Calcium/Calmodulin-Dependent Protein Kinase Is Negatively and Positively Regulated by Calcium, Providing a Mechanism for Decoding Calcium Responses during Symbiosis Signaling

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The establishment of symbiotic associations in plants requires calcium oscillations that must be decoded to invoke downstream developmental programs. In animal systems, comparable calcium oscillations are decoded by calmodulin (CaM)–dependent protein kinases, but symbiotic signaling involves a calcium/CaM–dependent protein kinase (CCaMK) that is unique to plants. CCaMK differs from the animal CaM kinases by its dual ability to bind free calcium, via calcium binding EF-hand domains on the protein, or to bind calcium complexed with CaM, via a CaM binding domain. In this study, we dissect this dual regulation of CCaMK by calcium. We find that calcium binding to the EF-hand domains promotes autophosphorylation, which negatively regulates CCaMK by stabilizing the inactive state of the protein. By contrast, calcium-dependent CaM binding overrides the effects of autophosphorylation and activates the protein. The differential calcium binding affinities of the EF-hand domains compared with those of CaM suggest that CCaMK is maintained in the inactive state at basal calcium concentrations and is activated via CaM binding during calcium oscillations. This work provides a model for decoding calcium oscillations that uses differential calcium binding affinities to create a robust molecular switch that is responsive to calcium concentrations associated with both the basal state and with oscillations.

INTRODUCTION

Calcium ions (Ca2+) play an essential and ubiquitous role as secondary messengers in many signaling pathways (Kudla et al., 2010). Decoding Ca2+ signaling via phosphorylation has been attributed to the action of several plant-specific proteins, including the Ca2+-dependent protein kinases (CDPKs; Harper et al., 2004) and the combined action of the calcineurin B-like proteins (CBLs) and CBL-interacting protein kinases (Batistic and Kudla, 2004). CBLs bind Ca2+ via EF-hand domains but rely on their interacting CBL-interacting protein kinases for a kinase domain to phosphorylate their targets (Batistic and Kudla, 2004). By contrast, CDPKs can directly phosphorylate their targets in response to Ca2+ binding since they possess EF-hands as well as a kinase domain (Harper et al., 2004). In animal systems, the Ca2+-decoding properties of calmodulin (CaM)–dependent protein kinases, such as CaMK-II, have been firmly established (e.g., during neuronal impulses). These proteins possess a CaM binding domain, which allows the protein to respond to Ca2+ via CaM (De Koninck and Schulman, 1998). Plants also possess CaM-responsive protein kinases, including a Ca2+/CaM-dependent protein kinase (CCaMK), which was first identified in lily (Lilium longiflorum; Patil et al., 1995), is present in legumes (Lévy et al., 2004; Mitra et al., 2004) and rice (Oryza sativa; Godfroy et al., 2006; Banba et al., 2008; Gutjahr et al., 2008), and functions during symbiosis signaling in these species. Phylogenetic analyses have also identified CCaMK in mosses, liverworts, and hornworts but not in members of the Brassicaceae (Lévy et al., 2004; Wang et al., 2010), which lack mycorrhizal associations. CCaMK has the exceptional ability among plant and animal proteins to bind Ca2+ through two different mechanisms, either directly via EF-hand domains or indirectly through a CaM binding domain (Patil et al., 1995).

In legumes, symbiosis signaling is initiated by the perception of diffusible signals released from the symbiotic microorganisms: lipochitooligosaccharides called Nod and Myc factors released by rhizobial bacteria or mycorrhizal fungi, respectively (Lerouge et al., 1990; Maillet et al., 2011). Nod factor is perceived by the plant via LysM receptor-like kinases (Broghammer et al., 2012), leading to the activation of the common symbiosis signaling pathway, for which Ca2+ oscillations are central (Ehrhardt et al., 1996; Oldroyd and Downie, 2006; Singh and Parniske, 2012). Many components of the symbiosis signaling pathway have been characterized in relation to the generation or perception of Ca2+ oscillations (Wais et al., 2000; Walker et al., 2000; Miwa et al.,...
RESULTS

Binding of Ca\textsuperscript{2+} to the EF-Hands Negatively Regulates CCaMK via Phosphorylation of Thr-271

Expression of the kinase domain of CCaMK alone gave rise to spontaneous nodulation 8 weeks after root transformation, but in the absence of rhizobia (Figure 1A), and we previously inferred that this resulted from the removal of an autoinhibition domain (Gleason et al., 2006). However, it was not clear how this hypothesis fitted with the autoactivation of CCaMK by mutation of Thr-271 (Tirichine et al., 2006). To rationalize between these apparently alternate modes of CCaMK activation, we undertook a more detailed deletion analysis of CCaMK, expressing deletions of CCaMK from the native promoter, which we previously showed to be important for activation of spontaneous nodulation (Gleason et al., 2006). This analysis revealed that in addition to the deletions that autoactivate CCaMK (Gleason et al., 2006), the removal of either EF-hand 3 or EF-hands 2 and 3 (1-477 and 1-435, respectively) gave rise to spontaneous nodulation in the absence of rhizobia (Figures 1A and 1B; see Supplemental Figure 1 online). This suggests that the EF-hand domains may negatively regulate the symbiotic activity of CCaMK. To validate this, we generated point mutations in the highly conserved aspartate residues of the EF-hand domains (Gifford et al., 2007), and these mutations in CCaMK were previously shown to limit Ca\textsuperscript{2+} binding (Swainsbury et al., 2012). Consistent with the deletion mutants, we observed spontaneous nodulation in the absence of rhizobia when the ccmk-1 null mutant was transformed with the single, double, or triple EF-hand point mutants (Figure 1B), with no apparent phenotypic difference with mutation of any or all of the EF-hand domains.

Ca\textsuperscript{2+} binding via the EF-hand domains promotes Thr-271 phosphorylation (Tirichine et al., 2006) and considering that mutation of either the EF-hands or Thr-271 autoactivate CCaMK, we hypothesized that these effects were linked. To quantify Thr-271 phosphorylation, we generated a state-specific antibody that could measure the phosphorylation status of Thr-271. This antibody was deemed specific to Thr-271 phosphorylation as demonstrated through in vitro kinase assays performed with wild-type CCaMK and the T271A (gain-of-function) and K47E (kinase-dead) mutants (Figure 2A). Furthermore, the dephosphorylation of CCaMK using λ protein phosphatase resulted in the inability of this antibody to detect CCaMK (Figure 2B). We conclude that this state-specific antibody detects only CCaMK phosphorylated at Thr-271.

