Simulating detailed Ca^{2+}-calmodulin-CaMKII network in a new way: biophysical attributes that affect CaMKII activation pattern

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Abstract

Calcium/calmodulin-dependent protein kinase II (CaMKII) holoenzymes play a critical role in decoding Ca^{2+} signals in neurons. But due to the multi-subunit nature of CaMKII, it is notoriously difficult to model and simulate. To study the Ca^{2+}-calmodulin-CaMKII network with detailed kinetics as well as the effect of diffusion, we customized an existing stochastic particle-based simulator Smoldyn to manage the combinatorial explosion problem. With the new method, spatial and temporal aspects of the signaling network can be studied without compromising the biochemical details. We examined how calmodulin molecules, both partially loaded and fully loaded with Ca^{2+}, choose their pathways to interact and activate CaMKII under various Ca^{2+} input conditions. We found that the frequency dependence of the CaMKII phosphorylation is not primarily determined by the subunits number, but is intrinsic to the network and can be modulated by the relative amount of Ca^{2+} to calmodulin and Ca^{2+} diffusion. Depending on whether Ca^{2+} amount is saturating or not, calmodulin molecules could choose different routes wthin the network to activate CaMKII subunits, resulting in varied frequency dependence pattern. The finding suggests particular intracellular environment factors such as crowding and limited calmodulin can play an important role in decoding Ca^{2+} signals. The tiny and confined structures such dendritic spines and Ca^{2+} channels nanodomains can give rise to distinct CaMKII activation patterns from cytoplasm.

Author summary

Ca^{2+} signals are commonly used by cells for various types of activities. In neurons, Ca^{2+} can regulate gene expression spine enlargement, increasing synaptic connectivity, 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). Calmodulin is a Ca^{2+} sensor protein, and CaMKII is a macromolecule consists of 12 subunits that interact with calmodulin. Experiments showed that CaMKII activity is sensitive to Ca^{2+} signal frequency. 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 cardiac muscles [1–3]. CaMKII is involved in intracellular Ca^{2+} signaling as an effector of a Ca^{2+} sensor protein calmodulin (CaM) [4–6]. In neurons, CaMKII molecules are epsecially invovled in long-term potentiation (LTP), which is 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 conductance [4, 6] or forming complexes with NMDA receptors [5]. In addition to synaptic functions, recent studies [8, 9] suggest that CaMKII in the soma can act as carriers to shuttle Ca^{2+}-CaM into the nucleus. Ca^{2+}-CaM molecules are unloaded in the nucleus, participating the CaMKIV (calcium/calmodulin-dependent protein kinase IV) cascade to activate nuclear transcription factors. Therefore CaMKII molecules play a key role in excitation-transcription coupling.

CaMKII molecule is a holoenzyme that consists of 12 subunits. Each subunit constains an association domain 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 phosphorylation result in various 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 Thr286/Thr287 site. Once phosphorylated, the CaM unbind rate decreases dramatcially, leading to a prolonged activation of the subunit; in other words, the CaM molecules 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 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 subunits number increases, the combinations of states for a holoenzyme grow rapidly. 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 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 distinct kinetic to interact with CaMKII subunits. Depending on the type of CaM being bound, a CaMKII subunit exhibits distinct phosphorylation rate. For simplicity, consider a 6-subunit CaMKII holoenzyme, each 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 would be extremely time-consuming for BioNetGen to generate the 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 fully-loaded CaM interacting with CaMKII subunits [11, 13, 14, 19].

Another complication is related with intracellular envrionment. Conventionally, biochemical reactions are modeled in a deterministic manner using Ordinary Differential Equations (ODE). The underlying assumptions are reactions occur in a spatially homogenous environment; reactants are abundant and not subject to stochasticity;

molecules diffuse sufficiently fast. The majority 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 a small space. Structures such as cytoskeletons, scaffold proteins or endoplasmic reticulum often act as diffusion barriers to slow down molecules. In addition, majority of the previous modeling studies focus on dendritic spine, where NMDA receptors are the main Ca^{2+} providers.

