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Inter-subunit capture of regulatory segments is a component of cooperative CaMKII activation

Luke H. Chao^{1,†}, Patricia Pellicena^{1,4,†}, Sebastian Deindl¹, Lauren A. Barclay¹, Howard Schulman², and John Kuriyan^{1,3,*}

¹Department of Molecular and Cell Biology, Department of Chemistry, California Institute for Quantitative Biosciences (QB3), Howard Hughes Medical Institute, University of California, Berkeley, CA, 94720 USA

²Allosteros Therapeutics, P.O. Box 743, Palo Alto, CA 94302-0743, USA

³Physical Biosciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA

SUMMARY

The dodecameric holoenzyme of Calcium-calmodulin dependent protein kinase II (CaMKII) responds to high frequency Ca^{2+} pulses to become Ca^{2+} independent. A simple coincidence detector model for Ca^{2+} -frequency dependency assumes non-cooperative activation of kinase domains. We show that activation of CaMKII by Ca^{2+} /calmodulin is cooperative, with a Hill coefficient of ~ 3.0 , implying sequential kinase domain activation beyond dimeric units. We present data for a model in which cooperative activation includes the inter-subunit capture of regulatory segments. Such a “capture” interaction is visualized in a crystal structure that shows extensive interactions between the regulatory segment of one kinase and the catalytic domain of another. These interactions are mimicked by a natural inhibitor of CaMKII. Our results show that a simple coincidence detection model cannot be operative, and point to the importance of kinetic dissection of the frequency response mechanism in future experiments.

INTRODUCTION

Calcium-calmodulin dependent protein kinase II (CaMKII) is unique among the protein kinases because it is known to respond not just to the strength of the activation signal but also to its frequency¹. This property, which is manifested in the ability of CaMKII to escape calcium-dependence at high calcium spike frequency, is likely to underlie the essential role for CaMKII in the strengthening of synaptic connections between neurons by Long Term Potentiation², and in the response to and control of heart rate³. The frequency response of CaMKII relies on the Ca^{2+} /calmodulin-dependent phosphorylation of a specific threonine residue (Thr286 in the mouse α isoform) in one kinase domain by another once a critical spike frequency is crossed, resulting in calcium-independent activity (autonomy)¹. Mutation

*Correspondence: kuriyan@berkeley.edu.

⁴Present Address: Amunix, Inc. 500 Ellis Street, Mountain View, CA 94043, USA

[†]These authors contributed equally to this work

Author Contributions Statement

L.H.C., P.P., H.S. and J.K. designed the experiments. L.H.C., P.P., L.A.B. performed the experiments and analyzed the data. S.D. performed the Hill coefficient analyses. L.H.C. and J.K. wrote the paper.

Accession codes

The coordinates for structures solved in this study were deposited in the Protein Data Bank with ID codes 3KK8 (substrate complex crystal form A), 3KK9 (substrate complex crystal form B), 3KL8 (CaMKIINtide complex).

of Thr286 in CaMKII has dramatic effects, most notably the impairment of spatial learning in mice that bear this mutation^{4,5}.

Another unique feature of CaMKII is its assembly into large symmetrical holoenzymes in which twelve kinase domains are tightly packed around a central ring-shaped scaffold^{6,8}. This raises a conceptual challenge in understanding how the enzyme is regulated. Since transphosphorylation of Thr286 is a key step in the activation process, it is difficult to understand how uncontrolled phosphorylation is prevented in such a holoenzyme assembly, which concentrates kinase domains to an extraordinary extent. One model for frequency decoding posits that CaMKII is a “coincidence detector”, in which the activation of kinase domains within a holoenzyme ring occurs stochastically as calcium levels rise⁹. Since adjacent kinase domains need to bind Ca²⁺/calmodulin in order to switch on^{10,11}, the time required for this to happen would be linked to the onset of transphosphorylation and the consequent acquisition of calcium independence (see Fig. 1a)¹²⁻¹⁵.

The simplest form of a coincidence detection model, illustrated in Figure 1a, is based on the fact that isolated and autoinhibited CaMKII kinase domains, separated from the holoenzyme assembly, bind to Ca²⁺/calmodulin in a 1:1 stoichiometry and are activated without cooperativity. It has also been observed, in studies using a particular synthetic peptide substrate known as autocamtide, that the activation of the holoenzyme by Ca²⁺/calmodulin occurs without apparent cooperativity¹⁶. This result was interpreted to mean that Ca²⁺/calmodulin binding to one kinase subunit within a holoenzyme is independent of binding to other subunits. The uncorrelated binding of Ca²⁺/calmodulin to the holoenzyme is the essential feature of this model that introduces the time delay before transphosphorylation can occur (Fig. 1a).

Despite the elegance of this model, other findings concerning CaMKII make it difficult to understand how such a coincidence detection mechanism might actually work. The binding of calcium-saturated calmodulin to the CaMKII holoenzyme shows clear evidence for positive cooperativity^{7,8}. This suggests that the binding of one molecule of Ca²⁺/calmodulin to a kinase subunit, which is expected to activate it, also potentiates adjacent kinase subunits for binding to Ca²⁺/calmodulin (Fig. 1b). A crystal structure of the autoinhibited kinase domain of CaMKII revealed that the autoinhibitory segment, which contains the Ca²⁺/calmodulin binding site, forms an antiparallel coiled coil within dimeric kinase domains⁸. The formation of autoinhibited dimers within the holoenzyme would explain why Ca²⁺/calmodulin binds cooperatively, because the first binding event would necessarily disrupt the coiled coil formed by the autoinhibitory segment and thereby increase the binding affinity of the holoenzyme for a second Ca²⁺/calmodulin (Figure 1b). These studies did not analyze the cooperativity of kinase activation by Ca²⁺/calmodulin, but because kinase activation results from Ca²⁺/calmodulin binding, it follows that activation should also be cooperative with respect to Ca²⁺/calmodulin. The acquisition of autonomy (i.e., the phosphorylation of Thr286 following activation) is indeed cooperative with respect to Ca²⁺/calmodulin¹, which is also difficult to understand in terms of the simplest coincidence detector model.

