Published in final edited form as:

J Comput Neurosci. 2009 December ; 27(3): 621–638. doi:10.1007/s10827-009-0173-3.

Dissecting cooperative calmodulin binding to CaM kinase II: a detailed stochastic model

Michael J. Byrne,

Department of Neurobiology and Anatomy, University of Texas Medical School, 6431 Fannin, Houston, TX 77030, USA

John A. Putkey,

Department of Biochemistry and Molecular Biology, University of Texas Medical School, 6431 Fannin, Houston, TX 77030, USA

M. Neal Waxham, and

Department of Neurobiology and Anatomy, University of Texas Medical School, 6431 Fannin, Houston, TX 77030, USA

Yoshihisa Kubota

Department of Neurobiology and Anatomy, University of Texas Medical School, 6431 Fannin, Houston, TX 77030, USA

Yoshihisa Kubota: Yoshihisa.Kubota@uth.tmc.edu

Abstract

Calmodulin (CaM) is a major Ca^{2+} binding protein involved in two opposing processes of synaptic plasticity of CA1 pyramidal neurons: long-term potentiation (LTP) and depression (LTD). The N- and C-terminal lobes of CaM bind to its target separately but cooperatively and introduce complex dynamics that cannot be well understood by experimental measurement. Using a detailed stochastic model constructed upon experimental data, we have studied the interaction between CaM and Ca^{2+} -CaM-dependent protein kinase II (CaMKII), a key enzyme underlying LTP. The model suggests that the accelerated binding of one lobe of CaM to CaMKII, when the opposing lobe is already bound to CaMKII, is a critical determinant of the cooperative interaction between Ca^{2+} , CaM, and CaMKII. The model indicates that the target-bound Ca^{2+} free N-lobe has an extended lifetime and may regulate the Ca^{2+} response of CaMKII during LTP induction. The model also reveals multiple kinetic pathways which have not been previously predicted for CaM-dissociation from CaMKII.

Keywords

Calmodulin; CaMKII; Synaptic plasticity; Gillespie algorithm; Particle swarm theory

1 Introduction

Calmodulin (CaM) is a dumbbell-shaped Ca^{2+} -binding protein that consists of N- and C-terminal lobes, each of which binds two Ca^{2+} ions in a cooperative fashion and the kinetics of the interaction are well-defined *in vitro* (Babu et al. 1985; Linse et al. 1991; Forsen and Linse 1995). In addition, the Ca^{2+} affinity of CaM is tuned when it binds to its target proteins (Keller et al. 1982; Olwin et al. 1982). The intra- and inter-molecular tuning of Ca^{2+} binding to CaM provide a mechanism by which CaM activation is optimized at the appropriate Ca^{2+} concentration and is selectively regulated by interacting molecules to perform its cellular function. Furthermore, the N- and C-terminal lobes of CaM bind to its target separately but cooperatively and may introduce complex dynamics of Ca^{2+} -CaM-target protein interactions which cannot be well-understood by experimental measurement.

In CA1 pyramidal neurons, Ca^{2+} and CaM are both important in synaptic plasticity (Malenka et al. 1989). Long-term potentiation (LTP) and long-term depression (LTD) require increases in Ca^{2+} and depend on Ca^{2+} activation of CaM (Malenka et al. 1989; Mulkey et al. 1994; Xia and Storm 2005). CaM then activates downstream targets such as Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) and calcineurin (PP2B). CaMKII is implicated in LTP induction (Malenka et al. 1989; Hudmon and Schulman 2002; Lisman et al. 2002) while calcineurin is involved in LTD (Mulkey et al. 1994) yet both are activated by Ca^{2+} /CaM. How CaM can lead to these two opposing processes of LTP and LTD is not well understood. Equally undefined is how the Ca^{2+} signal selectively activates one of the two enzymes, CaMKII or calcineurin, under different induction protocols for synaptic plasticity. In other words, how does CaM decode different temporal patterns of Ca^{2+} signals to activate the appropriate target enzymes? One possible approach to this problem is to dissect each individual step of Ca^{2+} -CaM-target interactions and understand how CaM-target protein complex entail different decoding capacity for Ca^{2+} signals during synaptic plasticity.

In this paper, we begin this analysis for the α -isoform of CaMKII (α CaMKII or CaMKII) using a comprehensive stochastic model that independently accounts for all four Ca^{2+} binding sites of CaM and the interactions of individual lobes with CaMKII. The model was constructed upon and constrained by stopped-flow fluorimetry data of Ca^{2+} -CaM-CaMKII interactions (Gaertner et al. 2004a). Experimentally, CaMKII was shown to cooperatively bind CaM and this may well contribute to the switch like activation of CaMKII (Bradshaw et al. 2003); however, the experimental dissection of such a kinetic pathway has been difficult. Full understanding of cooperative CaM binding requires a detailed mathematical model-based analysis.

The cooperativity of Ca^{2+} -CaM-CaMKII interaction can be divided into at least three different stages: 1) the cooperativity in the binding of Ca^{2+} within each lobe of CaM (Haiech and Kilhoffer 2002); 2) the cooperativity in lobe association with the target due to binding of one lobe and an increased effective concentration of the second lobe and 3) the cooperativity in the binding of CaM between subunits of CaMKII through an as yet structurally undefined mechanism (here we call it *intra-holoenzyme inter-subunit* cooperativity). The second form of cooperativity is a major focus of the current work. When one lobe binds to CaMKII, the opposing lobe is likely to associate to a CaMKII subunit due to its increased effective concentration. We hypothesized this accelerated binding of one CaM lobe is a critical determinant of cooperative interaction between CaM and CaMKII. Results from the stochastic model and theoretical analyses both support this hypothesis and accurately predict the experimental data of CaM-binding to CaMKII. The stochastic model also correctly simulates the experimentally observed Ca^{2+} dissociation process and reveals multiple dissociation pathways which have not been previously predicted. Another possible mechanism for the cooperativity is that the binding of one lobe of CaM to a CaMKII subunit

may change the local conformation of the second CaM binding site on the same CaMKII subunit having the effect of accelerating the binding of the opposing CaM lobe to the subunit. This *intra-subunit* cooperativity is conceptually different from the second (and third) type of cooperativity. However, the experimental distinction between these mechanisms can be quite complicated and quantitative data to demonstrate the difference is not yet available. Therefore, we did not address this issue in the current work.

The organization of this paper is as follows. First, we describe an experimentally constrained mathematical model of Ca^{2+} -CaM-CaMKII interactions (Section 2.1, Methods, Fig. 1 and Tables 1 and 2), followed by the outline of parameter optimization (Section 2.2–2.4). In order to accurately simulate the binding of each individual lobe to CaMKII, we used a modified version of the Gillespie algorithm (Gillespie 1976; Kubota et al. 2007) and constructed a single molecule level model of CaM. Section 2.5 explains the rationale of this algorithm. The parameter optimization using a stochastic model requires a dedicated numerical method described in Section 2.6. In Section 3.1 and 3.2, the model was compared to the experimental data used for the parameter optimization (Fig. 2). These simulations reveal the multiple Ca^{2+} dissociation pathways from each lobe that have not been well characterized by experimental measurements. In Section 4.1, we test the validity of the model by comparing the simulation results with experimental data that was not used during the initial parameter optimization. The model accurately predicts the experimentally measured dissociation constant of Ca^{2+} /CaM to CaMKII (Fig. 3). Without cooperativity described here, we have not been able to find a physically plausible parameter set for the model that fits these experimental data. With these analyses, we predict the cooperative binding may control the lifetime of CaMKII-bound CaM in the cellular interior. In Section 4.2, we test this possibility. The simulation results indicate that the Ca^{2+} free N-lobe remains bound to (un-phosphorylated) CaMKII for an extended period even at the Ca^{2+} concentration of cells at rest. This persistent CaM-CaMKII protein complex is distinct from the so-called “trapped” state of CaM bound to autophosphorylated CaMKII (Meyer et al. 1997) and may represent a different functionality of CaMKII. In fact, our model-based calculation shows that the unphosphorylated CaMKII holoenzyme can explore a large spatial area, close to or even larger than the size of dendritic spine head, while bound to Ca^{2+} -CaM. We illustrate these points in Section 4.3. Finally, we discuss the potential role of the N-terminal lobe of CaM in synaptic plasticity (Discussion).

2 Methods

2.1 Mathematical model and kinetic pathways

Each lobe of CaM binds two Ca^{2+} ions and the bindings are cooperative. We independently model binding of each Ca^{2+} ion at all four binding sites of CaM. Each lobe has four different states with respect to Ca^{2+} binding: Ca^{2+} freestate, state with one Ca^{2+} binding site occupied (two for each lobe) and Ca^{2+} saturated state (the upper diamond shaped pathways in Fig. 1(a)). The C-terminal lobe and N-terminal lobe of CaM bind CaMKII separately or cooperatively. Upon binding to a CaMKII subunit, CaM changes its Ca^{2+} binding kinetics (the lower diamond shaped pathways in Fig. 1(a)). Thus, each lobe can exist in eight possible states (Fig. 1(a)). Although there is a reported change in the Ca^{2+} affinity through interlobular interactions, the change is relatively small (1–1.5 kcal/mol) and would significantly complicate the model (Sorensen and Shea 1998). Therefore, we assume for the purpose of this study that Ca^{2+} bindings to the N- and C-terminal lobes are independent.

Another important issue is the stoichiometry of CaM binding to CaMKII. Recent experiments by Forest et al. (2008) showed that two molecules of physically separated lobes of CaM can simultaneously bind to and activate a single CaMKII subunit. This raises the possibility that two lobes from two different CaM molecules can potentially bind to a single

CaMKII subunit. Although such an exotic event is presumably rare for wild type CaM, we have created a stochastic modeling scheme (see below) that allows all possible reaction pathways, including two CaM molecules binding to a single CaMKII subunit.

