REVIEW ARTICLE

Structure–function of the multifunctional Ca^{2+}/calmodulin-dependent protein kinase II

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Ca^{2+}/calmodulin (CaM)-dependent protein kinase (CaMKII) is a ubiquitous mediator of Ca^{2+}-linked signalling that phosphorylates a wide range of substrates to co-ordinate and regulate Ca^{2+}-mediated alterations in cellular function. The transmission of information by the kinase from extracellular stimuli and the intracellular Ca^{2+} rise is not passive. Rather, its multimeric structure and autoregulation enable this enzyme to participate actively in the sensitivity, timing and location of its action. CaMKII can: (i) be activated in a Ca^{2+}-spike frequency-dependent manner; (ii) become independent of its initial Ca^{2+}/CaM activators; and (iii) undergo a 'molecular switch-like' behaviour, which is crucial for certain forms of learning and memory. CaMKII is derived from a family of four homologous but distinct genes, with over 30 alternatively spliced isoforms described at present. These isoforms possess diverse developmental and anatomical expression patterns, as well as subcellular locali-zation. Six independent catalytic/autoregulatory domains are connected by a narrow stalk-like appendage to each hexameric ring within the dodecameric structure. Ca^{2+}/CaM binding activates the enzyme by disinhibiting the autoregulatory domain; this process initiates an intra-holoenzyme autophosphorylation reaction that induces complex changes in the enzyme's sensitivity to Ca^{2+}/CaM, including the generation of Ca^{2+}/CaM-independent (autonomous) activity and marked increase in affinity for CaM. The role of CaMKII in Ca^{2+} signal transduction is shaped by its autoregulation, isoenzymic type and subcellular localization. The molecular determinants and mechanisms producing these processes are discussed as they relate to the structure–function of this multifunctional protein kinase.

Key words: autoinhibition, autophosphorylation, calmodulin, calcium oscillation, learning and memory.

INTRODUCTION

The cellular responses induced by many hormones, neurotransmitters and other signalling molecules follow a path from membrane-receptor activation to the elevation of second messengers. An elevation in the ion calcium (Ca^{2+}), like cAMP and diacylglycerol, functions as a major second messenger, because the intracellular concentration of Ca^{2+} can be maintained at extremely low levels, only to be increased following specific Ca^{2+}-mobilizing stimuli. Membrane pumps and Ca^{2+}-binding proteins rapidly buffer increases in intracellular Ca^{2+}, thereby allowing a discrete spatial control of its effectors and their targets. Temporal information in the frequency of the Ca^{2+} transients, or 'spikes', also may instruct the appropriate cellular response to Ca^{2+} [1,2].

Many of the cellular responses to Ca^{2+} signals are induced or modulated by a family of multifunctional Ca^{2+}/calmodulin (CaM)-dependent protein kinases (CaMKs): CaMKI, CaMKII and CaMKIV. These effectors translate and co-ordinate the dynamic second messenger, Ca^{2+}, into the appropriate cellular responses via phosphorylation, which is a rapid, highly specific and reversible post-translational modification involving a phosphate donor (ATP) and a phospho-acceptor amino acid (serine or threonine) in a substrate protein. These protein kinases, which are all linked to Ca^{2+} via the ubiquitous intracellular Ca^{2+} receptor CaM, have common as well as unique features with respect to their structure, regulation and activation (for reviews, see [3–5]). CaMKII, similarly to CaMKI and CaMKIV, has an autoregulatory domain that restricts or inhibits enzymic activity in the absence of Ca^{2+}/CaM. Ca^{2+}/CaM binding alone produces maximal activity of CaMKII, whereas CaMKI and CaMKIV have an activation loop that requires phosphorylation by an upstream CaMK kinase for maximal activity [6,7]. The multimeric CaMKII is phosphorylated by an intra-holoenzyme autophosphorylation reaction that is directed at either the autoregulatory domain or the CaM-binding domain, producing diverse effects in its autoregulation and sensitivity to Ca^{2+}/CaM (Figure 1). For example, autophosphorylation produces a state of CaMKII that retains enzymic activity even in the absence of Ca^{2+}/CaM, a form of activity known as Ca^{2+}/CaM-independent activity (autonomous activity), without affecting its maximal Ca^{2+}/CaM-stimulated activity. This phosphorylation involves a kinase cascade of sorts, with each subunit of the holoenzyme acting as both kinase and kinase of a kinase. Autophosphorylation is also associated with a 1000-fold increase in the affinity for its activator Ca^{2+}/CaM (CaM trapping); however, autophosphorylation within the CaM-binding domain following CaM dissociation of activated/autophosphorylated enzyme restricts or prevents CaM from rebinding (CaM capping). The mechanisms and consequences of autophosphorylation are central to this enzyme's complex autoregulatory behaviour, potentially underlying its ability to become differentially activated at different frequencies of calcium spikes. This, in turn, might enable CaMKII to act as a calcium spike frequency detector and to behave as a 'molecular switch' in learning and memory as a
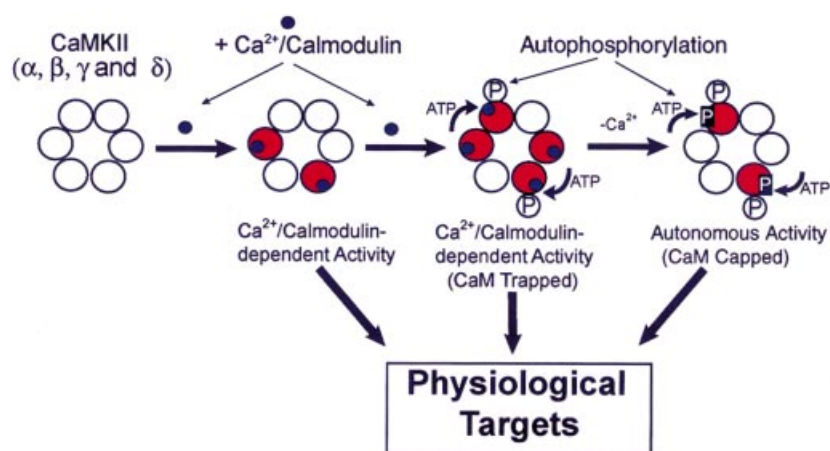
Figure 1   CaMKII undergoes multiple autoregulatory states that may have an impact on its function following Ca^{2+}/CaM activation

The multimeric holoenzyme structure of CaMKII is depicted as a 6-mer for simplicity, with activated catalytic subunits illustrated in red. In addition to the Ca^{2+}/CaM-dependent form of the enzyme phosphorylating physiological targets throughout the cell, autophosphorylation produces functional changes in CaMKII (autonomous activity, CaM trapping, CaM capping) that may alter further its activity, regulation and function.

readout of synaptic activity. Thus the functional properties of CaMKII are directly linked to its unique multimeric structure, autoregulation/activation and autophosphorylation. This review focuses on the molecular determinants of these features, as well as how these mechanisms impact the cellular function of CaMKII.

ISOFORM DIVERSITY AND STRUCTURE

Because CaMKII phosphorylates a variety of substrates, this multifunctional serine/threonine kinase has been implicated in regulating many aspects of cellular function in response to Ca^{2+} signalling, including the regulation of carbohydrate, amino acid and lipid metabolism, ion channels/receptors, neurotransmitter synthesis and release, transcription and translation, cytoskeletal organization and calcium homoeostasis (for reviews, see [8–13]). However, in examining the role of CaMKII in Ca^{2+} signalling, one must be aware that these assigned functions are contributed by a family of isoforms derived from four closely related yet distinct genes (α, β, γ and δ). CaMKII is highly enriched in brain tissue, where the brain-specific α (54 kDa) and β (60 kDa) isoforms constitute up to 2% of the total protein in the hippocampus of rodents and up to 1% of the total protein in the forebrain itself [14]. A cytosolic concentration of 19–37 μM

for the α subunit has been estimated in forebrain homogenate [14–16]. Its abundance in brain, relative to other tissues, probably contributed towards its initial discovery [17,18] and characterization in brain tissue [19–21]. CaMKII is expressed throughout the body; however, the γ and δ isoforms are present at approx. 0.02% of the level of the brain-specific forms [22].

CaMKII is a multi-subunit holoenzyme. The Stoke's radius [81.3–94.7 Å (1 Å = 0.1 nm)] and sedimentation coefficient (13.7–16.4 S) of purified brain CaMKII indicates that the holoenzyme is approx. 460000–654000 Da and is composed of 8–12 subunits [20,21]. Multiple populations of mixed holoenzymes have been purified (α:β ratios of 6:1, 3:1 and 1:1) from the rat forebrain [23]. Consistent with previous studies [20,21], an isoform ratio of 3:1 for α:β comprised the majority of the CaMKII activity, with other forms each contributing an estimated 5–10% [23]. In addition to mixed heteromultimers of CaMKII, homomers of the α isoform also have been identified from rat forebrain [24]. Thus, as discussed in detail in previous reviews, mixed and homomeric CaMKII holoenzymes have been purified from brain, with the isoform composition and ratio of the holoenzymes dictated by anatomical, developmental and local mRNA regulation within cells [8,25,26].

A linearized diagram for the domain structure of CaMKII is shown in Figure 2. The prototypical CaMKII subunit possesses
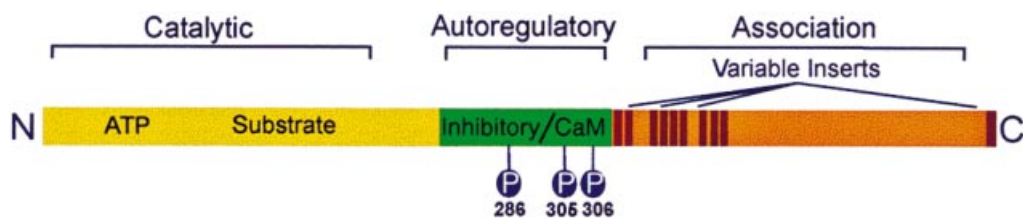

Figure 2   Linear diagram of a prototypical CaMKII subunit

The catalytic domain is autoinhibited by a pseudosubstrate autoregulatory sequence that is disinhibited following Ca^{2+}/CaM binding. The association domain produces the native form of the enzyme, a multimeric holoenzyme composed of 12 subunits. Isoform differences present in the α, β, γ and δ isoforms of CaMKII are contributed primarily by a region of multiple alternatively spliced sequences, termed variable inserts, which reside in the association domain. Conserved sites of autophosphorylation are indicated in the autoregulatory region.

a catalytic, autoregulatory and association domain, with all of the isoforms sharing approximately 89–93 % sequence similarity in their catalytic and autoregulatory domains [22]. CaMKII is maintained in an autoinhibited state by an autoregulatory domain that acts as a pseudosubstrate, preventing substrates from binding. The Ca²⁺/CaM-binding domain is contiguous to the autoregulatory domain. The isoforms all share autophosphorylation sites within the autoregulatory regions, including Thr²⁸⁶ (Thr²⁸⁷ in isoforms β, γ and δ) in the core regulatory domain, and Thr³⁰⁵/Thr³⁰⁶ (Thr³⁰⁶/Thr³⁰⁷ in isoforms β, γ and δ) in the CaM-binding domain. The association domain is necessary for formation and assembly of the CaMKII holoenzyme, the native form of the enzyme. The primary difference between the CaMKII isoforms results from a series of inserts C-terminal to the Ca²⁺/CaM-binding domain and within the association domain, termed the variable region. The CaMKII family is expanded further due to alternative processing of the α [27–30], β [31–35], γ [22,36–39] and δ [40,41] genes. The molecular masses of these subunits range from 25 kDa to 72 kDa. A summary of the genomic construction for the 30 alternatively spliced isoforms described to date for CaMKII is published elsewhere [11,42].