In this paper we reexamine the cooperativity of CaMKII activation by Ca²⁺/calmodulin by measuring the activity of the holoenzyme for two peptide substrates. One of these is autocamtide, which was used in the previous studies and is based on the sequence of a portion of the regulatory segment of CaMKII¹⁷. The other, referred to as syntide, is based on the sequence of a CaMKII phosphorylation site in glycogen synthase¹⁸. Our measurements using these substrates show that both the enzyme from *Caenorhabditis elegans* and the mammalian γ isoform are activated cooperatively by Ca²⁺/calmodulin. The Hill coefficients that we obtain from these measurements are as high as 4.3, depending on the construct used,

suggesting that the binding of the first Ca^{2+} /calmodulin molecule to one kinase subunit potentiates several other kinase subunits for Ca^{2+} /calmodulin binding (Figure 1c). Thus, binding and activation follow the same pattern, and the CaMKII subunits do not behave independently. Based on these results, CaMKII is unlikely to function as a simple coincidence detector.

Why did the earlier reports conclude that activation of CaMKII by autocamtide was not cooperative? We find that although both autocamtide and syntide show cooperative activation, the observed degree of cooperativity is lower for autocamtide. Since this peptide is based on the regulatory segment of CaMKII, we turned to an analysis of the role that this segment might play in the cooperativity of the activation process. We have determined a new crystal structure of a truncated form of CaMKII in which the regulatory segment of one kinase (the substrate, bearing the critical Thr286 residue) is bound at the active site of the other (the enzyme). This structure shows that the regulatory segment from the substrate interacts extensively with the kinase domain of the enzyme. This raises the possibility that Ca^{2+} /calmodulin-bound subunits with open active sites can capture the regulatory segments of adjacent autoinhibited kinase domains, thereby causing the activation to “spread” by potentiating the sequential binding of additional Ca^{2+} /calmodulin, in a manner extending beyond dimeric units (Fig. 1d). We present biochemical evidence that supports a role for such a substrate-capture mechanism in increasing the cooperativity of Ca^{2+} /calmodulin binding to CaMKII. The reduction of apparent cooperativity that is seen with autocamtide might arise from interference with this mechanism, and the cooperativity that is actually present might simply have been missed in previous analysis.

How does the enzyme prevent runaway transphosphorylation upon calcium stimulation, which would short circuit frequency detection? By showing that the simple coincidence detection model cannot be operative, our work emphasizes the need for future experiments aimed at explaining how the activity of the kinase domains is controlled in a way that sets the timing for transphosphorylation appropriately.

RESULTS

Activation of CaMKII by Ca^{2+} /calmodulin is cooperative when exogenous peptide substrate is used

The essential component of CaMKII activation is displacement by Ca^{2+} /calmodulin of a regulatory segment that otherwise blocks the active site⁹. The regulatory segment follows the kinase domain, and contains three elements, denoted R1, R2 and R3 (Figure 2a). Unlike many protein kinases, the catalytic activity of CaMKII does not depend on phosphorylation of the activation loop, located near the active site²⁰. Instead, the regulatory segment blocks the active site in the absence of Ca^{2+} /calmodulin and sequesters the R1 element, bearing Thr286, in a channel adjacent to helix α D of the catalytic domain (Figure 2a)⁸. The sequence motif recognized by calmodulin begins seven residues downstream of Thr286, and extends through the R3 element. The R2 and R3 elements form an α helix, with the R2 element clamping the regulatory segment to the kinase domain.

Phosphorylation of Thr286 requires two Ca^{2+} /calmodulin binding events: one resulting in activation of the subunit that serves as the enzyme and the other releasing Thr286 in the substrate subunit^{10, 11}. The crystal structure of the autoinhibited CaMKII kinase domain shows a dimer in which the R2 and R3 elements of the regulatory segment form an intermolecular, antiparallel coiled coil⁸. The C-terminal lobes (C-lobes) of the two kinase domains in the dimer are at either end of the ~40 Å long coiled coil, which serves to keep the Thr286 residue in one kinase domain far away from the active site of the other (Figure 2a). Isolated kinase domains without the association domain are monomeric in solution,

even when the regulatory segment is present. The crystallographic dimer may only be formed in the holoenzyme, where kinase domains are at high local concentration.

Calmodulin binding releases the regulatory segment from the kinase domain, thereby exposing Thr286 and making it available for phosphorylation. The rebinding of the regulatory segment to the catalytic domain is prevented by Thr286 phosphorylation, even in the absence of Ca^{2+} /calmodulin²¹. The R3 element also contains two autophosphorylation sites, Thr305 and Thr306, which prevent rebinding of Ca^{2+} /calmodulin when phosphorylated.

