The interaction of calmodulin with its target proteins is known to affect the kinetics and affinity of Ca²⁺ binding to calmodulin. Based on thermodynamic principles, proteins that bind to Ca²⁺-calmodulin should increase the affinity of calmodulin for Ca²⁺, while proteins that bind to apo-calmodulin should decrease its affinity for Ca²⁺. We quantified the effects on Ca²⁺-calmodulin interaction of two neuronal calmodulin targets: RC3, which binds both Ca²⁺- and apo-calmodulin, and CaM kinase II, which binds selectively to Ca²⁺-calmodulin. RC3 was found to decrease the affinity of calmodulin for Ca²⁺, whereas CaM kinase II increases the calmodulin affinity for Ca²⁺. Specifically, RC3 increases the rate of Ca²⁺ dissociation from the C-terminal sites of calmodulin up to 60-fold while having little effect on the rate of Ca²⁺ association. Conversely, CaM kinase II decreases the rates of dissociation of Ca²⁺ from both lobes of calmodulin and autophosphorylation of CaM kinase II at Thr²⁸⁶ induces a further decrease in the rates of Ca²⁺ dissociation. RC3 dampens the effects of CaM kinase II on Ca²⁺ dissociation by increasing the rate of dissociation from the C-terminal lobe of calmodulin when in the presence of CaM kinase II. This effect is not seen with phosphorylated CaM kinase II. The results are interpreted according to a kinetic scheme in which there are competing pathways for dissociation of the Ca²⁺-calmodulin target complex. This work indicates that the Ca²⁺ binding properties of calmodulin are highly regulated and reveals a role for RC3 in accelerating the dissociation of Ca²⁺-calmodulin target complexes at the end of a Ca²⁺ signal.

Calmodulin (CaM) is a small (16.8 kDa) ubiquitous Ca²⁺-binding protein that has been shown to play a central role in Ca²⁺ signaling in a wide variety of cell types. CaM leads to a conformational change in the protein, allowing it to bind to and activate a large number of intracellular target proteins, including enzymes (1–3), ion channels (4–6), cytoskeletal elements (7, 8), and transcriptional and translational machinery (9, 10). In this way, CaM is poised as a critical intermediate in numerous cell processes.

CaM binds four ions of Ca²⁺ via EF-hand domains, two in the C-terminal lobe, and two in the N-terminal lobe. Stopped flow fluorescence studies and ⁴⁴Ca²⁺-exchange experiments showed that two binding sites bind Ca²⁺ with high affinity and have slow off-rates (~10 s⁻¹), while the other two sites bind Ca²⁺ with low affinity and have fast off-rates (>500 s⁻¹) (11, 12). Using CaM fragments, the high affinity sites were mapped to the C-terminal lobe and the low affinity sites to the N-terminal lobe (12). These differences in Ca²⁺ binding kinetics of the two lobes provide CaM with the potential for lobe-specific tuning of its interactions with target proteins in response to rises and falls in Ca²⁺ levels.

Ca²⁺ levels in cells range from roughly 50 nM to tens of μM (13), with temporal dynamics that range from slow oscillations (min to h) to very fast repetitive spikes (ms to s). A fundamental issue is how CaM can decode such a wide range of Ca²⁺ signals to the multitude of CaM-dependent targets in order to provide a functionally integrated cellular response. Our work (14) and that of other groups (15–17) has shown that the Ca²⁺ binding properties of CaM are modulated by its interaction with target proteins, leading to the idea that the unique features of each CaM binding partner tune CaM to adapt to the frequency and amplitude of different Ca²⁺ signals. In the present work, we examine the effects of a member of two distinct classes of neuronal CaM targets, RC3, and CaM kinase II, on the interaction of Ca²⁺ with CaM.

RC3, also known as neurogranin, is a small neuronal IQ domain protein (SNIQ), found in the soma, dendrites, and dendritic spines of many neurons (18). Proteins of this family, which also includes GAP-43 and PEP-19 (19), contain an IQ-type CaM binding domain and bind to both Ca²⁺-bound and Ca²⁺-free forms of CaM (20). RC3, like other SNIQs, is hypothesized to function as a regulator of the availability of CaM, either by sequestering CaM to limit its abundance in the cytoplasm, or by acting as a source of CaM, by keeping it localized in specific regions of the cytoplasm (20–22). However, the mechanism by which RC3 modulates CaM function is unclear. Our recent work has shown that interaction of PEP-19 with CaM increases the rates of association and dissociation of Ca²⁺ from the C-terminal lobe of CaM (14). Therefore, we hypothesize that RC3 may act to alter the interaction of CaM with Ca²⁺. In this article, we investigate the effect of RC3 on the binding of Ca²⁺ to CaM and describe a new potential role for RC3 in regulating CaM function at the end of a Ca²⁺ transient.
Bidirectional Tuning of the Affinity of Calmodulin for Calcium

$a$CaM kinase II is the major neuronal isoform of this multifunctional CaM-dependent enzyme (1, 23). CaM binding in the regulatory domain of CaM kinase II activates the enzyme by relieving autoinhibition. CaM kinase II is then able to autophosphorylate at Thr286, which leads to both Ca$^2^+$/CaM independent activity and CaM-trapping, a phenomenon in which the affinity of the enzyme for CaM is increased by more than 1,000-fold (24). Our previous work identified that binding of Ca$^2^+$/CaM to unphosphorylated CaM kinase II increases the Ca$^2^+$ binding affinity of CaM by about 40-fold (14). Because phosphorylation increases the CaM binding affinity of CaM kinase II, we predict a further increase in the Ca$^2^+$ binding affinity of CaM when the kinase is in the phospho-Thr286 state.

We previously showed that the interaction of PEP-19 with CaM accelerates the exchange of Ca$^2^+$ from the C-terminal lobe of CaM (14). We now show that interaction of RC3 with CaM accelerates the dissociation of Ca$^2^+$ from the C-terminal lobe, but has no effect on association rates, leading to an up to 60-fold decrease in the affinity of Ca$^2^+$ for the C-terminal lobe of CaM. The different effects of RC3 and PEP-19 on the interactions of Ca$^2^+$ with CaM suggest that the IQ domain proteins have diverse modulatory effects on CaM function. Interaction of CaM with CaM kinase II causes a decrease in the rates of Ca$^2^+$ dissociation from both lobes. When present together, RC3 and CaM kinase II have non-linear effects on Ca$^2^+$ dissociation; RC3 dampens the effects of CaM kinase II by causing Ca$^2^+$ to dissociate more quickly from the CaM/CaM kinase complex, as we saw with PEP-19 (14). The data suggest that RC3 has the potential to decrease the lifetime of the Ca$^2^+$/CaM bound state of CaM kinase II presumably by preventing reassociation of CaM kinase with CaM. However, RC3 does not increase the rates of dissociation of Ca$^2^+$ from the CaM/CaM kinase II complex after autophosphorylation of the kinase, a state in which slower Ca$^2^+$ dissociation is seen, likely because the dissociation rate of CaM from phospho-CaM kinase is so slow that this dissociation pathway (kinase dissociates first, then Ca$^2^+$ is not favored. The refractory nature of the Thr$^{286}$ phosphokinase to RC3 adds an additional layer of complexity to the intradomain modulation of CaM function in neurons. The opposite effects of RC3 and CaM kinase II on the interaction of CaM with Ca$^2^+$ indicate that bidirectional tuning of the CaM affinity for Ca$^2^+$ plays an important role in regulating the CaM response to Ca$^2^+$ signals.

