Neurogranin Alters the Structure and Calcium Binding Properties of Calmodulin

Received for publication, February 27, 2014, and in revised form, April 4, 2014. Published, JBC Papers in Press, April 8, 2014, DOI 10.1074/jbc.M114.560656

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Background: Neurogranin is a IQ motif protein that binds to both apo- and calcium-saturated calmodulin.

Results: Neurogranin makes contacts with both the N- and C-domains of calmodulin that functionally leads to altered calcium binding kinetics.

Conclusion: The availability and calcium saturation of calmodulin is determined by neurogranin.

Significance: Neurogranin plays a major role in controlling the intracellular calcium–calmodulin–signaling pathway.

Neurogranin (Ng) is a member of the IQ motif class of calmodulin (CaM)-binding proteins, and interactions with CaM are its only known biological function. In this report we demonstrate that the binding affinity of Ng for CaM is weakened by Ca2+ but to a lesser extent (2–3-fold) than that previously suggested from qualitative observations. We also show that Ng induced a >10-fold decrease in the affinity of Ca2+ binding to the C-terminal domain of CaM with an associated increase in the Ca2+ dissociation rate. We also discovered a modest, but potentially important, increase in the cooperativity in Ca2+ binding to the C-lobe of CaM in the presence of Ng, thus sharpening the threshold for the C-domain to become Ca2+-saturated. Domain mapping using synthetic peptides indicated that the IQ motif of Ng is a poor mimetic of the intact protein and that the acidic sequence just N-terminal to the IQ motif plays an important role in reproducing Ng-mediated decreases in the Ca2+ binding affinity of CaM. Using NMR, full-length Ng was shown to make contacts largely with residues in the C-domain of CaM, although contacts were also detected in residues in the N-terminal domain. Together, our results can be consolidated into a model where Ng contacts residues in the N- and C-domains of both apo- and Ca2+-bound CaM and that although Ca2+ binding weakens Ng interactions with CaM, the most dramatic biochemical effect is the impact of Ng on Ca2+ binding to the C-terminal lobe of CaM.

Calmodulin is a small (16.8 kDa) ubiquitous protein responsible for transducing changes in intracellular Ca2+ concentrations for the activation of downstream targets. The concentration of CaM3 varies widely in tissues but typically ranges from 1 to 100 μM (1–3). Because each CaM molecule binds 4 Ca2+ ions, it has been postulated that CaM also functions as a buffer of intracellular Ca2+ (4–6). The four Ca2+ binding sites of CaM fall into the EF-hand motif class and are organized into two pairs; two low affinity sites (Kd ~ 10–12 μM) reside in the N-terminal domain, and two high-affinity sites (Kd ~ 1–2 μM) reside in the C-terminal domain (7). Ca2+ binding within each domain exhibits significant cooperativity, and although the N- and C-terminal domains are separated by a central flexible linker, interactions exist between the N- and C-domains that influence their Ca2+ binding properties (8–10). The kinetics of Ca2+ binding to each lobe are also dramatically different. The N-lobe exhibits association and dissociation rates for Ca2+ of >1 × 108 M−1 s−1 and >500 s−1, respectively, whereas the C-domain exhibits association and dissociation rates of ~ 0.05 × 108 M−1 s−1 and ~10 s−1, respectively (5, 11–14).

During the rising phase of intracellular Ca2+, the N-domain would fill before the C-domain but would also be the first to release Ca2+ if not bound to targets. This leads to the idea that binding of Ca2+ to the C-domain is the rate-limiting step in the activation of CaM-target proteins that bind to Ca2+-saturated CaM (12, 15).

CaM interacts with an extensive list (hundreds) of target proteins (16–20). Some preferentially bind to Ca2+-free (apoCaM) and others preferentially bind to Ca2+-saturated CaM (20). Following the principle of detailed balance, when target proteins bind CaM, there is a reciprocal impact on the Ca2+ binding properties of CaM. In this way the binding of target proteins can increase or decrease the Ca2+ binding properties of CaM and, if the target binds preferentially to the N- or C-domain, can selectively affect the Ca2+ sensitivity and or kinetics of the individual domains (11, 15, 21, 22).

Quantifying the reciprocal interactions between Ca2+, CaM, and individual targets is, therefore, essential in understanding how CaM achieves target selectivity.

* This work was supported, in whole or in part, by National Institutes of Health Grants GM097553 (NIGMS; to M. N. W.) and GM104290 (NIGMS; to M. N. W. and J. A. P.).

This article contains supplemental Fig. 1.

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The abbreviations used are: CaM, calmodulin; Ng, neurogranin; Nm, neuromodulin; apoCaM, calmodulin in the Ca2+-free state; C-CaM, isolated C domain of CaM; HEDTA, N-(2-hydroxyethyl)ethylenedinitriaci tic acid; ITC, isothermal titration calorimetry; HSQC, heteronuclear single quantum coherence.

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in the face of Ca\(^{2+}\) signals that fluctuate in frequency and amplitude.

It is also plausible that intracellular mechanisms exist to minimize inappropriate activation of CaM in the face of basal or spontaneous fluctuations that routinely occur in intracellular Ca\(^{2+}\), thereby providing a proofreading mechanism for ensuring CaM activation only under appropriate signaling situations. In fact, there is a class of proteins whose only identified role appears to be to control the level and activation state of CaM (14, 23–25). The best-characterized members of this class are Purkinje cell protein 4 (PEP-19), neuromodulin (Nm also called GAP-43), and neurogranin (Ng also called RC3). All three of these abundant proteins have similar CaM binding regions that fall into the IQ motif class, bind both apo- and Ca\(^{2+}\)/CaM, and where evaluated, impact the kinetics and/or binding affinity of CaM for Ca\(^{2+}\) (11, 14, 23, 26). These properties make them uniquely suited to provide roles in “buffering” the levels of available CaM and to reduce the probability that CaM will become Ca\(^{2+}\)-activated (supplying the proofreading function).

Nm and Ng are further subject to protein kinase C (PKC)-mediated phosphorylation of a specific Ser residue located in the middle of the CaM binding domain (23, 25, 26). Phosphorylation significantly weakens the binding of CaM to these proteins, freeing CaM to interact with other CaM-dependent targets. These three IQ motif proteins are all highly expressed in nervous tissue but have distinct anatomical localizations. Nm is largely a presynaptic protein, enriched in nerve terminals responsible for neurotransmitter release and is found widespread across the nervous system (27, 28). PEP-19 and Ng are also found widespread in the nervous system but are largely postsynaptic and are rarely, if ever, found to be co-expressed in the same neuron (23, 29–32). This leads to the hypothesis that Ng and PEP-19 may serve analogous roles in the postsynaptic cell but that they might possess unique properties (like cross-talk with the PKC pathway) that permits cell type-specific tuning of their impact on the Ca\(^{2+}\)/CaM signaling pathway.

We focus in this report on a quantitative understanding of the biochemical properties of Ng and its interactions with CaM, and we contrast these findings with published information on the related protein PEP-19 (14, 33). Ng (~7.8 kDa) and PEP-19 (~6.8 kDa) are small intrinsically disordered proteins that both contain an IQ motif (34, 35) preceded by an acidic region composed largely of Asp or Glu residues. The most significant sequence difference between these two proteins is in the region C-terminal to the IQ motif. The primary sequence of PEP-19 extends only 3 amino acids C-terminal to the IQ motif, whereas Ng has a 32-amino acid extension that is highly enriched in Gly and Pro residues. PEP-19 was previously shown to interact primarily with the C-terminal domain of CaM, and binding leads to a significant increase in both the Ca\(^{2+}\) association and dissociation rates from the C-terminal domain, with little impact on the overall \(K_d\) (14). The effects of full-length PEP-19 on the Ca\(^{2+}\) binding properties of CaM could only be mimicked by a peptide that encompassed both the IQ motif and the adjacent acidic sequence, whereas a peptide representing the IQ motif had little effect on the Ca\(^{2+}\) binding properties of CaM (21). One of the goals of the present study was to investigate whether the analogous IQ and acidic regions in Ng provide similar functions in modifying Ca\(^{2+}\) binding properties of CaM.

