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Ca^{2+} /Calmodulin-dependent protein kinase IV and calcium signaling

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Ca^{2+} /calmodulin dependent protein kinase IV (CaMKIV) is a multifunctional, serine-threonine protein kinase that is activated in the presence of increased intracellular calcium (Ca^{2+}). CaMKIV is a potent mediator of Ca^{2+} induced gene expression, primarily through its ability to phosphorylate and activate transcription factors such as CREB. CaMKIV-dependent activation of CREB is a key event in the expression of genes involved in the processes of T-cell activation and neuronal long term potentiation. The focus of this review is to describe the biochemical regulation of CaMKIV and examine how CaMKIV activates transcription in response to calcium in both cell and animal models.

Keywords: Ca^{2+}/calmodulin-dependent protein kinase IV, calcium, calmodulin, CREB, T lymphocyte

Introduction

Changes in intracellular Ca^{2+} concentration play a major role in a broad array of cell functions including transcription, cell cycle, apoptosis, exocytosis, protein synthesis, and motility, often having multiple roles within a single cell (A.R. Means, ed., 1995). Elevations in cytoplasmic Ca^{2+} concentration occur via diverse stimuli which activate voltage or ligand-gated Ca^{2+} channels in the surface membrane or by release from intracellular stores via second messengers. Ca^{2+} signals vary in both amplitude and duration and may form complex oscillating patterns. Although the regulatory mechanisms are controversial, the use of intracellular Ca^{2+} dyes provides strong evidence that changes in Ca^{2+} concentration also occur within the nucleus in response to cell stimulation by a variety of mechanisms (Himpens *et al.* 1994). A challenging issue to be resolved is how cells transduce the many Ca^{2+} signals generated into specific functions.

Calmodulin is a 17 kDa protein that serves as the cell's major Ca^{2+} receptor, where it is found in both cytoplasmic and nuclear compartments. The complex of Ca^{2+}/CaM regulates many types of downstream targets including protein kinases, protein phosphatases, adenylyl cyclases, and cyclic nucleotide phosphopdiesterases (Lu and Means 1993). One major family of Ca^{2+}/CaM effectors is the Ca^{2+}/calmodulin-dependent protein kinases (CaMKs), which can be divided into dedicated CaMKs that phosphorylate a single specific substrate such as myosin light chain kinase and the multifunctional CaMKs including CaMK I, CaMK II, and CaMK IV that phosphorylate a large number of proteins *in vitro* and *in situ* (Braun and Schulman 1995).

This review focuses on CaMKIV-mediated Ca^{2+} signaling, with special emphasis placed upon the biochemical regulation of CaMKIV and its role in transducing Ca^{2+}-activated gene transcription. CaMKIV is a serine/threonine protein kinase that has been localized in the nucleus (Jensen *et al.* 1991; Matthews *et al.* 1994; Bito *et al.* 1996). The tissue distribution pattern of CaMKIV is rather limited, with expression restricted primarily to discrete regions of the brain, T-lymphocytes, and post meiotic germ cells (Ohmstede *et al.* 1989; Frangakis *et al.* 1991; Means *et al.* 1991). It is especially enriched in cerebellar granule cells and for this reason is sometimes referred to as CaMK-Gr. The expression of CaMKIV is developmentally regulated in rodent brain and thymus. In both tissues, expression of CaMKIV mRNA and protein is not seen until embryonic day 16 of development, with maximum expression reached by embryonic day 18 (Krebs *et al.* 1996, 1997). CaMKIV is also inducible

in rat fetal telencephalon and murine fetal thymic organ cultures by the addition of T3 and 10% fetal calf serum, respectively, suggesting that hormones and growth factors can upregulate CaMKIV expression in a tissue specific manner (Krebs *et al.* 1997). CaMKIV migrates as a closely spaced doublet on SDS polyacrylamide gels. The faster and slower migrating species are referred to as CaMKIVα and CaMKIVβ, respectively, and are believed to represent two different isoforms of CaMKIV expressed from the same gene by means of alternative transcriptional initiation. The molecular cloning of CaMKIVβ by Sakagami and Kondo (1993) has shown that the β isoform of CaMKIV is identical to CaMKIVα except for the presence of an additional 28 amino acids at the amino-terminus. Alternatively, the two bands may also represent different phosphorylation states of the same isoform. *In vitro* transcription and translation of constitutively active forms CaMKIVα display two distinct bands, suggesting that the differential migration of the upper band may represent an autophosphorylated form of the protein (Chatila *et al.* 1996).

When studying a potential physiological role for CaMKIV, it has been important to consider the involvement of the other multifunctional CaMKs, CaMKI and CaMKII. Although each kinase optimally phosphorylates motifs with distinctive features (White *et al.* 1998), all three recognize the general consensus sequence R-X-X-S/T, which means there is considerable overlap in substrates. The compounds W7 and KN-93 inhibit CaM and CaMKs, respectively, but there are no pharmacological methods for distinguishing between CaMKI, CaMKII, and CaMKIV. Since CaMKI and CaMKII are both ubiquitously expressed throughout brain and in nonneuronal tissues, they are present in the cells which express CaMKIV and all three kinases can be potentially activated by the same intracellular Ca^{2+} signal. Therefore, defining CaMKIV function in some cases requires knowledge of CaMKI and CaMKII, which is provided in this review when relevant and available. This review has been divided into three major sections. The first section discusses the regulatory properties of CaMKIV, including mechanisms of activation and inactivation. This is followed by a discussion of how CaMKIV-dependent covalent modification of transcription factors results in the activation of transcription. The final section discusses the integration of CaMKIV function and calcium signaling in the context of physiological processes that occur in neuronal and lymphoid tissues.

