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Analysis of CaM-kinase Signaling in Cells

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Abstract

A change in intracellular free calcium is a common signaling mechanism that modulates a wide array of physiological processes in most cells. Responses to increased intracellular Ca^{2+} are often mediated by the ubiquitous protein calmodulin (CaM) that upon binding Ca^{2+} can interact with and alter the functionality of numerous proteins including a family of protein kinases referred to as CaM-kinases (CaMKs). Of particular interest are multifunctional CaMKs, such as CaMKI, CaMKII, CaMKIV and CaMKK, that can phosphorylate multiple downstream targets. This review will outline several protocols we have used to identify which members and/or isoforms of this CaMK family mediate specific cellular responses with a focus on studies in neurons. Many previous studies have relied on a single approach such as pharmacological inhibitors or transfected dominant-negative kinase constructs. Since each of these protocols has its limitations, that will be discussed, we emphasize the necessity to use multiple, independent approaches in mapping out cellular signaling pathways.

Introduction

Concentrations of free intracellular Ca^{2+}, which typically range from basal values of 50 nM to stimulated levels around 1-10 μM depending on the cell type, are finely regulated by a large variety of channels, exchangers and pumps on both the plasma membrane and intracellular storage organelles (e.g., endoplasmic reticulum, mitochondria) as well as low-affinity, highcapacity cytoplasmic buffer proteins (e.g., calsequestrin, calreticulin) [1]. Within cells, especially those such as neurons that have a complex morphology, Ca^{2+} signaling in microdomains, such as postsynaptic spines and presynaptic termini, can widely vary both temporally and spatially from the cell body [2]. Since prolonged Ca^{2+} elevation generally promotes cell death, increased intracellular Ca^{2+} is generally transient, lasting from milliseconds to minutes. Differences in amplitude, frequency and location of Ca^{2+} can encode a variety of messages that are deciphered by a number of different Ca^{2+}-binding proteins such as calmodulin (CaM) that has four EF-hand high-affinity Ca^{2+}-binding motifs. The Ca^{2+}/CaM complex binds to numerous target proteins, including a family of Ser/Thr protein kinases (CaMKs), thereby regulating their functionality. Some of these kinases, such

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as myosin light chain kinase, phosphorylase kinase, and CaMKIII (also known as eEF2-kinase), are dedicated to phosphorylation of only a single known protein substrate.

This paper will concentrate on the multifunctional CaMKs: CaMKII and the CaMK cascade in which CaMKK phosphorylates and activates CaMKI and CaMKIV. CaMKII exists as a heteromeric dodecamers of α, β, δ, and γ subunits with two hexameric rings stacked one on top of the other [3, 4]. Activation of CaMKII by Ca^{2+}/CaM allows intramolecular autophosphorylation of several sites; including Thr286, Thr305 and Thr306 (Figure 1A). Autophosphorylation of Thr286 in CaMKII generates autonomous or Ca^{2+}-independent activity (30-60%) that persists even after dissociation of Ca^{2+}/CaM (Figure 1B graph of activity). This allows a transient Ca^{2+} elevation to promote prolonged kinase activation. Members of the CaMK cascade—CaMKK (α, β), CaMKI (α, β, δ, γ), and CaMKIV (one gene, two splice variants)—are monomeric and, apart from activation by Ca^{2+}/CaM, show very different modes of regulation by phosphorylation compared to CaMKII. Like most other Ser/Thr protein kinases, CaMKI and CaMKIV have an "activation loop" phosphorylation site (Figure 1) that is absent in CaMKII. Binding of Ca^{2+}/CaM to CaMKI and CaMKIV exposes this activation loop site to allow phosphorylation by the upstream CaMKK when simultaneously activated by Ca^{2+}/CaM[5, 6]. Phosphorylation of the activation loop in CaMKI and CaMKIV primarily increases their Ca^{2+}/CaM-dependent activities - CaMKIV, but not CaMKI, can also exhibit significant Ca^{2+}- independent activity [7, 8] (Figure 1C and D) In neurons, CaMKK-mediated phosphorylation/activation of CaMKIV appears to last for only a few minutes [9, 10], whereas CaMKI phosphorylation can persist up to an hour or more [11]. For more extensive reviews on the properties and physiological functions of these kinases, see the following reviews: CaMKII [12-14] or the CaMK cascade [15-17].

In investigating the roles of these multifunctional CaMKs in physiological functions, it is essential to employ multiple, independent techniques since each experimental approach has its limitations as will be emphasized below. We utilize pharmacological reagents, transfections with dominant-negative or constitutive-active kinase constructs or a CaMKII inhibitor protein and RNAi suppression of the endogenous kinase. If these multiple techniques yield consistent results, we believe valid conclusions about the role of that kinase can be made. Of course, it is also important to realize that often multiple signaling pathways are employed to regulate complex physiology in a cellular context, so identification of a role for a particular CaMK does not exclude other pathways.

Mechanisms to activate CaMK signaling in cells

The primary way to increase CaMK activity in cells is to increase intracellular free Ca^{2+} by treatment, for example, with agonists that stimulate Ca^{2+}-permeable channels. In neurons, one can activate Ca^{2+}-permeable voltage- or ligand-gated channels in the plasma membrane by treating the culture with elevated K^+, NMDA, glutamate or glycine or by treatment with the GABA-A antagonist bicuculline that blocks inhibitory transmission, thereby elevating neuronal activity. Agonist treatments generally elevate Ca^{2+} throughout the cell, but application of glycine, an NMDA receptor co-agonist with glutamate, to mature cultured neurons only stimulates Ca^{2+}-permeable NMDA receptors at spontaneously active synapses also releasing glutamate. Two photon glutamate uncaging can activate receptors localized to a single spine [18]. Moreover, agonist treatments may selectively activate CaMKs that are spatially localized in proximity to the source of Ca^{2+} entry. CaMKs may be either directly or indirectly associated with these channels or microdomains containing these channels: CaMKII and Ca^{2+} channels [19, 20] and NMDA receptor [21, 22]; CaMKI and TRPC5 channel [23]. For cells lacking Ca^{2+} permeable channels, one could use ionophores, such as A23187 or ionomycin to increase intracellular Ca^{2+}. However, all these approaches will activate a plethora of Ca^{2+}-dependent mechanisms in the cell in addition to CaMKs, making

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interpretation of the signaling pathway responsible for the physiological response of interest difficult.