Autophosphorylation of lily CCaMK is Ca\textsuperscript{2+} dependent (Patil et al., 1995) and the Ca\textsuperscript{2+}-dependent autophosphorylation of Thr-271 was confirmed using the state-specific antibody (Figure 2C). CCaMK truncations (1-311, 328-355, and 1-435) and EF-hand point mutations that showed autoactivation (Figure 1) all showed low levels of Thr-271 phosphorylation in the presence of Ca\textsuperscript{2+} (Figure 2D; see Supplemental Figure 2 online). This suggests that the removal or mutation of the EF-hand domains blocks Ca\textsuperscript{2+} induction of Thr-271 phosphorylation and this in turn autoactivates the protein. The 1-346 truncation, which possesses just the kinase domain attached to the CaM binding domain, showed no gain-of-function activity and was constitutively phosphorylated at Thr-271 (Figure 2D). This suggests that the complete absence of the EF-hand domains leads to unregulated Thr-271 phosphorylation and deactivation of the protein. The constitutive Thr-271 phosphorylation and the inactivity of the 1-346 truncation correlate well with our hypothesis that Thr-271 phosphorylation deactivates CCaMK. This truncation also implies that a region between the EF-hand domains and the CaM binding domain is essential for regulation of Thr-271 phosphorylation.

Given the affinity of each EF-hand for Ca\textsuperscript{2+} (Swainsbury et al., 2012), we can predict the occupancy of the EF-hands under different Ca\textsuperscript{2+} concentrations (see Supplemental Figure 3 online).
At basal Ca\(^{2+}\) concentrations of ~150 nM (Felle et al., 1999; Swainsbury et al., 2012), 16% of CCaMK has all three of its EF-hands occupied and ~50% has two EF-hands occupied. Considering that mutation of a single EF-hand domain is sufficient to autoactivate CCaMK (Figure 1B), this suggests that Ca\(^{2+}\) binding to a single EF-hand domain is sufficient to activate Thr-271 phosphorylation. Therefore, the default state of CCaMK in vivo is likely to be Ca\(^{2+}\) bound to at least one of the EF-hand domains, with resultant Thr-271 phosphorylation. We propose that basal Ca\(^{2+}\) concentrations are sufficient to induce Thr-271 phosphorylation and this maintains CCaMK in the symbiotically inactive state.

**Figure 1.** Mutations at the EF-Hand Domains Autoactivate CCaMK.

(A) The kinase domain of CCaMK (1-311) gives rise to spontaneous nodulation in the absence of rhizobia, as do mutants lacking EF-hand 3 or EF-hands 2 and 3 (1-477 and 1-435, respectively). Bars = 0.5 mm.

(B) Quantification of spontaneous nodulation in the absence of rhizobia with CCaMK truncation mutants and single, double, and triple EF-hand point mutants. Schematic representation of CCaMK shows the kinase domain (light gray), CaM binding domain (dark gray), EF-hand domains (black), and location of point mutations (red stars). Data represent means and SE; all spontaneous nodulation phenotypes were significant relative to ccamk-1 mutants transformed with wild-type CCaMK (one-tailed binomial test; P < 0.005).

Shimoda et al. (2012) hypothesized that the first step in the activation of CCaMK in *Lotus japonicus* may be the disruption of a hydrogen-bond network predicted to exist between the auto-phosphorylation site Thr-265 (equivalent to *Medicago truncatula* Thr-271) and neighboring residues. It was proposed that this hydrogen-bond network stabilizes a conformation of CCaMK that keeps the kinase domain inactive through its association with the CaM binding domain (Shimoda et al., 2012). Analogous to the work in *L. japonicus* (Shimoda et al., 2012), we found that a hydrogen bond was predicted to form between Thr-271 and Arg-323 in *M. truncatula* CCaMK and that this interaction linked the kinase domain to the CaM binding domain (Figures 3A and 3B). The hypothesis put forward by Shimoda et al. (2012) stated that any perturbation to this network (i.e., phosphorylation or mutation) would break the link between these domains and result in kinase activation. This would suggest that Thr-271 phosphorylation should activate the protein, but our data show that it represses CCaMK. Using both the homology model of *L. japonicus* CCaMK (Shimoda et al., 2012) and our own *M. truncatula* CCaMK homology model (see Methods), we found that when molecular dynamics energy minimization was employed, with an appropriate force field for phosphothreonine, hydrogen bonds were predicted to form between phosphorylated Thr-271, Arg-323 (two bonds), and Ser-322 (Figure 3C), in contrast with what was previously reported (Shimoda et al., 2012). While care should be taken with interpreting such homology models, the prediction of hydrogen bonds to the phosphate is consistent with numerous structural studies of phosphate interactions with proteins (Luscombe et al., 2001) and phosphorylated amino acids (Kitchen et al., 2008).

To validate this model, we converted Arg-323 and Ser-322 to Ala and tested their ability to autoactivate CCaMK. We observed spontaneous nodules in the absence of rhizobia in plants transformed with the S322A mutant (see Supplemental Figure 1 online), but surprisingly not with the R323A mutant. However, Shimoda et al. (2012) showed that mutation of the Arg-323 equivalent in *L. japonicus* CCaMK to His led to an autoactive form of CCaMK. Perhaps the bulky side group associated with the His mutation at position 323 is necessary to destabilize the inactive fold in CCaMK. Our work implies that the hydrogen bond between phosphorylated Thr-271 and Ser-322 is the predominant stabilizing bond at the inactive fold in *M. truncatula* CCaMK. The fact that this hydrogen bond is only predicted when Thr-271 is phosphorylated highlights the importance of Thr-271 phosphorylation in stabilizing the inactive state of CCaMK.

CCaMK was autoactivated when Thr-271 was converted to Ala, Ile, or Asp (Gleason et al., 2006; Tirichine et al., 2006; Yano et al., 2008; Hayashi et al., 2010; Madsen et al., 2010; Takeda et al., 2012), and these results are puzzling since aspartate mutations are often used to mimic a phosphothreonine. In our homology model, no hydrogen bonds form when Thr-271 was converted to Ala or to Ile, while the Asp substitution was predicted to induce a hydrogen bond only to the backbone of Trp-272 present in the kinase domain (Figure 3D). Hence, the model offers an explanation for why both the phosphomimetic and phosphoablative substitutions at Thr-271 lead to gain-of-function phenotypes in
CCaMK: Both types of mutations destroy the hydrogen bond network that stabilizes the inactive state of the protein.