Regulatory properties

The CaMKs exhibit fascinating regulatory properties. The first to be discovered, CaMKII is the prototypical CaMK (reviewed in Braun and Schluman, 1995) to which other family members are often compared. Its regulatory properties are briefly summarized here followed by a discussion of the regulation of CaMKIV. Structurally, CaMKII is comprised of an amino-terminal catalytic domain, a central regulatory domain, and a carboxyl-terminal association domain. The enzyme homomultimerizes through the association domain to form a holoenzyme of 8–12 subunits. Within the regulatory domain are overlapping autoinhibitory and CaM-binding domains which have been precisely mapped. In the absence of Ca^{2+}/CaM, CaMKII is inhibited intrasterically by the autoinhibitory region which is believed to prevent both substrate and ATP from binding to the enzyme. The binding of Ca^{2+}/CaM relieves the autoinhibitory domain which leads to de-inhibition of the enzyme.

Once activated by Ca^{2+}/CaM, CaMKII autophosphorylates on Thr 286 located in the regulatory domain and although not necessary for enzyme activity, this event has two important consequences. First, the affinity of the enzyme for Ca^{2+}/CaM is increased in a process called 'CaM trapping'. Second, the autoinhibitory domain is further disrupted making CaMKII partially independent of Ca^{2+}/CaM or 'autonomous'. The mechanism of autophosphorylation has been studied in detail. Autophosphorylation occurs between two neighboring subunits in one holoenzyme in an intersubunit reaction. The process requires that Ca^{2+}/CaM be bound to both the kinase subunit and the substrate subunit. This particular set of properties has for years led to speculation that CaMKII is a suitable memory molecule for decoding Ca^{2+} oscillation frequency. CaM trapping prolongs binding of Ca^{2+}/CaM after intracellular Ca^{2+} concentration declines while autonomous subunits retain partial activity after Ca^{2+}/CaM dissociates. The total activity of one holoenzyme should thus be sensitive to the Ca^{2+} spike frequency. This was recently demonstrated *in vitro* (De Koninck and Schulman 1998), providing one example for how Ca^{2+} spike frequency may be translated into specific responses.

The regulation of CaMKIV has interesting parallels and differences with CaMKII. A schematic representation of the CaMKIV domain structure is shown in Figure 1. CaMKIV has catalytic and regulatory domains which are highly homologous to CaMKII. Truncation of CaMKIV within the regula-

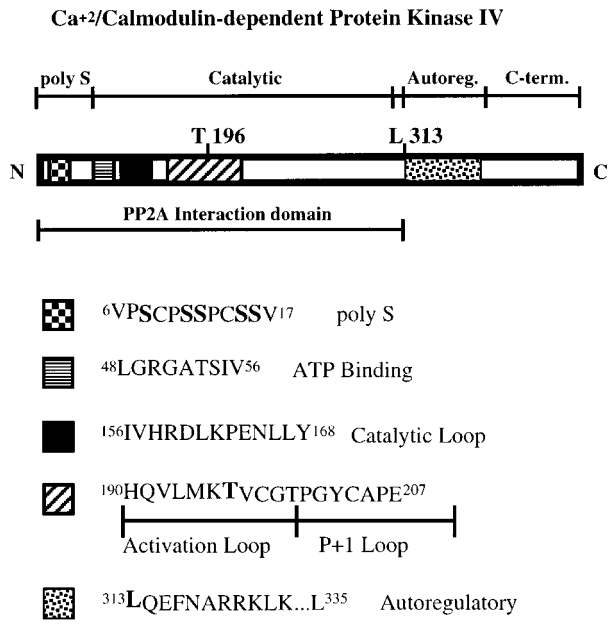

Figure 1. Schematic diagram depicting the domain structure of CaMKIV. The upper portion represents the linear diagram of the CaMKIV protein. The lower portion is a key, with the domain shading on the left and the amino acid sequence of each domain to the right (respectively).

tory domain, at Leu 317, generates a fully active, CaM-independent enzyme, suggesting that a common mechanism of intrasteric autoinhibition regulates the multifunctional CaMKs (Cruzalegui *et al.* 1993). Structural features unique to CaMKIV include a serine-rich amino-terminal domain believed to play a critical role in enzyme activation (see below) and a highly acidic carboxyl-terminal domain whose function has not been determined. In contrast to CaMKII, CaMKIV has not been observed to multimerize.

Early characterization of CaMKIV from brain indicated that the enzyme was activated by Ca^{2+}/CaM-dependent autophosphorylation which generated autonomous activity. The phosphorylated sites were mapped to Thr 196 and Ser 8, 11, 12, 15, and 16 (McDonald *et al.* 1993). Unlike tissue purified CaMKIV, recombinant CaMKIV expressed in Sf9 insect cells or in *E. coli* showed much lower Ca^{2+}/CaM-dependent activity, slow autophosphorylation on serine, and no autonomy; but could be activated by brain extract (Cruzalegui *et al.* 1993; Okuno and Fujisawa 1993). This led to the discovery of what is now known to be a family of activating CaM kinase kinases (CaMKKs). The critical residue phosphorylated by CaMKK and essential for CaMKIV activation is Thr 196, which is located in the activation loop of kinase subdomain VIII. The position of this activating threonine is conserved in several other protein kinases including PKA, CDK2, MAPK, and CaMKI (Selbert *et al.* 1995). Phosphorylation of Thr 196 results in a 10–25 fold increase in total CaMKIV activity, the generation of autonomy, and enhanced serine phosphorylation. In a manner somewhat similar to the autophosphorylation of CaMKII, phosphorylation of Thr 196 by CaMKK requires that Ca^{2+}/CaM be bound to CaMKIV. In addition, CaMKK is itself a CaM binding protein whose activity is enhanced by Ca^{2+}/CaM (Haribabu *et al.* 1995; Tokumitsu and Soderling 1996; Tokumitsu *et al.* 1997). The requirement of both CaMKIV and its upstream, activating CaMKK for Ca^{2+}/CaM could be expected to provide CaMKIV with an especially sharp sensitivity to Ca^{2+}.

