Cloning and Characterization of a novel family of inhibitory proteins of CaM-Kinase II

by

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A DISSERTATION

Presented to the Neuroscience Graduate Program and the Oregon Health Sciences University School of Medicine in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

February 2000

School of Medicine
Oregon Health Sciences University

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ACKNOWLEDGEMENTS

In order for me to fully acknowledge everyone who helped with this dissertation I would have to write another dissertation. Having said that, I will try to make this as brief as possible without leaving most of the important players out. First and foremost I cannot express enough gratitude and thanks to my mentor Tom. He always made himself available but never looked over my shoulder (even when that shoulder kept going to the mountains). Wherever my academic career may lead, it will be his example that I will try to follow. Also, I am deeply grateful for the members of my committee, Roger Cone, Stan Hollenberg, John Scott, and Charlie Meshul. They were always available for my questions either as a group or as individuals. They helped guide this thesis and trouble shoot the many stumbling blocks that arose.

Of course, the members (both new and old) of Tom's lab were invaluable to both my technical training and my knowledge base. It was Sucheta Mukherji who initiated this project by starting the Yeast two-hybrid screen that proved to be fruitful. Also, she always made sure that there were some cookies around to be eaten. I will forever remember the intellectual discussions and arguments with Gary Wayman, Herve Enslen, Andres Barria, Hiroshi Tokumitsu, and Eric Guire over a few yeast

byproducts. Debbie Brickey was the only one who was able to keep Tom's lab from losing everything.

Also, all the members of many labs of OHSU were invaluable to me. All the members of John Scott's lab were of great help and support. I should especially mention Maree Faux for her help in some of the biochemical data and Lorene Langberg who was always available to help with my confocal data. Ginger Withers and Chris Wallace from Gary Banker's lab were invaluable to this research with the neurons that were donated to this project and giving me pointers on the immunohistochemical data. Vivian Lee (not the actress) was of great help in using and trouble shooting the slice preparations. Of course, Brian Druker's lab and Richard Maurer's lab for allowing me to be an adjunct member for discussions. I am positive that I am forgetting many others who I have approached with questions on my project and I do apologize if I have forgotten to mention you.

Beyond the academic relations that have helped, I would also like to thank the many friends and family that have continued to support me and listen to my frustrations and my elations. Yoon-Jae Cho and Denis Glenn, who have been in the same program as me for many years, were always there to understand and listen to my rantings and found time to get out and ski, bike, surf and run. Jeremy Cooke who always pulled me out to go running with grandiose plans of running the Boston Marathon. Phuoc Tran for teaching me how to surf. Brian Druker for running the

M.D./Ph.D program with excitement and dedication. Finally, I would like to thank my family for always being there for me and supporting my decisions, even when they didn't quite understand why I wanted to spend so much time in school. In the end I hope that I have been able to convey that this project has been a collaborative dissertation and that it has been a pleasure to have been a contributor to this project.

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ABSTRACT

Ca^{2+} signaling plays many critical roles within the cell to activate signaling pathways. One of the downstream effectors of this second messenger is a Ca^{2+}/calmodulin-dependent multifunctional kinase known as CaM-kinase II. CaM-kinase II can phosphorylate *in vitro* a large number of proteins, but establishing its physiological functions are much more difficult. Recent reports are beginning to verify several important roles such as learning and memory in hippocampus.

In order to identify proteins that interact with CaM-kinase II, specifically within the catalytic domain, a yeast two-hybrid screen was performed on a rat brain cDNA library. This screen identified two small molecular weight proteins (CaM-KIINα, CaM-KIINβ) that directly interact with the catalytic domain. Biochemical studies have shown that these 7kD proteins both bind and inhibit the catalytic activity of CaM-kinase II. Furthermore, they have the same potency for both the Ca^{2+}-dependent and independent activities. Kinetic studies have shown that the inhibition is noncompetitive with substrate with a K_i of 10nM and an IC_{50} of 50nM. This family of CaM-kinase II inhibitors, known as CaM-KIIN, are specific for CaM-kinase II and has almost no effect on any other multifunctional kinase tested. A 27 residue portion

(CaM-KIINtide) of CaM-KIIN has been identified that retains the inhibitory specificity and potency.

Localization studies have shown that CaM-KIINα is expressed only in the brain whereas the message for CaM-KIINβ is expressed in brain and testes. Immunblotting shows 7kD and 19kD proteins that are identified only within the brain. *In situ* hybridization of the message for the two species shows that CaM-KIINα is strongly expressed in the cortex, hippocampus, and inferior colliculus whereas CaM-KIINβ is expressed evenly throughout the brain including the cerebellum and hindbrain. Immunohistochemical data would suggest that the proteins are expressed throughout the brain in a similar distribution as CaM-kinase II. Staining of primary hippocampal cultures show that CaM-KIIN is found throughout the cell body and dendrites but not in the dendritic spines, axons, or nerve terminal.