With the exception of α-KAP (α-CaMKII association protein; 25 kDa), an alternatively spliced isoform of the α gene, all CaMKII isoforms identified to date have regulatory and catalytic components necessary to function as a Ca²⁺/CaM-dependent kinase, and undergo the autoregulatory features described for the α and β isoforms. Thus, although the CaMKII isoforms appear to have the same capacity to function as a Ca²⁺/CaM-dependent kinase, isoforms may possess subtle yet unique differences that we currently are not able to fathom, as well as differences attributed to inserts or regions that are important for targeting and subcellular localization.

Currently, there are many isoforms that do not have any known functional differences resulting from their alternatively spliced sequence. For example, the β' form is alternatively spliced so that, unlike the β isoform (plus select γ and δ isoforms), it is missing an autophosphorylation site [32]. This autophosphorylation site (Thr³⁸² in the β subunit) is phosphorylated under limiting conditions and is therefore considered a fast autophosphorylation site (similar to Thr²⁸⁶α/Thr²⁸⁷$\beta\gamma\delta$) [26]. However, the consequence of spliced variants containing or missing this autophosphorylation site is unknown, because this site of autophosphorylation currently has no known function.

Several of the CaMKII isoforms have alternatively spliced domains or sequences conferring subcellular targeting on CaMKII. For example, an alternatively spliced form of the α gene, the α_B isoform, contains a nuclear-localizing sequence (NLS) that is functional [29] and is regulated by phosphorylation of a serine residing within the NLS [43]. Evidence for isoform ratio influencing subcellular targeting was observed using heterologous expression systems, whereby the ratio of transfected subunits containing an NLS relative to a non-nuclear insert was observed to influence the subcellular localization of CaMKII holoenzymes [29]. The extent to which isoform dominance contributes to this targeting, or whether different subcellular localization signals require different 'critical' ratios in either conferring or preventing a given subcellular localization, is not known. In addition, the complexity is enhanced further by the fact that multiple targeting domains can exist within the same subunit. For example, an isoform of the β gene, β_M, contains an NLS insert similar to the α_B isoform; however, it also contains a short hydrophobic membrane-targeting insert. The β_M isoform is expressed preferentially in muscle, where CaMKII holoenzymes appear to be targeted primarily to the sarcoplasmic reticulum, not the nucleus [35]. Further studies are needed to
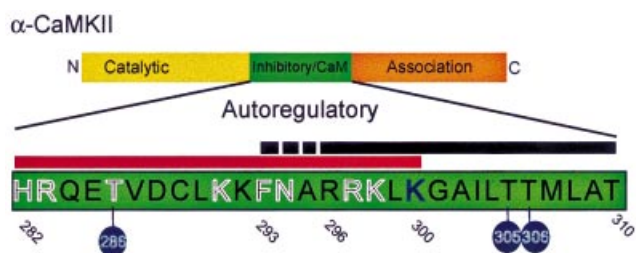

Figure 3   An expanded view of the α-CaMKII autoregulatory domain

The reddish-brown bar designates autoinhibitory region, whereas the black bar specifies the minimal CaM-binding domain. Residues important for autoinhibition are designated in white letters, with the P0 residue (P0 is the phospho amino acid acceptor, whereas P($-$/$+$ n) is the number (n) of amino acids either N- or C-terminal to P0 respectively) of the pseudosubstrate sequence indicated in blue. Sites of autophosphorylation that modulate the autoregulation of CaMKII are illustrated by the 'P' labelled blue pins.

determine how subunits containing different targeting motifs interact and function within the same subunit, as well as in mixed holoenzymes, where different isoform subunits, potentially with different subcellular targeting domains, interact and possibly compete to influence a given holoenzyme's subcellular distribution.

ACTIVATION AND AUTOINHIBITION OF CaMKII

Molecular determinants of autoinhibition

The autoregulatory domain of CaMKII ensures that basal kinase activity levels remain 100–1000-fold below the maximal Ca²⁺/CaM-stimulated value. A classic form of this autoinhibition is via a pseudosubstrate type of interaction (for reviews, see [44,45]). In this mode of inhibition, the autoinhibitory domain has residues that mimic a protein substrate or nucleotide to interact with the catalytic domain, thereby blocking access to the substrate-binding pockets. Binding of an allosteric activator alters the conformation of the autoinhibitory domain, and decreases its inhibitory potency to permit substrates' access to the catalytic site.

CaMKII displays a complex mode of pseudosubstrate inhibition, where both the ATP-binding pocket and the protein substrate-binding pocket are inhibited via the autoregulatory domain. Like all known Ca²⁺/CaMKs, the autoregulatory domain of CaMKII is N-terminal and contiguous with the CaM-binding domain. Peptides that include the minimal CaM-binding sequence (amino acids 296–309 of the α subunit), as well as amino acids 290–295, produced an inhibitor of CaMKII that is competitive with respect to peptide substrate, but non-competitive with respect to ATP (IC₅₀ = 25 μM) [46]. Further N-terminal extension of this inhibitor peptide to residue 281 produced a more effective inhibitor (K_i = 0.2 μM), and shifted the kinetics of inhibition to non-competitive with respect to peptide substrate and competitive with respect to ATP [47,48]. Figure 3 illustrates the autoinhibitory (red bar) and CaM-binding domain (black bar) for the α subunit, with key residues identified as being important for autoinhibition designated in white. For example, point mutations created in an autoinhibitory domain peptide (α281–302) was used to identify His²⁸², Arg²⁸³, Arg²⁹⁷, Lys²⁹¹ and Lys²⁹⁸ as being important for interaction with the catalytic domain [49]. Interestingly, although mutation to alanine for any one of these residues reduces the inhibitory potency of each peptide, only a basic amino acid substitution at His²⁸² was observed to shift the kinetics of inhibition, from competitive with
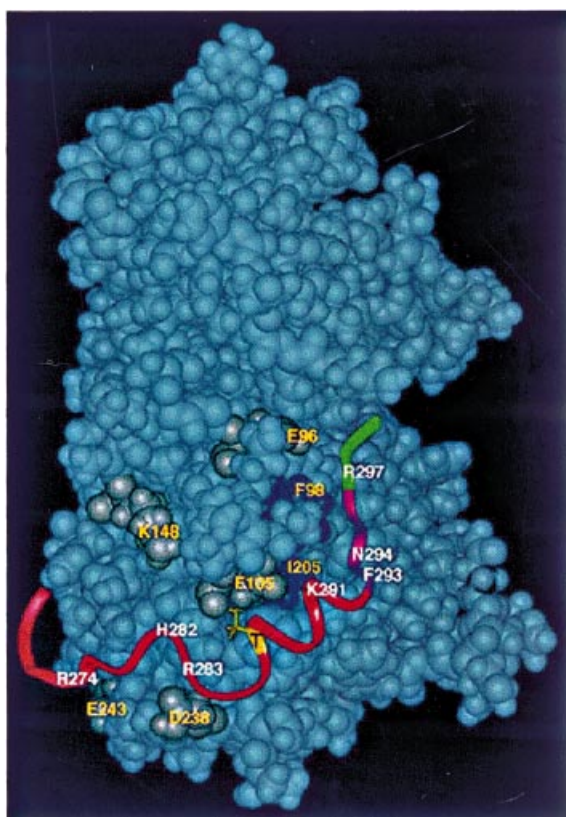
Figure 4   Molecular model of monomeric α-CaMKII

The catalytic domain (residues 3–271), shown in blue, was constructed by hybrid modelling on the basis of the co-ordinates of CaMKI and PKA. Residues in the catalytic domain involved in making electrostatic (grey) and hydrophobic (dark blue) interactions with the autoregulatory domain are labelled in yellow. The autoinhibitory domain (residues 272–300) are shown in ribbon format, with residues predicted to interact with the catalytic domain shown in white, as well as key residues discussed throughout the text: Thr[286] (yellow), Phe[293]/Asn[294] (magenta) and Arg[297] (green). Reproduced, with permission, from Yang, E. and Schulman, H. (1999) J. Biol. Chem. **274**, 26199–26208, © The American Society for Biochemistry & Molecular Biology.

ATP and non-competitive with respect to peptide substrate to non-competitive with ATP. His[282] clearly is an important residue; its protonation can alter the inhibitory potency of peptides and produce constitutive activity in the native enzyme [49]. Single-point mutations of His[282] (Gln, Arg and Glu) in CaMKII produced a 10-fold-lower K_m for ATP for basal (Ca^{2+}/CaM-independent) autophosphorylation [50]. These data may be interpreted to suggest that His[282] mimics the adenine of ATP to sterically block ATP binding in the autoinhibited state, and that pH and Ca^{2+}/CaM binding disinhibit His[282] to remove the ATP block and functionally decrease the K_m for ATP.

Molecular modelling of CaMKII autoinhibition

In the absence of a crystal structure and direct structural confirmation for CaMKII, it is not readily possible to determine whether His[282] makes a direct contact with the ATP-binding pocket, or whether it co-ordinates a series of contacts via the catalytic core to inhibit ATP binding in the inactive state. As such, molecular modelling studies of CaMKII have attempted to utilize the known structure of other kinases to generate predictive structural information about the autoregulation of CaMKII

[50,51]. A recent protein engineering and computer modelling study used a rational mutagenesis strategy to identify potential contacts between the catalytic and autoinhibitory domains [52]. This rational mutagenesis strategy, described by Gibbs and Zollar and applied to myosin light chain kinase [53], identified residues in the autoregulatory domain that might interact with the catalytic domain [54,55]. Using the constraints revealed by biochemical experiments, a hybrid model was constructed on the basis of the crystal structure of cAMP-dependent protein kinase (PKA) complexed with protein kinase inhibitor (PKI) and CaMKI [52]. This model, shown in Figure 4, identifies several key features of the autoregulatory domain (red ribbon) and its corresponding interactions (numbered residues shown in grey) with the catalytic lobes (space-filling model shown in blue). The core pseudosubstrate sequence was identified as residues 297–300 by mutational analysis, with Arg[297] at the P−3 position (see the legend to Figure 3) as being previously identified [50,56]. Other important residues identified in the autoregulatory domain included Arg[274], His[282], Arg[283], Lys[291], Arg[297], Phe[293] and Asn[294], and point mutations of these residues have been observed to increase constitutive activity or CaM binding [52]. Thus this study confirmed many of the residues identified using peptide-inhibition studies [49], as well as finding new residues reported previously as being important for high-affinity CaM binding [57]. In this model, the Thr[286] residue is buried within a hydrophobic pocket of the catalytic domain, producing a possible explanation for why mutations in this residue to hydrophobic amino acids have no effect on constitutive activity [58,59], whereas mutation of Thr[286] to charged residues (aspartate and lysine) produces an increase in constitutive activity [52,58,59]. In addition, many mutations that lead to constitutive activity do not result in Thr[286] autophosphorylation, supporting the hypothesis that Thr[286] autophosphorylation is not required for enzymic activity, and that this residue might have contacts with the catalytic core until Ca^{2+}/CaM binding exposes this site for autophosphorylation. Thus this molecular model provides an explanation for both autophosphorylation and the molecular basis of autonomous activity [52]. However, in contrast with a previous model [50], His[282], along with the other residues in the N-terminal region of the autoregulatory domain, are not positioned to interact directly with the ATP-binding pocket in this model, but rather make indirect contacts via the catalytic core to give rise to the competitive inhibition observed with ATP. Taken together, the biochemical, mutational and modelling studies described above have indicated a bi-substrate autoinhibitory interaction, whereby the ATP-binding pocket and the protein substrate-binding site are blocked by the N- and C-terminal regions of the autoregulatory domain respectively. Further experiments and/or structural information are needed to verify the precise nature of the ATP competitive mode of CaMKII autoinhibition.

