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Abstract

Calcium/calmodulin-dependent protein kinase II (CaMKII) holoenzymes play a critical role in decoding Ca^{2+} signals in neurons and understanding how this occurs has been the focus of many modeling studies. However,~~.~~ ~~But due to the multi-subunit nature of~~ CaMKII~~, it~~ is notoriously difficult to model and simulate in detail because of its multi-subunit nature and complex activation kinetics. To study the Ca^{2+}-calmodulin-CaMKII reaction network with detailed kinetics ~~as well as~~while including the effect of diffusion, we have customized an existing stochastic particle-based simulator Smoldyn to manage the ~~combinatorial explosion~~ problem of combinatorial explosion of the number of species. With ~~the~~ this new method, spatial and temporal aspects of the signaling network can be studied without compromising ~~the~~ biochemical details. We used this new method to examine~~d~~ how calmodulin molecules, both partially loaded and fully loaded with Ca^{2+}, choose ~~their~~ pathways to interact with and activate CaMKII under various Ca^{2+} input conditions. We found that the frequency dependence of ~~the~~ CaMKII phosphorylation with Ca^{2+} input is not primarily determined by ~~the~~ subunit~~s~~ number, but is intrinsic to the network and can be modulated by the relative amount of Ca^{2+} to calmodulin and by the rate of Ca^{2+} diffusion.

Depending on whether Ca^{2+} ~~amount~~ input is saturating or not, calmodulin molecules could choose different routes within the network to activate CaMKII subunits, resulting in ~~varied~~ different frequency dependence patterns. The findings suggest~~s~~ that particular intracellular environmental factors such as crowding and ~~limited~~ calmodulin availability can play an important role in decoding Ca^{2+} signals and can give rise to distinct CaMKII activation patterns in dendritic spines, Ca^{2+} channel nanodomains. ~~The tiny and confined structures such dendritic spines and Ca^{2+} channels nanodomains can give rise to distinct CaMKII activation patterns from~~ and cytoplasm.

Author summary

Ca^{2+} signals are commonly used by cells for various types of activities. In neurons, Ca^{2+} can regulate gene expression and dendritic spine enlargement, ~~increasing~~strengthen synaptic connectivity, and promote neural growth or even death. These strikingly different cell processes are possible because Ca^{2+} signals of different strengths and temporal patterns are interpreted differently by intracellular signaling pathways. In our study, we focus on one particular pathway that involves calmodulin and Calcium/calmodulin-dependent protein kinase II (CaMKII). We have developed a new computational method that gets around the problem of combinatorial explosion inherent with modeling this pathway in biophysical detail, and this allows us to model this pathway more realistically than conventional methods. ~~Calmodulin is a Ca^{2+} sensor protein, and CaMKII is a macromolecule consists of 12 subunits that interact with calmodulin.~~ Experiments have ~~showed~~ shown that CaMKII activity is sensitive to Ca^{2+} signal frequency and our models demonstrate how

this frequency dependence depends on Ca^{2+} input conditions, calmodulin availability and the Ca^{2+} diffusion rate. ~~We used a computational model to examine the issue and found that interactions between Ca^{2+}, calmodulin, and CaMKII subunits are the dominant factor. Our approach to model and simulate allows to observe more details of the network, therefore more biophysically realistic than conventional methods.~~

Introduction

Calcium/calmodulin-dependent protein kinase II (CaMKII) is an important enzyme widely distributed in the central nervous system and other tissues ~~such as~~including cardiac muscles [1-3]. CaMKII is involved in intracellular Ca^{2+} signaling as an effector of ~~a~~ the Ca^{2+} sensor protein calmodulin (CaM) [4-6]. In neurons, CaMKII molecules are ~~epsecially~~ especially ~~invovled~~ involved in long-term potentiation (LTP), which is our best model for the molecular basis of learning and memory [7]. In spines, activated CaMKII molecules interact with ~~the~~ postsynaptic density proteins, facilitating actins to reorganize, leading to spine enlargement and upregulation of *alpha*-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor numbers [4]. CaMKII molecules can also phosphorylate AMPA receptors to regulate ~~the~~ channel conductance [4,6] or form~~ing~~ complexes with NMDA receptors [5]. In addition to synaptic functions, recent studies~~-~~ suggest that CaMKII in the soma can act as carriers to shuttle Ca^{2+}-CaM into the nucleus [8,9]. Ca^{2+}-CaM molecules are then unloaded in the nucleus and~~,~~ participat~~e~~~~ing~~ in the ~~CaMKIV (~~calcium/calmodulin-dependent protein kinase IV (CaMKIV) cascade to activate

nuclear transcription factors. Therefore CaMKII molecules play a key role in excitation-transcription coupling.

The CaMKII molecule is a holoenzyme that consists of 12 subunits. Each subunit con~~s~~tains an association domain allowing the formation of ~~to form~~ multimers, a regulatory domain with CaM binding sites and phosphorylation sites, and a catalytic domain to act as a kinase. Activities such as CaM binding and auto-phosphorylation result in various subunit states. For example, in the presence of Ca^{2+}-CaM complexes, each subunit can bind a CaM molecule and expose its catalytic domain to phosphorylate the direct neighbor subunit at its Thr286/Thr287 site. Once phosphorylated, the CaM unbinding rate decreases dramatcially, leading to a prolonged activation of the subunit; ~~in other words~~we say~~,~~ the CaM molecule~~s~~ is "trapped" by the CaMKII subunit [10]. Even when the CaM molecule unbinds, the CaMKII subunit stays activated, entering an autonomous state. Finally, Thr305/Thr306 can also be phosphorylated. This theronine site overlaps with the CaM binding site and its phosphorylation blocks the binding of CaM molecules. In this case, the subunit becomes "capped".