In the present work, we focused 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 channels. This is an area less paid attention to in previous modeling works, nonethelss it plays a critical role in excitation-transcription coupling [9,24]. In order to cover the kinetic details of the network, we modified a published 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 a Ca^{2+}-CaM interaction network by comparing with COPASI [26]. Moreover, we added detailed components to the network and examined frequency dependence of CaMKII activation. As a result, we found the 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 of CaM molecules, 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 using a Ca^{2+}-CaM network (Fig 1B). The 4 Ca^{2+} binding sites on CaM gives rise to a total of 9 binding states of a CaM molecule.

Reactions occur in a $500 \times 500 \times 500$ nm^3 sized cube with all sides being reflective. The size of the compartment mimicks a Ca^{2+} channel nanodomain or a small spine head. Initially, the cube contains 3000 Ca^{2+} ions and 700 apoCaM molecules, equivalent to $3.986\,71 \times 10^1$ µM and 9.3023 µM respectively. The concentrations are chosen to result 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 $2.2 \times 10^{-6}\,\mathrm{cm^2\,s^{-1}}$ [27] and its half value $1.1 \times 10^{-6}\,\mathrm{cm^2\,s^{-1}}$.

We characterized the reactions in the network. A second order chemical reaction in the solvent phase consists of two steps: encountering and reacting. If the encountering step takes much longer time to occur, the reaction is diffusion-limited; otherwise, the reaction is activation-limited. Conventionally, an experimentally measured binding kinetic rate can be decomposed into an encountering rate and an intrinsic activation rate using the following equation

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

For diffusion-limited reactions, $K_a \gg K_{on}$; for activation-limited reactions, K_a is close to K_{on}. Given the diffusion coefficients and kinetic parameters, we calculated the $\frac{K_a}{K_{on}}$ ratios and concluded that the reactions in the network belong to the activation-limited regime. Thus this simple model resembles a well-mixed system and an ODE system simulated using COPASI is a good test standard. We set up a deterministic ODE system in COPASI using the same kinetics as in the simple stochastic model. The time courses of all CaM binding states species are shown in Fig 2. Slowing down Ca^{2+} diffusion does not significantly alter the time courses. The stochastic model exhibits consistent time courses as the ODE system does, and therefore verified our modifications of the simulator.

Analysis of the 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 (Fig 3). CaM molecules and CaMKII holoenzymes with 6-subunits are initially uniformly distributed in the $1\,\mu\mathrm{m} \times 1\,\mu\mathrm{m} \times 2\,\mu\mathrm{m}$ system. Ca^{2+} ions are released from the membrane surface as a single source. A previously generated 5 Hz Ca^{2+} input file is used to provide Ca^{2+} influx during the simulation. A 1.20×10^2 ms period prior to Ca^{2+} release is enforced to allow the system to reach an equilibrium before the Ca^{2+} influx starts. We simulated the model up to 3 s, recorded

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: Ca^{2+} 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 Ca^{2+}-CaM interactions the same as in figure B. Edges are labeled to indicate binding reactions. For example, N1 means binding of a Ca^{2+} ion on CaM N1 site. KN1 means CaM attached to a CaMKII subunit binds a Ca^{2+} ion on N1 site. KNxCy means CaMKII subunit binds a CaM has x Ca^{2+} 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 Ca^{2+}-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 Ca^{2+} and CaMKII attached CaM molecules. Layer3 represents interactions between Ca^{2+} and phosphorylated CaMKII attached CaM molecules.

Fig 2. Time courses of 9 states of CaM molecules. The smooth lines are simulated using COPASI, and the discontinous lines are from the simple stochastic models with two different diffusion coefficients.

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 1.10×10^2 ms till the Ca^{2+} trains are 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 considered as

the amount of molecule state change along the corresponding edge. When counting the events, the occurrences of unbinding are subtracted from that of the binding, resulting in a net occurrence. A negative number means the unbinding occurs more often than the binding within the given time span.

