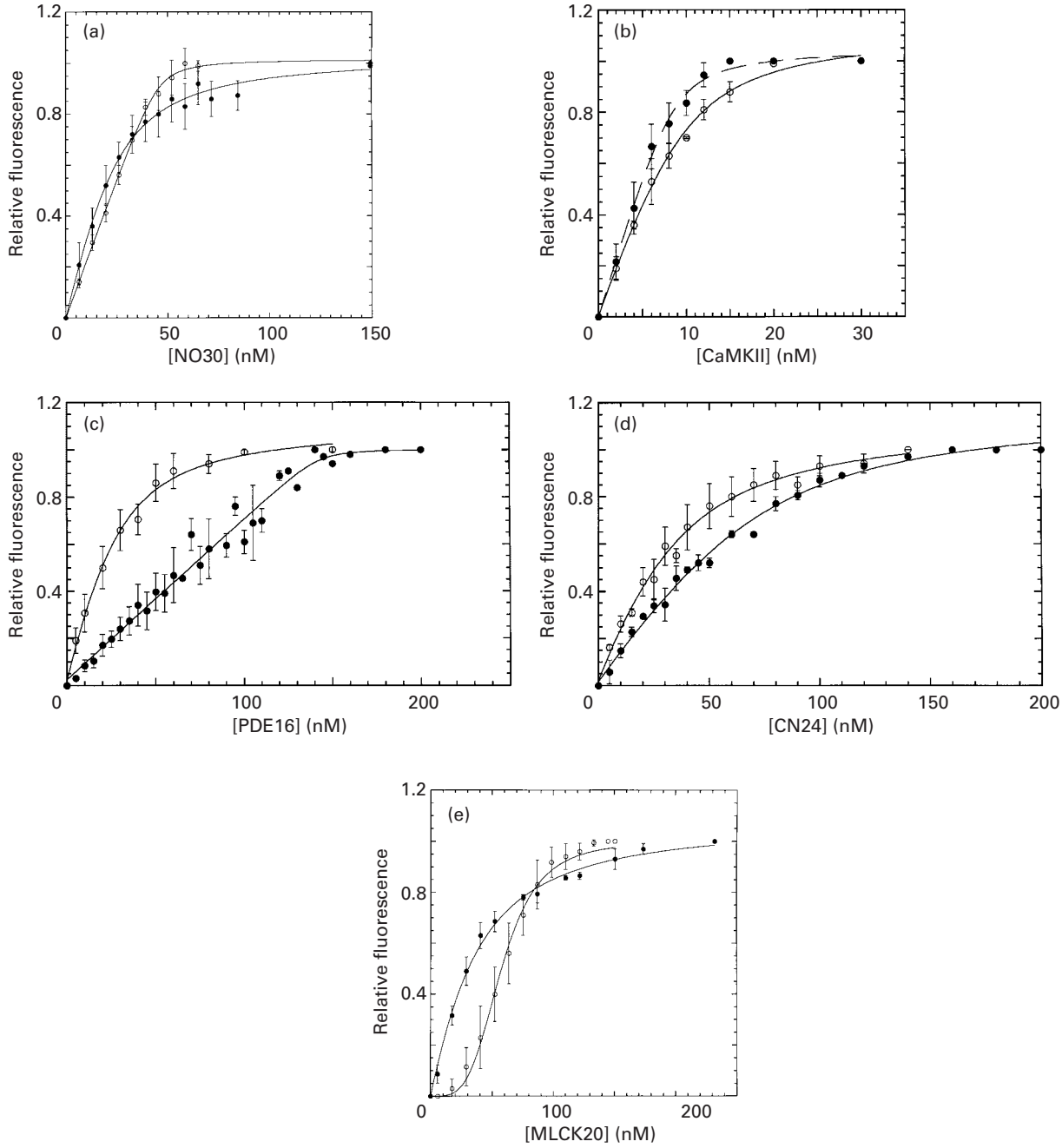

Figure 5 Titration of dans-CaM (○) and dans-PCaM (●) with five CaM-binding domains: NO30 (a), CaM kinase II (b), PDE16 (c), CN24 (d) and MLCK20 (e)

The continuous line corresponds to the fit to eqn. (2) except for the titration of MLCK20 with CaM (see the text for details). The initial concentrations of dans-CaM and dans-PCaM were 53 and 35 nM respectively, except for CaMKII24, which used 10 nM to avoid aggregation. The value of $[E_0]$ obtained from the fit should be compared with the initial concentration of dans-CaM or dans-PCaM. (**a**) CaM, $[E_0] = 45.7 \pm 2.5$ nM; PCaM, $[E_0] = 29.1 \pm 2.5$ nM. (**b**) CaM, $[E_0] = 9.5 \pm 11$ nM; PCaM, $[E_0] = 8.5 \pm 0.8$ nM. (**c**) CaM, $[E_0] = 142.2 \pm 5.9$ nM; PCaM, $[E_0] = 25.2 \pm 8$ nM. (**d**) CaM, $[E_0] = 68.1 \pm 10.7$ nM; PCaM, $[E_0] = 28.4 \pm 11.9$ nM. (**e**) CaM, the titration curve did not follow eqn. (2); PCaM, $[E_0] = 29.9 \pm 5.2$ nM. For experimental conditions, see the Materials and methods section. Values are means ± S.D. for three experiments. Excitation was at 340 nm and the fluorescence intensity was recorded at the emission maximum.