Researchers have long been interested in modeling and simulating CaMKII [11-19]. However, the structural complexity and multi-state nature of CaMKII ~~are~~ present a technical challenge. A major problem is combinatorial explosion [20]. One CaMKII subunit has only a countable number of states to consider, but ~~as the~~with 6 or 12 subunits on a holoenzyme~~ subunits number increases~~, the number of combinations of states for a holoenzyme ~~grow rapidly~~becomes extremely large. This is a common problem in systems biology since large proteins usually consist of multiple subunits. Software that adopts a

rule-based approach such as BioNetGen [21] can be used to expand the network based on a set of given reaction rules. But for the CaMKII holoenzyme, using BioNetGen to generate the network is still computationally intensive. After expansion with BioNetGen, a 6-subunit holoenzyme with four states for each subunit contains 700 unique species and 12192 unique reactions. The size of the network grows substantially when more detailed kinetics are involved. For example, each CaM has 4 Ca^{2+}-binding sites, giving rise to 9 distinct binding states; ~~for~~ each state ~~there is a~~has its own distinct kinetics to interact with a CaMKII subunit~~s. Depending on the type of CaM being bound, a~~ Then each CaMKII subunit exhibits a distinct phosphorylation rate depending on the state of CaM being bound. For simplicity, consider a 6-subunit CaMKII holoenzyme~~,~~ E~~e~~ach subunit would potentially have 20 states. Then for the holoenzyme, the total number of unique species reaches over $\frac{6^{20}}{6} = 609.36 \times 10^{12}$~~,~~ ~~which~~ It would be extremely time-consuming and not practical for BioNetGen to generate ~~the~~ this network. To overcome this problem, most ~~of the~~ previous modeling studies of CaMKII simplified the Ca^{2+}-CaM-CaMKII reaction network by either modeling CaMKII as monomers [15,18] or allowing only CaM fully-loaded ~~CaM~~ with Ca^{2+} to interact~~ing~~ with CaMKII subunits [11,13,14,19].

Another complication is related ~~with~~ to the intracellular envir~~r~~ironment. Conventionally, biochemical reactions are modeled in a deterministic manner using Ordinary Differential Equations (ODE). The underlying assumptions are that reactions occur in a spatially homogenous environment~~,~~ reactants are abundant and not subject to stochasticity~~,~~ and molecules diffuse sufficiently fast. The majority of previous modeling studies belong to this category except for a few that are stochastic or hybrid models- [12,15,17]. However,

intracellular space is highly heterogeneous and compartmentalized [22, 23]. Reactions are often restricted ~~in~~ to a small space. Structures such as the cytoskeleton~~s~~, scaffold proteins or endoplasmic reticulum often act as diffusion barriers to slow down molecules. In addition, most~~majority of the~~ previous modeling studies focus on the dendritic spine, where NMDA receptor-channels~~s~~ are the main Ca^{2+} providers.

In the present work, we focus~~ed~~ on the soma ~~area~~ where voltage-dependent Ca^{2+} channels (CaV) provide the Ca^{2+} influx. Specifically, we examined the Ca^{2+}-CaM-CaMKII network activities near ~~the~~ L-type Ca^{2+} channels. This is an area less ~~paid attention to in~~studied by previous model~~sing works~~, but nonethel~~e~~ss ~~it~~ plays a critical role in excitation-transcription coupling [9,24]. ~~In order to cover the kinetic details of the network~~To study this Ca^{2+}-CaM-CaMKII signaling network and deal with the problem of combinatorial explosion, we modified ~~a~~ the published and freely available simulator Smoldyn [25]. ~~to study the Ca^{2+}-CaM-CaMKII signaling network .~~ Smoldyn is a particle-based stochastic simulator and has been used for simulating reaction and diffusion processes in cells. It works well for relatively simple reaction networks but not for holoenzymes such as CaMKII. We added new data structures to describe the reaction network in a compact way. CaMKII holoenzymes are modeled as a collection of subunits. Each subunit has a set of binding sites. Subunits react independently and diffuse collectively. Reactions are defined between binding sites. The reaction network is stored in a hashtable to allow for lookup during simulations only when reactants collide, without expanding and loading a complete network. We tested and verified these modifications ~~using~~ by modeling a Ca^{2+}-CaM interaction network ~~by~~ and comparing results with COPASI [26]. Moreover, we added detailed components to the network and examined the frequency dependence of CaMKII

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activation. ~~As a result, we~~We found that the number of subunits ~~number~~ does not play a significant role in CaMKII activation. Instead, slow diffusion of Ca^{2+} can dramatically boost CaMKII activation. In the presence of a limited ~~amount~~ number of CaM molecules, CaM must make an intricate choice ~~it becomes intricated for CaM to choose~~ between binding ~~a~~ Ca^{2+} first or a CaMKII subunit first. Interestingly, a change in the decision pattern leads to an altered frequency dependence of the network.

Results

Testing of the simulator modifications

We first tested the modifications to Smoldyn used in this paper (see Methods) using a Ca^{2+}-CaM network (Fig [fig1]~~B~~). The 4 Ca^{2+} binding sites on CaM gives rise to a total of 9 different binding states of a CaM molecule. For the reaction volume we use ~~Reactions occur in~~ a 500 × 500 × 500 nm ~~sized~~ cube with all sides being reflective. The size of ~~the~~ this volume ~~compartment~~ mimics~~cks~~ that of a Ca^{2+} channel nanodomain or a small dendritic spine head. Initially, the cube contains 3000 Ca^{2+} ions and 700 apoCaM molecules, equivalent to 39.86 μM and 9.3 μM respectively. The concentrations are chosen to ~~result~~ produce ~~in~~ an observable amount of 4-Ca^{2+} bound CaM at the steady state. All molecules are initially uniformly distributed. We tested reactions using two different Ca^{2+} diffusion constants 2.2 x 10^{-6} cm^2s^{-1} [27] ~~and its~~ half of this value 1.1 x 10^{-6} cm^2s^{-1}~~.~~

We first characterized the reactions in the network to see if results of our stochastic model could reasonably be compared to results with standard ODE methods. A second order

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chemical reaction in the solvent phase consists of two steps: molecules encountering each other by diffusion followed by the molecules reacting with each other~~and reacting~~. If the encountering step takes a much longer time to occur than the reacting step, the reaction is diffusion-limited; otherwise, the reaction is activation-limited. Conventionally, an experimentally measured binding kinetic rate, k_{on}, can be decomposed into an encounter~~ing~~ rate, k_{enc}, and an intrinsic activation rate, k_a, using the following equation (ref)

$$\frac{1}{K_{on}} = \frac{1}{K_{enc}} + \frac{1}{K_a}$$

For diffusion-limited reactions, $K_a \gg K_{on}$ and $k_{on} \approx k_{enc}$; for activation-limited reactions, K_a ~~is close to~~ $\approx K_{on}$ as diffusion is sufficiently fast. Given the diffusion coefficients for Ca^{2+} and calmodulin and the ~~kinetic~~ parameter values for Ca^{2+} and calmodulin reaction kinetics in Table 1~~,~~ we calculated the $\frac{K_a}{K_{on}}$ ratios and concluded that the reactions in the network belong to the activation-limited regime. This was also true if diffusion constants were reduced 10-fold. Thus this simple model resembles a well-mixed system and a standard~~n~~ ODE model will ~~system simulated using COPASI is~~ provide a good test standard.