In the present report we used NMR to identify contacts between apoCaM and Ca\(^{2+}\)/CaM with Ng, a peptide representing its IQ motif, and a peptide representing the IQ motif peptide that includes the acidic region. These structural studies were complemented with an analysis of the binding affinity of Ng and Ng peptides for both apoCaM and Ca\(^{2+}\)/CaM, along with a quantitative assessment of their impact on Ca\(^{2+}\) binding properties of CaM. These studies revealed that, similar to PEP-19, the IQ motif peptide does not serve as a good mimetic of the intact Ng protein. The IQ motif peptide of Ng increased the Ca\(^{2+}\) binding affinity of CaM by decreasing the Ca\(^{2+}\) dissociation rate, whereas intact Ng decreased Ca\(^{2+}\) binding affinity by increasing the Ca\(^{2+}\) dissociation rate. Interestingly, the IQ peptide with the acidic region could largely, but not completely, mimic the effects of Ng on Ca\(^{2+}\) binding properties of CaM. For example, we found that there was a modest but reproducible increase in the cooperativity of Ca\(^{2+}\) binding to CaM in the presence of Ng, but this effect was not reproduced by either the IQ motif peptide or the IQ motif peptide that included the acidic sequence. The recently published crystal structure of the IQ motif peptide of Ng with Ca\(^{2+}\)/CaM reveals that the peptide makes contacts exclusively with the C-terminal domain of CaM (34). In contrast, our NMR results show that although Ng has extensive interactions with residues in the C-domain, it also makes significant contacts with the N-domain of CaM. Based on unique NMR signatures of the peptides versus the intact protein, we suggest that the C-terminal glycine-rich region of Ng likely makes contacts with the N-terminal domain of CaM.

Together, our results can be consolidated into a model where Ng contacts residues in the N- and C-domains of both apo- and Ca\(^{2+}\)-bound CaM. The binding affinity of Ng for CaM is weakened by Ca\(^{2+}\)-consistent with previous results and supporting the hypotheses that Ng can serve to sequester (and concentrate) CaM in the basal state. In turn, CaM becomes available for target binding through Ca\(^{2+}\)-dependent dissociation (36, 37). In addition, we demonstrate a modest, but potentially important, increase in the cooperativity in Ca\(^{2+}\) binding to the C-lobe of CaM in the presence of Ng, sharpening the threshold for the C-domain to become Ca\(^{2+}\) saturated. Finally, we show that Ng greatly decreases the affinity of Ca\(^{2+}\) binding to the C-domain of C-domain of CaM and that an acidic sequence just N-terminal to the IQ domain is essential for Ng ability to modulate Ca\(^{2+}\) binding to CaM.

These results reinforce that Ng plays a critical role in controlling the Ca\(^{2+}\)/CaM signaling pathway and, when the Ca\(^{2+}\)-buffering capacity of CaM is considered, also plays a role in establishing free Ca\(^{2+}\) levels inside cells (4, 24). Given these properties, it is not surprising that Ng has been documented as a key molecule regulating the induction of Ca\(^{2+}\)-dependent plasticity (38) in the nervous system and has been implicated in a growing number of neurological disorders (39–46).

**EXPERIMENTAL PROCEDURES**

**Protein Preparation**—Wild type mammalian CaM (47), \(^{15}\)N isotope-labeled CaM (33, 48), and truncated CaM (48) residues 76–149 (C-CaM), representing the C-terminal lobe were
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| Acridic motif | IQ motif | Glycine rich C-terminal region |
|--------------|----------|-------------------------------|
| GSHMCCCTENASCKPDDIDLIDPLDDPGANAAAAKIQASFRGHMARKKIKSGERGRKGPGPGPPGAGVARGGAGGGPSGD | ANAAAAKIQASFRGHMARKKIKSG | DDDILPDDPLDDPGANAAAAKIQASFRGHMARKKIKSG |

FIGURE 1. Ng protein sequence and peptides investigated in this work representing the IQ motif (center) and IQ motif plus acidic region (bottom). The glycine-rich C-terminal region is only represented in the full-length Ng protein.

expressed and purified as previously described. A full-length cDNA for human Ng was obtained from Open Biosystems and incorporated into pET23d expression vector (Novagen). An N-terminal His tag and tobacco etch virus protease cleavage site were engineered into the construct as well as a D2A point mutation for increased expression. Cloning was confirmed via DNA sequencing, and the resultant sequence of the translated protein product is as follows (the cleavage product in regular font, and tag is in italics): MGSSHHHHHHSSGGRENLYQFGSH-MACCTENACSPDDILDPLDDPGANAAAAKIQASFRG-HGMARKKIKSGERGRKGPGPGPPGAGVARGGAGGG-PSGD (see also Fig. 1). DNA was transformed into BL21 (DE3) pLysS Escherichia coli, and protein was expressed with a 1 mM isopropyl 1-thio-β-D-galactopyranoside induction. Protein was extracted from harvested cells by a freeze-thaw cycle followed by sonication in 20 mM PBS, 20 mM imidazole, pH 7.4. Cellular debris was separated by centrifugation, and supernatant was applied to a nickel-affinity resin for protein purification. Resin was washed with 20 mM PBS, 40 mM imidazole, pH 7.4, and protein was eluted in 20 mM PBS, 250 mM imidazole, pH 7.4. The six-histidine tag was cleaved with a tobacco etch virus protease kit (Promega), and digested material was separated via nickel affinity using the same buffers. Cleavage of the six-histidine tag was evaluated by Western blotting with a mouse penta-His antibody (Qiagen) at a 1:1000 dilution and a goat anti-mouse Cy5 secondary antibody (Jackson ImmunoResearch product number 115-175-146) at a 1:2000 dilution. Blots were imaged on a Typhoon Trio scanner (GE Healthcare). Cleaved protein was then further purified on a reverse phase C18 column (Vydac), loaded in 0.1% TFA, and eluted in 80% acetonitrile, 0.1% TFA. Protein was lyophilized and suspended as needed. Ng protein was identified by SDS-PAGE and Western blotting. Western analysis was carried out with a rabbit anti-Ng primary antibody (Chemicon (Millipore) Int., catalogue no. AB5620) at a 1:500 dilution and a goat anti-rabbit Cy3 fluorescent secondary antibody (Jackson ImmunoResearch product n. 111165-144) at a1:1000 dilution. Peptides Ng 26–49, comprising the IQ domain, and Ng 13–49, comprising the IQ domain and adjacent acidic region (see Fig. 1), were synthesized by LifeTein. Quantitative analysis of all protein and peptides was completed by the University of Texas Medical Branch Biomolecular Resource Facility to determine protein concentrations.

Steady State Ca\(^{2+}\) Affinity Measurements—Ca\(^{2+}\) binding to the CaM-Ng complex was monitored under steady state conditions where Ng protein or peptide was in 10-fold excess of CaM. This saturating concentration ensured that the CaM-Ng complex would remain associated through the range of titered Ca\(^{2+}\). Because the Ca\(^{2+}\) dissociation constants for the CaM-Ng complex are in the μM range, a concentration of 5 μM CaM was used in experiments to permit significant populations of Ca\(^{2+}\)-bound and Ca\(^{2+}\)-free forms at several titration points. Experiments were carried out in the presence of EGTA, nitro-2,2',2'-triacetic acid, and HEDTA so that the free Ca\(^{2+}\) concentration (i.e. Ca\(^{2+}\) available to bind the CaM-Ng complex) was controlled by the buffering capacity of the chelators. Ca\(^{2+}\) titrations were carried out in a 1-ml reaction volume consisting of 50 mM MOPS, 100 mM KCl, 1 mM EGTA, 1 mM HEDTA, 1 mM nitro-2,2',2'-triacetic acid, 5 μM CaM, pH 7.2, in the presence or absence of 50 μM Ng. A solution containing the same reagents plus 25 mM CaCl\(_2\) was titered from 0 up to 1.0 mM free Ca\(^{2+}\). To maintain a 1-ml reaction volume throughout the experiment, a volume equivalent to the titered volume was first removed from the reaction for each titration point. Values for free Ca\(^{2+}\) concentration were determined with the MaxChelator program (webmaxc extended version 2012) assuming an ionic strength of 0.133 M and temperature of 24 °C. Native Tyr fluorescence of CaM, shown to be sensitive to Ca\(^{2+}\) binding, was monitored on a PTI fluorometer with 276-nm excitation and 304-nm emission wavelengths with 1- and 10-nm slit widths, respectively. Fluorescence was monitored for 30 s at 10 points/s after each titration addition, and the average intensities at each point were plotted as a function of free Ca\(^{2+}\) concentration.

Data were fit with the Hill equation,

\[
y = \text{START} + (\text{END} - \text{START})(x^\prime/(K_\text{H}^\prime + x^\prime))
\]

where \(y\) is the fluorescence intensity average, \(x\) is free Ca\(^{2+}\), \(K_\text{H}\) is the microscopic dissociation constant and is equivalent to the concentration of Ca\(^{2+}\) at which the change in Tyr fluorescence is half-maximal, and \(n\) is the Hill coefficient. For comparisons, data were normalized such that the fits spanned intensities of 0 to 1.