**EXPERIMENTAL PROCEDURES**

**CaM and CaM Kinase II Expression and Purification**—A cDNA for sea urchin CaM was codon optimized for expression and mutated to produce an identical protein sequence to vertebrate CaM. The cDNA in pET-23d was expressed in *Escherichia coli* and purified as described (25). A rat aCaM kinase II cDNA was expressed in SF21 cells as previously described (26) and purified on a phosphocellulose cation exchange column as described by Bradshaw et al. (27).

**RC3 Expression and Purification**—A cDNA encoding the full-length RC3 protein from rat was originally obtained from Dr. Dan Gerendasy and the cDNA sequence was confirmed in our laboratory. RC3 was expressed from the pET23 vector in the BL21 DE3 pLys S strain of *E. coli* following the expression protocol detailed by Gerendasy et al. (28). To purify RC3, the following steps were performed at 4 °C. Cell pellets were lysed in a cold hypotonic lysis buffer (10 mM HEPES, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride), with sonication. The lysate was cleared by centrifugation at 25,000 × g, and the resulting supernatant was brought to 30% ammonium sulfate. The precipitate was removed by centrifugation at 10,000 × g for 30 min, and then the supernatant was brought to 65% ammonium sulfate, causing precipitation of RC3. After centrifugation at 10,000 × g for 30 min, the pellet was resuspended in 15 ml of 50 mM Tris, pH 6.8, 50 mM NaCl, 1 mM DTT, and perchloric acid was added to 2% final concentration. The mixture was centrifuged at 10,000 × g for 15 min, and then the supernatant from this spin containing RC3 was brought to 90% ammonium sulfate. After a final spin at 10,000 × g for 30 min, the pellet was resuspended in 10 mM HEPES, 200 mM KCl, 1 mM DTT, and dialyzed overnight into the same buffer. The RC3 was then subjected to high performance liquid chromatography using a Vydac C4 column and was eluted with a 0–60% gradient of water + 0.13% trifluoroacetic acid to acetonitrile + 0.13% trifluoroacetic acid, over 30 min. Peak fractions were detected by UV absorption and the peak containing RC3 (that eluted at 38 min) was identified by SDS-PAGE. Peak fractions were pooled and lyophilized, resuspended in water, and relyophilized. For each set of experiments, a 1 mM stock solution of RC3 was made in 20 mM MOPS, pH 7.5, 100 mM KCl, and 1 mM DTT. Before each experiment, the RC3 was treated with 50 mM DTT at 55 °C for 15 min to minimize the disulfide-bonded intermediate.

**Decalcification of Buffers and Proteins**—For Ca$^2^+$ titration experiments and Ca$^2^+$ association experiments, all solutions and proteins were decalcified before use by serial elution through columns of Calcium Sponge resin (Molecular Probes, Eugene, OR). All pipette tips, cuvettes, and other labware were rinsed with 0.1 mM HCl and MilliQ water to remove Ca$^2^+$. The absence of Ca$^2^+$ was confirmed by comparing Tyr fluorescence of diluted CaM before and after the sequential addition of 0.1 mM EGTA and 1 mM Ca$^2^+$. Residual Ca$^2^+$ in decalcified buffers was detected using Indo-1: 1 μM Indo-1 in a decalcified solution of 20 mM MOPS, pH 7.0, 100 mM KCl was excited at 355 nm and monitored emission was varied from 350 to 600. In the absence of Ca$^2^+$, a single peak was seen at ~475 nm. Binding of Ca$^2^+$ to Indo-1 (Kp = 0.23 μM) caused a decrease in this peak and an increase at ~400 nm. The above procedures remove essentially all Ca$^2^+$ from CaM (<0.1 μM total CaM), and reduced the background Ca$^2^+$ in buffers to <10−7 M.

**Stopped Flow Fluorometry**—All stopped flow experiments were performed on an Applied Photophysics Ltd. (Leatherhead, UK) model SV.17 MV sequential stopped flow spectrofluorometer with a dead time of 1.7 ms. For all experiments, data from five to six injections were averaged and then fit with either single, double, or triple exponential functions. All solutions were made in a buffer containing 20 mM MOPS, pH 7.5, 100 mM KCl and, where applicable, 1 mM MgCl2. The concentration of reagents given below are those in the syringes, before mixing. For Ca$^2^+$ dissociation using quin-2, the excitation wavelength was 334.5 nm, and the detection of fluorescence emission was controlled by a cut-off filter (Oriel) at 382 nm. Indo-1 (along with the CaM target and nucleotide, as indicated in the figure legends) were mixed with 150 μM quin-2 (Molecular Probes). For Tyr fluorescence, the excitation wavelength was 276 nm, and emission was monitored with a bandpass emission filter with peak transmission at 355 nm (Oriel 51662). To measure Ca$^2^+$ dissociation, 10 μM CaM and 100 μM CaCl2 with 50 μM RC3 where indicated was mixed with 5 mM EGTA. To measure rates of Ca$^2^+$ association with CaM, 2 μM CaM with or without 10 μM RC3 was mixed with 20–100 μM CaCl2.

**Steady State Ca$^2^+$ Binding**—Titration of CaM and 5.5 mM Br-BAPTA with CaCl2 was according to the method of Linse et al. (29) as used by us previously (14). CaCl2 was titrated into a cuvette containing 30 mM decalcified CaM, 30 mM 5.5 mM Br-BAPTA, with or without 60 μM RC3, all in 20 mM MOPS, pH 7.5 100 mM KCl. After each addition of CaCl2, the absorbance of the solution was measured at a wavelength of 263 nm using a Cary/Varian 100 spectrophotometer. Note that RC3 does not contain Tryp or Tyr residues and its presence contributes little to the absorbance at 263 nm.