A fusion construct with the DNA binding domain of Antennaepedia was constructed. This recombinant protein as well as the fusion with CaM-KIINtide have the ability to enter cells. Therefore, these constructs can be used to further dissect functions of CaM-kinase II within the cell.

CHAPTER I

INTRODUCTION

A. Historical Perspective.

For more than thirty years covalent protein phosphorylation and dephosphorylation has been known to play a role in the regulation of diverse cellular functions. The initial work by Krebs and Fischer (later awarded the Nobel Prize for their work) established the field of protein phosphorylation with their investigations of the protein kinases and phosphatases regulating muscle glycogen metabolism. It is currently thought that up to 20% of proteins in mammalian cells can be functionally regulated through phosphorylation of serine, threonine or tyrosine residues [1]. Most protein kinases in nonstimulated cells have low activity and become activated upon treatment by growth factors, hormones or neurotransmitter that elevate intracellular levels of signaling molecules such as cyclic nucleotides, Ca^{2+}, or phospholipids.

The first Ca^{2+} dependent protein kinase identified, phosphorylase kinase, [2] catalyzes the classical conversion of phosphorylase b to a. The mechanism of its Ca^{2+} activation wasn't understood until the discovery of the ubiquitous Ca^{2+} binding protein calmodulin [3]. During the early '80's, several investigators identified

Ca^{2+}/CaM dependent phosphorylation events within various tissues including rat brain, rabbit liver, and rabbit skeletal muscle ([4], [5] review). These kinases were originally named based on the identified substrates such as CaM-dependent synapsin I kinase, myosin light chain kinase, and CaM-dependent glycogen synthase kinase. Subsequent biochemical characterizations and sequencing indicate that some of these activities reside in the same enzyme termed CaM-kinase II. Other Ca^{2+}/CaM dependent enzymes were subsequently discovered that were very specific for certain substrates or had the ability to phosphorylate multiple substrates (Table 1). For example, Myosin light chain kinase appears to be very substrate specific as does CaM-kinase III (elongation factor 2). CaM-kinases I, II and IV can phosphorylate numerous substrates *in vitro* and probably in cells, and this family shares conserved catalytic and regulatory domains.

There are three fundamental strategies that the cell uses to increase the intracellular Ca^{2+} concentration (fig.1). Agonists such as hormones and some neurotransmitters act on G-protein coupled receptors to stimulate phospholipase C and elevate phosphatidylinositol 1,4,5-trisphoshate (IP_3) which releases Ca^{2+} from intracellular stores such as the endoplasmic reticulum. Many neurotransmitters stimulate ligand-gated ion channels that are permeable to Ca^{2+}. Excitable cells also have voltage-gated ion channels, some of which are Ca^{2+} permeable, that are responsive to changes in membrane potential. In most cells elevations of intracellular

Ca^{2+} are rather transient and last for seconds to several minutes due to elaborate mechanisms of pumps and exchangers that sequester the Ca^{2+} back to near basal values.

B. Background.

Calcium/Calmodulin-dependent protein kinase II (CaM-kinase II) belongs to a family of serine/threonine kinases that is regulated by the ubiquitous protein calmodulin. This family is comprised of four classes ($\alpha, \beta, \gamma, \delta$ encoded by different genes and expressed in different tissues. Alternative splicing of β gives rise to the β' isoform. α and β/β' are specific to nervous tissue while δ and γ are expressed in most tissues, including the brain ([6] review). CaM-kinase II is a multifunctional enzyme that, upon activation by Ca^{2+}/CaM, has the ability to autophosphorylate to become Ca^{2+} independent and phosphorylate a variety of protein substrates. Some identified substrates play roles in carbohydrate metabolism, protein synthesis, neurotransmitter biosynthesis and release, and ion channel functions. Therefore, CaM-kinase II is analogous to cAMP-kinase (PKA) and protein kinase C (PKC) in regulating numerous cellular functions.

Table 1. Brief overview of Ca^{2+}/CaM dependent protein kinases [7]

Enzyme (substrate)	**Properties** Size; activation and site; substrate site; location	**References**
CaM-KI (multifunctional)	Monomeric 37-42kD; potentiated by CaM-KK (T177); LRRRLSDSNF; cytosolic	[8, 9]
CaM-KII (multifunctional)	160kD, heteromeric and monomeric (α, β, γ, δ), 50-60kD; autophosphorylation (T286, T287); RXXSXD; membrane, cytosolic, nuclear	[10-13]
CaM-KIII, (elongation factor-2)	Monomeric, 95-103kD; no specific activation site; AGETRFTDTRK; cytosolic	[14, 15]
CaM-KIV (multifunctional)	Monomeric, 65-67kD; potentiated by CaM-KK; RXXS; predominantly nuclear, some cytosolic	[16]
CaM-KK (CaM-KI, CaM-KIV, PKB)	Monomeric, 68-70kD; no specific activation site; nuclear and cytosolic	[17]
Myosin Light chain kinase (skeletal and smooth muscle) (myson light chain)	Monomeric, 87-152kD; no specific activation site; KKXKRRX$_{5-7}$SXYY, KKRPQRATS; cytosolic	[18]
Phosphorylase kinase (phosphorylase)	Multimeric, (α,β,γ,CaM)4, 1,300kD; no specific activation site; KNISVRG; cytosolic	[19]