We ~~set up~~coded a deterministic ODE system in COPASI (ref) and compared results with our stochastic model using the same kinetics ~~as in the simple stochastic model~~. We found that the stochastic model and the ODE COPASI model exhibited similar time courses for all CaM binding state species as~~The time courses of all CaM binding states species are~~ shown in Fig [fig2]. Slowing down Ca^{2+} diffusion ~~does~~ did not significantly alter the time courses for the parameter values used. ~~The stochastic model exhibits consistent time courses as the~~

Comment [wh3]: Should these Ks be lower case ks? They are microscopic rate constants, not dissociation or association constants.

Comment [wh4]: This table should be part of the paper and not in the supplemental material

Comment [wh5]: Is it worth expanding on this to describe when changing kon or kenc would make it an issue? Maybe not.

Comment [wh6]: Need a label for CamN2C2 in the figure

~~ODE system does, and~~ These results suggest ~~therefore verified~~ that our modifications of ~~the simulator~~Smoldyn are working properly.

Analysis of the Ca^{2+}-CaM-CamKII network

We set up a prototype model to identify the major network branches that contribute to the phosphorylation of CaMKII subunits. The prototype model comprises Ca^{2+}-CaM-CaMKII interactions as shown in (Fig [fig3]). In this network diagram molecule species are represented as vertices and reactions are represented as the network edges. CaM molecules and CaMKII holoenzymes with two 6-subunit rings ~~are~~ were initially uniformly distributed in ~~the~~ a 1 µm x 1 µm x 2 µm box. ~~$\SI{1}{\um}\times\SI{1}{\um}\times\SI{2}{\um}$ system~~ . Ca^{2+} ions ~~are~~ were released as a single source from the top 1 µm x 1 µm membrane surface. ~~as a single source.~~ A previously generated input file having a total of 6 Ca^{2+} bursts delivered at 5 Hz ~~Ca^{2+} input file~~ was~~is~~ used to provide Ca^{2+} influx during the simulation (see Methods~~. A period prior to Ca^{2+} release is enforced to allow~~ T~~t~~he system was allowed to ~~reach~~ settle into an~~an~~ equilibrium state ~~before~~ prior to the start of the Ca^{2+} influx ~~starts~~. We ran the simulation ~~simulated the model~~ up to 3 s, recorded the molecule numbers at every 1 ms and logged all reaction events. Using the events log, we counted the accumulated occurrences of each reaction type at every 5 ms, starting from the initial steady-state until the Ca^{2+} ~~trains~~ bursts ~~are~~ were over. ~~In a graph, molecule species can be represented as vertices and reactions can be represented as the network edges.~~ For a particular reaction, the number of occurrences during a time span ~~can be~~is considered ~~as~~ to be the net ~~amount~~ number of molecule state changes along the corresponding edge, i.e., ~~. When counting the events,~~ the

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Comment [wh8]: Is this true? Did you cease counting reaction occurrences once the ca influx stopped?

number of ~~the occurrences of~~ unbinding events ~~are~~ is subtracted from the number of ~~that of the~~ binding events~~, resulting in a net occurrence~~. A negative number means ~~the~~ that unbinding occurs more often than ~~the~~ binding within the given time span.

We analyzed the edges in the Ca^{2+}-CaM-CaMKII network (Fig [fig3~~1~~]). The network consists of three layers. ~~(Fig [fig1]D).~~ The back layer (Layer1) describes interactions between Ca^{2+} and the 9 states of CaM (NxCy, x,y=0,1,2) as shown separately in Fig. 1. The front layer (Layer2) is for reactions of Ca^{2+} with CaM attached to unphosphorylated CaMKII (KNxCy). The middle layer (Layer3) is for reactions of Ca^{2+} with CaM attached to phosphorylated CaMKII~~s~~ (KpNxCy). Starting i~~I~~n Layer1, there are at most 4 possible ways for each CaM species to change state: binding ~~a~~ Ca^{2+} at a C site, binding Ca^{2+} at an N site, binding to an unphosphorylated CaMKII subunit or binding to a phosphorylated CaMKII~~.~~ subunit. However, binding to a phosphorylated CaMKII subunit requires a bound subunit to be phosphorylated first and then lose the bound CaM. This type of reaction rarely occurs during the early stage of the simulation since phosphorylation is slow and CaM unbinding from a phosphorylated subunit is uncommon. Thus we focus on the first 3 types of reactions.

For each CaM state, we counted the accumulated occurrences of the possible reaction types. Results are shown in Fig. 4. The plot ~~revealed~~ reveals a predominant pathway for apoCaM (N0C0) to become fully Ca^{2+} loaded and attached to ~~phosphorylated~~ CaMKII subunits (K~~p~~N2C2). Starting as ~~an~~ apoCaM, a CaM molecule tends to bind a Ca^{2+} ion on the C1 site and then on the C2 site, entering the N0C2 state. CaM in the N0C2 state has a strong preference to enter layer 2 by binding to a CaMKII subunit, after which it quickly binds Ca^{2+}

Comment [wh9]: It might be helpful to put a title above each graph saying starting at N0C0, starting at N0C1, etc.

Comment [wh10]: Nowhere in this figure is any indication of subunits being phsophosylated

ions to the N1 and N2 sites to become KN2C2 (Fig. 4B). Also evident from Fig. 4 is that once CaM has two Ca^{2+} ions on either lobe, the preference is to bind to a CaMKII subunit before binding additional Ca^{2+} ions. Once bound to a CaMKII subunit, additional Ca^{2+} ions bind to CaM more quickly. ~~. Afterwards, the CaM molecule can either enter Layer2 by binding a CaMKII subunit to form KN0C2 or stay in Layer1, continue to bind Ca^{2+} ions on the N sites and enter Layer2 to interact with CaMKII as N1C2 or N2C2.~~ In this scenario, it is rare for CaM to become fully bound with Ca^{2+} ions prior to binding with a CaMKII subunit. Nevertheless, ~~In either case,~~ phosphorylation of CaMKII subunits occurs most often when ~~the~~ subunits are bound with fully loaded CaM, but still often with CaM having C sites loaded, ~~:~~ KN0C2 and~~,~~ KN1C2, as shown in Fig. 4C~~, and N2C2~~. This preferred pathway is consistent with a hypothetical major pathway that leads to CaMKII autophosphorylation shown in Pepke et al. [18]. This observation ~~also agrees~~is also consistent with (refs) in showing that partially loaded CaM molecules ~~are~~ may be very important in activating CaMKII subunits. We note that t~~T~~he way ~~a~~ the predominant pathway is chosen at each ~~vertice~~ vertex is ~~related to~~a consequence of reaction affinities. For example, even though N sites bind Ca^{2+} faster, the C sites have higher affinity making ~~in Layer1, since C sites have higher affinities for Ca^{2+} than N sites,~~ Ca^{2+} binding to C sites ~~are~~ preferred.