2.2 Parameter optimization: overview

The model contains 48 total parameters, 24 for each lobe of CaM (Fig. 1(a), Tables 1 and Table 2). A systematic analysis and numerical parameter optimization is required to deduce these parameter values. In this section, we summarize the rationale and assumptions that we made during the parameter optimization. First, we have sixteen parameters for the Ca^{2+} association to and dissociation from CaM (not bound to CaMKII) (the top pathway in each panel of Fig. 1(a)). These 16 parameter values are estimated from experimental data and kept unchanged throughout the parameter optimization process. Derivation of these CaM parameter values will be explained in the next section (Section 2.3). Ca^{2+} association and dissociation rates to (each lobe of) CaM-CaMKII (8 for each lobe) are largely unknown and they will be determined through parameter optimization.

Ca^{2+} binding of CaM (the vertical arrows in Fig. 1(a)) increases the affinity of CaM toward CaMKII. We assume that the difference in the affinity is due to the change in the dissociation rate of (each lobe of) CaM from CaMKII as supported by experimental data (Gaertner et al. 2004a). We assume the association rates of each lobe of CaM toward CaMKII are not affected by bound Ca^{2+} . There is no biochemical data that we are aware of to contradict this assumption. When one lobe binds to CaMKII, the opposing lobe is more likely to associate due to its close proximity to a CaMKII subunit: the free lobe is confined in a small volume near the subunit (Fig. 1(b)). The rate of this “intra-molecular” binding reaction can be calculated as a product of the effective concentration of the free lobe in this small volume and the rate of CaM binding of the free lobe (e.g., k_{on}^{N} = (the effective concentration) $\times k_{\text{on}}^{\text{N}}$ for the apo-N lobe, see Fig. 1(a)). The effective concentration is calculated to be 25,000 μM (25 mM) by assuming that the small volume is approximately equal to a half sphere with the radius of gyration of CaM (~ 2 nm) (Seaton et al. 1985). Mori et al. (2004) employed a classical formula developed by Flory (see Cantor and Schimmel 1980) to calculate the (bulk) local concentration of *untethered* CaM in the vicinity of the mouth of the Ca^{2+} channel and obtained an estimated concentration of 2.5 mM. In their experiment, a CaM molecule was attached to the ion channel via a glycine linker. It is not clear if we can directly apply their calculation to our case. Furthermore, we do not know the impact of intra-subunit cooperativity (see Introduction). It is unclear one can directly apply the estimation made by Mori et al. (2004) to the present model. We decided to use 25 mM for the effective concentration and confirmed that the qualitative conclusion of the present work remained unchanged even if the effective concentration is lowered to 1.0–2.5 mM. Finally, we assume that the dissociation of each CaM lobe from CaMKII is independent of the binding status of the opposite lobe. Thus, we are left with 10 unknown parameters for CaM-CaMKII interactions ($= (1 + 4) \times 2$, one unknown association rate and four unknown dissociation rates for each lobe).

In total, we have 26 unknown parameters: 16 (8×2) for Ca^{2+} binding to CaM-CaMKII and 10 (5×2) for CaM binding to CaMKII. We can reduce the number of these unknown parameters to 18 by applying the principle of microscopic reversibility (Colquhoun et al. 2004). In this thermodynamic principle, the product of the rates going clockwise is the same as the product going anticlockwise for any closed reaction pathway. For example, applying this principle to the Ca^{2+} binding cycle to CaM (C-lobe)-CaMKII complex (the bottom diamond shaped cycle in Fig. 1(a)), we arrive at $k_{\text{ck45}} \cdot k_{\text{ck57}} \cdot k_{\text{ck76}} \cdot k_{\text{ck64}} = k_{\text{ck46}} \cdot k_{\text{ck67}} \cdot k_{\text{ck75}} \cdot k_{\text{ck54}}$, which allows one rate constant to be calculated from the other seven. Thus we can reduce the unknown parameters for the target bound Ca^{2+} binding cycle by 2

(1×2). Applying the microscopic reversibility to CaM (lobe)-CaMKII-Ca²⁺ binding cycles (four vertical facets in Fig. 1(a) for each lobe), we can further reduce 6 (3×2) more parameter values. Note the microscopic reversibility of the forth facet is automatically fulfilled if other three are satisfied. The remaining 18 parameter values were optimized using five different sets of experimental data. We describe the experimental data used for the parameter optimization with the simulation results in Section 3.1 and 3.2 (Fig. 2). The numerical algorithm used for optimization is explained in Section 2.6.

2.3 Parameter optimization: Ca²⁺-CaM interaction

Assignment of microscopic kinetic and equilibrium constants for binding Ca²⁺ to the C-domain of CaM is based on several experimental observations and has been published earlier (Putkey et al. 2008). In this section, we reproduce and explain this calculation with a minor modification. First, we measured the macroscopic binding constants for intact CaM (K1 to K4) which were very close to those measured in a classical study by Linse et al. (1991). They reported that $K1=12\text{ }\mu\text{M}$ and $K2=0.4\text{ }\mu\text{M}$ corresponded to binding of the first and second Ca²⁺ ions to the C-domain of CaM.

The microscopic dissociation constants $KIII = kc20/kc02$ and $KIV = kc10/kc01$, correspond to binding the first Ca²⁺ to so-called site III or IV, respectively, while $KIII/IV = kc31/kc13$ and $KIV/III = kc32/kc23$ correspond to binding to the second Ca²⁺ when the other site is already occupied (Fig. 1(a), C-lobe, the upper diamond shape pathway). The macroscopic dissociation constants and microscopic dissociation constants are constrained by the following algebraic relations where c is the coupling factor (Haiech and Kilhoffer 2002):

$$1/K1 = 1/KIII + 1/KIV \quad (1)$$

$$1/(K1 \cdot K2) = c/(KIII \cdot KIV) \quad (2)$$

$$1/KIII/IV = c/KIII \quad (3)$$

$$1/KIV/III = c/KIV \quad (4)$$

In addition, Evenas et al. (1997) used Ca²⁺ binding mutants to show that the relative Ca²⁺ binding affinity of site IV is approximately 6.3-fold greater than site III in both the 0-Ca²⁺ and 1-Ca²⁺ states of the C-lobe. These five algebraic relationships (for five unknown variables) allow for the calculation of microscopic equilibrium binding constants from the macroscopic equilibrium constants. Thus, $KIII = kc20/kc02 \sim 18.5\text{ }\mu\text{M}$, $KIV = kc10/kc01 = 116.4\text{ }\mu\text{M}$, $KIII/IV = kc31/kc13 = 0.34\text{ }\mu\text{M}$, $KIV/III = kc32/kc23 = 0.06\text{ }\mu\text{M}$ and the coupling factor is $c=250$.

The individual microscopic association and dissociation rates are derived as follows. Malmendal et al. (1999) used NMR relaxation methods to show that the first Ca²⁺ bound to the C-domain with a dissociation rate $\sim 5,115\text{ s}^{-1}$, which we assigned as $kc10=5,100\text{ s}^{-1}$. Then, $kc01=275\text{ }\mu\text{M}^{-1}\text{s}^{-1}$. If the 6.3-fold difference in the affinity of binding Ca²⁺ to sites IV and III were due exclusively to the on-rates and there is no change in the off rates, then $kc20=5,100\text{ s}^{-1}$ and $kc02=43.8\text{ }\mu\text{M}^{-1}\text{s}^{-1}$. If it is due exclusively to off-rates and there is no change in the on-rates, then $kc20=32,000\text{ s}^{-1}$ and $kc02=275\text{ }\mu\text{M}^{-1}\text{s}^{-1}$. We chose the second set of parameters for C-lobe as a starting point. Microscopic rate constants for binding the second Ca²⁺ ion were constrained by the fact that the observed dissociation of both Ca²⁺ ions from the C-lobe measured using stopped-flow experiments best fit a single exponential

rate of around $\sim 8.5 \text{ s}^{-1}$. Since the rate of dissociation of Ca^{2+} from the 1- Ca^{2+} state is very fast, then the observed dissociation rate reflects the rate-limiting dissociation of the first Ca^{2+} from the 2- Ca^{2+} state followed by very rapid release of the second Ca^{2+} . This means the observed dissociation rate is the sum of the dissociation rates from the fully Ca^{2+} saturated C-lobe ($kc31+kc32$). Together with Eqs. (1–4), these experimental values allow us to derive all microscopic rates for the C-lobe of CaM.

As for the N-terminal lobe, the available experimental data are limited except for the macroscopic dissociation constants for Ca^{2+} (Putkey et al. 2003). We assume the ratios of microscopic dissociation constants are similar to the C-lobe, $kn10/kn01 = 6.3 \cdot kn20/kn02$. We also assume the binding rate of the first Ca^{2+} is similar to the N-lobe. For the second Ca^{2+} binding, we assume the association rates are $500\sim 500 \mu\text{M}^{-1}\text{s}^{-1}$ ($kn13, kn23$) similar to the C-lobe and close to the diffusion limited binding rate. In addition, we assume the ratio of Ca^{2+} dissociation rates are similar to C-lobe, $kn32/kn31=kc32/kc31$. These assumptions allow us to calculate all microscopic reaction rates for N-lobe.

Finally, we used the Ca^{2+} chelator, quin-2, in the simulations of Fig. 2. Quin-2 tightly binds Ca^{2+} and upon Ca^{2+} binding, changes its fluorescence. The Ca^{2+} association and dissociation rates for quin-2 are $250 \mu\text{M}^{-1}\text{s}^{-1}$ and 35 s^{-1} , respectively (Champeil et al. 1997).

2.4 Steady-state CaM-CaMKII interaction

Several groups measured the steady state binding of CaM to CaMKII using different methods (Meyer et al. 1997; Torok et al. 2001; Gaertner et al. 2004a) and the reported dissociation constant ($45 \text{ nM}\sim 74 \text{ nM}$) was used to validate the model in Fig. 3(a). In the experiment (Gaertner et al. 2004a) analyzed in Fig. 7 (Appendix), 100 nM of CaM-C75-IAEDANS, $500 \mu\text{M}$ Ca^{2+} are mixed with varying concentration of CaMKII. After reaching the steady state, the relative fraction of CaMKII bound CaM was plotted against the free CaMKII concentration in the system. In the experiment, the CaM binding to CaMKII was monitored by the fluorescence signal from CaM-C75-IAEDANS. However, the origin and efficiency of this fluorescence signal is unclear. The fluorescence probe may not report all CaM-CaMKII complexes with 100 % efficiency. See Appendix for more detailed discussion of this issue.