Binding of CaM Blocks Phosphorylation of Thr-271 and Positively Regulates CCaMK

Considering that the binding of Ca\(^{2+}\) negatively regulates CCaMK by enhancing Thr-271 phosphorylation, we considered the effect of CaM binding on the activation of CCaMK. Addition of CaM greatly decreased the total autophosphorylation of CCaMK (Figure 4A), as described previously (Takezawa et al., 1996), and specifically blocked the phosphorylation of Thr-271 (Figure 4B). The level of Thr-271 phosphorylation was not altered if CaM was added after Ca\(^{2+}\) (see Supplemental Figure 4 online), suggesting that CaM only prevents Ca\(^{2+}\)-dependent...
Thr-271 phosphorylation. These in vitro analyses do not have a phosphatase present, and we presume that phosphorylated Thr-271 will be hydrolyzed in vivo by the action of phosphatases. In such a scenario, CaM binding, along with the action of phosphatases, would shift the balance of CCaMK species toward the unphosphorylated active form. Based on the affinity and kinetics of Ca^{2+} binding to CaM and of CaM binding to CCaMK (Swainsbury et al., 2012), it is likely that CaM binds to CCaMK in significant levels only during Ca^{2+} oscillations.

To further assess the function of the CaM binding domain in the activation of CCaMK, we attempted to identify the amino acid residues involved in the regulation of this domain by substitution of each amino acid residue in the CaM binding domain to an Ala residue. This Ala-scanning approach identified several residues in the CaM binding domain that were required for both nodulation and mycorrhization (Figure 4C). The mutants that were least able to complement the ccamk-1 null mutant, following 4 weeks of inoculation with Sinorhizobium meliloti or 8 weeks with Rhizobagrus irregularis (E319A, L324A, L333A, and S343A) were characterized further. Thr-271 phosphorylation was completely abolished in the L324A mutant, even upon Ca^{2+} addition, while the L333A mutant showed enhanced Thr-271 phosphorylation even in the absence of Ca^{2+} (Figure 4D), suggesting a constitutively deactivated protein. Based on our hypothesis, the lack of Thr-271 phosphorylation in the L324A mutant should lead to autoactivation, and consistent with this, we observed spontaneous nodules in the absence of rhizobia in ccamk-1 transformed with CCaMK L324A, but not in plants transformed with the E319A, L333A, or S343A mutants (see Supplemental Figure 1 online). Note that spontaneous nodules in the absence of rhizobia take many weeks to emerge and will not be included in the nodulation analysis performed in the presence of rhizobia (Figure 4C), since rhizobia-induced nodulation was measured just 4 weeks after inoculation.

Neither the E319A nor L324A mutants supported the binding of M. truncatula CaM 1 as determined by fluorescence anisotropy spectroscopy using a His-tagged construct comprising the CaM binding and EF-hand domains (K_{d} >1500 nM; see Supplemental Figure 5 online). By contrast, the L333A and S343A mutations showed only modest decreases in the affinity for CaM (K_{d} = 14 and 38 nM, respectively; see Supplemental Figure 5 online). The fact that Glu-319 and Leu-324 are essential for both CaM binding and symbiotic function highlights the positive role that CaM binding plays in the activation of CCaMK. Interestingly, Glu-319 and Leu-324 sit on either side of Ser-322 and Arg-323 that are proposed to hydrogen bond with phosphorylated Thr-271 (Figure 3C; see Supplemental Figure 6 online). This mutational analysis suggests that CaM binding activates CCaMK through the protection of Thr-271 from phosphorylation and possibly also through structural changes imposed on the form of CCaMK phosphorylated at Thr-271.

Opposing Regulation of CCaMK by Ca^{2+} Provides a Robust Inactive State That Can Be Temporally Overridden during Ca^{2+} Spiking

To study the overall kinetic behavior of CCaMK, we formulated the proposed reactions as ordinary differential equations. Ca^{2+} binding is rapid (Swainsbury et al., 2012) compared with the time scales of phosphorylation and was therefore approximated by steady state kinetics (see Methods). The key reaction steps are the binding of Ca^{2+} to CCaMK, Ca^{2+}-saturated CCaMK catalytically phosphorylating Ca^{2+}-unsaturated CCaMK, Ca^{2+} binding to CaM, Ca^{2+}-saturated CaM binding to both phosphorylated and unphosphorylated CCaMK (with different affinities according to published observations; Sathyanarayanan et al., 2000), and the catalytic phosphorylation of the target protein (Figure 5A).

Although our in vitro data shows that the binding to CaM prohibits the autophosphorylation of Thr-271 in the Ca^{2+}-bound form of CCaMK (Figure 4B), this blockage is only sufficient to explain a slight activation of CCaMK in the model leading to target phosphorylation (see Supplemental Figures 7A and 7C online). If we add the action of phosphatases to the model, then there is a greater discrimination between the active and inactive states (see Supplemental Figures 7B and 7D online), but the model demands a phosphatase with a finely balanced activity to remain robust. An alternative model allows CaM binding to override the effect of the hydrogen bond network and thus activate the phosphorylated Thr-271 form of CCaMK. As discussed previously, the residues likely involved in CaM binding support such a hypothesis. When we assume this to be the case, the model is
Figure 4. CaM Binding Blocks Thr-271 Autophosphorylation and Is Required for Mycorrhization and Nodulation.

(A) Total autophosphorylation of CCaMK is decreased by the presence of 0.5 μM CaM. CBB, Coomassie blue.

(B) Ca²⁺-induced Thr-271 phosphorylation of CCaMK is decreased by the addition of CaM at the time of Ca²⁺ treatment.

(C) Ala scanning of the CaM binding domain reveals that residues Glu-319, Leu-324, Leu-333, and Ser-343 are important during mycorrhization and nodulation. The ccamk-1 mutant was transformed with the different CCaMK genes, and lines were tested for levels of mycorrhizal colonization (8 weeks after inoculation; black) and numbers of nodules following *S. meliloti* inoculation (4 weeks after inoculation; gray). Spontaneous nodules only occur at later time points, and in this analysis the nodules observed will be only those induced by *S. meliloti*. Data represent means and SE; asterisks denote mutants showing a significant nodulation and mycorrhization phenotype relative to ccamk-1 mutants transformed with wild-type CCaMK (two-tailed Student’s t test; *P* < 0.05). In total, an average of 23 independent transformed plants was scored per construct, per phenotype. These transformed plants were generated from at least three independent transformation experiments. EV, empty vector; WT, wild type.

(D) Immunoblots assessing Thr-271 phosphorylation of CCaMK. Thr-271 phosphorylation is abolished in the L324A mutant, while the L333A mutant showed enhanced Thr-271 phosphorylation, suggesting a constitutively deactivated protein. The E319A and S343A mutants are able to phosphorylate Thr-271, but to lower levels than the wild-type protein.

