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Ca^{2+}/CaM-DEPENDENT KINASES: From Activation to Function

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■ **Abstract** Calmodulin (CaM) is an essential protein that serves as a ubiquitous intracellular receptor for Ca^{2+}. The Ca^{2+}/CaM complex initiates a plethora of signaling cascades that culminate in alteration of cellular functions. Among the many Ca^{2+}/CaM-binding proteins to be discovered, the multifunctional protein kinases CaMKI, II, and IV play pivotal roles. Our review focuses on this class of CaM kinases to illustrate the structural and biochemical basis for Ca^{2+}/CaM interaction with and regulation of its target enzymes. Gene transcription has been chosen as the functional endpoint to illustrate the recent advances in Ca^{2+}/CaM-mediated signal transduction mechanisms.

INTRODUCTION

Calcium Signaling and Homeostasis

Calcium (Ca^{2+}) is an important intracellular second messenger in such processes as growth factor and hormone signaling, cell cycle regulation, gene expression, and apoptosis, among others (for reviews see 1–5). A resting cell has a cytosolic Ca^{2+} concentration of roughly 100 nM, a concentration approximately 20,000-fold lower than that of extracellular Ca^{2+}, and thus cells have an intricate network by which to precisely control cytoplasmic Ca^{2+} levels (for review see 6). ATP-dependent Ca^{2+} pumps shuttle Ca^{2+} into the two major Ca^{2+} sinks, the endoplasmic reticulum (ER) and the extracellular space (7). Various signals stimulate either waves or spikes of increased intracellular Ca^{2+} with concentrations reaching 1–2 μM. The source of Ca^{2+} depends on the stimulus. Receptor tyrosine kinases and G-protein-coupled receptors classically increase Ca^{2+} levels by producing IP3, which induces Ca^{2+} release from the ER via the IP3 receptor (8, 9; for review see 10). Similarly, cyclic ADP ribose can release Ca^{2+} from intracellular stores. Ligand gated ion channels and voltage-dependent ion channels in the plasma membrane, however, initiate

Ca^{2+} entry via extracellular stores. Subsets of each of these types of channels have also been shown to cause Ca^{2+} release from intracellular stores via either the IP3 receptor or the ryanodine receptor (for review see 11).

The origin of nuclear Ca^{2+} signals has remained somewhat controversial (for review see 12). Some suggest that nuclear Ca^{2+} is regulated independently from cytoplasmic Ca^{2+} (for review see 13). The inner and outer leaflets of the nuclear membrane are separated by what is known as the nuclear envelope lumen or perinuclear space. The outer leaflet of the nuclear membrane is contiguous with the endoplasmic reticulum, and thus the perinuclear space is likely to contain very high levels of Ca^{2+}. The nuclear membrane not only contains ATP-dependent Ca^{2+} pumps (14) as does the plasma membrane, but it is unique in also having IP4 receptors (15, 16), both of which could funnel Ca^{2+} back into the perinuclear space. In addition, the inner side of the nuclear membrane contains IP3 receptors (17, 16) that may generate nuclear Ca^{2+} spikes. Indeed, Ca^{2+}-mobilizing agents, when introduced into the nucleus or isolated nuclei, generate increased nuclear Ca^{2+} in the absence of cytosolic Ca^{2+} changes (18–20). Interestingly, despite the large channel size of the nuclear pore complex, it seems to display ion channel activity (21, 22).

Although nuclei apparently contain transmembrane proteins known to regulate Ca^{2+} homeostasis, some groups maintain that nuclear Ca^{2+} increases are due to rapid diffusion of Ca^{2+} from the cytosol. Allbritton and collegues (23) have shown that in rat basophilic leukemia cells cytoplasmic IP3 generates cytoplasmic Ca^{2+} spikes that are transduced through the nucleus with a less than 200 ms delay. Blocking cytosolic IP3 receptors by microinjection of heparin-dextran ablated Ca^{2+} transients both in the cytoplasm and nucleus. These data suggest that cytosolic Ca^{2+} is required for nuclear transients. Lipp and co-workers (24) have shown that individual calcium "puffs" stimulated by physiological levels of histamine resulted in nuclear Ca^{2+} transients only if the puff was in close proximity (2–3 μm) to the nuclear envelope. Although neither of these studies could conclusively demonstrate that nuclear Ca^{2+} stores did not contribute to nuclear Ca^{2+} transients, both groups did conclude that nuclear Ca^{2+} signals arise in the cytoplasm. Thus, the complexity by which cells regulate both Ca^{2+} homeostasis and signaling between the cytoplasm and nucleus is only beginning to be realized.

Calmodulin

One of the key proteins that transduces a signal in response to increases in intracellular Ca^{2+} is calmodulin (CaM) (for review see 25). CaM is a 148–amino acid protein (16,680 daltons) comprised of 4 helix-loop-helix protein folding motifs called EF hands, with two making up the N-terminal domain and two comprising the C-terminal domain (for review see 26). Each of the four EF hands binds one Ca^{2+} ion. Nuclear magnetic resonance structures of the N- and C-terminal domains of CaM in the absence of Ca^{2+} indicate that these domains adopt superimposible conformations because the root mean square difference of the α carbon backbone is only 2 Å (27, 28) (Figure 1, left;). Despite having similar structures, sites III

and IV in the C-terminal domain have a 10-fold higher affinity for Ca^{2+} than do sites I and II in the N-terminal domain (29). Ca^{2+} binding is a cooperative event (30), and within the C-terminal domain Ca^{2+} might first bind to site III in vivo (31), although in vitro it binds to sites III and IV nearly simultaneously (32). Ca^{2+} serves to slightly modify the EF hand conformation by pulling the α helices towards one another. This subtle movement, however, is transduced through a short β sheet connecting the EF hand to the second Ca^{2+}-binding site within the domain, giving it an increased binding affinity. Binding of the second Ca^{2+} ion results in the two EF hands being pulled away from one another so as to expose a hydrophobic pocket within the domain, which enables CaM to recognize the binding domains of target proteins. In addition, the "cooperativity of Ca^{2+}-binding affinity" is enhanced in the presence of a peptide from a target CaM-binding protein (33).

Ca^{2+} binding to CaM not only induces local conformational changes within the EF hands, but also causes a global structural change where the α helical content of the protein is increased (34). Most significant is the formation of an eight turn alpha helix, helix IV, which connects the N-terminal and C-terminal globular domains to form a dumbbell-shaped conformation (35, 36) (Figure 1, middle). The 3-dimensional structure of Ca^{2+}-bound CaM with target peptides (37–39) suggests that in order for the hydrophobic patches on the N- and C-terminal domains to interact, the central helix must undergo a 120° unwinding and a 100° bend (Figure 1, right). This helix allows CaM to accommodate the structures of many different CaM-binding proteins (37, 39). The CaM-binding domains are 9–26 residues in length and typically form amphipathic alpha helices that traverse the hydrophobic tunnel created by the two hydrophobic patches of CaM. Each of the hydrophobic regions of CaM contain four conserved M residues, some of which make critical contacts with the target sequences, as will be discussed later.

CaM KINASES

One action of CaM is to activate members of a family of S/T protein kinases called Ca^{2+}/CaM-dependent protein kinases or CaM kinases. This family includes kinases such as phosphorylase kinase, myosin–light chain kinase (MLCK), and CaM kinases I, II, III, and IV (reviewed in 40, 41). These kinases are grouped according to whether they are dedicated kinases having a single substrate (phosphorylase kinase, CaM KIII, and MLCK) or whether they are multifunctional (CaM KI, II, and IV) and have several substrates. Members of both groups of CaM kinases have very similar domain structures. Some of the comparative properties of CaM KI, II, and IV are listed in Table 1.

Domain Structure

The general architecture of the CaM kinases includes an N-terminal kinase domain, followed by an autoinhibitory domain and overlapping CaM-binding domain, and

TABLE 1 Properties of the multifunctional Ca^{2+}/CaM-dependent protein kinases

	CaMKI	**CaMKIV**	**CaMKII**
Tissue distribution	Ubiquitous	Limited	Ubiquitous overall, but with isoform/splice variant specific distributions
Subcellular localization	Cytoplasmic	Nuclear and cytoplasmic	Cytoplasmic, except α_B, γ_A, and δ_B forms can be nuclear
Subunit composition	Monomeric	Monomeric	Homo- or heteromultimeric
Known requirements for complete activation	Ca^{2+}/CaM binding, activation loop phosphorylation	Ca^{2+}/CaM binding, autophosphorylation, activation loop	Ca^{2+}/CaM binding phosphorylation
Gains Ca^{2+}/CaM-independence	No	Yes (up to 20% of Ca^{2+}/CaM-dependent activity)	Yes (with autophosphorylation, up to 80% of Ca^{2+}/CaM-dependent activity)
K_{CaM}	14 nM (unphosphorylated) 4 nM (activation loop phosphorylated) (73)	158 nM (recombinant) 32 nM (tissue purified) (77)	20–100 nM decreases to 60 pM with autophosphorylation (83)
Inhibition by KN-62 (K_I) (KN-93 similar) (157a)	0.8 μM	3 μM	0.8 μM
Substrate consensus sequence (133)	Hyd-X-R-X-X-S/T-X-X-X-Hyd	Hyd-X-R-X-X-S/T	Hyd-X-R-NB-X-S/T

in the cases of phosphorylase kinase and CaM KII, a C-terminal association domain essential for multimerization (Figure 2B). Ca^{2+}/CaM activates the enzyme by binding to it and removing the autoinhibitory domain from the catalytic pocket that allows substrate access. Localization of these domains was largely achieved through studies of mutated, truncated, or proteolyzed forms of MLCK and CaM KII. More recently, these domains have also been identified in the newest cloned member of the multifunctional kinase family, CaM KI. CaM KI is a 374/370 amino acid protein (rat/human, respectively) (42, 43) that when truncated at residue 332,

completely retains dependence on Ca^{2+}/CaM (44). Serial truncations at amino acids 321, 314, and 309 result in decreasing ability to bind CaM in a gel overlay assay (44). Proteins truncated between residues 306 and 299 are unable to bind CaM and are completely inactive (43–45). These data suggest that the CaM-binding domain of CaM KI lies between residues 306 and 321 (Figure 2). Truncation at residue 294 produces a protein that is constitutively active (43). Thus, removing amino acids 295–299 of CaM KI generates an active kinase, indicating that these few amino acids are sufficient for autoinhibition.