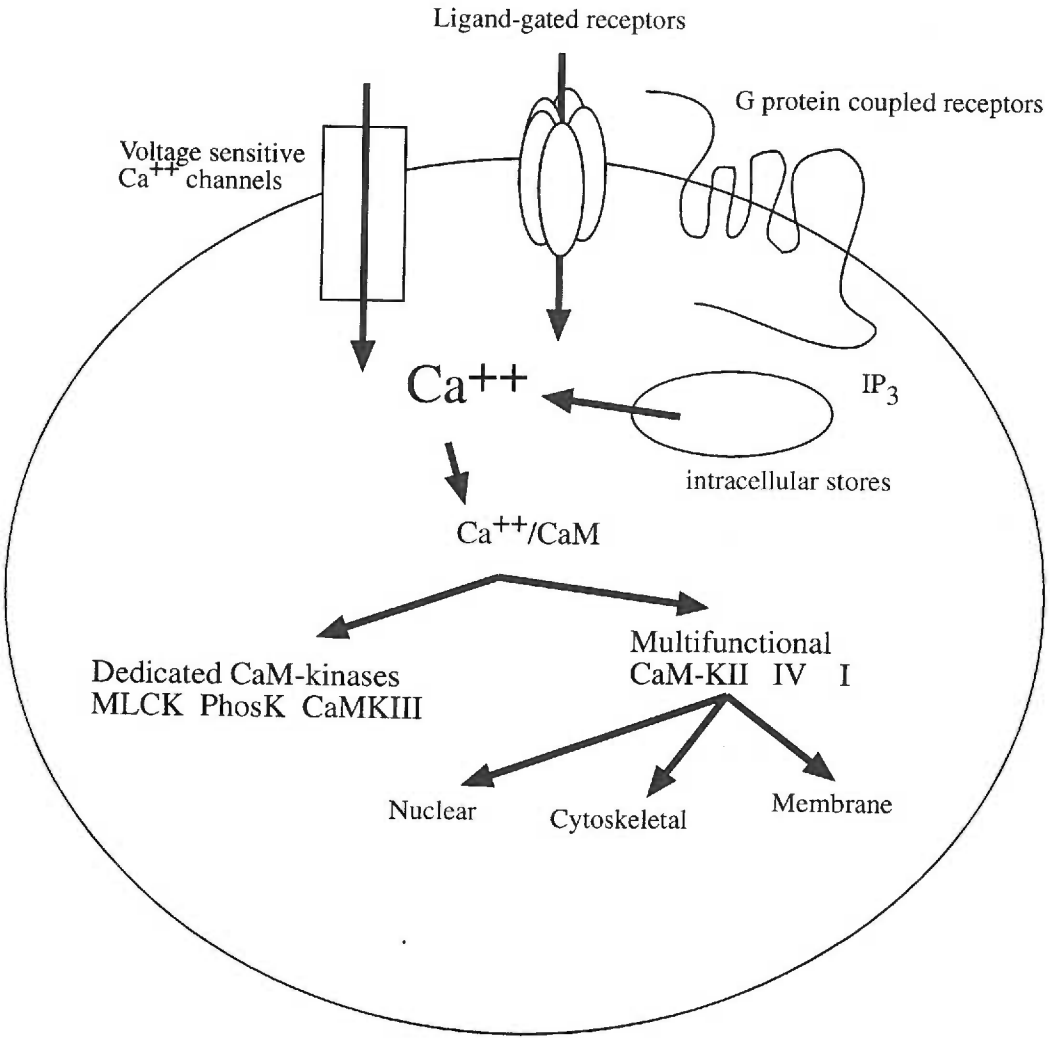

Figure1. Schematic representation of the activation of the classes of CaM-dependent kinases. Signals act upon voltage–gated Ca^{2+} channels, ligand receptor channels and G-protein coupled receptors to increase intracellular Ca^{2+}. This then can activate Ca^{2+}/CaM dependent protein kinases that can have specific down stream effects or generalized effects. Adapted from Braun and Schulman [6].