Isothermal Titration Calorimetry—ITC measurements were performed in a Microcal VP-ITC instrument in a buffer consisting of 25 mM HEPES buffer, pH 7.2, in the presence of either 1 mM EDTA or 2 mM CaCl\(_2\) and in the presence or absence of KCl. Samples were degassed and brought to 25 °C before starting titrations. All titrations were carried out by injecting 5 μl of 150 μM peptide or protein into the cell containing 1.8 ml of 5 μM CaM at 25 °C with constant stirring. Each experiment consisted of 28 injections with a 20-s injection duration and 210-s spacing between injections. The raw data were base-line corrected, and integrated peak areas were calculated using Microcal software then plotted as a function of mole ratio. Data were fit with single binding site models to determine the binding stoichiometry, \(N\), association constant, \(K\), enthalpy, \(\Delta H\), and entropy, \(\Delta S\).

Nuclear Magnetic Resonance—NMR experiments were carried out with a Bruker DRX 600MHz spectrometer equipped with a 5-mm triple-resonance cryoprobe or broadband probe at 298 K. \(^1\)H heteronuclear single quantum coherence (HSQC) spectra for 0.2–0.5 mM \(^{15}\)N isotope-labeled CaM were collected.
in buffer consisting of 10 mM imidazole, pH 6.3, 100 mM KCl, and 5% D_2O in the presence of either 5 mM CaCl_2 or 5 mM EDTA. Titration of 15N-labeled CaM with Ng or Ng peptides was performed as described previously for PEP-19 and PEP-19 peptides (21). All NMR spectra were processed and analyzed using Topspin 3.2 (Bruker). H chemical shifts were referenced to 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). Residue assignments for backbone amide chemical shifts were made as described previously for apoCaM (35) and Ca^2+/-CaM (14). Binding of Ca^2+/-CaM to Ng or Ng peptides showed characteristics of fast exchange on the NMR time scale, which allowed assignment of backbone amides for Ca^2+/-CaM in the bound form by tracking chemical shift changes during titration with Ng or Ng peptides. Changes in chemical shifts comparing unbound to Ng-saturated CaM were determined using the formula

\[
\Delta \delta = \sqrt{(\Delta \delta H)^2 + (\Delta \delta N/5)^2}/2
\]  
(Eq. 2)

where \(\Delta \delta\) is the total change in chemical shift, \(\Delta \delta H\) is the change in the proton chemical shift, and \(\Delta \delta N\) is the change in the 15N chemical shift.

**Kinetic Rate Measurements**—Rates for Ca^2+ dissociation from the CaM-Ng complex were measured using an Applied Photophysics Ltd model SV.17 MV sequential stopped flow spectrofluorimeter with an instrument dead time of 1.7 ms. The time course of Ca^2+ dissociation was monitored after the addition of excess chelator by observing the native tyrosine fluorescence of CaM, shown to decrease upon Ca^2+ dissociation. Reactions were carried out by rapidly mixing solutions from two syringes in equal volumes at room temperature. One syringe contained 10 \(\mu\)M CaM and 100 \(\mu\)M CaCl_2 in a standard buffer (50 mM MOPS, 100 mM KCl, pH 7.2), and the other contained an excess of 10 mM EGTA chelator in the same standard buffer resulting in final reaction concentrations of 5 \(\mu\)M CaM and 5 mM EGTA. Reactions were carried out in the presence or absence of 50 \(\mu\)M full-length Ng or Ng peptides. Samples were excited at 276 nm, and fluorescence emission was detected with a bandpass filter with peak transmittance at 355 nm (Oriel 51662). Off-rates were additionally evaluated using the fluorescent chelator Quin-2, which increases in fluorescence, which is sensitive to Ca^2+ binding to the C-lobe of CaM, was monitored as a function of free Ca^2+ concentration. Data were fit with a Hill function, and \(K_0\) values were determined. Results show that the CaM-Ng complex has a >10-fold reduction in Ca^2+ affinity as compared with unbound CaM (Fig. 2A, Table 1) consistent with previous work (11). We are able to determine the cooperativity of Ca^2+ binding to CaM by fitting the steady state data to the Hill equation. Cooperativity has been attributed to structural changes that occur after binding an initial Ca^2+ ion, which serves to stabilize subsequent ion binding (51). Cooperative Ca^2+ binding within lobes has been characterized in the absence of target, and cooperativity between lobes has been observed in the presence of targets (52, 53). Fig. 2A shows a change in the slope of steady state Ca^2+ binding curves for CaM in the absence (black) and presence of Ng (red). The cooperativity (n) of Ca^2+ binding was increased from 1.5 to 2.0 for CaM and CaM-Ng, respectively (Table 1). Overall, the change in Ca^2+ affinity and cooperativity indicate that Ng acts as a modulator of CaM-dependent signaling.

We then determined if the change in affinity was due to modified Ca^2+ dissociation by measuring Ca^2+ off-rates from the CaM-Ng complex. Calmodulin was incubated with Ng in the presence of Ca^2+ and then rapidly mixed with excess EGTA using a stopped flow experimental setup. Native Tyr fluorescence was monitored as a function of time, and data were fit with a single exponential equation to determine \(k_{off}\). It is important to note that because of the cooperative binding of Ca^2+ within CaM lobes, the release of one Ca^2+ ion is tightly coupled to release of the other within the same domain. Therefore, dissociation of two Ca^2+ ions from the same lobe is observed as one rate. We discovered that the off-rate was increased by 50-fold for Ng-CaM compared with CaM alone (Fig. 2B). These results are corroborated by rate measurements using the fluorescent chelator Quin-2 (Fig. 2C). Experiments yielded similar rates of 11.1 and 511 s^-1 for CaM and CaM-Ng, respectively.

**RESULTS**

Ng Promotes Rapid Dissociation of Ca^2+ from CaM and Necessitates Increased Ca^2+ Concentrations for Saturation—We first determined how Ng affects Ca^2+ binding to CaM. If apoCaM is sequestered by Ng at basal Ca^2+ levels in vivo, then the capacity for CaM to respond to increased Ca^2+ will be dependent on the Ca^2+ affinity of the CaM-Ng complex as well as the affinity of CaM for Ng. Because Ng has been shown to prefer binding to apoCaM rather than Ca^2+-saturated CaM (34, 49, 50), the CaM-Ng complex should have a reduced affinity for Ca^2+ relative to free CaM. Our group has reported that Ng indeed decreased the affinity of CaM for Ca^2+; however, binding affinities were not previously quantified (11). We, therefore, first quantified the steady state Ca^2+ affinity for the CaM-Ng complex. For these experiments, apoCaM was complexed with Ng protein, and Ca^2+ concentration was systematically increased under steady state conditions. Native Tyr fluorescence, which is sensitive to Ca^2+ binding to the C-lobe of CaM, was monitored as a function of free Ca^2+ concentration. Data were fit with a Hill function, and \(K_0\) values were determined. Results show that the CaM-Ng complex has a >10-fold reduction in Ca^2+ affinity as compared with unbound CaM (Fig. 2A, Table 1) consistent with previous work (11). We are able to determine the cooperativity of Ca^2+ binding to CaM by fitting the steady state data to the Hill equation. Cooperativity has been attributed to structural changes that occur after binding an initial Ca^2+ ion, which serves to stabilize subsequent ion binding (51). Cooperative Ca^2+ binding within lobes has been characterized in the absence of target, and cooperativity between lobes has been observed in the presence of targets (52, 53). Fig. 2A shows a change in the slope of steady state Ca^2+ binding curves for CaM in the absence (black) and presence of Ng (red). The cooperativity (n) of Ca^2+ binding was increased from 1.5 to 2.0 for CaM and CaM-Ng, respectively (Table 1). Overall, the change in Ca^2+ affinity and cooperativity indicate that Ng acts as a modulator of CaM-dependent signaling.