We analyzed the edges in the Ca^{2+}-CaM-CaMKII network (Fig 1). The network consists of three layers (Fig 1D). The back layer (Layer1) describes interactions between Ca^{2+} and the 9 states of CaM (NxCy, x,y=0,1,2). The front layer (Layer2) is for CaM attached to unphosphorylated CaMKII (KNxCy). The middle layer (Layer3) is for CaM attached to phosphorylated CaMKIIs (KpNxCy). In Layer1, there are at most 4 possible ways for each CaM species to change state: binding a Ca^{2+} at a C site, at an N site, binding to a unphosphorylated CaMKII or a phosphorylated CaMKII. 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.

Fig 3. Accumulated reaction occurrences over time for nine states of CaM molecules. For each type of CaM, three types of reactions can occur: binding of Ca^{2+} at N site, at C site or binding a CaMKII subunit. N1 represents Ca^{2+} binding on the N1 site; N2 means Ca^{2+} binding on the N2 site; C1 means Ca^{2+} binding on the C1 site; C2 means Ca^{2+} binding on the C2 site; K-NxCy means binding between CaMKII and CaM NxCy. Green arrows indicate the path consists of most likely chosen reactions starting from N0C0. Following the green arrows, labeled reaction leads to constructing the predominant pathway.

For each CaM state, we counted the accumulated occurrences of the possible reaction types. The plot revealed a predominant pathway for apoCaM (N0C0) to become fully Ca^{2+} loaded and attached to phosphorylated CaMKII subunits (KpN2C2). Starting as an apoCaM, a CaM molecule tends to bind a Ca^{2+} ion on the C1 site and then on the C2 site. 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 either case, phosphorylation of CaMKII occurs most often the subunits bound with CaM having C sites loaded: N0C2, N1C2, 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 that partially loaded CaM molecules are

important in activating CaMKII subunits. The way a predominant pathway is chosen at each vertice is related to reaction affinities. For example, 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 activating CaMKII, we set up two modified reaction schemes (Fig 1D,E). In Scheme1, only CaMKII subunits bound with NxC2 are allowed to become phosphorylated. In Scheme2, phsophorylation is allowed only for subunits bound with N2Cx. In particular, the phosphorylation rates of the two schemes are consistent. The k_{on} of reaction KN2C0 is the same as that of KN0C2, and the k_{on} of KN2C1 equals that of KN1C2. Not surprisingly, the two schemes give rise to different phosphorylation levels. Scheme 1 performs slightly worse than the whole network; whereas Scheme 2 produces much lower phosphorylation level. Therefore, edges of the network are not equally invovled in CaMKII phosphorylation. Phosphorylations from NxC2 are especially important.

CaMKII frequency dependence is not primarily determined by CaMKII subunits

A classic experiment by DeKonick and Schulman [28] showed that *in vitro* CaMKII holoenzymes activation are sensitive to the frequency of Ca^{2+}-CaM pulses. A recent study *in vivo* also noticed glutate uncaging frequency affects CaMKII activation in spine [29]. It has been hypothesized that the holoenzyme structure requires cooperative phosphorylation between neighboring subunits and thus the structure contributes to the observed frequency dependence. However several other studies [13, 18, 19] suggest that the number of subunits of a holoenzyme does not result in significant difference in CaMKII activation pattern, therefore should not be related to the frequency dependence. Since multi-subunit CaMKII has not been modeled in fine details, we used the customized simulator to test the effects of subunits number by varying the number from 2, 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 generated from a 5 Hz and a 10 Hz theta-burst voltage file respectively, simulated using a stochastic Ca^{2+} channel model as described in Methods.

For a given subunit number, we consistently observed significantly higher phosphorylation level with 10 Hz than 5 Hz (Fig 4B,C). Typically, 10 Hz input allows more accumulation of NxC2 type CaM, leading to more bound CaMKII subunits and thus more phosphorylations (Fig 4A). The number of subunits affects the phosphorylation level but does not significantly contribute to the observed frequency

effect(Fig 4C). Specifically, the 2-subunits 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.

Fig 4. A: Time courses of CaMKII subunits and CaM molecules when given 5 Hz 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 Ca^{2+} 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.

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 5). 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 5F).