The ATP-binding domain of α-CaMKII is located between amino acids 19–46, and includes the consensus ATP-binding motif (GK[42]GAFS) [28]. A 12-amino-acid peptide (residues 23–34 of CaMKII) generated from this region was recently reported to inhibit Ca^{2+}/CaM-dependent substrate phosphorylation, as well as autophosphorylation, with an IC[50] of 3 μM for CaMKII, 12.5 μM for CaMKI and 85 μM for CaMIV [60]. Interestingly, this peptide did not inhibit autonomous activity. No inhibition was observed for PKA in that study, suggesting that this peptide may have specificity for Ca^{2+}/CaM-dependent kinases, particularly CaMKI and CaMKII [60]. This peptide did not appear to bind either ATP directly or Ca^{2+}/CaM, and kinetic analysis indicated a mixed-type pattern of inhibition with respect to ATP and substrate. Although the binding site for this peptide in

producing this inhibition is unknown, comparisons with the structure of CaMKI [61], as well as the model of CaMKII [52], suggest that this region may be involved in an autoinhibitory interaction with domains other than the pseudosubstrate region of CaMKII, yet which, like the pseudosubstrate region, appears to be influenced by the autophosphorylation state of CaMKII [60].

CaMKII activation by Ca²⁺/CaM

Displacement of the autoregulatory region from interaction with the ATP- and protein substrate-binding sites in the catalytic domain occurs by Ca²⁺/CaM activation. Half-maximal activation of CaMKII occurs at a concentration of 0.5–1 μM free Ca²⁺ [26], whereas at saturating Ca²⁺, half-maximal activity is observed at 25–100 nM CaM [21,62–64]. Although studies initially reported that the enzyme behaved in a 'switch-like' manner, with maximal enzymic activity occurring at sub-stoichiometric Ca²⁺/CaM binding [65,66], the binding of Ca²⁺/CaM to the subunits of CaMKII in solution is 1:1 [67], and maximal activation of the enzyme appears to require stoichiometric Ca²⁺/CaM binding [64]. Unlike CaMKII purified from brain cytosol, CaMKII purified from cytoskeletal preparations reportedly exhibits positive co-operativity in the Ca²⁺/CaM activation for substrate phosphorylation and autophosphorylation [68], suggesting that CaM activation of CaMKII may be influenced by its subcellular targeting.

CaM contains four Ca²⁺-binding sites. Two Ca²⁺ ions are co-ordinated via two EF hand-like structures within each lobe, with the N- and C-terminal lobes connected by a flexible linker. The conformational change in CaM associated with Ca²⁺-binding produces an increased affinity for its target helix (for reviews, see [4,69]). The CaM-binding domain is highly conserved among the various isoforms of CaMKII, and is predicted to form an amphipathic α helix, a common motif among Ca²⁺/CaM-binding peptides [46]. Ca²⁺/CaM-binding peptides from multiple proteins are highly variable, and yet the sequences possess several common features: (i) patches of basic amino acids at the beginning and at the end of the sequence; and (ii) hydrophobic residues within the intervening sequence, as well as from the N- and C-terminal anchor points. Complementary patches of hydrophobic and acidic amino acids in CaM interact with the acceptor residues to stabilize the complex. The central 'tether' of CaM produces the flexibility to accommodate different sequences and structural variations observed in target domains of CaM-binding proteins [70].

As illustrated by the black bar in Figure 3, residues α296–309 comprise the minimal core CaM-binding domain of CaMKII [46,71]. The crystal structure (Protein Data Bank #1CDM) of Ca²⁺/CaM binding to a peptide derived from the CaM-binding domain of CaMKII (α290–314) indicates that the lobes of Ca²⁺/CaM wrap around the target sequence in a head-to-tail orientation, with the C-terminal lobe of Ca²⁺/CaM interacting with the N-terminal lobe of the Ca²⁺/CaM-binding sequence of CaMKII [72]. In the crystallized complex, contacts between CaM and the peptide appeared to occur in all of the residues 293–310, except for Asn²⁹⁴, which N-caps the target α-helix by hydrogen-bonding with Arg²⁹⁷ [72]. Because the crystal was generated using an α-CaMKII peptide sequence displaying picomolar binding affinity [73], this structure is presumed to represent the high affinity (CaM-trapped) form. As described below, autophosphorylation alters the affinity of CaMKII for Ca²⁺/CaM by more than 3 orders of magnitude, rendering this one of the highest affinities reported to date for Ca²⁺/CaM target interaction.

Ca²⁺/CaM and Mg²⁺/ATP binding

The half-maximal value for CaMKII activation by Ca²⁺/CaM is influenced by the ATP concentration [64]. Differential poly-(ethylene glycol) precipitation of CaMKII indicated that Ca²⁺/CaM binding is stoichiometric in the presence of ATP, but sub-stoichiometric in its absence [74]. The K_d in this assay was determined to be 150 pM in the presence of ATP, and 2 μM in the absence of ATP, with a half-maximal increase in CaM binding at 10 μM ATP. This was also observed under conditions not supporting autophosphorylation, including both ADP and a non-hydrolysable analogue of ATP. ATP binding has been reported recently to stabilize bound Ca²⁺/CaM. Using a double-fluorophore-labelled CaM that produces fluorescence energy transfer depending on the conformation or distance of the N- and C-terminal lobes of CaM, Torok et al. [75] recently reported that Ca²⁺/CaM activation of CaMKII is associated with at least two distinct conformational states of bound CaM. The fully compact state, as defined in that study by the inter-probe distance between lobes of CaM and the peptide-binding domain of CaMKII, is partially assumed for full-length CaMKII in the presence of non-hydrolysable ATP analogues; however, the production of the most compact conformer requires ATP and might be a consequence of Thr²⁸⁶ autophosphorylation. In these studies, the net on-rate for Ca²⁺/CaM was not affected by ATP; however, the net Ca²⁺/CaM compaction was approximately an order of magnitude slower in the presence of ATP (or ADP), suggesting that unproductive complexes, possibly due to an ordered-ligand binding reaction, occur in the activation mechanism of CaMKII by Ca²⁺/CaM [75].

In addition to Ca²⁺/CaM binding being influenced by ATP concentration [64,67,74], Ca²⁺/CaM binding also influences the binding of ATP. Although the K_d for ATP binding is not known either in the presence or absence of Ca²⁺/CaM, the K_m for ATP is reported to be in the range of 10–20 μM for substrate phosphorylation [21]. Because CaMKII undergoes autophosphorylation in the presence and absence of Ca²⁺/CaM, both the influence of CaM binding on the enzyme's affinity for ATP and the rate of autophosphorylation can be measured. Basal autophosphorylation of CaMKII occurs at Thr³⁰⁶ within the CaM-binding domain [76,77]. This process appears to result from Thr³⁰⁶ having access to the catalytic cleft in the autoinhibited state [76,77]. The K_m for ATP for basal autophosphorylation is 150 μM, whereas in the presence Ca²⁺/CaM the K_m for autophosphorylation is 20 μM [77]. Thus CaMKII can bind and hydrolyse ATP in the absence of Ca²⁺/CaM binding; however, Ca²⁺/CaM binding influences this process, possibly via an increase in the affinity of ATP for the enzyme. In addition to Ca²⁺/CaM, bivalent ions in the absence of ATP have also been reported to increase the exposure or accessibility of the ATP-binding site [78,79]. Whether this is mechanistically similar to bivalent co-ordination of ATP binding and catalysis, as described for PKA, is currently unknown [80,81]. Thus the ATP-binding domain and the Ca²⁺/CaM-binding domain appear to communicate at the molecular level via disinihibition of the autoinhibitory domain to reciprocally enhance each other's binding; interactions that also appear to be influenced by autophosphorylation within the autoregulatory domain occur via a process discussed later in this review.

Reaction mechanism for CaMKII

Detailed kinetic analysis of the CaMKII activation mechanism has yielded different results that are probably a function of enzyme sources and substrates examined. Initial studies using casein as a substrate favoured a rapid-equilibrium random
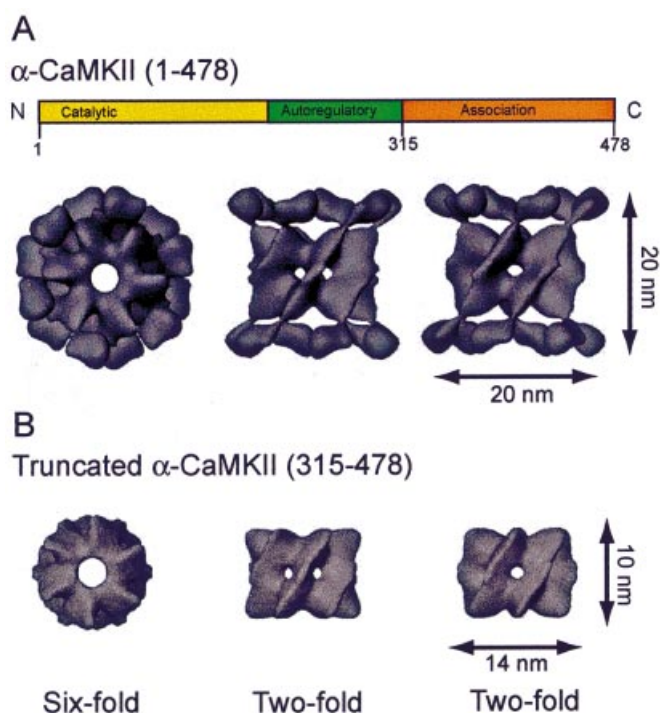
A

α-CaMKII (1–478)

B

Truncated α-CaMKII (315–478)

Six-fold   Two-fold   Two-fold

Figure 5   Shaded surface views for three-dimensional structures of (A) α-CaMKII (1–478) and (B) truncated α-CaMKII (315–478), obtained following transmission electron microscopy and image reconstruction

The resolution of the α-CaMKII (**A**) and truncated α-CaMKII (**B**) structures were 25 and 28 Å respectively. The 6-fold view was rotated 90° about a horizontal axis through the centre of the molecule to produce the 2-fold view, whereas the second 2-fold view was generated by rotating this view 30° about a vertical axis through the centre of the molecule. The absence of the foot-like extensions on the truncated α-CaMKII indicates that the catalytic/autoregulatory domains reside in this region of the molecule. Adapted, with permission, from Kolodziej, S. J., Hudmon, A., Waxman, M. N. and Stoops, J. K. (2000) J. Biol. Chem. **275**, 14354–14359, © The American Society for Biochemistry & Molecular Biology.

mechanism [21]. A similar mechanism was also obtained using microtubule-associated protein 2 as a substrate in a detailed kinetic study under steady-state conditions [64]. In the presence of saturating levels of Ca^{2+}/CaM, a rapid-equilibrium random Bi Bi mechanism was favoured, due to the capacity for CaMKII to bind protein substrates in the absence of ATP. This conclusion was supported by additional studies whereby substrates of CaMKII appear to co-purify with the kinase [62,82–85]. A rapid-equilibrium random mechanism was also reported for other Ca^{2+}/CaM-dependent protein kinases, including skeletal muscle myosin light chain kinase and phosphorylase kinase [86,87]. These results are inconsistent with the reaction mechanism reported for a proteolytically cleaved constitutively active CaMKII using a synthetic peptide substrate, syntide-2 [88]. In that study, a rapid-equilibrium ordered mechanism was most consistent with the data, with nucleotide binding first in both the forward and reverse reactions. The use of synthetic peptides, such as syntide-2, would appear to reduce the complicating properties of native polypeptide substrates, such as microtubule-associated protein 2, which also binds Ca^{2+}/CaM. However, synthetic peptides do not have the steric constraints of native protein substrates. Further studies are necessary to determine how the presence of an autoregulatory domain and/or substrate differences used in these studies might influence the observed reaction mechanism of CaMKII.