Final concentrations of 10 mM Mg²⁺ and 0.2 mM Ca²⁺ were used in (A), (B), and (D).
capable of producing enhanced phosphorylation of CCAMK targets (Figure 5B) and can do so without the need for a phosphatase. The fact that target phosphorylation can occur in vitro in the absence of a phosphatase, but in the presence of Ca\(^{2+}\) and CaM (Takezawa et al., 1996; Gleason et al., 2006; Tirichine et al., 2006; Shimoda et al., 2012), provides further support for this model. This model allows CCA MK to remain inactive at basal Ca\(^{2+}\) concentrations and to become activated during Ca\(^{2+}\) spiking (see Supplemental Figure 8 online), leading to an increase in target phosphorylation. Once Ca\(^{2+}\) spiking has stopped, CCA MK returns to the inactive state (Figure 5B).

The CaM dissociation rate constant for CCA MK was estimated at 0.1 s\(^{-2}\) (Swainsbury et al., 2012). However, this is based on the unphosphorylated form of CCA MK, and we know that phosphorylated CCA MK has a greater affinity for CaM (Sathyanarayanan et al., 2000), suggesting that the CaM dissociation rate is likely to be slower in the Thr-271 phosphorylated form. The CaM dissociation rate has a profound impact on the responsiveness of the system, such that an estimated rate of 0.01 s\(^{-2}\) greatly enhances the active state relative to the inactive state (Figure 5C). Interestingly, if we mimic the T271A behavior in silico and prevent autophosphorylation, we observe that the system remains in a state that has low activity but over time can achieve significant target phosphorylation even in the absence of Ca\(^{2+}\) oscillations (Figure 5C), thus reproducing the experimental findings. This modeling work shows that the predicted negative and positive impacts of Ca\(^{2+}\) and CaM binding, respectively, can create a robust molecular switch that is finely tuned to the Ca\(^{2+}\) concentration within the cell.

DISCUSSION

CCA MK is unique among Ca\(^{2+}\)-regulated proteins in having the ability to bind both free Ca\(^{2+}\) and Ca\(^{2+}\) in complex with CaM (Patil et al., 1995; Lévy et al., 2004; Mitra et al., 2004). The Ca\(^{2+}\) binding affinities of the EF-hands and CaM (Swainsbury et al., 2012) allow CCA MK to discriminate between basal Ca\(^{2+}\) concentrations and those during Ca\(^{2+}\) spiking. The deletion or mutation of the EF-hand domains led to autoactivity of CCA MK with resultant spontaneous nodulation in the absence of rhizobia (see Supplemental Figure 1 online; Figure 1), strongly suggesting that binding of Ca\(^{2+}\) to the EF-hands is involved in the suppression of CCA MK activity. That Ca\(^{2+}\) binding to the EF-hands promotes Thr-271 phosphorylation and that mutation of Thr-271 autoactivates CCA MK is consistent with Ca\(^{2+}\)-induced

Figure 5. Activation of CCA MK during Ca\(^{2+}\) Spiking and Target Phosphorylation.

(A) A schematic network of reactions regulating the activity of CCA MK showing all the chemical reactions that were taken into account in the mathematical model of the system. The complexes that are active for target phosphorylation are shown in the gray box, and only Ca\(^{2+}\)-saturated CaM is assumed to bind to Ca\(^{2+}\)-saturated CCA MK.

(B) and (C) The simulation results of the system of ordinary differential equations of the reactions in (A) for different dissociation rate constants of CaM for CCA MK\(_{0}\)Ca\(^{2+}\) and CCA MK\(_{0}\)Ca\(^{2+}\) at basal Ca\(^{2+}\) concentrations 0.1 s\(^{-1}\) in (B) and 0.01 s\(^{-1}\) in panel (C). With the experimentally determined kinetic parameters, CCA MK complexes (green) and pT271 CCA MK (blue) rise and fall with oscillations in Ca\(^{2+}\) concentrations. The complexes assumed to be active for target phosphorylation (green) lead to an increase in the level of phosphorylated target during Ca\(^{2+}\) spiking (provided that the target dephosphorylation rate does not reduce the phosphorylated target concentration to that prior to the previous Ca\(^{2+}\) spike). The kinase activity factor was set to 0.09 in (B) and 0.01 in (C) (see Methods). The equations and kinetic parameters are given in Methods and Supplemental Table 2 online, respectively.
phosphorylation of Thr-271 suppressing CCaMK activity. We propose that the EF-hands bind Ca$^{2+}$ at basal concentrations, and this ensures the default state is inactive (Figure 6). This symbiotic inactive state is stabilized by a hydrogen bond network between the CaM binding and the kinase domains, which is promoted by Thr-271 phosphorylation. Mutations that block phosphorylation disrupt the hydrogen bond network, sufficiently releasing the kinase domain for target phosphorylation (Figure 6).

The binding of CaM is associated with the activation of CCaMK (Figure 6), and we have found that the residues in CCaMK that are essential for CaM binding are adjacent to the residues associated with the hydrogen bond network. Interestingly, autophosphorylation promotes CaM binding (Sathyanarayanan et al., 2001), implying that while Ca$^{2+}$ binding to the EF-hands inactivates the protein, it also primes the protein for activation, once Ca$^{2+}$ levels rise to sufficient concentrations to bind CaM. The modeling of CCaMK showed how important this primed state was for the overall level of kinase activation. Our in vitro data show that CaM binding can protect Thr-271 from Ca$^{2+}$-induced phosphorylation. However, our modeling suggests that this activity alone is not sufficient to explain the activation of CCaMK. Rather, we propose that CaM binding overrides the impact of Thr-271 phosphorylation (Figure 6). Considering the location of the residues associated with CaM binding, it seems likely that CaM binding will induce conformational changes that may disrupt the hydrogen bond network and disassociate the CaM binding domain from the kinase domain, thus leading to its activation. This is consistent with in vitro analyses that show target phosphorylation in the presence of Ca$^{2+}$ and CaM, but without a phosphatase to hydrolyze phosphorylated Thr-271 (Takezawa et al., 1996; Gleason et al., 2006; Tirichine et al., 2006; Shimoda et al., 2012).

Shimoda et al. (2012) concluded that CaM binding was redundant for CCaMK activation of mycorrhization, but essential for nodulation. However, this was based on a form of CCaMK that was autoactive and, thus, independent of both Ca$^{2+}$ and CaM. Interestingly this autoactive mutation not only blocks CaM binding, but also blocks autophosphorylation and renders the EF-hand domains redundant (Shimoda et al., 2012), consistent with our conclusions. Our own data reveal two mutations (E319A and L324A) that block CaM binding and abolish both the capability to support nodulation and mycorrhization, and these are inconsistent with the conclusions by Shimoda et al. (2012). We believe that the relevance of CaM binding should not be assessed on the activity of a mutation that renders CCaMK autoactive in a CaM-independent manner, but rather should be based on mutations that abolish CaM binding without autoactivating CCaMK. That residues associated with CaM binding and with suppression of CCaMK appear to be overlapping, renders the discrimination between these issues challenging and this is highlighted by the L324A and the FN-ED mutations that block CaM binding but autoactivate CCaMK giving rise to spontaneous nodulation in the absence of rhizobia. The E319A mutation appears to block CaM binding without autoactivating

Figure 6. Schematic Overview of CCaMK Activation.