Alternatively, one can promote the cellular activity of a specific CaMK by expressing constitutively-active forms of the CaMK of interest (caCaMK) (see details below). Although we use this approach, there are serious caveats in expressing constitutively-active constructs of any signaling protein including CaMKs. One concern is that since they are constantly active in the cell, they can overpower opposing protein phosphatases resulting in non-physiological levels of phosphorylation. Moreover, some studies use expression of truncated CaMKs containing only the catalytic domain that may result in subcellular mislocalization. For example, full-length CaMKIα is predominantly cytoplasmic, whereas the truncated, constitutively-active construct (e.g., residues 1–293) is also nuclear due to the loss of the nuclear export signal in the regulatory domain [24]. Another concern of overexpressed caCaMKs is loss of substrate specificity that is often achieved by subcellular localization of the kinase in a multiprotein complex. Thus, continuous, high-levels of mislocalized activated kinase may promote phosphorylation of nonphysiological substrates resulting in aberrant cellular signaling.

In spite of these multiple caveats, the use of caCaMKs is not without merit as a complement to dnCaMKs, si/shRNA, and pharmacological inhibitors. Most of our caCaMKs are constructed by mutating and inactivating the autoinhibitory domain of the kinase (see details below), thereby allowing activity in the absence of Ca^{2+}/CaM-binding. Our caCaMKs are generated by the following mutations: CaMKI 286IHQS to 286EDDD, 307F to A; CaMKIV HMDT308 to DEDD; and CaMKII H282 to R. Our caCaMKK (CaMKKa1-434, Figure. 1A) is the exception as it is a truncation of the C-terminus including the autoinhibitory domain. If the physiological function under study is modulated by expression of a given caCaMK, we conclude that CaMK may potentially be involved. However, this tentative conclusion has to be substantiated by other independent approaches as will be detailed.

Monitoring cellular activation of CaM-kinases

Most physiological processes are subject to homeostatic regulation that is, adapting to alterations in extracellular or intracellular conditions. These adaptations often utilize multiple signal transduction pathways, the most common involving protein phosphorylation [25]. The question at hand for this review is how to determine if CaMKs are involved in modulating cellular functions known to be regulated by intracellular Ca^{2+} and phosphorylation.

Fluorescent Approaches—If one has preliminary data suggesting a role for a CaMK in regulating a Ca^{2+}-dependent physiological function in response to some type of stimulus, the first task is to determine which CaMKs are activated by that stimulus. The ideal method for determining spatial and temporal activation of protein kinases in living cells is to utilize Fluorescent Resonance Energy Transfer (FRET) of appropriate kinase constructs or engineered kinase substrates. Either the kinase or the substrate or both often undergo substantial conformational changes upon activation or phosphorylation, respectively, that alter the degree of FRET [26, 27]. In the inactive states of the various CaMKs, an autoinhibitory domain is thought to interact with and suppress catalytic elements [28], and this interaction is disrupted upon binding of Ca^{2+}/CaM, thereby achieving kinase activation [29]. Indeed, when CFP is fused to the C-terminus and YFP to the N-terminus of CaMKII, this construct exhibits a basal FRET signal that is quenched upon activation by Ca^{2+}/CaM-binding and subsequent autophosphorylation of Thr286 which maintains constitutive activity of CaMKII [30]. FRET analysis has been used to measure the temporal and spatial kinetics of CaMKII activation within a single dendritic spine stimulated by two-photon glutamate

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uncaging [18]. The transient CaMKII activation was restricted to the stimulated spine and was associated with the prolonged phase of spine enlargement in LTP. The Thr286Ala mutant was only partially effective because autophosphorylation enhances and prolongs CaMKII activation, probably by suppressing dissociation of bound Ca^{2+}/CaM [31]. FRET analysis of activation of other CaMKs in cells should be a powerful emerging approach now that these kinases have established physiological roles [14].

Analysis with anti-phosphoCaMK antibodies—The most commonly used technique for assessing kinase activation in cells due to a particular stimulation paradigm is to use phosphospecific antibodies and either immunocytochemistry or Western blot analysis. As mentioned in the Introduction, activation of CaMKII is accompanied by autophosphorylation of Thr286 (Fig. 1B) whereas activation of CaMKI and CaMKIV by CaMKK can be monitored by the phosphorylation status of their respective activation loop sites (Figs. 1C, D). Antibodies specific to these phosphorylation sites are commercially available.

