A New Role for IQ Motif Proteins in Regulating Calmodulin Function

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IQ motifs are found in diverse families of calmodulin (CaM)-binding proteins. Some of these, like PEP-19 and RC3, are highly abundant in neuronal tissues, but being devoid of catalytic activity, their biological roles are not understood. We hypothesized that these IQ motif proteins might have unique effects on the Ca$^{2+}$-binding properties of CaM, since they bind to CaM in the presence or absence of Ca$^{2+}$. Here we show that PEP-19 accelerates by 40 to 50-fold both the slow association and dissociation of Ca$^{2+}$ from the C-domain of free CaM, and we identify the sites of interaction between CaM and PEP-19 using NMR. Importantly, we demonstrate that PEP-19 can also increase the rate of dissociation of Ca$^{2+}$ from CaM when bound to intact CaM-dependent protein kinase II. Thus, PEP-19, and presumably similar members of the IQ family of proteins, has the potential to alter the Ca$^{2+}$-binding dynamics of free CaM and CaM that is bound to other target proteins. Since Ca$^{2+}$ binding to the C-domain of CaM is the rate-limiting step for activation of CaM-dependent enzymes, the data reveal a new concept of importance in understanding the temporal dynamics of Ca$^{2+}$-dependent cell signaling.

Fluctuation in Ca$^{2+}$ levels defined by its amplitude and frequency of oscillation provides a universal intracellular signal, which can be highly variable between cells or even within different compartments of the same cell (1, 2). Although the ubiquitous protein calmodulin (CaM) is in large part responsible for decoding Ca$^{2+}$ signals, its Ca$^{2+}$ binding properties seem too restricted to accommodate the diversity of Ca$^{2+}$ signals. Thus far, changes in the Ca$^{2+}$ binding properties of CaM have been observed only as an increase in affinity due to a decrease in the Ca$^{2+}$ dissociation rate upon binding Ca$^{2+}$-CaM to target proteins and peptides (3–6,9). The magnitude of this effect is dependent on a given target enzyme and can be so large as to promote constitutive association of CaM with targets even at basal Ca$^{2+}$ levels.

It would be of great functional significance if proteins were discovered that could increase, rather than decrease, the rate of dissociation of Ca$^{2+}$ from free or target-bound CaM or potentially modulate the rate of Ca$^{2+}$ association. This would be particularly important for the C-domain of CaM, which exhibits Ca$^{2+}$ binding kinetics that are too slow to respond to rapid Ca$^{2+}$ transients such as those found in excitable cells. Our search for such proteins led to the analysis of the small, neuronal IQ motif proteins, or SNIQs, that include neuromodulin (Nm or GAP-43), neurogranin (Ng or RC3), and PEP-19 (for review see, Refs. 10 and 11). The SNIQs are highly abundant (up to 50% of neuronal tissues but have no known catalytic activity. Their ability to bind to apo-CaM supports the idea that SNIQs modulate effective levels and/or distribution of free CaM at basal Ca$^{2+}$ levels (12). However, Nm binds CaM equally well in the presence or absence of Ca$^{2+}$ at physiological salt concentrations (13). We hypothesized that these CaM binding properties could potentially affect the rates of both association and dissociation of Ca$^{2+}$ from CaM and thus provide an alternate function for the SNIQs that could have pervasive effects on CaM activity. We show here that PEP-19 accelerates the rates of association and dissociation of Ca$^{2+}$ from the C-domain of free CaM, and of CaM when bound to CaM-dependent protein kinase II α (CaMKII).

EXPERIMENTAL PROCEDURES

Expression of PEP-19—A human cDNA for PEP-19 was purchased from ResGen (IMAGE expressed sequence tag clone 4792589). The amino acid coding reading for PEP-19 was amplified using a 5’-primer (TTGGTGATTAGACCCCATGCTGGTAGG1ACAGTGG1TGTC), the 3’-primer (CCGAAGCTGCTACCGACCTCAGGACTGAGACCAGCC1), and subcloned into the NcoI and BamHI sites of the pET23d expression plasmid. The N terminus was modified from MSERQ to MAEQR to enhance expression levels of the recombinant protein.