STRUCTURE OF CaMKII HOLOENZYME

Consistent with the hydrodynamic and biophysical properties of CaMKII, single particle images of CaMKII viewed by negative staining or rotary shadowing indicate that this enzyme is a multimeric holoenzyme composed of 'lollipop'-like extensions that elaborate from a central core complex [89–91]. The three-dimensional (3D) structure for α-CaMKII was resolved recently [92] using transmission electron microscopy and image analysis of both the full-length α-CaMKII and a truncated fragment of α-CaMKII containing only the association domain (amino acids α315–478). The 3D structure of CaMKII displays $6 \times 2 \times 2$ symmetry, as shown in Figure 5, where the reconstruction of the truncated association domain is shown below the full-length reconstruction of α-CaMKII. The association domain comprises the dodecameric core of the holoenzyme, with the catalytic domain contained within the peripheral particles or 'foot'-like structures and stalk that decorate the top and bottom of the hexameric rings. On the basis of the comparison of the truncated fragment and the full-length reconstruction of CaMKII, the stalk-like tether radiating out from the association domain appears to result from the C-terminal residues of the Ca^{2+}/CaM-binding domain [92]. However, deleting this (residues α317–328) did not produce any apparent change in the morphology of single-particle images [91], indicating that the linker segment hypothesized to connect the association and catalytic domains must be explored further [90].

The 12 subunits of CaMKII are organized into a ring-like structure, whereby the catalytic/autoregulatory components of each subunit are attached to the hexameric ring by a stalk-like appendage that presumably allows subunits to behave independently of one another for activity and Ca^{2+}/CaM binding, but in concert with one another for an intra-holoenzyme autophosphorylation reaction – a process discussed in the next section. The mechanism of subunit assembly in forming the holoenzyme is unknown. However, thresholding the 3D reconstruction of α-CaMKII until only the highest areas of protein density remain visible suggests that dimers of subunits between rings initially interact to assemble the dodecameric structure [92]. Molecular determinants of holoenzyme assembly have been studied using functional expression and gel filtration; however, yeast two-hybrid binding assays as well as *in situ* biophysical and functional assays have also been employed [93–95]. Shen and Meyer [95] reported that a 135-amino-acid stretch within the C-terminal domain (residues 344–478) of α-CaMKII were necessary and sufficient for holoenzyme formation [95]. In addition, deletion analysis identified six residues (α344–350) from the N-terminal end of the association domain (α350–478) important for multimerization, as well as 11 residues from the C-terminus. Kolb et al. [94] reported that the minimal regions required for oligomerization in the α and β isoforms resided between residues 315–478 and 314–542 respectively. C-terminal truncations of the α subunit indicated that amino acids 382–478 are necessary for holoenzyme formation, whereas residues between 427 and 478 contribute primarily to holoenzyme formation by stabilizing the complex [94]. Interestingly, the formation of β homomers or mixed α/β holoenzymes appears to require additional sequence requirements that are not required in the formation of homomeric α holoenzymes. Although residues between α355 and 478 formed homo- and hetero-α/β oligomers, further truncation analysis revealed that α382–478 failed to produce heteromultimers of α and β subunits, although still fully capable of forming homomers of α subunits. As expected, truncation analysis of the β subunit by removing an analogous region resulted in the production of only monomeric β-CaMKII,

indicating that this domain required for β holoenzyme assembly is conserved between the α and β isoforms. Thus the assembly of 12 subunits into the CaMKII holoenzyme requires relatively small domains within the context of the 15 kDa association domain, with residues residing at both the N- and C-terminal ends of the association domain important for assembly and/or stability of the holoenzyme complex.

MOLECULAR MECHANISM OF Thr²⁸⁶ AUTOPHOSPHORYLATION

Fully active catalytic fragments of CaMKII produced by limited proteolysis generated some of the first insights into the mechanism of disinhibition via Ca²⁺/CaM activation and autophosphorylation. These kinase fragments have no endogenous autoinhibitory domain; nor do they require Ca²⁺/CaM for enzymic activity [96,97]. The synthetic peptide (residues 281–309) derived from the autoinhibitory domain of CaMKII is a potent inhibitor of enzyme activity, and inhibits the catalytic fragment with a K_i of 0.2 μM [47,48]. Addition of Ca²⁺/CaM was observed to alter the properties of peptide 281–309, turning it from an inhibitor into a substrate. The conformational change in the peptide induced by Ca²⁺/CaM binding exposes a site of phosphorylation on the 281–309 peptide at Thr²⁸⁶ [98]. Phosphorylation of this residue decreased the inhibitory potency of the autoinhibitory peptide by over 10-fold [47]. Thus peptide 281–309 is an inhibitor that becomes a substrate following Ca²⁺/CaM binding. This peptide study characterized an important feature in the formation of Ca²⁺/CaM-independent activity. The region surrounding Thr²⁸⁶ is inhibitory, and Ca²⁺/CaM binding alters the conformation of this region to turn it into a substrate, a process that ultimately decreases the inhibitory potency of the autoinhibitory-like sequence. It is this molecular mechanism that disrupts the autoinhibitory interaction with the catalytic domain following Ca²⁺/CaM binding, and continues to produce such a disinhibition in the absence of Ca²⁺/CaM to produce autonomous activity.

This Thr²⁸⁶ residue, as shown in Figure 3, is present in all isoforms of CaMKII (Thr²⁸⁷ in β, γ and δ subunits), except α-KAP. Phosphopeptide analysis identified Thr²⁸⁶ and Thr²⁸⁷ (α and β subunits respectively) following autophosphorylation and generation of autonomous activity [99–103], and dephosphorylation of Thr²⁸⁶ converts the enzyme back into a Ca²⁺/CaM-dependent state [65,104,105]. Site-specific mutagenesis of this site to non-phosphate-accepting residues, such as alanine or leucine, resulted in enzyme that could be fully activated by Ca²⁺/CaM, but which exhibited no autonomous activity, demonstrating the necessity of Thr²⁸⁶ autophosphorylation in the generation of autonomous activity [58,106,107]. Substitution of the Thr²⁸⁶ residue with a charged amino acid, such as aspartate, produced constitutive activity [58,59]. Thus Thr²⁸⁶ autophosphorylation is both necessary and sufficient for the generation of autonomous activity. Mutagenesis of Arg²⁸³ also disrupts the generation of autonomous activity [107,108]. This site is in the consensus phosphorylation sequence for CaMKII, sitting at the P$-$3 position, and is therefore important for substrate recognition of the Thr²⁸⁶ site.

Does autophosphorylation occur within a holoenzyme, or do CaMKII holoenzymes autophosphorylate one another? Initial investigations of this question suggested an intra-holoenzyme autophosphorylation mechanism. For instance, mixing holoenzymes with a catalytically inactive kinase domain (Lys⁴² → Met) with fully functional CaMKII holoenzymes did not produce autophosphorylation on Thr²⁸⁶ of the inactive holoenzymes [109–111]. Furthermore, the lack of a dependence of the CaMKII autophosphorylation rate on concentration also supported an intra-holoenzyme process [104,112].
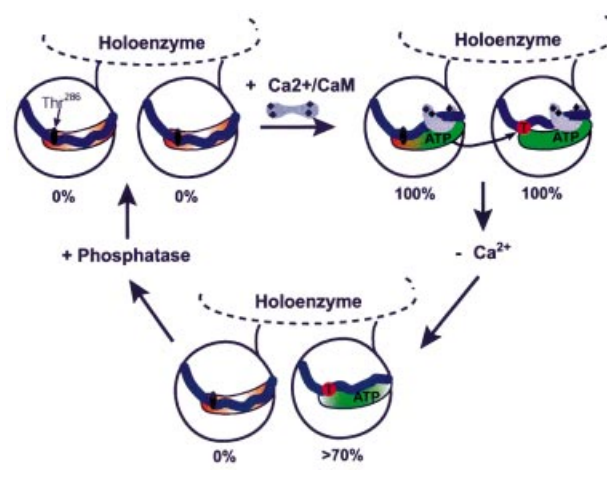

Figure 6   The autophosphorylation of CaMKII requires coincident Ca²⁺/CaM binding between neighbouring subunits within the holoenzyme

The catalytic/autoregulatory region is shown for each subunit. In the absence of Ca²⁺/CaM, the blue autoregulatory domain inhibits catalytic activity (inactivity illustrated in red). Ca²⁺/CaM binding to each subunit independently disinhibit the autoregulatory region to produce catalytic activity (green). Activated subunits may then act as both kinase and substrate in an intersubunit–intraholoenzyme autophosphorylation reaction of Thr²⁸⁶ (shown as 'T' within the red circle). Only the autophosphorylated subunit retains activity in the absence of Ca²⁺/CaM. Although this autonomous activity is uncoupled from its dependence on Ca²⁺/CaM, this enzymic state is sensitive to phosphatase activity.

Previous investigations have also suggested that autophosphorylation occurs between different subunits of a CaMKII holoenzyme. Mixed holoenzymes engineered to contain both catalytically inactive and active subunits within the same molecular assembly showed Thr²⁸⁶ autophosphorylation on functional and dead subunits [110,111]. Furthermore, the autophosphorylation of a monomeric form of CaMKII revealed a concentration-dependence on its reaction rate, indicating that this process is a bimolecular reaction, and hence is intermolecular in nature [109].

While the biochemical investigations of CaMKII autophosphorylation described above suggested an intra-holoenzyme, intersubunit autophosphorylation reaction, the recent 3D electron microscopy structure of CaMKII did not favour this mechanism because of the large distance ($\approx$ 40–50 Å) between kinase subunits [92]. How might the structural and biochemical data be reconciled? One hypothesis involved the recently described propensity of CaMKII holoenzymes to self-associate into supra-molecular complexes [113–116]. If CaMKII was in an aggregated form during past investigations of autophosphorylation, the results of those experiments might be ambiguous: it is possible that the autophosphorylation was occurring between different holoenzymes, but appeared to be intra-holoenzyme owing to the supra-molecular, or aggregated, state of the kinase. However, a recent study using chemical quench-flow kinetics has examined the mechanism of autophosphorylation using dynamic light scattering to verify that the preparations of CaMKII are mono-dispersed [117]. In that study, the autophosphorylation rate of CaMKII holoenzymes (12 s⁻¹ at 30 °C) was independent of concentration, whereas the rate of CaMKII monomer autophosphorylation was dependent on concentration, corroborating previous findings of an intra-holoenzyme, intersubunit autophosphorylation mechanism. The distance between each catalytic foot of the holoenzyme reconstruction shown in Figure 5 suggests

that extensive subunit rearrangements are necessary for auto-phosphorylation. In the native state, a translation of ≈ 40–50 Å for one subunit to reach its neighbour is expected, assuming that one measures the closest distance between adjacent catalytic feet (M. N. Waxham, personal communication). It is conceivable that Ca^{2+}/CaM binding somewhere in the vicinity of the stalk region induces a dramatic structural rearrangement, permitting intersubunit autophosphorylation to proceed among neighbouring subunits. How Ca^{2+}/CaM binding induces autophosphorylation between neighbours, in addition to the question of whether subunits have either complete accessibility to neighbouring subunits or a preferred pairing in the autophosphorylation reaction, remains to be determined.