To confirm the critical role of NxC2 (x=0,1,2) CaM in activation~~ng~~ and phosphorylation of CaMKII, we set up two modified reaction schemes (Fig [fig3~~1~~]B~~D~~,C~~E~~). In Scheme1, only CaMKII subunits bound with NxC2 are allowed to become phosphorylated. In Scheme2, phos~~so~~phorylation is allowed only for subunits bound with N2Cx. In particular, the phosphorylation rates ~~of~~ in the two schemes are ~~consistent~~equivalent, i.e.. ~~The~~ k_{on} of reaction KpN2C0 is the same as that of KpN0C2, and the k_{on} of KpN2C1 equals that of

Comment [wh11]: Is this an accurate description of what the model shows? If so, a figure with layer 2 reactions needs to be shown.

Comment [wh12]: Again, is this what you see? Figures have to be made to show this. The next comment is old and may no longer be relevant.

Comment [wh13]: You do not show this with data. You do not show phosphorylation. Why should this be true? The bottom plots in your Fig. 3 (my Fig. 4) show high numbers of KN2C0 and KN2C1. It seems easier to bind to CaMKII from N2C0 than to add a C1 or later a C2. Why is this?

Comment [wh14]: See if my latest changes make this comment moot.
This is not phosphorylation is it? Isn't this binding of N2C0 with CaMKII?

This whole section mixes up binding with CaMKII with autophosphorylation. This needs to be cleared up.

KpN1C2. Not surprisingly, the two schemes give rise to different phosphorylation levels as shown in Fig. 5?. Scheme 1 performs slightly worse than the whole network~~,~~ whereas Scheme 2 produces much lower phosphorylation levels. Therefore, edges of the network are not equally ~~invovled~~ involved in CaMKII phosphorylation. Phosphorylation~~s~~ from KNxC2 ~~are~~ is especially important.

CaMKII activation frequency dependence is not primarily determined by CaMKII ~~subunit~~holoenzyme structure~~s~~

A classic experiment by DeKoninick and Schulman [28] showed that *in vitro* CaMKII holoenzyme~~s~~ activation ~~are~~ is sensitive to the frequency of Ca^{2+}-CaM pulses. A recent *in vivo* study ~~*in vivo* also noticed~~ found that glutamate uncaging frequency affects CaMKII activation in dendritic spines [29]. Numerous modeling studies have also reported a dependence of CaMKII activation on the frequency of calcium signals. It has been hypothesized that the observed frequency dependence arises from cooperative autophosphorylation because of the requirement for neighboring subunits to be activated by CaM before autophosphorylation can occur and that this depends on the number of subunits in a holoenzyme. ~~holoenzyme structure requires cooperative phosphorylation between neighboring subunits and thus the structure contributes to the observed frequency dependence.~~ However ~~several others~~some modeling studies [13, 18, 19] suggest that the number of subunits ~~of a~~in a CaMKII holoenzyme does not result in a significant difference in the CaMKII activation pattern, implying that holoenzyme structure ~~therefore~~ should not be related to the frequency dependence.

Comment [wh15]: some combination fo the upper 2 plots in your Fig 4

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Comment [wh18]: Is this an accurate statement?

Comment [wh19]: Requires for what? This sentence needs to be rewritten to be more clear.

Comment [wh20]: (This may be moot now) Tell me, what specifically did these other studies do to reach this conclusion. Were they modeling studies? What did they do?

Comment [wh21]: (This may be moot now) What does this mean?

Since multi-subunit CaMKII has not been modeled in fine detail~~s~~ to investigate frequency dependence, we used the customized simulator to test the effect~~s~~ of subunit~~s~~ number by varying the number of subunits in a ring from 2~~,~~ to 6 to 12. For each subunit number, we used 5 Hz and 10 Hz Ca^{2+} input and simulated 10 trials for each frequency. The Ca^{2+} input files ~~are~~ were generated from neuron models subjected to ~~from a~~ 5 Hz and ~~a~~ 10 Hz theta-burst stimulation ~~voltage file~~ respectively, ~~simulated using a stochastic Ca^{2+} channel model~~ as described in Methods. For a given number of subunit~~s number~~, we consistently observed significantly higher phosphorylation level~~s~~ with 10 Hz than with 5 Hz input (Fig [fig4]B,C). Typically, 10 Hz input allows more accumulation of NxC2 type CaM, leading to more bound CaMKII subunits and thus more phosphorylations (Fig [fig4]A). The number of subunits affects the phosphorylation level but does not significantly contribute to the observed frequency effect(Fig [fig4]C). Specifically, the 2-subunit~~s~~ condition makes it easier for a holoenzyme to propagate phosphorylation between neighbors, thus resulting in the highest phosphorylation level under 10 Hz and 5 Hz. However, the fewer-subunits-easier-propagation advantage is not consistent between the 6-subunits and 12-subunits conditions.

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CaMKII frequency dependence results from Ca^{2+}-CaM-CaMKII interactions

The observed frequency preference for 10 Hz resides in Ca^{2+}-CaM kinetics and can be modulated by slowing down Ca^{2+} diffusion and limiting the total amount of CaM. In the presence of moderate amount of Ca^{2+}, CaM molecules follow the predominant pathway by choosing reaction paths according to affinities. As a result, CaMKII interactions with CaM

and Ca^{2+} binding to CaM are relatively independent. In contrast, when Ca^{2+} influx becomes saturating, the flush of Ca^{2+} quickly takes up the CaM, leaving CaMKII temporarily lack of binding partners. This temporary shortage of CaM results in an alternative pathway decision and ultimately a suboptimal phosphorylation level. Given the same total amount of Ca^{2+}, 10 Hz is easier to saturate CaM in a short time than 5 Hz. To demonstrate the effect of saturating Ca^{2+}, we increased the Ca^{2+} channel number from 16 channels as in the prototype model to allow more Ca^{2+} ions to flow in per action potential (Fig [fig5]). In the mean time, the total Ca^{2+} influx is kept consistent for 5 Hz and 10 Hz. As Ca^{2+} level increases, the network produces more phosphorylated CaMKII subunits, and the phosphorylation level difference between 5 Hz and 10 Hz conditions diminishes. Eventually, the 10 Hz input becomes saturating and the network generates less phosphorylation under 10 Hz than the 5 Hz stimulus (Fig [fig5]F).