The amino acids 294–299 of CaM KI, IKKNFA are homologous to CaM KIIα residues LKKFNA, the first five of which, in the context of a truncated protein, are also sufficient for CaM KII autoinhibition (46) (Figure 2). Further deletion analysis of CaM KI reveals the remarkable importance of F298 for autoinhibiton, in that a 1–298 truncation was completely inactive whereas removal of only F298 (1–297) results in a protein that exhibits 70% constitutive activity as compared to wild-type (47). These data should be interpreted with caution, as residues sufficient for autoinhibition in the context of truncated proteins are not necessarily "required" in full-length proteins. For example, amino acid substitutions of full-length CaM KII in the region, LKKFN, clearly show that these residues are not essential, indicating that other contacts between the autoinhibitory domain and catalytic domain were functionally important (46). Likewise, CaM KI in which I294 and F298 are mutated to A showed only slight increases in Ca^{2+}/CaM-independent activity, a parameter that is an indirect measure of autoinhibition. In fact, the crystal structure of 1–320 CaM KI reveals that not only F298 but also I286, V290, and I294 pack against the D α helix in the catalytic core (48) (Figure 3). Interestingly, point mutation of F307 to A results in CaM-independent activity that is 30% of the maximal CaM-dependent activity. Thus, this residue is also important for the complex interactions that occur between the autoinhibitory and catalytic domains. This complexity is further substantiated by the observation that an autoinhibitory domain from one CaM-dependent kinase cannot be substituted for the same domain in another in a manner that maintains autoinhibition (49).

Several CaM-dependent kinases are thought to have overlapping autoinhibitory and CaM-binding domains, and this appears to be the case for CaM KI as well. Collectively, many studies suggest that residues I286 through F307 are important for autoinhibition (Figure 2). Based on the crystal structure of Ca^{2+}/CaM bound to peptides derived from the autoregulatory regions of CaM KIIα, the CaM-binding domain of CaM KI would be from N297 to R314 (39). Alternatively, if the enzyme binds Ca^{2+}/CaM more similarly to smMLCK, residues A299 to R317 would be involved (38). Regardless of which prediction is correct, W303 has definitively been shown to be required for CaM binding (47). The overlapping autoinhibitory and CaM-binding domain concept is further supported by studies using a synthetic peptide to CaM KI residues 294–321. This peptide inhibited wild-type CaM KI in a manner competitive with Ca^{2+}/CaM but was competitive with substrate and non-competitive with ATP when assayed against the constitutively active 1–293 CaM

KI (44). The later observation raised the possibility that CaM KI autoinhibition is of the pseudosubstrate type.

Kemp and co-workers (50) had postulated that the autoinhibitory domain of MLCK was a pseudosubstrate in that it lies, in the absence of Ca^{2+}/CaM, in the substrate-binding region. The pseudosubstrate idea was confirmed with the crystallization of twitchin, a *Caenorhabditis elegans* kinase with homology to the MLCK family (51). Twitchin's autoregulatory domain consists primarily of an α helix and a long loop that traverses the entire catalytic pocket. The α helix occupies the substrate-binding groove and the loop swings up to make contacts with catalytic residues and part of the ATP-binding loop (Figure 4, middle;). The crystal structure of the 1–320 fragment of rat CaM KI reveals that the autoinhibitory domain forms a helix-loop-helix structure that blocks the substrate-binding groove but then makes a nearly 90° turn away from the catalytic cleft towards the back side of the ATP-binding loop (48) (Figure 4, left). Despite the structures of CaM KI and twitchin having very different conformations of their autoregulatory regions, these amino acids in CaM KI also make contacts the way a substrate would. CaM KI has a strict substrate requirement for a basic residue at P-3 relative to the phosphoacceptor site (52). K300 within the autoinhibitory domain of CaM KI mimics R at P-3 by interacting with E102 within the catalytic domain (Figure 3). Likewise, mutation of E102 alters the enzyme specificity at P-3 (48). CaM KI has a preference for a hydrophobic residue at P-5 within the substrate (52, 53), which is illustrated by the key autoinhibitory domain residue F298 being buried within a deep hydrophobic pocket formed by F104, I210, and P216 (48) (Figure 3). A similar substrate-mimicking interaction of the analogous F residue in CaM KII had been previously postulated based on the crystal structure of protein kinase A (PKA) (46). Recall that for truncated forms of CaM KI, F298 could inhibit enzyme activity, whereas its removal allowed substrate access and hence, constitutive kinase activity (47).

In addition to insights into CaM KI regulation by autoinhibition, the crystal structure revealed a very interesting hint as to how CaM kinases bind CaM. CaM binds in an antiparallel fashion such that the C-terminal domain makes initial contact with a hydrophobic "anchor" in the N-terminus of the target sequence, and the N-terminal domain then binds the C-terminal hydrophobic "anchor." CaM KI residue W303 resides on a loop between the autoinhibitory α helix, and the CaM-binding α helix and is completely solvent exposed (Figure 3). Based on the structure alone, one might predict that W303 is the N-terminal hydrophobic anchor of CaM KI that would interact with M124 within the C-terminal domain of CaM. Indeed, replacement of W303 with S results in a $>$3300-fold increase in the K_{CaM} of CaM KI (47). Likewise, mutational analysis of individual M mutants within CaM confirm that M124Q raised the CaM KI K_{CaM} $>$50-fold, and the maximal enzyme activity at 20 μM is only 70% of wild-type (54). Corroborating evidence that W303 in CaM KI binds M124 in CaM comes from the crystal structure of either smMLCK or CaM KII peptides complexed with Ca^{2+}/CaM. The analogous residues W800 (MLCK) and L299 (CaM KII) both make direct contacts with M124 (38, 39). The

C-terminal hydrophobic anchor of CaM KI is predicted by the structure to be M316, a residue that is buried within the interface of the ATP-binding loop (Figure 3). In fact, the contacts between the CaM-binding domain and the ATP-binding loop are so extensive that F31, a residue that in PKA is important in maintaining the structural integrity of the active site, is shifted 15 Å away from the catalytic cleft towards F301 in the regulatory region (48). Thus, the distorted conformation of the ATP-binding loop combined with the inaccessibility of the C-terminal hydrophobic anchor indicates that the N-terminal domain of CaM may play a very significant role in activating CaM KI. Mutations of hydrophobic residues in the N-terminal domain of CaM increased the K_m ATP of CaM KI by up to 10-fold, whereas they have no effect on K_m for peptide (54). Thus, these CaM mutants are able to relieve autoinhibition of the peptide-binding groove, but are not able to derepress interaction between the C-terminal portion of the CaM-binding domain with those from the ATP-binding loop. Further studies are needed to determine whether any of the other CaM kinases are regulated in a similar manner.

In comparing the overall structure of CaM KI crystallized in the absence of Mg^{2+}, ATP, and CaM (48) with the structure of PKA crystallized in the presence of Mg^{2+}, ATP, and the substrate analogue, PKI (55, 56), several differences are apparent. The structure of PKA reflects an active kinase with a closed conformation in which the residues involved in catalysis are clustered in a small three-dimensional space (Figure 4, right). In addition, the threonyl phosphate of T197 within the activation loop makes critical contacts with R165, one residue away from D166, the proposed catalytic base, and with K189, a residue in the β sheet following the Mg^{2+} chelating-loop conformation (56–58). Therefore, this phosphate may be critical for maintaining the structural integrity conducive for catalysis. In contrast, the catalytic domain of CaM KI is distended, and the small and large lobes of the kinase are twisted 18° from one another so that the catalytic cleft is solvent exposed (48) (Figure 4, left). Furthermore, the activation loop of CaM KI is disordered. The structure indicates that in order for CaM KI to be an active kinase, relief of autoinhibition of the peptide- and ATP-binding regions is needed, as well as a rotation between the small and large lobes of the kinase, and possibly even activation loop phosphorylation.