Fig 5. A: Using Ca^{2+} input generated with double times $Ca2+^{2+}$ amount, accumulated reaction occurrences for autophosphorylation of CaMKII bound with N0C2, N1C2 and N2C2 respectively. Thick lines are for 10 Hz input; thin lines are for 5 Hz. B-E: The same as in A with Ca^{2+} input generated with 3×. 3.5×, 4× and 6× channels respectively. F: Summary plot of phosphorylation level with 10 Hz and 5 Hz input when various numbers of Ca^{2+} channels are involved in Ca^{2+} influx generation.

We examined the reaction occurrences profile over time on Layer1 edges (Fig 6) 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 6A-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 6D-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 6G-I). In breif, this switch of dominant pathway occurs for 10 Hz, correlating with the suboptimal phosphorylation level observed for 10 Hz.

Fig 6. A-C: Reaction occurrences of N0C0 binding Ca^{2+} at the N1 site and reaction occurrences of N0C0 directly binding with CaMKII. Thick lines are for 10 Hz input, and thin lines are for 5 Hz input. D-F: Reaction occurrences for CaMKII binding with all 9 CaM states. 5 Hz input generated with 1×, 3× and 6× Ca^{2+} influx are used. N0C2 are the dominant CaM state binding with CaMKII. Colored lines are for frequently occurred reactions. Grey lines are for infrequently occurred reactions. G-I: The same as in D-F except that 10 Hz input are used.

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 7). 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 $1.1 \times 10^{-6}\,cm^2\,s^{-1}$, 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 7C,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 7A,B). To demonstrate, we compared simulations using 3.5× Ca^{2+} input with the default amount of CaM (5 µM) and limited CaM (2.5 µM). 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 7E,F).

Fig 7. A: Using 3.5× Ca^{2+} input, accumulated reaction occurrences for autophosphorylation of CaMKII bound with N0C2, N1C2 and N2C2 respectively. Thick lines are for 10 Hz input; thin lines are for 5 Hz. B: The same as in panel A except that the total CaM available in the system is decreased to half amount. C: Reaction occurrences using Ca^{2+} input generated with 3× Ca^{2+} input. D: The same as in C except that Ca^{2+} diffusion is slowed to $1.1 \times 10^{-6}\,cm^2\,s^{-1}$. E: Reaction occurrences using twice Ca^{2+} input. F: The same as in C except with slowed Ca^{2+} diffusion and half amount of CaM.

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 8B). 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 $1\,s^{-1}$. 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 8A). 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 7B,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 8A). But the trend is considerable for 10 Hz and only slight for 5 Hz. Such an effect is mostly contributed from Path2 (Fig 8B). 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.

Fig 8. A: The reduced reaction network derived from the whole network. CaMKII are modeled as monomers and can undergo phosphorylation at an arbitrary rate $1\,s^{-1}$ as long as bound with a CaM N1C2. B: Summary plot of phosphorylation level by varying Ca^{2+} ions amount per pulse from 10000 ions/pulse to 50000 ions/pulse. Dashed lines represent the slow of phosphorylation level growth as a function of Ca^{2+} influx. C: Summary plot of phosphorylation level with the various amount of Ca^{2+} ions delivered at either 5 Hz or 10 Hz. Phosphorylation levels are generated through Path1 or Path2 alone. D: In the presence of non-saturating Ca^{2+}(ca10000), time courses of N0C2 molecules when Path1 or Path2 alone is present, or both are present. E: The same as in D except that in the presence of saturating Ca^{2+}(ca50000).

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 8D) and saturating Ca^{2+} influx (50000 ions per pulse, Fig 8E). 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 [13,18,19]. Third, CaM availablility is factor for CaMKII frequency response, as suggested in [28]. Given that free diffusible CaM molecules are limited *in vivo* [30–32], this conclusion further implies the regulatory role of of many endogenous CaM binding proteins [33]. 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 [15]. In addition, a recent biophysical study [34] 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 [35].