C. Structural characteristics of CaM-kinase II.

The CaM-kinase II holoenzyme is a floral shaped 670kD oligomeric protein comprised of 10-12 subunits (fig. 2)[20]. The structural design of the holoenzyme is proposed to have the C-terminal domain within the core of the floral design and the N-terminus facing outward. Each subunit of 49 to 63kD is composed of three domains; an N terminal catalytic domain, a central regulatory domain composed of an autoinhibitory domain (AID) and an overlapping calmodulin binding region, and a variable C terminal domain [21]. The catalytic domain probably has a globular, bimodal conformation, in analogy with other crystallized Ser/Thr protein kinases. It has been proposed that the AID either sterically occupies the catalytic cleft [22] or the AID interacts with the upper and lower lobes and distorts their orientation to prevent ATP binding [23], [24]. In this model, the autoinhibitory domain lies within the catalytic cleft competitively inhibiting both the substrate and ATP binding sites when calmodulin is not bound [22]. A crystal structure for CaM-kinase II has not been solved yet.

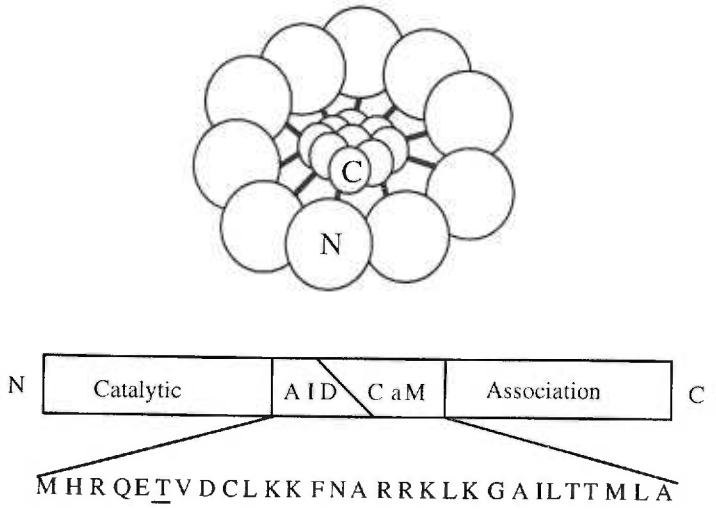

Figure 2. Diagrammatic representation of CaM-Kinase II. The holoenzyme is approximately 10 to 12 subunits arranged in a floral pattern. Each subunit is organized with a catalytic N terminal domain, an autoinhibitory domain with an overlapping CaM binding domain and a variable C terminal domain that contains the association domain. The amino acid sequence for the regulatory domain is shown. T^{286} is under-lined.

D. Biochemical characteristics of CaM-kinase II.

Each subunit of CaM-kinase II is able to bind Ca^{2+}/calmodulin with an apparent K_a of 100nM [25]. Upon binding Ca^{2+}/CaM, the AID dissociates from the catalytic domain, allowing binding of ATP [26]. Removal of the autoinhibitory

domain decreases the K_M for ATP from 145μM to 10-20 μM [26]. The activated kinase immediately autophosphorylate at Thr^{286} [27] by an intramolecular, intersubunit reaction [28], [29]. Autophosphorylation requires that Ca^{2+}/CaM be bound to adjacent subunits – one subunit catalyzes the reaction and the other subunit is the substrate. The primary consequence of Thr^{286} autophosphorylation is to increase the Ca^{2+} independent activity to 30-50% of the total activity. A second consequence of this autophosphorylation is a 1000-fold decrease in the rate of Ca^{2+}/CaM dissociation [30]. Therefore, it is believed that this enzyme has the capacity to "remember" the quality of the Ca^{2+} signal by the amount of its Ca^{2+} independent activity. A recent study has confirmed that the extent of Ca^{2+} independent CaM-kinase II is dependent on the frequency and magnitude of Ca^{2+} oscillations [31].

The Ca^{2+} independent form of CaM-kinase II has also been shown to autophosphorylate at another site, $Thr^{305/306}$, which is in the CaM-binding domain [26]. This phosphorylation event is an intrasubunit reaction in contrast to autophosphorylation of Thr^{286}, and it blocks subsequent binding of Ca^{2+}/CaM, thereby inhibiting total activity. Although this Ca^{2+} independent autophosphorylation is a potentially interesting regulatory mechanism, it has not been documented to occur in cells.

E. Localization of CaM-kinase II within neurons.

CaM-kinase II is highly localized in the dendrites but is also found in the cell body and presynaptic terminal of pyramidal neurons of the hippocampus [32]. There have been studies to identify how the α-isoform of CaM-kinase II localizes at the dendritic spines. Some evidence suggests that the mRNA itself for CaM-kinase IIα is localized to the dendrites [33]. This localization of the mRNA may be specified by the 3'-untranslated region [34]. Dendrites contain all the machinery necessary for localized protein synthesis [35], and certain paradigms that induce synaptic plasticity cause increases in CaM-kinase II protein. For example, induction of LTP (see next section) produces an increase in dendritic CaM-kinase II, probably due to localized synthesis [36]. Exposure of dark-reared rats to light produces extensive synaptic remodeling and increased CaM-kinase II protein. In this case the likely mechanism of protein synthesis is thought to involve polyadenylation of the CaM-kinase II mRNA in dendrites [37].