CaM KI

The first hints that CaM KI was regulated by phosphorylation came from studies of the multiple polypeptides purified from bovine and rat brain (59). The enzyme activity of all the proteins was significantly increased by autophosphorylation in the presence of Ca^{2+}/CaM. However, the most telling observation was made with the 43-kDa rat protein designated CaM KIa. During the extensive purification CaM KIa lost activity after CaM-Sepharose chromatography (60). Adding fractions from previous chromatographic steps could restore this activity (61). Thus, the apparent activation by autophosphorylation could be explained by a copurifying "activator" protein. Mochizuki and collegues had also purified an "activator" for CaM KV,

an enzyme that has recently been shown to be a splice variant of CaM KI (62–64). Two lines of evidence suggested that the activator was itself a protein kinase. First, the activator fractions could phosphorylate kinase-inactive (K49A) recombinant CaM KI (43). Likewise, treatment of the activator with the ATP-affinity analogue fluorosulfonylbenzoyl adenosine (FSBA) could block CaM KI phosphorylation, whereas treatment of CaM KIa had no effect on phosphorylation (65). Careful purification, extensive biochemical analysis, and protein sequencing ultimately revealed the existence of two CaM kinase kinases (66–68), CaM KKα (64 kDa), which had been characterized as the CaM KIV kinase (69, 70), and CaM KKβ (68 kDa) (71, 72). Both kinase kinases phosphorylate CaM KI on the predicted site of autophosphorylation, T177 (66) (Figure 2). T177 within the activation loop of CaM KI is in the equivalent position as T197 in PKA and, as the CaM KI structure may have predicted, is regulated by phosphorylation.

Activation-loop phosphorylation of some kinases dramatically enhances their activity such that unphosphorylated forms are considered "inactive" (58). Likewise, it is the phosphorylated kinases that are thought to be physiologically relevant. Phosphorylation of CaM KI correlates with a 25–50-fold increase in enzyme activity (43). The mechanism by which CaM KI activity increases in response to phosphorylation is primarily reflected in a 44-fold decrease in the K_m for peptide substrate, although the K_{CaM} is also decreased by 4-fold (73).

The need for CaM KI phosphorylation can be overcome by the sequence contained within the peptide substrate. For example, a peptide variation on the sequence surrounding S9 of synapsin, LRRRLSDANF, has a K_m and V_{max} of 209 μM and 26.1 μmol/min/mg, respectively, using dephosho-CaM KI. After phosphorylation these values change to 4.7 μM and 65.5 μmol/min/mg, respectively. Phosphorylation of CaM KI therefore correlates with an increase in catalytic efficiency, expressed as V_{max}/K_m, of 107-fold. Remarkably, the addition of one hydrophobic and two basic residues, LKK, at P-8 through P-6 onto the synapsin peptide imparts a very low K_m and high V_{max} such that the additional residues increase the catalytic efficiency of dephospho-CaM KI 45-fold relative to the synapsin peptide. Phosphorylation of CaM KI increases the catalytic efficiency of the LKK-synapsin peptide a mere threefold. Individual point mutations suggest that the L at P-8 (relative to the phosphoacceptor site, P0) is dispensable for "activation-independent substrate specificity," whereas the K at both P-7 and P-6 contribute to this effect. Thus, the addition of basic residues onto the synapsin peptide creates a substrate that is largely independent of activation-loop phosphorylation in that it is 30-fold less responsive to CaM KI T177 phosphorylation (73). CaM KI is the first example in which activation-loop phosphorylation may not be essential for kinase activity but rather, may serve to widen the enzyme's substrate specificity. Recently, Jakobi et al (73a) have added a second enzyme to this list by showing that substrates enhance autophosphorylation and activation of the p21-activated protein kinase in the absence of activation loop phosphorylation.

In our discussion of the crystal structure of inactive CaM KI relative to the active structure of PKA, we stated that CaM KI would need relief of autoinhibition in order for ATP and peptide to bind, as well as a rotation and closure of the catalytic

cleft in order for catalysis to occur. For PKA, activation-loop phosphorylation is thought to be a key mediator of this "closed conformation" (Figure 4, compare left and right). How then can CaM KI efficiently phosphorylatate some substrates in the absence of activation-loop phosphorylation but not others? The answer to this question may come from studies using small-angle X-ray and neutron scattering with skeletal muscle MLCK. Krueger and co-workers (74) have shown that Ca^{2+}/CaM binding to MLCK causes the autoinhibitory domain to swing away from the kinase domain. The addition of a nonhydrolyzable ATP analogue, AMPPNP, or peptide substrate causes a compaction of the catalytic core. In addition, complete closure between the small and large lobes of the kinase as well as the accompanying shift of the CaM and MLCK centers of mass towards one another requires both Mg^{2+}/ATP- and peptide-substrate binding (75). Although MLCK is not regulated by activation-loop phosphorylation, its enzyme activation by Ca^{2+}/CaM is similar to that of CaM KI. If we extend the findings from MLCK to CaM KI, we can hypothesize that the conformational change due to peptide substrate binding may be influenced by T177 phosphorylation. "Activation-independent" substrates can induce the requisite conformational change, whereas "activation-dependent" substrates would require T177 phosphorylation to induce the conformational change. Alternatively, T177 phosphorylation could serve to induce a conformational change that can be mimicked by binding of a high-affinity substrate. Further experimentation will be required to determine if this model is correct.

CaM KIV

Another multifunctional Ca^{2+}/CaM-dependent kinase, CaM KIV has a very similar domain structure to that of CaM KI (reviewed in 76). Truncation of CaM KIV at L317, the residue equivalent to I294 in CaM KI (Figure 2), generates a Ca^{2+}/CaM-independent kinase, which suggests that this enzyme is also regulated by intrasteric autoinhibiton (77). Indeed, introduction of negative charges within the autoinhibitory sequence, HMDT308 to DEDD308, interferes with autoinhibition and thus increases CaM KIV Ca^{2+}/CaM-independent activity (70). These mutations are likened to autophosphorylation of T286 within the CaM KII holoenzyme, which generates up to 80% Ca^{2+}/CaM-independent activity (78–83).

Similar to CaM KI, CaM KIV is phosphorylated within its activation loop on T196 in the rat enzyme and T200 in the human enzyme by both CaM KKα and CaM KKβ (66, 84) (Figure 2). As is the case for CaM KI, the primary kinetic parameter modulated by CaM KIV phosphorylation is a decrease in the substrate K_m (70). In addition, activation-loop phosphorylation serves to widen the substrate specificity of this kinase and is not a strict requirement for high kinase activity (73). In contrast to CaM KI, phosphorylation of CaM KIV generates Ca^{2+}/CaM-independent activity. In response to Ca^{2+}/CaM binding, CaM KIV can be phosphorylated on its activation loop T by the CaM KKs but also undergoes intramolecular autophosphorylation on S12 and 13. However, phosphorylation of S12 and 13 is not responsible for Ca^{2+}/CaM-independent activity because enzymes containing a deletion of residues 1–21 gain Ca^{2+}/CaM independence just as the wild-type

enzymes do (85). The site responsible for generation of Ca^{2+}/CaM-independent activity may be T200 or another yet to be determined site.

Intriguingly, CaM KIV is the only CaM kinase regulated by two modes of autoinhibition (85). In order for CaM KI or IV to be phosphorylated by one of the CaM KKs, autoinhibition must be relieved either by Ca^{2+}/CaM binding to full-length CaM KI or IV or by truncation of either enzyme N-terminal to the autoinhibitory/CaM-binding domains (43). The existence of a second autoinhibitory domain was detected by using the 1-317 Ca^{2+}/CaM-independent form of CaM KIV as a substrate for the CaM KKs. Although CaM KIV 1-317 containing both S12A and S13A point mutations is phosphorylated by CaM KK (KA Anderson & AR Means, unpublished data), it is not activated (85). Individual point mutations of S12 or S13 within the context of CaM KIV 1-317 were able to be activated by CaM KK, which indicates that autophosphorylation of either residue 12 or 13 is sufficient to relieve this unusual form of intrasteric autoinhibition by the N-terminal region of the protein (85).

In addition to showing how CaM KIV is activated, recent studies have begun to shed light on how it is inactivated. CaM KIV that has been phosphorylated by CaM KK can be dephosphorylated in vitro by protein phosphatase 2A but not protein phosphatase 1 (86). Westphal and collegues (87) have shown that CaM KIV copurifies in a complex that contains all three subunits of PP2A through four chromatographic steps. This interaction requires the catalytic domain of CaM KIV (1-313), but the catalytic function of either CaM KIV or PP2A or phosphorylation of CaM KIV on T196 is not essential. PP2A dephosphorylates CaM KIV that has been phosphorylated by CaM KK, although it is unclear whether the PP2A target is T196, S12, or S13. Moreover, this interaction seems to have in vivo functional significance. Phospho-CaM KIV is capable of stimulating cAMP-response element–binding protein (CREB)–mediated transcription in Jurkat T cells. Coexpression of the PP2A inhibitor, SV40 small T antigen, potentiates CaM KIV induction of CREB-dependent transcription, which indicates that PP2A was likely to dephosphorylate and inactivate CaM KIV in vivo. The effects of SV40 small T antigen were specific for CaM KIV induction of CREB because the antigen had no effect on PKA regulation of CREB (87).

In addition to PP2A-dephosphorylating CaM KIV, another CaM kinase phosphatase has been cloned (88). This phosphatase has biochemical properties that appear distinct from the previously identified PP2A, 2B, and 2C, although it is somewhat similar in sequence homology to PP2C (89). The phosphatase can dephosphorylate CaM KI and IV that have been phosphorylated by CaM KK in vitro (90). Whether this new phosphatase regulates CaM KI and IV in vivo, however, remains to be established.