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Data were fit to a single exponential equation to determine \(k_{off}\)

\[
F = (F_{init}\ e^{-k_{off}t}) + F_{final}
\]  
(Eq. 3)

where \(F\) is the observed fluorescence intensity at time \(t\), and \(k_{off}\) is the Ca^2+ dissociation rate. Although CaM can coordinate four Ca^2+ ions, data were best fit with a single rate model because 1) Ca^2+ dissociation rates from the N-terminal lobe are too fast to accurately measure at room temperature, and 2) the cooperativity within lobes tightly couples release of one Ca^2+ ion to release of the other.
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![Graphs and figures](image)

FIGURE 2. Steady state calcium binding and kinetics of calcium dissociation. A, steady state Ca\(^{2+}\) binding affinities were determined by titrating Ca\(^{2+}\) into CaM (black circles) or the CaM-Ng complex (red, solid circles). Fluorescence intensities are plotted as a function of free Ca\(^{2+}\) concentration, fit with a Hill function (solid lines), and normalized such that the fits spanned intensities from 0 to 1. Comparisons of Ca\(^{2+}\) dissociation rate measurements are shown for CaM and the CaM-Ng complex measured via Tyr (B) and Quin-2 (C) fluorescence. Fluorescence intensities were plotted as a function of time and fit with single exponential decay functions to determine \(k_{off}\). Every 50th data point is depicted for simplicity. D, Ca\(^{2+}\) binding affinity experiments are shown for the CaM-Ng (red open circles) and C-CaM-Ng (red solid circles) complexes. Comparisons of Ca\(^{2+}\) dissociation rates for CaM-Ng and C-CaM-Ng measured by Tyr (D) and Quin-2 (F) experiments are shown.

TABLE 1

Calcium affinity and kinetics for CaM and CaM-Ng

| Protein         | \(K_A\) (n = 2) | \(n\) (n = 2) | \(k_{off}\) (n \(\geq\) 3) |
|-----------------|-----------------|--------------|--------------------------|
| CaM             | 1.78 ± 0.18     | 1.5 ± 0.03   | 10.7 ± 0.47              |
| CaM-Ng          | 11.1 ± 0.12     | 2.0 ± 0.09   | 556 ± 34.7               |
| C-CaM           | 3.18 ± 0.002    | 1.6 ± 0.06   | 13.2 ± 1.01              |
| C-CaM-Ng        | 13.4 ± 0.57     | 1.9 ± 0.05   | 177 ± 6.60               |

(Table 1), and are in agreement with Tyr fluorescence data (within a 10% error). From the Quin-2 experimental system, we were also able to determine the stoichiometry of Ca\(^{2+}\) ions released. To do so, the fluorescence intensity was calibrated with known ion concentrations to determine the relationship between the Ca\(^{2+}\) concentration and fluorescence intensity. Results report that the release of two Ca\(^{2+}\) ions from the CaM-Ng complex is related with the measured dissociation rate. We can, therefore, infer that we are only observing C-lobe dissociation because 1) Quin-2 rates are in agreement with rates measured via Tyr fluorescence, which exclusively reports C-lobe binding, and 2) data are best fit with a single exponential function, indicating that only one rate is detected. In the case of CaM in the absence of target, all four Ca\(^{2+}\) sites are occupied, but dissociation from the N-lobe is not observed because rates are too large to measure at 24 °C. We speculate that this is also the case for CaM-Ng. Overall, these results indicate that association of Ng with CaM decreases the Ca\(^{2+}\) affinity in the C-domain by increasing the rate of Ca\(^{2+}\) dissociation.

Intra- and Inter-lobe Interactions of CaM Modulate Ca\(^{2+}\) Association and Dissociation from the CaM-Ng Complex—Because Ng has previously been described to interact with the C-lobe of CaM (11, 34, 54), the increase in cooperativity we observed (Table 1) may be due to intradomain structural changes within the C-lobe. However, this result does not necessarily exclude the possibility that the N-lobe contributes to the cooperativity. To investigate whether or not this increase results from intra-lobe or inter-lobe interactions, we utilized a truncated version of CaM consisting of the C-terminal half of the protein (C-CaM residues 76–148), thereby eliminating contributions from the N-lobe. The \(K_A\) for Ca\(^{2+}\) binding to the C-lobe was weaker (3.18 \(\mu\)M) than that measured for intact CaM (1.78 \(\mu\)M; Table 1), consistent with earlier reports (9). Again, Ng binding weakened the Ca\(^{2+}\) binding affinity to the C-lobe (13.4 \(\mu\)M) as in wt CaM (11.1 \(\mu\)M). Fitting of data resulted in an \(n\) value of 2.0 for C-CaM in the presence of Ng, similar to wt CaM (Fig. 2D). Because CaM-Ng and C-CaM-Ng have the same \(n\) value, we conclude the N-lobe does not contribute to the increase in Ca\(^{2+}\) cooperativity of binding. This identifies that Ng causes intra-lobe rather than inter-lobe struc-
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We also used C-CaM to examine the roles of the N- and C-lobes of CaM in the rate of Ca\(^{2+}\) dissociation. The rate of Ca\(^{2+}\) dissociation from C-CaM-Ng was 177 s\(^{-1}\), which is -3-fold lower than that of the full-length CaM-Ng complex (Fig. 2, E and F; Table 1). These results indicate that when the N-lobe is omitted, Ca\(^{2+}\) dissociation from the C-lobe is considerably slower. This implies that the N-lobe serves to increase rates of Ca\(^{2+}\) dissociation from the CaM-Ng complex. Taken together, our investigations with C-CaM indicate that although the N-lobe does not play a role in modulating cooperativity of Ca\(^{2+}\) binding, the presence of the N-lobe increases the rate of Ca\(^{2+}\) dissociation from the C-lobe. Thus the full impact of the effect of Ng on the Ca\(^{2+}\) binding properties of the C-domain of CaM are realized only in the context of intact CaM.

Ca\(^{2+}\) and Ionic interactions Influence Stability of the CaM-Ng Complex—Ng has been previously indicated to preferentially bind Ca\(^{2+}\)-free CaM (49, 50), and recently Kumar et al. (34) reported that Ng has an association constant for apoCaM that is significantly higher than for Ca\(^{2+}\)-saturated CaM, indicating a strong preference for apoCaM. Similarly, Nm was reported to bind more tightly to apoCaM than Ca\(^{2+}\)/CaM, although the difference in affinity was appreciably less in the presence of increasing ionic strength (55). To quantitatively assess CaM-Ng interactions, we also measured the steady state affinity of binding in the presence and absence of Ca\(^{2+}\) using isothermal titration calorimetry. Data were fit with a single binding site model to derive dissociation constants. We observed an ~2-fold difference in affinity with \(K_d\) values of 139 and 302 nM for Ng binding to apoCaM and Ca\(^{2+}\)/CaM respectively (fitting parameters delineated in Table 2). These results indicate that Ng indeed has a decreased affinity for Ca\(^{2+}\)/CaM.

![FIGURE 3. Ng-CaM binding affinities.](image)

**FIGURE 3. Ng-CaM binding affinities.** Representative ITC experiments of Ng titrated into apoCaM (A) or Ca\(^{2+}\)/CaM (B). Upper panels show raw thermogram data plotted as a function of experimental time, and lower panels show integrated peak intensities as a function of the Ng/CaM molar ratio. Data have been fit to single site binding models to determine \(K_d\) values of 139 and 302 nM for apoCaM and Ca\(^{2+}\)/CaM respectively (fitting parameters delineated in Table 2).
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TABLE 3

Comparison of binding affinities of Ng peptides to apoCaM and Ca\(^{2+}\)/CaM

ITC was accomplished at 25 °C as described under "Experimental Procedures" in 25 mM HEPES, pH 7.2, with 1 mM EDTA (apoCaM) or with 2 mM CaCl\(_2\) (Ca\(^{2+}\)/CaM) in the absence of presence of 150 mM KCl. Values of \(N\) (stoichiometry of binding), \(K_a, \Delta H,\) and \(\Delta S\) were obtained by fitting the heat signature of a series of injections of Ng or Ng peptides into solutions of CaM (5 μM) with a single site binding model in the Microcal software. \(K_a\) (in μM) was calculated from 1/\(K_p\). Dashes indicate that experiments were attempted, but no change in heat signature was detected.

| Sample           | KCl  | \(N\) | \(K_a\) \(\mu M\) | \(\Delta H\) cal/mol | \(\Delta S\) cal/mol
|------------------|------|-------|-----------------|----------------------|------------------|
| ApoCaM-Ng 26–49 | 0    | 0.950 ± 0.003 | 3.42 \(\times 10^6\) ± 1.12 \(\times 10^5\) | 0.29 | −14,930 ± 70 | −20.2
| ApoCaM-Ng 13–49 | 0    | 0.956 ± 0.002 | 10.5 \(\times 10^6\) ± 5.18 \(\times 10^5\) | 0.09 | −12,060 ± 48 | −8.33
| Ca\(^{2+}\)/CaM-Ng 26–49 | 0 | 0.986 ± 0.002 | 1.0 \(\times 10^6\) ± 1.56 \(\times 10^5\) | 1.01 | −7,988 ± 96 | 18.1
| ApoCaM-Ng 13–49 | 150  | –       | 8.03 \(\times 10^6\) ± 7.00 \(\times 10^4\) | 1.25 | −4,630 ± 139 | 11.5
| ApoCaM-Ng 13–49 | 150  | 1.10 ± 0.012 | 1.48 \(\times 10^6\) ± 9.93 \(\times 10^4\) | 0.68 | −7,189 ± 110 | 4.11
| Ca\(^{2+}\)/CaM-Ng 26–49 | 150 | –       | 22.0 \(\times 10^6\) ± 2.91 \(\times 10^4\) | 4.50 | −3,795 ± 355 | 11.7

Sensitivity to salt has previously been documented for other members of the small neuronal IQ motif family. For example, increasing the ionic strength causes Nm, which has a nearly identical IQ domain to Ng, to dissociate from apoCaM (55, 56).