In addition to Thr 196, a critical regulatory role for Ser 11 and Ser 12 has been demonstrated (Chatila *et al.* 1996). Singly mutating Ser 11 or Ser 12 to Ala results in an enzyme indistinguishable from the wild type, as does deletion of the 20 amino-terminal residues. However, doubly mutating Ser 11 and 12 to Ala produces an enzyme which both lacks Ca^{2+}/CaM-dependent basal activity and is unresponsive to CaMKK. The amino-terminus has been suggested to produce a second type of CaMKIV autoinhibition that can be relieved by Ser 11 or 12 phosphorylation. There is believed to be slow Ca^{2+}/CaM-dependent Ser 11 and 12 autophosphorylation that occurs in the absence of CaMKK and which parallels the low basal activity. Upon activation by CaMKK, phosphorylation of serine increases in rate and extent.

The autonomy generated during activation is believed to result directly from phosphorylation of Thr 196 or of another currently undefined site. It is interesting to note that although there is a threonine in the regulatory domain of CaMKIV similar to CaMKII Thr 286, mutation to Ala has no effect on total or autonomous activity (Chatila *et al.* 1996).

In summary, maximal activation of CaMKIV *in vitro* occurs via a CaM kinase cascade requiring three steps: 1) Ca^{2+}/CaM binding, 2) phosphorylation of Thr 196 by CaM-bound CaMKK and, 3) autophosphorylation of Ser 11 and 12. There is evidence for CaMK cascades *in situ* as well. Partial purification of endogenous CaMKIV from Jurkat cells exhibits 17-fold greater activity after T cell receptor (TCR)-mediated stimulation of the cells, indicating that the enzyme has undergone activation (Hanissian *et al.* 1993). CaMKIV activated in this manner can be additionally activated only slightly by purified CaMKK and is inactivated by phos-

phatase treatment. Once inactivated by phosphatase, CaMKIV can then be reactivated by CaMKK (Park and Soderling, 1995). Transient transfection assays using a CRE reporter as an indicator of CaMKIV activity demonstrate that the T196A CaMKIV mutant is unable to stimulate CREB activity in response to stimuli that increase intracellular Ca^{2+}, while wild type CaMKIV induces a 10-fold activation (Chatila *et al.* 1996). Collectively, these data strongly indicate the presence of physiological CaMK cascades and have stimulated interest in this new family of activating CaMKKs.

There are two distinct CaMKKs, referred to as CaMKKα and CaMKKβ, which have been purified and cloned from rat brain (Tokumitsu *et al.* 1995; Edelman *et al.* 1996; Kitani *et al.* 1997; Anderson *et al.* in press). These kinases share greatest sequence homology with the CaMK family. CaMK1aK, originally characterized as a kinase which activates CaMKI by phosphorylating Thr 177 (DeRemer *et al.* 1992), has been shown to be comprised of a mixture of CaMKKα and CaMKKβ. The CaMKIVK described by Tokumitsu *et al.* (1995) is identical to CaMKKα. Both CaMKKs activate CaMKI and CaMKIV by phosphorylating the activation loop Thr 177 and Thr 196 of these kinases, respectively. CaMKKα and CaMKKβ differ significantly in peptide sequence at their amino- and carboxyl-termini, and in their tissue distribution patterns. CaMKKα expression is restricted to brain and thymus while CaMKKβ is found in a broader array of tissues (Anderson *et al.* in press). Interestingly, within the brain CaMKKβ expression most closely parallels that of CaMKIV. Both are highly expressed in the granule cell layer of the cerebellum where little CaMKKα is detected (Sakagami *et al.* 1998; Anderson *et al.* in press). CaMKKβ is also expressed in all noneuronal tissues which express CaMKIV. The CaMKKs bind and are stimulated by Ca^{2+}/CaM both *in vitro* and *in situ*, while CaMKKα is reportedly downregulated by PKA *in situ* (Wayman *et al.* 1997). *In vitro* characterization of CaMKKα and CaMKKβ with respect to substrate specificity or regulation have revealed no differences between these enzymes. Both CaMKKs will also undergo autophosphorylation, although this occurs without any observable effect on *in vitro* function. It will be of interest to determine whether autophosphorylation or signals other then Ca^{2+} play a role in the physiological regulation of CaMKK.

Given the multiple phosphorylation steps required to activate CaMKIV, it would be logical to predict that once activated, CaMKIV may be returned to the resting, unactivated state by dephosphorylation. The protein phosphatase type 2A (PP2A) will dephosphorylate and inactivate CaMKIV which has been previously activated by CaMKK *in vitro*, while PP1 is unable to do so (Park and Soderling 1995). In addition, PP2A coimmunoprecipitates with CaMKIV from Jurkat T cells and the PP2A heterotrimer copurifies with CaMKIV from brain extract through four sequential columns (CaM sepharose, ion exchange, phenyl sepharose, and gel filtration) (Westphal *et al.* 1998)! These data imply a strong *in situ* physical interaction between the kinase and phosphatase. A GST-CaMKIV fusion protein containing residues 1–313 of CaMKIV is sufficient to co-precipitate PP2A from cell lysates (Fig. 1). Of interest is the fact that neither Ca^{2+}/CaM binding, Thr 196 phosphorylation, kinase activity, nor phosphatase activity are required for complex formation to occur. In addition to a physical interaction, there is evidence for an *in situ* functional interaction between CaMKIV and PP2A (Westphal *et al.* 1998). The small t antigen, a specific inhibitor of PP2A, enhances CaMKIV-mediated activation of CREB in Jurkat cells while having no effect on CREB induction by PKA. This is consistent with a model in which PP2A negatively regulates CaMKIV. We are currently in the process of testing this hypothesis. Likely candidates of PP2A dephosphorylation include Ser 11 and 12 and/or Thr 196, based on these residues' importance in CaMKIV activation. Another important issue concerns the regulation of PP2A activity. TCR-mediated stimulation of CaMKIV activity in Jurkat cells peaks and then returns to a basal level within 5 minutes post stimulation, although the intracellular Ca^{2+} concentrations remains elevated for up to 1 hour post stimulation. The level of PP2A/CaMKIV complex appears to be independent of the state of cell stimulation. Thus, PP2A activity towards CaMKIV is either constitutive or regulated, perhaps by post-translational modification or by other currently undefined mechanisms.