CaM KK and the CaM Kinase Cascade

The exciting discovery of the CaM KKs led to a flurry of research that ultimately advanced our understanding of how CaM KI and IV function biochemically (for review see 91). The CaM kinase kinases are themselves regulated by intrasteric

autoinhibition as well as by Ca^{2+}/CaM binding (43, 69, 72, 84, 92–94). Both CaM KKα and KKβ contain phosphorylatable residues within their activation loops and thus, may be substrates of upstream protein kinases as well. To date, however, there is no experimental evidence to suggest that these residues become phosphorylated. The kinase kinases are unusual in the way they recognize their substrates. Most protein kinases phosphorylate short peptides derived from known substrate proteins. CaM KKα, however, only phosphorylates a peptide from CaM KIV with a K_m 370-fold higher than that of the CaM KIV protein (95). It appears that both CaM KKα and β recognize a tertiary structure in their substrates, in that heat denaturation of CaM KI or IV prevents phosphorylation by CaM KK (95; SS Hook, KA Anderson, & AR Means, unpublished data). CaM KIV seems to bind to a unique region in CaM KK that is positioned after the ATP-binding loop between kinase subdomains II and III and is called the RP domain (for arg/pro rich domain). Deletion of this domain in CaM KK inhibits interaction with GST-CaM KIV, and consequently CaM KIV is no longer a substrate (96). The deletion, however, does not affect CaM KK activity, as the kinase was able to autophosphorylate and phosphorylate another putative substrate, PKB. Collectively, these data indicate that the RP domain in CaM KK recognizes a tertiary structure motif in CaM KIV. This idea correlates with the observation that the phosphorylation sites in CaM KI and IV are not very similar in sequence. Thus, it is unclear whether CaM KK also recognizes primary amino acid sequences in its substrates.

There is no doubt that in vitro CaM KKα and β each activate CaM KI and IV. In addition, overexpression studies show that CaM KKs can increase CaM KI and IV activity and in turn, increase CREB-mediated transcription (72). Very little is known, however, regarding how this cascade functions in vivo. The tissue distribution/subcellular localization of these kinases presents something of a conundrum. CaM KIV has limited tissue distribution in that it is found in T cells, thymus, bone marrow, adrenal gland, skin, testis, ovary, and distinct regions of the brain (reviewed in 97), whereas CaM KI is found to varying levels in all cells and tissues (43, 45). Interestingly, the CaM KKs are primarily expressed in neuronal tissues, although both isoforms are expressed at lower levels in thymus, testis, and spleen (72, 92). The expression of CaM KKβ in the brain strikingly tracks that of CaM KIV, with the highest levels found in cerebellar granule cells and outer cortex (72, 98, 99). However, because the tissue distribution of CaM KI is much wider than either CaM KKα or β, the possibility exists that other CaM KKs have not yet been identified. Alternatively, in those tissues in which CaM KKα and β are not expressed CaM KI may have activation-independent substrates, as discussed above. Neither CaM KKα or β nor CaM KI or IV contain obvious nuclear localization signals, yet clearly CaM KIV, at least, is nuclear, although it is also found in the cytoplasm (100). Subcellular localization of the CaM KKs is still under debate, as one study indicates that CaM KKα is nuclear (101) and one indicates that both KKα and KKβ are cytoplasmic (102). Our unpublished studies support the latter, as we do not find CaM KKβ to be nuclear under any experimental manipulations we have tried (S Lemrow & AR Means, unpublished data). Suprisingly, although truncated forms of mammalian CaM KI can stimulate transcription from CREB

reporter genes (72, 103), it is not localized to the nucleus (104). Thus, in order for CaM KI to stimulate CRE-dependent transcription CaM KI must either translocate into the nucleus by some phantom mechanism or activate a downstream protein kinase(s), which results in CREB phosphorylation. It would be very worthwhile to address where CaM KI and IV phosphorylation occurs within the cell using phospho-specific antibodies directed to the activation loop threonines.

The question of what extracellular signalling pathways activate the "CaM kinase cascade" is only beginning to be addressed. Aletta and co-workers (105) have shown that KCl depolarization of PC12 cells results in phosphorylation and enzymatic activation of endogenous CaM KI. Likewise, CaM KIV is phosphorylated in response to KCl, glutamate, and N-methyl-D-asparate (NMDA) treatment of cultured rat hippocampal neurons (106, 107). As discussed previously, CaM KIV is also phosphorylated and activated in response to stimulation of the T cell receptor (72, 86, 108, 109). It appears that at least in PC12 cells, the cAMP pathway can cross-talk to the Ca^{2+} pathways such that activation of CaM KI and IV is inhibited by treatment with the PKA activator forskolin. PKA phosphorylates CaM KKα on T108, which decreases its activity towards CaM KI and IV (110, 111).

The possibility exists that CaM KK may phosphorylate targets other than CaM KI and IV. The AMP kinase is phosphorylated and activated by AMP KK in a manner similar to that of CaM KI and IV. CaM KK can also activate AMP K but not as efficiently as it activates CaM KI (112). Secondly, Ca^{2+} was suggested to promote cell survival through CaM KKα, which phosphorylates and activates protein kinase B (or c-Akt). PKB then phosphorylates the apoptotic inducer, BAD, on S136. This phosphorylation inactivates BAD function by promoting its interaction with 14-3-3 (113).

The recent isolation of CaM KI/IV and CaM KK homologues in *C. elegans* (114), *Aspergillus nidulans*, and *Schizosaccharomyces pombe* (115) will certainly advance our understanding of what these kinase family members do physiologically. The mRNA of the *S. pombe* homologue of CaM KI, *cmk1*, is expressed in a cell cycle–dependent manner with a dramatic peak at the G1/S transition. Overexpression of wild-type *cmk1* has no effect on organismal growth; however, overexpression of a T192D (the equivalent of T177 in mammalian CaM KI) causes cell cycle arrest (115). These data suggest that CaM KI/IV homologue activity needs to be downregulated in order for the cells to progress through the cell cycle. In contrast, data from Joseph & Means (115a) suggests that both CaM KK and CaM KI in *A. nidulans* are required for cell cycle progression. Thus, genetic studies in these organisms have provided the first clues that the CaM kinase cascade can potentially regulate progression through the cell cycle.

CaM KII

Clearly, the most well characterized of the multifunctional CaM kinases is CaM KII (for review see 116). CaM KII is encoded by 4 separate genes (α, β, γ, and δ), and alternate splicing of these genes produces the 24 polypeptides seen in

vivo (for review see 117). Every cell type has at least one isoform of CaM KII. Through a C-terminal association domain that follows the CaM-binding domain CaM KII assembles into homo- or heteromultimers of 8–12 subunits (118–120), forming a pinwheel-like structure (121). Brocke and collegues (122) have recently shown that rat forebrain CaM KII holoenzymes contain α:β subunits at a ratio of 2:1. When these subunits are expressed in Cos-7 cells they form heteromultimers containing a 1:3–4 stoichiometry of α:β. Thus, it appears that composition of the heteromultimers may be influenced by the availability of individual subunits. This is supported by the observation that in rat forebrain $\sim$45% of the α subunits are associated with all the available β subunits and the remaining α subunits assemble into homomultimers (122).

Recently, several alternatively spliced variants of CaM KII that reside in the nucleus owing to a nuclear localization signal (NLS) within their association domains have been identified (123, 124). The locale of heteromultimers is determined by whether the majority of the isoforms are nuclear or cytoplasmic (123). Ca^{2+}/CaM-independent forms of CaM KI and IV can phosphorylate CaM KII S332, the residue immediately C-terminal to the NLS (Figure 2). This phosphorylation serves to exclude CaM KII from the nucleus by preventing its interaction with one of the nuclear import receptors (125). The in vivo significance of nuclear exclusion of CaM KII is currently unknown.

As indicated above, much of what is known about the regulation of CaM KI and IV by autoinhibition and CaM-binding was first shown for CaM KII, and thus the principles governing this regulation will not be reiterated here. CaM KII is distinct from CaM KI and IV, however, in that it is not regulated by activation-loop phosphorylation but by autophosphorylation within the pseudosubstrate domain (Figure 2). One catalytic subunit phosphorylates the autoinhibitory domain of the neighboring subunit on T286 (number in the α isoform). This intersubunit event requires that both the catalytic subunit and the substrate subunit have Ca^{2+}/CaM bound to them (126, 127). T286 phosphorylation then results in 20–80% Ca^{2+}/CaM-independent activity (78–83, 128).

Recent modeling studies of CaM KII paint a convincing structural picture of the importance of T286. Yang & Schulman (129) used a rational mutagenesis strategy (130) to identify residues within the autoinhibitory sequence that might interact with specific residues within the catalytic domain based on earlier mutational studies with MLCK (131). The mutagenesis data indicated that the CaM KII structure could not be modeled exactly like the crystal structure of CaM KI. Several of the loops and α helices were positioned more like those in PKA. Thus, the final modeling structure of CaM KII was a hybrid based on both the CaM KI and PKA structures. T286 is buried within a hydrophobic pocket within the catalytic domain. Phosphorylation of this residue, mutation to D (78, 81), or mutation to K (129) disrupts the interaction between the pseudosubstrate domain and the catalytic domain, thus generating Ca^{2+}/CaM-independent activity. Mutation to a hydrophobic residue, however, has no effect (78, 81). Reciprocal mutations introducing charged residues in the catalytic domain also generated

Ca^{2+}/CaM independence (129). This structure, as well as a model based solely on that of PKA (46), predicts why T286 phosphorylation is an intersubunit reaction and that both the kinase and substrate subunits require bound Ca^{2+}/CaM. T286 is not positioned such that it can be phosphorylated intramolecularly. Because the side-chain is facing the catalytic domain, Ca^{2+}/CaM would need to relieve its interactions with hydrophobic residues, thus allowing the neighboring subunit to phosphorylate it. This model is very reminiscent of how Ca^{2+}/CaM is required to bind to CaM KI in order for it to be phosphorylated by CaM KK (43, 112).