We examined the reaction occurrences profile over time on Layer1 edges (Fig [fig6]) and noticed that the reaction pathway choice deviates from the predominant pathway when Ca^{2+} level becomes saturating. This trend can be revealed from the pathway choice for CaM N0C2 (Fig [fig6]A-C). At nonsaturating Ca^{2+} level, N0C2 molecules are more likely to bind with CaMKII (reaction KN0C2) than binding a Ca^{2+} ion on the N1 site (reaction N1). When Ca^{2+} becomes saturating, N0C2 molecules tends to choose N1 binding over reaction KN0C2. This switch of reaction choice is observed for 10 Hz but not for 5 Hz. Likewise, we analyzed the profile of all possible CaMKII-CaM binding reactions. For 5 Hz conditions, binding of N0C2 consistently dominates over other CaMKII-CaM binding reactions, regardless of the amount of Ca^{2+} influx (Fig [fig6]D-F). However for 10 Hz, as Ca^{2+} influx increases, binding of N2C2 (reaction KN2C2) gradually replaces binding of N0C2 (reaction KN0C2) and becomes

the dominant choice for CaM to enter Layer2 (Fig [fig6]G-I). In breif, this switch of dominant pathway occurs for 10 Hz, correlating with the suboptimal phosphorylation level observed for 10 Hz.

The saturating Ca^{2+} effects on frequency preference can also be facilitated by slowing Ca^{2+} diffusion and limiting the available CaM in the system (Fig [fig7]). A slowed Ca^{2+} diffusion allows more interactions between Ca^{2+} and CaM, effectively mimicking a saturating level Ca^{2+}. To demonstrate, we set Ca^{2+} diffusion as , which is half of the default speed and does not change the activation-limited regime of the network. Slow diffusion results in a dramatic increase of CaMKII phosphorylation regardless of Ca^{2+} channels and input frequencies. Also, the frequency preference for 10 Hz is reversed at 3 × of 6 × Ca^{2+} influx condition(Fig [fig7]C,D). In other words, with slow diffusion, Ca^{2+} influx provided by 3 × becomes sufficiently saturating to change the frequency preference. On the other hand, the same effect can be artificially created by limiting the total amount of CaM in the system (Fig [fig7]A,B). To demonstrate, we compared simulations using 3.5 × Ca^{2+} input with the default amount of CaM () and limited CaM (). Even though the Ca^{2+} amount does not change, by limiting CaM we noticed a reverse of frequency preference from 10 Hz to 5 Hz. It is also worth noting is that by combining slow diffusion and limited CaM, reversing the frequency preference is possible with merely twice amount of Ca^{2+} influx (Fig [fig7]E,F).

CaM changing dominant pathway can be demonstrated using a reduced network

To better understand what happens during the process, we used a reduced reaction network to capture the observed frequency prefrence reversion (Fig [fig8]B). The network is derived from the initial steps in the whole Ca^{2+}-CaM-CaMKII network. It is simple enough to be simulated using the original Smoldyn. We used 5 pulses of instantaneous Ca^{2+} release as the input instead of realistic Ca^{2+} influx and varied the amount of Ca^{2+} influx per pulse. The CaMKII molecules are modeled as monomers with a phosphorylation rate of . The 10 Hz stimulus becomes saturating and fails to generates more phosphorylation than 5 Hz when Ca^{2+} amount reaches 40000 ions per pulse. We separated the reduced network into two paths based on the sequence of Ca^{2+}-CaM-CaMKII binding (Fig [fig8]A). Simulations were carried out using each path alone to compare with the reduced network. When Ca^{2+} amount is not saturating, the reduced network shows a comparable phosphorylation level with that of Path2 (Fig [fig7]B,C). This pathway choice is consistent with the "follow-the-affinity" principle. Also the phosphorylation level increases linearly with the Ca^{2+} input amount. However when Ca^{2+} becomes saturating, the growth of phosphorylation level with incrasing Ca^{2+} amount slows down for both 10 Hz and 5 Hz (Fig [fig8]A). But the trend is considerable for 10 Hz and only slight for 5 Hz. Such an effect is mostly contributed from Path2 (Fig [fig8]B). When Path2 alone is present, the phosphorylation level as function of increasing Ca^{2+} input shows a faster increase for 5 Hz than 10 Hz.

The two paths branch from the node N0C2. That is, CaM N0C2 molecules need to make choices for binding parterners. How choices are made depend on the amount of each

binding partner. When Ca^{2+} amount is scarce, Path2 dominates; when Ca^{2+} becomes saturating, Path1 competes with Path2. Hence inherently, there is a competition invovled between the two paths, which can alter the time course of N0C2 and eventually the phosphorylation level. To visualize the competition, we compared the time courses of N0C2 in the reduced network and those when Path1 or Path2 are recruited alone. Hypotheticall, when competitions exhist, the N0C2 molecules are in high demand. Hence the consumption of N0C2 increases when both Path1 and Path2 are present. In other words, the amount of N0C2 decreases more than when either Path is present alone. This is indeed the case when we examined the N0C2 amount in nonsaturating (10000 ions per pulse, Fig [fig8]D) and saturating Ca^{2+} influx (50000 ions per pulse, Fig [fig8]E). In the nonsaturating case, the time course of N0C2 of the reduced network matches well with that of the Path2 alone condition. N0C2 molecules in Path2 are not affected by the presence of Path 1. In contrast, in the saturating case, N0C2 amount becomes considerably lower when both paths are recruited together than when either path is present alone. This implies that the two paths negatively affect each other. Hence confirms the competition.

Discussion

In the study, we presented an efficient approach to simulate multi-subunit molecules and reaction network in detailed kinetics, as well as the effects of diffusion on the reaction network. We also introduced an intuitive approach to analyzing the pathway choices of a network based on the reaction history of a simulation, allowing us to grasp insights of a large network quickly.