If the phospho-specific antibody is suitable in terms of specificity and sensitivity, immunocytochemistry is the procedure of choice as it can potentially provide information on subcellular localization of kinase activation. Immunocytochemistry showed that depolarization of hippocampal neurons results in Thr286 autophosphorylation of CaMKII – this was blocked in cells that were transfected with the specific CaMKII inhibitor protein CaMKIIN (Figure 2). Numerous studies have shown that stimulation of Ca^{2+}-permeable NMDA receptors in hippocampal neurons results in translocation of CaMKII to the postsynaptic density (PSD) in the spine head [32]. Its association with the PSD can be either transient or prolonged [33]. The association of CaMKII with the PSD is facilitated by interactions with NR2B and enhanced by the autophosphorylation status of T286 [33, 34]. The stable translocation may also be associated with “synaptic memory” formation [35]. This translocation of GFP-CaMKII to the PSD has been observed in intact zebrafish upon repeated sensory stimulation [36]. A recent study shows that moderate NMDA receptor stimulation selectively targets translocation of autophosphorylated CaMKII to inhibitory synapses whereas stronger stimuli promote translocation to excitatory synapses [37]. Immunocytochemical analysis has demonstrated in cultured hippocampal neurons the presence of CaMKK-phosphorylated CaMKI in dendritic spines where, as part of a multi-protein complex, it promotes formation of excitatory synapses [38].

Western blot analysis of cell lysates with phospho-specific antibodies has more commonly been used to demonstrate cellular activation of various CaMKs by agonists. Some information on subcellular activation can be achieved by isolation, in the presence of protein phosphatase inhibitors, of specific organelles (e.g., PSDs) or by co-immunoprecipitation with other cellular proteins known to associate with the kinase prior to western blot analysis.

Mechanisms to inhibit CaM kinases in cells

Having identified which CaMKs are activated in response to a particular protocol that stimulates the Ca^{2+}-dependent physiological response of interest, the next question is whether inhibition of those CaMKs suppresses the stimulatory effect. As stated above, it is necessary to use multiple, independent approaches to make valid conclusions.

Pharmacological inhibitors—The easiest, and therefore most common, approach to inhibiting kinases is to use cell-permeable pharmacological inhibitors. However, seldom are these inhibitors selective, in part because many are competitive with ATP, a required substrate for all kinases. Our working hypothesis is that pharmacological kinase inhibitors may be selective but are rarely specific [39]; their specificity is generally inversely related to

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how extensively they have been tested against other kinases! Pharmacological inhibitors that are not competitive with ATP should also be used with great caution. For example, KN-62 and KN-93 were initially described as specific CaMKII inhibitors that act by blocking binding of Ca^{2+}/CaM, but we now know they inhibit other CaM-kinases [40-42] and some voltage-gated K^{+} and Ca^{2+} channels [43, 44]. Furthermore, since the KN drugs interfere competitively with the activation of the kinases by Ca^{2+}/CaM, they do not inhibit Ca^{2+}/CaM-independent (i.e., autonomous activity) CaMKII [42, 45]. For example, depending on the age and density of neuronal cultures, they exhibit spontaneous neuronal activity associated with partial autonomous CaMKII that is not blocked by acute treatment (5-20 min) with KN compounds. However, prolonged treatment (several hours) will allow endogenous phosphatases to dephosphorylate T286 and render it CaM-dependent. Unfortunately, the involvement of CaMKII in numerous physiological pathways has been and continues to be erroneously based on the exclusive use of KN compounds (KN-62, KN-93) and the assumption that these inhibitors are CaMKII-specific inhibitors. Our approach is to use KN-93 as an initial tool to implicate potential involvement of one of the CaMK members in a particular mechanism, but other more specific techniques are needed to verify such a role.

We have used STO-609 (2-10 μM) extensively as an initial probe for involvement of CaMKK in cellular functions, especially neuronal development. STO-609 is a selective, cellpermeable CaMKK inhibitor, [46, 47] it has an *in vitro* IC_{50} of 0.32 μM (120 ng/ml) and 0.11 μM (40 ng/ml) for CaMKKα and CaMKKβ, respectively whereas a 100~250-fold higher concentration (26μM) inhibits CaMKII by only 50%, with essentially no effects on CaMKI, CaMKIV, PKA, PKC, or Erk1. Incubation of hippocampal neurons with 2.6 μM STO-609 blocks CaMKK, assessed by its activation of CaMKI and CaMKIV upon KCl treatment, but no inhibition of CaMKII is observed [48]. Recently it has been reported that STO-609 can activate the Aryl hydrocarbon receptor [49]. Fortunately, non-specific effects of STO-609 can be evaluated in cells by rescue experiments with a mutant of CaMKKα isoform (Leu233Phe) that is approximately 10-fold less sensitive to inhibition by STO-609. If expression of CaMKKα (L233F) attenuates or reverses the inhibition by STO-609, this is strong evidence that the phenotype is CaMKK-dependent. Furthermore, we have also found it valuable to attempt reversal of STO-609 inhibition by expression of constitutively-active (ca) downstream effectors of CaMKK such as caCaMKI or caCaMKIV [48, 50].

Protein/peptide inhibitors—There is an endogenous PKA inhibitor protein [51] in cells, and a peptide derived from that protein has high specificity and potency for inhibiting PKA [52]. Inhibition of PKA by this peptide has been very useful in identifying PKA-dependent functions. Synthetic peptides can be made cell-permeable through attachment of the Antennapedia peptide (Ant) or multiple Args [53]. Similar approaches have been explored for inhibitors of CaMKII. Although a peptide based on the autoinhibitory domain of CaMKII is not specific for CaMKII [54] [55], a modified autoinhibitory peptide termed AC3-I [56] appears to be quite specific for CaMKII versus PKC, CaMKI or CaMKIV [57]. There is also a brain-enriched protein termed CaMKIIN that is a highly specific endogenous inhibitor of CaMKII, as are peptides derived from CaMKIIN [58, 59]. Expression of CaMKIIN in neurons blocks CaMKII activity but does not suppress formation of axons or their outgrowth [23, 48] (Figure 2), suggesting that CaMKII does not exert an essential role in this process. Several studies have utilized bath applied CaMKIIN-derived peptides fused to either antennapedia (Ant) or Tat, highly basic peptides that promote cell-permeability. The Ant-inhibitory peptides suppressed outgrowth of immature neurites [60] and it blocked maintenance of long-term potentiation (LTP) [61]. These two studies included appropriate controls of either the reverse inhibitory peptide sequence fused to Ant [60] or Ant alone [61], neither of which exhibited effects on the physiological readouts. However, a recent study challenges these conclusions since Ant itself binds CaM, thereby confounding

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interpretation of results [62]. The Tat-inhibitory peptide, which does not bind CaM, inhibited LTP induction but did not reverse established LTP in a hippocampal slice [62]. Thus, the role of activated CaMKII in LTP maintenance needs further investigation. Moreover, the Tat-inhibitory peptide, which permeates the blood-brain barrier, inhibited learning but not memory storage or retrieval in vivo.