Previous results suggest that there may be an associating protein within the postsynaptic density (PSD) for CaM-kinase II [38]. A 190kD protein may bind activated CaM-kinase II at the PSD. One putative protein that associates with activated CaM-kinase II is the NMDA receptor itself [39]. It has been shown that the enzyme directly interacts with both the NR1 and NR2B subunits of the NMDA receptor. Furthermore, activation of the enzyme by stimulating the NMDA receptor

increases the association. Autophosphorylated CaM-kinase II has been shown to directly interact with only the NR2B subunit [40]. Also, CaM-kinase IIβ binds to F-actin that localizes the holoenzyme to the cytoskeleton. Upon activation, the enzyme autophosphorylates and dissociates from F-actin. The Ca^{2+}/CaM bound enzyme will then translocate to the PSD [41]. At the postsynaptic spines CaM-kinase II can phosphorylate and modulate numerous proteins including the AMPA-type glutamate receptor [42], [43], a small GTPase activating protein (SynGAP) [44], and a scaffold protein (DLG) in Drosophila [45]. Not only is there an increase in association, there is some evidence that there is also an increase in the synthesis of CaM-kinase II at the dendrite after tetanic stimulation [36].

In addition, other associating proteins localize different enzymes to distinct compartments. For example, A kinase anchoring protein (AKAP79) has been shown to localize PKA, phosphatase 2B (CaN), and protein kinase C (PKC) to the PSD [46]. For PKC, AKAP 79 also has the ability to inhibit its activity in a mixed competitive form. Therefore, this localization of PKC may keep it inactive until a further signal is produced. Besides AKAP's, there are other proteins that bind PKA. For example, there are several isoforms of a heat stable inhibitor protein (PKI) that may play a role in removing activated PKA from the nucleus [47]. Also, there are proteins known as RACKS for PKC that allows the active kinase to be in specific locations [48].

F. CaM-kinase II and Learning and Memory

CaM-kinase II is best characterized within the central nervous system. It constitutes approximately 1-2% of the total protein found in the hippocampus [49]. Within neurons, the kinase is further concentrated at synaptic sites. At excitatory synapses that utilize glutamate as a neurotransmitter, CaM-kinase II is moderately localized in the nerve terminal and highly localized within PSD's [32]. Pre-synaptically, it is thought to play key regulatory roles in both neurotransmitter biosynthesis and release. There is early evidence that it may directly phosphorylate and activate tyrosine hydroxylase, thereby increasing catecholamine synthesis [50]. Furthermore, it has been shown to phosphorylate synapsin I, which may increase the amount of available vesicles at the active zone of the nerve terminal [51].

There is evidence that CaM-kinase II may have a direct role in modulating synaptic responses within the hippocampus. It has previously been hypothesized that the hippocampus is required for the formation of new memories. Removal of this region disrupts the ability to learn new facts and faces in humans and disrupts the ability of rats to map new environments [52], [53]. A model has been proposed for this initial memory storage by the synaptic change known as long-term potentiation (LTP) ([54] review). LTP is thought to reflect a use-dependent strengthening of the synaptic connection both pre- and post-synaptically. Although this phenomenon has been observed in many areas of the mammalian nervous system, different

mechanisms have been observed. Briefly, it has been shown in hippocampal slice preparations that tetanic stimulation or theta burst stimulation in the afferent axons of the Schaffer collateral pathway will enhance the response of the post-synaptic pyramidal neurons in the CA1 region (fig. 3). This enhancement occurs at fast excitatory synapses that utilize glutamate as the neurotransmitter.

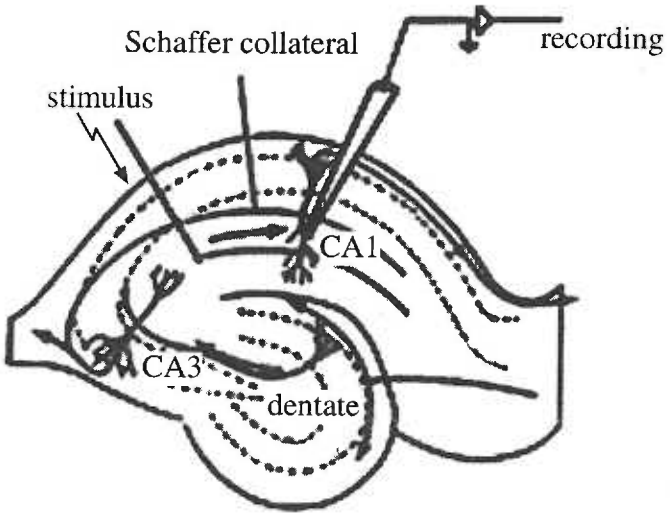

Figure 3. Diagrammatic representation of a hippocampal transverse slice. Arrows mark the orientation of the connections. Pyramidal CA3 neurons send their axons to the pyramidal neurons of the CA1 region along the schaffer collateral pathway. A stimulating electrode is usually placed within the schaffer collateral and a recording pipette within the dendritic arbors of the CA1 region. Adapted from Ouyang, *et al.* [55].

