Minireview

Defining Ca\(^{2+}\)/Calmodulin-dependent Protein Kinase Cascades in Transcriptional Regulation*

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Calcium is a well established regulator of transcription. Modulation of responses to this ubiquitous second messenger can occur by superposition of coincident Ca\(^{2+}\)-independent signals, but there is also growing evidence that the strength, frequency, source, and location of the Ca\(^{2+}\) signal are determinants for specific transcriptional results. These complex variations must be translated into changes in protein function that preserve and process the information conveyed by the original signal. The Ca\(^{2+}\) receptor calmodulin (CaM)\(^\dagger\) is involved in many of these changes through its effects on a variety of CaM-binding proteins (1). Among these, the multifunctional Ca\(^{2+}\)/calmodulin-dependent protein kinases (CaMKs) are notable for their effects on components of transcription complexes, directly connecting Ca\(^{2+}\) with changes in gene expression. The highly homologous CaMKI and CaMKIV are distinct from the multimeric CaMKII, although all have broad and overlapping substrate preferences, because their activation is greatly enhanced following phosphorylation catalyzed by "upstream" kinases in a manner analogous to the mitogen-activated protein kinase cascade.

Based on an evolving understanding of CaMKII/IV regulation and cloning of the CaMKII/IV kinases (CaMKks), a Ca\(^{2+}\)/CaM-dependent protein kinase I/IV cascade (CaMK cascade) has been proposed (2, 3). This review will discuss the biochemical and physiologic basis for the existence of this cascade and its potential for mediating Ca\(^{2+}\) regulation of transcription.

Identification and Biochemical Characterization of a CaMK Cascade

CaMKI and CaMKIV are closely related protein kinases with many similarities in mode of activation and substrate preferences in vitro but with different tissue distributions. The kinases are regulated by Ca\(^{2+}\)/CaM binding, which relieves intramolecular steric inhibition of the active site by a C-terminal autoinhibitory domain (Fig. 1). A second autoinhibitory mechanism unique to CaMKIV is relaxed by the autophosphorylation of Ser-12 and Ser-13 (4). In addition to deinhibition, CaMKI and CaMKIV are activated 10–50-fold by trans phosphorylation on a single Thr residue in the activation loop. Once activated, CaMKIV acquires Ca\(^{2+}\)/CaM independence, whereas CaMKI remains Ca\(^{2+}\)/CaM-dependent (5). Dephosphorylation and inactivation of activated CaMKIV can be catalyzed in vitro by PP1, PP2A, calcineurin (CaN), and the CaMK phosphatase, but the relevant phosphatase(s) in vivo is still unclear (6, 7). CaMKI is ubiquitously expressed, whereas CaMKIV has a more limited distribution, although both enzymes are strongly expressed in the brain.

Recognition of the ability of kinases in brain extract to phosphorylate and activate CaMKIV led to the cloning of two upstream kinases, CaMKK\(\alpha\) and CaMKK\(\beta\) (8, 9). In addition to the brain, where both CaMKKs are highly expressed, CaMKK\(\alpha\) mRNA is found in thymus and ilepen, whereas CaMKK\(\beta\) is present at lower levels in all tissues that express CaMKIV. Although derived from distinct genes, rat CaMKKs are 80% similar, and either CaMKK can phosphorylate and activate CaMKI and CaMKIV in vitro. Both CaMKKs bind and are positively regulated by Ca\(^{2+}\)/CaM in vitro, and although their CaM binding site is different from the other CaMKs, the autoinhibitory mechanism functions in a similar manner to that of other Ca\(^{2+}\)/CaM-dependent kinases (10). Importantly, Ca\(^{2+}\)/CaM binding to CaMKI/IV is a prerequisite to phosphorylation by the CaMKks (5). Thus, in theory Ca\(^{2+}\)/CaM could relegate CaMKIV/IV activity on many levels.

The substrate preferences of CaMKI and CaMKIV are similar and intersect with CaMKII. In vitro, all three can phosphorylate the transcription factor CREB on the activating site Ser-133, only CaMKII phosphorylates aN inhibitor site, Ser-142 (13).