profile was obtained when the dans-CaM concentration was decreased to 10 nM, suggesting that the K_d of CaM for the peptide was less than 1 nM. In contrast with the sigmoid titration curve obtained with dans-CaM, the titration curve for dans-PCaM was hyperbolic and showed only one binding site with a K_d of 6.2 ± 2.4 nM.

DISCUSSION

Phosphorylation of CaM by CK II has been shown repeatedly to alter the activation of CaM target enzymes [7,8,15,17]. It was thus of interest to investigate the effect of CaM phosphorylation on its interaction with the CaM-binding domains. In the present paper we have studied the interaction of PCaM with synthetic

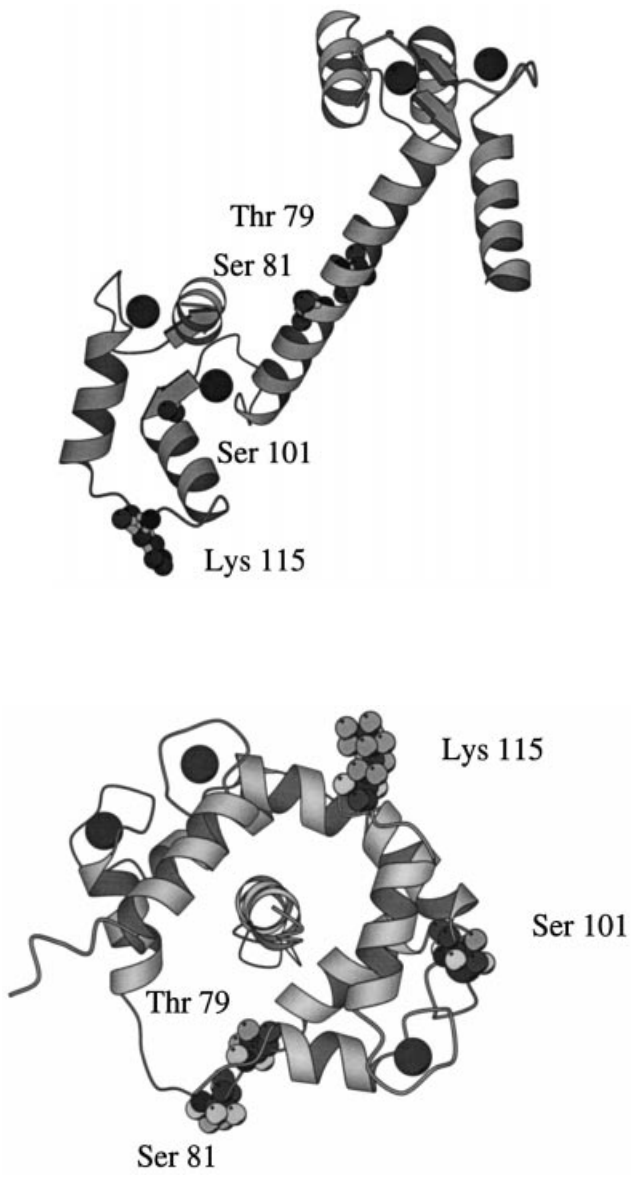

Figure 6 Schematic representations of the structures of free and peptide-bound CaM

Upper panel: three-dimensional representation of the structure of Ca^{2+}-bound CaM [65] generated with MOLSCRIPT. The three phosphorylation sites *in vivo* (Thr-79, Ser-81 and Ser-101) and the site of dansylation (Lys-115) are indicated in ball-and-stick form. Lower panel: three-dimensional structure of Ca^{2+}-bound CaM complexed with the CaM-binding domain of CaM kinase II [31]. The phosphorylation sites and the position of Lys-115 are indicated.

CaM-binding domains of Ca^{2+}-ATPase, calcineurin, CaM kinase II, MLCK, neuronal nitric oxide synthase and phosphodiesterase. Two strategies were chosen for studying the interaction. Peptides C20W and C21W of the Ca^{2+}-ATPase were dansylated at their N-termini and the protein–peptide interaction was followed by measuring the change in the fluorescence properties of the dansyl group of the peptide. Because the dansylation induced instability in some peptides, the second strategy was to dansylate CaM and PCaM and to follow the protein–peptide interaction by the change in the fluorescence properties of the dansyl group of the protein.