**Free Energy Calculations**—The free energy coupling of Ca$^2^+$ binding to CaM associated with CaM kinase II was calculated as described in Keller et al. (30). The free energies for the interaction of Ca$^2^+$ with CaM ($\Delta G_{Ca/CaM}$), the interaction of Ca$^2^+$ with the CaM/CaM kinase II complex ($\Delta G_{Ca/CaM/CK}$) and the interaction of Ca$^2^+$–CaM with CaM kinase II ($\Delta G_{Ca/CaM/CK}$) were calculated. The formula $\Delta G_{Ca/CaM/CK} = \Delta G_{Ca/CaM} - R T \ln K_{Ca/CaM}$ is the temperature constant (0.586135 kcal/mol) and $T$ is the temperature in K. The free energy for the interaction of apo-CaM with CaM kinase II ($\Delta G_{Ca/CaM}$) was calculated using Equation 1,

$$\Delta G_{Ca/CaM} = \Delta G_{Ca/CaM/CaM} - \Delta G_{Ca/CaM}$$

(1) and the $K_p$ for this interaction was calculated using the above relationship between $\Delta G$ and $K_p$. The magnitude of free energy coupling ($\Delta G_{Ca/CaM}$) was calculated according to Equation 2.

$$\Delta G_{Ca/CaM/CaM} = \Delta G_{Ca/CaM}$$

(2)

For all these calculations, the free energy of Ca$^2^+$ binding used was the average free energy of the four sites, using $K_p$ values of 13 μM and 2 μM for the N-terminal and C-terminal lobes of uncomplexed CaM, respectively (17), and assuming that the rate of association of Ca$^2^+$ with CaM was not altered by the binding of CaM kinase II.
Bidirectional Tuning of the Affinity of Calmodulin for Calcium

RESULTS

Because RC3 can bind to the Ca\(^{2+}\)-free form of CaM, we reasoned that it might stabilize this form of CaM and decrease its affinity for Ca\(^{2+}\), either by increasing its rate of dissociation and/or decreasing its rate of association. To address these possibilities, two different stopped flow methods were used to assess the rate of Ca\(^{2+}\) dissociation from CaM: quin-2 fluorescence and Tyr fluorescence. Quin-2 is a fluorescent Ca\(^{2+}\) chelator, which exhibits an increase in fluorescence upon binding Ca\(^{2+}\). Free Ca\(^{2+}\) associates with quin-2 so quickly that the reaction is complete within the 1.7 ms dead time of the fluorometer leaving a fluorescence increase that results from the slower release of Ca\(^{2+}\) from CaM and thus the rate of dissociation of Ca\(^{2+}\) from CaM can be determined. As shown in Fig. 1 and summarized in Table I, the rate of Ca\(^{2+}\) dissociation from CaM is dramatically increased in the presence of RC3. Without RC3, the dissociation of Ca\(^{2+}\) from CaM at 3 °C fits to 2 exponentials, with rates of 476 ± 18 s\(^{-1}\) and 1.9 ± 0.2 s\(^{-1}\), which correspond to the rates of Ca\(^{2+}\) dissociation from the N-terminal and C-terminal lobes of CaM respectively (Fig. 1A). The entire amplitude of fluorescence change associated with the slower rate was easily captured in the stopped flow instrument, and we used this amplitude to calibrate the amount of Ca\(^{2+}\) dissociation in the quin-2 experiments: it was considered to be equal to the dissociation of 2 mol of Ca\(^{2+}\) per mole of CaM (11, 17, 31). Dissociation rates were confirmed by performing stopped-flow experiments while monitoring the intrinsic Tyr fluorescence of CaM, which decreases upon release of Ca\(^{2+}\). These experiments gave rise to a single rate of 1.69 ± 0.02 s\(^{-1}\) for the dissociation of Ca\(^{2+}\). Because the Tyr residues in CaM all reside in the C-terminal lobe, this rate represents the dissociation of Ca\(^{2+}\) from the C-terminal lobe.

In the presence of RC3 at 3 °C, the quin-2 fluorescence data (Fig. 1A) was fit with a triple exponential curve, with rates of >500 s\(^{-1}\), 98.6 ± 9.1 s\(^{-1}\), and 9.7 ± 0.7 s\(^{-1}\). The Tyr fluorescence data was fit with a double exponential, with rates of 115 ± 1 s\(^{-1}\) and 12.0 ± 2.7 s\(^{-1}\). Because the fast rate measured with quin-2 fluorescence is absent from the Tyr fluorescence data, it can be assumed to correspond to the dissociation of Ca\(^{2+}\) from the N-terminal lobe. The other two rates correspond to Ca\(^{2+}\) release from the C-terminal lobe. By setting the amplitude of the Ca\(^{2+}\) release from the C-terminal lobe of CaM alone (without RC3) to be approximately equal to the release of 2 mol of Ca\(^{2+}\) per mole of CaM (11, 17, 31), we calculate that in the presence of RC3, 1.64 ± 0.16 mol of Ca\(^{2+}\) dissociate from the C-terminal lobe at the faster rate of about 100, while 0.46 ± 0.06 mol dissociate with the slower rate of about 10 s\(^{-1}\). We also measured Ca\(^{2+}\) dissociation from CaM at room temperature and looked at the effect of RC3 interacting with CaM. Tyr fluorescence stopped-flow experiments using CaM alone gave a C-terminal dissociation rate of 9.54 ± 0.06 s\(^{-1}\). In the presence of RC3, the fluorescence data fit with a double exponential as it did at 3 °C. The two rates were 557 ± 55 s\(^{-1}\) and 71.2 ± 2.8 s\(^{-1}\). The dissociation of Ca\(^{2+}\) from CaM in the presence of RC3 as measured by Tyr fluorescence is shown in Fig. 1B. Quin-2 experiments performed at room temperature were not able to separate the accelerated release of Ca\(^{2+}\) from the C-terminal lobe in the presence of RC3 from the N-terminal Ca\(^{2+}\) dissociation, and the data fit with double exponential curves, giving rates of >500 s\(^{-1}\) and 60.7 ± 5.3 s\(^{-1}\). At room temperature, the slower rate represented a greater proportion of the amplitude than at 3 °C, when measured with either quin-2 or Tyr fluorescence. The quin-2 data indicate that at room temperature, the slower rate represents the release of ~1.4 mol of Ca\(^{2+}\) per mol of CaM.

We next determined whether binding to RC3 causes a change in the overall affinity of CaM for Ca\(^{2+}\), using a steady state titration method. 5,5'-Br\(_2\)-BAPTA absorbance decreases when the chelator binds Ca\(^{2+}\), and this can be used to quantify the amount of free Ca\(^{2+}\) in solution (29). By titrating Ca\(^{2+}\) into a solution containing both CaM and 5,5'-Br\(_2\)-BAPTA, and measuring the absorbance, we can derive the affinity of Ca\(^{2+}\) for CaM. As shown in Fig. 2A, the presence of RC3 causes a significant left shift of this binding curve. The data could not be fit to a model of competition for Ca\(^{2+}\) between CaM and 5,5'-Br\(_2\)-BAPTA, which suggests that binding to RC3 decreases the affinity of CaM for Ca\(^{2+}\) so that it is much lower than the affinity of 5,5'-Br\(_2\)-BAPTA for Ca\(^{2+}\) (K\(_D\) = 1.6 μM).