Autophosphorylation of T286 increases affinity for CaM by decreasing the rate of CaM dissociation. CaM is trapped by autophosphorylation so that even when Ca^{2+} levels are reduced, the kinase is fully active until CaM dissociates (several hundreds of seconds) (83). This could serve as a mechanism to increase sensitivity of CaM KII to changes in intracellular Ca^{2+} concentration (83, 126). There is an increasing body of evidence that suggests CaM KII autonomous activity is important in such neuronal processes as long-term potentiation (LTP) and long-term depression (LTD) (for review see 132).

REGULATION OF TRANSCRIPTION

cAMP-Response Element Binding Protein/ CREB-Binding Protein

Because CaM kinases I, II, and IV have quite similar substrate specificity determinants (52, 133), it is not completely surprising that they sometimes phosphorylate the same proteins. One such in vitro substrate for all three of these kinases is the cAMP-response element binding protein, CREB (134; for review see 135). CREB is a ubiquitous transcription factor that binds consensus cAMP-response element (CRE) DNA sequences, TGACGTCA (136, 137). CREB binds DNA through its basic region and homodimerizes or heterodimerizes with closely related family members through its leucine zipper motif. CREB acquired its name because it is a target of the cAMP-dependent protein kinase (PKA). PKA phosphorylates CREB on a single S residue, S133 (137), which promotes recruitment of the transcriptional co-activator CBP (CREB-binding protein) (138), a protein that has recently been shown to display acetyltransferase activity towards both histones and other transcriptional regulatory proteins (139–142). The phospho-CREB/CBP complex then interacts with multiple basal transcriptional proteins to initiate RNA synthesis (for review see 143).

One of the first gene promoters shown to be regulated by CREB and the CRE was that of *c-fos* (for review see 144). The *c-fos* CRE is very similar in sequence to a region of the promoter, TGACGTTT, that had been termed the Ca^{2+} response element (CaRE). In fact, the *c-fos* Ca^{2+} response element and CRE are functionally indistinguishable in cell transfection studies (145, 146). The mechanism by

which Ca^{2+} induces transcription through Ca^{2+} response element/CRE is very reminiscent to that of PKA. CaM KI and IV can phosphorylate the activating S133 of CREB (134, 147) and consequently, can markedly stimulate CRE-mediated transcription. Most of these transcription studies have employed truncated Ca^{2+}/CaM-independent CaM kinases that are small enough to readily diffuse into the nucleus. The literature reveals that CaM KI is predominantly cytoplasmic (104) and the question as to whether it directly or indirectly phosphorylates CREB remains controversial. In contrast, endogenous CaM KIV is present in the nucleus, and T cell experiments indicate that it could be a relevant CREB kinase for interleukin-2 (IL-2) production (109). T cell receptor stimulation initiates lymphocyte proliferation, a process that requires an increase in intracellular Ca^{2+} and an increase in IL-2 mRNA. Several lines of evidence suggest that the CRE within the IL-2 promoter is crucial for gene transcription. Transgenic mice expressing a dominant-negative CREB, containing the S119A mutation (the mouse equivalent to S133A), are not able to transcribe the IL-2 gene in response to PMA/ionomycin stimulation of thymocytes (148). Likewise, transgenic mice with targeted expression of kinase-inactive CaM KIV to thymocytes have shown an inability to produce IL-2 after PMA/ionomycin treatment or T cell receptor stimulation, and this effect correlates with dramatic decreases in the levels of both phospho-CREB and *c-fos* mRNA (109).

In neuronal cell lines truncated (constitutively active) CaM KII (1-290) can increase transcription of the *c-fos* gene (149). Intriguingly, the only response element in the *c-fos* promoter that was not stimulated by CaM KII was the CaRE/CRE. CaM KII in fact, does phosphorylate CREB on S133 but does not induce CRE-mediated transcription (150, 151). Phosphopeptide mapping indicated that CaM KII phosphorylates another site in CREB, S142. Mutation of S142 to A renders CREB transcriptionally responsive to CaM KII (151). Phosphorylation of S142 markedly decreases the in vitro binding affinity of CREB for CBP. In addition, the nuclear magnetic resonance structure of CREB (amino acids 101–160) complexed with the CREB binding domain of CBP suggests that the extensive interactions between the amphipathic helix B in CREB with hydrophobic residues in CBP would be sterically strained by a phosphate at S142 (152), thus providing a mechanism by which CaM KII negatively regulates CREB-dependent transcription.

Because the CaM kinases have opposing effects on gene expression, the question arises of how Ca^{2+} might both stimulate and inhibit CRE-mediated transcription. Recall that both CaM KI and IV can inhibit nuclear translocation of CaM KII by directly phosphorylating S332, the residue immediately C-terminal to the NLS (125). CaM KI and IV have lower K_{CaM}s than CaM KII, and it has been suggested that these enzymes are activated in vivo before CaM KII. Thus, increased intracellular Ca^{2+} may serve to stimulate transcription through CREs and inhibit a signal that would decrease transcription through CREs. Many labs have implicated protein phosphatases in terminating CRE transcription by dephosphorylation of CREB. An equally viable possibility is that phosphatases are acting on CaM KII so that it can be imported into the nucleus to inhibit transcription.

Although S133 phosphorylation of CREB is required to induce gene expression through CREs, this phosphorylation is not sufficient for gene expression (for review see 153). For instance, both neurotrophins and N-methyl-D-asparate receptors stimulate CREB phosphorylation, but neither induces CRE-mediated gene transcription effectively (154–157). In addition to CaM KI, II, and IV, a nuclear target of the MAP kinase cascade, $pp90^{rsk}$, also phosphorylates CREB on S133. However, the other "regulatory" event for stimulating gene expression is thought to be mediated only by nuclear CaM kinase in response to nuclear Ca^{2+} transits. Treatment of neurons with the CaM kinase I, II, and IV inhibitor, KN-62 (157a; see Table 1), or the MAP kinase inhibitor, PD98059, does not significantly alter phospho-CREB levels in response to Ca^{2+} mobilization (KCl), although KN-62 alone inhibited CRE-mediated transcription (158, 159). Cytoplasmic Ca^{2+} alone is sufficient for CREB phosphorylation, but in the absence of nuclear Ca^{2+} this CREB is transcriptionally inert (158). Microinjection of constitutively active forms of CaM KII, CaM KIV, or activated ras (to stimulate $pp90^{rsk}$) reveals that all three kinases induced phosphorylation of S133 of CREB, but only CaM KIV stimulated CREB-mediated transcription (158).

The focus then shifted to the possibility that CBP may be a target for Ca^{2+} induction of gene transcription. Clearly, full-length CBP and portions of CBP (amino acids 1–460, 1678–2441, and 1892–2441), when fused to the GAL4 DNA binding domain (DBD), are transcriptionally responsive to increases in intracellular Ca^{2+} (158, 160). The requirement for CaM kinases, however, is questionable. Although constitutive CaM kinase can induce transcription from fragments of CBP, it is still unclear whether CaM kinases are essential for Ca^{2+} regulation of CBP. For instance, although microinjection of Ca^{2+}/CaM-independent CaM KIV (1–313 of the rat isoform) into AtT20 pituitary cells could stimulate GAL4-CBP 1892–2441 transcription, Ca^{2+}-induced CBP phosphorylation was not detected, and potential CaM KIV phosphorylation sites within the 1892–2441 fragment of CBP did not affect its transcriptional activation. The possibility that Ca^{2+} may be regulating CBP in a manner distinct from that of the CaM kinases is supported by a report from Hu and co-workers (160). They found that in E18 rat cortical neurons, 1–313 CaM KIV as well as 1–290 CaM KII can induce GAL4-CBP 1678–2441, although glutamate activation of CBP 1678–2241 is not affected by the CaM kinase I, II, or IV inhibitor, KN-93. The idea that glutamate could stimulate CBP through $pp90^{rsk}$ seems unlikely because MAP kinase inhibitors also had no effect. In constrast, stimulation of transcription by the N-terminal fragment of CBP, 1–460, in response to glutamate was blocked by KN-93. Because 1–290 CaM KII and 1–313 CaM KIV could stimulate GAL4-CBP 1–460 transcription, these were presumed to be the relevant kinases activated by glutamatergic stimulation (160). The question as to why CaM KII would negatively regulate CREB but positively regulate its co-activator CBP is unclear. In addition, definitive evidence that CaM kinases regulate CBP function in the context of either a promoter or within chromatin is still lacking.

Activating Transcription Factor-1, CAAT-Enhancer Binding Protein, and Serum Response Factor

The mechanisms by which CaM kinases are thought to regulate activating transcription factor-1 (ATF-1), CAAT-enhancer binding protein (c/EBP), and serum response factor (SRF) transcription factors have been extensively reviewed (91, 161–163) and will be covered only briefly here.