Our findings agree with observations from previous studies. First, under physiological conditions when Ca^{2+} influx is moderate, CaM molecules partially loaded with Ca^{2+} are important for CaMKII activation. In particular, the pathway through N0C1, N0C2, and KN0C2 plays a crucial role. Second, when studying the effects of Ca^{2+} input frequency, we noticed that the number of subunits does not significantly affect the patterns of CaMKII. The mechanism should reside within the network kinetics. These notions are consistent with previous modeling studies . Third, CaM availablility is factor for CaMKII frequency response, as suggested in . Given that free diffusible CaM molecules are limited *in vivo* , this conclusion further implies the regulatory role of of many endogenous CaM binding proteins . Fourth, factors such as crowding and spatial homogeneity can slow down molecule diffusion and thus may have substantial effects on CaMKII phosphorylation. In our simulations, Ca^{2+} diffusion slowed down by half could lead to a dramatic increase of phosphorylation level. A similar effect of slow diffusion on CaMKII activation has also been shown in a spine model . In addition, a recent biophysical study suggests that the diffusion of Ca^{2+} ions can be reduced by ten times in a nanodomain around the Ca^{2+} channel mouth.

Our model does not contain Thr305/Thr306 phosphorylation and some newly discovered mechanisms of CaMKII, which may explain the special multi-subunit strucutre of the holoenzyme. For example, it has been found that there exists a compact autoinhibition state, which occurs through dimerization of adjacent subunits from top and bottom rings. Once Ca^{2+} bound to a dimerized subunit, the dimer dissembles and the two subunits are released. Another recent study indicated that phosphorylated CaMKII subunits can undergo subunits exchange to faciliate proprogating the activation triggered by Ca^{2+}-CaM .

One technical challenge for particle-based simulation is to handle diffusion-limited reactions, especially in the presence of high molecule concentrations. One recent experimental study estimated that the N sites of CaM are acting very fast to bind Ca^{2+}, much faster than previously cited for CaM-N lobe binding kinetics in modeling studies. These binding kinetics are in the diffusion-limited regime, rendering traditional mass action based methods inaccurate. However for these fast kinetics, there currently lacks an efficient method to simulate. If to simulate with the original Smoldyn algorithm, the simulation time step has to be considerablly reduced to obtain the correct steady state . Another software package using an enhanced Green's Function Algorithm can handle the high concentration diffusion-limited reactions accurately, but it takes an impractically long time to simulate. It is interesting to examine the Ca^{2+}-CaM-CaMKII using the fast kinetics parameters and we anticipate to address this question in future development.

Methods

Simulator modifications

We expanded the molecule data structure in Smoldyn to include complexes, molecules and binding sites. A complex may contain multiple molecules and a molecule may contain multiple binding sites. Reactions are specified between binding sites. Each binding site has binary states. For example, bound is coded as 1 and unbound as 0; phosphorylated as 1 and unphosphorylated as 0. Each molecule has a vector to store the states of binding sites and the vector can be calculated into an integer. All reactions are stored in a hashtable with reactants and binding states as entry keys. As an example, binding reactions involving the N

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and C lobes of a CaM molecule can be coded as in Fig [fig10]A. A CaMKII holoenzyme is modeled as ~~a~~ two 6-subunit~~s~~ ring~~s~~. Each subunit is sep~~a~~~~e~~rated from neighbors at a fixed distance of 8 nm [39] ~~-~~and has a distinct physical location.

In the original version of Smoldyn, each reaction generates new molecules as products and removes reactants. In our case, since one molecule can have multiple binding sites and ~~are~~ is potentially associated with multiple partners, entirely removing a molecule is not practical because other attached molecules would also be affected. Also, removing and generating new molecules makes it difficult to track the reaction history of a molecule. Therefore, during reactions we keep ~~these~~ molecules ~~and~~ but change species identities from reactants to products. Molecules bound together physically overlap and automatically synchronize their locations, with the diffusion coeffi~~e~~cient determined by the dominant molecule. For example, when Ca^{2+} and CaM are bound, Ca^{2+} molecules diffuse with CaM.

Macromolecules usually have multiple binding sites, and sometimes these sites compete for the same ligand. For example, CaM has 4 Ca^{2+} binding sites. Since the N and C sites act independently, the N1 and C1 site~~s~~ compete~~s~~ for ~~binding~~ Ca^{2+} and an apoCaM, N0C0, can become either N1C0 or N0C1, resulting in a branching reaction scheme. Thus a decision process is needed to choose a reaction ~~channel~~ path when a binding event occurs.

As an example, consider the following two reactions

```
rxn1: camN0C0 + ca <-> camN1C0
rxn2: camN0C0 + ca <-> camN0C1
```

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Comment [wh25]: This is not clear

Rxn1 has a forward rate constant k_{f1} and a backward rate constant k_{b1}. Rxn2 has rate constants k_{f2} and k_{b2}. The two reactions can be viewed together as rxn3, which has an overall kinetics k_{f3} and k_{b3}. According to the law of mass action, the following relations hold:

$k_{f3} = k_{f1}+k_{f2}$ and k_{b3} (CamN1C0 + CamN0C1) = k_{b1}CamN1C0 + k_{b2}CamN0C1, so $k_{b3} = pk_{b1} + (1 - p)k_{b2}$

$$\frac{k_{f3}}{k_{b3}} = \frac{k_{f2}}{k_{b2}} + \frac{k_{f1}}{k_{b1}}$$

$$k_{b3} = pk_{b1} + (1-p)k_{b2}$$

where p represents the proportion of (CamN1C0 + CamN0C1) that is CamN1C0 and (1-p) is the proportion that is CamN0C1. in k_{b3} is due to unbinding of camN1C0. ~~Knowing that $\frac{p}{1-p} = \frac{[camN1C0]}{[camN0C1]} = \frac{k_{f1}/k_{b1}}{k_{f2}/k_{b2}}$, we obtain $k_{f3} = k_{f1} + k_{f2}$~~. Smoldyn uses binding radii to implement second order reactions. If two molecules are spatially separated by a distance smaller than the corresponding binding radius, then the reaction proceeds. In Smoldyn, a special algorithm is used to calculate the binding radius, which depends on the kinetic rate constant, simulation time step and total diffusion rate of reactants. In case of a branched binding scheme sharing common reactants, we first calculate a binding radius r_3 based on k_{f3}. If the distance between a molecule pair is smaller than r_3, binding happens. To make a reaction choice, we generated a uniformly distributed random number from 0 to 1. If the number falls in the range $(0, \frac{k_{f1}}{k_{f3}}]$, then rxn1 is chosen; instead if the number falls in the

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range ($\frac{kf_1}{kf_3}$, 1], we pick rxn2. Following this approach, the steady state of the reaction network can be kept consistent with the prediction by the mass action law.