2.5 Modified Gillespie algorithm

We adopted the Gillespie-type stochastic algorithm (Gillespie 1976) to model transitions between all possible internal states of each individual CaM molecule based on the kinetic scheme shown in Fig. 1(A) and parameter values shown in Tables 1 and 2. As stated earlier, we need to keep track of the binding state of each lobe of CaM and CaMKII molecule in the simulation. In a conventional Gillespie algorithm, one keeps track of the number of molecular species in each activation state but not the internal state of each individual molecule. A modification of the Gillespie algorithm is required to extend it to the single molecule level. This extended version of the Gillespie algorithm has been developed and used earlier in Kubota et al. (2007) to analyze the spatio-temporal pattern of Ca^{2+} /CaM activation at a single molecule level. Here we explain the mathematical principle underlying this modification.

Suppose we model a binary association reaction $A+B \xrightarrow{k} C$ with a rate constant k . The propensity of this reaction, defined in a conventional Gillespie scheme, is proportional to the product of the current number of A and B molecules and the reaction probability determined from the macroscopic reaction rate k . The Gillespie algorithm assumes a Poisson (exponential) distribution of the next expected reaction time for this chemical process

(Gillespie 1976). If there are other types of chemical reactions, we calculate the propensity function for those reactions and take the sum of all the propensity functions to calculate the next reaction time step. Which reaction will happen is determined according to the propensity of each chemical reaction in the system: the higher the propensity the more likely the reaction is selected. In fact, the Gillespie algorithm or its equivalent, the Bortz-Kalos-Lebowitz (BKL) (Bortz et al. 1975) algorithm, relies on the fact that the sum of multiple independent Poisson processes becomes one big Poisson process (Fichthorn and Weinberg 1991).

We apply this principle to model individual CaM and CaMKII molecules. The rationale is as follows. In the above example of binary association reactions $A+B \xrightarrow{k} C$, suppose we have n molecules of A (say A_1 to A_n) and m molecules of B . Instead of calculating the total propensity function of this reaction, we can calculate the propensity function for each A_i molecule ($i = 1, \dots, n$) and carry out the Gillespie algorithm as usual. In this particular example, each A_i molecule becomes a C_i molecule if they react with one of the B molecules. One can think of this reaction as a change of the state of the i -th molecule (from the state “A” to “C”) with a probability proportional to the product of the reaction rate k and the number of B molecules. As stated, the resultant algorithm is identical to the original Gillespie scheme but this way we can keep track of each individual A molecule in the system as they react with B molecules. In our algorithm, we calculate individual propensity functions of each chemical reaction involving each individual CaM molecule and/or CaMKII subunit of each holoenzyme, take a sum of all of these propensity functions, and execute Monte Carlo simulations as in the original Gillespie algorithm.

This extended Gillespie-type scheme is as exact as the original Gillespie algorithm. It allows keeping track of the internal state of each Ca^{2+} binding site of each individual lobe of each CaM molecule and the state of each subunit of each CaMKII holoenzyme and modeling all possible cooperative mechanisms discussed in the Introduction. In these stochastic simulations, we use the molecular number and reaction probabilities instead of molecular concentration and reaction rate. In Figs. 4 and 5, we used the volume of a medium sized dendritic spine head ($\sim 0.1 \mu\text{m}^3$) as a reference: in such a small volume, $1 \mu\text{M}$ of any chemical species corresponds to ~ 60 molecules (Franks and Sejnowski 2002). This number is used to convert molecular concentration and reaction rates to molecular number and reaction probabilities.

2.6 Numerical methods

Particle Swarm (Eberhart and Kennedy 1995), a stochastic global optimization method was used to determine values for the 18 free parameters. The details of this optimization method are described in the next section. The objective function used to minimize the error was the absolute difference between the model result and empirical data for the five data sets. During the optimization process, each simulation was averaged up to 50 times to minimize fluctuations during error comparisons. Optimization was performed on a Penguin 16 node cluster with two dual-core Opteron 280s processors per node. All codes for parameter optimization were written in Java (compiled with the 64-bit JDK 1.6.0_10).

As described below, each set of experimental data was fitted with a triple exponential curve (with three time constants and three amplitudes for each data set). The stopped-flow experiment often contains a relatively large noise/error during the first few milliseconds due to mixing artifacts in the dead time of the instrument. We have determined that the “fitted-curve” provides the most reliable and consistent representation of the experimental traces which are the average of five experimental runs for each condition. We have tried single-, double-, triple-, quadratic-or higher multi-exponential curve fits for each set of experimental

data. The triple-exponential curve fit was chosen because it is the simplest model that gives rise to the best fit to all experimental data.

We used these fitted curves, defined by these 30 numerical values, to constrain 18 unknown parameter values. Under the model assumption described above, we have successfully identified a unique region in parameter space to minimize the error function. We took advantage of the efficient multidimensional matrix array computation in the MATLAB environment (The MathWorks, Natick, MA) for simulations in Figs. 3, 4 and 5.

2.7 Particle swarm optimization

Particle Swarm Optimization is a stochastic process where particles cluster around points in the parameter space which minimize an error function (Eberhart and Kennedy 1995). The optimizer is initiated with n ($=20$ by default) particles distributed randomly in the 18-dimensional parameter space (we have 18 unknown parameters). The position (coordinate) of each particle represents values of these 18 parameter values. Each particle contains a current position, velocity and memory of its position where the error function was minimal. At each iteration, every particle's position is updated based upon its current velocity and velocity is updated based on a delta value. The delta value is determined by the following equation:

$$\Delta = \text{Random} * (P_{\text{BEST}} - P_{\text{CURRENT}}) + \text{Random} * (N_{\text{BEST}} - P_{\text{CURRENT}}) \quad (5)$$

Random is a number between 0 and 2, P_{BEST} is the previous best location of the particle, N_{BEST} is the neighboring particle's best position, and P_{CURRENT} is the current location of the particle. This steering behavior causes a particle to swarm about two locations in the parameter space which are known to that particle to minimize the error function. Each particle is assigned two neighbors out of n total particles so multiple local minima can be searched simultaneously.

2.8 3D Single particle trajectory of CaMKII molecules

The time evolution of the Ca^{2+} -CaM binding state of a given CaMKII holoenzyme was taken from the corresponding simulations in Fig. 5. The Brownian motion of this molecule was created using a Monte Carlo scheme. At each time step ($dt=100$ ns), we draw two unit-interval uniform random numbers, r_1 and r_2 which define the direction vector ($\sin\theta\cos\phi$, $\sin\theta\sin\phi$, $\cos\theta$) where $\theta=\pi r_1$ and $\phi=2\pi r_2$, respectively. The mean displacement of a Brownian particle over a time dt is $\sqrt{6Ddt}$, where D ($1\text{ }\mu\text{m}^2\text{s}^{-1}$) is the diffusion coefficient of CaMKII holoenzyme in the cytoplasm (Kim et al. 2004, 2005). A Gaussian distribution (0 mean and $\sqrt{6Ddt}$ variance) was used to determine the jump length (third random number). The size and geometry of the simplified model spine used in our simulations is shown in Fig. 6(b). When a CaMKII molecule collides into the wall (boundary), we impose an elastic collision rule to calculate its trajectory. For this set of simulations, we assume that the diffusion coefficient of CaMKII molecules is independent of its Ca^{2+} -CaM binding state.

3 Parameter optimization

3.1 Ca^{2+} Dissociation from CaM

In the following two sections (3.1 and 3.2), we describe the experimental data that we used to optimize the model parameters. The model with the final parameter values will be shown here with these experimental data (Fig. 2). The first set of experimental data involves the chelator (quin-2) induced Ca^{2+} dissociation from CaM-CaMKII complex (Gaertner et al. 2004b). In this experiment $2\text{ }\mu\text{M}$ of CaM was mixed with varying concentrations of CaMKII

subunit (2, 3, 4, or 6 μM) with saturating amounts of Ca^{2+} (20 μM). After reaching steady state, 150 μM of quin-2 was rapidly mixed to initiate Ca^{2+} dissociation. Quin-2 tightly binds Ca^{2+} with a dissociation constant of 140 nM (Champeil et al. 1997) and upon Ca^{2+} binding, changes its fluorescence. The change in quin-2 fluorescence was measured as an indicator of Ca^{2+} dissociation from the CaM or CaM-CaMKII complex. As stated before, these experiments monitor Ca^{2+} dissociation from CaM with or without bound CaMKII. Note that the dissociation (and re-association) of each lobe from CaMKII also influences the overall kinetics. In other words, all kinetic rates in Fig. 1(a) may have a potential impact on Ca^{2+} dissociation kinetics.

In the simulation, we mixed all molecules (Ca^{2+} , CaM, and CaMKII) except quin-2 and the simulation was run for 5 s until the system reached steady-state. After adding 150 μM of quin-2, we waited 1.6 ms (i.e., the dead time of the stopped flow spectrofluorimeter used for the experiment) and the normalized quin-2 fluorescence change was fitted to the data. The same procedure was used during the parameter optimization. As pointed out above, the quin-2 fluorescence signal reflects a convolution of multiple kinetic pathways. Each experimental data (average of five runs) was fitted to a triple exponential model (Gaertner et al. 2004b). During the parameter optimization, we used this triple exponential curve fit as experimental data. Figure 2(a) shows the goodness of fit of the model using the final optimized parameter values (Tables 1 and 2).