LTP appears to require at least two types of glutamate-gated ionotropic receptors, NMDA-Rs and AMPA-Rs ([54] review). Rapid excitatory synaptic transmission is mediated largely by the AMPA-R because the NMDA-R is subject to a voltage-dependent block by Mg^{2+}. The strong stimuli used for LTP induction produces sufficient postsynaptic depolarization through the AMPA-Rs to remove the blockage of the NMDA-Rs. Whereas the AMPA-Rs are impermeable to Ca^{2+}, the NMDA-Rs are permeable, and their activation with resultant Ca^{2+} influx is essential to LTP induction. For example, postsynaptic chelation of Ca^{2+} prevents LTP induction [56]. Whereas NMDA-Rs are essential for LTP induction, the potentiated postsynaptic current during LTP expression is predominantly due to AMPA-Rs. A central question is the mechanism of AMPA-R potentiation.

A molecular model for LTP has been proposed involving CaM-kinase II [57]. It is hypothesized that the influx of Ca^{2+} through the NMDA-Rs results in activation of CaM-kinase II with autophosphorylation of Thr^{286} and generation of Ca^{2+} independent activity. This would allow a transient elevation of Ca^{2+} in the dendritic spine to be transduced into a prolonged readout through CaM-kinase II. Furthermore, since CaM-kinase II is oligomeric and autophosphorylation is intersubunit and requires binding of Ca^{2+}/CaM to adjacent subunits [28], generation of Ca^{2+} independent activity may depend on synaptic frequency and strength. Indeed, a recent study has shown that CaM-kinase II activation is sensitive to the frequency of

Ca^{2+} oscillations [31]. These two properties, sensitivity to synaptic frequency and strength and prolonging a transient Ca^{2+} elevation, make CaM-kinase II a very attractive candidate for mediating LTP.

Evidence for CaM-kinase II's role in LTP has been shown in both tissue and cell preparations. First, LTP in hippocampal slices can be blocked by inhibiting post-synaptic elevation of intracellular calcium ([6] review) or by microinjecting inhibitors of CaM-kinases into the pyramidal cell body in the CA1 region [58]. Also, increases in CaM-kinase II activity within the CA1 pyramidal neurons, either by microinjection or vaccinia infection of a constitutively active form of CaM-kinase II, will enhance synaptic transmission [59], [60]. This enhancement by CaM-kinase II cannot be further potentiated by tetanic stimulation, suggesting that the effect utilizes similar mechanisms to LTP. Furthermore, slices stimulated with theta bursts have a CaM-kinase II dependent phosphorylation of the AMPA type glutamate receptor [42].

Second, in transfection studies in both HEK 293 cells and microinjections in *Xenopus* oocytes, activated CaM-kinase II can increase the AMPA and kainate currents [61], [42]. This increase in the current has been correlated with a phosphorylation site on an AMPA channel subunit, GluR1. Phosphorylation at Ser831 recruits a new high-conductance-state of the AMPA channel [62]. Also, recordings from primary hippocampal cultured neurons with activated CaM-kinase II within the recording pipette showed an increase in the AMPA current [63].

There are also *in vivo* transgenic data that supports the role of CaM-kinase II in LTP and learning and memory. A CaM-kinase IIα knockout mutant mouse was generated in 1992 by Silva, *et al* [64]. This appeared to be a nonlethal mutation and did not affect long term survival. In fact, these mice showed very little abnormalities including neuroanatomical structures. In contrast, these mutant mice did show a deficit in LTP in comparison to wild-type mice. Furthermore, these animals also showed an impairment in spatial learning as tested by the Morris water maze. Briefly, this is a behavioral task whereby a hidden platform is placed in a fluid tank and the animals are trained to find the platform. Later they are tested for how quickly they are able to find the platform [53].

Using gene targeting techniques, a transgenic mouse was also created that substituted a Thr^{286} to Ala mutant for CaM-kinase IIα [65]. These mutants showed a 50% decrease in the amount of Ca^{2+} independent activity, but no effect on total activity. Again, these mice showed a loss in NMDA mediated LTP and also a deficiency in spatial learning.

Therefore, it is proposed that a transient Ca^{2+} signal through NMDA receptors may activate CaM-kinase II which can then autophosphorylate and become Ca^{2+} independent. This independent activity is then able to phosphorylate the AMPA channels for a sustained period after the initial activation, thereby enhancing the

channel current [66]. This enhancement of the response is the initial plastic response of the neurons to change the characteristics of the connections.