Isolation of CaM and PEP-19—Recombinant CaM was expressed and isolated as described previously (14). CaM was labeled with $^{15}$Na as described previously for cardiac troponin C (15). PEP-19 was isolated as described in the Supplemental Material.

Equilibrium Ca$^{2+}$ Binding—Macroscopic equilibrium Ca$^{2+}$ binding constants were determined using the competitive binding assay described by Linse et al. (16).

Stopped-flow Measurements—Stopped-flow fluorescence experiments were acquired using an Applied Photophysics Ltd. (Leatherhead, UK) model SX.18 MV sequential stopped-flow spectrophotometer with a 150-watt xenon/mercury lamp and a dead time of 1.7 ms. We found that concentrations of 2 μM CaM, 20 μM Ca$^{2+}$, and 300 μM Quin-2 were sufficient to fully extract Ca$^{2+}$ from CaM complexes and the CaM-PEP-19 complex. All solutions contained a base buffer of 20 mM MOPS, pH 7.5, 100 μM KCl.

Fluorescence from Quin-2 was detected using excitation wavelengths of 334 or 334.5 nm and an Oriem emission cut-off filter 51282. Tyrosine fluorescence was detected using an excitation wavelength of 286 nm.

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The equilibrium calcium binding constant ($K_{eq}$) was derived from the data in Fig. 1B as described in the on-line Supplemental Material. The dissociation rate ($k_{off}$) was derived from the data in Fig. 1A. The association rate was calculated from $K_{eq} = k_{on}/k_{off}$.

| Domain  | $K_{eq}$ ($10^4$ M$^{-1}$) | $k_{on}$ (s$^{-1}$) | $k_{off}$ (s$^{-1}$) | $k_{off}/k_{on}$ |
|---------|-------------------------|--------------------|---------------------|------------------|
| N-domain | -PEP19 9.1 ± 1.3        | >1000              | >100                | 0.00001          |
|         | +PEP19 8.7 ± 1.1        | >1000              | >100                | 0.00001          |
| C-domain | -PEP19 50 ± 2.5         | 8.5 ± 0.2          | 4                   | 0.0006           |
|         | +PEP19 38 ± 4.4         | 430 ± 50           | 170                 | 0.00012          |

and Oriel filter 51662. All stopped-flow experiments were done at room temperature (23 °C). CaM$_{DANS}$ was excited at 334 nm, and fluorescence was monitored at 485 nm.

**RESULTS**

CaM has four Ca$^{2+}$ binding sites: two in the N-domain and two in the C-domain. Fig. 1A shows the rate of dissociation of Ca$^{2+}$ from CaM detected using the Ca$^{2+}$-sensitive fluorescent dye Quin-2. Release of Ca$^{2+}$ from the N-domain is too rapid (>1000 s$^{-1}$) to observe using conventional stopped-flow at room temperature (4). Thus, only the release of 2 mol of Ca$^{2+}$ is observed per mol of free CaM with a $k_{off}$ of 8.5 s$^{-1}$, which is identical to the value reported by Brown et al. (6). This observed rate of release of Ca$^{2+}$ was increased by a factor of 50 in the presence of PEP-19. Tyrosine fluorescence is a marker for Ca$^{2+}$ binding to the C-domain of CaM. The inset to Fig. 1A uses Tyr fluorescence to confirm that PEP-19, which has no Tyr residues, greatly accelerates the dissociation of Ca$^{2+}$ from the C-domain of CaM.