The autophosphorylation of Thr[286] is an intersubunit reaction within the holoenzyme, a process that requires Ca^{2+}/CaM binding to a subunit that is acting as the 'kinase', as well as the subunit acting as 'substrate'. This was demonstrated experimentally by recombinantly producing CaMKII holoenzymes whereby subunits with these heteromeric holoenzymes were mutated to serve as obligatory substrates or kinases [111]. Obligatory substrates were produced by the Lys[42] → Met mutation, rendering the kinase inactive. Obligatory kinase subunits were produced by mutating Thr[305]/Thr[306] to aspartate residues, a process that prevents CaM binding. In addition, the Thr[286] → Asp mutation ensured catalytic activity in the absence of CaM binding. Phosphorylation at Thr[286] of the substrate subunits was completely dependent upon Ca^{2+}/CaM binding, consistent with both the kinase and substrate presentation of Thr[286] being co-operative for Ca^{2+}/CaM binding. The schematic model in Figure 6 illustrates two subunits within a holoenzyme in their basal autoinhibited state. Following Ca^{2+}/CaM binding, catalytic domains are disinhibited, and the autoregulatory region becomes exposed to a neighbouring subunit for phosphorylation. For simplicity, the kinase subunits are shown with a right-handed catalytic orientation in this illustration, with the subunit on the left behaving in a kinase-directed role and the subunit on the right behaving in a substrate-directed role. In essence, the multimeric structure of the CaMKII holoenzyme increases the relative subunit concentration for autophosphorylation, and enables a built-in kinase cascade. Activation of CaMKI and CaMKIV has both similarities and differences to that of CaMKII (for reviews, see [3,4]). CaMKI and CaMKIV are both monomers that require phosphorylation by an upstream kinase kinase in order to exhibit maximal activity. In contrast, CaMKII expresses full activation by Ca^{2+}/CaM alone. CaMKI and CaMKIV have a requirement for Ca^{2+}/CaM to be bound to allow a Ca^{2+}/CaM-activated kinase kinase to phosphorylate their activation loop. Like CaMKII, however, coincident binding of Ca^{2+}/CaM on both the protein serving as substrate and that serving as kinase is required to permit the phosphorylation reaction.

A high local concentration of catalytic sites and substrate targets (Thr[286]) within the holoenzyme appear to make the autophosphorylation reaction particularly susceptible to 'auto-dephosphorylation', a reaction that requires Mg^{2+}/ADP and depends upon the ratio and concentration of the reactant products, ATP : ADP [118]. This study demonstrated that auto-dephosphorylation occurs for both the α and β isoenzymes, is nucleotide-specific, primarily occurred at Thr[286], and leads to a loss of autonomous activity. Autophosphorylated [^{32}P]CaMKII also undergoes dephosphorylation in the presence of ATP; however, unlike in the presence of ADP, this process appears to maintain the kinase in a phosphorylated state. Thus CaMKII is able to reverse its autophosphorylation state in an ADP-dependent manner, and appears to undergo cycles of autophosphorylation–autodephosphorylation in the presence of ATP.

FUNCTIONAL CONSEQUENCES OF Thr[286] AUTOPHOSPHORYLATION

Multiple lines of evidence indicate that autophosphorylation of CaMKII at Thr[286] alters synaptic correlates of learning and memory, such as long-term potentiation (LTP) and long-term depression (LTD), as well as the behaviour itself. CaMKII is activated following LTP synaptic stimulation protocols, auto-phosphorylates at Thr[286] and becomes autonomous of Ca^{2+} [119–122]. CaMKII is sufficient to mimic the effect of LTP induction on the response of neurons to glutamate. Introduction of a constitutively active form of the kinase into hippocampal neurons increases the response of neurons to glutamate 2–3-fold [123,124]. Furthermore, synaptic potentiation by LTP occludes a subsequent effect by constitutive CaMKII and vice versa, suggesting that the two are acting via the same mechanism [125,126]. Transgenic studies specifically targeting the effects of Thr[286] autophosphorylation have demonstrated that the substitution of Thr[286] with an alanine [127], making a non-phosphorylatable form, or an aspartate [128], making constitutively active subunits, has profound effects on synaptic plasticity and learning and memory. Mice expressing the mutation of Thr[286] → Ala failed to show LTP and spatial learning [127]. Transgenic mice expressing both wild-type and an Asp[286] transgene displayed an altered frequency response function for the generation of LTP and LTD: a shift that favoured LTD generation over LTP [128]. This mutant also displayed impaired spatial learning without a loss of contextual or fear-conditioned learning; processes that are both hippocampal-dependent forms of learning [129]. Thus, in addition to the knock-out of α-CaMKII being deleterious to synaptic plasticity and learning and memory, single-point mutations at Thr[286] to either alanine or aspartate alters these processes as well.

Autophosphorylation at Thr[286] produces the autonomous form of CaMKII

The capacity for CaMKII to autophosphorylate and generate autonomous activity has intrigued enzymologists and neurobiologists for over a decade. The presence of CaMKII enzymic activity in the absence of Ca^{2+}/CaM was first reported using lysates from the invertebrate ganglia of *Aplysia californica* [130]. These authors reported that, following stimulation by Ca^{2+}/CaM in the presence of Mg^{2+}/ATP, the enzyme retained 'autonomous' activity in the presence of the Ca^{2+} chelator, EGTA. The generation of the autonomous form of CaMKII has been observed in intact synaptosomes [131], PC12 cells [132], primary cultures of dissociated neurons [133] and hippocampal slices [134,135]. A low 'basal' or non-stimulated autonomous activity ($> 10\%$) is typically observed, indicating that CaMKII is partially autonomous, even before Ca^{2+} stimulation in these preparations. The percentage of autonomous activity generated (15–50%) following an increase in intracellular Ca^{2+} with various cellular stimulation protocols varies greatly, and recent data have indicated that the preparation of the tissue has an impact on the autonomous activity measurement [136]. However, these values are still submaximal relative to the values of autonomous activity produced *in vitro*, indicating that the generation of Thr[286] autophosphorylation is submaximal following Ca^{2+}-mobilizing stimulation in cells. Interestingly, the treatment of neurons with EGTA prior to raising the level of intracellular Ca^{2+} dramatically increases the extent of autonomous activity and Thr[286] autophosphorylation, leading to an approximation of the value (90–100%) that can be generated *in vitro* [133].

CaMKII has been demonstrated to undergo autophosphorylation of Thr[286] and to generate the autonomous form of the
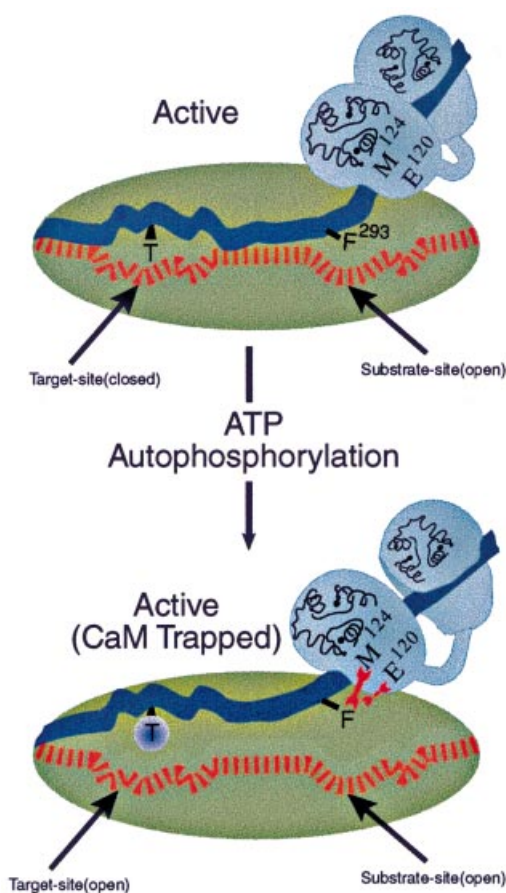
Figure 7   Interactions that promote CaM trapping. Ca²⁺/CaM binding displaces the autoregulatory region (shown in blue) to expose the substrate binding pocket to produce kinase activity

In the absence of Thr²⁸⁶ autophosphorylation, residues within the autoregulatory CaM-binding domain (e.g. Phe²⁹³), important for stabilizing the high-affinity form (CaM trapped), are unavailable for interaction with CaM, even though CaMKII is catalytically active. However, autophosphorylation of Thr²⁸⁶ further displaces the autoregulatory region, ultimately exposing Phe²⁹³ to make interactions with CaM residues Met¹²⁴ and Glu¹²⁰ to contribute to CaM trapping. Thr²⁸⁶ autophosphorylation also exposes a 'targeting' site whereby proteins that have sequence similarity to this region of the autoregulatory region can form complexes and stable interactions.

enzyme in many different cell types. Moreover, the significance of this process is exemplified by single-point mutations of Thr²⁸⁶ in mouse models discussed above that have impaired Ca²⁺-dependent alterations in synaptic plasticity, as well as in learning and memory itself. Thus a critical question is concerned with how Thr²⁸⁶ autophosphorylation is important to the cellular function of CaMKII. For example, how could autonomous activity affect cellular function differently from kinase activity coupled with Ca²⁺/CaM? The role of autophosphorylation and the generation of autonomous activity have been the subject of multiple models, which have postulated that this form of the enzyme could function as a molecular switch of learning and memory by retaining a 'memory' of past Ca²⁺ transients in the form of autonomous activity [137–142]. Autonomous CaMKII activity would still be regulated, however, instead of being strictly dependent on the binding of Ca²⁺/CaM for its activity; the extent of this autonomous activity would be regulated by the action of phosphatases.

Autophosphorylation increases the affinity of the kinase for CaM

Autophosphorylation at Thr²⁸⁶ produces both the autonomous form of the enzyme following CaM dissociation, as discussed above, and a dramatic increase in the affinity of the enzyme for CaM. Autophosphorylation of Thr²⁸⁶ increases the affinity of CaMKII for CaM over 1000-fold, converting it from a relatively poor CaM-binding enzyme into one with an extremely high affinity, an effect termed 'CaM trapping' [67]. This increase in affinity of CaM for CaMKII is due primarily to changes in the off-rate of CaM. For example, autophosphorylation decreased the off-rate of CaM by > 20000-fold in the presence of saturating Ca²⁺ in a recent study [143]. The low affinity (15–45 nM) reported for Ca²⁺/CaM binding to the native enzyme is ascribed to steric constraints or the accessibility of Ca²⁺/CaM to the binding site, because a peptide containing the Ca²⁺/CaM-binding domain from CaMKII (residues 290–309) has a K_d for CaM of 0.1–0.3 nM [46,71]. The affinity of Ca²⁺/CaM binding to the native enzyme is influenced by both Mg²⁺/nucleotide binding and autophosphorylation.

How does autophosphorylation produce such dramatic changes in the Ca²⁺/CaM binding affinity? Interestingly, the peptide α290–314 binds with high affinity (100 pM), whereas the α296–314 does not, suggesting that autophosphorylation alters the conformation of CaMKII to expose additional stabilizing interactions in the residues 293–298 that ultimately produce high-affinity binding [73]. The N-terminal residues of the CaM target helix of CaMKII appear to be critical for CaM-trapping, since deletions of residues Arg²⁹³, Asn²⁹⁴ and Ala²⁹⁵ from a peptide derived from the CaM-binding domain of CaMKII resulted in low-affinity Ca²⁺/CaM binding [57]. Alanine-replacement studies within the CaM-binding peptide indicated that the basic residues Arg²⁹⁶, Arg²⁹⁷ and Lys²⁹⁸ appeared to contribute primarily to Ca²⁺/CaM trapping only when the CaM target peptides were extended to include the 293–295 N-terminal residues, leading those authors to propose that autophosphorylation permits CaM trapping by exposing residues 293–295 on the target helix, which, in turn, stabilizes the basic residues at 296–298 to make optimal contacts with Ca²⁺/CaM. This mechanism proposed for CaM trapping was investigated recently in the context of the full-length protein using functional mutagenesis studies, as well as mutant cycle analysis to identify residues in Ca²⁺/CaM and CaMKII that interact in the trapped state [143]. In that study, Phe²⁹³ of CaMKII and Glu¹²⁰/Met¹²⁴ of CaM were observed to interact and contribute specifically to the high-affinity-binding state [143]. Residues Asn²⁹⁴ and Arg²⁹⁶ also contribute to the binding energy of the 'trapped' state; however, their contacts with CaM were not identified. The 23000-fold decrease in the dissociation constant for Ca²⁺/CaM following autophosphorylation is equivalent to a $\Delta\Delta G$ of 25.5 kJ/mol. These mutants may fully account for the trapped state of CaMKII, because the effect of autophosphorylation was reduced by 12.6, 10.9 and 4.6 kJ/mol for the Phe²⁹³ → Ala, Asn²⁹⁴ → Ala, and Arg²⁹⁶ → Glu mutants respectively.