We measured the activity of full-length wild type *C. elegans* CaMKII holoenzyme (residues 1-482) towards two different peptide substrates as a function of Ca^{2+} /calmodulin concentration, using a continuous spectrophotometric assay to measure reaction progress (see Methods). One of these peptides, known as autocamtide, was used in the earlier work and its sequence (KKALRRQETVDAL, with phosphorylation site underlined) is derived from the Thr286 autophosphorylation site of CaMKII¹⁷. The other peptide, known as syntide (PLARTLSVAGLPGKK, with phosphorylation site underlined), is derived from glycogen synthase, an exogenous CaMKII substrate¹⁸. We use saturating levels of Ca^{2+} (200 μM), so that the measurements report on the cooperativity of Ca^{2+} /calmodulin binding to CaMKII, rather than that of Ca^{2+} binding to calmodulin. Although the activation of CaMKII under sub-saturating calcium concentrations is likely to be relevant^{22,23}, it has been demonstrated that the frequency response of CaMKII is preserved under conditions where calmodulin is saturated with Ca^{2+} (Ref¹). Standard Hill analysis was used to determine cooperativity (Supplementary Discussion 1), and the Hill coefficient was determined by a numerical fit to the reaction velocity as a function of Ca^{2+} /calmodulin concentration (see Methods).

The EC_{50} value for Ca^{2+} /calmodulin is 55 ± 1.0 nM when syntide is used as a substrate, with a Hill coefficient of 3.0 ± 0.3 (Figure 2b). For a system with two coupled binding sites, positive cooperativity results in Hill coefficients greater than 1.0 but less than 2.0 (Supplementary Discussion 2). The observation that the Hill coefficient is greater than 2.0 indicates that three or more subunits of the holoenzyme are coupled in the activation process.

There are more than 20 different forms of mammalian CaMKII as a result of alternative splicing insertions and deletions in the linker region between the kinase and association domains²⁴. Different isoforms are expressed in a tissue-specific and developmentally timed manner⁹, and have been shown to have different Ca^{2+} frequency responses to Ca^{2+} /calmodulin²⁵. To examine the effect of linker length, we made constructs of *C. elegans* CaMKII in which we shortened the linker between the regulatory segment and the association domain, either by deleting 17 residues or by deleting the linker completely. We also lengthened the linker by inserting six residues that are expected to be flexible (with the sequence SAGSAS) between residues 327 and 328, as indicated in Fig. 2c. The length of the linker has a marked effect on the Hill coefficient, with the shorter linkers yielding higher Hill coefficients (Figure 2c). The maximum value of the Hill coefficient was obtained for the CaMKII $\Delta 17$ construct in which 17 residues are deleted (Hill coefficient of 4.3 ± 0.2 compared to 2.7 ± 1.0 for the wild type enzyme). Making the linker longer and more flexible decreases the Hill coefficient to 1.7 ± 0.2 . The linker length varies in different isoforms of mammalian CaMKII, and our results suggest that this may reflect the tuning of the Ca^{2+} responsiveness between different isoforms.

We measured the activity of full length mammalian CaMKII (*S. scrofa* γ isoform) towards syntide as a function of increasing Ca^{2+} /calmodulin concentration. The Hill coefficient is 2.0

± 0.2 , showing that cooperative activation for syntide is also observed in a mammalian isoform (Supplementary Fig. 1a). The *S. scrofa* γ isoform linker region is 15 residues longer than the wild type *C. elegans* CaMKII, consistent with the observed inverse correlation between linker length and Hill coefficient (Figure 2c). We also measured the activity of *C. elegans* CaMKII with all the major regulatory phosphorylation sites (Thr286, Thr305 and Thr306) mutated to alanine. The Hill coefficient for Ca^{2+} /calmodulin activation of this mutant form is 2.9 ± 0.6 , indicating that cooperative activation is a process that does not require autophosphorylation (Supplementary Fig. 1b).

Previous measurements of the activation of the mouse α isoform of the CaMKII holoenzyme by Ca^{2+} /calmodulin utilized an end-point radiometric assay and autocamtide as a substrate, and yielded an EC_{50} value of 48 ± 6 nM and a Hill coefficient of 1.1 ± 0.2 ¹⁶. By repeating the syntide measurements with a radiometric assay we verified that our observation of positive cooperativity for syntide phosphorylation is not due to differences between the spectrophotometric and radiometric assays (Supplementary Fig. 1c). We used the spectrophotometric assay to compare the degree of cooperativity in Ca^{2+} /calmodulin activation of the *C. elegans* $\Delta 17$ construct with syntide and autocamtide as substrates. The Hill coefficient derived from these measurements is 3.9 ± 0.2 for syntide and 1.8 ± 0.3 for autocamtide (Figure 2d). Thus, our new data show that the activation of CaMKII by Ca^{2+} /calmodulin is cooperative for both substrates, although the apparent degree of cooperativity is somewhat lower with autocamtide.

To visualize the degree of cooperativity more directly, a graph of $\log_{10}\left(\frac{f}{1-f}\right)$ versus $\log_{10}[\text{CaM}]$ where f is the fraction of maximal specific activity, is shown in Figure 2d for the activity of the *C. elegans* CaMKII $\Delta 17$ construct towards syntide. The Hill coefficient is the slope of $\log_{10}\left(\frac{f}{1-f}\right)$ versus $\log_{10}[\text{CaM}]$, and the graph confirms that the Hill coefficient is ~ 3.0 for syntide as a substrate, and ~ 2 for autocamtide.

We also measured the activity of *C. elegans* CaMKII holoenzyme towards autocamtide and syntide at saturating concentrations of Ca^{2+} /calmodulin. The K_M values for CaMKII towards autocamtide and syntide are 6.4 ± 0.8 μM and 150.0 ± 24 μM , respectively (Supplemental Fig. 1d). These results show that the reduction in cooperativity observed for autocamtide is correlated with a lower K_M value for autocamtide as a substrate. Both sequences contain the core canonical Ser/Thr recognition motif: $\text{RXX}(\text{T/S})\Phi$ (where X represents any amino acid, and Φ represents a hydrophobic amino acid), indicating that interactions outside this motif are responsible for a lower K_M value for autocamtide as a substrate. Autocamtide is an artificial substrate based on the regulatory segment of CaMKII¹⁷, and so we wondered whether the observed differences between autocamtide and syntide could reflect a role for a regulatory segment in cooperativity. As noted above, phosphorylation of Thr286 is not required for cooperative activation, and so the regulatory segment is not necessarily playing a role as a substrate in whatever process underlies cooperativity. The studies discussed below were aimed at dissecting the role of the regulatory segment in cooperativity.