Because it is possible that the shift in the 5,5'-Br\(_2\)-BAPTA absorbance curve results from a nonspecific effect of RC3 on 5,5'-Br\(_2\)-BAPTA absorbance; we decided to quantitate the RC3 effect on the affinity of CaM for Ca\(^{2+}\) by directly determining the association rate constant for Ca\(^{2+}\) binding to CaM. Fig. 2B shows representative stopped flow fluorometry data measuring the increased intrinsic Tyr fluorescence of CaM upon Ca\(^{2+}\) association with the C-terminal lobe. This change in fluores-
Bidirectional Tuning of the Affinity of Calmodulin for Calcium

Ca\textsuperscript{2+} dissociation from CaM or from CaM bound to RC3 was measured at 3 °C or 22 °C using quin-2 fluorescence and Tyr fluorescence stopped flow methods as described under "Experimental Procedures," and the data were fit with single, double, or triple exponential equations using SigmaPlot. Values shown represent mean ± S.D. of the fits to at least four curves.

|                  | N-term rate |  | C-term Rate 1 |  | C-term Rate 2 |  |
|------------------|-------------|---|---------------|---|---------------|---|
| CaM 3 °C         |             |   |               |   |               |   |
| Quin-2           | 476 ± 18    |   | 1.9 ± 0.2     |   | 2             |   |
| Tyr              | n/a         |   | 1.69 ± 0.02   | 1 | n/a           | n/a|
| CaM + RC3 3 °C   |             |   |               |   |               |   |
| Quin-2           | >500        |   | 98.6 ± 9.1    | 1.64 ± 0.16 | 9.7 ± 0.7 | 0.46 ± 0.06 |
| Tyr              | n/a         |   | 115 ± 1       | 0.925 ± 0.002 | 12.0 ± 2.7 | 0.075 ± 0.002 |
| CaM 22 °C        |             |   |               |   |               |   |
| Quin-2           | >500        |   | 9.1 ± 0.8     |   | 2             |   |
| Tyr              | n/a         |   | 9.54 ± 0.06   | 1 | n/a           | n/a|
| CaM + RC3 22 °C  |             |   |               |   |               |   |
| Quin-2           | >500        |   | >500          |   | 60.7 ± 5.3    | 1.39 ± 0.08 |
| Tyr              | n/a         |   | 557 ± 55      | 0.74 ± 0.02 | 71.2 ± 2.8  | 0.26 ± 0.02 |

For quin-2 experiments, amplitudes are given as the number of moles of Ca\textsuperscript{2+} released per mole of CaM. For Tyr fluorescence experiments, amplitudes are given as a fraction of the total amplitude.

Concentration was measured in the presence or absence of RC3 for a range of Ca\textsuperscript{2+} concentrations, and the observed rates of binding (determined by monoeXponential fits of averaged data) were plotted against the concentration of Ca\textsuperscript{2+}. The slope of a linear fit to these data gives the association rate constant, which was 6.0 × 10\textsuperscript{6} M\textsuperscript{-1} s\textsuperscript{-1} for CaM alone, and 6.5 × 10\textsuperscript{6} M\textsuperscript{-1} s\textsuperscript{-1} for CaM plus RC3. Therefore, the rate of association of Ca\textsuperscript{2+} with the C-terminal lobe of CaM is not altered by CaM interaction with RC3.

Because RC3 does not alter the rate of association of Ca\textsuperscript{2+} with CaM, the change in affinity of CaM for Ca\textsuperscript{2+} when bound to RC3 is proportional to the quantified changes in dissociation rates. The fast rate of dissociation of Ca\textsuperscript{2+} from CaM in the presence of RC3 is more than 60-fold faster than in the absence of RC3; the slower rate is 8-fold faster. Therefore, the affinity of CaM for Ca\textsuperscript{2+} can be decreased up to 60-fold by CaM interaction with RC3.

We have shown that proteins such as RC3 and PEP-19 (14) that bind to apo-CaM can lead to an increase in the rate of dissociation of Ca\textsuperscript{2+} from CaM and/or a decrease in the affinity of CaM for Ca\textsuperscript{2+}. In contrast, Ca\textsuperscript{2+}-dependent targets of CaM lead to an increase in the affinity of CaM for Ca\textsuperscript{2+} (15). We demonstrated previously that CaM binding to CaM kinase II significantly increases CaM affinity for Ca\textsuperscript{2+} (14). However, the additional impact that CaM kinase II autophosphorylation might have on CaM Ca\textsuperscript{2+} binding affinity was not assessed. In the absence of any interacting protein partner, dissociation of Ca\textsuperscript{2+} from CaM at room temperature occurs at two rates: one which is too fast to measure practically, and a second rate of 9.1 ± 0.6 s\textsuperscript{-1} (Fig. 3). As discussed above, these are the rates of dissociation from the N-terminal and C-terminal lobes, respectively. In the presence of CaM kinase II, the data fit best to a triple exponential curve, as evidenced by the residuals shown in Fig. 3, panels B and C. About 1 mol of Ca\textsuperscript{2+} dissociates at a rate of 48.9 ± 15.2 s\textsuperscript{-1}, roughly 2 mol dissociate with a rate of 7.6 ± 0.9 s\textsuperscript{-1}, and less than 1 mol of Ca\textsuperscript{2+} dissociates with a rate of 0.45 ± 0.06 s\textsuperscript{-1} (Fig. 3). These data are summarized in Table II.

When CaM kinase II is autophosphorylated at Thr\textsuperscript{286}, it has a much higher affinity for CaM than unphosphorylated CaM kinase II (24). Based on thermodynamic principles, phosphorylated CaM kinase II should therefore stabilize the Ca\textsuperscript{2+}-bound form of CaM to an even greater extent than unphosphorylated CaM kinase II. We measured Ca\textsuperscript{2+} dissociation from CaM bound to CaM kinase II phosphorylated in the presence of ATP\textsubscript{γ}S, which promotes phosphorylation only at Thr\textsuperscript{286} (32). We again measured three rates of dissociation: less than 1 mol of Ca\textsuperscript{2+} dissociates at a rate of 11.2 ± 2.8 s\textsuperscript{-1}, less than 1 mol of Ca\textsuperscript{2+} dissociates at a rate of 0.9 ± 0.2 s\textsuperscript{-1}, and approximately 3 mol of Ca\textsuperscript{2+} dissociate at 0.38 ± 0.03 s\textsuperscript{-1} (Fig. 3A and Table II). Phosphorylation of CaM kinase II in the presence of ATP (as opposed to ATP\textsubscript{γ}S) can lead to phosphate incorporation at a number of sites in addition to Thr\textsuperscript{286} (33, 34). Using CaM kinase II phosphorylated in this manner, the Ca\textsuperscript{2+} dissociation data was less consistent than with the ATP\textsubscript{γ}S phosphorylated kinase. In about half of the experiments, the rates were similar to those with ATP\textsubscript{γ}S, while in the other half the rates were considerably slower (data not shown). The cause of this variability is not readily discernible; however, variable autophosphorylation of other sites on the kinase might produce effects on its interaction with CaM that are yet to be characterized.