A hydrogen bond network between the kinase domain and the CaM binding domain is strengthened by Ca$^{2+}$-induced Thr-271 phosphorylation at basal Ca$^{2+}$ concentrations; in this state, CCaMK is inactive for target phosphorylation. With relatively low dephosphorylation rates, phosphorylated CCaMK is likely to be the dominant species at basal Ca$^{2+}$ concentrations. At elevated Ca$^{2+}$ concentrations during Ca$^{2+}$ spiking, CaM binds to CCaMK, and this overrides the negative regulation caused by Thr-271 phosphorylation, making the kinase active for target phosphorylation. Phosphorylation of Ser-343/Ser-344 inhibits CaM binding, and this negatively regulates CCaMK activity (Liao et al., 2012; Routray et al., 2013). The hydrogen bond network in the gain-of-function T271A mutant (yellow star) is disrupted, rendering the protein active for target phosphorylation.
Such an opposing modality for a Ca²⁺-regulated protein provides new insights into the regulation of CaMK activity by decreasing the affinity for CaM binding. This phosphorylation of two neighboring residues within the CaM binding domain of CaMK is reminiscent of regulation of CaMK-II in mammalian systems (Colbran, 1993). Our structural model suggests that Ser-343 and Ser-344 are located far from Thr-271 and the hydrogen bond network (see Supplemental Figure 6C online), so it is perhaps unlikely that phosphorylation of Ser-343/Ser-344 influences the hydrogen bond network that we observed. However, it should be noted that the structural study presented here, and those presented by previous publications, are only predicted structures based on proteins related to CaMK. Although our genetic and biochemical data agree with our structural modeling, a high-resolution crystallographic structure of CaMK will be critical for underpinning future research on this protein. Understanding the significance of Thr-271, Ser-343, and Ser-344 phosphorylation, and any potential interplay between the phosphorylation statuses of these residues, will be essential to fully dissect the molecular mechanisms by which CaMK is regulated during symbiosis signaling. Many CaM binding proteins display an enhanced affinity for CaM in the presence of their target proteins (Bayer et al., 2001). In time, it will therefore be important to include in the model of CaMK activation, its negative regulation through Ser-343 and Ser-344 phosphorylation, and the effect of substrate (such as IP3/CYCLOPS) phosphorylation on CaMK dynamics.

Our model predicts two stable states for CaMK: an inactive form that predominates at basal Ca²⁺ concentrations and involves a hydrogen bond network between the CaM binding and kinase domains and an active form that predominates during Ca²⁺ oscillations associated with CaM binding. In our model, the frequency of the Ca²⁺ oscillations dictates the rate of CaMK inactivation, but we see no evidence for frequency modulation analogous to that proposed for CaMK-II (De Koninck and Schulman, 1998). The capability of CaMK to respond both positively and negatively to Ca²⁺ allows the kinase to be activated in response to Ca²⁺ oscillations during symbiosis signaling, but inactivated at basal Ca²⁺ concentrations. This dual regulation by Ca²⁺ provides a robust molecular switch that is attuned to the dynamic Ca²⁺ concentrations that occur in the cell and suggests that CaMK not only measures the induced Ca²⁺ concentrations but is also sensitive to the basal state. Such an opposing modality for a Ca²⁺-regulated protein provides a new model for regulation by Ca²⁺.

**METHODS**

**Plant Material and Bacterial Strains**

Seeds of Medicago truncatula cv Jemalong A17 and ccamk-1 (dmi3-1; TRV25) were scarified with sandpaper or 98% H₂SO₄, surface-sterilized in 10% sodium hypochlorite solution, imbibed in sterile water, and plated on 1% deionized water agar. After stratification at 4°C for a minimum of 4 d, seeds were germinated overnight at room temperature. Seedlings were transformed with Agrobacterium rhizogenes strain AR1193 carrying the appropriate binary vector as described by Boisson-Dernier et al. (2001). Three weeks after transformation, plantlets were screened for positive DsRed expression, transferred to a 1:1 mix of sterilized sand:terragreen watered with sterile buffered nodulation medium (Oil-Dri Company), and inoculated with Sinorhizobium meliloti strain 1021 (OD₆₀₀ = 0.03) or Rhizosphagrus irregularis (Endorize). Nodulation and mycorrhization were scored 4 and 8 weeks after inoculation, respectively. Mycorrhized roots were ink stained (Vierheilig et al., 1998), and percentage of root length colonization was determined using the grid-line intersect method (Giovannetti and Mosse, 1980). An average of 23 independent transformed plants was scored per construct, per phenotype. These transformed plants were generated from at least three independent transformation experiments. Spontaneous nodulation in the absence of rhizobia was performed in sterile growth pouches (Mega International) or a 1:1 mix of sterilized sand:terragreen watered with sterile buffered nodulation medium, and scored after 8 weeks.

**Expression and Purification of CaMK**

For in vitro work the full-length M. truncatula CaMK (Dmi3) cDNA sequence was synthesized for optimal codon usage for Escherichia coli expression (GenScript) (Swainsbury et al., 2012) and amplified by PCR (see Supplemental Table 1 online for primer sequences). The PCR product was ligated into the maltose binding protein (MBP) fusion vector, pMal-c2X (New England Biolabs) using BamHI and EcoRV sites, resulting in MBP-CaMK. Mutations including MBP-Δ328-355 were introduced to MBP-CaMK constructs using PCR-based mutagenesis (Adereth et al., 2005). The K47E and T271A CaMK mutant constructs were also generated using this PCR-based method. The CaMK truncation mutant constructs (1-311, 1-346, and 1-435) were generated by PCR (see Supplemental Table 1 online).