Activation of transcription

The elevation of nuclear Ca^{2+} is a potent inducer of gene transcription. CaMKIV is thought to be a major mediator of Ca^{2+} induced gene expression given its activation in response to Ca^{2+} as well as its ability to phosphorylate and activate various transcription factors. This section will discuss how CaMKIV dependent phosphorylation of CREB and other proteins serves as a general paradigm for the activation of transcription. When applicable, references to the other members of the CaM kinase

family will be made in order to illustrate how CaMKIV is a key mediator of Ca^{2+} induced gene expression in certain cell types.

CREB. The cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) is an important transcription factor responsible for inducing expression of genes containing a cAMP response element (CRE) in their proximal promoter. While CREB was first identified as a cAMP inducible factor that is activated by protein kinase A (PKA) (Gonzalez and Montminy 1989), subsequent studies have shown that it is also responsive to calcium and can be activated by calmodulin-dependent kinases (Sheng *et al.* 1990; Sheng *et al.* 1991; Dash *et al.* 1991).

The initial question of how CREB becomes activated in response to phosphorylation has ignited a flurry of research that continues on to today. The seminal observation was that CREB becomes activated after it is phosphorylated on Ser 133 by PKA. (Gonzalez & Montminy 1989). This phosphorylation event is absolutely required for CREB mediated transcription, as mutation of Ser 133 to Ala produces a transcriptionally inactive molecule. The placement of a negative charge at this position is also insufficient for transcriptional activation, as mutation of Ser 133 to Asp also produces a transcriptionally inert factor. Recent biochemical experiments have shown that Ser 133 phosphorylation is the trigger for assembling a multi-protein complex at the site of the CRE promoter. At the core of this complex is the CREB binding protein (CBP), a transcriptional coactivator that is recruited to CREB after Ser 133 has been phosphorylated (Chrivia *et al.* 1993). CBP exhibits histone acetylase activity and also serves as a scaffold for other coactivators such as SRC/p160 family members as well as for components of the basal transcription machinery such as TFIIB and the TATA-binding protein (TBP), a component of TFIID (Kamei *et al.* 1996; reviewed in Shikama *et al.* 1997).

While PKA was the first CREB kinase to be identified, initial studies characterizing Ca^{2+} mediated activation of CREB suggested that CaMKII may be the regulatory kinase based upon its ability to phosphorylate Ser 133 *in vitro* (Sheng *et al.* 1991; Dash *et al.* 1991). However, transient transfection studies using CaMKII failed to show significant CREB-mediated transcription *in situ* (see below). More recently, a large amount of experimental evidence points to CaMKIV as a bona fide CREB kinase. Like PKA, CaMKIV recognizes the same minimum substrate recognition motif of R-X-X-S, the context in which Ser 133 is found in CREB (White *et al.* 1998). CREB is also a substrate of CaMKIV *in vitro* and *in situ*, with phosphoamino acid analysis showing a single phosphorylation at Ser 133 (Cruzalegui & Means 1993; Sun *et al.* 1994). While CaMKII can phosphorylate Ser 133, careful phosphopeptide mapping of CREB after CaMKII phosphorylation both *in vitro* and *in situ* has shown that CaMKII can phosphorylate CREB on an additional site of Ser 142 (Sun *et al.* 1994). The second phosphorylation event at Ser 142 is inhibitory and dominant, suggesting that CaMKII may not be an inducer of CREB mediated gene activation in all cell types.

Transient transfection experiments have verified the ability of CaMKIV to drive CREB mediated transcription in response to a Ca^{2+} signal *in situ* (Matthews *et al.* 1994; Sun *et al.* 1994; Sun *et al.* 1996). Cells that are not normally Ca^{2+} responsive can recapitulate Ca^{2+} dependent activation of CREB mediated transcription via the introduction of CaMKIV expression vectors (Matthews *et al.* 1994). In these experiments, production of an intracellular Ca^{2+} signal by the use of a Ca^{2+} ionophore or through membrane depolarization will result in CaMKIV-dependent phosphorylation of CREB on Ser 133 and a concomitant increase in CRE-dependent transcription. Transcriptional activation is independent of PKA and requires Ser 133, as the Ser 133 Ala mutant is not responsive to CaMKIV (Matthews *et al.* 1994; Sun *et al.* 1994; Sun *et al.* 1996). CREB dependent transcription can also be obtained in the absence of a Ca^{2+} signal by the use of a Ca^{2+}/CaM independent and constitutively active truncation mutant of CaMKIV (Matthews *et al.* 1994; Sun *et al.* 1994; Sun *et al.* 1996). In addition, a further increase of CREB transcription can be obtained if the CaMKIV upstream activating kinase, CaMKK, is cotransfected with CaMKIV (Tokumitsu *et al.* 1995). This implies the existance of a CaM kinase activation pathway which may fine tune the activity of CREB *in situ*. Given these findings, it appears that CaMKIV can act as an important mediator of CREB activation *in situ*.

What is the role of the other CaM kinases in regulation of CREB transcription? While both CaMKI and CaMKII can phosphorylate Ser 133 *in vitro*, neither is thought to be a predominant mediator of CREB activation in certain cell types. The strongest argument for this idea rests with the immunolocalization of both enzymes and the inhibitory phosphorylation of Ser 142 in CREB by CaMKII.