We next considered the fact that both CaM kinase II and RC3 are known to be present at high concentrations in neurons, and both reside in the same compartments: soma, dendrites, and dendritic spines (35, 36). We previously showed that PEP-19 can increase the rates of Ca\textsuperscript{2+} dissociation from CaM bound to CaM kinase II (14). Therefore, we asked the question: if both RC3 and CaM kinase II are present, how does this tripartite system affect Ca\textsuperscript{2+} dissociation from CaM? Representative results from quin-2 stopped flow experiments are shown in Fig. 4. Panel A shows that Ca\textsuperscript{2+} dissociation from CaM in the presence of both RC3 and CaM kinase II occurs at rates intermediate between those in the presence of RC3 or in the presence of CaM kinase II alone. Changing the ratio of RC3 to CaM kinase II did not alter the measured rates of Ca\textsuperscript{2+} dissociation (data not shown). When we examined the effect of thiophosphorylated CaM kinase II and RC3 on Ca\textsuperscript{2+} dissociation from CaM, however, different results were obtained, as shown in Fig. 4B. The presence of RC3 did not affect Ca\textsuperscript{2+} dissociation from CaM when CaM was associated with the thiophosphorylated form of CaM kinase II. The measured dissociation rates were not significantly different from those measured in the presence of thiophosphorylated CaM kinase II alone.

DISCUSSION

In this work, we present data showing that RC3 and CaM kinase II have opposing effects in altering CaM affinity for Ca\textsuperscript{2+}, and that RC3 can lead to an increase in the observed rates of dissociation of Ca\textsuperscript{2+} from CaM complexed with CaM kinase II. RC3 causes a significant decrease in CaM affinity for Ca\textsuperscript{2+} by increasing the rate of dissociation of Ca\textsuperscript{2+} from the C-terminal lobe of CaM. CaM kinase II causes an increase in the affinity of CaM for Ca\textsuperscript{2+} by decreasing the rate of dissociation of Ca\textsuperscript{2+} from both the N-terminal and C-terminal lobes.
Bidirectional Tuning of the Affinity of Calmodulin for Calcium

A Kinetic Scheme for Dissociation of Ca\(^{2+}\) and Target from CaM—The dissociation of Ca\(^{2+}\) and Ca\(^{2+}\)-dependent target peptides from CaM induced by chelating Ca\(^{2+}\) has been studied in detail for peptides derived from the CaM binding domain of skeletal muscle myosin light chain kinase (37, 38). A major conclusion from this work was that the observed dissociation rates cannot be directly assigned to any kinetic step in the dissociation pathway. This is because several competing dissociation pathways exist in the Ca\(^{2+}\)-CaM-target system, as shown in Fig. 5. This scheme assumes that the two Ca\(^{2+}\) ions in each lobe bind cooperatively, and so the dissociation of two Ca\(^{2+}\) ions from one lobe can be considered as a single step. The first step in the dissociation can be either Ca\(^{2+}\) dissociation from the N-terminal lobe (Fig. 5, step 1), Ca\(^{2+}\) dissociation from the C-terminal lobe (Fig. 5, step 2), or target dissociation from Ca\(^{2+}\)-CaM (Fig. 5, step 3). The target protein can reassociate with (Ca\(^{2+}\))\(_{2}\)-CaM or the (Ca\(^{2+}\))\(_{2}\)-CaM intermediates, altering the overall observed rates of Ca\(^{2+}\) dissociation, depending on the relative rates of Ca\(^{2+}\) dissociation and target dissociation and reassociation. The dissociation of the Ca\(^{2+}\)-CaM-target complex is therefore under kinetic control, in which the order of dissociation steps and the overall speed of the dissociation depend on the relative kinetics of the individual steps in the pathways. This idea of kinetic control of the dissociation process applies to the dissociation of Ca\(^{2+}\) from CaM bound to both Ca\(^{2+}\)-dependent targets like CaM kinase II, and those like RC3, which interact with both Ca\(^{2+}\)-bound and Ca\(^{2+}\)-free CaM.

Dissociation of Ca\(^{2+}\) from CaM Bound to RC3—Previous work from our laboratories has shown that PEP-19, which contains an IQ-type CaM binding domain homologous to that in RC3, also increases the rate of dissociation of Ca\(^{2+}\) from the C-terminal lobe of CaM (14). However, while PEP-19 causes an increase in the rate of Ca\(^{2+}\) association with CaM, RC3 does not. Because of this difference, PEP-19 does not alter the overall affinity of Ca\(^{2+}\) for CaM, while RC3 causes a significant decrease in CaM affinity for Ca\(^{2+}\). When CaM is bound to RC3...
Bidirectional Tuning of the Affinity of Calmodulin for Calcium

Effect of CaM kinase II on the rates of dissociation of Ca\(^{2+}\) from CaM

| Rate 1  | Rate 2  | Rate 3  |
|--------|--------|--------|
| s\(^{-1}\) | s\(^{-1}\) | s\(^{-1}\) |
| CaM    | >500   | 9.1 ± 0.6 | 2    |
| CaM + CaM kinase II | 48.9 ± 15.2 | 2    |
| CaM + phospho-CaM kinase II | 11.2 ± 2.8 | 2    |
| CaM + CaM-kinase II + RC3 | 272 ± 83 | 2    |
| CaM + phospho-CaM kinase II + RC3 | 8.24 ± 1.03 | 2    |

Ca\(^{2+}\) dissociation from CaM or from CaM bound to CaM kinase II was measured at 22 °C using quin-2 stopped-flow fluorometry as described under "Experimental Procedures" and as shown in Fig. 3. The data were fit with single or triple exponential equations using Sigma Plot. Values shown represent mean ± S.D. of the fits to at least four curves.

TABLE II

Ca\(^{2+}\) dissociation from CaM in the presence of both CaM kinase II and RC3. Ca\(^{2+}\) dissociation from CaM was measured using quin-2 fluorescence at 22 °C as described for Fig. 3. Panel A shows Ca\(^{2+}\) dissociation from 2 μM CaM in the presence of 10 μM RC3, 2 μM CaM kinase II, or a mixture of 10 μM RC3 with 2 μM CaM kinase II. Panel B shows Ca\(^{2+}\) dissociation from 2 μM CaM in the presence of 2 μM phospho-CaM kinase II or 2 μM phospho-CaM kinase II with 20 μM RC3.