Light scattering measurements indicate that the R1 element mediates inter-subunit interactions upon calmodulin binding

A CaMKII construct containing the kinase domain and intact regulatory segment (but lacking the association domain) is monomeric in solution at concentrations below 100 μM , as observed previously⁸. The addition of Ca^{2+} /calmodulin to this construct shifts the population towards a predominantly dimeric form, as determined by multi-angle light scattering coupled to gel filtration (Supplementary Fig. 2). When the C-terminal portions of the regulatory segment (the R2 and R3 elements) are removed, the shorter construct (kinase

and R1 element) is a multimer. A construct containing only the kinase domain, with the R1 element also removed, is monomeric at 100 μ M concentration.

Structure of an enzyme-substrate complex of CaMKII kinase domains

The light scattering measurements suggest that release of the R1 element from the autoinhibitory interaction by Ca^{2+} /calmodulin allows it to form an alternative intermolecular interaction. In order to define the nature of this interaction we crystallized a CaMKII construct containing the kinase domain and the R1 element of the regulatory segment, but lacking the R2 and R3 elements. This construct crystallized readily in multiple crystal forms, and the structures of two crystal forms were determined in the absence of nucleotide (Forms A and B, Supplementary Table 1). The structure in crystal form A was determined at 1.7 Å resolution, and refined to a conventional R-value of 17.2% (free R-value of 20.7%). The structure in crystal form B was refined at 3.2 Å (conventional and free R-values of 27.0% and 32.9%, respectively).

In both structures the R1 element of the regulatory segment of one kinase domain is presented for phosphorylation at the active site of another (Figure 3a). This interaction is repeated in a chain throughout the crystal lattice, with the R1 element of one kinase (referred to as the “substrate-kinase”) inserted into the active site of the next one (referred to as the “enzyme-kinase”). The *in trans* interaction of the regulatory segment with a second kinase domain is essentially the same in the two crystal forms, although the orientation between the enzyme-kinase and the substrate-kinase is different. Unambiguous features in electron density maps indicate that the Thr286 residue is phosphorylated, which presumably occurred during protein expression or purification.

All eighteen residues of the R1 element (residues 274-291) are visualized in electron density maps, allowing identification of the connection between this element and the main body of the kinase domain from which it emanates. Sixteen of these residues (Arg276 to Val291) make contacts with the C-lobe of the enzyme-kinase. Asparagine 273 forms a pivot between the substrate-kinase and the R1 element. This pivot is flexible, as indicated by comparison with the form B crystal structure.

While this manuscript was being prepared a structure of a complex between calmodulin and a human δ CaMKII kinase domain construct with all three regulatory elements present was deposited in the Protein Data Bank (PDB code 2WEL) by the Structural Genomics Consortium. Intriguingly, this structure reveals a very similar chain of “enzyme-substrate” interactions, although the segment connecting the R1 element to the kinase domain (residues 275-280) is apparently disordered. Calmodulin is bound to the R3 element, not present in our structure, and does not make significant contact with the kinase domain. Along with other structures determined by our group and the Structural Genomics Consortium, a consistent picture of the interactions made by the regulatory segment emerges. When the intact regulatory segment (R1, R2 and R3) is present, but without calmodulin, the regulatory segments form a dimeric coiled-coil⁸. When the calmodulin recognition element (R3) is deleted, but R1 and R2 are present, the R2 element enters and blocks the active site because a coiled-coil can no longer be formed (seen in PDB entries 2VN9, 2VZ6, 2V70 and 3BHH). If calmodulin is bound to the R3 element, or if the R2 and R3 elements are deleted, an activated form of the kinase is obtained (the structures discussed in this paper, and PDB entry 2WEL).

The kinase domain is in an active conformation in our new structures^{26,27} (Figure 3b). The last eight residues of the R1 element of the substrate-kinase (Arg283 to Leu291) bind to the active site of the enzyme-kinase as seen previously for other kinase-substrate complexes²⁷. The last eight residues of the R1 element therefore form a canonical substrate-

docking interaction, and we refer to the region of the kinase domain that engages this portion of the R1 element as docking site A (Figure 3c).

There are two other interactions made by the R1 element on the enzyme-kinase. A hydrophobic pocket is located in the C-lobe of the kinase domain, between helix α D and the C-terminal end of the activation loop, and it cradles Ile280 from the R1 element (Figure 3c). This site, referred to as docking site B, is occluded in the autoinhibited form of the kinase due to rotation of helix α D⁸.

A cluster of acidic residues is located at the very base of the kinase domain, near the C-terminal end of helix α D and alongside helix α G, and interacts with Arg274 and Arg276 of the R1 element. This region, referred to as docking site C, orients the R1 element as it leaves the substrate-kinase and enters the channel leading into the active site of the enzyme-kinase (Figure 3c). Autocamtide contains several basic residues that are absent in syntide, and we speculate that these residues interact with docking sites B and C and are responsible for a lower K_M value for autocamtide as a substrate and, anticipating the discussion that follows, a reduction in cooperativity. Basic residues N-terminal to the canonical recognition sequence are also found in high-affinity cellular CaMKII substrates such as the N-methyl-D-aspartate glutamate (NMDA) receptor^{28,29}. Utilization of docking site C in a high affinity substrate interaction is consistent with the observation that autocamtide can compete with binding of the NMDA receptor tail, while syntide cannot³⁰.