Dominant-negative kinases—Another approach we and others use is to generate dominant-negative interfering mutants of CaMKs (dnCaMKs). This approach appears to achieve more specific inhibition of CaM kinase signaling, although there are some caveats. In general, dnCaMKs have a series of mutations, described in detail below, which renders the kinase catalytically inactive. For CaMKI and CaMKIV there are also mutations within their activation loop that render these mutants incapable of being phosphorylated by CaMKK as well as mutations in their autoinhibitory domain to render them Ca^{2+}/CaM-independent. These expressed dnCaMKs could function in one or more possible ways to inhibit endogenous kinase activity. They could 1) block the activation of endogenous kinases by binding to and quenching the available active population of the upstream CaMKK; 2) disrupt the subcellular localization (e.g., multiprotein signalsome) of the endogenous CaMK and thereby block localized CaMK signaling; or 3) compete with the endogenous active CaMK for binding to downstream substrates. With these possibilities in mind, we and others have engineered a series of mutant CaMKs that inhibit endogenous neuronal CaMK signaling. If mechanism 1 were correct, then dnCaMKI and dnCaMKIV could inhibit CaMKK and suppress its activity towards all downstream targets (CaMKI, CaMKIV, PKB/Akt, AMP-kinase) unless there is compartmentalization involved. If this is the case then this would represent a limitation to dnCaMKI/IV as they would function as a compartmental inhibitor of CaMKI/IV signaling. Some data supporting this conclusion is that, endogenous CaMKIV is predominantly nuclear [63] whereas overexpressed dnCaMKIV is present throughout the neuron (Figure 3). Expression of dnCaMKIV can inhibit CaMKI-dependent axonal outgrowth [48]. However, when a nuclear localization signal is attached to dnCaMKIV to restrict it to the nucleus, it no longer suppressed axonal outgrowth even though it still blocked CREB-dependent transcription. This result highlights the importance of establishing the subcellular localization of overexpressed kinase constructs. If mechanism 3 were correct, lack of substrate specificity between CaMKs (and other kinases such as PKA), especially at high kinase concentrations, would limit the specificity of the dnCaMK. However, these concerns can be largely obviated by confirming results using RNAi as described below.

dnCaMKK: A dnCaMKK was constructed by mutating the catalytic Lys157 to Ala, rendering CaMKK catalytically inactive (Figure 1). Expression of dnCaMKK blocks multiple aspects of neuronal development such as axon formation [23] and outgrowth [48], dendritic arborization [50] and spine/synapse formation [38]. Corroborating evidence (e.g., RNAi against CaMKK or CaMKI isoforms) indicate that these processes are indeed regulated in part by the CaMK cascade. How does dnCaMKK work? Since there is no known upstream activator of CaMKK (except Ca^{2+}/CaM), it likely acts by interacting with downstream substrates (CaMKI, CaMKIV, PKB, AMP-kinase). This conclusion is supported by the fact that an affinity column of the catalytic domain of CaMKK bound two substrates, AMP-kinase and SAD-B. AMP-kinase is a known substrate for CaMKK [64], and SAD-B is activated by CaMKKα phosphorylation at the activation-loop Thr (Thr189) *in vitro* and in transfected cells [65]. Thus, it is important to demonstrate that suppression of a cellular function by dnCaMKK can be reversed by transfection with a constitutively-active downstream effector (e.g., caCaMKI).

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dnCaMKI: The dnCaMKI was generated by a series of point mutations: K49E, T177A, 286IHQS to 286EDDD, and 307F to A. The Lys49 (catalytic lysine) to Glu mutation disrupts catalytic activity (Figure 1B). The T177 (activation loop threonine) to A mutation prevents CaMKK phosphorylation of the activation loop of CaMKI. Mutations 286IHQS to EDDD and 307F to A [66] disrupt the autoinhibitory domain, thereby mimicking the effect of Ca^{2+}/CaM-binding and allowing the dnCaMKI to be a constitutive inhibitor in the absence of Ca^{2+}/CaM. Expression of dnCaMKI blocks the phosphorylation of Thr177 of endogenous wildtype CaMKI in NG108 cells, indicating that it acts by blocking CaMKK [48]. It may additionally function by binding to downstream substrates, thereby suppressing their phosphorylation by active endogenous CaMKI. As with CaMKK, an affinity column was made containing the catalytic domain of inactive (Lys49Glu mutation) CaMKI. The CaMKI was previously phosphorylated by CaMKK, as phosphorylation of its activation loop increases its apparent affinity for substrates. This affinity column binds CaMKI substrates such as synapsin I, CREB and Numb/Numbl [67].