Interestingly, the presence of two additional basic amino acids 6 and 7 residues N-terminal to the phosphorylation site in certain peptide substrates allows phosphorylation by CaMKIV/IV equally well with or without activation by a CaMKK (14). This "activation independence" has not yet been demonstrated toward protein substrates. Nonetheless, because CaMKIV/IV requires Ca\(^{2+}\)/CaM for deinhibition in addition to activation loop phosphorylation, substrates of this type would not be phosphorylated by CaMKIV until a Ca\(^{2+}\) signal was initiated and so could represent Ca\(^{2+}\)-dependent but activation-independent signaling targets. Subsequent activation by a CaMKK would increase the number of available substrates by enhancing CaMKIV activity toward a second set of substrates (Fig. 1).

Does the Cascade Function in Cells?

The first reconstruction of a CaMK cascade in cells used transient transfection experiments with CREB as the transcriptional target. CaMKI and CaMKIV phosphorylate CREB on its activating Ser-133 in vitro and stimulate Gal4-CREB-dependent transcription in response to a rise in intracellular Ca\(^{2+}\) when cells are cotransfected with Gal4-CREB and a Gal4 reporter gene (15). Additional cotransfection with a CaMKK increases reporter activity more than 10-fold (8, 9). Mutation of the CaMKIV activation loop T to A abolishes CaMKK enhancement.

To be a signaling cascade, kinase activation loop phosphorylation must be dependent upon induction of CaMKK activity. Phosphatase incorporation into endogenous CaMKIV in Jurkat cells is induced rapidly following T-cell receptor stimulation and is blocked by chelation of extracellular Ca\(^{2+}\) (16). This is accompanied by 8–14-fold increases in immunoprecipitated CaMKIV activity that is refractory to further activation by exogenous CaMKK and can be reversed by in vitro treatment with PP2A (16). Recombinant CaMK IV transfected into BJAB cells, which lack endogenous CaMKIV, demonstrates similar activation following anti-IgM stim-

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‡ The abbreviations used are: CaM, calmodulin; CaMK, Ca\(^{2+}\)/CaM-dependent protein kinase; CaMKK, CaMK/IV kinase; CaN, calcineurin; CREB, cAMP response element-binding protein; CBP, CRE-binding protein; CRE, cAMP response element; dn, dominant negative; ROR, retinoic acid-related orphan receptor; LBD, ligand binding domain.
The evolutionary conservation of CaMKI/IV and CaMKK suggests a fundamental biological role. From Aspergillus nidulans to Caenorhabditis elegans to mammals, the cascade members are highly conserved and biochemically interchangeable in gross assays of cascade function in vitro (28, 29). Potential homologues have also been identified in Schizosaccharomyces pombe (30, 31) and Drosophila melanogaster (32). Investigating the CaMK cascade in genetically tractable systems will help determine whether it is a physiologic pathway and what biological functions it might regulate.

However, to assess the biological significance of the cascade, cellular targets must be identified. CREB is a likely possibility, as it can be activated by the CaMK cascade in transfection experiments (Fig. 2), but many kinases can be induced to phosphorylate CREB (33, 34). It seems likely that the physiologic kinase(s) depends on the situation. For example, expression of catalytically inactive, dominant negative CaMK (dnCaMK) specifically in developing thymocytes blocks CREB phosphorylation on Ser-133, leading to effects including decreased interleukin-2 production that are reminiscent of thymocytes from mice expressing dnCREB S133A (35). Yet, in initial experiments with thymocytes from CaMKIV-null mice, Ser-133 phosphorylation is subtly decreased but not prevented, and total interleukin-2 production appears normal. The most parsimonious explanation for these conflicting observations is that the dnCaMKIV prevented CREB phosphorylation not only by blocking CaMKIV but also by other thymic CREB kinases. In contrast, CREB phosphorylation is severely reduced in both cerebellar extracts and stimulated hippocampal neurons of CaMKIV knockout mice but is unaffected in the testis (36–38). These results suggest that CaMKIV functions as a CREB kinase in at least but not all tissues. Evaluation of CREB phosphorylation in the absence of CaMKs awaits development of appropriate genetic models.