Capture of the regulatory segment during Ca^{2+} /calmodulin binding results in cooperative activation of a monomeric kinase domain

The extensive interactions between the R1 element of the substrate-kinase and the three docking sites on the enzyme-kinase suggests that the R1 element can play two distinct roles in the regulation of CaMKII. In addition to its role in sequestering Thr286 in the autoinhibited state, the R1 element in one subunit might serve as a molecular grappling hook that can be captured by an adjacent activated kinase domain. Such a capture would potentiate Ca^{2+} /calmodulin binding to the first subunit, increasing the cooperativity of the process.

To test this idea we measured the activity of isolated catalytic subunits, without the association domain. Note that the isolated kinase domain, even with the regulatory elements R1 to R3 present, is monomeric in solution^{8,31,32}. Monomeric subunits are not capable of Thr286 trans-phosphorylation at the low concentrations (10 nM) we use in our assays.

Activity towards syntide substrate peptide was measured for the construct lacking the association domain, yielding an EC_{50} value of 803 nM with no cooperativity (Hill coefficient of 0.9 ± 0.1 ; Fig. 4a). Mass spectrometric analysis showed that although our preparations of holoenzyme are not phosphorylated, constructs of the kinase domain lacking the association domain are partially phosphorylated on Thr305 and Thr306 (data not shown). This is likely to account for the increased value of EC_{50} for activation by Ca^{2+} /calmodulin, which is ~10-fold higher than that observed for Ca^{2+} /calmodulin binding to a kinase-dead form of this construct⁸.

We added a 10-fold excess (100 nM) of a kinase domain inactivated by mutation (D135N, K42M), that lacks the regulatory segment. This “decoy” kinase domain has docking sites A, B and C unoccupied, and cannot bind to Ca^{2+} /calmodulin. The presence of the decoy lowers the EC_{50} values of Ca^{2+} /calmodulin to 380 nM, consistent with capture of the R1 element by the decoy and facilitation of Ca^{2+} /calmodulin binding to the autoinhibitor (Figure 4b). Activation of the kinase domain shows apparent cooperativity with respect to the concentration of Ca^{2+} /calmodulin, with a Hill coefficient of 1.4 ± 0.1 .

The non-hyperbolic response of the CaMKII kinase domain to Ca^{2+} /calmodulin in the presence of the decoy protein implies that the decoy protein and Ca^{2+} /calmodulin mutually facilitate the binding of each other to the enzyme. Binding of calmodulin to the enzyme releases its R1 element, which can then bind to the alternative docking sites on the decoy protein. In a reciprocal fashion, binding of the decoy protein to the R1 element releases the R2 and R3 elements for interaction with Ca^{2+} /calmodulin. We have modeled this system with a kinetic scheme, which predicts that the Hill coefficient for Ca^{2+} /calmodulin activation of the enzyme should be greater than 1.0, but less than 2.0 (Supplementary Discussion 3).

If the decoy subunit is mutated in docking site B to prevent binding of the R1 element (I205K, I101D, F98E, each mutation introduced separately), the activation by Ca^{2+} /calmodulin is no longer cooperative in the presence of the decoy (Figure 4c). Mutation of residues in the R1 element that interact through docking site B (A280D, I281D, introduced together) in the full length *C. elegans* holoenzyme likewise results in a reduction for the Hill coefficient for Ca^{2+} /calmodulin dependent activation to 1.5 ± 0.1 (compared to a Hill coefficient of 3.0 ± 0.3 for the wild type holoenzyme), consistent with capture of the R1 element being one component of cooperative activation (Figure 4d).

At the high local concentrations of the subunits within the holoenzyme, the R1 element could block the access of exogenous substrates by acting as a product inhibitor. This is consistent with the observation that the activity of Thr286 phosphorylated CaMKII in the absence of Ca^{2+} /calmodulin is only 30%-70% that of fully activated Ca^{2+} /calmodulin bound CaMKII¹¹.

A feedback inhibitor of CaMKII binds to the kinase domain by mimicking interactions made by the R1 element

There is considerable similarity between the substrate-like binding mode of the R1 element of CaMKII and the docking of the protein kinase inhibitor PKI to protein kinase A (PKA)³⁴. CaMKIINtide is a peptide inhibitor of CaMKII derived from the protein CaMKIIN, whose tissue-specific expression correlates tightly with that of CaMKII³⁵. A peptide inhibitor corresponding to residues 281-309 of the regulatory segment inhibits the activity of the isolated CaMKII kinase domain towards syntide with a K_i value of $0.2 \mu\text{M}$ ²¹. We also measured CaMKIINtide inhibition of CaMKII kinase domain activity towards syntide, and obtained an IC_{50} value of $0.12 \mu\text{M}$. We observe that CaMKIINtide is a competitive inhibitor of *C. elegans* CaMKII with respect to autocalmodulin (Supplementary Fig. 3b), as reported by others for the mammalian enzyme³⁴. Mutational analysis has indicated that CaMKIINtide interacts with docking site B³⁶, but the primary sequence of CaMKIINtide does not indicate an obvious mode of inhibition.