Dans-CaM has been used extensively to study the interaction of CaM with drugs or peptides [35–38], CaM-binding domains [22,27,28,39–41]) or CaM target proteins [30,36,42]. The recombinant CaM used in this study was dansylated on Lys-115, which is normally trimethylated in native mammalian CaM. Lys-115 is located in a loop connecting two α-helices (residues 102–112 and 119–128) and is exposed to the solvent (Figure 6, upper panel). Trimethylation at Lys-115 has previously been shown not to have a role in the modulation of the activity of most CaM target enzymes, except for NAD^+ kinase [43]. The dansyl-group at this position should therefore not interfere in the binding of the peptides. Dans-PCaM was found to be a suitable tool for studying the interaction of PCaM with target peptides or proteins because of the identical site of dansylation. Moreover, the Ca^{2+}-induced conformational changes in dans-CaM and dans-PCaM are likely to be similar, as detected by fluorescence spectroscopy.

The binding parameters of the dans-CaM–peptide complexes obtained in this study, in particular the dissociation constants (K_d) and the binding stoichiometry, agree with published values, with the exception of those for peptide MLCK20.

The binding parameters of peptides C20W and NO30 were similar to those obtained when studying the interaction of the dansylated peptide with bovine or recombinant CaM [22,27,41].

The peptide PDE16, an analogue of the CaM-binding domain of PDE, has not been studied previously. However, two very similar peptides (residues 23–44 and 20–41) were studied by Charbonneau et al. [44]. These authors reported a high affinity (K_d 30 nM) for CaM and a stoichiometry of two peptides per CaM. In the present study, we also found a binding stoichiometry of two peptides per CaM and a very high affinity (K_d 0.5 nM) for peptide PDE16. The peptide of sequence Q^{22}TEKMWQRLK-GILRCLVKQL[41] has recently been reported to interact with CaM with a relatively low affinity (K_d 224 nM) [45]. However, the author used an indirect method, competition with MLCK, to determine the affinity. The differences in methodology are probably responsible for the differences in the K_d. The relatively high affinity of the peptide PDE16 for CaM found in this study is not fully understood yet. However, one has to consider that the replacement of a cysteine residue with serine at position 36 may have unexpectedly enhanced the interaction of CaM with the peptide.

The affinity of dans-CaM for peptide CN24 determined in this study (K_d 20 nM) was of the same order of magnitude as that reported by Takano et al. [46] and was in agreement with the affinity for the full enzyme (K_d 6 nM) [1].

The interaction of CaM to the CaM-kinase-II-binding domain has been previously reported by two groups with the use of slightly different peptides. Putkey and Waxham [47] measured the kinetic parameters of the binding of CaM to a 25-residue peptide (residues 290–314) and found a K_d of approx. 0.01 nM. Sacks et al. [15] reported a K_d of 100 nM for a peptide five residues shorter (residues 290–309). We have measured a K_d of 1.9 nM with the 24-residue peptide CaMKII24 (residues 290–313). The reason for these large differences in K_d for very similar peptides is not clear. Sacks et al. [15] have also shown that a mixture of Ser/Thr-phosphorylated CaM containing 15% non-phosphorylated CaM had a affinity towards the short peptide (residues 290–309) that was one-eighth that of non-phosphorylated CaM. We show, on the contrary, that PCaM has a 3-fold higher affinity for the peptide CaMKII24 than does non-phosphorylated CaM. These opposite findings might be related to the differences in the lengths of the peptides or, more probably, to differences in the compositions of the PCaM samples used and in assay methods.

The peptide derived from MLCK has been studied previously and our results on high-binding affinity to CaM are in good agreement with those from other groups (K_d 1 nM) [23–25,48].

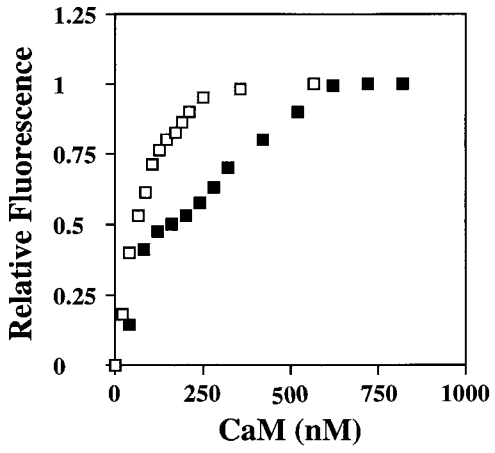

Figure 7 Titration of MLCK20 with bovine CaM (□) or recombinant CaM (■)

Experimental conditions: 240 nM MLCK20 in 20 mM Hepes (pH 7.2)/1 mM $CaCl_2$/130 mM KCl. Excitation of tryptophan was performed at 295 nm.