![Figure 4](image)

Fig. 4. Ca\(^{2+}\) dissociation from CaM in the presence of both CaM kinase II and RC3. Ca\(^{2+}\) dissociation from CaM was measured using quin-2 fluorescence at 22 °C as described for Fig. 3. Panel A shows Ca\(^{2+}\) dissociation from 2 μM CaM in the presence of 10 μM RC3, 2 μM CaM kinase II, or a mixture of 10 μM RC3 with 2 μM CaM kinase II. Panel B shows Ca\(^{2+}\) dissociation from 2 μM CaM in the presence of 2 μM phospho-CaM kinase II or 2 μM phospho-CaM kinase II with 20 μM RC3.

at 3 °C, we measure two rates of dissociation of Ca\(^{2+}\) from the C-terminal lobe of CaM: one of about 100 s\(^{-1}\), with an amplitude of 1.6 mol of Ca\(^{2+}\) released per mole of CaM, and a second slower rate of about 10 s\(^{-1}\), with an amplitude of 0.4 mol of Ca\(^{2+}\) released per mole of CaM. We could not resolve whether RC3 led to any change in the dissociation of Ca\(^{2+}\) from the N-terminal lobe, due to the extremely fast dissociation rates. Our previous NMR studies of Ca\(^{2+}\)/CaM bound to PEP-19 showed that PEP-19 interacts with residues on the C-terminal lobe of CaM that border the hydrophobic pocket (14). Others showed that a peptide derived from the CaM binding domain of RC3 interacted with similar sites in the C-terminal lobe of apo-CaM, as well as a small region in the N-terminal domain (39). Therefore, RC3 may interact with CaM in a similar manner as PEP-19, with the major interactions occurring with the C-terminal lobe of CaM. This would be consistent with the observed effects on Ca\(^{2+}\) dissociation occurring predominantly in the C-terminal lobe.

Because the dissociation of Ca\(^{2+}\) from the N-terminal lobe of the Ca\(^{2+}\)-CaM-RC3 complex is so fast, it is likely that the first step in the dissociation pathway is the release of the N-terminal Ca\(^{2+}\) ions from this complex (Fig. 5, step 1). The remaining Ca\(^{2+}\) dissociation occurs therefore through dissociation of Ca\(^{2+}\) from the C-terminal lobe of RC3-bound CaM (step 7) or through dissociation of RC3 followed by dissociation of Ca\(^{2+}\) from the C-terminal lobe of uncomplexed CaM (steps 5 and 6). Based on our quin-2 data, we propose that the dissociation rate in step 7 is very fast (~500 s\(^{-1}\) at room temperature), and this step is responsible for the observed fast rate of Ca\(^{2+}\) dissociation from the C-terminal lobe. The observed slow rate of Ca\(^{2+}\) dissociation is not due simply to dissociation of RC3 followed by dissociation of Ca\(^{2+}\) from the uncomplexed C-terminal lobe, because that rate would be at most 9 s\(^{-1}\), which is much slower than we observe. Therefore, the observed slow rate is likely also in part due to dissociation and reassociation of RC3 with CaM before dissociation of Ca\(^{2+}\).

At higher temperatures, the relative amplitude of the slower observed rate of dissociation from the C-terminal lobe of CaM complexed with RC3 increases, and the amplitude of the faster rate decreases. This indicates that at higher temperatures, the direct dissociation pathway through steps 7 → 8 decreases in prominence, which implies that temperature does not affect all rates equally. The dissociation rate of RC3 from (Ca\(^{2+}\))\(_{2}\)-CaM and/or the dissociation of Ca\(^{2+}\) from uncomplexed CaM increases more than rate of reassociation of RC3 with (Ca\(^{2+}\))\(_{2}\)

![Figure 5](image)

Fig. 5. Kinetic scheme for the chelator-induced dissociation of Ca\(^{2+}\) and target from CaM. A schematic diagram shows the possible pathways of dissociation of the Ca\(^{2+}\)-CaM-target complex. CaM is represented by the letter C, and subscripts indicate the interactions of CaM with Ca\(^{2+}\) and target. The subscript N indicates Ca\(^{2+}\) is bound to the N-terminal lobe, the subscript C indicates Ca\(^{2+}\) is bound to the C-terminal lobe, and the subscript T indicates target is bound to CaM. This scheme assumes that the Ca\(^{2+}\) ions dissociate in pairs and that their dissociation is effectively irreversible due to immediate chelation of Ca\(^{2+}\) upon dissociation from CaM. Adapted from Brown et al. (38).
CaM. The differential effect of temperature on these rates could also be due to the fact that RC3 largely exists as an unstructured molecule (40), i.e. sampling a large variety of conformations, and the kinetics of interaction of RC3 with CaM may be different for the different conformers. Lower temperatures may preferentially stabilize certain conformers, leading to differences in the effects of temperature on the kinetic steps described in Fig. 5.

**Ca2⁺ Dissociation from CaM Bound to CaM Kinase II**—Our studies on the effect of the interaction of CaM kinase II with CaM are consistent with the idea that Ca2⁺-dependent targets of CaM stabilize the Ca2⁺-bound form of CaM (30). Dissociation of Ca2⁺ from CaM when bound to CaM kinase II was slower than from CaM in the absence of target protein. We measured three dissociation rates: for each mole of CaM, 1 mol of Ca2⁺ dissociates at a rate of ~50 s⁻¹, and about 2 mol of Ca2⁺ dissociate at 8 s⁻¹, and less than 1 mol of Ca2⁺ dissociates at a rate of 0.4 s⁻¹. If these data are considered in terms of the dissociation scheme presented in Fig. 5, the rates of dissociation of CaM kinase II from (Ca2⁺)₂-CaM (step 3) must be compared with the fast rate of dissociation of Ca2⁺ from the full complex (step 1 or 2) in order to see whether dissociation of the kinase is an important pathway. The first step in the dissociation pathway cannot logically be slower than the fastest observed rate. The kinase dissociation in step 3 occurs at a rate of 1.6 s⁻¹, which is much slower than the observed fast dissociation rate of ~50 s⁻¹, leading to the conclusion that the pathway through step 3 will not be significant in the overall dissociation. Therefore, most of the release occurs through pathways starting with dissociation of Ca2⁺ from CaM complexed with CaM kinase II (steps 1 or 2). A reasonable assumption is that the dissociation of Ca2⁺ from the N-terminal lobe is faster than dissociation from the C-terminal lobe, causing step 1 to predominate (38). Dissociation therefore occurs through steps 1, 7, and 8 (i.e. Ca2⁺ dissociates from each lobe, then CaM kinase II dissociates from 2-CaM-CaM kinase II) (steps 1 or 2). The interaction of Ca2⁺ from CaM while it is bound to CaM kinase II (step 1) is known to be extremely slow, on the order of 10⁻³ to 10⁻⁵ s⁻¹ (24, 41), therefore dissociation of this complex must occur through pathways starting with steps 1 or 2. Again, we will assume that the N-terminal Ca2⁺ ions have a faster dissociation rate, however, the entire description that follows could apply with the C-terminal dissociation occurring first. Since the fastest observed rate must be because of the first step in the pathway, one explanation of our data is that there is altered cooperativity in the interaction of Ca2⁺ to CaM in the presence of phospho-CaM kinase II. The first step is the dissociation of one Ca2⁺ ion at a rate of about 11 s⁻¹ (step 1, but with only one Ca2⁺), leading to the formation of a Ca2⁺³-CaM-CaM kinase II intermediate. Further dissociation occurs by release of the 3 remaining Ca2⁺ ions at rates of about 1 s⁻¹ and 0.4 s⁻¹ (via step 7 and/or steps 5 and 6) followed by phospho-CaM kinase II dissociation from Ca2⁺³-CaM.