![Fig. 1A](image1.png)

**Fig. 1. Effect of PEP-19 on the Ca$^{2+}$ binding properties of CaM.**

A shows the rate of dissociation of Ca$^{2+}$ from free CaM in the absence (open circles) or presence (closed circles) of PEP-19 detected using the Ca$^{2+}$-sensitive fluorophore Quin-2. Note the discontinuous time scale used to highlight the early time points in the Quin-2 experiment. The offset in the y-axis in the presence of PEP-19 is due to substantial release of Ca$^{2+}$ that occurs during the deadtime of the stopped-flow instrument (1.7 ms). A, inset, shows an experiment using intrinsic Tyr fluorescence as a specific marker for release of Ca$^{2+}$ from the C-domain of CaM. B shows equilibrium Ca$^{2+}$ binding to CaM detected using the competitive dye-binding assay (16) in the absence (open circles) or presence (closed circles) of PEP-19. The lines show a least squares fit of the data to an algorithm that specifies four Ca$^{2+}$ binding sites. B, inset, shows the rate of association of Ca$^{2+}$ with CaM detected using intrinsic Tyr fluorescence. C shows the rate of dissociation of Ca$^{2+}$ from the CaM-CKIIa complex in the absence (open circles) or presence (closed circles) of PEP-19. C, inset, shows the effect of PEP-19 on fluorescence from IAEDANS-labeled CaM (CaM$_{DANS}$) (14) in the absence (open circles) or presence (closed circles) of CKIIa. The percent change in fluorescence is relative to the fluorescence intensity observed for free Ca$^{2+}$/CaM$_{DANS}$. The arrowheads indicate the concentration of PEP-19 used for stopped-flow experiments.
of the solvent exposed surface of the C-domain of Ca²⁺-CaM. The figures were generated using Insight II 2000.

PEP-19 on CaM amide chemical shifts was determined by sites of interaction between CaM and PEP-19. The effect of ping of amide chemical shift perturbations was used to localize vented assignment of amide chemical shifts by titration with change on the NMR time scale (data not shown), which pre-

vented assignment of amide chemical shifts by titration with PEP-19. Association of PEP-19 with Ca²⁺-CaM showed char-

acteristics of fast exchange and a dominant Ca²⁺-CaM/PEP-19 conformer. This allowed assignment of CaM amides in the complex of Ca²⁺-CaM-PEP-19 by titration of Ca²⁺-CaM with PEP-19 as illustrated in the inset to Fig. 2A.

The histogram in Fig. 2A summarizes the effect of PEP-19 on CaM amide chemical shifts. All changes of greater than 1 S.D. are found in the C-domain and are clustered in two regions. The first cluster includes amino acids 105–117 spanning helix F and the linker between helix F and G. Binding of PEP-19 to this region on CaM could be responsible for affecting the Ca²⁺-binding properties of sites III and IV. The second cluster includes amino acids 142–148 at the very C terminus of CaM. Fig. 2B illustrates the location of these clusters on the solvent accessible surface of the C-domain of Ca²⁺-CaM. The structure on the left emphasizes the hydrophobic surface of CaM shown in blue, which is now known to play a critical role in binding a variety of Ca²⁺-dependent CaM-binding proteins (18). Residues that are affected by PEP-19 are shown in red. This structure was rotated 180° to obtain the view on the right. Helix F is indicated in both views as a point of reference. Two regions on CaM are affected by PEP-19. Although these surfaces appear to be distinct, and to border the central portion of the hydrophobic pocket, this analysis does not account for potential conformational changes in CaM induced by PEP-19, which could present a contiguous binding surface. Nevertheless, it is clear from the two views of CaM that the most expansive surface that is affected by PEP-19 includes helix F and extends well away from the hydrophobic pocket. This suggests that PEP-19 could form ternary complexes with CaM and other binding proteins and potentially affect the Ca²⁺-binding properties of the complex.

We used recombinant CaM-dependent protein kinases II α (CKIIα) to determine the potential of PEP-19 to affect the Ca²⁺-binding properties of CaM when bound to another target protein. CKIIα binds CaM with high affinity (K_d = 10⁻⁷ to 10⁻⁸ M) that can be further enhanced by CaM-dependent autophosphorylation, but CaM/CKIIα is fully active in the absence of auto-

phosphorylation (19). Fig. 1C shows that 4 mol of Ca²⁺/mol of CaM are released when CaM is bound to CKIIα, with rate constants of 0.9 and 12 s⁻¹, respectively. Thus, association of CaM with CKIIα must greatly slow the release of Ca²⁺ from the N-domain, since this event is too fast to be observed with free CaM. We cannot currently assign these two rates to spe-

cific domains in CaM; however, the presence of PEP-19 greatly increased the fast rate from 12 to ~400 s⁻¹.