We investigated the CaM-Ng affinity dependence on ionic strength by determining affinities at increasing concentrations KCl (see Table 2). The affinity of binding Ng to apoCaM and Ca\(^{2+}\)/CaM decreased in a nonlinear fashion as salt concentration increased. Data follow an exponential-like trend where the difference in measured affinities. We then determined the difference in affinity for apoCaM and determined a peptide containing the CaM recognition motif as well as the N-terminally adjacent acidic domain, which contains an abundance of aspartic acid residues. We first investigated the IQ domain by engineering a peptide consisting of only residues of the IQ domain, termed Ng 26–49 (Fig. 1). We measured its affinity for apoCaM and determined a \(K_a\) value of 292 nm (Table 3). This is a 2-fold lower affinity than the full-length Ng protein, signifying that other domains of Ng contribute to the stability of the CaM-Ng complex. We considered if the acidic domain of Ng might serve to stabilize the interaction with CaM to explain the difference in measured affinities. We then determined the \(K_a\) for a peptide composed of both the IQ domain and the acidic domain, Ng 13–49 (Fig. 1), and interestingly the affinity is 3-fold higher than that of the IQ domain (Ng 26–49; Table 3). This implies that the acidic domain indeed stabilizes the interaction between apoCaM and Ng. In comparison to the full-length protein, the \(K_a\) is 1.5 times lower, meaning this peptide actually has a higher affinity for apoCaM than the intact protein. We suggest that the C-terminal glycine-rich domain in Ng may be the reason for this difference.

We then determined if the peptides would follow the same trends in the presence of Ca\(^{2+}\). We first investigated the affinity of the IQ domain peptide Ng 26–49 for Ca\(^{2+}\)/CaM and found it follows the same trend as for apoCaM. The \(K_a\) value was determined to be 1.01 \(\mu M\), a decreased affinity as compared with the full-length protein (Table 3). Thus the isolated IQ domain has a slightly decreased affinity for CaM compared with full-length Ng in either the presence or absence of Ca\(^{2+}\). However, when the Ng 13–49 peptide corresponding to the IQ plus acidic domains was evaluated in the presence of Ca\(^{2+}\), we observed an opposite trend. Instead of stabilizing the CaM-Ng interaction, the affinity was substantially reduced to 1.25 \(\mu M\), corresponding to a 4-fold decrease in affinity as compared with the intact protein. Of significance, this peptide has a 13-fold difference in affinity for apoCaM versus Ca\(^{2+}\)/CaM. These results indicate that the Ng acidic domain appears to play a modest role in stabilizing the CaM-Ng interaction in the absence of Ca\(^{2+}\) and destabilize it in the presence of Ca\(^{2+}\).

The Ng Acidic Domain Impacts Ca\(^{2+}\) Binding to CaM-Ng—We have determined that affinities are substantially influenced by ionic strength and propose that charge-charge interactions will be integral in switching between high affinity binding to apoCaM and low affinity binding to Ca\(^{2+}\)/CaM. To begin to decipher the structural mechanism of how this switching occurs, we focused on the role of charged residues in the IQ domain containing the CaM recognition motif as well as the N-terminally adjacent acidic domain, which contains an abundance of aspartic acid residues. We first investigated the IQ domain by engineering a peptide consisting of only residues of that domain, termed Ng 26–49 (Fig. 1). We measured its affinity for apoCaM and determined a \(K_a\) value of 292 nm (Table 3). This is a 2-fold lower affinity than the full-length Ng protein, signifying that other domains of Ng contribute to the stability of the CaM-Ng complex. We considered if the acidic domain of Ng might serve to stabilize the interaction with CaM to explain the difference in measured affinities. We then determined the \(K_a\) for a peptide composed of both the IQ domain and the acidic domain, Ng 13–49 (Fig. 1), and interestingly the affinity is 3-fold higher than that of the IQ domain (Ng 26–49; Table 3). This implies that the acidic domain indeed stabilizes the interaction between apoCaM and Ng. In comparison to the full-length protein, the \(K_a\) is 1.5 times lower, meaning this peptide actually has a higher affinity for apoCaM than the intact protein. We suggest that the C-terminal glycine-rich domain in Ng may be the reason for this difference.

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We next assessed the influence of Ng peptides on the affinity and kinetics of Ca\(^{2+}\) binding to CaM. The CaM-Ng 26–49 IQ domain demonstrated a >30-fold decrease in \(K_a\) for Ca\(^{2+}\) as compared with the CaM-Ng complex (Fig. 4A, Table 4). In fact, the impact on the Ca\(^{2+}\) binding affinity of the IQ domain peptide is actually higher than for CaM alone and drives the affinity in the opposite direction than intact Ng. This further establishes that the IQ motif is a poor mimetic for the full-length Ng protein. The Ng 13–49 peptide corresponding to the IQ and acidic domains showed a \(K_a\) for Ca\(^{2+}\) binding of ~7.8 \(\mu M\), which is ~40% higher affinity for Ca\(^{2+}\) compared with the intact protein. These results corroborate data obtained from ITC experiments and likewise suggest that the C-terminal glycine-rich domain plays a role in Ng’s modification of the affinity.
of CaM for Ca\(^{2+}\). Interestingly all peptides have comparable \(n\) values around 1.5 that is similar to CaM in the absence of target. Only the full-length protein produces an increase in cooperativity of \(-2.0\). That glycine-rich domain is present only in the full-length protein suggests the domain is responsible for the increase in cooperativity of Ca\(^{2+}\) binding. We also evaluated Ca\(^{2+}\) off-rates from each of these peptides complexed with CaM (Fig. 4B). The CaM-Ng 26 – 49 IQ motif has a slowed rate, whereas the rate is substantially increased for the CaM-Ng peptide complex corresponding to the IQ and acidic domain (CaM-Ng 13–49; Table 4). These results indicate that off-rates largely drive the changes in affinity, and the presence of the acidic domain drives fast Ca\(^{2+}\) dissociation.

**TABLE 4**

Ca\(^{2+}\) binding properties for CaM bound to Ng and Ng peptides

The macroscopic dissociation constant, \(K_a\), and the Hill coefficient, \(n\), was determined by monitoring Tyr fluorescence during titration with Ca\(^{2+}\) and fitting the data to the Hill equation as described under “Experimental Procedures.” Values and errors were determined from two independent experiments for each condition. Calcium dissociation rates \((k_{off})\) were derived by fitting Ca\(^{2+}\) dissociation curves to a monoeponential equation. Values for \(k_{off}\) are the average of 3–5 determinations in the presence or absence of 50 \(\mu\)M Ng or the specified peptides, repeated three independent times.

| Sample          | \(K_a\) \((\mu\text{M})\) | \(n\) \((n = 2)\) | \(k_{off}\) \((s^{-1})\) |
|-----------------|--------------------------|------------------|--------------------------|
| CaM             | 1.78 ± 0.18              | 1.5 ± 0.03       | 10.7 ± 0.47              |
| CaM-Ng          | 11.1 ± 0.12              | 2.0 ± 0.09       | 556 ± 34.7               |
| CaM-Ng 26–49    | 0.336 ± 0.011            | 1.5 ± 0.03       | 2.14 ± 0.04              |
| CaM-Ng 13–49    | 7.78 ± 0.74              | 1.4 ± 0.08       | 340 ± 15.3               |

**FIGURE 4.** Steady state Ca\(^{2+}\) affinity and Ca\(^{2+}\) off-rates from the CaM-Ng peptide complexes. A, steady state Ca\(^{2+}\) affinity measurements are shown for CaM (black), CaM-Ng full-length protein (red), CaM-Ng 26 – 49 corresponding to the IQ domain (orange), and CaM-Ng 13–49 corresponding to the IQ and acidic domains (blue). Data were normalized such that the fits spanned 0 to 1. B, off-rates are shown for the corresponding protein-peptide complexes. The inset illustrates the same data on a shorter timescale for visualization of the faster rates. \(K_a\) values and \(k_{off}\) rates are detailed in Table 4.