Another issue that must be considered is that in our experiments it is difficult to assess whether all subunits of CaM kinase II are in the phosphorylated state. If not, the faster rates of dissociation that we see might be because of dissociation of Ca2⁺ from unphosphorylated subunits. If true, this would imply that all dissociation of Ca2⁺ from CaM bound to phospho-CaM kinase II occurs at a rate of about 0.4 s⁻¹.

**Thermodynamic Coupling of Ca2⁺ and Target Binding to CaM**—The effect of binding to target protein on CaM affinity for Ca2⁺ has been investigated previously with respect to the free energy coupling of Ca2⁺ binding to CaM. The binding of CaM to target reduces the free energy for the interaction of Ca2⁺ and CaM. This free energy coupling was first demonstrated by Keller et al. (30) for the binding of CaM to tropomyosin I (TnI). They calculated that the energy for binding for four Ca2⁺ ions and TnI binding to CaM was −5 kcal/mol, which indicated that the affinity of TnI for CaM was increased 4,500-fold by the binding of Ca2⁺ to CaM. Similarly, Olwin et al. (42) calculated the free energy coupling for the binding to CaM of skeletal muscle myosin light chain kinase (skMLCK) and concluded that the difference in free energy for Ca2⁺ binding to CaM was −8.44 kcal/mol. They determined from this that the affinity of skMLCK for apo-CaM was about 28 mm. Perschini et al. (43, 44) re-investigated the free energy coupling in CaM/skMLCK complex and determined that the change in Ca2⁺ affinity was due mostly to changes in dissociation rate, with little effect on association rates of Ca2⁺ with CaM. If we assume that there are negligible changes in the rate of association of Ca2⁺ with CaM in the presence of CaM kinase II, the calculated average free energy coupling is −7.12 kcal/mol for unphosphorylated CaM kinase II and −11.22 kcal/mol for phosphorylated CaM kinase II. This leads to the conclusion that the affinity of Ca2⁺⁻free CaM for unphosphorylated CaM kinase II is about 11 mM, while for phosphorylated CaM kinase II it is 0.41 mM. It should be noted that these calculations used the observed dissociation rates, which represent the upper limit for the actual dissociation rates for each step. If the actual rates of dissociation of Ca2⁺ from CaM complexed to CaM kinase II are slower, the free energy coupling is even greater and the affinity of CaM kinase II for apo-CaM is even weaker.

**Effect of RC3 on Dissociation of the Ca2⁺⁻CaM-CaM Kinase II Complex**—The interaction of RC3 with CaM while it is bound to CaM kinase II is consistent with our experiments on PEP-19, which similarly increases the rate of dissociation of Ca2⁺ from CaM complexed with CaM kinase II (14). Depending on the rates of each dissociation step, it may be possible to explain this result with a kinetic argument, by suggesting that RC3 and PEP-19 act as a shunt in the dissociation pathway. The dissociation of the complex of Ca2⁺⁻CaM with CaM kinase II has a large component that occurs through step 5: dissociation of the kinase from (Ca2⁺)₂-CaM. What then follows is dissociation of Ca2⁺ from uncomplexed CaM or reassociation of (Ca2⁺)₂-CaM with CaM kinase II. In the presence of RC3, what happens instead is that (Ca2⁺)₂-CaM binds to RC3, which induces a fast dissociation of Ca2⁺ from the C-terminal lobe of CaM. Ca2⁺ dissociation occurs before CaM dissociates from RC3, so it does not have the chance to reassociate with CaM kinase II. The result is an overall increase in the rates of dissociation of Ca2⁺. This interpretation of the results is likely an oversimplification, because the presence of RC3 appears to accelerate the dissociation of Ca2⁺ from all four binding sites on CaM, but it illustrates the principle that RC3 has an ability to change the rates of

---

*Footnote:* T. R. Gaertner and M. N. Waxham, unpublished observation.
dissociation of the Ca\textsuperscript{2+}-CaM-CaM kinase II complex through a kinetic competition for CaM.

RC3 has no effect on the observed rates of dissociation of Ca\textsuperscript{2+} from CaM complexed with phosphorylated CaM kinase II. The dissociation of this complex occurs mainly through dissociation of Ca\textsuperscript{2+} from CaM complexed to CaM kinase II because the dissociation of the phosphorylated kinase from Ca\textsuperscript{2+}-CaM is so slow as to be negligible. Therefore, RC3 cannot bind to Ca\textsuperscript{2+}-CaM and has no effect on the rates of dissociation.

A second possible explanation of this data is that RC3 (or PEP-19) and CaM kinase II can bind to CaM at the same time. The N-terminal lobe of CaM could remain bound to CaM kinase II while the C-terminal lobe dissociates from CaM kinase II and is then available to bind to RC3 or PEP-19. Phosphorylated CaM kinase II makes additional contacts with CaM, which may preclude the binding of RC3 (45, 46). This may occur through a phospho-CaM kinase II interacting competitively with sites on CaM that are required for interaction with RC3, or by altering the conformation of CaM so that RC3 no longer has access to its binding sites. Alternatively, RC3 may interact with CaM bound to phospho-CaM kinase II, but have no effect on Ca\textsuperscript{2+} dissociation.

**Cellular Effects of Observed Ca\textsuperscript{2+} Dissociation Rates**—The ability of RC3 to accelerate Ca\textsuperscript{2+} release from CaM bound to CaM kinase II indicates that in the cell, RC3 has a role at the end of a Ca\textsuperscript{2+} stimulus in increasing the rate at which Ca\textsuperscript{2+}-dependent targets release CaM. The impact of RC3 at the end of the Ca\textsuperscript{2+} stimulus is likely critical for pulsatile Ca\textsuperscript{2+} stimuli, in which the decrease in binding of CaM to Ca\textsuperscript{2+}-dependent target sets the startpoint for the response to the next pulse of Ca\textsuperscript{2+}. An important cellular phenomenon that depends on such pulsatile stimulation is synaptic plasticity, which is induced experimentally by stimulating neurons at frequencies from 1 to 100 Hz. Stimulation at low frequencies has been shown to lead to long-term depression of synaptic strength, while high frequency stimulation leads to long-term potentiation of synaptic strength. A role for RC3 at the end of a Ca\textsuperscript{2+} pulse is supported by the observation that mice lacking RC3 have altered synaptic plasticity (47, 48), but the details of the role of RC3 in plasticity remain to be determined. Krucker et al. (48) propose that preferential activation of the Ca\textsuperscript{2+}-dependent phosphatase calcineurin over activation of CaM kinase II may explain the results they observe. However, it is also clear that the RC3− mice have widespread alterations in intracellular signaling (49) and Ca\textsuperscript{2+} dynamics (50), suggesting that it is not possible to assign the deficits in synaptic plasticity to the effect of RC3 on any one particular Ca\textsuperscript{2+}-dependent target. Indeed, because CaM is an activator in so many pathways, one would expect that removal of a protein such as RC3, which dynamically regulates the ability of CaM to interact with Ca\textsuperscript{2+} and target proteins, would lead to broad signaling deficits.