The structure of CaMKI suggests a molecular mechanism that explains why the critical residues Phe²⁹³ and Asn²⁹⁴ are not exposed until Thr²⁸⁶ autophosphorylation. The corresponding residues for Thr²⁸⁶ and Leu²⁹⁰ of α-CaMKII are Val²⁹⁰ and Ile²⁹⁴ in CaMKI; residues which are buried in a hydrophobic groove of the catalytic core of the enzyme in the autoinhibited state [61]. Similarly, the molecular model presented in Figure 4 shows the corresponding residues in CaMKII packed into a similar hydrophobic groove in CaMKII [52]. Because low-affinity binding was not disrupted by mutants at the N-terminus of the CaM-binding domain, such as Phe²⁹³ and Asn²⁹⁴ [143], one explanation for
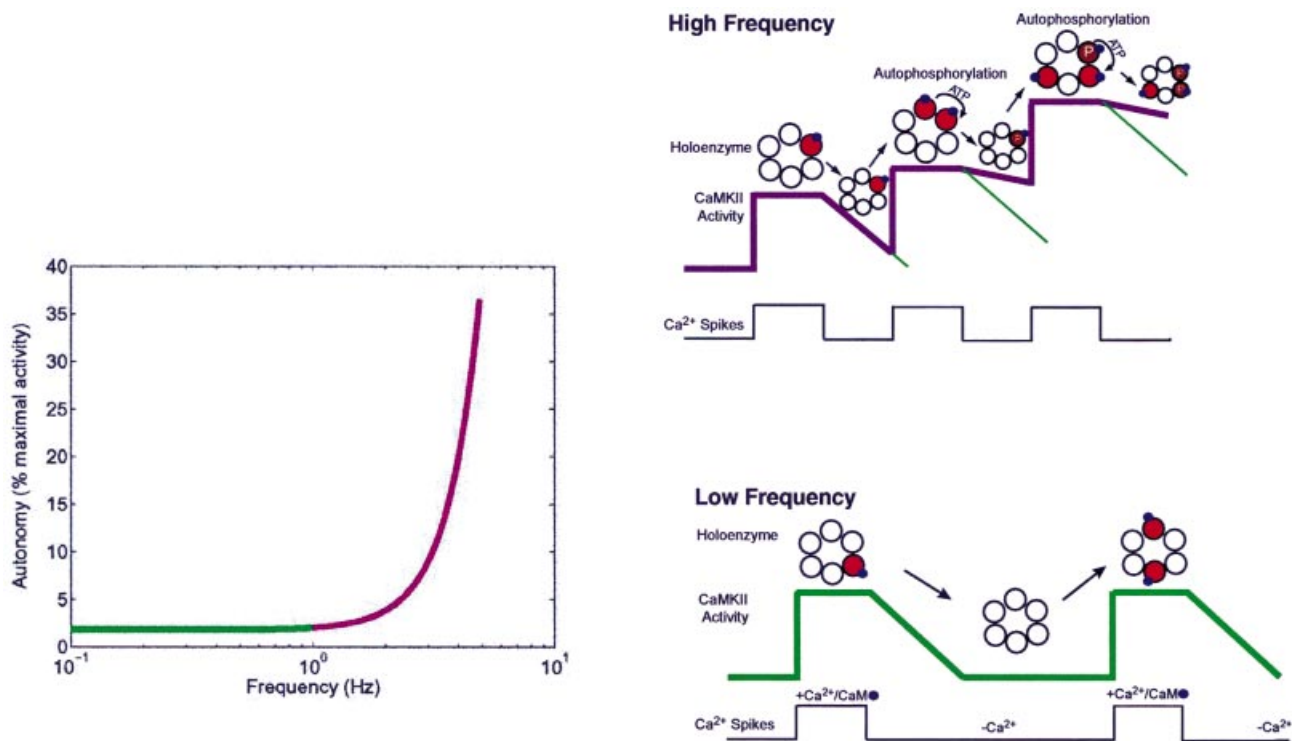
Figure 8   A model encompassing the biophysical and enzymic characteristics of CaMKII displays a non-linear Ca^{2+} spike frequency-dependence in the generation of autonomous activity (left) and the role of coincident CaM binding, autophosphorylation and CaM trapping in Ca^{2+}-spike frequency detection (right)

Inactive subunits within CaMKII holoenzymes (shown as 6-mers for simplicity) are represented by open circles. Ca^{2+}/CaM (blue circle) binding activates a given subunit (shown in red) and coincident Ca^{2+}/CaM binding results in autophosphorylation and CaM trapping ('P' inside a filled dark red circle). During low-frequency Ca^{2+} spikes, CaM completely dissociates between Ca^{2+} spikes, effectively producing 'naïve' CaMKII subunits at each inner spike interval. However, at high Ca^{2+} spike frequencies, CaM does not completely dissociate between Ca^{2+} spikes, which increases the probability that coincident CaM binding, autophosphorylation and CaM trapping may occur; a process that effectively increases the probability that a neighbouring subunit will also bind Ca^{2+}/CaM during successive spikes to produce the dramatic non-linear increase in autonomous activity.

CaM trapping is that autophosphorylation is needed to fully expose the CaM-binding domain; CaM binding alone may only partially disrupt the autoregulatory domain. Thus residues critical for trapping (Phe^{293}/Asn^{294}) remain partially or completely sequestered due to their interactions with the catalytic core (presumably, residues Phe^{98} and Ile^{205} [52]). As shown in Figure 7, complete dissociation and displacement of the autoregulatory domain follows autophosphorylation of Thr^{286}, permitting Phe^{293} to make contacts with Glu^{120} and Met^{124} in CaM, as well as residues not shown in Figure 7 that either interact directly with CaM, such as Arg^{296}, or indirectly stabilize the trapped state (such as Asn^{294}) to ultimately maximize its interactions to produce the trapped state. In accordance with this model, the observation that mutations in residues Phe^{293} or Asn^{294} actually enhance low-affinity binding [143] is probably caused by disruption of these autoregulatory interactions with the catalytic domain, resulting in increased exposure of the N-terminal residues of the CaM-binding domain to increase the stability of the low-affinity state (absence of autophosphorylation).

On the basis of positions of conserved hydrophobic residues (Leu^{299}, Ile^{303} and Leu^{308}), all of the CaM-binding domains of the CaMKII isoforms fall into a 1–5–10 category, as defined previously [144]. Utilizing this classification scheme and the Ca^{2+}/CaM–CaMKII-peptide crystal structure [72], the C-terminal anchor for high-affinity Ca^{2+}/CaM binding is probably at position Leu^{308} with 14 intervening residues, ending at what is likely to be the N-terminal anchor point for high-affinity binding,

Phe^{293} [57]. Although the anchor points for low-affinity binding to CaMKII are unknown, the C-terminal anchor is probably unchanged. The N-terminal anchor is C-terminal to Phe^{293}, possibly residing at position Leu^{299} [73], but further studies are necessary to confirm these predictions.

FREQUENCY DETECTION

The activity of each subunit within the holoenzyme is independent of other subunits. Ca^{2+}/CaM-dependent substrate phosphorylation is directly proportional to the degree of Ca^{2+}/CaM binding, with stoichiometric Ca^{2+}/CaM producing maximal enzymic activity. However, a dual requirement for Ca^{2+}/CaM for the generation of autonomous activity and Ca^{2+}/CaM trapping produces effective co-operativity for the Thr^{286} autophosphorylation reaction. Stimuli that would only slightly increase Ca^{2+}/CaM would be likely to favour enzyme activation rather than processes requiring autophosphorylation of Thr^{286}. Thus one might consider what benefit or consequence might be associated with generation of autonomous activity and Ca^{2+}/CaM trapping? Because Ca^{2+} signals can be episodic and rapid (of the order of 100 ms), one obvious benefit is that autophosphorylation of Thr^{286} serves to potentiate the Ca^{2+} signal. The nature of the extracellular stimulus appears to produce Ca^{2+} spikes of different frequencies, and the frequency of the Ca^{2+} signal carries important information for differentially influencing gene transcription [2]. Thus the downstream effectors of Ca^{2+} must be

capable of decoding frequency as well as graded and amplitude information.

The autophosphorylation of CaMKII *in vitro* was observed to follow a frequency–response function, whereby the generation of autonomous activity was sensitive to the frequency of Ca^{2+}/CaM spikes [145]. CaMKII immobilized to plastic tubing was subjected to various frequencies of high and low Ca^{2+} pulses via a high-pressure, computer-controlled valve assembly. Distinct amounts of autonomous activity resulted from various frequencies of Ca^{2+} pulses. The frequency–response function was also modulated by the amplitude and duration of individual Ca^{2+} spikes, as well as previous states of activation [145]. In addition, the α and β isoenzymes appear to have different sensitivities to the frequency of Ca^{2+}/CaM pulses [145], indicating that the isoforms of CaMKII may be differentially tuned to Ca^{2+}-spike frequency.

The molecular mechanism of the frequency-sensitivity of CaMKII autophosphorylation has not been formally demonstrated. However, theoretical modelling studies have generated unique insights into potential molecular mechanism(s) of CaMKII's capacity to function as a Ca^{2+}-spike integrator [109,146–154]. Extrinsic factors (e.g. phosphatases), as well as intrinsic properties of CaMKII itself, have been proposed by these modelling studies, including one study proposing that the observed frequency-sensitivity of CaMKII is due to a form of 'interval detection', which assumed that dephosphorylation between stimulus intervals was the critical step [147], whereas another model indicated the sequential order of 'site-selective autophosphorylation' of Thr²⁸⁶ and a 'burst' of Thr³⁰⁵/ Thr³⁰⁶ autophosphorylation as being responsible for this process [149]. To assess the critical step in CaMKII's activation and autoregulation that imparts frequency-sensitivity, a cell-free model enzyme assay on the basis of known biophysics and enzyme kinetics reported previously for purified CaMKII was used to recreate the behaviour of the enzyme [155]. The non-linear Ca^{2+}-spike frequency-dependence in the generation of autonomous activity is shown in Figure 8 (left panel). The predicted curve fits well with previous experimental data [145] performed under similar conditions (30 Ca^{2+} spikes of 200 ms duration) over a range of frequencies. This modelling study indicated that the frequency-sensitivity described previously *in vitro* [145] probably resides between autophosphorylation and coincident Ca^{2+}/CaM binding, which are intrinsic properties of CaMKII. The role of coincident Ca^{2+}/CaM binding and autophosphorylation as a potential explanation for the generation of this frequency–response function during repetitive Ca^{2+} spikes at high and low frequencies is shown in Figure 8 (right panels). The holoenzyme is depicted as a hexamer for simplicity, with open circles representing the catalytic domain. At a high frequency of stimulation, the interstimulus interval between Ca^{2+} pulses is too short to allow full dissociation of CaM (top right panel). This situation increases the probability that successive Ca^{2+} spikes produce autophosphorylation, a process that results in an increase in CaM affinity to potentiate further the probability that CaM remains associated with activated subunit(s) during subsequent pulses. This entire process hinges upon the fact that the probability and efficiency of autophosphorylation and CaM trapping depends on whether neighbouring subunits within the holoenzyme are activated by Ca^{2+}/CaM coincidentally. At low frequencies, a supralinear increase in autonomous activity is not achieved, because subunits within the holoenzyme fail to recruit CaM molecules with successive Ca^{2+} spikes owing to CaM completely dissociating between Ca^{2+} spikes. Importantly, such model-based predictions are tractable, and future experiments using mutant forms of CaM and/or CaMKII that have impaired or augmented Ca^{2+}/CaM binding and/or autophosphorylation

could be employed as described previously [145] to experimentally test this hypothesis for the mechanism of Ca^{2+}-spike frequency-sensitivity displayed by CaMKII.