G. CaM-kinase II and excitotoxicity

Certain regions of the brain, such as the hippocampus have been shown to be particularly vulnerable to delayed neuronal cell death due to ischemia [67]. Ischemia produces an excess amount of glutamate released leading to a large increase in intracellular Ca^{2+}. It has been shown that antagonists that prevent Ca^{2+} influx through NMDA receptors have a neuroprotective effect [68]. There are two main processes that may explain the Ca^{2+}-mediated cytotoxic effects. The first is the calcium activated proteases such as calpain I [69]. The loss of cytoskeletal integrity as seen by the degradation of spectrin, MAP2 and MAP1A is attributed to this protease activity. The second is the Ca^{2+} activated kinases. PKC and CaM-kinase II are both thought to play a role in excitotoxicity. Inhibitors to PKC have been shown to be neuroprotective from ischemia *in vivo* [70]. Furthermore, PKC activity has been shown to decrease during ischemic paradigms [71].

It was shown in early experiments that CaM-kinase II activity decreases during ischemia [72]. This decrease in activity is both an early (within 10sec) and long lasting phenomenon [73]. Two possibilities as to the decrease in activity are the

degradation of the protein and an inhibition of activity. There is a dramatic loss of CaM-kinase II activity within the cytosol early in ischemia [74]. Furthermore, there is a loss of CaM-kinase II immunoreactivity within the hippocampus that precedes the death of the neurons [75]. One possibility for the loss of immunoreactivity is a degradation of CaM-kinase II. *In vitro*, CaM-kinase II is a substrate for calpain which first generates a 30kD fully active fragment that can further become degraded to inactive fragments [76]. The other possibility is a posttranslational modification occurring near the ATP binding domain. There may have been a modification of the enzyme at the recognition site of the monoclonal antibody. In fact, there are studies that suggest that there is decrease in activity before there is an actual loss of protein [73]. This is not thought to be an autophosphorylation event or binding of a soluble inhibitor [73].

Although there is no direct evidence as to the role of this loss of activity, CaM-kinase II is known to be able to phosphorylate many substrates that are important for cell homeostasis. Proteins such as synapsin I, tyrosine hydroxylase, MAP2, cyclic nucleotide phosphodiesterase, and inositol triphosphate receptors that are important for transmitter systems, cytoskeletal structure, and transport mechanism could be adversely affected by CaM-kinase II activity. Furthermore, inhibition of CaM-kinases with KN-62, appears to attenuate cell death induced by NMDA toxicity or hypoxia induced neuronal injury in rat cortical cultures [77]. In contrary, the CaM-

kinase IIα knockout mice have an increase in infarct volume as compared to wild-type litter mates after cerebral ischemia [78]. These knockout mice still produce the beta isoform of CaM-kinase II within the brain. This may suggest that localization and activity of CaM-kinase II may play specific roles in the response to hypoxia.

H. Hypothesis

With the current understanding of the crucial role of CaM-kinase II in multiple neuronal functions, the existence of additional anchoring and/or other regulatory proteins for CaM-kinase II was hypothesized. Briefly, the yeast two-hybrid approach was performed using an inactive mutant form of the catalytic domain of CaM-kinase II to screen a rat brain cDNA library. It was believed that this mutant should be able to bind to proteins but would not phosphorylate them. Therefore, this bait could have the potential for identifying *in vivo* substrates as well as other interacting proteins. The next few chapters will show that this screen identified two small molecular weight novel proteins (CaM-KIINα, and CaM-KIINβ) that bind to CaM-kinase II and inhibit its kinase activity. Furthermore, the protein is located in areas of the brain where CaM-kinase II is present. A fusion construct was developed using the inhibitory domain of the CaM-KIIN and the third alpha helix of antennapedia homeodomain. This construct has the ability to enter cells and possibly

inhibit CaM-kinase II activity. Therefore, this protein will be a useful probe to help identify new physiological roles of CaM-kinase II. For example, chapter IV describes the use of CaM-KIIN to identify CaM-kinase II's role in the goldfish Mauthner cell.

CHAPTER II

RESULTS

CHARACTERIZATION OF A CALMODULIN-KINASE II INHIBITOR PROTEIN IN BRAIN

Bill H. Chang, Sucheta Mukherji, and Thomas R. Soderling

Published in *Proceedings in the National Academy of Sciences, USA*

Data Deposition: The nucleotide sequence reported in this manuscript has been submitted to the GenBank™ /EMBL Data Bank with accession number AFO41854.