Activating transcription factor-1, like CREB, is a leucine zipper transcription factor that can heterodimerize with other ATF family members as well as with CREB to stimulate transcription through CREs. The regulation of ATF-1 by CaM kinases is remarkably similar to that of CREB. The equivalent residue to S133 in CREB is S63 in ATF-1. S63 is phosphorylated by CaM KI, II, and IV although only CaM KI and IV activate ATF-1-dependent transcription. As was the case with CREB, CaM KII phosphorylates a second site in ATF-1, S72. The transcriptional activity of ATF-1 proteins is enhanced by CaM KII in pituitary GH3 cells only when they contain a S72 to A mutation (103). Phosphorylation of this residue, however, may be dependent on the cell type because in F9 cells, transcription by wild-type ATF was stimulated by CaM KII and S72 phosphorylation was undetectable (164).

In hippocampal neurons, c/EBP DNA binding is stimulated by increases in intracellular Ca^{2+}, and this stimulation is blocked by the CaM kinase I, II, IV inhibitor, KN93 (165). In G/C pituitary cells Ca^{2+}/CaM-independent CaM KIIα stimulated transcription from reporter genes containing c/EBPβ (but not c/EBPα) sites within their promoters. CaM KII phosphorylates S276 in vitro, and c/EBPβ mutants in which S276 was changed to A are markedly attenuated in their ability to be stimulated by CaM KII (166). Because c/EBPβ is nuclear in G/C cells (167), it is unclear whether CaM KII stimulation is specific to the 1–290 truncated kinase or whether full-length nuclear CaM KII isoforms also regulate c/EBPβ. The same argument could be made for CaM KIV because truncated forms of the kinase can stimulate transcription via both c/EBPβ and c/EBPδ in hippocampal neurons (165).

Serum response elements are bound by homodimers of serum response factor (SRF) as well as by ternary complex factor (TCF), which is comprised of one subunit of SRF and one subunit of the ets family member, elk-1. SRF was thought to be a potential target for CaM kinases because it is rapidly phosphorylated in PC12 cells after KCl-mediated depolarization (168). Likewise, constitutively active CaM KII (1–290) can increase transcription of the *c-fos* gene, and deletion analysis revealed that one of the DNA sequences stimulated comprised the serum response element (149, 169). Indeed, CaM KII and IV can phosphorylate SRF on S103 in vitro. However, although this site is phosphorylated in vivo (170) and seems to increase DNA binding (171), suprisingly, it is not responsible for conferring Ca^{2+} sensitivity (168). Conversely, CaM KII is reported to phosphorylate SRF on T160 within the MADS box (DNA-binding domain) (172). Presumably, a phosphate at

this position would negatively affect DNA binding (173). Nonetheless, the first demonstration that a full-length nuclear form of CaM KII regulated transcription was through the serum response element in the atrial natriuretic factor promoter (174). The predominant CaM KII isoform found in the heart is δ (175). The nuclear spliced variant, δB could increase transcription of the atrial natriuretic factor promoter in cultured ventricular myocytes in response to the α1-adrenergic receptor agonist, phenylephrine, whereas cytoplasmic CaM KIIα or δC could not (174). The molecular mechanism by which CaM kinase regulates SRF is still only speculative. One report suggests that CaM KIV may stimulate transcription of the SRF binding protein elk-1 when fused to the GAL4 DNA binding domain fusions of the SRF binding partner, elk-1 (176). CaM kinase regulation of SRF, however, seems to be independent of elk-1 phosphorylation (168). Given that elk-1 interacts with CBP (177), it is possible that Ca^{2+} and/or the CaM kinases indirectly regulate SRF through CBP (as discussed above).

Orphan Receptors

Recently, CaM KI and IV have been shown to potentiate transcription mediated by nuclear hormone receptors, specifically some of the orphan receptors. Unlike classical steroid receptors, orphan receptors are thought to be "constitutively active" in that they apparently do not need a ligand for their transcriptional activity. Because CaM KIV has been shown to be a key kinase involved in transcriptional activation and because its tissue distribution overlapped that of RORα1 (178–180), a functional relationship between the two proteins was hypothesized. More importantly, mice null for the CaM KIV or RORα (181, 180) genes display strikingly similar phenotypes. The spontaneous mutation of the *staggerer* mouse (182), named after its ataxic phenotype, maps to a mutation preventing translation of the ligand-binding domain (LBD) of RORα (179).

These similarities prompted Kane & Means (183) to address whether CaM KIV may affect RORα-mediated transcription. Indeed, truncated versions of CaM KIV and CaM KI could markedly potentiate RORα-dependent transcription in multiple cell lines. On the other hand, a 1–290 CaM KIIα potently repressed transcription consistent with its ability to negatively regulate transcription in several different systems (150, 184). Supporting a role for CaM KIV in RORα-mediated transcription, transfection of a full-length CaM KIV was also effective, but only in response to a rise in intracellular Ca^{2+} (183).

CaM KIV can stimulate transcription not only via RORα1, but also via a spliced variant RORα2, RORγ, and a related orphan receptor, COUP-TF1. The effect of CaM KIV is unique to this class of orphan receptors, as neither thyroid hormone receptor, estrogen receptor, androgen receptor or glucocorticoid receptor (GR), either in the presence or absence of ligand, was potentiated by CaM KIV (183; CD Kane & AR Means, unpublished data). The LBD of RORα, when fused to the GAL4 DNA binding domain, is stimulated by a magnitude similar to that by which a full-length RORα stimulates an RORE reporter gene. However, because

the LBD is not a substrate for CaM kinase, co-activator molecules for RORα function may be targets for CaM KIV. Co-activators for nuclear receptors typically contain LXXLL motifs required for their interaction with LBD of the receptors. Mammalian two-hybrid assays revealed that RORα interacts with LXXLL peptides containing a novel consensus sequence, and this interaction is markedly potentiated by CaM KIV (183). Because blast search analysis indicated that no known co-activators contain similar amino acid sequences to these peptides, RORα may use an unidentified co-activator. The precise mechanism by which CaM KIV functions to increase RORα's transcriptional functions is unclear. Regardless, careful analysis of the RORα (180, 181), RORγ (185), COUP-TF1 (186) knock-out mice compared with those null for CaM KIV (187, 187a,b) may reveal which phenotypic effects that occur in the absence of CaM KIV are due to compromised transcription mediated by one of these orphan recptors.

Myocyte Enhancer Factor-2/Histone Deacetylase

One of the most exciting involvements of Ca^{2+}/CaM and CaM KI/IV has been in the proposal that they can regulate activity of the myocyte enhancer factor-2 (MEF2) family of transcription factors. MEF2 proteins (reviewed in 188) were first identified because they were required for muscle morphogenesis (189), but now have been shown to be expressed in many tissues. Vertebrates have four MEF2 genes designated MEF2A through D. MEF2 contains a MADS box (named for the first four members of this family of transcription factors: MCM1, agamous, deficiens, and SRF) that is a 57-amino-acid motif in the extreme N-terminus of the protein that serves as a minimal DNA-binding motif. Immediately C-terminal to the MADS box is the MEF2 domain, which enhances DNA binding and is responsible for homodimerization and heterodimerization (190), as well as dimerization with basic helix-loop-helix proteins such as MyoD (191, 192).

Several lines of evidence suggest that in both cardiac muscle and T cells, CaM KI/IV may synergize with the Ca^{2+}/CaM-dependent phosphatase, calcineurin, in activating transcription through MEF2. Overexpression of calcineurin both in mouse ventricular myocytes and in transgenic mice in which ventricular expression is controlled by the α-myosin heavy chain promoter results in cardiac hypertrophy (191). Likewise, Ca^{2+}/CaM-independent forms of CaM KI or IV can activate promoters sensitive to hypertrophic stimuli, such as atrial natriuretic factor and α skeletal actin, in transfected cardiomyocytes. In addition, transgenic mice expressing 1–317 CaM KIV from the α-myosin heavy chain promoter display cardiac hypertrophy and a 100-fold increase in MEF2 transcriptional activity (193). The hypertrophic effect of CaM KIV can be enhanced by calcineurin. Likewise, calcineurin and CaM KIV synergize in activating MEF2A enhancers in the desmin gene (194). The synergism between these two Ca^{2+}/CaM-dependent enzymes is not limited to muscle cells but is also found in T cells. The steroid receptor Nur77 is transcribed in a Ca^{2+}-dependent manner in response to stimulation of the T cell receptor in a process that induces apoptosis during negative selection. The Ca^{2+}

responsive element in the Nur77 promoter was mapped to two MEF2 binding sites (195). This promoter, as well as GAL-MEF2D, is synergistically activated by calcineurin and a constitutively active fragment of CaM KIV (196). Although calcineurin can dephosphorylate MEF2A, it does not affect its DNA-binding affinity in muscle nuclear lysates (194), although it may increase binding affinity in cerebellar granulocytes (197). Thus, the functional significance of a direct regulation of MEF2 by calcineurin is somewhat unclear. In vivo MEF2 interacts with a known target of calcineurin, NFAT (nuclear factor of activated T cells) (196). NFAT is a cytoplasmic transcription factor that is dephosphorylated by calcineurin and subsequently enters the nucleus to stimulate transcription (for review see 198). The synergism between CaM KIV and calcineurin in α-myosin heavy chain transgenic mice is due in part to NFAT3 (193). In T cells calcineurin and CaM KIV appear to have nonredundant roles in activating MEF2D. Calcineurin effects can be mimicked using constitutively active NFAT2 and inhibited by dominant negative NFAT. In contrast, neither pharmacological inhibitors of calcineurin nor dominant negative NFAT have effects on CaM KIV–stimulated transcription through MEF2D (196).