Reaction network

We focus on the interactions among Ca^{2+}, CaM, and CaMKII. We first used a Ca^{2+}-CaM network for testing to confirm that modifications on the simulator are valid. Then we added CaMKII holoenzymes to study the signaling network in detailes (Fig [fig1]C). Our rule-based approach allows reactions to be described at the binding site level and perform simulations without compromising the resolution of chemical kinetics. We set up a cube-shaped model to represent a chunk from a cell body. The cell is assumed as a sphere with radius . The cube has a dimension in width and in depth (Fig [fig9]A). The top surface of the cube is the cell membrane, reflective to all molecules. The four sides are also reflective to prevent molecules from escaping laterally through membrane surface of side compartments. The reflective boundary is essentially equivalent to periodic boundary in terms of conservation of molecules in lateral direction. The bottom surface is partially absorbing to Ca^{2+} ions but reflective to CaM. Reflective to CaM guarantees a steady state initial condition. The partial absorption is built-in in Smoldyn to resemble an unbounded diffusion situation .

Voltage-gated Ca^{2+} channels (presumably L-type CaL) are located on the top surface to provide Ca^{2+} influx. For simplicity, these channels are clustered at the center of the membrane. The channels open and close depending on a time-varying membrane voltage file generated from a neuron model. CaMKII subunits (β-subunits) are uniformly present at

a concentration of . They are also immobilized presumably by attaching to actins . Free diffusible CaM molecules are uniformly distributed at a concentration of . This is consistent with the notion that at the cell resting level free diffusible CaM is considerably limited . Table S1 lists all the reactions with corresponding kinetic parameters involved in the network. Kinetic parameters are integrated from various sources and are adjusted to satisfy microscopic reversibility.

Ca^{2+} input conditions

We used a previously built multi-compartment neuron model to generate the somatic action potential trains at frequencies of 5 Hz and 10 Hz . The action potentials are activated by synaptic stimulations, following a series of presynaptic release of neurotransmitters which was modeled with a probability profile measured in experiments . Presynaptic stimulations activate spine AMPA receptors, elevate the membrane potential and open NMDA receptors, allowing Ca^{2+} ions to enter the spines. Accumulated Ca^{2+} influx further depolarizes the dendrite tree and rapidly propagate toward the soma, initiating somatic action potentials. The pattern of action potentials follows the synaptic stimulation. We varied the intervals of synaptic release to generate action potentials in the form of theta-burst with different inter-bursts intervals, which provide different Ca^{2+} influx frequencies for the signaling network.

The cell level neuron model was constructed using NEURON with a detailed morphology and ion channel conductances. Its membrane voltage output was applied to the Ca^{2+} signaling model (Fig [fig10]A,C). Since L-type Ca^{2+} channels are relatively fast, the membrane potentials are unaffected by the channel activities, and the neuron model and

the signaling model can be safely decoupled. The L-type channels in the signaling model are implemented as single channels with voltage-depdendent stochastic opening and closing. The rates of open and close are functions of membrane voltage and calculated using varialbles of n and τ (Fig [fig9]C) similar to Hudgkin-Huxley equations .

```
CaL{gate==0} <-> CaL[gate=1].
```

Then the following set of equations are used to decribe voltage-gated Ca^{2+} channels:

$$rate_{open}(V) = \frac{n_{\inf}}{\tau_n}$$

$$rate_{close}(V) = \frac{1 - n_{\inf}}{\tau_n}$$

$$n_{\inf} = \frac{1}{1 + exp(\frac{V + V_{half}}{slope})}$$

$$\tau_n = \tau_0 + \frac{4\tau_a\sqrt{\tau_g(1-\tau_g)}}{exp(-D_V(1-\tau_g)k_f/s) + exp(D_V\tau_g k_f/s)}$$

The $rate_{open}$ and $rate_{close}$ are used to calculate conditional probabilities to determine the state of a channel for the next time step in the following way

$$P(C|O) = 1 - exp(-rate_{close}(V)dt)$$

$$P(O|C) = 1 - exp(-rate_{open}(V)dt)$$

$$P(O|O) = 1 - P(C|O)$$

For each channel, at a given time, a probability is calculated based on the membrane voltage to decided whether a channel opens. If it opens, a varying number of Ca^{2+} ions are

generated. To calculate how many ions, we used the following equation to obtain the unitary current for a single channel

$$i_{ca} = -g(V - V_s)\frac{\exp\frac{-(V - V_s)}{RT/zF}}{1 - \exp\frac{-(V - V_s)}{RT/zF}}$$

where g is chosen as and RT/zF equals . A current density is calculated using GHK current equation assuming an extracellular [Ca^{2+}] of and an intracellular concentration of . Since the membrane surface is , the current density is converted to a total current I_{ghk} for the area. Assuming a total number of N channels are present, for each channel $\frac{I_{ghk}}{N}$ should equal i_{ca}. By fitting the two expressions, we obtained a N of 16 and a V_s of (Fig [fig9]B). Using the i_{ca}, the number of ions entered during one time step is calcuated as $\frac{i_{ca}}{2e}dt$, where 2 is the valence of Ca^{2+} and e is the elementary charge. To speed up the simulation, Ca^{2+} influx patterns were generated in a separate simulation using the voltage file (Fig [fig10]B,D). Then the generated Ca^{2+} influx files were fed into the signaling network. To guarantee a consistent amount of total Ca^{2+} for a given set of 5 Hz and 10 Hz voltage files, we generated 40 trials of Ca^{2+} influx files for each frequency and then matched them by total Ca^{2+}.

Conclusion

Supporting information

S1 Table.

The total amount of Ca^{2+} influx of selected files for 5 Hz and 10 Hz.

S2 Table.

Kinetic parameters of all reactions.

S3 Table.

Reaction characterizations.