Much like CaMKIV, CaMKI will phosphorylate Ser 133 *in vitro* and *in situ* as well as drive CREB-mediated transcription using either wild type or constitutively active CaMKI expression vectors in transfection assays (Sheng *et al.* 1991; Sun *et al.* 1996). These results are not unexpected, as both

CaMKI and CaMKIV share a common minimum substrate recognition motif and recognize many of the same substrates. However, immunostaining of CaMKI in hippocampal neurons argues for a primarily cytoplasmic localization in these cells (Bito *et al.* 1996; Deisseroth *et al.* 1998). While its small molecular weight (40 kDa) and its existence as a monomeric protein suggest that it is capable of entering the nucleus, the preponderance of data to date suggests that CaMKI is cytoplasmic and is therefore unlikely to activate CREB in a direct manner in cells such as hippocampal neurons.

Arguments against CaMKII as being a CREB kinase *in situ* also rely upon the cytoplasmic compartmentalization of the various CaMKII isoforms. Several studies have shown that CaMKII is a cytoplasmic enzyme (reviewed in Braun & Schulman 1995). In addition, the oligomerization of CaMKII into aggregates with an observed molecular weight in excess of 400 kDa indicates that in this multimeric state, these complexes are likely to be excluded from the nucleus. Recent evidence also suggests that phosphorylation of the nuclear-localized isoforms of CaMKII by CaMKIV negatively effects CaMKII nuclear localization sequences, therefore actively blocking CaMKII access to the nucleus (Heist *et al.* 1998). The colocalization of CaMKIV and CREB in the nuclear compartment indicates that CaMKIV, but not CaMKII, should have access to CREB as a substrate.

Although early studies of Ca^{2+} mediated activation of CREB were thought to be carried out by CaMKII, transient transfection experiments failed to confirm this hypothesis. Several groups have shown that CaMKII fails to stimulate CREB mediated transcription from CRE based reporters using either wild type or constitutively active CaMKII cDNAs (Matthews *et al.* 1994; Sun *et al.* 1994; Sun *et al.* 1996). This appears due to CaMKII's ability to phosphorylate CREB on Ser 142, in addition to Ser 133 (Sun *et al.* 1994). Phosphorylation of Ser 142 appears as a dominant and inhibitory effect on CREB activation, as cotransfection of PKA or CaMKIV while in the presence of CaMKII fails to activate CREB mediated transcription (Sun *et al.* 1994). Mutation of Ser 142 to Ala produces a CREB molecule which can be activated by CaMKII or by PKA and CaMKIV while in the presence of CaMKII; (Sun *et al.* 1994). Based upon these studies, it was suggested that the phosphorylation of Ser 142 by CaMKII may serve as a negative regulator of CREB activity in some cell types.

Neurons and T-lymphocytes are two types of cells that undergo CREB phosphorylation and express CRE containing genes in response to elevated intracellular Ca^{2+}. Although CaMKIV can act as a CREB kinase *in vitro* and *in situ*, the question remains if CaMKIV is a CREB kinase *in vivo*. Recent evidence has shown that nuclear Ca^{2+} signals, CaM, and CaMKIV are all required components for Ca^{2+} dependent activation of CREB-mediated transcription. Hardingham *et al.* (1997) used microinjection of a nuclear retained, Ca^{2+} chelator BAPTA into murine pituitary AtT20 cells in order to produce a 50% decrease in nuclear Ca^{2+} after activation of L-type Ca^{2+} channels. Use of this agent decreased CRE mediated transcription of the c-fos promoter significantly, in addition to blocking the activity of a Gal4-CREB fusion gene on a heterologous Gal4 UAS reporter. The BAPTA chelator could effectively eliminate Ca^{2+} stimulation of Gal4-CREB while leaving it fully responsive to cAMP-induced transcription activation, demonstrating CREB as a nuclear Ca^{2+} responsive transcription factor.

The requirement for nuclear Ca^{2+} has lead to the discovery that the cell's primary Ca^{2+} receptor, CaM, also translocates to the nucleus in support of CREB phosphorylation (Deisseroth *et al.* 1998). Immunocytochemical staining of cultured rat hippocampal neurons after synaptic stimulation or direct depolarization showed a rapid translocation (within 1–2 minutes) of CaM to the nucleus with a concomitant decrease in cytoplasmic CaM. Translocation was sharply diminished by L-type Ca^{2+} channel antagonists, but not by N or P/Q channel antagonists, suggesting that distinct channel activity and not just bulk increases of $[Ca^{2+}]_i$ are required for CaM translocation. A strong correlation was found between the presence of nuclear CaM and the extent of Ser 133 phosphorylation, as pharmacological agents which antagonized either L-type Ca^{2+} channel activity or CaM function both decreased the appearance of phosphoCREB.

Taken together, these experiments suggest how an extracellular stimulus can induce Ca^{2+} mediated gene expression in neuronal cells. Membrane depolarization causes an increase of nuclear Ca^{2+} and a concomitant increase of nuclear CaM. These components are able to activate the endogenous pool of nuclear localized CaMKIV, which then phosphorylates and activates CREB, resulting in Ca^{2+} induced, CREB-mediated gene transcription.

ATF-1. Activating Transcription Factor-1 (ATF-1) is a member of the CREB family of transcription factors that is responsive to both cAMP and Ca^{2+} (Liu *et al.* 1993). ATF-1 can heterodimerize with CREB and may therefore affect CRE mediated transcription by altering the equilibrium of CREB

homodimers versus CREB/ATF-1 heterodimers. Transient transfection experiments which assessed the ability of constitutively active CaM kinases I, II, and IV to potentiate ATF-1 activity showed that like CREB, both CaMKI and IV could activate ATF-1 -mediated transcription while CaMKII could not (Sun *et al.* 1996). Alignment of the primary amino acid sequences of CREB and ATF-1 showed that the Ser 133 activation site of CREB was conserved as Ser 63 in ATF-1 while the inhibitory site of Ser 142 in CREB was also maintained as Ser 72 in ATF-1. Mutation of Ser 63 to Ala blocked the ability of CaMKI and IV to activate ATF-1 while a Ser 72 to Ala mutant allowed CaMKII to activate ATF-1 (Sun *et al.* 1996). *In vitro* phosphorylation assays using ATF-1 and the CaM kinases demonstrated that all of the CaM kinases can phosphorylate ATF-1 at Ser 63, but only CaMKII could phosphorylate Ser 72 (Sun *et al.* 1996). This result, coupled with the transfection data suggests that like CREB, CaMKI and IV can activate ATF-1 by phosphorylation of Ser 63, while CaMKII fails to activate ATF-1 because it additionally phosphorylates Ser 72, which acts as an inhibitory event. Based on these studies, it seems that the regulation of the transcriptional activity of ATF-1 mirrors that of CREB. This may be of biological significance when considering how a given cell will respond to a Ca^{2+} signal given the differential expression patterns of ATF-1/CREB and CaM kinases that arise as a result of tissue specific or developmental factors.