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 [36] 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 [37]. Another software package using an enhanced Green's Function Algorithm [38] 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 and C lobes of a CaM molecule can be coded as in Fig 1A. A CaMKII holoenzyme is modeled as a 6-subunits ring. Each subunit is seperated from neighbors at a fixed distance 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 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 change species identities from reactants to products. Molecules bound together physically overlap and automatically synchronize their locations, with the diffusion coeffecient 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 competes 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 when a binding event occurs.

As an example, consider the following two reactions

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rxn1: camN0C0 + ca <-> camN1C0
rxn2: camN0C0 + ca <-> camN0C1
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Rxn1 has a forward rate constant k_{f1} and a backward rate constant k_{b1}. Rxn2 has 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:

$$\frac{k_{f3}}{k_{b3}} = \frac{k_{f2}}{k_{b2}} + \frac{k_{f1}}{k_{b1}} \tag{2}$$

$$k_{b3} = pk_{b1} + (1-p)k_{b2} \tag{3}$$

where p represents the proportion 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 range $(\frac{k_{f1}}{k_{f3}}, 1]$, 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 1C). 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 5 µm. The cube has a dimension 1 µm in width and 2 µm in depth (Fig 9A). 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 [40].

Fig 9. A: Dimension of the cube-shaped model. Ca^{2+} channels are located on the top surface. B: Find the number channels by fitting the single channel current to the GHK equation. N equals 6 by fitting. C: $n_{inf}(v)$ and $\tau_n(v)$ used in the voltage-gated Ca^{2+} channels.

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 1.0×10^1 µmol. They are also immobilized presumably by attaching to actins [9]. Free diffusible CaM molecules are uniformly distributed at a concentration of 5 µM. This is consistent with the notion that at the cell resting level free diffusible CaM is considerably limited [30, 32, 41]. 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 [42]. 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 [43]. 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 [44] with a detailed morphology and ion channel conductances. Its membrane voltage output was applied to the Ca^{2+} signaling model (Fig 10A,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 9C) similar to Hudgkin-Huxley equations [45].

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CaL{gate==0} <-> CaL[gate=1].
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Then the following set of equations are used to decribe voltage-gated Ca^{2+} channels:

$$rate_{open}(V) = \frac{n_{\inf}}{\tau_n} \tag{4}$$

$$rate_{close}(V) = \frac{1 - n_{\inf}}{\tau_n} \tag{5}$$

$$n_{\inf} = \frac{1}{1 + exp(\frac{V+V_{half}}{slope})} \tag{6}$$

$$\tau_n = \tau_0 + \frac{4\tau_a\sqrt{\tau_g(1-\tau_g)}}{exp(-D_V(1-\tau_g)k_f/s) + exp(D_V\tau_g k_f/s)} \tag{7}$$

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

$$P(C|O) = 1 - exp(-rate_{close}(V)dt) \tag{8}$$

$$P(O|C) = 1 - exp(-rate_{open}(V)dt) \tag{9}$$

$$P(O|O) = 1 - P(C|O) \tag{10}$$

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 [34] to obtain the unitary current for a single channel

$$i_{ca} = -g(V - V_s)\frac{\exp\frac{-(V-V_s)}{RT/zF}}{1 - \exp\frac{-(V-V_s)}{RT/zF}} \tag{11}$$

where g is chosen as 5 pS and RT/zF equals 1.2×10^1 mV. A current density is calculated using GHK current equation [46] assuming an extracellular $[Ca^{2+}]$ of 2 m M and an intracellular concentration of 5.0×10^1 nM. Since the membrane surface is 1 µm, 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 $-1.909\,55$ mV (Fig 9B). 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 10B,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+}.

Fig 10. A: A 5 Hz action potential trains voltage file generated from the NEURON model. B: Ca^{2+} influx generated using the 5 Hz action potentials voltage file. Ca^{2+} influx is stochastic. There are spontaneous firings in the absence of action potentials. C: 10 Hz action potential trains voltage file generated from the NEURON model. D: Ca^{2+} influx generated using the 10 Hz action potential file. Accumulated Ca^{2+} influx for 5Hz, 10 Hz, 8 and 16 channels respectively. The total Ca^{2+} amount is matched between Ca^{2+} influx files.

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

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