Situation-specific signal regulation could be accomplished, in part, through regulated association of CaMKIV with other cellular components. An avid association between CaMKIV and PP2A has been demonstrated by copurification and coprecipitation; immunoprecipitated CaMKIV is nearly stoichiometrically associated with PP2A, although the reverse is not true because of the large excess of PP2A (39). Inhibition of PP2A with adenosine virus small t antigen or okadaic acid increases CaMKIV-dependent activation of Gal4-CREB transcription, which could result from blocking dephosphorylation of CaMKIV, CREB, and/or CBP. In contrast to the apparently constitutive association of CaMKIV with a potential deactivator, attempts to coprecipitate CaMKIV and CaMKH have been unsuccessful, suggesting that the preformed signaling complex may not include the known activators. Although its significance is not understood, biochemical fractionation of testis extract indicates that a hyperphosphorylated form of CaMKIV is associated with the nuclear matrix (40). Targeting CaMKIV to appropriate loci in a preformed signaling complex could be an important factor in its effects on transcription. As the example of CREB phosphorylation in CaMKIV-null mice poignantly demonstrates, we cannot yet predict when a transcription factor that can be regulated by CaMKIV in transfected cells will be regulated by CaMKIV in vivo and how that specificity is determined.

Other Transcriptional Targets of CaMKIV
CaMKIV has been linked with the regulation of many transcription factors other than CREB, including AP-1, serum response.
factor, and activating transcription factor 1, and may have multiple modes of regulating CREB-dependent transcription (Fig. 2) (12). CREB transcriptional activation occurs through its binding to the coactivator protein CBP/p300, which links many transcription factors to components of the general transcriptional machinery. Phosphorylation of CREB on Ser-133 increases its affinity for CBP but whether CREB Ser-133 phosphorylation is sufficient to induce CRE-dependent transcription without other regulatory signals to CBP itself is controversial. Microinjection of phosphoserine 133-CREB or transfection with the Y134P CREB mutant that is constitutively phosphorylated induces CRE-dependent reporter plasmid transcription without additional stimuli (41, 42). Seemingly contradictory experiments demonstrate that induction of CRE-mediated transcription by depolarization of AtT20 cells requires nuclear Ca\(^{2+}\) but not increased CREB Ser-133 phosphorylation (43).

Moreover, reporter gene transcription driven by a CREB-independent Gal4-CBP fusion is enhanced by constitutively active CaMKIV. Sites in CBP are inducibly phosphorylated, which has led to the hypothesis that CaMKIV regulates CBP by direct phosphorylation, but this remains to be demonstrated.

CaMKIV stimulation of transcription by the orphan receptor RO\(\alpha\) may also be related to effects on coactivators. A CaMKIV effect on ROR was investigated because of the similarities in phenotypes between CaMKIV and RO\(\alpha\) knockout mice. In transfection assays, CaMKIV induces a 20–30-fold increase in ROR-dependent transcription of a reporter plasmid (44). The RO\(\alpha\) ligand binding domain (LBD) is not a substrate for CaMKIV in vitro, but LBD binding peptides that disrupt LBD association with endogenous coactivators abrogate the CaMKIV effect. Although the mechanism remains to be elucidated, these results and the CaMKIV effects on CBP suggest a new role for CaMKIV in recruiting or stabilizing coactivator-containing transcriptional complexes.