SRF. The serum response factor (SRF) is another nuclear-localized transcription factor that has been shown to be the critical mediator of serum stimulation of the c-fos promoter. The SRF binds its cognate response element (SRE), which is upstream of the CRE element found in the c-fos promoter. The c-fos SRE can be bound by either the SRF homodimer or the ternary complex factor (TCF) composed of an SRF homodimer and an ELK-1 monomer (an ETS family transcription factor). As is the case with CREB, SRF is a substrate of CaMKIV *in vitro*, with phosphorylation occurring on Ser 103 (Misra *et al.* 1994). Phosphorylation of Ser 103 occurs rapidly after an increase of $[Ca^{2+}]_i$ driven by membrane depolarization and precedes c-fos induction. Transient transfection experiments which utilized a c-fos reporter demonstrated that the SRF, but not Elk-1, mediated the Ca^{2+} regulated, SRE-dependent transcription in PC12 cells (Miranthi *et al.* 1995). The same results were obtained by Johnson *et al.* (1997), who showed that in the context of AtT20 and hippocampal neurons using a wild type c-fos reporter, that Ca^{2+} can stimulate transcription via a TCF-independent (ie: Elk-1 independent) mechanism that requires the SRF binding site. In addition, a constitutively active CaMKIV expression vector is sufficient for driving SRE dependent transcription via the SRF (Miranthi *et al.* 1995). However, the mechanism of Ca^{2+} mediated activation of the SRF is still not completely defined. Quite unexpectedly, mutation of Ser 103 to Ala resulted in an SRF that was found to be equally as effective in transcriptional activation as the wild type protein, suggesting either limitations of the transient transfection approach or implying that other as yet undefined Ca^{2+} dependent mechanisms are in effect (Miranthi *et al.* 1995). In addition, recent experiments by Hardingham *et al.* (1997) demonstrated that the reduction of nuclear Ca^{2+} concentration in the presence of elevated cytoplasmic Ca^{2+} was sufficient for the expression of a c-fos promoter driven by the SRE element, implying that Ca^{2+} responsive factors other than CaMKIV were at work. The implications are that in the context of complex promoters as exemplified by c-fos, spatially distinct pools of $Ca^{2+,}$ currently defined as being either nuclear or cytoplasmic can control transcription via distinct mechanisms (Hardingham *et al.* 1997).

CBP/P300. The transcriptional coactivators CBP and P300 have come under intense scrutiny as of late due to the essential role of these proteins in mediating the activity of a diverse group of transcription factors including CREB, STATs, and the steroid/thyroid hormone family of nuclear receptors. In addition to physically interacting with both these and other transcription factors, CBP and P300 can also bind other coactivators such as SRC1, PCIP, and PCAF as well as components of the basal transcription machinery (Korzus *et al.* 1998). CBP and p300 are also phosphoproteins, given their ability to change electrophoretic mobility patterns after phosphatase treatment (Eckner *et al.* 1996). Analysis of the primary amino acid sequence of CBP and P300 results in the identification of several putative CaMKIV phosphorylation sites. Given these factors, it seems reasonable to expect that CBP/P300 may be the target for regulation via phosphorylation.

Recent evidence from our laboratory suggests that this is the case (C.D. Kane and A.R. Means, unpublished). Transient transfection experiments utilizing the orphan receptor RORα1 display synergistic activation of transcription in the presence of constitutively active CaMKIV. This activity is completely inhibited by E1A, while a mutant of E1A that can not bind CBP/P300 partially restores activity, suggesting that CBP/P300 is involved in RORα-mediated transcription and that CaMKIV could be

acting on the level of the coactivator. Expression of the N-terminal region of P300 as a Gal4 fusion protein demonstrated CaMKIV inducible activity on a heterologous promoter, while this same fragment of P300 could act as an *in vitro* substrate of CaMKIV (C.D. Kane and A.R. Means, unpublished). While the mechanism of how phosphorylation of CBP/P300 supports transcription is unknown, possible scenarios include the stabilization of receptor-coactivator interactions, recruitment of other coactivator molecules to the CBP/P300 core, or possibly regulation of the histone acetylase activity of the coactivators themselves.

Other targets of CaMKIV. Although the search for substrates of CaMKIV continues, there are reports of a number of proteins which appear to be substrates of CaMKIV *in vitro*. These include the synaptic vesicle protein synapsin I (Miyano *et al.* 1992), the neuronally derived, Ras-related protein Rap1-b (Sayhoun *et al.* 1991), the neuronal type I adenylyl cyclase (Wayman *et al.* 1996), and the cytosolic microtubule regulator oncoprotein-18 (Op-18) (Gradin *et al.* 1997). In addition, transient transfection experiments have suggested that the MAP kinases JNK-1 and p38, and to a lesser extent ERK-2, are factors whose activity may be regulated by CaMKIV (Enslen *et al.* 1996). While many of these findings are tantalizing, further studies regarding these putative substrates should be undertaken. For example, detailed kinetic analysis of putative phosphorylation events *in vitro* and identification of CaMKIV-dependent phosphorylation *in vivo*, establishment of appropriate subcellular localization and tissue distribution patterns of both substrate and kinase, and characterization of direct or indirect involvement of the kinase are just some of the steps needed for the verification of these proteins as targets of CaMKIV. Until there is significant evidence to characterize a given protein as a legitimate substrate, the decision to comment on the physiological relevance of a given protein should be made with caution.