We determined the crystal structure of a 21 residue segment of CaMKIINtide bound to the kinase domain of CaMKII at 3.4 Å resolution, and observe a mode of interaction that is very similar to that observed for the *in trans* interaction of the R1 element (Figure 5). Instead of threonine, the inhibitor has an Arg residue at the P0 site, and therefore cannot be phosphorylated. Several hydrophobic and basic residues are present in the N-terminal portion of the inhibitor, and confer specificity for the CaMKII kinase domain by using interactions in docking sites B and C (Figure 5). The visualization of a mode of inhibition that is similar to the R1 interaction suggests that CaMKIINtide, in addition to inhibiting the activated subunit, would also block the capture of adjacent regulatory segments. While CaMKIINtide can block peptide substrate phosphorylation, it apparently does not block autophosphorylation³⁶, suggesting that high CaMKIINtide concentrations would be necessary to block inter-subunit capture interaction from occurring within the holoenzyme.

At sub-saturating levels of inhibition, it is conceivable that different expression levels of CaMKIINtide could tune the cooperative response of CaMKII.

DISCUSSION

The observation that the activation of CaMKII by Ca^{2+} /calmodulin is cooperative means that adjacent kinase domains are activated preferentially over those located more distantly within the holoenzyme. This rules out a simple coincidence detection mechanism such as the one depicted in Figure 1a. Our previous analysis of the structural basis for cooperativity in CaMKII focused on the role of autoinhibited kinase domain dimers in the process⁸. If the binding of two molecules of Ca^{2+} /calmodulin affects only two kinase subunits, then the value of the Hill coefficient should be between 1.0 and 2.0. Our new data, which show that the Hill coefficient for activation is greater than 2.0 for many CaMKII constructs suggests that there are additional interactions between dimers in the holoenzyme, such that disruption of one dimer facilitates the disruption of additional dimers.

A reduction in the Hill coefficient is observed when autocamtide, the peptide derived from the regulatory segment, is used as a substrate. For the construct with the greatest degree of cooperativity (CaMKII $\Delta 17$, see Fig. 2d), the Hill coefficient is reduced from ~4.0 with syntide to ~1.8 with autocamtide. This is consistent with a role for autocamtide in weakening interactions between dimers. Our finding that the R1 element of the regulatory segment makes extensive interactions with another kinase domain in a *trans* configuration suggests that an activated kinase domain can capture the regulatory segment of an adjacent kinase domain, potentiating the binding of Ca^{2+} /calmodulin to it. This is supported by the results of experiments in which a decoy kinase domain provide open binding sites for the regulatory segment, resulting in cooperativity for the activation process. The higher affinity of autocamtide compared to syntide for the substrate binding site is expected to impair the efficiency of this process.

Our results bring to the forefront the question of how an appropriate time delay is introduced in the transphosphorylation of Thr286, a critical parameter in determining the sensitivity of the CaMKII holoenzyme to the frequency of Ca^{2+} spikes¹. Resolution of this issue awaits further experimentation, but one clue is provided by dramatic structural differences between two states of CaMKII that have been noted in electron microscopic reconstructions from different groups^{37,38}. In one set of reconstructions the kinase domains appear to be arranged in the central plane of the holoenzyme assembly³⁸. This is consistent with SAXS analysis of inactive CaMKII⁸, which led to a model in which dimeric autoinhibited kinase domains form an outer ring around the central hub of association domains. In another set of electron microscopic reconstructions the kinase domains are located in two rings, one well above the central plane, and one below it³⁷.

We speculated previously that Ca^{2+} /calmodulin binding and the subsequent disruption of kinase domain dimers might result in these domains moving apart from each other, above and below the association domain ring⁸. This is consistent with the increased distance between kinase domains that results from activation, as inferred from FRET measurements³⁹, and with the second set of electron microscopic reconstructions³⁷. In such a mechanism for setting an autophosphorylation time delay, the first pair of kinase domains that are activated are prevented from phosphorylating each other because they are located on opposite sides of the central plane (Figure 6). Transphosphorylation would await the release of the next pair of kinase domains, aided by the substrate capture mechanism that is suggested by our data. A clearer picture as to how the timing of this step is determined will emerge after the kinetic rate constants for the various steps are measured. One exciting possibility for the future is that the frequency-dependent process may be monitored directly

in neuronal dendrites, as highlighted by a recent study on the *in situ* activation of CaMKII by laser stimulated Ca^{2+} spikes⁴⁰.

METHODS

Protein Expression and Purification

We developed a bacterial expression system for CaMKII by coexpression with Lambda Phosphatase, using a strategy similar to that for bacterial expression of certain tyrosine kinases¹⁹. We obtained yields of 2.5 mg of pure active holoenzyme per liter of bacterial culture, compared to 5-10 mg per liter of insect cell culture⁸. Bacterial expression of CaMKII in *E. coli* was accomplished by coexpression with lambda phosphatase (kind gift of J. Dixon, University of California, San Diego) in Tuner(DE-3)pLysS cells (Novagen). Lambda Phosphatase was subcloned into a pCDFDuet1 vector (Novagen) and C-terminally 6-histidine tagged CaMKII and its mutants forms were cloned into a pET-20b vector (Novagen). Protein expression in bacteria was induced by the addition of 0.4 mM IPTG and 0.5 mM Mn^{2+} . Cells were grown overnight at 20°C and flash frozen until used. The average yield was 2 mg of protein per liter of cells at >95% purity as judged by SDS/PAGE and Comassie stain. Autoinhibited constructs of CaMKII (residues 1-340), and other truncations (residues 1-274 and residues 1-291) of the *C. elegans* unc-43 gene were fused to an N-terminal cleavable 6-histidine tag and cloned as described previously.