dnCaMKIV: The dominant negative CaMKIV was constructed analogous to dnCaMKI with the following mutations: 1) Lys75Ala in the ATP-binding site was mutated to Ala, 2) the activation loop phosphorylation site (Thr196) was mutated to Ala, and 3) the autoinhibitory domain was inactivated by the triple mutation HMDT308 to DEDD (Figure 1A). Expression of this construct in COS-7 cells co-transfected with His-tagged-wtCaMKIV and CaMKK blocked Ca^{2+}-dependent phosphorylation of His-tagged wtCaMKIV (Wayman unpublished observations). Several investigators have used various constructs of CaMKIV, but our experience highlights a special concern. When expressed in neurons, the EGFP-dnCaMKIV mislocalized in the cell body rather than being restricted to the nucleus as is endogenous CaMKIV [63], and the dnCaMKIV suppressed axon outgrowth (Figure 3A upper panels). Lemrow et. al. [68] demonstrated that basal catalytic activity is required for CaMKIV to enter the nucleus - inhibition of CaMKIV by mutation of Lys75Ala blocks nuclear entry into the nucleus. However, when expression was restricted to the nucleus by attachment of an NLS, the EGFP-dnCaMKIVnuc no longer inhibited axon elongation, but it did still block CREB-dependent transcription [48] (Figure 3A lower panels and B). This result highlights a potential caveat of over-expressed and mutated proteins – namely, mislocalization within the cell. Since signaling specificity often depends on subcellular compartmentalization, this concern needs to be addressed. However, a role for CaMKIV in homeostatic synaptic depression, due to elimination of spines/synapses, has been established using both dnCaMKIV and shRNA against CaMKIV – the effect of the shRNA was reversed by expression of a CaMKIV construct not sensitive to the shRNA [69].

dnCaMKII: The dnCaMKII contains a single point mutation at Lys43Met that renders the subunit catalytically dead (Figure 1A). Several labs have used these mutants as dominant-negatives [70, 71] - however, the efficiency of this approach in neurons we believe is questionable. The dnCaMKII must compete with endogenous CaMKII to work effectively as a dominant-negative. Thus, it must be expressed at significantly higher concentrations than the endogenous CaMKII. Given the fact that in neurons CaMKII comprises approximately 1-2% of total brain protein [13], it seems unlikely that one would be able to express enough of the exogenous dnCaMKII to effective inhibit endogenous CaMKII activity. Alternatively, if the expressed dnCaMKII subunit oligomerizes with endogenous active CaMKII subunits, this may suppress autophosphorylation of Thr286 in the active subunits of the oligomeric holoenzyme since this process is intersubunit (i.e., requires kinase activity in adjacent subunits). When active CaMKIIα is co-expressed with inactive CaMKIIβ, the two subunits are incorporated into a heteromeric complex, and the inactive β subunit is phosphorylated on Thr286 by the active α subunit [72]. Unfortunately, it was not determined if the phosphorylation of Thr286 was suppressed in this construct by the presence of the inactive β subunit. If this were the case, the heteromeric (i.e., containing

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active endogenous subunits and inactive dn subunits) holoenzyme may not achieve sufficient autonomous activity to support the physiological function under investigation. If this mechanism were operative for the functionality of dnCaMKII in neurons, very high level of over-expression of the dnCaMKII relative to endogenous CaMKII would not be required. However, because of this uncertainty we utilize expression of the endogenous inhibitor protein of CaMKII, CaMKIIN, to block CaMKII activity in neurons (Figure 2) [23, 48].

Si/Sh-RNA inhibition of CaMK signaling—As an alterative to inhibiting CaMK signaling by either pharmacological or dominant negative methods, RNAi approaches to suppress levels of endogenous CaMKs have become popular. A major benefit to this method is that specific isoforms of CaMKs, which can not be selectively suppressed by either pharmacological or dominant negative inhibitors, can be individually suppressed using si/sh-RNA methods (small interfering RNA/short hairpin RNA). For our studies we routinely utilize sh-RNAs. These RNAs are expressed using mU6pro or pSuper vectors. Table 1 lists the sequence we have successfully used to inhibit the expression of CaMKs in rat neurons [23, 38, 50, 73]. When the appropriate antibodies are available, knockdown should be verified by immunostaining. When antibodies for the various CaMKs are not available, we validate the sh-RNA constructs by co-transfecting either Flag or Myc-tagged CaMKs, along with the sh-RNA to be tested, into either HEK-293 cells or neurons. Following transfection (24-48hrs) the expression levels of the tagged CaMKs were monitored by western blot. All of the sh-RNAs listed in table 1 have been validated using these methods. Effective knockdown of endogenous CaMKs in rat hippocampal neurons normally occurs within 48hrs following transfection. To control for nonspecific effects of the si/sh-RNA constructs, it is important to demonstrate rescue through the expression of a homolog that is resistant to knock RNAi.

Choice of vector to be used to express mutant CaMKs and morphological markers

A critical parameter when one studies signal transduction pathways, and more specifically CaM kinase signaling, is the maintenance of equal cDNA expression under all conditions tested (e.g., control vs agonist). Over the years we have observed in our experiments that cDNA constructs which are driven by CMV promoters in hippocampal neurons can show dramatic upregulation (greater than 10 fold induction, personal observation) following stimuli such as increased synaptic activity (bicuculline or KCl stimulation) or neurotrophic stimulation (BDNF) as well as other stimuli that elevate intracellular Ca^{2+} in hippocampal neurons. This can greatly complicate the interpretation of experiments in which these stimuli are used. We have tried numerous expression vectors and have concluded that in hippocampal and cortical neurons the pCAGGS vector gives the most consistent results with very slight to no modulation of the transgene expression under any culture or stimulation condition tested. The pCAGGS vector consists of the CMV immediate early enhancer and the chicken β-actin promoter [74]. When expressed in hippocampal or cortical neurons, stimuli such as bicuculline or BDNF do not significantly affect the expression cDNAs contained within the pCAGGS vector. Furthermore, this vector has the added benefit of restricted expression. Within a mixed culture of neurons and glia, this expression vector only detectably expresses cDNA inserts in neurons and not glia. We have taken advantage of this fact to express both CaMK mutants and our morphological markers (EGFP-MAP2B and mRFP-β-Actin) using this vector (Figure 4)[23, 38, 50, 75, 76]. This allows us to image just neurons in our morphological assays. A potential disadvantage of this vector is that in neurons it may not express cDNA inserts to the same high level in the first 12-48 hrs after transfection as seen when using a CMV driven expression system. This has not been a

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limitation for our experiments. To the contrary, we have found that the modest expression level of pCAGGS based transfection is advantageous as it reduces the possible non-specific effects and/or toxicity of very high levels of transgene expression.