Physiological functions

Role in brain. Long-term potentiation (LTP) is an adaptive neuronal response in which long-lasting changes in neuronal cell structure and function lead to an increase in synaptic strength. This process, which is required for long-term memory and learning, is triggered by Ca^{2+} influx via repetitive stimulation of the synapse. Although the molecular mechanism is not well understood, studies with Aplysia, Drosophila, and transgenic mice have established a central role for CREB in LTP (Alberini *et al.* 1994; Yin *et al.* 1995; Bourtchuladze *et al.* 1994). Recent work suggests that it is CaMKIV, at least in mammals, which functions as the CREB kinase in this process. Using a pharmacological approach, Deisseroth *et al.* (1996) show that stimulation of LTP in hippocampal neurons in primary culture results in CREB phosphorylation on Ser 133 via a CaMK and interestingly, show also the involvement of a submembranous Ca^{2+} sensor. Deisseroth *et al.* (1998) demonstrate that CaM can function as this sensor by rapidly translocating from the surface membrane to the nucleus to mediate CaMK-dependent CREB phosphorylation. Two subsequent results led Bito *et al.* (1996) to identify CaMKIV as the CaMK responsible for phosphorylating CREB in this pathway. First, CaMKIV was the only family member clearly localized in the nucleus. Second, a CaMKIV antisense oligonucleotide decreased both CaMKIV expression and CREB phosphorylation. Bito *et al.* (1996) also showed that not only must CREB become phosphorylated, but phosphoCREB levels must remain elevated a set period of time in order for the activation of gene expression to occur. The decay of phosphoCREB is regulated by dephosphorylation catalyzed by the protein phosphatase type I (PPI) whose activity may in turn be potentiated by calcinuerin (CN). CN is sensitive to the duration of the initial synaptic input, with longer stimuli resulting in its inactivation and thus a lengthening of the time that phosphoCREB remains high. Thus, it is the initial stimulus duration together with CaMKIV, PP1, and CN which bring about CREB-mediated gene expression. One of the downstream targets of CREB is hypothesized to be brain-derived neurotrophic factor (BDNF). Its expression, at the mRNA and protein level, is Ca^{2+}-dependent and induced by stimuli that evoke LTP. The BDNF promoter has a CRE element essential for its activity in the mature neuron, in addition to a novel Ca^{2+}-response element (Tao *et al.* 1998; Shieh *et al.* 1998). In cortical neurons maintained in primary culture, the promoter linked to a reporter gene can be activated by CREB and becomes Ca^{2+}-independent in the presence of constitutively active CaMKIV (Shieh *et al.* 1998). BDNF is believed to act directly at the synapse by increasing both the presynaptic firing rate and the postsynaptic response. Knockout of the BDNF gene in mice results in severely impaired LTP which can be rescued by exogenous application of the factor to hippocampal slices. It is thus possible that CaMKIV, through CREB, activates expression of proteins which act at the synapse to modulate LTP.

Role in T lymphocytes. Studies with transgenic mice that express a catalytically inactive form of CaMKIV suggest that CaMKIV may play a role in thymocyte development and that it may function as a physiologically relevant CREB kinase required for activation of mature T cells (Anderson *et al.* 1997). Driven by the lck promoter, transgene expression in these mice is restricted to thymocytes and begins at embryonic day 15, one day prior to the first detectable expression of endogenous CaMKIV. The developmental phenotype is characterized by a dramatic reduction in thymic cellularity, although the distribution of cells at different developmental stages in the remaining population is normal, as determined by CD4 and CD8 surface antigen distribution. The cells also show a decreased survival rate when cultured in nonactivating conditions. The molecular mechanism responsible for this phenotype is not yet understood. Since CaMKIV has been implicated as the CREB kinase in brain (described above) and in the activation of mature T cells (see below), CREB should logically be considered a potential target during thymocyte development as well. In this respect it is interesting that CREB null mice also exhibit a thymocyte developmental defect characterized by reduced cellularity (Rudolph *et al.* 1998). However, in contrast to the mutant CaMKIV phenotype, the CREB deficient cells apparently accumulate at the immature double negative stage, unable to progress further. Thus, if CREB is a target of CaMKIV in this process it would appear to have additional CaMKIV-independent functions, perhaps prior to the time that CaMKIV expression begins.

The second major phenotype of the CaMKIV transgenic mice is characterized by a failure of the mature T cells to activate properly. T cell activation refers to the cascade of events which occur in response to engagement of the TCR that result in activation of gene expression and ultimately in cytokine production and expansion of the T cell clone by cell proliferation (Fig. 2) (Crabtree and Clipstone, 1994). T-cell activation requires the integration of multiple signal transduction components, such as the Ca^{2+}/CaM and PKC pathways, in order to coordinate cytokine expression, with expression of the IL-2 gene serving as perhaps the best understood model. Expression of IL-2 requires newly synthesized fos and jun in combination with NFAT, whose translocation from cytoplasm to nucleus is mediated by CN. It has been proposed that fos and jun induction in this pathway requires CREB. CREB is rapidly activated following TCR stimulation and mice expressing a dominant negative form of the factor show markedly reduced fos and jun induction and an absence of IL-2 production (Barton *et al.* 1996). T cells isolated from the CaMKIV transgenic mice exhibit a phenotype in which CREB phosphorylation, fos induction, and IL-2 expression are all markedly reduced in response to TCR stimulation, consistent with CaMKIV acting at the level of CREB (Anderson *et al.* 1997). Cells that have exited the thymus, by which point the proximal lck promoter is no longer active, no longer express mutant CaMKIV protein and activate normally. The reversibility of the phenotype suggests a direct effect of CaMKIV. These data have led to the hypothesis that CaMKIV functions as a CREB kinase during the early stage T cell activation. It is consistent with the observation that the activation kinetics of