CaMKII has intrinsic properties that result in this kinase having Ca^{2+}-spike frequency-dependent activation. Situations that may cause submaximal activation of CaMKII following individual Ca^{2+} spikes include: (i) rapid Ca^{2+} spikes on the millisecond-to-second time scale; (ii) relatively low Ca^{2+}/CaM affinity of the native kinase; (iii) high expression levels of the kinase; and (iv) limiting free Ca^{2+}/CaM levels. Probably all these situations have an impact on the dynamics of CaMKII activation *in situ*. Ca^{2+} spike frequency decoding in dorsal root ganglion neurons to a physiological stimulus was reported recently [156]. Although spike frequencies between 1–10 Hz appeared to saturate autonomous activity after only 45 pulses, a frequency-dependent activation of CaMKII was observed at spike frequencies between 0.1 and 1 Hz. These data indicate that, in living cells, an optimal spike frequency relationship might exist, and further experiments will certainly need to explore whether this frequency–response function is unique to cell types or to subcellular compartments, such as observed in the dendritic arbors and boutons of highly polarized cells. e.g. the pyramidal neurons in the brain, as well as how the various isoforms of CaMKII differentially have an impact on this function.

AUTOPHOSPHORYLATION OF THE Ca²⁺/CaM-BINDING DOMAIN

There are many examples of proteins whose Ca^{2+}/CaM binding is regulated by phosphorylation [67,157,158]. In these cases, phosphorylation of a serine/threonine within the CaM-binding domain results in an inability of CaM to bind and activate its target protein. CaMKII, which is phosphorylated (autophosphorylated) within its CaM-binding domain, is one such example. Following autophosphorylation of Thr²⁸⁶ and Ca^{2+}/CaM dissociation, CaMKII undergoes a 'burst' of further autophosphorylation that inhibits subsequent reactivation of the enzyme by Ca^{2+}/CaM, a process termed 'CaM capping' [76]. The sites of this 'burst' autophosphorylation are primarily Thr³⁰⁵, Thr³⁰⁶ and Ser³¹⁴, residues within the Ca^{2+}/CaM-binding domain. However, only phospho-Thr³⁰⁵ or phospho-Thr³⁰⁶ appear functionally capable of preventing Ca^{2+}/CaM from rebinding. The mechanism of this 'burst' autophosphorylation is proposed to involve a mixed mechanism, including both inter- and intra-subunit modes [110].

Autophosphorylation in the Ca^{2+}/CaM-binding domain might also occur in the basal or inactive state of CaMKII. Autophosphorylation in the basal state preferentially results in Thr³⁰⁶ being autophosphorylated, a process that also blocks Ca^{2+}/CaM binding [76,77]. Unlike the 'burst' reaction, the rate of this basal autophosphorylation is low and appears to occur by a predominantly intrasubunit mechanism within the holoenzyme [76,77,110]. This reaction is probably a result of the Thr³⁰⁶ residue being in close proximity to the catalytic cleft (recall that P0 for the pseudosubstrate is presumed to reside at Lys³⁰⁰, and is shown in blue in Figure 3) in the inactive or autoinhibited state. It is currently unknown how this mode of basal autophosphorylation contributes to CaM-binding dynamics *in situ*, where phosphatases would be expected to overwhelm this relatively low reaction rate. A potential function for burst autophosphorylation might be to functionally 'mark' activated/ autophosphorylated autonomous subunits of CaMKII, so that these subunits are no longer effectively competing for free CaM with inactive subunits for CaM binding and activation.

The free Ca^{2+}/CaM level is a function of both the relative CaM concentration and that of its targets. Both the heterogeneity

A

NR2B   KAQKKNRNKLRRQHSYDTFVDL   Ser^{1303}

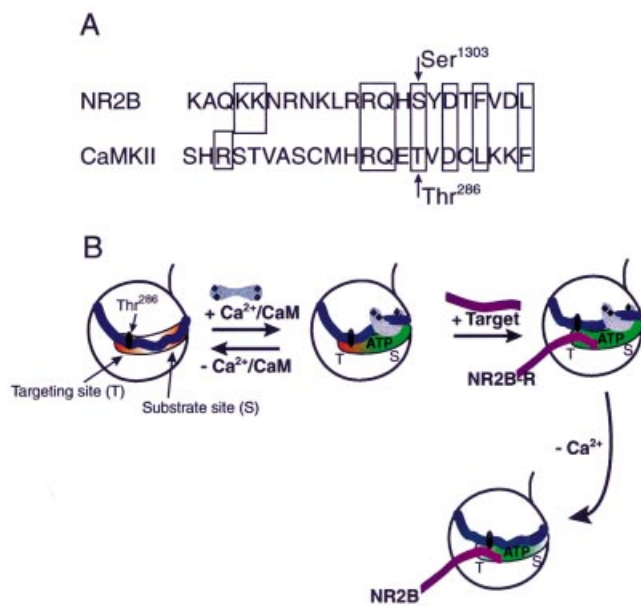CaMKII   SHRSTVASCMHRQETVDCLKKF   Thr^{286}

B

Figure 9   Displacement of the autoregulatory domain exposes a region within the catalytic lobe of CaMKII that can target and interact with proteins that have sequence homology with the autoregulatory region

(**A**) Sequence alignment from a region of the NR2B subunit (NMDA subtype of glutamate receptor) and the autoregulatory domain of α-CaMKII. (**B**) In the absence of Ca^{2+}/CaM, the autoregulatory domain interacts with the catalytic lobe to maintain an inactive enzyme (orange). Ca^{2+}/CaM binding displaces the autoregulatory region to expose the substrate binding site, to produce kinase activity (green). In addition, Ca^{2+}/CaM exposes a binding site on the catalytic lobe for proteins that have sequence similarity to the autoregulatory region of CaMKII around Thr^{286}. CaMKII interaction with the NR2B subunit of the NMDA receptor requires Ca^{2+}/CaM to bind; however, following CaMKII activation, this protein is able to interact with the catalytic lobe to act as a 'wedge' to displace the autoregulatory domain to maintain kinase activity, even in the absence of Ca^{2+}/CaM.

of CaM target proteins and their expression levels, as well as their subcellular compartmentalization, could influence the local supply of free Ca^{2+}/CaM, and therefore the capacity to regulate CaM binding and its subcellular localization becomes critically important. CaMKII might be unique in that autophosphorylation within the autoregulatory domain (Thr^{286}) enhances CaM binding. Thus by CaM capping, basal autophosphorylation and CaM trapping, autophosphorylation might dynamically alter and regulate CaM availability in cells. By autophosphorylation of sites within its own CaM-binding domain, CaMKII might be effectively removing this population of CaMKII subunits from competing with a localized or subcellular pool of CaM targets. Autophosphorylation of Thr^{286} and CaM trapping would produce the opposite result by limiting CaM to its target proteins.

CaMKII TARGETING VIA AUTOREGULATORY–CATALYTIC DOMAIN INTERACTIONS

Subcellular targeting and dynamic alterations in the subcellular localization of kinases and other regulatory proteins might produce specificity in the substrates of multifunctional regulatory proteins, as well as a rapid response to signal-transduction pathways. The multifunctional protein kinases, PKA and protein kinase C (PKC), have regulatory binding modules, AKAPs (A-kinase anchoring proteins) [159,160] and RACKs (receptors for activated C-kinase) [161,162] respectively, which determine the subcellular localization of these kinases and appear to be

functionally important for proper transduction of their second-messenger systems. As discussed previously, isoenzyme structure and differential splicing might produce subcellular targeting to achieve the rapid kinetics and substrate specificity important for stimulus-specific Ca^{2+} signalling. Although no adapter protein (excluding isoforms of CaMKII) has been reported for CaMKII, this enzyme might have a 'conserved' targeting mechanism intrinsic to all of the various isoforms, a targeting mechanism that benefits from CaMKII's multimeric structure and its mode of autoinhibition (for a review, see [163]).

The interaction between the catalytic and autoregulatory domains of CaMKII appears to afford the enzyme with a targeting mechanism that may reflect a general mode of subcellular targeting for this enzyme. When Ca^{2+}/CaM binds, the autoregulatory domain is displaced from its catalytic interactions to allow CaMKII access to Mg^{2+}/ATP and substrate proteins. However, one can envision that such a displacement would also reveal site(s) for anchoring or targeting proteins to CaMKII. Just as the binding site for substrates is occluded in the basal state by a pseudosubstrate sequence, the targeting site, termed a 'pseudo-anchor' sequence, is available only following kinase activation. An example of this mechanism was recently described for the interaction between CaMKII and the NR2B subunit of the N-methyl-D-aspartate (NMDA) receptor [164]. A sequence comparison from a region within the C-terminal tail of the NR2B receptor and the autoregulatory domain of CaMKII is shown in Figure 9. The homology includes only the N-terminal region and not the pseudosubstrate region, suggesting that binding this region of NR2B would not block access of kinase substrates. As shown in Figure 9, the catalytic residues important for contact with the autoregulatory domain can be divided into what may be considered as substrate binding and targeting contacts. Substrate-binding contacts compete for activity with other substrates, whereas targeting contacts do not [164]. The autoregulatory-like sequence of the NR2B subunit does not appear to have residues important for ATP inhibition as seen in the CaMKII N-terminal autoregulatory sequence, partially explaining why interaction of CaMKII spares enzymic activity. Thus a consequence of targeting CaMKII via a pseudosubstrate anchor to the NR2B subunit of the NMDA receptor is to effectively 'wedge open' the kinase, i.e. a conformational or steric wedge, so that, although requiring Ca^{2+}/CaM to bind initially, once complexed, the NR2B association with CaMKII is stable even in the absence of Ca^{2+}/CaM [164]. Moreover, this protein–protein interaction between CaMKII and NR2B produces an increase in Ca^{2+}/CaM affinity, as shown by data demonstrating that this interaction decreases the dissociation rate of CaM from the complex, even in the absence of autophosphorylation [164]. A second functional consequence of this binding interaction is that, following Ca^{2+}/CaM dissociation, the enzyme retains partial activity even in the absence of Thr^{286} autophosphorylation [164], indicating that CaMKII binding to the NR2B subunit would generate a state of enzymic activity that is subsequently independent of Ca^{2+}/CaM, autophosphorylation and, consequently, phosphatase action.

Multiple studies have examined the interaction of CaMKII with NMDA receptor subunits, and explored its regulation [165–172]. Dynamic translocations to NR2B subunits have been observed in heterologous expression systems [164,166,170], as well as to synaptic sites in neurons using green fluorescence protein (GFP)-labelled CaMKII [173–175]. Those studies have demonstrated a remarkable complexity in the regulation of this targeting interaction, with extrinsic factors, such as other kinases (PKC) [171] or other targeting proteins (PSD-95) [172], as well as intrinsic properties of CaMKII itself, including site-specific autophosphorylation [166,175] and phosphorylation of the re-

ceptor directly [170]. Further studies are required to determine whether the different isoforms of CaMKII exploit equally this targeting mechanism, and whether other substrate proteins of CaMKII are targeted via a similar pseudosubstrate type of anchor.

If CaMKII interacts with other proteins (e.g. the NR2B subunit of the NMDA receptor) via an 'autoregulatory-like' sequence to catalytic domain form of interaction, what prevents holoenzymes or subunits within the same holoenzyme from undergoing this process? CaMKII undergoes a form of aggregation, termed 'self-association', whereby the assembly of large complexes of holoenzymes is observed both *in vitro* [113,115] and in neurons [114,116]. Self-association might also occur between subunits within the same holoenzyme. This form of intra-holoenzyme self-association appears to produce a form of inactivation that blocks self-association between holoenzymes [115], and might be responsible for the thermal instability observed following incubation in Ca²⁺/CaM in the absence of ATP, or under low ATP conditions [104,113,176–178]. Self-association is influenced by the isoform composition of the holoenzyme. *In vitro*, α holoenzymes or mixed α/β holoenzymes undergo robust self-association, whereas holoenzymes composed of only β subunits do not. This observation is consistent with data *in situ*, which showed that CaMKII holoenzyme complexes or aggregates of predominantly α subunit are isolated from neurons [114].