Conclusions

CaMKs regulate numerous physiological processes within the cell by phosphorylating multiple downstream targets. In the past, the study of CaMK signaling was largely restricted to pharmacological methods that are not specific and often lead to erroneous conclusions. To rigorously investigate the roles of multifunctional CaMKs in physiological functions, it is essential to employ multiple, independent techniques since each experimental approach has its limitations. In this review we describe the use of not only pharmacological reagents, but methods to manipulate CaMK signaling using transfections of dominant-negative or constitutive-active kinase constructs or a CaMKII inhibitor protein and RNAi suppressors of the endogenous kinases. When used correctly, this multifaceted approach can yield consistent results that validate conclusions about the roles of specific CaMKs in physiological functions.

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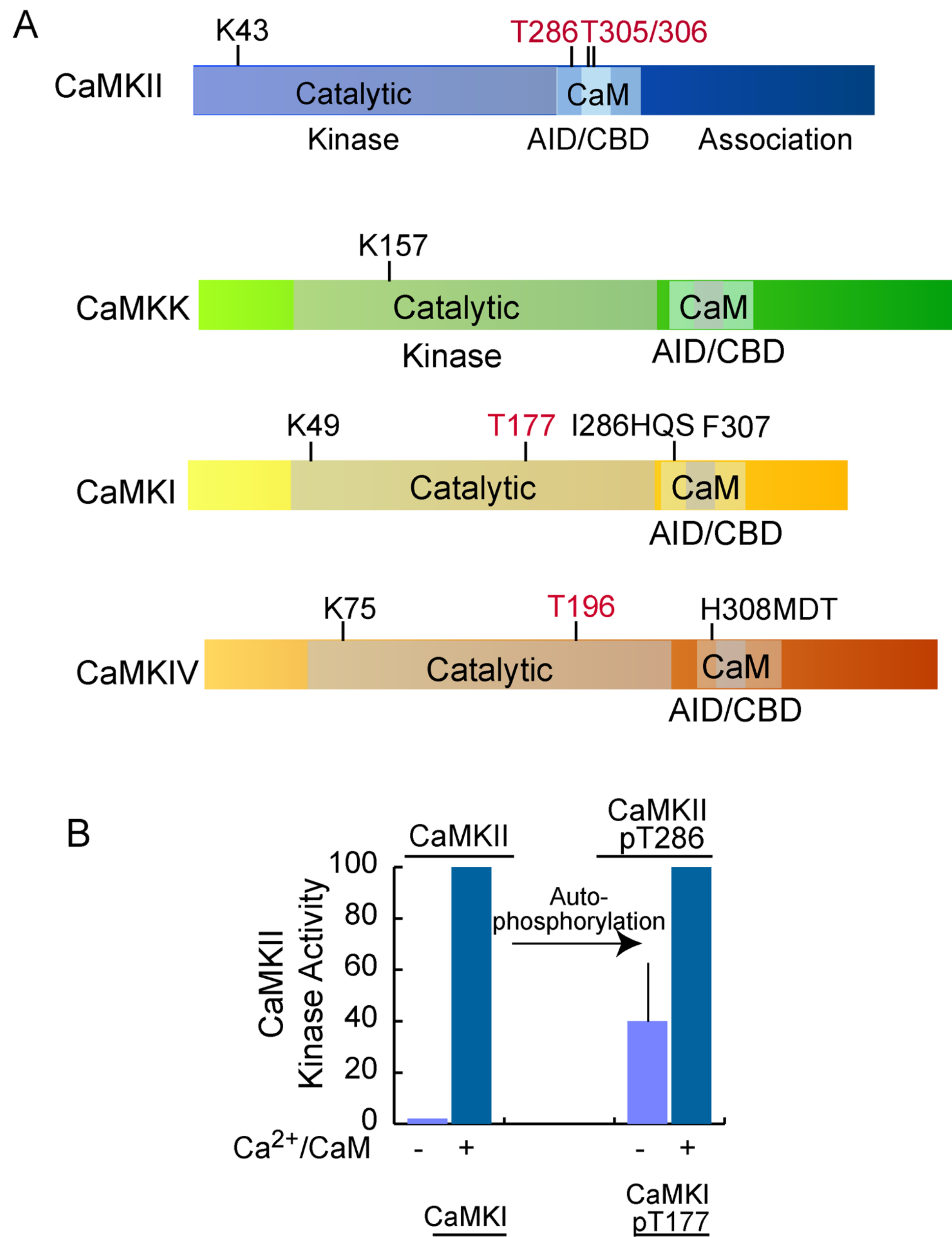

Figure 1.