CaMKIV has also been implicated in the Ca\(^{2+}\)-dependent regulation of MEF2 family transcription factors (Fig. 3). In cardiomyocytes and neurons, Ca\(^{2+}\) influx leading to MEF2 activation correlates with activation of p38, which phosphorylates MEF2 on multiple sites in vitro including those in its activation domain (45, 46). MEF2 has also been reported to be a substrate for CaMKIV in vitro (47). Constitutive forms of CaMKIV and CaN independently activate MEF2 reporter genes but synergize when cotransfected. There is evidence for two complementary mechanisms for the effect of CaN: dephosphorylation of NFAT, promoting its nuclear translocation and allowing it to synergize with MEF2 to activate reporter genes; and dephosphorylation of MEF2, which enhances its DNA binding (48). The coactivator CBP/p300 can bind both NFAT and MEF2 and may be involved in stabilizing a NFAT-MEF2 complex on the promoter and/or may itself be a target of a Ca\(^{2+}\)-dependent stimulatory signal (49). Furthermore, Cabin-1, originally identified as a CaN inhibitor, also binds and suppresses MEF2 transcriptional activity in T-cells (50). Cabin-1 binding to MEF2 is competitive with p300 and is relieved by Ca\(^{2+}/\)CaM binding to Cabin-1 (50, 51). Because Cabin-1 also associates with mSin3A and histone deacetylases 1 and 2 (HDAC1/2), its dissociation from MEF2 may replace a repressing complex with an activating complex (51). In a like manner, MEF2 binds and is repressed by HDAC4 but is released by Ca\(^{2+}/\)CaM binding to HDAC4 (52). Association of MEF2 with HDAC4/5 can also be inhibited by co-transfection with a constitutively active fragment of CaMKIV, which may provide an indirect mechanism for CaMKIV enhancement of MEF2 transcription (53). Clearly, Ca\(^{2+}\) regulation of MEF2 is multifaceted, involving both stimulatory phosphorylation/dephosphorylation and dissociation from transcriptional repressors.

Missing from many studies of CaMKIV and transcription is a characterization of CaMKK effects and differentiation between CaMKI and CaMKIV. Unfortunately, the kinase inhibitors currently available, KN62 and KN93, inhibit CaMKI, II, and IV similarly and so they cannot be used alone to define a role for a specific CaMK (54). Moreover, these drugs block voltage-dependent K\(^{+}\) currents at concentrations comparable with those used for CaMK inhibition, so results from the use of these inhibitors should be interpreted cautiously (55). Truncations of the kinases to form constitutively active forms are also frequently used to study transcription, but because this removes domains of the kinase whose functions are unknown, these proteins could be inappropriately localized or regulated. For example, the CaMKII(Δ1–290) activating truncation also removes the association domain and thus both changes its activation biochemistry and permits inappropriate nuclear entry (2, 11). For CaMKI and CaMKIV, functions of the C-terminal region are not as well defined but may also affect subcellular distribution and substrate preference. One alternative approach that circumvents these pitfalls but has not yet been extensively employed is to use mutations in the autoinhibitory domains identified for CaMKII, CaMKIV, and CaMKKs that allow Ca\(^{2+}/\)CaM-independent activity without truncation (10, 56, 57).

Finally, because understanding of the cascade is still so skeletal, it is not reasonable to assume that transcriptional effects attributed to CaMKIV are also regulated by the CaMKK without directly testing that hypothesis. These caveats should be kept in mind in interpreting the results of experiments designed to implicate one of the CaMKs in a CaMK cascade.

**Evidence for a CaMK Cascade**

The evidence for a working CaMK cascade regulating Ca\(^{2+}\)-dependent transcription in cells is largely favorable but still circumstantial. CaMKI and CaMKIV are excellent substrates of the CaMKKs and are dramatically activated by activation loop phosphorylation that occurs following stimulation of intact cells. The tissue distributions of the CaMKKs appropriately overlap those of CaMKI and CaMKIV, but the question of subcellular localization...
still needs to be resolved. Whether the CaMKs so far identified are the only kinases capable of activating CaMKI and CaMKIV is unknown.

If this cascade is physiologic, does it function as a signal integrator or as an amplification circuit? Of course, inducible phosphorylation by CaMKs provides a mechanism for amplification. However, like CaMKII, these kinases have elaborate activation mechanisms that rely on a Ca\(^{2+}\) signal for multiple steps. For CaMKII, this complex activation biochemistry has been shown to differentiate stimulation frequencies (58), but no such evidence yet exists for the CaMK cascade. The suggestion from peptide experiments that CaMKI/IV might exhibit different substrate specificities and integration are possible roles for a CaMK cascade. The suggestion from peptide experiments that CaMKI/IV might exhibit different substrate specificities and integration are possible roles for a CaMK cascade. The suggestion from peptide experiments that CaMKI/IV might exhibit different substrate specificities and integration are possible roles for a CaMK cascade. 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