In the simulation, 99% of CaM molecules are fully Ca^{2+} saturated and CaMKII bound after 5 s of simulation before adding quin-2. Within 0.5 s after initiating Ca^{2+} buffering by quin-2, ~85% of the bound Ca^{2+} dissociates from CaM. It is illustrative to go over the possible Ca^{2+} dissociation pathways as shown in Fig. 1(a) with the final parameter set (Tables 1 and 2). The initial Ca^{2+} dissociation in the model may take place from the N-terminal lobe because this shows the highest dissociation rate ($kn75=140.4\text{s}^{-1}$, $kn76=45.8\text{s}^{-1}$) from CaM when it is bound to CaMKII. However, the C-terminal lobe dissociation from CaMKII can happen relatively quickly ($kc73=213.1\text{s}^{-1}$) and Ca^{2+} dissociation from the CaMKII-free C-terminal lobe of CaM then can occur ($kc32=7.1\text{s}^{-1}$). The N-terminal lobe dissociation from CaMKII can also happen relatively quickly ($kn73=24.4\text{s}^{-1}$) and Ca^{2+} dissociation from the CaMKII-free N-terminal lobe of CaM then can occur ($kn32=250\text{s}^{-1}$). These multiple pathways contribute to the Ca^{2+} dissociation which has not been explicitly predicted from previous experimental data.

3.2 Dissociation of CaM-C75-DANS from CaMKII

The second experimental data used was CaM dissociation from CaMKII (Gaertner et al. 2004b). In this experiment, 0.3 μM of CaM-C75-DANS and 0.3 μM of CaMKII subunits were mixed in the presence of saturating amounts of Ca^{2+} (500 μM). After reaching steady state, CaM dissociation was initiated by rapidly mixing with excess amount (15 μM) of unlabeled CaM at saturating Ca^{2+} . The dissociation of CaM from CaMKII was monitored by the decrease in the normalized fluorescence signal from CaM-C75-DANS using stopped-flow fluorimetry. As in Section 3.1, we used a single exponential curve fit to the data (which is the average of five experimental runs) for the parameter optimization. Figure 2(b) shows the goodness of fit of the model with the final parameter sets (the black solid line). Interestingly, the time constants revealed by the single component fit to the experimental data, 1.6s^{-1} , does not correspond to the Ca^{2+} saturated N-lobe dissociation rates from CaMKII ($kn73=24.4\text{s}^{-1}$) nor to the Ca^{2+} saturated C-lobe dissociation rates from CaM-CaMKII complex ($kc73=213.1\text{s}^{-1}$). One possible explanation is that the CaM dissociation may involve multiple dissociation and re-association events of each lobe while the other lobe is still bound to CaMKII. Thus, the dissociation is much slower than expected from the dissociation rate of each lobe from CaMKII. This is related to the molecular brachiation idea proposed by Levin et al. (2002).

4 Cooperative CaM binding to CaMKII

4.1 Steady-state Ca⁴⁺-CaM binding to CaMKII

Having established the model structure and its final parameter values, we test the validity of the model by simulating an overall affinity of α CaMKII for Ca²⁺-saturated CaM. Several groups reported the measurement of this affinity (45 nM~74 nM) using different methods (Meyer et al. 1997; Torok et al. 2001; Gaertner et al. 2004a). In addition, recent experimental data suggested the stoichiometry of binding (the ratio of CaM and CaMKII subunit) is close to 1 (Forest et al. 2008). We have not used this information to constrain the parameters. In order to validate the model parameters, we simulate interaction of CaMKII (100 nM) with varying concentrations of CaM (0.1 nM~1 μ M) in the presence of 500 μ M Ca²⁺. We plot the steady-state level of CaM-bound CaMKII (average of 50 simulation runs) against (calculated) free CaM (Fig. 3(a), the circles). These results were fitted with the Hill

function (of the form $\frac{a_{\max} \cdot [\text{freeCaM}]^h}{k_d^h + [\text{freeCaM}]^h}$) where [free CaM] is the free CaM concentration, h is the Hill coefficient, K_d is the dissociation constant, and $a_{\max}$ is the maximum value, the solid line in Fig. 3(a)). The simulation produced a dissociation constant of ~49 nM and a Hill coefficient of ~1.1. The model accurately reproduced the experimental data (there is one published work regarding this CaM binding stoichiometry that requires additional analyses with simulation; see Appendix for more details).

To test the importance of cooperativity, we have repeated the entire parameter optimization using a (mutant) CaM-CaMKII model in which there is no increased effective concentration of the free lobe while the other lobe is bound to CaMKII. Using this mutant, we have not been able to find a physically plausible parameter set that fits the experimental data of both Figs. 2 and 3 simultaneously. Especially, without cooperativity, the physiological parameter results for the K_d value in Fig. 3 is much (>~10 fold) larger than the model with cooperative lobe interactions. The only parameter set that fits the Fig. 2 data contains Ca²⁺ binding rates larger than the diffusion limited reaction rate and this parameter set still predicts the K_d value (~160 nM), three times larger than the wild type CaM-CaMKII model (see Supplementary Figures 1 and 2). We conclude that the cooperativity is critical to explain the experimental data in Figs. 2 and 3.

4.2 CaM remains bound to CaMKII at physiological Ca²⁺ concentrations

Having established the mechanism of the cooperative CaM binding to CaMKII, we would like to know whether this cooperativity plays any physiological role in the cellular environment. So far, we have analyzed the dynamics of Ca²⁺-CaM-CaMKII interaction in the presence of 20~500 μ M of Ca²⁺ (e.g., Figs. 2 and 3). These saturating *in vitro* conditions are necessary to properly constrain the parameter optimizations to determine the rate constants for the model. We now use this model to make predictions in more physiological ranges of Ca²⁺ concentrations. For example, the basal cytosolic Ca²⁺ concentration is ~50–100 nM and the Ca²⁺ decay time constant in the spine might be slower than the quin-2 induced Ca²⁺ decay in Fig. 2 (Majewska et al. 2000). We predict, under the physiological condition, the Ca²⁺ and CaM dissociation processes would be very different from those observed in the *in vitro* experiments with Ca²⁺ chelator. We next examine the lifetime and the dynamics of CaMKII bound CaM molecules during a slow Ca²⁺ decay process similar to that predicted for the intracellular environment.

In Fig. 4, 20 μ M of CaM and 10 μ M of CaMKII (as subunit) were mixed with 20 μ M of Ca²⁺ and after reaching the steady state (indicated by the vertical arrow at time 0 in Fig. 4 (a–c)), the free Ca²⁺ concentration was reduced to 50 nM, close to the basal Ca²⁺ level in the cytoplasm. We assume that the CA1 pyramidal neuron contains ~20 μ M of CaM and

$\sim 10 \mu\text{M}$ of CaMKII (Kubota et al. 2007). The Ca^{2+} ions released from CaM and CaMKII were cleared from the system with a decay time constant ~ 15 ms until the free Ca^{2+} level reached the basal concentration (50 nM). We chose this value to mimic the Ca^{2+} decay time constant (~ 15 – 50 ms) in the dendritic spine (Majewska et al. 2000) following a brief influx of Ca^{2+} .

In Fig. 4(a), we plot the number of CaM molecules free of CaMKII (black line), only with the N-lobe bound to CaMKII (blue line), only with the C-lobe bound (red line) or when both lobes are bound to CaMKII (green line). The number of CaM molecules with only C-lobe bound to CaMKII (red line) is always smaller than other molecular species. The number of both lobes bound to CaMKII (green line) decreases as CaMKII free CaM (black line) increase as Ca^{2+} is cleared from the system. The number of CaM molecules with only the N-lobe bound to CaMKII (blue line) slightly increases.

Figure 4(b) and (c) show the number of bound Ca^{2+} ions for each lobe (Fig. 4(b) for the N-lobe and c for the C-lobe). As predicted, the fully Ca^{2+} saturated and one Ca^{2+} bound form of CaM decays during this process while apo-lobe (bound to CaMKII) and CaMKII free CaM increase. Note that there is an overshoot of apo-N-lobe bound to CaMKII. The results in Fig. 4(a–c) indicate that, the apo-N-lobe bound to CaMKII persists even at the basal Ca^{2+} concentration ($=50$ nM which correspondes to ~ 3 Ca^{2+} molecules in a spine volume similar to the one in Fig. 6).

Figure 4(d) is a snapshot of a randomly selected single CaM molecule between time $=3.0$ (s) and time $=3.05$ (s) (x-axis) changing its CaMKII binding states. In this figure, the vertical axis represents four possible states of this CaM molecule. State 1 is the CaMKII free state. State 2 is only the N-lobe bound to CaMKII. State 3 is both lobes bound, while state 4 is only the C-lobe bound. What is clear is that the binding of CaM to CaMKII starts with the N-lobe binding in most of cases ($>90\%$). This is because the N-lobe has a higher affinity toward CaMKII than the C-lobe. Once bound, this CaM molecule alternates between State 2~4. The impact from the effective concentration of the free lobe results in multiple binding and rebinding events before this CaM molecule dissociates from CaMKII. This alternating dynamics persists for this CaM molecule beyond the time period shown (3 – 3.05 s) in Fig. 4(d) (data not shown).

In the present work, we focus on the interaction of CaM and unphosphorylated CaMKII but not with phosphorylated CaMKII. CaM remains bound to Thr286 phosphorylated CaMKII for an extended period. This phenomenon (CaM trapping) may well play a significant physiological role; however, Fig. 4 suggests, even without phosphorylation, the lifetime of CaM bound to unphosphorylated CaMKII at basal Ca^{2+} is already significant.

4.3 Life-time and mean cycle time of Ca^{2+} -CaM bound active CaMKII holoenzyme: single molecule level analysis

So far, we have used the stochastic simulation of Ca^{2+} -CaM-CaMKII and analyzed the data in terms of bulk number (or concentration) of molecules in each different binding state. For example, the simulation predicted that the concentration of CaMKII bound apo-N-lobe is $\sim 4.1 \mu\text{M}$ (~ 247 molecules) and is higher than the concentration of CaMKII bound apo-C-lobe $\sim 3.2 \mu\text{M}$ (~ 190 molecules) at basal Ca^{2+} concentrations (Fig. 4(b) and (c), blue lines). At the single molecule level, however, each CaMKII subunit alternates between Ca^{2+} -CaM-bound and Ca^{2+} -CaM-free states. In a small sub-cellular compartment, such as a dendritic spine, a limited number of CaMKII molecules explore the cellular interior to find its downstream target via random walk (Brownian motion). The frequency and lifetime of the Ca^{2+} -CaM bound active state are both critical in determining the spatial aspects of the CaMKII signaling system. The shorter the life time of the Ca^{2+} -CaM bound active state, the

lower the probability that a CaMKII molecule can encounter and phosphorylate its target and the smaller the subcellular space that a single CaMKII holoenzyme can explore in the active state.