Subunit structure and regulation of CaMKs. A. Schematic diagrams of CaMKs with key residues involved in their regulation by phosphorylation (red font) or that are mutated in making dominant-negative or constitutively-active constructs (black font). See text for details. The ∇ in CaMKK at D434 indicates the site of truncation for the constitutively-active construct. AID, autoinhibitory domain; CBD, calmodulin-binding domain. B-D. Regulation of CaMKs by phosphorylation. Autophosphorylation of Thr286 in CaMKII (B) generates Ca^{2+}/CaM-independent activity (30-70% of total) whereas phosphorylation of the activation loop sites in CaMKI (C) or CaMKIV (D) by CaMKK primarily increases total activity (i.e., with Ca^{2+}/CaM) although CaMKIV exhibits some (~ 10-20%) independent activity.

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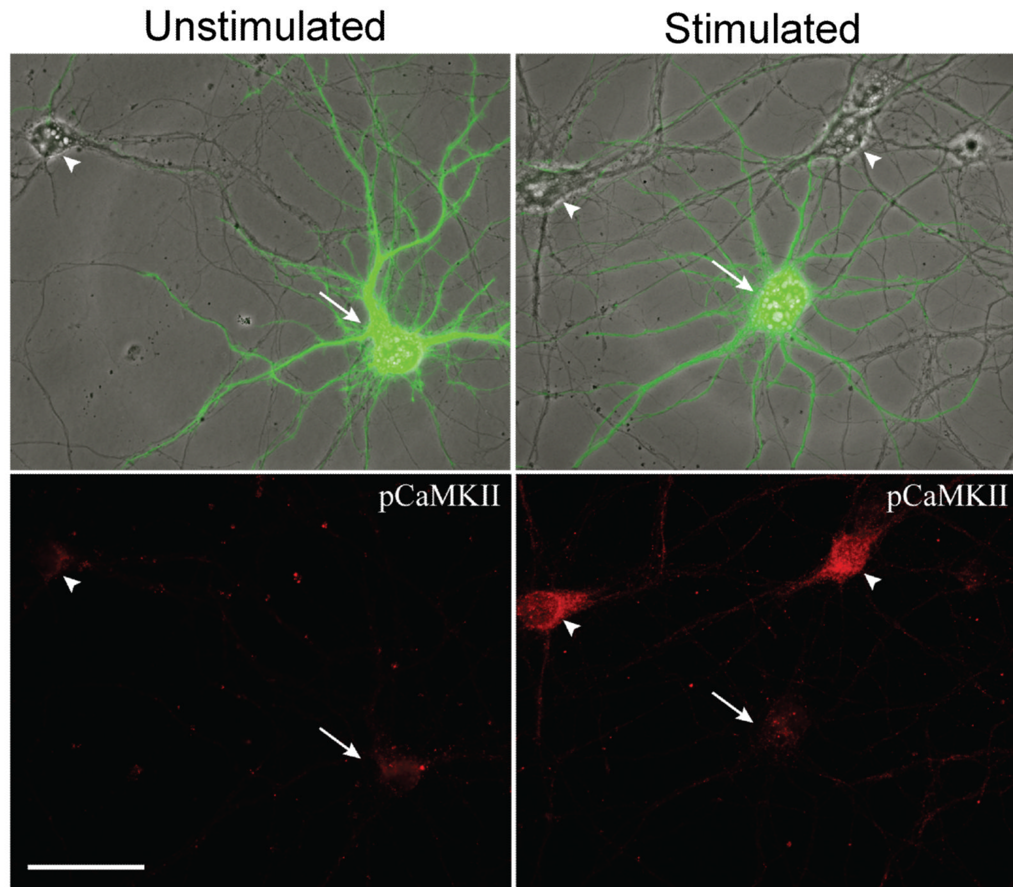

Figure 2.

Immunocytochemical analysis of CaMKII activation in hippocampal neurons is blocked by the CaMKII inhibitor CaMKIIN. Low-density hippocampal cultures were transfected on day 5 with plasmid encoding EGFP-CaMKIIN. The neurons were cultured an additional 9 d and then stained with antibody specific for phospho-Thr286 in CaMKII (pCaMKII), either with (right panels) or without (left panels) prior stimulation with 90 mM KCl (5 min). After KCl stimulation (right panel), nontransfected neurons (arrowheads) show enhanced staining for activated CaMKII (autophosphorylated at Thr286) throughout their cell bodies and processes (bottom panel). EGFP-CaMKIIN-expressing neurons (arrows) show no increase in anti-phospho-CaMKII staining over levels seen in unstimulated cells, demonstrating that overexpression of CaMKIIN inhibited activation of endogenous CaMKII. Scale bar; *B*, 50 μm. Reproduced from Wayman et al. 2004 by permission from the Journal of Neuroscience.

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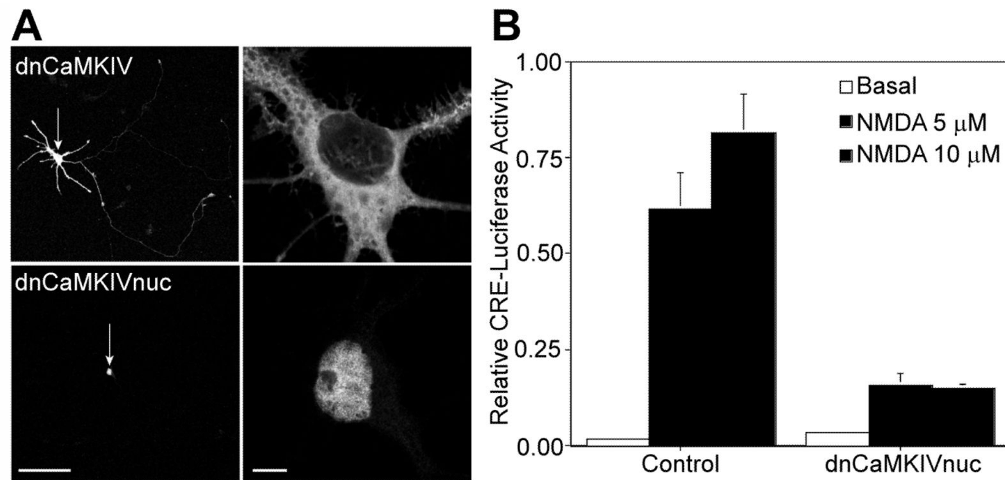

Figure 3.