The CaM KIV–responsive region of MEF2C was primarily the MADS box/MEF2 domain (199). This region of MEF has been shown to interact with the histone acetyltransferases p300 and CBP (200) as well as class II histone deacetylases (HDACs) (199, 201, 202). Histone acetyltransferases and HDACs are thought to either activate or repress transcription primarily through altering the acetylation state of histones and thereby modifying chromatin structure. Lu and co-workers (199) have shown that constitutively active CaM KI/IV can dissociate HDAC5 from MEF2C (Figure 5, left;). Exactly how CaM KIV mediates this dissociation is unclear because the MADS box/MEF2 domain is not a substrate (199). It is possible that CaM KIV phosphorylates HDAC5, although if it does, there is no effect on its deacetylase activity (199). These novel findings indicate that CaM KIV may activate transcription by "relief of repression." Loss of HDAC5 binding to MEF2 exposes the p300/CBP binding site (203). Because CaM KIV seems to stimulate CBP activity (see above) and also may potentiate MEF2 transactivation by phosphorylating the transactivation domain (196), the kinase may play multiple roles in regulating MEF2.

The complexity by which Ca^{2+} regulates the MEF2 family of transcription factors is further illustrated by the effects of the Cabin1 protein. Cabin1 was originally identified as a Ca^{2+}-dependent inhibitor of calcineurin (204). Interestingly, in T cells Cabin1 represses MEF2 function in the absence of Ca^{2+} by binding to its MADS box/MEF2 domain. Stimulation of the T cell receptor leads to increases in intracellular Ca^{2+} such that Ca^{2+}-loaded CaM frees MEF2 by competitively binding to Cabin1 (205) (Figure 5, right).

The results with Cabin1 prompted Youn and collegues (203) to address whether HDAC 4 binding to MEF was also regulated by Ca^{2+}/CaM. By sequence alignment with known CaM-binding proteins, HDAC 4 contains a potential CaM-binding domain. Indeed, HDAC4 binds CaM-sepharose in a Ca^{2+}-dependent manner.

Because the putative CaM binding domain of HDAC4 (203) overlaps the MEF2 interaction domain (199), Ca^{2+}/CaM may titrate HDAC4 from MEF2 (Figure 5, middle). Immunoprecipitates of MEF2D from T cells contain HDAC only in the presence of the Ca^{2+} chelator, EGTA. In the presence of Ca^{2+}, however, CBP co-immunoprecipitates with MEF2 (203). These results could suggest that Ca^{2+}/CaM acts to derepress MEF2, although the possible involvement of CaM kinase cannot be excluded.

The question arises of why nature would use both Ca^{2+}/CaM and a Ca^{2+}/CaM-dependent kinase to regulate MEF2 interaction with HDAC. Three simplistic possibilities exist. (*a*) CaM kinase may specifically relieve repression mediated by HDAC5, whereas Ca^{2+}/CaM targets repression by HDAC4. (*b*) Alternatively, HDAC repression of MEF2C and MEF2D is somehow functionally distinct and thus requires different mechanisms to terminate it. (*c*) There is also the possibility of tissue and/or cell-type specificity. CaM KIV (and possibly also CaM KI) are not expressed in ventricular myocytes. Thus, although overexpression of truncated CaM KI/IV can relieve MEF2C repression (199), perhaps Ca^{2+}/CaM is the physiologically relevant regulator. Conversely, in T cells in which CaM KI and IV are expressed, perhaps they are the primary regulators of MEF2. Regardless, because this area of research has sparked considerable excitement, answers to this dilemma are sure to come swiftly.

CONCLUDING REMARKS

The study of the multifunctional CaM kinases I, II and IV have elucidated how Ca^{2+}/CaM can bind to a target and relieve an intramolecular autoinhibition that results in activation of the enzyme. In the case of CaM KII this results in autophosphorylation that generates a Ca^{2+}/CaM autonomous kinase activity (78–83, 128). CaM KIV also can become autonomous of Ca^{2+}/CaM, although the precise mechanism responsible for generating this activity state has yet to be worked out (84, 206). On the other hand, CaM KI is always Ca^{2+}/CaM-dependent, even when phosphorylated on its activation loop (43). Common to all three enzymes is the fact that phosphorylation sensitizes the protein kinase to subsequent Ca^{2+} signals (73, 207). However, despite the vast literature elucidating the principles governing activation in vitro, surprisingly little is known about how these principles may be relevant in vivo. For example, the values for K_{CaM} and K_{Ca2+} suggest that CaM kinases I, IV, and II could be activated sequentially (in that order) in the cell in response to a rise in Ca^{2+}. This possibility has yet to be addressed, and in fact, the origins of the Ca^{2+} transients responsible for CaM kinase activation are only beginning to be explored. Secondly, biochemical data suggest that CaM kinases may be "primed" to respond to a Ca^{2+} spike owing to the fact that CaM might already be associated with CaM kinases at ambient Ca^{2+} concentrations (208). Cell biological data addressing this idea are lacking. Third, why would two members of a CaM kinase cascade, one a direct target of the other, both need Ca^{2+}/CaM

binding to become active? Is this a way to amplify the Ca^{2+} signal? Are CaMKK and CaMKI/IV components of a larger signaling complex by analogy to the MAP kinase complex? Little has been done to explore whether CaM KI and IV shuttle from cytoplasm to nucleus. Where in the cell are CaM KI and IV activated? Do the CaMKKs remain in the cytoplasm and phosphorylate CaM KIV as a part of the mechanism for nuclear translocation of this enzyme? CaM has been reported to move into the nucleus in response to a rise in Ca^{2+} (209–211). Is this an active process? Does Ca^{2+}/CaM piggyback with any of its kinase targets (or other binding proteins) for the ride into the nucleus?

Certainly, understanding how Ca^{2+} regulates gene transcription will require elucidation of the movement of CaM and its targets in and out of the nucleus. Although CaM kinases have been clearly implicated in regulating gene transcription, Ca^{2+} and Ca^{2+}/CaM also appear to be capable of participating in transcriptional regulation without the involvement of a CaM-dependent enzyme (203, 205, 212, 213; for review see 214). Thus, the task of teasing apart these various mechanisms becomes a daunting undertaking. Fortunately, the use of pharmacological inhibitors of CaM and the CaM kinases, constitutively active and dominant negative forms of the kinases, and genetic manipulation of the kinases in cells and experimental organisms will make this task much less formidable.

We now know of mechanisms whereby CaM kinases stimulate transcription by direct phosphorylation of a transcription factor (e.g. CREB) (134, 147) or by phosphorylating unknown components of the transcription complex that are subject to regulation (e.g. in the case of the ROR family of transcriptional activators) (183). Presumably, in the latter case, phosphorylation stabilizes protein/protein interactions. Regardless of the mechanism involved, both of these modes of regulation enhance transcriptional activation rather than facilitate transcription factor dimerization or DNA binding. Most recently, although again the precise mechanism remains to be established, CaM KI and/or IV have also been implicated in the relief of transcriptional repression (199). Interestingly, this is also an endpoint that can be accomplished by Ca^{2+} or Ca^{2+}/CaM without the need of a kinase intermediate (203, 205). However, in all these instances, gene transcription is increased. Conversely, CaM KII can negatively regulate transcription of CRE-mediated genes by phosphorylating CREB on a specific S residue that decreases its affinity for the obligate coactivator CBP/p300 (151). In addition, at least in one specific promoter, multiple response elements appear to be repressed by CaM KII, raising the possibility that CaM KII may repress transcription through more than one mechanism (184). It will require a great deal of effort to sort out the various mechanisms by which Ca^{2+}, Ca^{2+}/CaM and CaM kinases regulate transcription. However, this should be a fertile field to plow for years to come.

Calcium is without question the most versatile of the second messengers. It is hard to identify a cellular process that does not require Ca^{2+} input. Calmodulin is also ubiquitous and regulates a remarkable array of physiological events by binding to one or more of its impressive array of binding proteins. In many ways CaM is the prototypical ligand-dependent receptor. However, because of its broad

distribution, it has been considered by some as a "housekeeping" protein. Nothing could be further from the truth. The ability to precisely regulate Ca^{2+} transients and CaM movement, coupled with the large number of target proteins, has endowed this receptor with exquisite specificity control. Elucidating the components of the signaling cascades by which Ca^{2+} regulates transcription of important cellular genes is a challenge worth the efforts of many talented investigators. Are you up to this challenge?

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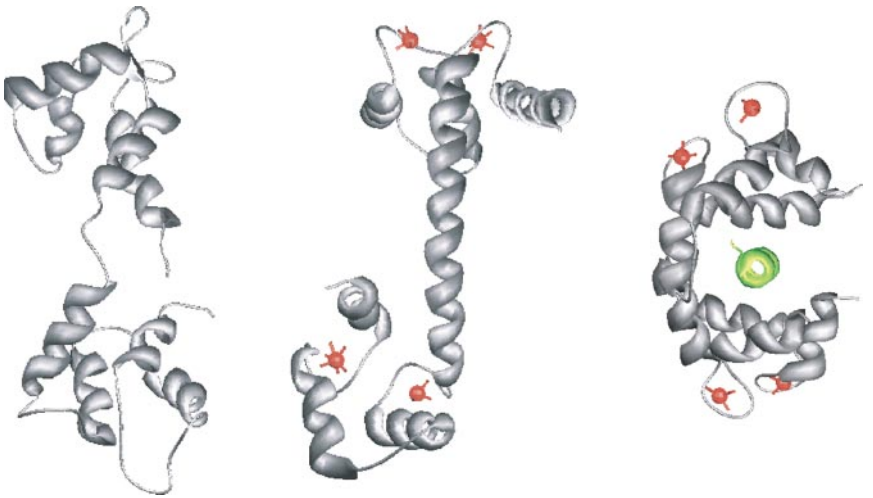

Figure 1 Calmodulin structures. NMR structure of apo CaM (left) (27); crystal structure of Ca^{2+}/CaM (middle) (35, 36); and crystal structure of Ca^{2+}/CaM bound to the CaM KII peptide (right) (39). In all three structures the N-terminal domain is on top.