Our experiments focused on the *C. elegans* enzyme because crystal structures were determined initially using this enzyme⁸. Key experiments are repeated using mammalian enzyme, from *Sus scrofa*. Crystal structures for truncated forms of the human α , β , δ and γ isoforms of the human CaMKII autoinhibited kinase domain, determined by the Structural Genomics Consortium, are also available (PDB codes 2VN9, 2VZ6, 2V70 and 3BHH). The sequence identity within the kinase domain and the association domain between the *C. elegans* and human enzymes are ~80% and ~50%, respectively.

Purification of the CaMKII holoenzyme and the kinase domain constructs was accomplished by Ni-NTA affinity chromatography using a HiTrap HisBind column (Pharmacia) followed by anion exchange and S200 size exclusion chromatography. The final buffer from the gel filtration was 25 mM tris(hydroxymethyl)aminomethane hydrochloride (pH 8.3), 250 mM potassium chloride, 10% (v/v) glycerol, and 1mM tris(2-carboxyethyl)phosphine. Wild type, full length *C. elegans* CaMKII (encoded by the unc-43 gene) was also expressed in Sf9 cells using a baculovirus expression system as described previously⁸. The enzyme purified from the bacterial source, used in the experiments described here, behaves similarly to that purified from insect cell culture, as assessed by mass spectrometry, analytical gel filtration and enzyme activity assays (data not shown). *Gallus gallus* calmodulin was expressed and purified as previously described⁴³ and its final concentration was determined by amino acid analysis (Molecular Structure Facility, University of California, Davis). The *S. scrofa* γ isoform of CaMKII was expressed in Sf9 cells using a baculovirus expression system⁸ and purified as described above.

Crystallographic analysis

Crystals of CaMKII (residues 1-289) were grown using sitting drop vapor diffusion. Crystals were obtained with 0.1 M potassium chloride, 5 mM magnesium sulfate, 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.0 and 15% (v/v) 2-methyl-2,4-pentanediol. Crystals of CaMKII (1-272) and CaMKIIntide were grown in 20% (v/v) 2-methyl-2,4-pentanediol. Crystals were cryoprotected in 25% (v/v) glycerol prior to being frozen in liquid nitrogen. X-ray diffraction data were collected at the Advanced Light Source beamline 8.2.2 at 100°K, at the wavelength .9537Å.

Structures were solved by molecular replacement using Phaser⁴⁴, using the autoinhibited kinase domain of CaMKII (residues 1-278) as a search model. Refinement was performed with CNS⁴⁵ and PHENIX⁴⁶ and model building with O⁴⁷ and Coot⁴⁸.

Enzyme Activity Assays

Kinase activity was monitored using a continuous spectrophotometric assay as described earlier⁴⁹. In this assay, ADP that is produced as a result of phosphorylation by the enzyme is coupled to the oxidation of NADH to NAD⁺, which produces a decrease in absorbance at 340 nm. The assays were carried out in 100 mM tris(hydroxymethyl)aminomethane hydrochloride (pH 7.5), 150 mM potassium chloride, 0.2 mM calcium chloride, 10 mM magnesium chloride, 0.5 mM adenosine 5'triphosphosphate, 1 mM phosphoenolpyruvate, 0.28 mM nicotinamide adenine dinucleotide, 89 units/ml pyruvate kinase, 124 units/ml lactate dehydrogenase, 0.3 - 0.5 mM peptide substrates autocalmodulin AC-3 (KKALHRQETVDAL) or syntide (PLARTLSVAGLPGKK) and various concentrations of calmodulin, at 30°C in a 150 µl reaction volume. Peptides were prepared by David King (Howard Hughes Medical Institute). Reactions were initiated by the addition of 10 - 20 nM CaMKII to the mix and the decrease in absorbance was monitored at 340 nm at 30°C in a microtiter plate spectrophotometer (SpectraMax). The enzyme concentration is expressed in terms of the concentration of kinase units and not holoenzymes. In addition, CaMKII activity was also measured using a phosphocellulose filter binding assay and ATP-³²P as previously described¹⁶.

Cooperativity curves were plotted and analyzed using the program Prism (version 5, GraphPad Software). The data was fit to the Hill equation:

$$Y = Y_{\min} + \frac{(Y_{\max} - Y_{\min})}{1 + \left(\frac{10^{(\log_{10} EC_{50})}}{10^{(\log_{10} [L])}} \right)^n}$$

Where Y is the maximal velocity, EC₅₀ is the concentration at half maximal velocity, [L] is the ligand concentration of calmodulin, and *n* is the apparent Hill coefficient.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Cartoon schematic of models for CaMKII activation. **(a)** In a coincidence detection model, Ca^{2+} /calmodulin binding and activation occurs stochastically. Activation without cooperativity allows for CaMKII transphosphorylation to occur only in response to coincident and adjacent Ca^{2+} /calmodulin binding events. It is not known if autophosphorylation is bidirectional for nearest-neighbor subunits. **(b)** Binding of Ca^{2+} /calmodulin is cooperative^{7,8}, and autoinhibited kinase domains form a dimer in the crystal⁸. In a holoenzyme comprising autoinhibited dimers, Ca^{2+} /calmodulin binding to one kinase in the dimer would release the Ca^{2+} /calmodulin binding site of a second kinase domain. **(c)** This work demonstrates that activation of CaMKII is cooperative, thus CaMKII is not a simple coincidence detector. The level of cooperativity observed indicates sequential activation of kinase domains beyond dimers, where activation of a kinase subunit potentiates activation beyond a second subunit (shown as grey-blue subunits). **(d)** The capture of regulatory segments as substrates for adjacent kinase domains increases the cooperativity of activation. Only the activation and potentiation through subunit capture are shown, with any subsequent autophosphorylation omitted for simplicity.

Figure 2.