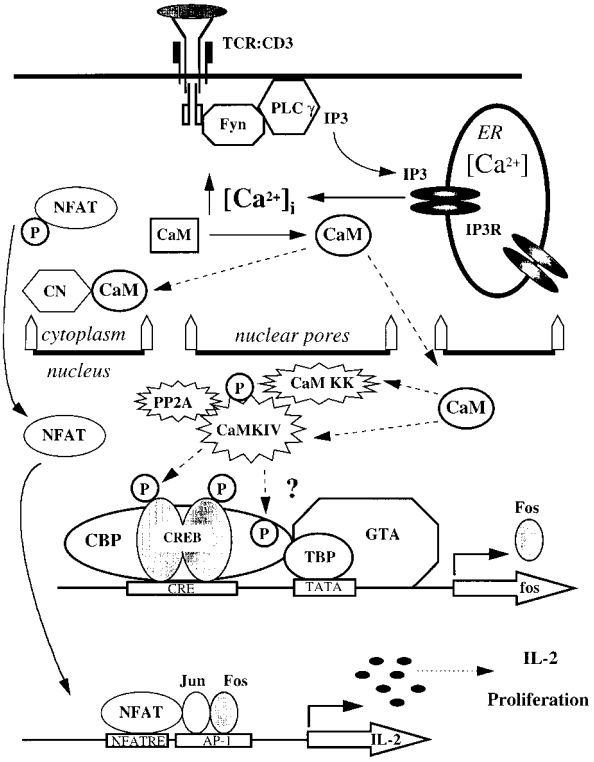

Figure 2. Schematic diagram depicting Ca^{2+} -mediated signaling in the activated T-cell. The abbreviations used are: AP-1, activating protein -1 response element; $[Ca^{2+}]i$, intracellular calcium; CaM, calmodulin; CaMKIV, CaM-dependent protein kinase IV; CaMKK, CaM kinase kinase; CBP, CREB binding protein; CRE, cAMP response element; CREB, cAMP response element binding protein; CN, calcinuerin; ER, endoplasmic reticulum; GTA, general transcription apparatus; IL-2, interleukin-2; IP3, inosital 3,4,5 phosphate; IP3R, IP3 receptor; NFAT, nuclear factor of activated T-cells; NFATRE, NFAT response element; PLCγ, phospholipase C γ; PP2A, protein phosphatase 2A; TBP, TATA binding protein; TCR-CD3, T-cell receptor-CD3 complex.

CaMKIV in Jurkat cells parallels that of CREB phosphorylation (Hanissian *et al.* 1993). Gringhuis *et al.* (1997) have proposed that CaMKIV also mediates the enhanced IL-2 production observed when T cell activation occurs in the presence of CD5 costimulation. They show that IL-2 promoter CAT reporter activity in primary T cells is blocked by CaM kinase antagonists or by a dominant negative form of CaMKIV. CaMKIV was suggested to function in this pathway downstream of Rac1 by enhancing AP-1 activity and by stabilizing IL-2 mRNA transcripts (Gringhuis *et al.* 1997, 1998). These hypotheses can be further tested in future studies with CaMKIV null mice.

The Ca^{2+}-dependent switch of EBV from Latency to Viral Replication. CaMKIV is normally found in T-lymphocytes but is absent from primary human B-lymphocytes (Hanissian *et al.* 1993). However, in Epstein-Barr virus (EBV) transformed B-lymphoblastoid cell lines, human CaMKIV is upregulated in response to the EBV gene product latent-infection membrane protein (LMP-1) (Mosialos *et al.* 1994). In LMP-1 transformed B-cells, CaMKIV is expressed and is functional, being activated by increased $[Ca^{2+}]_i$ produced by crosslinking B-cell surface IgM. Further studies have identified CaMKIV as a component of Ca^{2+} signaling pathway which causes the switch from latent EBV virus infection to active viral amplification (Chatila *et al.* 1997). Processes which produce increases of $[Ca^{2+}]_i$ and the activation of CaMKIV causes the expression of the EBV gene product Zta, presumably due to the activation of CREB and/or CREB family members which drive Zta expression from an AP-1/CRE site harbored in the Zta promoter. Expression of Zta results in the further expression of other viral early genes and ultimately results in activation of the EBV lytic cascade. It is of interest to note that Zta expression can be significantly increased by the Ca^{2+}/CaM dependent phosphatase CN, suggesting that those two different signaling molecules converge at a similar point during the transition from latency to viral production. The EBV system serves as an excellent model of how a virus has compromised the Ca^{2+}/CaMKIV signaling pathway for its own propagation.

Conclusions and future directions

CaMKIV is a multifunctional, serine-threonine protein kinase which becomes activated in response to Ca^{2+}/CaM binding, phosphorylation by an upstream CaMKK, and by autophosphorylation of its amino-terminus. Activated CaMKIV serves as a critical mediator of Ca^{2+} induced gene transcription in both neurons and T-cells. CaMKIV-dependent transcription occurs through its ability to phosphorylate and activate transcription factors such as CREB. By doing so, CaMKIV plays a central role in the expression of genes required for cellular processes such as long-term potentiation and T-cell activation.

While significant progress has been made in recent years regarding the biochemical regulation of CaMKIV and how it can influence the activity of specific transcription factors, several important questions remain regarding the function of CaMKIV *in vivo*. What are the roles of CaMKIV during development of the nervous, reproductive, and immune systems? What are the implications of the association of CaMKIV and PP2A *in vivo*? Can aberrant CaMKIV activity be linked to disease states? These are the questions that can be addressed using gene replacement and removal technologies in whole mouse models in order to obtain a greater understanding of how CaMKIV is involved in Ca^{2+} mediated signaling.

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