Figure 5 calculates this (mean) lifetime of Ca^{2+} -CaM-bound active and inactive states of CaMKII holoenzymes at different steady-state concentrations of free Ca^{2+} . The (mean) lifetime of the Ca^{2+} -CaM bound active state refers to the time duration of a given CaMKII having at least one CaM bound to one of its twelve subunits with at least two Ca^{2+} ions bound to the lobe in the complex with CaMKII. In other words, during this time period, the CaMKII holoenzyme has at least one subunit being active. From now on, we refer to this form as an active state. The binding of at least two Ca^{2+} ions are required for CaM for activation of CaMKII (Shifman et al. 2006; but also see Forest et al. 2008 for an alternative interpretation of Shifman et al. 2006). On the other hand, the inactive state refers to the time interval between two consecutive active states. During this time period, no subunit of a given CaMKII holoenzyme enters the active state. Our simulation algorithm keeps track of the Ca^{2+} , CaM binding state of each subunit of each CaMKII holoenzyme in the system so the calculation of the lifetime is straightforward.

In Fig. 5, we used the same total concentration of CaM (20 μM) and CaMKII (10 μM as subunit) to determine the mean lifetime within the synthetic dendritic spine compartment. We then added different concentrations of Ca^{2+} and ran the simulation until it reached steady state (> 15 s). After reaching steady state, we start calculating the lifetime of the active and inactive states of each CaMKII holoenzyme for another 400 s. At the end of each simulation, we record the Ca^{2+} and CaM binding and unbinding statistics of each CaMKII subunit of each CaMKII holoenzyme and the final steady-state level of free Ca^{2+} . Note even at the lower free Ca^{2+} concentration, a significant amount of Ca^{2+} are already bound to CaM-CaMKII complex. We repeated this simulation experiments 50 times for different total Ca^{2+} concentration and obtained the mean lifetime averaged over the entire CaMKII holoenzyme population. Figure 5(a) is the plot of the lifetime of the active state and Fig. 5(b) is the duration of the interval (lifetime of the inactive state) plotted against the steady-state free Ca^{2+} in the simulation.

Figure 5(a) shows a sharp dependence of the lifetime of the active state on the steady-state free Ca^{2+} concentration. At basal concentration (~ 50 nM), the lifetime of (at least one subunit) active state is ~ 5 ms. In the presence of lower Ca^{2+} , apo-CaM or partially Ca^{2+} saturated CaM lobes quickly dissociate from CaMKII and thus the lifetime of the Ca^{2+} -CaM bound state is shorter than at higher Ca^{2+} concentrations. The lifetime sharply increases as the free Ca^{2+} concentration is raised beyond ~ 0.5 μM . The lifetime of the active state is ~ 0.22 s at 0.5 μM free Ca^{2+} and it reaches ~ 24 s at 0.75 μM free Ca^{2+} . Even though the active state of each subunit may have a limited lifetime, the duration of time (or probability) that all twelve subunits simultaneously reside in an inactive state sharply decrease as the free Ca^{2+} concentration increases. Figure 5(b) illustrates this point and shows the time duration of the inactive state. It starts with ~ 0.065 s at 50 nM free Ca^{2+} concentration and decreases as the free Ca^{2+} concentration increases.

How does this flip-flopping behavior between active and inactive states influence the spatial pattern of CaMKII dynamics? To illustrate this, we generated a trajectory for a CaMKII holoenzyme following simple Brownian motion, alternating between the active state (i.e., at least one subunit active state, drawn in red) and Ca^{2+} -CaM-free states (i.e., all subunits inactive, drawn in blue). The Brownian motion of this molecule was created using a Monte Carlo scheme described in Section 2.8. The diffusion coefficient of CaMKII holoenzyme used here is $1 \mu\text{m}^2\text{s}^{-1}$ (Kim et al. 2004, 2005). To illustrate the flip-flopping dynamics of CaMKII, we take a snapshot of the activation state of CaMKII at every 100 ns. As shown in

Fig. 6(a), we start with the inactive state and plot the location of a given CaMKII holoenzyme in the spine at 100 ns intervals with different colors (blue for all subunits inactive state and red for at least one subunit in its active state). The CaMKII changes its location via diffusion and alternates between these two states (see snapshots at 4, 16, and 400 ms). The time evolution of the Ca^{2+} binding state of this molecule was taken from our simulation in Fig. 5.

Comparing the 3D trajectory of a CaMKII holoenzyme at 50 nM (Fig. 6(a)) and 0.75 μM (Fig. 6(b)) Ca^{2+} concentration, demonstrates that free Ca^{2+} controls the effective range of action of CaM-bound CaMKII. As stated earlier (Fig. 5), the lifetime of the active state dramatically increases as free Ca^{2+} concentration reaches 0.75 μM . In fact, the lifetime of the CaM-bound form at this Ca^{2+} concentration is ~ 24 s and long enough for CaMKII to not only explore the entire dendritic spine but also it can reach the neighboring dendritic shaft and neighboring spines (Supplementary Fig. 3).

5 Discussion

Calmodulin (CaM) is a ubiquitous Ca^{2+} binding protein involved in the regulation of a range of cellular functions. The kinetic dissection of Ca^{2+} -CaM-target interaction has been a focus of numerous experimental and modeling studies (Bayley et al. 1996; Brown et al. 1997; Barth et al. 1998; Black et al. 2006). Experimental technique used to study such an interaction includes atomic scale NMR study to stopped-flow fluorescence spectroscopy as well as macroscopic steady-state analysis for both intact protein and peptides representing the CaM-binding domains of target proteins (Bayley et al. 1996; Brown et al. 1997; Putkey et al. 2008). As for the modeling approach, Black et al. (2006), used an ODE model to study the interaction of individual lobes of CaM with its target and examined target binding induced changes in Ca^{2+} affinity of CaM using IQ-motif proteins. Brown et al. (1997) used a detailed kinetic model and systematically analyzed multiple dissociation pathways for Ca^{2+} -CaM-target (peptide) complex with stopped-flow fluorimetry data.

The modeling scheme presented here differs from previous models of CaM-target interactions in several ways. First, all four Ca^{2+} binding sites and the individual lobes of CaM are explicitly modeled. Second, a stochastic algorithm was used to model CaM and CaMKII at the single molecule level. Third, a dedicated parameter optimization strategy was used to constrain kinetic rate constants in the model from experimental data. The model-based analyses presented here suggest that Ca^{2+} -CaM-CaMKII complex obeys kinetic control mechanism for dissociation similar to Brown et al (1997). However, it has been difficult to predict such complex dissociation processes for CaMKII from experimental measurements (Figs. 2 and 4). Furthermore, the modeling scheme of Brown *et al.* assumes Ca^{2+} ions dissociate as pairs, an assumption that might overlook important kinetic steps involved in Ca^{2+} or CaM dissociation if, for example, there is an alteration in the cooperativity of Ca^{2+} binding in either lobe of CaM when it is bound to a target like CaMKII. Therefore, we have refined the modeling scheme of Brown et al. (1997) by incorporating all Ca^{2+} binding sites and by treating each lobe of CaM as an independent structure. This stochastic modeling scheme allowed us to investigate all dissociation pathways and examine the cooperative binding of the N- and C-lobes of CaM to CaMKII.

Using this stochastic model, we investigated a mechanism of cooperative CaM binding to CaMKII. The model suggests the cooperative binding of CaM lobes to CaMKII is a critical determinant of the overall Ca^{2+} -CaM-CaMKII interaction. The free lobe of CaM is likely to bind CaMKII when the opposing lobe is already bound to the subunit. A mutant CaM which lacks this cooperativity fails to explain the macroscopic binding kinetics of CaM to CaMKII and exhibits dissociation kinetics from CaMKII distinct from wild type CaM (Fig. 3). The

accelerated binding of a free lobe is due to its high effective concentration when the opposing lobe is bound to CaMKII. The simulation and theoretical analyses indicate that a fraction of CaM molecules constantly alternate between partially CaMKII bound (mostly N-lobe bound) and fully CaMKII bound states (Section 3.1). A partially Ca^{2+} saturated (or Ca^{2+} free) and partially CaMKII bound CaM plays an important role in the kinetics of CaM-CaMKII interaction. This cooperativity relies on the bi-lobular structure of CaM and may well apply to other CaM-targets.

Another important finding is that the Ca^{2+} dissociation from CaM, especially from the N-lobe, is significantly reduced when CaMKII is bound. More specifically, CaMKII association disrupts the cooperative Ca^{2+} binding of CaM. In addition, we discovered that the binding affinity of the N-lobe is much higher than the C-lobe. Consequently, at a basal cytosolic Ca^{2+} level (~50 nM), CaM molecules remained bound to CaMKII via N-lobe and alternate between one lobe bound state and both lobes bound state (Fig. 4). If already bound to CaMKII, a CaM molecule can facilitate CaMKII's temporal response to Ca^{2+} signals. Furthermore, the model predicts the range of action of Ca^{2+} -CaM-bound CaMKII sharply depends on the free Ca^{2+} level and can be as large as the entire spine volume within the physiological range of basal Ca^{2+} concentrations (Figs. 5 and 6). Note again this prolonged lifetime of CaM-bound CaMKII observed in Fig. 4 depends on the cooperative binding of the two lobes of CaM and it does not require the CaM trapping previously described for phosphorylated CaMKII. The model suggests that the N-terminal lobe and cooperative binding kinetics to CaMKII plays an important role in the Ca^{2+} -CaM dependent activation of CaMKII in the neuron. It would be also interesting to see how autophosphorylation of CaMKII and phosphatase activity in neurons may influence the range of action of Ca^{2+} -CaM-bound CaMKII and its dependence on the free Ca^{2+} level.