The full-length CaMK (Dmi3) cDNA sequence synthesized for optimal E. coli expression (Swainsbury et al., 2012) was also amplified by PCR (see Supplemental Table 1 online) and ligated into the glutathione S-transferase (GST) fusion vector, pGEX4T1 (GE Healthcare), using BamHI and NotI sites. The K47E and T271A mutations were introduced into the CaMK sequence as described above (see Supplemental Table 1 online). All mutant variants of CaMK were sequenced to confirm the presence of the correct mutations. Each MBP-CaMK construct for protein expression was used to transform E. coli strain BL21 (DE3) (Merck Chemicals), and the GST-CaMK constructs were used to transform Rosetta (DE3) pLysS (Merck Chemicals). MBP-CaMK protein was induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside for 2 h at 28°C and purified as described previously (Tirichine et al., 2006). Proteins were eluted in buffer containing 20 mM Tris, pH 7.4, 200 mM NaCl, and 10 mM maltose. GST-CaMK protein was induced with 0.1 mM isopropyl β-D-1-thiogalactopyranoside for 16 h at 16°C and purified as described previously (Miyahara et al., 2008). Protein concentration was determined by Bradford assay (Sigma-Aldrich).

**In Vitro Kinase Assays**

Kinase assays were performed by incubating 0.3 μM MBP-CaMK protein in 50 mM HEPES, pH 7.5, 1 mM DTT, 200 μM ATP, and 5 μCi [γ-³²P]ATP in a 20-μL reaction at 30°C for 30 min. Treatments involved the addition of 2.5 mM EGTA, 10 mM MgCl₂ (Sigma-Aldrich; catalog number...
255777, 10 mM MgCl₂, and 0.2 mM CaCl₂ (Sigma-Aldrich; catalog number 499609), or 10 mM MgCl₂, 0.2 mM CaCl₂, and 0.5 µM bovine CaM (Millipore; catalog number 14-368A), as indicated. No premixing of Ca²⁺ and CaM was performed. Kinase assays with GST-CCaMK was performed by incubating 0.3 µM GST-CCaMK protein in 50 mM HEPES, pH 7.5, 1 mM DTT, 5.0 mM MgCl₂, 0.5 mM MnCl₂, and 5 µCi [γ-³²P]ATP in a 20-µL reaction at 30°C for 30 min. The reactions were terminated by adding SDS-PAGE sample buffer and boiling at 95°C for 5 min, and then protein samples were separated by 10% SDS-PAGE. The gels were stained by Instant Blue (Expedeon) and dried using a gel dryer. The radioactivity was quantified by a phosphor imager (FLA-7000; Fujifilm), and data were analyzed using the MultiGauge program (Fujifilm).

Immunoblot Analysis

A custom phosphorylation state-specific antibody for Thr-271 (pT271) of M. truncatula CCaMK was produced by Eurogentec against the following sequence: CSFYKpTKWGISQ. Kinase reactions were performed as described above without 5 µCi [γ-³²P]ATP, and the reaction samples separated by SDS-PAGE were transferred to polyvinylidene difluoride membranes. Immunodetection of phosphorylated proteins was performed using anti-pT271 (1:150) and a secondary antibody, horseradish peroxidase–conjugated anti-rabbit antibody (1:15,000) as described in the PhosphoProtein Handbook (Qiagen). Chemiluminescent signal generated by ECL Plus Western Blotting Detection Reagents (GE Healthcare) was either visualized by autoradiography or quantified by FLA-7000. The data were analyzed using the MultiGauge program. GST-CCaMK (1.2 µM) was dephosphorylated by 80 units of protein phosphatase (New England BioLabs) at 30°C, and the aliquots were taken at time intervals of 0, 10, 20, 30, and 60 min.

Expression of CCaMK in Plants

The full-length M. truncatula CCaMK (DM3) cDNA sequence was amplified by PCR using Gateway-modified primers (see Supplemental Table 1 online). This PCR product was cloned into the Gateway donor vector pDONR207 by BP recombination reaction (Invitrogen) and subsequently used for creating point mutations by either extension overlap PCR (Ho et al., 1989; Horton et al., 1989) or QuikChange site-directed mutagenesis (Stratagene; see Supplemental Table 1 online). CCaMK truncations were prepared as previously described by Gleason et al. (2006). Constructs were recombined into the appropriate Gateway destination vectors by LR recombination reaction (Invitrogen). The pK7FWG2-R destination vector was prepared as previously described by Gleason et al. (2006). Constructs were first subjected to vacuum minimization to remove major structural problems and then placed in a dodecahedron box of water, ions were added to neutralize the system, and the solvated systems were equilibrated (Sathyanarayanan et al., 2000), and titration data were used to improve this and complete a number of missing fragments and chain breaks. Gromacs version 4.1 (Hess et al., 2008) was used to carry out energy minimization of the resulting homology structures. We installed the forcefield ffG43a1p that can be obtained from the Gromacs Users’ Web pages and includes the atomistic parameters and topological descriptions of phosphoresidues, including phosphothreonine. All models were first subjected to vacuum minimization to remove major structural problems and then placed in a dodecahedron box of water, ions were added to neutralize the system, and the solvated systems were equilibrated to room temperature and pressure. The resulting homology model for the Ca²⁺-bound form has high structural similarity in the kinase domain with the autoinhibited CaMK-II structure 2bdw.B (2.4 Å RMSD).

CaM Binding Analysis by Fluorescence Anisotropy Spectroscopy

Canonical M. truncatula CaM 1 was expressed in E. coli and purified using hydrophobic interaction and size exclusion chromatographies as described previously (Swainsbury et al., 2012). The M. truncatula CCaMK (DM3) cDNA sequence was synthesized for optimal E. coli expression (Swainsbury et al., 2012) and was used as a PCR template to generate a construct suitable for the production of protein consisting of the EF-hands (visinin-like domain) and CaM binding domain (residues 317 to 523) with an N-terminal His-tag using the pET101/D-TOPO kit (Invitrogen) together with the forward and reverse primers 5’-CAC-CATGGAATACCACTACCGGAAATTGTGCTGCG-3’ and 5’-CTAGCAGCCGGATTGACGACAGA-3’, respectively. Site-directed mutants of CCaMK 317-523 (E319A, L324A, L333A, S343A, D413A, D449A, and D491A) were generated using the QuikChange Lightning site-directed mutagenesis kit (Stratagene; see Supplemental Table 1 online). His-CCaMK 317-523 proteins were expressed in E. coli BL21 Star (DE3) grown in Auto-Induction Medium (Formentum) at 37°C for 3 h followed by 18°C for a 18 h. Cells were harvested at 5000g for 20 min at 4°C, resuspended in 50 mL 15 mM Tris-HCl, pH 7.5, containing 300 mM NaCl, 10 mM imidazole, 6 mM mL⁻¹ DNase 1, and a Complete protease inhibitor cocktail tablet (Roche), and disrupted as described above. The lysate was centrifuged as described above, and the supernatant was passed through a 0.45-µm filter before being loaded on to a 1-mL HisTrap fast flow column (GE Healthcare), previously equilibrated with 10 mL of 15 mM Tris-HCl, pH 7.5, containing 300 mM NaCl and 10 mM imidazole. A 10-µL gradient of 0 to 500 mM imidazole in the above buffer eluted the proteins of interest at ~200 mM imidazole. A HiLoad 16/600 Superdex 75 gel filtration column (GE Healthcare), previously equilibrated with 350 mL of 15 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 1 mM DTT, was used to further purify the proteins. Protein concentration was determined by absorbance at 280 nm using a predicted extinction coefficient of 9970 M⁻¹ cm⁻¹ (Gasteiger et al., 2005).