The results in Fig. 1C could be due to formation of a ternary complex between CaM, CKIIα, and PEP-19 or due to displace-

ment of CaM from CKIIα by PEP-19. We used IAEDANS-
labeled CaM (CaM_DANS) to distinguish between these possibilities. Fluorescence from CaM_DANS is increased upon binding to CKIIα (14, 20), but association with PEP-19 has little effect on fluorescence intensity. If PEP-19 displaced CaM_DANS from CKIIα, then it should reverse the CKIIα-induced increase in fluorescence. The results of this experiment are shown in the inset to Fig. 1C. Addition of up to 40 μM PEP-19 does not reverse the CKIIα-induced increase in fluorescence from CaM_DANS, which supports the formation of a ternary complex between CaM, CKIIα, and PEP-19. Together, these data demon-

strate that PEP-19 can affect the kinetics of Ca²⁺ binding to CaM even when it is bound to another protein.

**DISCUSSION**

The rates of association and dissociation of Ca²⁺ at the C-domain of CaM are up to 2 orders of magnitude slower than the N-domain (see Table I). Thus, Ca²⁺ binding to the C-domain is rate-limiting for activation of targets that require Ca²⁺-saturated CaM and will dictate its temporal response to Ca²⁺ signals. The effect of PEP-19 on the C-domain of CaM, and localization of PEP-19 and other SN1Qs to neurons, sug-

gest that these proteins play a role in modulating the Ca²⁺-binding properties of CaM so it can respond to challenging
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Ca²⁺ signals, such as high frequency action potentials, which control Ca²⁺-dependent processes in pre and post-synaptic compartments. Fig. 3 simulates the effect of PEP-19 on Ca²⁺ binding to the C-domain of CaM during a train of Ca²⁺ pulses of 50 Hz and amplitude of 1.5 μM. In the absence of PEP-19, intrinsic slow on and off Ca²⁺ binding kinetics lead to a gradual increase in the percent saturation of the C-domain of CaM with Ca²⁺. In the presence of PEP-19, Ca²⁺ binding to the C-domain of free CaM more closely parallels the rise and fall of free Ca²⁺. This illustrates that the C-domain of CaM could respond to Ca²⁺ in distinct ways depending on the presence or absence of PEP-19. The effect of other SNIQs on CaM will likely be fine-tuned by differences in primary sequences. For example, Ng accelerates the dissociation of Ca²⁺ from CaM but has little effect on the association rate, which results in decreasing the overall Ca²⁺ binding affinity of the C-domain of CaM. Post-translational modification, such as phosphorylation of Ng and Nm that inhibits association with CaM (13, 21, 22), would provide another level of regulation. These data and simulations emphasize that the influence of PEP-19, and other proteins with similar activity toward CaM, should be incorporated into models of neuronal Ca²⁺ dynamics and CaM activation that use stochastic approaches to account for CaM diffusion and spatial constraints.

Current structural models for the regulation of target proteins by CaM invoke distinct functions for its N- and C-domains. IQ CaM binding motifs found in diverse proteins may help facilitate these interactions. For example, Ca²⁺-dependent facilitation and inactivation of L-type and P/Q-type voltage-dependent Ca²⁺ channels (23–27) is due largely to the temporal and differential binding of the N- and C-domains of CaM to the IQ motif of these channel proteins. DeMaria et al. (27) proposed that the C-domain of CaM mediates channel facilitation by responding to millisecond spike-like elevations in local Ca²⁺ that result from opening of individual channels. The data presented here suggest that the IQ motif in voltage-operated Ca²⁺ channels could modulate the Ca²⁺ binding properties of the C-domain of CaM such that it can appropriately respond to calcium spikes.

Calcium binding is the fundamental property of CaM that allows it to function as a central regulatory protein in a plethora of signal transduction pathways. From a general perspec-

T. R. Gaertner, J. A. Putkey, and M. N. Waxham, unpublished observation.

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