Self-association appears to result from a catalytic-to-autoregulatory-domain type of interaction, as described above for the CaMKII NR2B interaction; however, self-association between holoenzymes is sensitive to biochemical parameters, including pH and ATP concentration [113,115]. Exposure of the autoregulatory domain to permit or stabilize holoenzyme self-association appears to require a pH-sensitive (< pH 7.0) conformational change. The ATP sensitivity of the self-association reaction might occur due to sub-stoichiometric Thr²⁸⁶ autophosphorylation, a process that is supported by the fact that this process occurs in ADP and that Thr²⁸⁶ → Ala mutations can no longer be protected from self-association by ATP (A. Hudmon and N. Waxham, unpublished work). Thus the CaMKII self-association reaction appears to be regulated by both pH and the extent of Thr²⁸⁶ autophosphorylation. The formation of CaMKII enzyme complexes in neurons after NMDA application was recently reported to be reversible [116]. Thus two potential physiological roles for CaMKII self-association between holoenzymes are: (i) to isolate or contain CaMKII activity in regions of the brain where the α subunit is the predominant isoform, to prevent aberrant Ca²⁺ stimulated kinase activity during conditions associated with dysfunctional Ca²⁺-signalling, such as ischaemia [114,116]; or (ii) this process may reflect a physiological accumulation of CaMKII, possibly as a 'cytoskeletal' enzyme network, which is more readily observed and exacerbated during pathological processes.

Before it was identified as an enzyme, CaMKII was first described as the 'major post-synaptic density protein (mPSDp)', and was considered by many to have the potential of being a cytoskeletal protein because of its relative abundance in brain tissue. The contamination of post-synaptic density (PSD) preparations produced by ischaemia-induced self-association probably contributes to the overestimation of CaMKII in these preparations [179–181]. However, depolarization of hippocampal neurons by glutamate was recently reported to produce a morphological thickening directly to the PSD, as revealed by electron microscopy [182]. The mechanism or binding proteins recruiting CaMKII translocation to the PSD is unknown; however, protein components of the PSD complex, such as

NMDA receptors, probably contribute to this translocation by recruiting active cytoplasmic CaMKII. In addition, the ability of CaMKII to form holoenzyme complexes at synaptic sites by self-association could contribute to descriptions of GFP–CaMKII 'spots' forming in neurons following increases in intracellular Ca²⁺ [173,174]. The self-association of CaMKII holoenzymes is a form of holoenzyme polymerization that appears to be conditional and regulated by autophosphorylation *in vitro*, and could conceivably function in conjunction with CaMKII targeting to other proteins (e.g. NMDA receptors), possibly to form cytoskeletal lattices of CaMKII holoenzymes that could exploit this enzyme's multimeric structure and autoregulation; a process intriguing enough to warrant further study.

PROTEIN PRODUCTS AS INHIBITORS OF CaMKII

Like PKA and other kinases [183–186], CaMKII has specific protein products expressed in cells that probably function to inhibit activated enzyme. Ca²⁺ signalling can lead to very diverse functional outcomes in both physiological and pathological contexts. One mechanism that may facilitate signalling diversity is via the expression of inhibitor proteins that function to oppose or inactivate the Ca²⁺ signal from producing activation of ubiquitous CaMKII. For example, PEP-19 is a small protein inhibitor that differentially antagonizes CaMKII activation, depending on the extracellular stimulus used to mobilize intracellular Ca²⁺ [187]. One successful strategy to identify specific inhibitors of CaMKII used a yeast two-hybrid approach to clone proteins that specifically interact with activated or constitutively active CaMKII. A unique clone encoding a 79-amino-acid polypeptide was pulled out using the catalytic domain (residues 1–269) of β-CaMKII as bait [188]. This protein bound to both the catalytic domain of α- or β-CaMKII and inhibited enzymic activity with an IC_{50} of 50 nM. Little cross-reactivity was observed with other protein kinases, including CaMKI, CaMKIV, CaMK kinase, PKA and PKC. This protein, designated βCaMKIIN, binds only to Ca²⁺/CaM-bound or autophosphorylated forms of the enzyme. Further research is necessary to determine the mechanism of inhibition; however, it is tempting to speculate that this polypeptide may behave analogously to regions of the autoregulatory domain, binding to the substrate and/or ATP-binding pockets to block catalytic activity. This mechanism would not only influence CaMKII catalytic activity, but also may prevent CaMKII targeting to substrate proteins via the pseudosubstrate anchor mechanism (see Figure 9). It is currently unclear how β-CaMKIIN activity is regulated in the brain; however, recent cloning of α-CaMKIIN suggests that these two CaMKIIN isoforms are differentially expressed in the brain [189]. Their expression correlates with the immunoreactivity of CaMKII, suggesting that differential cellular expression and potentially developmental regulation may provide a localized function of CaMKIIN in inhibiting specialized pools of CaMKII in the brain.

IDENTIFYING CELLULAR FUNCTIONS OF CaMKII

Substrate recognition

Substrates for CaMKII have been identified using many experimental paradigms. Historically, equilibrating ATP pools with ³²P-labelled orthophosphate, protein separation with SDS/PAGE and sequencing of ³²P-labelled proteolysed fragments of candidate proteins has been successfully employed to identify multiple CaMKII substrates. This process can be refined further by first determining whether a protein is a substrate of CaMKII *in vitro*; however, the ability of CaMKII to phosphorylate any

given protein must ultimately be confirmed either in its cellular context or following specific signalling cues. The core consensus sequence of substrates for CaMKII is Φ-Xaa-Arg-NB-Xaa-Ser*/Thr*-Φ, where Xaa, NB and Φ represent any amino acid, non-basic and hydrophobic amino acids respectively, and the asterisks denote sites of phosphorylation [190]. This information has been compiled from: (i) comparative analysis of many protein substrates; (ii) detailed mutagenesis studies of peptide substrates; and (iii) a degenerate peptide library technique [190–192]. Like all kinases, CaMKII prefers a basic residue at position $P-3$ (P0 is the phosphorylated serine/threonine residue). The nature of residues occupying the $P-4$, $P-1$ and $P+4$ positions exert relatively little influence on phosphorylation kinetics [190]. The presence of an arginine is only slightly better (2–3-fold) than a lysine at the $P-3$ position [193,194]. A hydrophobic amino acid is preferred at the $P+1$ position, and is often found in the $P-5$ position [190,194]. However, a basic residue at $P+1$ appears to never be tolerated [194]. The preferable residue at the $P-2$ position for CaMKII is a non-basic amino acid [190,192]; however, it is not obligatory. Common to many substrates is an acidic residue at $P-2$ position, an important residue for CaMKII phosphorylation of vimentin (an example of an anomalous substrate lacking a $P-3$ basic residue; [195]). Thus substrate recognition is complex, and although the consensus sequence may apply in most cases, there are probably many determinants other than those listed in this consensus sequence that will ultimately determine whether a protein is a substrate of CaMKII, including the relative and effective concentration of substrates produced by targeting, and the subcellular localization of CaMKII in cells.

Designing and utilizing inhibitors to understand CaMKII function

The use of inhibitors to block CaMKII activity has been instrumental in understanding this enzyme's role in Ca^{2+} signal transduction. Classical inhibitors to kinases, or, more aptly, ATP-binding sites, such as staurosporine and its derivatives (K252a, K252b and UCN-01), and isoquinolinesulphonamides, such as H-7, inhibit CaMKII very effectively. As expected, CaM antagonists, including the neuroleptics (trifluoperazine), miconazoles (calmidazolium), napththalenesulphonamides (W-7), as well as CaM-binding peptides, have also successfully been utilized to examine the role of Ca^{2+}/CaM-dependent protein kinases in cellular function. To examine the role of CaMKII specifically in signal transduction, reagents that possess relative specificity for the isoforms of CaMKII were identified. A family of reagents is known as the KN series of inhibitors, with KN-62 being the first described CaMKII inhibitor: an isoquinoline-sulphonamide derivative {1-[N,O-bis(5-isoquinolinesulphonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine} with a K_i value of 0.9 μM [196]. KN-93, a methoxybenzenesulphonamide, has improved solubility with a slightly better inhibitory potency (K_i of 0.37 μM; [197]). Although the binding site for KN-62/KN-93 is unknown, the mechanism of this inhibition appears to be competitive for Ca^{2+}/CaM activation, and therefore has little potency on autophosphorylated or 'active' CaMKII [196] and, $in\ situ$, its inhibitory efficacy would be influenced by local CaM concentration. Peptide inhibitors designed on the basis of the CaMKII autoregulatory domain are relatively selective for CaMKII, and are capable of inhibiting both the native and autophosphorylated forms of the enzyme [198–201]. However, unlike the KN-family of compounds, these peptides do not readily cross the plasma membrane, and therefore have to be either chemically modified (e.g. myristoylated) and bulk-loaded into cells, or micro-injected. Because kinases have many similar

features in their catalytic domains and cross-reactivity among peptide inhibitors is more of a reality than a possibility, conclusions drawn solely on the basis of the use of these inhibitors must be interpreted carefully [202–204]. Thus cell permeability, general toxicity, reversibility and, of course, specificity have always been a concern when using many of these compounds and peptides that inhibit CaMKII activity.

The design of unnatural ligands for engineered proteins holds considerable promise for determining the cellular function of a protein of interest. Combining genetic and chemical methodologies, this strategy complements traditional biochemical, as well as both forward and reverse genetic, techniques. On the basis of the design of functionally silent mutations that 'sensitize' a target protein to a novel small molecule, this approach has been shown recently to hold considerable promise for kinases [205–208]. Although initially described for the tyrosine protein kinase v-Src, this paradigm appears equally potent with serine/threonine protein kinases, and has several advantages over traditional biochemical or transgenic/knock-out types of studies, because: (i) it is potentially delivered very rapidly; (ii) it is potentially reversible by metabolic or renal clearance; (iii) dose-dependent application of the inhibitor is capable of generating graded phenotypes; and (iv) since the inhibitor molecule can be introduced at any time, developmental defects are not a concern. A conserved residue in the ATP-binding pocket was identified in all eukaryotic protein kinases that, when mutated, sensitizes the modified kinase to unnatural ligands, and yet does not compromise the biological activity [209–212]. This modification and approach has been successfully tested for several protein kinases, including the α isoform of CaMKII [205]. A substitution of Phe[68] with glycine in α-CaMKII produced a 200–2000-fold decrease in the IC_{50} for the synthetic compound, pyrazolo[3,4-d]pyrimidine, indicating that inhibitory compounds that exploit this inhibitory mechanism might have a bright future in inhibitor-based studies designed to access the function of CaMKII in cells.

In addition to simply ablating CaMKII enzymic activity to understand its role in cellular function, tools or reagents must be developed to study how this enzyme's complex autoregulatory properties contribute to its function. The role of CaMKII in transducing synaptic activity, presumably via differential Ca^{2+} signalling, into changes in synaptic strength and learning and memory is dependent upon the autophosphorylation of Thr[286], a process that produces multiple changes in the autoregulatory properties of CaMKII. Therefore inhibitors or mutants of CaMKII must be discovered, whereby specific functional lesions in such properties as autonomous activity, CaM trapping, CaM capping and/or frequency detection can be functionally isolated and disrupted to determine their individual contributions to the function and regulation of CaMKII in these cellular processes. These functional changes in CaMKII associated with autophosphorylation are complex, and appear to be interconnected at the structural level; however, mutations in CaMKII that affected CaM trapping without influencing autonomous activity have been identified recently [143], suggesting that similar or new mutations in the autoregulatory/CaM-binding domain and/or possibly in the catalytic core, as indicated by molecular modelling studies [52], may prove useful in dissecting autophosphorylation in itself and enzymic activity from the complex autoregulation associated with these processes.

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