The binding affinity of M. truncatula CaM to wild-type and mutated CCaMK 317-523 was determined using fluorescence anisotropy spectroscopy as described previously for potato (Solanum tuberosum) CaM and lily (Lilium longiflorum) CCaMK (Sathyanarayanan et al., 2000). CaM was labeled with a fourfold molar excess of dansyl chloride to yield dansyl CaM (D-CaM). D-CaM concentration was determined as described above for CaM. CCaMK 317-523 was titrated in duplicate into 2 nM D-CaM in 50 mM Tris-HCl, pH 7.5, containing 200 mM NaCl and either 0.2 mM CaCl₂ or 0.1 mM EDTA at 30°C. Fluorescence anisotropy spectroscopy was recorded with a Perkin-Elmer LS 55 fluorescence spectrometer using excitation and emission wavelengths of 335 and >390 nm, respectively. The ratio of the quantum yields of the bound and free D-CaM was 1.9 as determined previously (Sathyanarayanan et al., 2005), and titration data were fitted using a simple noncooperative binding model using Origin 7 software.

Structural Analysis and Energy Minimization

We used DeepView (Guex and Peitsch, 1997) to build homology models of M. truncatula CCaMK based largely on the structural scaffolds of C3h4 for the activated form and C3u2 for the inactive form. These are structures of CDPKs (Wernimont et al., 2010, 2011) with and without Ca²⁺ that had a high structural similarity (Cα root mean square deviation [RMSD] of 0.81 Å) to the top ab initio–predicted structure from I-Tasser (Zhang, 2008; Roy et al., 2010). Despite some key differences, such as the lack of a CaM binding domain, CDPKs have some striking features in common with CCaMK, such as the kinase domain, a linker region, and an EF-hand domain. Given these observations and the recent availability of high-resolution X-ray structures of CDPK in both inactive and Ca²⁺–activated states, we built homology models of M. truncatula CCaMK based on CDPK structure scaffolds. The structural results presented in this article are derived from the model built for the form without Ca²⁺ based on C3u2, which has a 30% sequence identity to M. truncatula CCaMK. The DeepView tools (Guex and Peitsch, 1997) were employed to get an initial reasonable alignment and the automated refinement Web server was used to improve this and complete a number of missing fragments and chain breaks. Gromacs version 4.1 (Hess et al., 2008) was used to carry out energy minimization of the resulting homology structures. We installed the forcefield ffG43a1p that can be obtained from the Gromacs Users’ Web pages and includes the atomistic parameters and topological descriptions of phosphoresidues, including phosphothreonine. All models were first subjected to vacuum minimization to remove major structural problems and then placed in a dodecahedron box of water, ions were added to neutralize the system, and the solvated systems were equilibrated to room temperature and pressure. The resulting homology model for the Ca²⁺-bound form has high structural similarity in the kinase domain with the autoinhibited CaMK-II structure 2bdw.B (2.4 Å RMSD).
and the CCaMK homology model (2.3 Å RMSD) of Shimoda et al. (2012). Color coding in the structural models follows numbering from the UniProt database for M. truncatula CCaMK: kinase domain (red), residues 12 to 306; predicted CaM binding domain (blue), residues 329 to 342; linker region (yellow), residues 307 to 328.

**CCaMK Autophosphorylation Modeling**

We assumed that each CCaMK molecule must be fully Ca\(^{2+}\) saturated in order to become active for autophosphorylation. This is based on in vitro studies that have shown that autophosphorylation of Thr-271 is Ca\(^{2+}\)-dependent (Takezawa et al., 1996) and by analogy to structural studies of CDPK (Wernimont et al., 2010 2011) that reveal that Ca\(^{2+}\) binding can cause a large conformational change, moving the C-terminal domain away from the active site enabling ATP and substrate access. Following Shimoda et al. (2012), we assumed that the phosphorylatable species is in the non-Ca\(^{2+}\)-activated form. As Ca\(^{2+}\) binding is fast (Swainsbury et al., 2012) compared with Ca\(^{2+}\) oscillations and phosphorylation, we used the steady state approximation, thus giving rise to the following expression for the fraction of CCaMK with Ca\(^{2+}\) bound to all three EF-hands,

\[
p_{\text{all}} = \frac{\left[ \frac{\text{Ca}^{2+}}{\text{K}_{\text{CaM}}^1} \right]}{\left[ \frac{\text{Ca}^{2+}}{\text{K}_{\text{CaM}}^1} \right] + \left[ \frac{\text{Ca}^{2+}}{\text{K}_{\text{CaM}}^2} \right] + \left[ \frac{\text{Ca}^{2+}}{\text{K}_{\text{CaM}}^3} \right]}
\]

which assumes that the binding of Ca\(^{2+}\) to CCaMK EF-hands shows no cooperativity (Swainsbury et al., 2012). The equilibrium dissociation constants \(K_{\text{CaM}}^1\), \(K_{\text{CaM}}^2\), and \(K_{\text{CaM}}^3\) for the EF-hands 1, 2, and 3 are those determined by Swainsbury et al. (2012) (see Supplemental Table 2 online).

The fraction of Ca\(^{2+}\) unsaturated phosphorylated CCaMK by

\[
x_1 = \frac{k_1 (1 - x_0) (1 - x_1)}{k_{x0} x_0 + k_{x1} x_1}
\]

where \(x_0\) is the initial concentration of Thr-271 phosphorylated CCaMK. The concentrations of Ca\(^{2+}\) and CCaMK used in the assays were 0.1 mM and 0.3 \(\mu M\), respectively (see in vitro kinase assays in Methods). This model was fit to the averages of the data points obtained from five repeats of the phosphorylation time-course experiments. We sought to minimize the difference between the data points and the model values by varying the phosphorylation rate constants. The optimization was performed in MATLAB using simulated annealing within the SBML toolbox (Keating et al., 2006). The resulting residual 0.0114 for this model, gave a phosphorylation rates of \(k_1 = 0.0015 \text{ M}^{-1} \text{s}^{-1}\), \(k_2 = 0.7150 \text{ M}^{-1} \text{s}^{-1}\), and an initial concentration \(x_0 = 0.014 \text{ M}\).