NMR Studies Show That Ng Interacts with Both the N-lobe and C-lobe of CaM—We show that Ca\(^{2+}\) association and dissociation from the CaM-Ng complex can be influenced by the N-terminal lobe of CaM. The N-lobe could affect kinetics in two possible ways depending on whether or not the N-lobe interacts with Ng. Previous structural (34, 54) and biochemical (11) studies indicate that the IQ motif from Ng, as well as intact Ng, interact primarily with the C-lobe of CaM. In this case unconstrained tumbling of the tethered N-domain could propagate an increase in dynamics in the C-lobe. Increased CaM dynamics have previously been associated with rapid dissociation of Ca\(^{2+}\) (33, 57–59). Alternatively the N-lobe of CaM may make contacts with Ng. This has been described for a different CaM target where the primary binding interface is in the C-lobe, and the N-lobe has been inferred to interact transiently (60). Although N-lobe contact was not observed in the crystal structure of the Ng IQ motif peptide bound to CaM (34), it is possible that the N-terminal acidic and/or the C-terminal glycine-rich regions of Ng interact with the N-domain of CaM. For a structural analysis of CaM-Ng interactions, we evaluated amide chemical shift perturbations to compare structural changes induced in CaM upon binding Ng 26–49, Ng 13–49, or intact Ng. \(^{1}H,^{15}N\) HSQC spectra of \(^{15}N\)-labeled CaM were collected at increasing concentrations of ligands in the presence or absence of Ca\(^{2+}\). Because Ng increases the rate of dissociation of Ca\(^{2+}\) from CaM, we first compared the effects of ligands on CaM in the presence of Ca\(^{2+}\). The addition of Ng or Ng peptides caused chemical shift changes that were fast on the NMR time scale, which allowed assignment of backbone amide chemical shifts in bound CaM by following changes in chemical shifts during titration of Ca\(^{2+}\)/CaM with ligand (data not shown).

Fig. 5 shows the effects of Ng peptides and intact Ng on backbone amides of Ca\(^{2+}\)/CaM. The peptide corresponding to the IQ domain, Ng 26–49, affected residues in both the N- and C-domain of Ca\(^{2+}\)/CaM, especially within helices D, F, and H. In addition, Ng 26–49 binding significantly affected the amides of hydrophobic residues Phe-19, Val-55, and Phe-92 located just before Ca\(^{2+}\) binding sites I, II, and III respectively (see residues marked by arrows in Fig. 5, bottom panel). In contrast, the peptide corresponding to the IQ and acidic domains, Ng 13–49, had little effect on residues in the N-domain of Ca\(^{2+}\)/CaM but significantly affected Phe-92 as well as residues in helix F and H and the linker between helices F and G (Fig. 5, center panel). Residues Gly-113, Glu-114, and Lys-115 in the linker, which are not greatly affected by the IQ motif, are all broadened beyond detection by chemical exchange processes.
Figure 5. Amide chemical shift perturbations of Ca2+/CaM with Ng. HSQC experiments were completed for Ca2+/CaM in the presence and absence of Ng protein and peptides, and CaM amide chemical shift assignments were compared between unbound Ca2+/CaM and Ng-bound Ca2+/CaM. Histograms summarize Ng-induced changes in chemical shifts for fully intact protein (top panel), a peptide corresponding to the IQ and acidic domains, Ng 13–49 (center), and a peptide comprising the IQ domain, Ng 26–49 (bottom). Arrows indicate large changes in chemical shifts, and asterisks indicate chemical shifts broadened beyond detection. CaM helices (black boxes) and Ca2+ binding sites (roman numerals) are indicated in linear sequence (top).

Similar to the Ng 26–49, intact Ng also affects backbone amides in both the N- and C-domain of Ca2+/CaM, including Phe-19, Val-55, and Phe-92 (Fig. 5, top panel). However, the peptide corresponding to the IQ and acidic domains does not show interaction with the N-lobe, whereas the intact protein does. These results suggest that although the acidic domain confers specificity for the C-lobe of CaM, the Gly-rich domain interacts with the N-lobe of Ca2+/CaM.

We next examined the effect of Ng and Ng peptides on backbone amide chemical shifts in apoCaM. Intact Ng, Ng 13–49, and Ng 26–49 induced changes in amide chemical shifts in apoCaM that were characteristic of slow-to-intermediate exchange on the NMR time scale. Slow-to-intermediate exchange prevents unambiguous assignment of backbone amides in the bound form. However, comparing spectra in the absence and presence of ligand allowed us to identify residues affected by ligand binding. Specifically, residues in apoCaM were identified as being affected by binding to Ng or Ng peptides only if the cross-peak was shifted and showed no significant overlap when overlaid with spectra collected in the presence of ligand. This conservative approach does not account for the magnitude of changes in chemical shifts or broadening but can distinguish if Ng and Ng peptides affect amide chemical shifts in one or both domains of CaM. Fig. 6A shows that both intact Ng and Ng 13–49 have extensive effects on backbone amides in the C-domain of apoCaM but also affect cross-peaks associated with residues in the N-domain. In contrast, Ng 26–49, which includes only the core IQ CaM binding motif, has selective effects on backbone amides only for residues in the C-domain of CaM. This is consistent with a recent crystal structure that shows that Ng 26–49 associates with the C-domain of apoCaM (34). Potential interaction of Ng and Ng 13–49 with both the N- and C-domains can account for higher affinity of binding to apoCaM relative to the Ng 26–49.

Chemical shifts for at least 50 residues in apoCaM are different when bound to intact Ng versus Ng 26–49, but fewer differences are observed when apoCaM is bound to Ng versus Ng 13–49 (data not shown). Fig. 6B illustrates these observations in a region of the spectra where cross-peaks for down-field-shifted amides for Gly residues in apoCaM are typically seen. Amides for Gly-25 and Gly-61 at the 6th position in Ca2+/binding loops I and II have essentially the same chemical shifts for Gly-96 and Gly-134 in Ca2+/CaM. In contrast, the peptides corresponding to the IQ Ng 26–49 and acidic domains Ng 13–49 are dominated by interactions with residues within the C-lobe of CaM. These experiments indicate that Ng interacts with residues in both the N- and C-lobes of CaM in both the absence and presence of Ca2+. The differences between the contact maps of Ng and peptides representing the IQ domain and IQ and acidic domains imply that the Gly-rich domain at the C terminus of Ng is likely responsible for the interactions detected in the N-lobe of CaM.
DISCUSSION

Our analysis provides novel insights into how the small regulators of CaM signaling, Ng and PEP-19, can differentially affect fundamental biochemical properties of CaM. We showed previously that PEP-19 has little effect on the affinity of Ca\(^{2+}\) binding to CaM, but it increases both the \(k_{on}\) and \(k_{off}\) for Ca\(^{2+}\) binding to its C-domain by 40–50-fold (14). In contrast, Ng decreases Ca\(^{2+}\) binding affinity largely by increasing the Ca\(^{2+}\) \(k_{off}\). Additionally PEP-19 was shown to decrease cooperativity of Ca\(^{2+}\) binding, whereas Ng increases cooperativity. We showed previously that the effects of PEP-19 on CaM require an acidic sequence that is just N-terminal to the IQ motif (21, 61).

We now show that the Ng acidic sequence is also necessary to mediate its effects on the biochemical properties of CaM. The composition and distribution of acidic residues in Ng and PEP-19 are quite different and must be responsible for the different effects that Ng and PEP-19 have on Ca\(^{2+}\) binding to the C-domain.

Because the IQ peptides from both PEP-19 and Ng have very similar effects on the Ca\(^{2+}\) binding properties of CaM and because a peptide that spans only the acidic sequence of PEP-19 has no intrinsic affinity for CaM, we propose that the IQ motifs of PEP-19 and Ng provide a docking function that tethers the acidic sequence to CaM, where it can modulate and tune Ca\(^{2+}\) binding to CaM. We propose that synergy between the acidic sequence and the IQ CaM motif is a structural hallmark of small regulators of CaM signaling. Sequence divergence in the acidic region confers diversity in the effects of Ng and PEP-19 on the biochemical properties of CaM and may be why these small regulators of CaM signaling have different tissue and subcellular distributions.