Acknowledgments

Fig 1. Ca^{2+} and CaM interactions can produce 9 different states of CaM. CaM has two lobes, an N lobe and a C lobe, each of which has 2 Ca^{2+} binding sites. Binding sites on the lobes are designated N1, N2, C1 and C2. The N lobe and C lobe bind Ca^{2+} independently of each other, but Ca^{2+} binding within a lobe is cooperative. Thus site N2 cannot bind Ca^{2+} unless N1 has already bound Ca^{2+} and a similar rule applies to sites C2 and C1. In the figure N0C0 represents apo-CaM (no Ca^{2+} bound), "N2" implies both binding sites N1 and N2 are both bound with Ca^{2+} and "C2" implies similarly that both ~~binding sites~~ C1 and C2 have Ca^{2+} ~~are~~

~~both~~ bound. ~~with Ca^{2+}, while~~ "N1" and "C1" imply only the "N1" site and only the "C1" site have Ca^{2+} bound. Arrows represent the direction of binding reactions.

Fig 2. Test of Smoldyn modifications with a simple Ca^{2+}-CaM reaction network. Plots compare the number of CaM molecules in each of the 9 possible states as computed with the modified stochastic simulator and COPASI. Thick s~~S~~mooth lines are results using COPASI and ~~noisy~~ thin black and red lines are results with the modified simulator for two different values of the calcium diffusion coefficient.

Fig 3. The complete Ca^{2+}-CaM-CaMKII network. A: The layer shaded in blue represents Ca2+-CaM interactions as in Fig. 1. Species are represented by vertices. Edges are labeled to indicate binding reactions. For example, N1 means binding of a Ca^{2+} ion on CaM at the N1 site. KN1 means CaM attached to a CaMKII subunit binds a Ca^{2+} ion on its N1 site. KNxCy means a CaMKII subunit binds a CaM that has x Ca^{2+} ions bound on the N-lobe and y Ca^{2+} ions bound on the C-lobe (x and y range from 0 to 2). KpNxCy means a phosphorylated CaMKII subunit binds a CaM with x and y Ca^{2+} ions bound on the N and C lobes respectively. Red edges mark the preferred pathway. B: Reaction scheme1. From top to bottom, the blue edges on the right represent autophosphorylation of CaMKII bound with CaM in states N0C2, N1C2 and N2C2 respectively. The layer shaded in yellow represents Ca2+-CaM interactions as in Fig. 1. C: Reaction scheme 2. From left to right, the blue edges on the bottom represent autophosphorylation of CaMKII bound with CaM in states N2C0, N2C1

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and N2C2 respectively. The front layer (Layer2) shaded yellow represents interactions between Ca2+ and CaM attached to a CaMKII subunit. Layer3 represents interactions between Ca2+ and CaM attached to a phosphorylated CaMKII subunit.

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Fig 4. Accumulated reaction occurrences over time for the nine binding states of CaM (the back layer in Fig. 3). For a CaM binding state, three types of reactions may occur: binding of Ca^{2+} at an N site, binding of Ca^{2+} at a C site or binding of CaM to an unphosphorylated CaMKII subunit. In the figure legends N1 represents Ca^{2+} binding on the N1 site; N2 is Ca^{2+} binding on the N2 site; C1 is Ca^{2+} binding on the C1 site; C2 is Ca^{2+} binding on the C2 site; K-NxCy means binding of CaMNxCy to CaMKII. Green arrows indicate reactions participating in the predominant pathway starting from N0C0.

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Fig 4. A: Time course~~s~~ of bound and phosphorylated CaMKII subunits ~~and CaM molecules~~with ~~when given~~ 5 Hz Ca^{2+} bursts. Ca^{2+} influx generated from 16 channels. `camkii b' means CaM bound CaMKII subunits. `camkii p' means phosphorylated CaMKII subunits. `KN2C2 + KpNxC2' represents CaM molecules bound to either CaMKII or phosphorylated CaMKII and are fully Ca2+ loaded on C sites. B: Time courses of phosphorylated CaMKII subunits for 10 Hz (smooth lines) and 5 Hz input (dashed lines) with the complete network, reaction scheme1 and reaction scheme2 respectively. C: Time courses of phosphorylated CaMKII

subunits for 10 Hz(fast rising) and 5 Hz input (slow rising) when one holoenzyme contains 2, 6 and 12 subunits respectively. D: With 5 Hz input, time courses of molecule number for subunits that are activated themselves (bound or phosphorylated) and together their neighbors. E: The same as in D except that 10 Hz input is used.

Original Fig 1

Fig 1. A: In this example, N1 and N2 are the two sites of N-lobe; C1 and C2 are the two sites of C-lobe. The binding and unbinding rate constants are labeled at the end of reactions. Between lobes reactions are independent. Within each lobe bindings are cooperative. Thus N2 binding cannot happen without N1 being bound; a similar rule applies to C sites. `+' represents a binding reaction; ` ' represents molecules that are bound. `' on the left-hand side specifies the binding sites states of reactants or the conditions for a reaction to occur. On the right-hand side, binding sites involved in the reaction are assigned to new values to change state. Notice the `==` sign on the left-hand side, and the `=` sign on the right-hand side are different. The `==` represents True or False of equality, whereas the `=` denotes an assigning operation. B: Ca2+ and CaM interactions, resulting in 9 different states of CaM. Arrows represent the direction of binding reactions. C: The complete Ca^{2+}-CaM-CaMKII network. The layer in blue represents Ca2+-CaM interactions the same as in figure B. Edges are labeled to indicate binding reactions. For example, N1 means binding of a Ca2+ ion on CaM N1 site. KN1 means CaM attached to a CaMKII subunit binds a Ca2+ ion on N1 site. KNxCy means CaMKII subunit binds a CaM has x Ca2+ ions bound on N-lobe and y Ca2+ on C-lobe, x and y are from 0 to 2. KpNxCy means phosphorylated CaMKII subunit binds a

CaM. Red edges mark the preferred pathway. D: Reaction scheme1. From top to bottom, the blue edges represent autophosphorylations of CaM N0C2, N1C2 and N2C2 respectively. The layer colored yellow represents Ca2+-CaM interactions, the same as in panel B. E. Reaction scheme22. From left to right, the blue edges represent autophosphorylations of CaM N2C0, N2C1 and N2C2 respectively. The front layer(Layer2) colored yellow represents interactions between Ca2+ and CaMKII attached CaM molecules. Layer3 represents interactions between Ca2+ and phosphorylated CaMKII attached CaM molecules.