**CCaMK Substrate Phosphorylation Modeling**

A system of coupled ODEs was used to describe the network of reactions regulating the kinase activity of CCaMK shown schematically in Figure 5A. Experiments have shown that the rate of Ca\(^{2+}\) binding to CCaMK (Swainsbury et al., 2012) and CaM is much faster compared with all other the rates of reaction described in Figure 5A: therefore, the steady state approximation was used to model Ca\(^{2+}\) binding to CCaMK and CaM. The Ca\(^{2+}\) binding equilibrium constants for the EF-hands are assumed to be the same for phosphorylated and nonphosphorylated CCaMK (Swainsbury et al., 2012). Our experimental investigations found no significant evidence to suggest any binding cooperativity between N- and C-terminal lobes of CaM. The Ca\(^{2+}\) binding to CaM was therefore modeled by assuming mutually independent steady state occupancy of EF-hand pairs situated at N- and C-terminal lobes. The expression used to calculate the steady state fraction of the population of CaM, which is bound to Ca\(^{2+}\) at all four EF-hands at a given Ca\(^{2+}\) concentration is therefore be given by

\[
\rho_{\text{CaM}} = \frac{\left[ \frac{\text{Ca}^{2+}}{\text{K}_{\text{CaM}1}^1} \right]}{\left[ \frac{\text{Ca}^{2+}}{\text{K}_{\text{CaM}1}^1} \right] + \left[ \frac{\text{Ca}^{2+}}{\text{K}_{\text{CaM}2}^2} \right] + \left[ \frac{\text{Ca}^{2+}}{\text{K}_{\text{CaM}3}^3} \right]}
\]

Here, the \(K_{\text{CaM}1}\) and \(K_{\text{CaM}2}\) are the experimentally determined dissociation constants for Ca\(^{2+}\) ions binding to N- and C-terminal lobes (see Supplemental Table 2 online).

The affinity for CaM was observed to decrease substantially for all EF-hand CCaMK point mutants (D413A, D449A, and D491A) in our experiments, suggesting that the CaM/Ca\(^{2+}\) complex preferentially binds to Ca\(^{2+}\)-saturated CCaMK. For simplicity, the model only considers the binding of CaM to Ca\(^{2+}\)-saturated CCaMK.

Surface plasmon resonance investigations into the effect of dissociation of Ca\(^{2+}\) ions from CaM on the binding of CaM to CCaMK suggested that the CaM-bound CCaMK complexes are Ca\(^{2+}\)-saturated (Swainsbury et al., 2012). Introduction of a chelator in the surface plasmon resonance experiment showed up to a 3 orders of magnitude increase in the rate of dissociation of CaM from CCaMK (Swainsbury et al., 2012). To account for this observation, only Ca\(^{2+}\)-saturated CaM complexes were assumed to bind to CCaMK in the model. The two different rates of dissociation for CCaMK/CaM complex corresponding to basal and spike level Ca\(^{2+}\) concentrations were incorporated in the ODEs by assigning different dissociation rates to Ca\(^{2+}\)-saturated and Ca\(^{2+}\)-unsaturated CCaMK/CaM complex populations. The rate of Thr-271 dephosphorylation of CCaMK is assumed to be comparable to the slower of the two phosphorylation rates.

All of the above key experimental observations are summarized in the reaction rate Equations 1 to 3 below, which were used to model the time evolution of the concentration of various forms of CCaMK, based on the reaction schemes described in Figure 5A. Equation 4 below was used to analyze the target phosphorylation via the kinase active forms of CCaMK, namely, Ca\(^{2+}\)-saturated CaM-bound CCaMK, Ca\(^{2+}\)-saturated CaM-bound Thr-271 phosphorylated CCaMK complex, and Ca\(^{2+}\)-saturated CCaMK.

\[
\frac{dx_1}{dt} = \rho_{\text{CaM}} (1 - p_{\text{ph}}) \left( k_1 (1 - x_1 - x_2 - x_3) + k_2 x_2 + k_3 x_3 + k_4 x_4 \right)
\]

\[
\frac{dx_2}{dt} = k_{\text{onpCaM}} (1 - p_{\text{ph}}) x_0 - k_{\text{offpCaM}} x_2 + k_{\text{onpCaM}} x_3 - k_{\text{offpCaM}} x_2
\]

\[
\frac{dx_3}{dt} = k_{\text{onpCaM}} (1 - p_{\text{ph}}) x_0 - k_{\text{offpCaM}} x_3
\]

\[
\frac{dx_4}{dt} = k_{\text{onpCaM}} (1 - p_{\text{ph}}) x_0 - k_{\text{offpCaM}} x_4
\]

Here, \(x_1\), \(x_2\), \(x_3\), and \(x_4\) represent the concentrations of Thr-271 phosphorylated CCaMK, CaM-bound CCaMK, CaM-bound Thr-271, phosphorylated CCaMK,
and phosphorylated target substrate. In our model, we assume that the total concentration of CCaMK (T) and CaM (C) is constant. This allows us to evaluate the concentration of nonphosphorylated CCaMK relatively easily from the expression: $T = x_1 - x_2 - x_3$. The other symbols and their biological meaning can be found in Supplemental Table 2 online.

The different activities of the three forms of active kinases for target phosphorylation have been reported previously (Takezawa et al., 1996). The activity of Ca$^{2+}$-saturated CaM-bound CCaMK complex and Ca$^{2+}$-saturated CaM-bound Thr-271 phosphorylated CCaMK complex was shown to be similar to within an order of magnitude, while Ca$^{2+}$-saturated CCaMK was found to have comparatively lower activity for target phosphorylation. A relative kinase activity factor $a$, was used in Equation 4 to reflect these observations. The lower activity of CCaMK relative to the CaM-bound form is key to understanding the CCaMK T271A mutant behavior. The hydrogen bond network between the kinase and CaM binding domain cannot be reinforced by phosphorylation in the mutant. This mutant can therefore be seen to behave like Ca$^{2+}$-saturated CCaMK. We simulated these effects of the T271A mutant, and the results are shown in Figure 5. The numerical analysis of the model ODEs and was performed using the ode45 solver on MATLAB.

**Supplemental Data**

Sequence data from this article can be found in GenBank/EMBL databases under the following accession numbers: M. truncatula CCaMK (OM3), Q6RE7T.1; M. truncatula CaM 1, AAM81202.1.

**References**


Calcium/Calmodulin-Dependent Protein Kinase Is Negatively and Positively Regulated by Calcium, Providing a Mechanism for Decoding Calcium Responses during Symbiosis Signaling

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