Previous work showed that the core IQ peptide from Ng interacts predominantly with the C-lobe of CaM (11, 34, 54). In the present report we showed that the Gly-rich C-terminal domain of intact Ng permits contacts to be made with both the N-lobe and C-lobes of CaM. Congruently, N-lobe interaction was not detected when bound to PEP-19, which does not have a homologous Gly-rich domain. The significance of N-lobe interaction only with Ng may indicate that Ng has a greater capacity to tune Ca\(^{2+}\) responses on CaM.

The present structural analyses are consistent with our previous studies of the small neuronal IQ motif protein PEP-19 (for comparison of chemical shifts, see supplemental Fig. 1; Ref. 21). All Ng and PEP-19 proteins and peptides that include the respective acidic sequences have similar patterns of effects on amides in the C-domain of CaM that include large changes for Phe-92 and for residues in helices F and H and the linker between helices F and G. The most striking difference in amide chemical shift perturbations induced by proteins and peptides with and without acidic sequences is in the linker between helices F and G, which are greatly affected by Ng and PEP-19 but not by the corresponding IQ peptides that do not include the acidic sequence. We showed previously that these residues also exhibit conformational exchange on the \(\mu\)s to ms time frame when bound to intact PEP-19 (33). This ligand-induced conformational exchange may play a role in gating the release of Ca\(^{2+}\) from the C-domain of CaM by Ng and PEP-19.

Binding of Ca\(^{2+}\) exposes hydrophobic residues of CaM essential for recognition of Ca\(^{2+}\)-dependent targets; however, ionic interactions tend to dominate in the recognition of targets that bind apoCaM (28, 36, 62). Indeed we found the CaM-Ng interaction to be dependent on ionic conditions with increased salt concentrations resulting in decreased stability of the CaM-Ng complex. Interestingly, our HSQC analysis shows that hydrophobic residues Phe-19, Val-55, and Phe-92 located just before Ca\(^{2+}\) binding sites undergo substantial changes in chemical shifts when bound to CaM. Additionally, IQ motif domain interaction with the CaM C-lobe was shown to contain several important hydrophobic interactions (34). Like other IQ motif proteins that are thought to interact with both apo- and Ca\(^{2+}\)/CaM (63), we suggest that both hydrophobic and ionic forces are important for Ng regulation of CaM.

The changes in Ca\(^{2+}\) affinity and cooperativity upon Ng association with CaM indicate that Ng acts as a modulator of CaM-dependent signaling by (at least) two mechanisms. First, binding of Ng to CaM decreases its affinity for Ca\(^{2+}\), leading to a circumstance where a higher Ca\(^{2+}\) concentration is required for CaM to transition to the fully Ca\(^{2+}\)-bound state. Additionally the Ca\(^{2+}\) concentration range at which CaM responds is narrowed due to increased cooperativity in the C-lobe so that when Ca\(^{2+}\) levels reach a high enough concentration to bind, saturation will occur over a narrow range of Ca\(^{2+}\). By increasing the cooperativity, the CaM-Ng complex has increased sensitivity with tighter allosteric control over Ca\(^{2+}\) binding to CaM. These characteristics may be consequential in cellular signaling where CaM must “choose” among diverse targets in response to varying Ca\(^{2+}\) signals. These properties allow for transient interactions and efficient competition, and we therefore suggest that tuning the dynamics of Ca\(^{2+}\) binding by Ng promotes prompt responses necessary to maintain signaling fidelity.

In addition, we and others (49, 50) showed that Ca\(^{2+}\) binding serves to stimulate dissociation of Ng from CaM. At rest, Ng will sequester and compete with other targets that have preferential association with apoCaM. When intracellular Ca\(^{2+}\) increases, Ng will dissociate from CaM due to weakened binding affinity when Ca\(^{2+}\) binds CaM and become available for activating downstream targets. However, some refinement in this simple model is required. We showed that in the presence of Ng, the binding affinity of Ca\(^{2+}\) to CaM is greatly weakened, mandating that a higher level of Ca\(^{2+}\) be achieved before CaM becomes Ca\(^{2+}\)-saturated. This “kinetic” model requires integrating the three-way interaction between Ca\(^{2+}\), CaM, and Ng to fully encompass the biochemical data from earlier and the present studies. In addition, because Ng preferentially affects the slow Ca\(^{2+}\) binding kinetics of the C-terminal domain of CaM, its effect is to modulate the rate-limiting step for saturation of CaM with Ca\(^{2+}\). In total, Ng can release CaM depending on the amplitude and frequency of the Ca\(^{2+}\) flux and has been described to function as a capacitor (36, 37).

We also predict Ng will have a substantial impact on the amount of free Ca\(^{2+}\) in cells. CaM is at high (10–100 \(\mu\)M) concentration and, given its capacity to bind four Ca\(^{2+}\) ions, can function as an effective buffer for Ca\(^{2+}\) (4–6). Our results suggest that Ng would significantly weaken the capacity of CaM to buffer Ca\(^{2+}\) at lower Ca\(^{2+}\) levels as it decreases the affinity of
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CaM for Ca²⁺ by >10-fold. It is also important to note that Ng is the target for a number of post-translational modifications that alter its capacity to interact with CaM. PKC phosphorylation of a Ser-36 in the center of the IQ domain significantly weakens CaM binding. A similar PKC-mediated phosphorylation site is present in the IQ domain of the related protein Nm. Ng is also susceptible to oxidation, which also weakens its interactions with CaM (64). Finally, phosphatidic acid binds to Ng and has been postulated to play a role in targeting Ng to cellular membranes (65). Phosphatidic acid binding also weakens binding to CaM and decreases susceptibility to PKC phosphorylation, which adds a further level of regulation to the highly regulated Ng/CaM system.

The cellular role for the small neuronal proteins, Ng and PEP-19, bears some similarities but also exhibits unique features. Biochemically, they have very different effects on affinity and kinetics of Ca²⁺ binding to the C-domain of CaM. Additionally, the two proteins are rarely if ever expressed in the same cells. For example, Purkinje cells in the cerebellum and the CA2 pyramidal cells in the hippocampus express high levels of PEP-19, whereas most other pyramidal cells in the hippocampus and cortex express high levels of Ng. It appears that the Ca²⁺/CaM signaling pathway in these different cell types must be tuned in unique ways, and Ng and PEP-19 play these important functions.

Acknowledgments—We thank Dr. Margaret Cheung for helpful discussions during the course of this work. The NMR instrument was supported, in part, through the Structural Biology Center at UT Health Science Center-Houston.