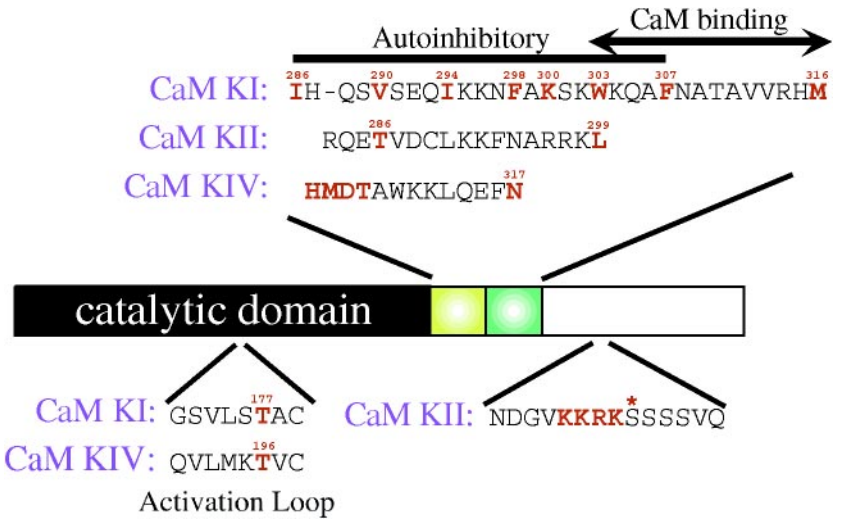

Figure 2 Schematic diagram of the CaM kinases highlighting their domain structure. The sequences of the overlapping autoinhibitory and CaM-binding domains of CaM KI and homologous regions of CaM KII and IV are shown. Important residues discussed in the text are denoted in red. Within the catalytic domain of CaM KI and IV is the activation loop containing the T residues phosphorylated by CaM KK. The C-terminal NLS of CaM KII is shown with the S phosphorylated by CaM KI and IV specified with an asterisk.

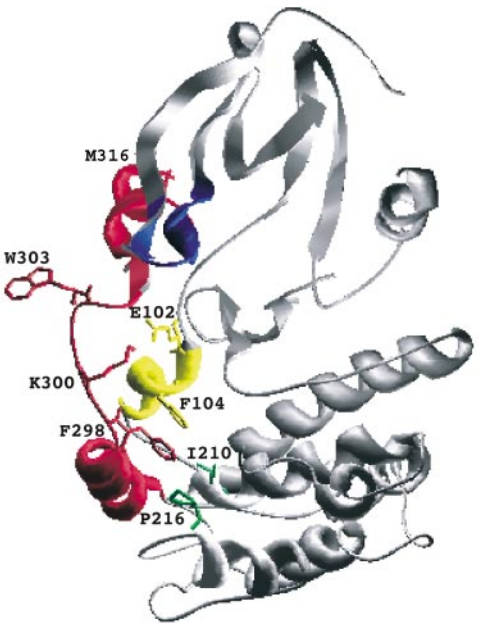

Figure 3 Crystal structure of 1-320 CaM KI (48). The autoinhibitory and CaM-binding domains are shown in red whereas the ATP-binding loop is in blue. The C-terminal hydrophobic anchor for CaM interaction, W303, is completely solvent exposed whereas the N-terminal anchor, M316, is buried in the ATP-binding loop. Autoinhibitory residues, F298 and K300 (both side chains in red) interact with F104 and E102, respectively, on the D α helix (in yellow). In addition, F298 interacts with I210 and P216 (shown in green).

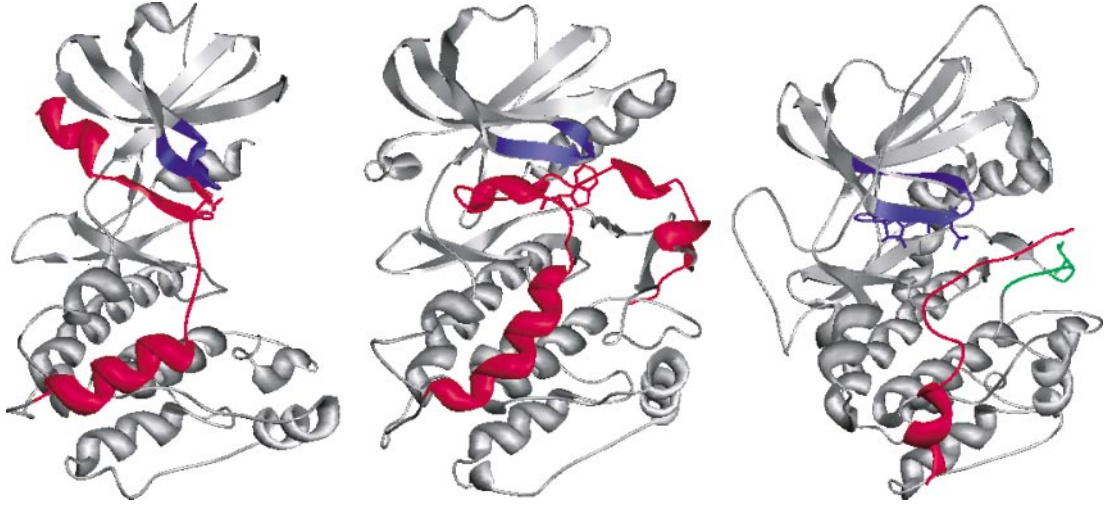

Figure 4 Crystal structures of CaM KI (48), twitchin (51), and PKA (56). Notice that here CaM KI has been rotated about the y axis approximately 80° counterclockwise compared with the view shown in figure 3. The ATP-binding loop of each kinase is shown in blue. The autoinhibitory segment of CaM KI (left) and twitchin (middle) and the PKI pseudosubstrate bound to PKA (right) are shown in red. In this view, W303 of CaM KI (left) is near the ATP binding loop as well is the homologous residue, W6228 in twitchin (middle). The C-terminal portion of the autoregulatory segment in CaM KI makes an abrupt turn away from the catalytic cleft, whereas in twitchin it swings in between the small and large lobes of the kinase, over the activation loop. In PKA the activation loop and T197 are shown in green. Notice the activation loop in CaM KI is missing due to disorder in this region of the structure.

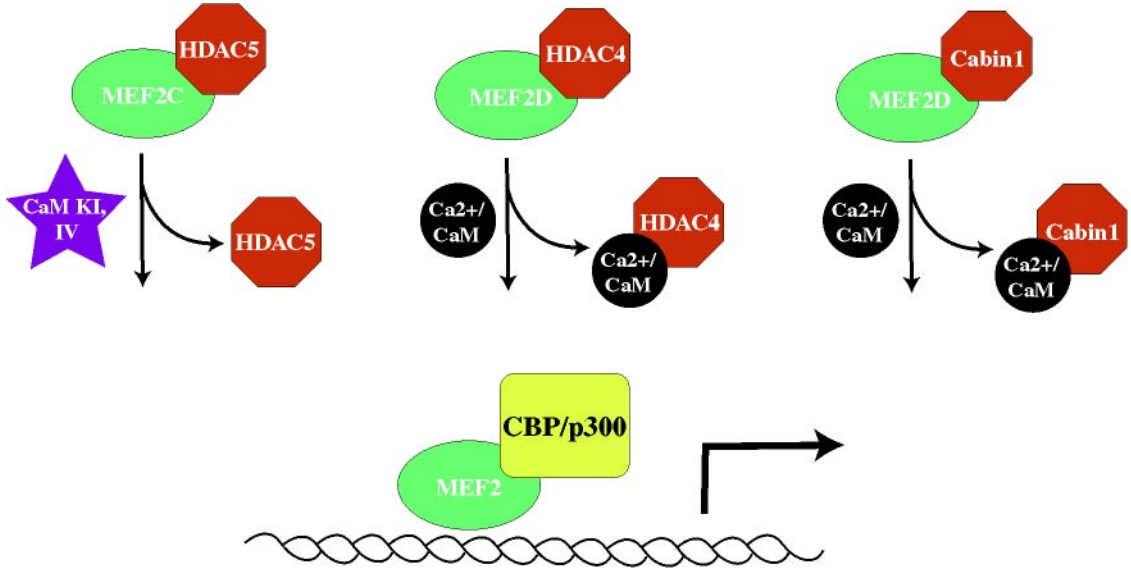

Figure 5 Schematic denoting proposed mechanisms of relieving MEF2 repression. The model on the left was postulated in cardiomyoctes using constitutively active CaM KI and IV (199). The models in the middle (203) and right (205) are thought to occur in T cells.