Dominant-negative CaMKIV distributes throughout the cytoplasm of transfected neurons. A. Low-density hippocampal cultures were transfected on day 3 with plasmid encoding EGFP-dnCaMKIV (upper panels) or a construct modified by addition of a nuclear localization signal, dnCaMKIVnuc (lower panels). Low magnification confocal images of the EGFP signal are shown in the left panels. While the dnCaMKIV lacking a nuclear targeting signal filled the cytoplasm of the cell body (arrow), the whole length of the axon and dendrites, it was largely excluded from the nucleus (right panel). In contrast, EGFP-dnCaMKIVnuc (lower panels) was found exclusively in the nucleoplasm of the transfected neuron (arrow). Scale bars: 50 μm and 5 μm. B. Nuclear restricted dnCaMKIVnuc inhibits NMDA-stimulated CRE-mediated transcription. Hippocamapal neurons (10 DIV) were transfected with a CRE-regulated luciferase reporter, a β-actin promoter-driven β-galactosidase reporter, and a 10-fold excess of empty vector or dnCaMKIVnuc. After 48 hours the neurons were stimulated with 5 or 10 μM NMDA in the presence of 1 μM glycine for 5 hours and assayed for luciferase activity. β-galactosidase assay was conducted using the Galacton substrate (Tropix) according to the manufacturers instructions. The data (±SEM) are expressed as the ratio of luciferase to galactosidase (n = 4).

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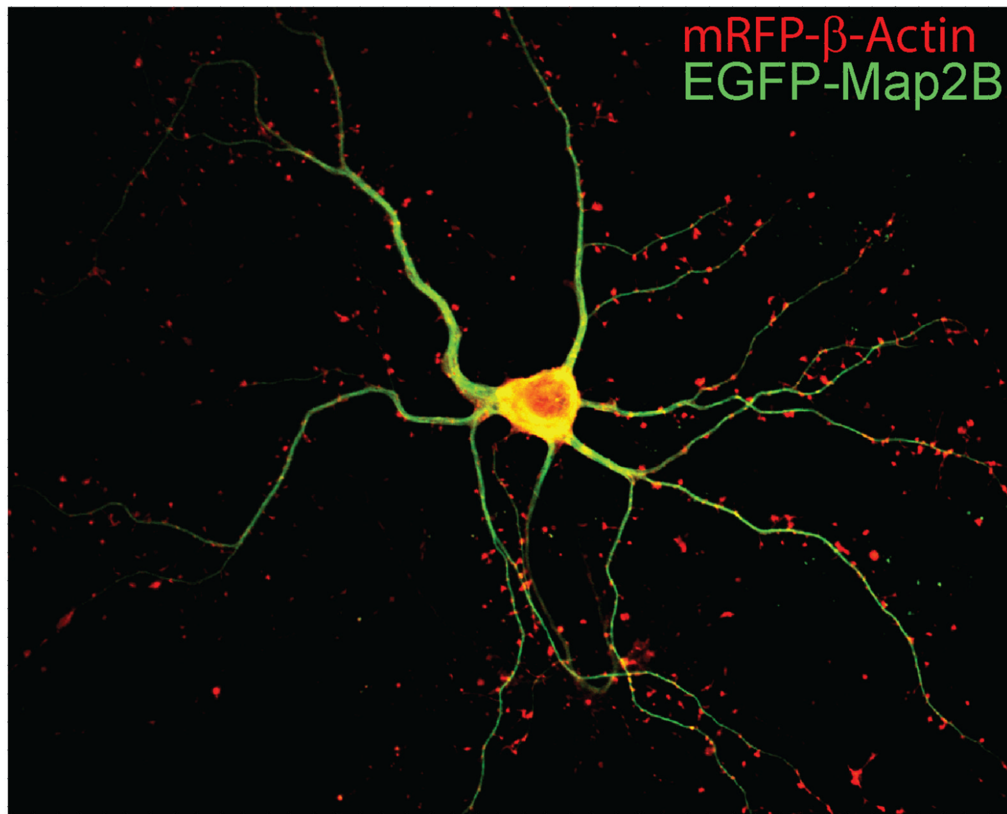

Figure 4.
pCAGGS efficiently expresses dendritic morphology markers in hippocampal neurons. DIV7 hippocampal neurons were transfected with pCAGGS-mRFP-β-Actin and pCAGGSEGFP- Map2B. On DIV12 the neurons were fixed and imaged by confocal microscopy. EGPFMap2B highlights the dendritic arbor whereas mRFP-β-Actin highlights the dendritic spines. Neither of these markers showed detectable expression in glia.

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Table 1

sh-RNA constructs targeting CaM kinases in rat neurons.

CaM kinase	sh-RNA	Reference
CaKKα	TGTTTGACCTCCTGAGAA	[38]
CaMKKβ	GGTCGAGAATTCAGTCAACA	[38]
CaMKIα	CTTGTGTAAGACGGCGATCTC	[23, 50]
CaMKIβ	CCAAGTGGACTGACTCCTA	[23, 50]
CaMKIγ	CCGAGCACTTCCATGAAGATG	[23, 50]
CaMKIδ	ATGGATCGCTGGTGACACA	[23, 50]
CaMKIIα	GAATGATGGCGTGAAGGAA	[73]
CaMKIIβ	GAGTATGCAGCTAAGATCA	[73]