This N-lobe dominated interaction between CaM and (unphosphorylated) CaMKII may have an important implication in the induction of synaptic plasticity. As stated earlier (Section 1, Introduction), a confound for conceptualizing the induction of synaptic plasticity in CA1 pyramidal neurons is how the same molecule, CaM, can lead to two opposing processes as distinct as LTP and LTD. The work presented here indicates a distinctive role for the N-terminal lobe in the CaM-CaMKII interaction. The N-lobe of CaM has a poorer affinity for Ca^{2+} than the C-lobe but its Ca^{2+} association rate and CaMKII binding rate are both higher than the C-lobe. During the brief high-frequency stimuli used to induce LTP there is a steep rising phase of the Ca^{2+} transient of higher amplitude. In this case, the N-lobe will be filled with Ca^{2+} before the C-lobe and it will initiate the subsequent interaction with CaMKII that will lead to the productive activation of CaMKII and LTP induction. The C-lobe requires a longer Ca^{2+} stimulation due to its slow association rate, but not necessarily high amplitude, before it is filled with Ca^{2+} and binds tightly to its target. It would be interesting if the C-terminal lobe turns out to play an equally critical role in LTD induction, e.g., CaM-calcineurin interaction. In other words, there may be unique lobe specific effects that dictate how CaM transmits its activated status to different CaM-dependent targets such as CaMKII and calcineurin.

In fact, this lobe specific interaction of CaM with its target has been well documented in other physiological system. For example, the N-terminal lobe and C-terminal lobe of CaM interact differentially with Ca^{2+} channels (Liang et al. 2003). The N-terminal lobe and C-terminal lobe selectively respond to global and local Ca^{2+} signals of varying magnitudes and frequencies (Dick et al. 2008; Tadross et al. 2008). Through this lobe-specific mechanism, CaM serves both as a Ca^{2+} sensor and as a feedback controller of its binding target, the Ca^{2+} channel, and modulate channel function. The model-based analysis shown here may reveal a similar but distinctive role of CaM in the bidirectional synaptic plasticity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by US National Institutes of Health grants NS26086, NS038310, and GM069611 and Robert A. Welch Foundation Grant AU1144. Y. Kubota also gratefully acknowledges support from the Institutional Training Grant on Neuroplasticity (NS 041226) during the early phase of this work.

References

- Babu YS, Dack JS, Greenhough TJ, Bugg CE, Means AR, Cook WJ. Three-dimensional structure of calmodulin. *Nature*. 1985; 315:37–40. [PubMed: 3990807]
- Barth A, Martin SR, Mayley PM. Specificity and symmetry in the interaction of calmodulin domains with the skeletal muscle light chain kinase target sequence. *Journal of Biological Chemistry*. 1998; 273:2174–2183. [PubMed: 9442059]
- Bayley PM, Findlay WA, Martin SR. Target recognition by calmodulin: dissecting the kinetics and affinity of interaction using short peptide sequences. *Protein Science*. 1996; 5:1215–1228. [PubMed: 8819155]
- Black DJ, Leonard J, Persechini A. Biphasic Ca^{2+} -dependent switching in a calmodulin-IQ domain complex. *Biochemistry*. 2006; 45:6987–6995. [PubMed: 16734434]
- Bortz AA, Kalos MH, Lebowitz JL. A new algorithm for Monte Carlo simulation of Ising Spin Systems. *Journal of Computational Physics*. 1975; 17:10–18.
- Bradshaw JM, Kubota Y, Meyer T, Schulman H. An ultrasensitive Ca^{2+} /calmodulin-dependent protein kinase II-protein phosphatase 1 switch facilitates specificity in postsynaptic calcium signaling. *Proceeding of the National Academy of Science USA*. 2003; 100:10512–10517.
- Brown SE, Martin SR, Bayley PM. Kinetic control of the dissociation pathway of calmodulin-peptide complexes. *Journal of Biological Chemistry*. 1997; 272:3389–3397. [PubMed: 9013581]
- Cantor, CR.; Schimmel, PR. *The Behavior of Biological Macromolecules*. Vol. Part III. San Francisco, CA: Freeman; 1980. *Biophysical Chemistry*.
- Champeil P, Henao F, de Foresta D. Dissociation of Ca^{2+} from sarcoplasmic reticulum Ca^{2+} -ATPase and changes in fluorescence of optically selected Trp residues. Effect of KCL and NaCl and implications for substeps in Ca^{2+} dissociation. *Biochemistry*. 1997; 36:12383–12393. [PubMed: 9315879]
- Colquhoun D, Dowsland KA, Beato M, Plested AJR. How to impose microscopic reversibility in complex reaction mechanism. *Biophysical Journal*. 2004; 66:3510–3518. [PubMed: 15189850]
- Dick IE, Tadross MR, Liang H, Tay LH, Yang W, Yue DT. A modular switch for spatial Ca^{2+} selectivity in the calmodulin regulation of Ca_v channels. *Nature*. 2008; 451:830–835. [PubMed: 18235447]
- Eberhart, RC.; Kennedy, J. A new optimizer using particle swarm theory. Nagoya, Japan. *Proceedings of the Sixth International Symposium on Micro Machine and Human Science*; 1995. p. 39–43.
- Evenas J, Thulin E, Malmendal A, Forsen S, Carlstrom G. NMR studies of the E140Q mutant of the carboxy-terminal domain of calmodulin reveal global conformational exchange in the Ca^{2+} -saturated state. *Biochemistry*. 1997; 36:3448–3457. [PubMed: 9131994]
- Fichthorn KA, Weinberg WH. Theoretical foundations of dynamical Monte Carlo simulations. *Journal of Chemical Physics*. 1991; 95:1090–1096.
- Forest A, Swulius M, Bradshaw M, Gaertner TR, Waxham MN. Role of the N- and C-Lobes of Calmodulin in the Activation of Ca^{2+} /calmodulin-dependent Protein Kinase II. *Biochemistry*. 2008; 47:10587–10599. [PubMed: 18795794]
- Forsen S, Linse S. Cooperativity: over the Hill. *Trends in Biochemical Sciences*. 1995; 20:495–497. [PubMed: 8571449]
- Franks KM, Sejnowski TJ. Complexity of calcium signaling in synaptic spines. *Bioessays*. 2002; 24:1130–1144. [PubMed: 12447978]

- Gaertner TR, Kolodziej SJ, Wang D, Kobayashi R, Koomean JM, Stoops JK, et al. Comparative analyses of the three-dimensional and enzymatic properties of α , β , γ , and δ isoforms Ca^{2+} -calmodulin-dependent protein kinase II. *Journal of Biological Chemistry*. 2004; 279:12484–12494. [PubMed: 14722083]
- Gaertner TR, Putkey JA, Waxham MN. RC3/Neurogranin and Ca^{2+} /calmodulin-dependent protein kinase II produce opposing effects on the affinity of calmodulin for calcium. *Journal of Biological Chemistry*. 2004; 279:39374–39382. [PubMed: 15262982]
- Gillespie DT. A general method for numerically simulating the stochastic time evolution of coupled chemical reactions. *Journal of Computational Physics*. 1976; 22:403–434.
- Haiech J, Kilhoffer MC. Deconvolution of calcium-binding curves. Facts and fantasies. *Methods in Molecular Biology*. 2002; 173:25–42. [PubMed: 11859766]
- Hudmon A, Schulman H. Neuronal Ca^{2+} /calmodulin-dependent protein kinase II: the role of structure and autoregulation in cellular function. *Annual Review of Biochemistry*. 2002; 71:473–510.
- Keller CH, Olwin BB, LaPorte DC, Storm DR. Determination of the free-energy coupling for binding of calcium ions and troponin I to calmodulin. *Biochemistry*. 1982; 21:156–62. [PubMed: 7059575]
- Kim SA, Heinze KG, Waxham MN, Schuille P. Intracellular calmodulin availability accessed with two-photon cross-correlation. *Proceeding of the National Academy of Science USA*. 2004; 101:105–110.
- Kim SA, Heinze KG, Bacia K, Waxham MN, Schuille P. Two-photon cross-correlation analysis of intracellular reactions with variable stoichiometry. *Biophysical Journal*. 2005; 88:4319–4336. [PubMed: 15792970]
- Kubota Y, Putkey JA, Waxham MN. Neurogranin controls the spatiotemporal pattern of postsynaptic Ca^{2+} /CaM signaling. *Biophysical Journal*. 2007; 93:3848–3859. [PubMed: 17704141]
- Levin MD, Shimizu TS, Bray D. Binding and diffusion of CheR molecules within a cluster of membrane receptors. *Biophysical Journal*. 2002; 82:1809–1817. [PubMed: 11916840]
- Liang H, DeMaria CD, Erickson MG, Mori MX, Alseikhan BA, Yue DT. Unified mechanisms of Ca^{2+} regulation across the Ca^{2+} channel family. *Neuron*. 2003; 39:951–960. [PubMed: 12971895]
- Linse S, Helmersson A, Forsen S. Calcium binding to calmodulin and its globular domains. *Journal of Biological Chemistry*. 1991; 266:8050–8054. [PubMed: 1902469]
- Lisman J, Schulman H, Cline H. The molecular basis of CaMKII function in synaptic and behavioral memory. *Nature Reviews Neuroscience*. 2002; 3:175–190.
- Majewska A, Brown E, Ross J, Yuste R. Mechanisms of calcium decay kinetics in hippocampal spines: role of spine calcium pumps and calcium diffusion through the spine neck in biochemical compartmentalization. *Journal of Neuroscience*. 2000; 20:1722–1734. [PubMed: 10684874]
- Malenka RC, Kauer JA, Perkel DJ, Mauk MD, Kelly PT, Nicoll RA, et al. An essential role for postsynaptic calmodulin and protein kinase activity in long-term potentiation. *Nature*. 1989; 340:554–557. [PubMed: 2549423]
- Malmendal A, Evenas J, Forsen S, Akke M. Structural dynamics in the C-terminal domain of calmodulin at low calcium levels. *Journal of Molecular Biology*. 1999; 293:883–899. [PubMed: 10543974]
- Meyer T, Hanson PI, Stryer L, Schulman H. Calmodulin trapping by calcium-calmodulin-dependent protein kinase. *Science*. 1997; 256:1199–1202. [PubMed: 1317063]
- Mori MX, Erickson MG, Yue DT. Functional stoichiometry and local enrichment of calmodulin interacting with Ca^{2+} channels. *Science*. 2004; 304:432–435. [PubMed: 15087548]
- Mulkey RM, Endo S, Shenolikar S, Malenka RC. Involvement of a calcineurin/inhibitor-1 phosphatase cascade in hippocampal long-term depression. *Nature*. 1994; 369:486–488. [PubMed: 7515479]
- Olwin BB, Keller CH, Storm DR. Interaction of a fluorescent N-dansylaziridine derivative of troponin I with calmodulin in the absence and presence of calcium. *Biochemistry*. 1982; 21:5669–5675. [PubMed: 6293554]
- Putkey JA, Kleerekoper Q, Gaertner TR, Waxham MN. A new role for IQ motif protein in regulating calmodulin function. *Journal of Biological Chemistry*. 2003; 278:49667–49670. [PubMed: 14551202]