We made an unexpected observation during the titration of the peptide MLCK20 regarding its binding to CaM: the titration curve was sigmoidal, indicating positive co-operativity in the binding. Because such a behaviour had never been observed previously, we performed control experiments to investigate it. We speculated that the dansylation of CaM at Lys-115 might be responsible and repeated the titration with non-dansylated (recombinant) CaM. Because the peptide MLCK20 contains a tryptophan residue, the change in its fluorescence was used to follow the binding to non-dansylated CaM. In this case the titration was biphasic, suggesting two binding sites per molecule of CaM. We then performed the same experiment with bovine brain CaM instead of recombinant CaM. We observed a hyperbolic titration curve with saturation for one peptide per CaM (Figure 7), as reported previously [23]. It therefore seems that the interaction of MLCK20 with the recombinant CaM is different from that with bovine CaM. The lysine residue at position 115 might therefore be involved in the binding of MLCK20 to CaM, because this is the only difference between the three CaM species used (non-dansylated recombinant CaM, dansylated recombinant CaM and trimethylated bovine CaM). More detailed studies will be necessary to answer the remaining questions.

Two of the four phosphorylation sites are located in the central helix of CaMl the phosphorylation of these residues (Ser-81 and Thr-79) might influence the plasticity of CaM by modifying the flexibility of the helix. This helix is the key element, allowing significant conformational changes in CaM between an extended and a compact conformation [49,50]. When bound to a target peptide, the central helix is disrupted and forms an expansion joint between residues 73 and 77 (smooth-muscle MLCK peptide) [32] or 73 and 83 (CaM kinase II peptide; Figure 6, lower panel) [31]. The role of the central helix has been investigated by several methods, including mutations and deletions of one or several residues [24,51]. The results of these studies show that modifications in the central helix affect mainly the interaction of CaM with phosphodiesterase and MLCK, but had no significant effect on calcineurin or Ca^{2+}-ATPase. For example, the replacement of Thr-79 with a helix-breaking proline residue caused a 2-fold change in the activation constant for phosphodiesterase and MLCK, whereas no effects were observed with calcineurin and Ca^{2+}-ATPase. The insertion of an additional turn into the helix caused a more pronounced effect (a 9-fold increase) on the activation constant of phosphodiesterase and MLCK, whereas again no effect was observed with Ca^{2+}-ATPase and calcineurin [24]. Deletions of one to four residues ($S^{81}EEE^{84}$) also had specific effects [52]. The activation constant for MLCK was increased 5–7-fold, whereas a smaller increase (1–3-fold) was observed for calcineurin. This shows that the central helix has enzyme-specific roles, i.e. the helix has a greater importance in the activation of MLCK and phosphodiesterase than in that of calcineurin and Ca^{2+}-ATPase. Phosphorylation of CaM resulted in very similar behaviour: phosphorylation significantly decreased the binding affinity for the CaM-binding domains of MLCK and phosphodiesterase (to 10%). A minor effect was observed for the binding domains of Ca^{2+}-ATPase (the affinity was reduced by a factor of between 1.3 to 2.9) and no significant change was detected with the calcineurin peptide.

Whereas our results are in good agreement with structural studies described above, they show some discrepancy with the enzyme assays performed previously in our laboratory [17] (Table 2). For nitric oxide synthase, Ca^{2+}-ATPase, phosphodiesterase and MLCK, the increase in the dissociation constant was correlated with the increase in the activation constant of the full enzyme. The same was not true of calcineurin or CaM kinase II. The differences in the results might reflect the flexibility in the PCaM molecule, which could adopt slightly different conformations when interacting either with the full enzyme or with the CaM-binding domains.

CK II is believed to phosphorylate CaM *in vivo*. The phosphorylation of CaM *in vitro* by this kinase has been studied in detail [10,53]. However, the role of CK II on the phosphorylation of CaM *in vivo* is still unclear. CK II has been shown to phosphorylate more than 160 cellular proteins *in vitro*, including enzymes that participate in nucleic acid synthesis, transcription and translation factors, oncogenes and tumour suppressor proteins, cytoskeleton proteins and proteins that participate in signal transduction [54,55]; the list is still being added to [56–60]. Several substrates *in vitro* have also been shown to be phosphorylated *in vivo* by CK II: this includes c-Fos [61] and acetyl-CoA carboxylase [62] and very probably CaM [7,16]. Although the cellular events leading to the activation of CK II in the cell are still unclear, the kinase has been shown to be activated by insulin. In rat hepatocytes, the activation of the kinase was correlated with an increase of the level of PCaM [16,62–64].

Now that it has been clearly demonstrated that CaM is phosphorylated *in vivo* and that the activation of some CaM target enzymes is affected strongly by its phosphorylation, it is necessary to elucidate the molecular events leading to CaM phosphorylation.

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