We calculate that RC3 causes an up to 60-fold increase in the $K_{D}$ for the interaction of Ca\textsuperscript{2+} with the C-terminal lobe of CaM. This increase in $K_{D}$ affects both the activation of CaM by Ca\textsuperscript{2+}, and, additionally, the levels of free Ca\textsuperscript{2+} in the neuron. RC3 decreases CaM's affinity for Ca\textsuperscript{2+}, so it will bind less Ca\textsuperscript{2+} for a given influx of Ca\textsuperscript{2+} into the cell. There will therefore be more free Ca\textsuperscript{2+}. Indeed, Van Dalen et al. (50) found that in neurons from transgenic mice lacking RC3, Ca\textsuperscript{2+} transients in response to NMDA or the metabotropic glutamate receptor agonist DHPG were significantly smaller than in neurons containing RC3. Our results provide a possible explanation for this finding, by suggesting that in the neurons containing RC3, less of the Ca\textsuperscript{2+} that enters is able to bind to CaM, and so the free Ca\textsuperscript{2+} levels are higher; conversely, in neurons without RC3, more of the entering Ca\textsuperscript{2+} binds to CaM and free Ca\textsuperscript{2+} levels are lower.

How might the CaM kinase II effect on the dissociation rate of Ca\textsuperscript{2+} from CaM affect cellular signaling? When CaM is bound to phosphorylated CaM kinase II, the slow rate for Ca\textsuperscript{2+} dissociation is 0.4 s\textsuperscript{-1}. If this corresponds to Ca\textsuperscript{2+} dissociation from the C-terminal lobe, then the $K_{D}$ for Ca\textsuperscript{2+} interaction with this lobe of CaM is about 40 nM, assuming that CaM kinase II does not alter the association rates of Ca\textsuperscript{2+} with CaM. This means that at a resting Ca\textsuperscript{2+} level of 50–100 nM, most of the CaM bound to phospho-CaM kinase II will still be in the Ca\textsuperscript{2+}-bound form. The presence of RC3 acts to mitigate this effect, however, because once Ca\textsuperscript{2+} dissociates from CaM, CaM will be bound up by RC3, making it less likely for Ca\textsuperscript{2+} to reassociate with it.

Further analysis of the data presented in this manuscript is ongoing in our laboratories. We aim to fit these data to the kinetic scheme shown in Fig. 5 and thereby gain an estimate of the kinetics of the individual steps in the dissociation pathways for the Ca\textsuperscript{2+}-CaM-RC3 and Ca\textsuperscript{2+}-CaM-CaM kinase II complexes. This computational effort will allow us to better predict the dynamics of the interaction of Ca\textsuperscript{2+} with CaM in the context of the cell.

The effect of RC3 and CaM kinase II in altering CaM affinity for Ca\textsuperscript{2+} clearly alters the level of activation of CaM by a given Ca\textsuperscript{2+} transient. A kinetic explanation of the observed rates is useful for understanding the dissociation pathways important for each protein, and shows that RC3 can have an important role in terminating the activity of Ca\textsuperscript{2+}-CaM-dependent enzymes when Ca\textsuperscript{2+} levels fall. These two CaM-binding proteins are examples of two classes of CaM binding interactions: Ca\textsuperscript{2+}-dependent and Ca\textsuperscript{2+}-independent. There are many other proteins which fall into these two classes, each with slightly different affinity for CaM; each probably alters Ca\textsuperscript{2+} binding to CaM in a slightly different manner. In this way, the response of CaM to Ca\textsuperscript{2+} signals is tuned by the binding proteins that are present, so that the appropriate response to a given Ca\textsuperscript{2+} signal is achieved. This response is also affected by the state of the proteins, since phosphorylation and other modifications of the proteins are known to alter their CaM binding properties, which in turn may alter their effect on Ca\textsuperscript{2+} dissociation from CaM. This allows the cell to dynamically regulate the response to a Ca\textsuperscript{2+} signal by regulating the Ca\textsuperscript{2+} binding properties of CaM. Because Ca\textsuperscript{2+} signaling is critical to many neuronal functions, it is important for the neuron to be able to carefully modulate its response to Ca\textsuperscript{2+} signals. Regulation of the Ca\textsuperscript{2+} binding properties of CaM by interaction with CaM-binding proteins may be a central mechanism for tuning the cellular response to Ca\textsuperscript{2+} influx.

Acknowledgments—We thank Dawn Wang for assistance in expressing and purifying proteins used throughout this study. The PTI fluorometer was made available through funding from the W. M. Keck Foundation.

REFERENCES
1. Bennett, M. K., Ercndu, N. E., and Kennedy, M. B. (1983) J. Biol. Chem. 258, 12733–12744
2. Sharma, R. K., Wang, T. H., Wirch, E., and Wang, J. H. (1980) J. Biol. Chem. 255, 5916–5925
3. Perrino, B. A., Ng, L. Y., and Soderling, T. R. (1995) J. Biol. Chem. 270, 340–346
4. Zhang, S., Ehlers, M. D., Bernhardt, J. P., Su, C. T., and Huganir, R. L. (1998) J. Biol. Chem. 273, 443–453
5. Fanger, C. M., Ghanshani, S., Logsdon, N. J., Rauer, H., Kalman, K., Zhou, J., Beckingham, K., Chandy, K. G., Cahalan, M. D., and Aiyar, J. (1999) J. Biol. Chem. 274, 5746–5754
6. DeMaria, C. D., Soong, T. W., Alseikhan, B. A., Alvania, R. S., and Yue, D. T. (2001) Nature 411, 484–489
7. Job, D., Fischer, E. H., and Margolis, R. L. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 4670–4672
8. Sobue, K., Fujita, M., Muramoto, Y., and Kakikuchi, S. (1981) FEBS Lett. 132, 137–140
RC3/Neurogranin and Ca\textsuperscript{2+}/Calmodulin-dependent Protein Kinase II Produce Opposing Effects on the Affinity of Calmodulin for Calcium

Tara R. Gaertner, John A. Putkey and M. Neal Waxham

J. Biol. Chem. 2004, 279:39374-39382.
doi: 10.1074/jbc.M405352200 originally published online July 15, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M405352200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 50 references, 28 of which can be accessed free at http://www.jbc.org/content/279/38/39374.full.html#ref-list-1