- Putkey JA, Waxham MN, Gaertner TR, Brewer K, Goldsmith M, Kubota Y, et al. Acidic/IQ motif regulator of calmodulin. *Journal of Biological Chemistry*. 2008; 283:1401–1410. [PubMed: 17991744]
- Rosenberg OS, Deindl S, Sung RJ, Nairn AC, Kuriyan J. Structure of the autoinhibited kinase domain of CaMKII and SAXS analysis of the holoenzyme. *Cell*. 2005; 123:849–860. [PubMed: 16325579]
- Schuhmeier RP, Dietze B, Ursu F, Lehmann-Horn F, Melzer W. Voltage-activated calcium signals in myotubes loaded with high concentrations of EGTA. *Biophysical Journal*. 2003; 84:1065–1078. [PubMed: 12547788]
- Seaton BA, Head JF, Engelman DM, Richards FM. Calcium-induced increase in the radius of gyration and maximum dimension of calmodulin measured by small-angle X-ray scattering. *Biochemistry*. 1985; 24:6740–6743. [PubMed: 4074724]
- Shifman JM, Choi MH, Mihalas S, Mayo SL, Kennedy MB. Ca^{2+} /calmodulin-dependent protein kinase II (CaM-KII) is activated by calmodulin with two bound calciums. *Proceeding of the National Academy of Science USA*. 2006; 103:13968–13978.
- Sorensen BR, Shea MA. Interactions between domains of apo calmodulin alter calcium binding and stability. *Biochemistry*. 1998; 37:4244–4253. [PubMed: 9521747]
- Tadross MR, Dick IE, Yue DT. Mechanism of local and global Ca^{2+} sensing by calmodulin in complex with a Ca^{2+} channel. *Cell*. 2008; 133:1228–1240. [PubMed: 18585356]
- Torok K, Tzortzopoulos A, Grabarek Z, Best SL, Thorogate R. Dual effect of ATP in the activation mechanism of brain Ca^{2+} /calmodulin-dependent protein kinase II by Ca^{2+} /calmodulin. *Biochemistry*. 2001; 40:14878–14890. [PubMed: 11732908]
- Xia Z, Storm DR. The role of calmodulin as a signal integrator for synaptic plasticity. *Nature Reviews Neuroscience*. 2005; 6:267–276.

Appendix (Stoichiometry of CaM Binding)

As stated earlier, our recent experimental data (Forest et al. 2008) confirmed that the stoichiometry of CaM binding to CaMKII is close to 1. However, there is experimental data (Gaertner et al. 2004a; Rosenberg et al. 2005) suggesting the Hill coefficient of CaM binding to CaMKII is ~1.9. The data reported by the same group indicates the CaMKII activation requires only one CaM molecule bound to the subunit. The observation by Gaertner et al. (2004a) of Hill coefficient ~1.9 data seems to contradict the CaMKII activation data as well as our recent CaM-CaMKII binding stoichiometry data (Forest et al. 2008). In this Appendix, we propose a possible explanation for these seemingly conflicting observations.

In their experiment, Gaertner et al. (9) mixed 100 nM of CaM-C75-IAEDANS, 500 μM Ca^{2+} , and varying concentration of CaMKII (Section 2.4). After reaching the steady state, the relative fraction of CaMKII-bound CaM was plotted against the free CaMKII concentration in the system. Note, in Fig. 3, the fraction of CaMKII-bound CaM was plotted against the free CaM not the free CaMKII. In order to calculate the free CaMKII concentration, one must accurately measure the actual concentration of CaMKII bound CaM from the fluorescence signals. However, as we pointed out in Section 2.4 the fluorescence signal used in this experiment may not necessarily report all CaMKII-CaM complexes with 100% efficiency.

If the fluorescence signal does not report the accurate concentration of CaMKII bound CaM molecules, then it would result in an over-estimate of free CaMKII concentration, which in turn may potentially lead to a higher Hill coefficient. Figure 7(a) strongly supports this hypothesis. In this figure, we faithfully reproduced the experiment of Gaertner et al. (2004a) and plotted the fraction of CaMKII bound CaM as a function of *real* concentration of free CaMKII in the simulation (the circles). The Hill function fitted to this data (the dotted line) results in the Hill coefficient of ~1 and the dissociation constant of ~40 nM. If, however, we

assume that only 10 % of CaMKII-bound CaM is reported by the fluorescence signal and if the fraction of CaMKII-bound CaM is plotted against the erroneously estimated concentration of free CaMKII (the crosses), the Hill coefficient will become ~2.1 (the Hill function curve fit is represented by the solid line). The resultant dissociation constant is ~65–70 nM. This simulation experiment seems to explain the higher Hill coefficient and higher dissociation constant reported by Gaertner et al (2004a) and by Rosenberg et al. (2005).

In fact, the efficiency of fluorescence signal does not have to be 10%. The efficiency less than 100 % always results in a Hill coefficient larger than 1 and a dissociation constant higher than the expected value. Figure 7(b) illustrates the underlying principle. In this figure, we analyze a simple binding reaction:

in which a fluorescence labeled ligand **A** binds to **X** with a dissociation constant of K_D . The fraction of **A**-bound **X** molecule expressed by the free **A** concentration is

$$\frac{A}{K_D + A} \quad (7)$$

In this and following equations, the symbols for the chemical species, e.g., **A**, **X**, and **Y** also denote the concentration of corresponding molecules. The fraction of **X**-bound **A** molecule expressed by the free **X** concentration is

$$\frac{X}{K_D + X} \quad (8)$$

Both of these expressions result in the curves of a Hill coefficient=1 and of the same dissociation constant K_D .

Suppose we assume only a fraction **k** ($0 < k < 1$) of **X**-bound **A** molecule is detected by the fluorescence signal, the steady state concentration of free **A**, **X**, and **Y** obey:

$$X_T = \frac{K_D Y}{A_T - Y} + Y \quad (9)$$

$$X_{estimated} = X_T - kY = \frac{K_D Y}{A_T - Y} + (1-k)Y \quad (10)$$

where X_T and A_T are the total concentrations of molecular species **X** and **A**, respectively. Note $X_{estimated}$ in Eqs (10 and 11 below) is the (erroneously) estimated concentration of the molecular species **X** from the fluorescence signal. Then the fraction of **X**-bound **A** molecule expressed by the free **X** concentration is

$$\frac{2X_{estimated}}{X_{estimated} + K_D + A_T(1-k) + \sqrt{[X_{estimated} + K_D + A_T(1-k)]^2 - 4A_T X_{estimated}(1-k)}} \quad (11)$$

Equation (11) may result in a steady-state curve that fits to a Hill function with a coefficient higher than 1. Without going through further analysis (e.g., asymptotic expansion), Fig. 7(b)

already shows this is the case with a numerical example for $K_D=0.01 \mu\text{M}$ and $k=0.1$. Even for this simple binding reaction, the incorrect estimate of X-bound **A** by fluorescence signal leads to a Hill coefficient ~ 2 and an overestimate of dissociation constant (the crosses in Fig. 7(b) is the plot of Eq. (11) and the solid line is the Hill function fit to the analytical results). The analytical result for Eq. (8) is also shown (the circles and dotted line) for comparison.

Fig. 1.

(a) Kinetic diagram of the model. The reaction scheme of CaM, Ca^{2+} and CaMKII where C- and N-terminal lobes of CaM are treated as independent structures. Each lobe of CaM binds two Ca^{2+} ions while they are in CaMKII-bound or unbound states. CaMKII binding decreases the rate of Ca^{2+} dissociation from CaM. The association rate of each lobe to CaMKII depends on the binding state of the opposing lobe of the same CaM molecule (e.g., k_{n04} and k_{n04k} for apo N-lobe, see Section 2.2). If the opposing lobe is already bound to CaMKII, the association reaction rate is considered as an intra-molecular (=CaM-CaMKII complex) reaction (e.g., k_{n04k}). The reaction rate constants for the C-lobe and N-lobe are summarized in Tables 1 and 2, respectively. (b) A free lobe (in this example, C-terminal lobe) is confined in a small volume (shaded region) when the opposing lobe of the same CaM molecule is bound to CaMKII. We assume the radius of this (half-sphere shaped) shaded region is equal to the radius of gyration of CaM (~ 2 nm)

Fig. 2.