REFERENCES

1. Klee, C. B., and Vanaman, T. C. (1982) Calmodulin. Adv. Protein Chem. 35, 213–321
2. Biber, A., Schmid, G., and Hempel, K. (1984) Calmodulin content in specific brain areas. Exp. Brain Res. 56, 323–326
3. Kakiuchi, S., Yasuda, S., Yamazaki, R., Teshima, Y., Kanda, K., Kakiuchi, R., and Sobe, K. (1982) Quantitative determinations of calmodulin in the supernatant and particulate fractions of mammalian tissues. J. Biochem. 92, 1041–1048
4. Kubota, Y., Putkey, J. A., Shouval, H. Z., and Waxham, M. N. (2008) IQ-motif proteins influence intracellular free Ca²⁺ in hippocampal neurons through their interactions with calmodulin. J. Neurophysiol. 99, 264–276
5. Faas, G. C., Raghavachari, S., Lisman, J. E., and Mody, I. (2011) Calmodulin as a direct receptor of Ca²⁺ signals. Nat. Neurosci. 14, 301–304
6. Baimbridge, K. G., Celio, M. R., and Rogers, J. H. (1992) Calcium-binding proteins in the nervous system. Trends Neurosci. 15, 303–308
7. Linse, S., Helmersson, A., and Forsén, S. (1991) Calcium binding to calmodulin and its globular domains. J. Biol. Chem. 266, 8050–8054
8. Sorensen, B. R., Faga, L. A., Hultman, R., and Shea, M. A. (2002) An interdomain linker increases the thermostability and decreases the calcium affinity of the calmodulin N-domain. Biochemistry 41, 15–20
9. Sorensen, B. R., and Shea, M. A. (1998) Interactions between domains of apo calmodulin alter calcium binding and stability. Biochemistry 37, 4244–4253
10. Xiong, L. W., Kleerekoper, Q. K., Wang, X., and Putkey, J. A. (2010) Intra- and interdomain effects due to mutation of calcium-binding sites in calmodulin. J. Biol. Chem. 285, 8094–8103
11. Gaertner, T. R., Putkey, J. A., and Waxham, M. N. (2004) RC3/neurogranin and Ca²⁺/calmodulin-dependent protein kinase II produce opposing effects on the affinity of calmodulin for calcium. J. Biol. Chem. 279, 39374–39382
12. Peersen, O. B., Madsen, T. S., and Falke, J. J. (1997) Intermolecular tuning of calmodulin by target peptides and proteins: differential effects on Ca²⁺ binding and implications for kinase activation. Protein Sci. 6, 794–807
13. Johnson, J. D., Snyder, C., Walsh, M., and Flynn, M. (1996) Effects of myosin light chain kinase and peptides on Ca²⁺ exchange with the N- and C-terminal Ca²⁺ binding sites of calmodulin. J. Biol. Chem. 271, 761–767
14. Putkey, J. A., Kleerekoper, Q., Gaertner, T. R., and Waxham, M. N. (2003) A new role for IQ motif proteins in regulating calmodulin function. J. Biol. Chem. 278, 49667–49670
15. Kubota, Y., and Waxham, M. N. (2010) Lobe specific Ca²⁺-calmodulin nano-domain in neuronal spines: a single molecule level analysis. PLoS Comput. Biol. 6, e1000987
16. Chinn, D., and Means, A. R. (2000) Calmodulin: a prototypical calcium sensor. Trends Cell Biol. 10, 322–328
17. Tidow, H., and Nissen, P. (2013) Structural diversity of calmodulin binding to its target sites. FEBS J. 280, 5551–5565
18. Yap, K. L., Kim, J., Truong, K., Sherman, M., Yuan, T., and Ikura, M. (2000) Calmodulin target database. J. Struct. Funct. Genomics 1, 8–14
19. Zhang, M., and Yuan, T. (1998) Molecular mechanisms of calmodulin’s functional versatility. Biochem. Cell Biol. 76, 313–323
20. Crivici, A., and Ikura, M. (1995) Molecular and structural basis of target recognition by calmodulin. Ann. Rev. Biophys. Biomol. Struct. 24, 85–116
21. Putkey, J. A., Waxham, M. N., Gaertner, T. R., Brew, K. J., Goldsmith, M., Kubota, Y., and Kleerekoper, Q. K. (2008) Acidic/IQ motif regulator of calmodulin. J. Biol. Chem. 283, 1401–1410
22. Theoharis, N. T., Sorensen, B. R., Theisen-Toupal, J., and Shea, M. A. (2008) The neuronal voltage-dependent sodium channel type II IQ motif lowers the calcium affinity of the C-domain of calmodulin. Biochemistry 47, 112–123
23. Baudier, J., Deloume, J. C., Van Dorsselaer, A., Black, D., and Matthes, H. W. (1991) Purification and characterization of a brain-specific protein kinase C substrate, neurogranin (p17). Identification of a consensus amino acid sequence between neurogranin and neuromodulin (GAP43) that corresponds to the protein kinase C phosphorylation site and the calmodulin-binding domain. J. Biol. Chem. 266, 229–237
24. Gerendasy, D. (1999) Homeostatic tuning of Ca²⁺ signal transduction by members of the calpastatin protein family. J. Neurosci. Res. 58, 107–119
25. Huang, K. P., Huang, F. L., and Chen, H. C. (1993) Characterization of a 7.5-kDa protein kinase C substrate (RC3 protein, neurogranin) from rat brain. Arch. Biochem. Biophys. 305, 570–580
26. Apel, E. D., Byford, M. F., Au, D., Walsh, K. A., and Storm, D. R. (1990) Identification of the protein kinase C phosphorylation site in neuromodulin. Biochemistry 29, 2330–2335
27. Basi, G. S., Jacobson, R. D., Virág, I., Schilling, J., and Sene, H. J. (1987) Primary structure and transcriptional regulation of GAP-43, a protein associated with nerve growth. Cell 49, 785–791
28. Alexander, K. A., Cimler, B. M., Meier, K. E., and Storm, D. R. (1987) Regulation of calmodulin binding to P-57. A neurospecific calmodulin-binding protein. J. Biol. Chem. 262, 6108–6113
29. Ziai, R., Pan, Y. C., Hulmes, J. D., Sangameswaran, L., and Morgan, J. I. (1986) Isolation, sequence, and developmental profile of a brain-specific polypeptide, PEP-19. Proc. Natl. Acad. Sci. U.S.A. 83, 8420–8423
30. Sangameswaran, L., Hempstead, J., and Morgan, J. I. (1989) Molecular cloning of a neuron-specific transcript and its regulation during normal and aberrant cerebellar development. Proc. Natl. Acad. Sci. U.S.A. 86, 5651–5655
31. Sangameswaran, L., and Morgan, J. I. (1993) Structure and regulation of the gene encoding the neuron-specific protein PEP-19. Brain Res. Mol. Brain Res. 19, 62–68
32. Watson, J. B., Battenberg, E. F., Wong, K. K., Bloom, F. E., and Sutcliffe, J. G. (1990) Subtractive cDNA cloning of RC3, a rodent cortex-enriched mRNA encoding a novel 78-residue protein. J. Neurosci. Res. 26, 397–408
33. Wang, X., Kleerekoper, Q. K., Xiong, L. W., and Putkey, J. A. (2010) Intrinsically disordered PEP-19 confers unique dynamic properties to apo and calcium calmodulin. Biochemistry 49, 10287–10297
34. Kumar, V., Chichili, V. P., Zhong, L., Tang, X., Velazquez-Campoy, A., Sheu, F. S., Seetharaman, J., Gerges, N. Z., and Sivaraman, J. (2013) Structural basis for the interaction of unstructured neuron specific substrates
neuromodulin and neurogranin with calmodulin. *Sci. Rep.* 3, 1392
35. Kleerekoper, Q. K., and Putkey, J. A. (2009) PEP-19, an intrinsically disordered regulator of calmodulin signaling. *J. Biol. Chem.* 284, 7455–7464
36. Gerendas, D. D., Herron, S. R., Watson, J. B., and Sutcliffe, J. G. (1994) Mutational and biophysical studies suggest RC3/neurogranin regulates calmodulin availability. *J. Biol. Chem.* 269, 22420–22426
37. Gerendas, D. D., and Sutcliffe, J. G. (1997) RC3/neurogranin, a postsynaptic calpain for setting the response threshold to calcium influxes. *Mol. Neurobiol.* 15, 131–163
38. Zhong, L., and Gerges, N. Z. (2012) Neurogranin targets calmodulin and lowers the threshold for the induction of long-term potentiation. *PloS ONE* 7, e41275
39. Broadbelt, K., Ramlasaud, A., and Jones, L. B. (2006) Evidence of altered neurogranin immunoreactivity in areas 9 and 32 of schizophrenic prefrontal cortex. *Schizophr. Res.* 87, 6–14
40. Ruano, D., Aulchenko, T. S., Macedo, A., Soares, M. I., Valente, J., Azevedo, M. H., Hutz, M. H., Gama, C. S., Lobato, M. I., Monte-de-Abrue, P., Goodman, A. B., Pato, C., Heutink, P., and Falha, J. A. (2008) Association of the gene encoding neurogranin with schizophrenia in males. *J. Psychiatry Res.* 42, 125–133
41. Pohilack, S. T., Nee, F., Ruttorf, M., Witt, S. H., Nieratschker, V., Rujescu, D., Werge, T., Pietila¨inen, O. P., Mors, O., Mortensen, P. B., Bo¨ttcher, Y., Olesen, J., Breuer, R., Mo¨ller, H. J., Børglum, A., Hansen, T., Suvisaari, J., Lonnqvist, J., Paunio, T., Børglum, A. D., Macedo, A., Soares, M. J., Valente, J., Costas, J., Jonsson, E. G., Terenius, L., Agartz, I., Petursson, H., Nothen, R. M., Murray, R., Kong, A., Golimbet, V., Carracedo, A., Arango, C., Vassos, E., Fraser, G., Ehrhardt, M. R., Erijman, L., Weber, G., and Wand, A. J. (1999) Molecular recognition by calmodulin: pressure-induced reorganization of a novel calmodulin-peptide complex. *Biochemistry* 35, 1599–1605
42. Chapman, E. R., Au, D., Alexander, K. A., Nicolson, T. S., and Storm, D. R. (1991) Characterization of the calmodulin binding domain of neurogranin. Functional significance of serine 41 and phenylalanine 42. *J. Biol. Chem.* 266, 207–213
43. Andreason, T. J., Luetje, C. W., Heideman, W., and Storm, D. R. (1983) Purification of a novel calmodulin binding protein from bovine cerebral cortex membranes. *Biochemistry* 22, 4615–4618
44. Neuner-Jehle, M., Denizot, J. P., and Mallet, J. (1996) Role for BSX and neurogranin in neurocognitive and behavioral defects in the 11q terminal deletion disorder (Jacobsen syndrome).