Cooperativity of CaMKII activation by Ca^{2+} /calmodulin. **(a)** Schematic diagrams of the CaMKII domain structure and regulatory segment arrangement in the autoinhibited state. Shown at left, the domain structure of CaMKII: the regulatory segment is enlarged, highlighting three elements: the R1 element, which contains the regulatory phosphorylation site Thr286; the R2 element, which clamps the regulatory segment to the kinase domain in the autoinhibited state; and the R3 element, which includes the calmodulin recognition motif. At right, a schematic diagram of the structure of the autoinhibited kinase domain: schematic of arrangement of autoinhibited dimers of the kinase domain in the CaMKII holoenzyme, with individual kinase domain enlarged. In the autoinhibited state, the R1 element is sequestered in a cleft below helix α D, and the R2 element positions helix α D to prevent substrate access to the active site. **(b)** Cooperative activation of *C. elegans* CaMKII holoenzyme occurs with a Hill coefficient of 3.0 ± 0.3 . *C. elegans* holoenzyme phosphorylation of the peptide syntide was measured as a function of calmodulin concentration. **(c)** The Hill coefficient for *C. elegans* CaMKII activation, for constructs with various linker lengths. The velocity of substrate phosphorylation at varying calmodulin concentrations is shown. The linker modifications within residues 314-340 are indicated in the schematic shown at left. In the +6 linker six flexible residues were introduced to the middle of the linker region. In the $\Delta 17$ linker, 17 residues were deleted from the middle of the linker region. In the $\Delta 26$ linker all 26 residues were removed from the linker region. **(d)** Cooperativity of calmodulin activation is reduced by autocamtide for the *C. elegans* CaMKII $\Delta 17$ deletion construct. Activity of CaMKII $\Delta 17$ was measured towards syntide and

autocamtide, and plotted on a velocity (v) vs. $\log_{10}[\text{CaM}]$ plot (right panel), and $\log_{10} [v/(V_{\max}-v)]$ vs. $\log_{10} [\text{CaM}]$ plot (left panel). All error bars and $\pm$ terms expressed are s.e.m.

Figure 3.

Crystal structure of the CaMKII enzyme-substrate complex. **(a)** Schematic diagram of the crystallized CaMKII enzyme-substrate complex. The construct contains the kinase domain of CaMKII and the R1 portion of the regulatory segment. The structure shown (at right) is that of crystal form A. **(b)** The CaMKII enzyme-substrate complex active site is in the active conformation. Comparison of the major active site components of the CaMKII enzyme-substrate complex with that of Protein Kinase A in the active state³⁴. **(c)** Docking sites utilized by the R1 element are indicated as docking sites A, B and C and colored red, green and gold, respectively on a surface representation of the kinase domain. The R1 element is shown in a sticks representation. At right, an electrostatic surface potential representation (produced with APBS (Adaptive Poisson-Boltzmann Solver) tools⁴¹) of the CaMKII kinase domain in the enzyme-substrate complex illustrates a negatively charged region (red) encompassing docking sites B and C that is utilized by residues in the R1 element (basic residues shown in blue, hydrophobic residues shown in black).

Figure 4.

Capture of the regulatory segment results in cooperative activation of a monomeric kinase domain. **(a)** The activity of monomeric *C. elegans* CaMKII kinase domain towards syntide at varying calmodulin concentrations. The monomeric kinase domain does not exhibit cooperative activation (data from a representative Ca²⁺/calmodulin activation response shown). **(b)** Presence of a decoy kinase domain that is competent for capture of the R1 element results in cooperativity in the calmodulin activation of monomeric kinase domain. The decoy was added in 10X molar excess (100 nM), and has no enzyme activity because of mutations (D135N, K42M) introduced in the active site. **(c)** Mutations in docking site B of the decoy eliminate cooperative activation of the monomeric kinase domain. Data for three mutations (I205K, I101D and F98E; each introduced separately) are shown. **(d)** Mutation of residues (A280D, I281D, introduced together) in the R1 element in the *C. elegans* CaMKII holoenzyme holoenzyme results in a reduction in the Hill coefficient for Ca²⁺/calmodulin activation. All error bars and $\pm$ terms expressed are s.e.m.

Figure 5.

Structure of the CaMKII inhibitor CaMKIINTide bound to the kinase domain. At left is shown a schematic diagram of the CaMKII kinase domain and the docking regions occupied by CaMKIINTide. At right, a surface representation of the CaMKII kinase domain with the CaMKIINTide peptide bound is shown, with critical residues highlighted. Basic residues (Arg43 and Lys46 (rat numbering for CaMKIINTide⁴²) occupy docking site C and B, Leu47 and Ile50 occupy docking site B, and a pseudosubstrate recognition mode of interaction is observed in docking site A (Arg52 at the P-3 site, Val56 at the P+1 site).

Figure 6.

A hypothetical mechanism for CaMKII activation. If the time interval between Ca²⁺ spikes is long compared to the dissociation time of Ca²⁺ calmodulin (low frequency), initial binding are presumed to lead to a separation of kinase domains, however transphosphorylation of Thr286 does not occur before Ca²⁺/calmodulin dissociates. If the interval between Ca²⁺ spikes is short compared to the Ca²⁺/calmodulin dissociation time (high frequency regime). Ca²⁺/calmodulin remains bound to the first pair of kinase domains long enough for a second, slow, step to occur in which the activated kinase domains capture the regulatory segments of adjacent kinase domains. This potentiates the binding of Ca²⁺/calmodulin to those domains with increased affinity, resulting in the phosphorylation of Thr286 and acquisition of autonomy (Ca²⁺/calmodulin-independent activity).