Ca^{2+} chelator induced Ca^{2+} and CaM dissociation from CaMKII. **(a)** Quin-2 induced Ca^{2+} dissociation from the Ca^{2+} -CaM-CaMKII complex. We mix 2 μM of CaM with varying concentrations of CaMKII subunits (2, 3, 4, or 6 μM) in a saturating amount of Ca^{2+} (20 μM) (Gaertner et al. 2004b). After reaching steady state, 150 μM of quin-2 was rapidly mixed to initiate Ca^{2+} dissociation (at time zero). Quin-2 tightly binds Ca^{2+} and upon Ca^{2+} binding, increases its fluorescence. The normalized change in quin-2 fluorescence was plotted as a function of time. The *black solid line* is the experimental data (triple exponential curve fitted to the average of five experimental runs) and the *red solid line* is a simulation run using the parameters established in Tables 1 and 2. **(b)** CaM dissociation from CaMKII. 0.3 μM of CaM-C75-DANS and 0.3 μM of CaMKII subunits were mixed in the presence of saturating amount of Ca^{2+} (500 μM). After reaching steady state, CaM dissociation was initiated by rapidly mixing 15 μM of unlabeled CaM saturated with Ca^{2+} at time zero. The dissociation of CaM from CaMKII was monitored by the normalized fluorescence decreased in signal from CaM-C75-DANS. The *black solid line* is experimental data (single exponential curve fitted to the experimental runs) and the *red solid line* is a simulation using the parameters established in Tables 1 and 2

Fig. 3. Steady-state CaM binding to CaMKII. We mix 100 nM of CaMKII and 500 μM Ca^{2+} with varying concentration of CaM. After reaching steady state, the relative fraction of CaMKII bound CaM was plotted against the free CaM concentration in the system. In the experiment, the CaM binding to CaMKII was monitored by the fluorescence signal from CaM-C75-DANS. The *open circle* is an average of 50 simulation runs and the *solid line* is the Hill function fit

Fig. 4. Dissociation dynamics of Ca^{2+} -CaM from CaMKII. In these simulations, $20\ \mu\text{M}$ of CaM and $10\ \mu\text{M}$ of CaMKII were mixed with $20\ \mu\text{M}$ of Ca^{2+} and after reaching steady state, the free Ca^{2+} concentration was reduced to $50\ \text{nM}$ (the time designated with the *vertical arrows*), close to the basal Ca^{2+} level in the dendritic spine of hippocampal CA1 pyramidal neurons. The Ca^{2+} ions released from CaM and CaMKII were cleared from the system with a decay time constant $\sim 15\ \text{ms}$ until the free Ca^{2+} level reaches the basal concentration ($50\ \text{nM}$). The (average) volume of the dendritic spine is $0.1\sim 0.125\ \mu\text{m}^3$: $1\ \mu\text{M}$ of any chemical species corresponds to ~ 60 molecules. In **(a)** we plot the number of CaM molecules free of CaMKII (*black line*), only the N-lobe bound to CaMKII (*blue line*), only the C-lobe bound to CaMKII (*red line*) or when both lobes are bound to CaMKII (green line). **(b and c)** examine the number of bound Ca^{2+} ions for each lobe (**b** for the N-lobe and **c** for the C-lobe). CaMKII free (black), CaMKII bound but no Ca^{2+} (cyan), CaMKII bound with one Ca^{2+} (magenta), and CaMKII bound with two Ca^{2+} ions (yellow). **(d)** is a snapshot of a randomly selected single CaM molecule between time=3 and time=3.05 (x-axis) alternating between its CaMKII binding states. State 1 is the CaMKII free state, state 2 is only the N-lobe bound, state 3 is both lobes bound and state 4 is only the C-lobe bound

Fig. 5. Mean lifetime of the Ca^{2+} -CaM-bound and the Ca^{2+} -CaM-free states of CaMKII. In these simulations, 20 μM of CaM and 10 μM of CaMKII (as subunit) were mixed with varying concentrations of Ca^{2+} and after reaching steady state (>15 s), the lifetime of the CaMKII holoenzyme (at least one subunit is) in the Ca^{2+} -CaM-bound state **(a)** and (all subunits are) in Ca^{2+} -CaM-free state **(b)** was calculated. The average of the lifetime was calculated over 50 simulations and over all CaMKII holoenzymes in the system and plotted against the free Ca^{2+} (50 nM~1 μM) in the simulation. The inset of **(a)** shows the lifetime of Ca^{2+} -CaM-bound state for lower Ca^{2+} concentrations (50 nM~0.5 μM). Note the steep dependence of the average lifetime on free Ca^{2+} starting at approximately 0.4~0.5 μM

Fig. 6.

Single particle trajectory of a CaMKII molecule alternating between active and inactive states, bounded within a model synaptic spine. The time evolution of a single CaMKII holoenzyme taken from the simulation (Fig. 5) was used to generate a 3D trajectory of its Brownian motion (over 400 ms) in the postsynaptic spine. The intracellular diffusion coefficient of CaMKII was $1 \mu\text{m}^2\text{s}^{-1}$. At every 100 ns, we plot the location of the CaMKII molecule and draw in red when it is in the active state and in blue when inactive. The concentrations of Ca^{2+} are 50 nM (a) and $0.75 \mu\text{M}$ (b). We show snapshots of the trajectory at 4 ms and 16 ms in (a) (two small illustrations on the top) and the entire 400 ms trajectory in (a) and (b). Two snapshots in (a) at 4 ms and 16 ms are to show the intermediate trajectory of a single CaMKII holoenzyme. The spatial dimension of a model synaptic spine is shown in (b). See Supplementary Fig. 3 for a longer (24 s) simulation

Fig. 7.

Stoichiometry of CaM Binding to CaMKII. (a) The steady-state binding kinetics of CaM to CaMKII. In this simulation experiment, 100 nM of CaM-C75-IAEDANS, 500 μM Ca^{2+} are mixed with varying concentration of CaMKII. After reaching the steady state, the relative fraction of CaMKII bound CaM was plotted against the free CaMKII concentration in the system. Note in Fig. 3, the fraction of CaMKII-bound CaM was plotted against the concentration of free CaM but not free CaMKII. The average of 50 simulations (*the circles*) was fitted with the Hill function (*the dotted line*) revealing a Hill coefficient 1 and a dissociation constant of 40 nM. If, however, we assume that only 10 % of CaMKII-bound CaM is reported by the fluorescence signal, the fraction of CaMKII-bound CaM is plotted against the erroneously estimated concentration of free CaMKII (*the crosses*) was best fit by the Hill function with the coefficient of ~ 2.1 (*the solid line*). The resultant dissociation constant is $\sim 65\text{--}70$ nM. **(b)** A simple binding reaction $A + X \rightarrow Y$ can result in a Hill coefficient 2. The analytical result of Eq. (8) (*the open circles*) for this simple binding

reaction was plotted with a Hill function curve fit (the *dotted line* of Hill coefficient 1). In Appendix, we derived an analytic formula for the fraction of X-molecule bound A as a function of erroneously estimated concentration of free X. This analytical result (*the crosses*) was plotted with a Hill function curve fit (of the coefficient 2, *the solid line*)

Table 1**C-Terminal Lobe Parameters**

Parameter	Symbol	Value	Units
Ca ²⁺ binding to CaM	kc01	275.0	$\mu\text{M}^{-1}\text{s}^{-1}$
	kc02	275.0	$\mu\text{M}^{-1}\text{s}^{-1}$
	kc13	3.71	$\mu\text{M}^{-1}\text{s}^{-1}$
	kc23	118.0	$\mu\text{M}^{-1}\text{s}^{-1}$
Ca ²⁺ dissociation from CaM	kc10	5,100.0	s^{-1}
	kc20	32,000.0	s^{-1}
	kc31	1.4	s^{-1}
	kc32	7.1	s^{-1}
Ca ²⁺ binding to CaM/CaMKII	kck45	450.0	$\mu\text{M}^{-1}\text{s}^{-1}$
	kck46	311.9	$\mu\text{M}^{-1}\text{s}^{-1}$
	kck57	9.96	$\mu\text{M}^{-1}\text{s}^{-1}$
	kck67	106.0	$\mu\text{M}^{-1}\text{s}^{-1}$
Ca ²⁺ dissociation from CaM/CaMKII	kck54	710.6	s^{-1}
	kck64	3,520.3	s^{-1}
	kck75	1.34	s^{-1}
	kck76	3,520.3	s^{-1}
CaMKII binding to CaM	kc04	1.0	$\mu\text{M}^{-1}\text{s}^{-1}$
	kc15	1.0	$\mu\text{M}^{-1}\text{s}^{-1}$
	kc26	1.0	$\mu\text{M}^{-1}\text{s}^{-1}$
	kc37	1.0	$\mu\text{M}^{-1}\text{s}^{-1}$
CaMKII dissociation from CaM	kc40	7,008.0	s^{-1}
	kc51	596.8	s^{-1}
	kc62	679.8	s^{-1}
	kc73	213.1	s^{-1}

Table 2**N-Terminal Lobe Parameters**

Parameter	Symbol	Value	Units
Ca ²⁺ binding to CaM	kc01	275.0	$\mu\text{M}^{-1}\text{s}^{-1}$
	kc02	275.0	$\mu\text{M}^{-1}\text{s}^{-1}$
	kc13	507.2	$\mu\text{M}^{-1}\text{s}^{-1}$
	kc23	500.0	$\mu\text{M}^{-1}\text{s}^{-1}$
Ca ²⁺ dissociation from CaM	kc10	9,148.0	s^{-1}
	kc20	63,218.0	s^{-1}
	kc31	1,750.0	s^{-1}
	kc32	250.0	s^{-1}
Ca ²⁺ binding to CaM/CaMKII	kck45	300.0	$\mu\text{M}^{-1}\text{s}^{-1}$
	kck46	517.6	$\mu\text{M}^{-1}\text{s}^{-1}$
	kck57	324.2	$\mu\text{M}^{-1}\text{s}^{-1}$
	kck67	491.3	$\mu\text{M}^{-1}\text{s}^{-1}$
Ca ²⁺ dissociation from CaM/CaMKII	kck54	468.7	s^{-1}
	kck64	3,749.0	s^{-1}
	kck75	140.4	s^{-1}
	kck76	45.8	s^{-1}
CaMKII binding to CaM	kc04	20.0	$\mu\text{M}^{-1}\text{s}^{-1}$
	kc15	20.0	$\mu\text{M}^{-1}\text{s}^{-1}$
	kc26	20.0	$\mu\text{M}^{-1}\text{s}^{-1}$
	kc37	20.0	$\mu\text{M}^{-1}\text{s}^{-1}$
CaMKII dissociation from CaM	kc40	4,138.8	s^{-1}
	kc51	194.4	s^{-1}
	kc62	130.4	s^{-1}
	kc73	24.4	s^{-1}