A. Abstract

Ca^{2+}/calmodulin-dependent protein kinase II (CaM-KII) regulates numerous physiological functions including neuronal synaptic plasticity through the phosphorylation of AMPA-type glutamate receptors (AMPA-Rs). To identify proteins that may interact with and modulate CaM-KII function, a yeast two-hybrid screen was performed using a rat brain cDNA library. This screen identified a unique clone of 1.4 kb which encoded a 79 amino acid brain-specific protein that bound the catalytic domain of CaM-KII α and β and potently inhibited kinase activity with an IC_{50} of 50 nM. The inhibitory protein (CaM-KIIN), and a 28 residue peptide derived from it (CaM-KIINtide), was highly selective for inhibition of CaM-KII with little effect on CaM-KI, CaM-KIV, CaM-KK, PKA or PKC. CaM-KIIN interacted only with activated CaM-KII (i.e., in the presence of Ca^{2+}/CaM or after autophosphorylation) using GST/CaM-KIIN precipitations as well as co-immunoprecipitations from rat brain extracts or from HEK293 cells co-transfected with both constructs. Co-localization of CaM-KIIN with activated CaM-KII was demonstrated in COS-7 cells transfected with green fluorescent protein fused to CaM-KIIN. In COS-7 cells phosphorylation of transfected AMPA-R by CaM-KII, but not by PKC, was blocked upon co-transfection with CaM-KIIN. These results

characterize a novel, potent and specific cellular inhibitor of CaM-KII that may have an important role in the physiological regulation of this key protein kinase.

B. Introduction

Calmodulin-dependent protein kinase II (CaM-KII) is a widely-distributed protein kinase that is particularly abundant in neuronal tissues where it can constitute up to 1-2% of total protein [6]. *In vitro* it can phosphorylate up to 40 proteins including enzymes, ion channels, transcription factors, etc., and a number of these proteins appear to be physiological substrates. For example, CaM-KII is highly concentrated in the postsynaptic density of glutamatergic synapses where it phosphorylates and potentiates current through the AMPA-type glutamate receptor ion channel (AMPA-Rs) [42], [60], [59]. This phosphorylation of AMPA-Rs occurs upon induction of long-term potentiation (LTP), a model of cellular learning and memory, in region CA1 of hippocampus and is thought to contribute to the postsynaptic current potentiation [60].

Oligomeric CaM-KII is comprised of multiple subunits each composed of an NH_2-terminal catalytic domain, a central regulatory motif that includes an autoinhibitory (AID) and overlapping CaM-binding elements, and a COOH-terminal region involved in subunit assembly [6], [21]. The kinase is maintained in an inactive

form due to interaction of the AID with the catalytic domain. Binding of Ca^{2+}/CaM conformationally disrupts the AID, allowing access of the catalytic domain to substrates. CaM-KII has the unusual property that upon activation by Ca^{2+}/CaM it exhibits rapid intersubunit autophoshorylation on Thr^{286}, generating constitutive kinase activity [29], [28]. This constitutive activity prolongs the kinase function beyond the transient elevations of intracellular Ca^{2+}, an essential characteristic for forms of synaptic plasticity such as LTP. A recent study shows that CaM-KII can also act as a sensor to decode the frequency of Ca^{2+} spikes [31].

It is likely that CaM-KII may interact with proteins, other than Ca^{2+}/CaM, which can either anchor it to subcellular organelles or directly regulate its activity. For example, a 190 kDa protein may localize activated CaM-KII to the PSD[38], [40]. In an attempt to identify proteins that interact with CaM-KII we used the yeast two-hybrid screen with the COOH-terminus or the catalytic domain of CaM-KII as bait. This protocol identified a specific and potent cellular inhibitor protein for CaM-KII in brain. Inhibitory proteins that exert critical physiological roles are known for several protein kinases such as PKA [79], the cyclin-dependent kinases [80], [81] and the MAP-kinase JNK [82].

C. Experimental Methods

Yeast 2-hybrid Screen. The screen was performed as described [83]. The DNA sequence (1-830 bp) corresponding to the catalytic domain (amino acids 1-269) of the inactive β CaM-KII mutant (K43A) was inserted into the pBTM116 vector in frame with the LexA-DNA binding domain. The COOH-terminal bait was constructed using the sequence (975-1650 bp) encoding the regulatory and association domains (amino acids 282-478) of the α subunit into the pBTM116 vector. The rat brain matchmaker cDNA library (Clontech) was constructed in a pGAD10, GAL4 activation domain vector. Interaction with the bait protein was monitored by prototrophy for histidine and/or β-galatosidase activity.

Northern Analysis. cDNA insert was isolated from the pGAD10 vector and labeled with [α-^{32}P]dCTP by random priming. Total RNA from rat tissues was isolated with Trizol (Gibco-BRL), and poly A RNA was further purified using Oligotex mRNA mini kit (QIAGEN). Either 2μg of poly A RNA or 20μg of total RNA were subjected to electrophoresis on a formaldehyde-containing 1.2% agarose gel and then blotted onto Hybond-N (Amersham). The blot was hybridized in Rapid-H4B hybridization buffer (Amersham) with the radioprobe at 65° C for 2 hr and was washed once in 2xSSC, 0.1% SDS at 25 °C for 15 min. and then twice in 1xSSC, 0.1% SDS at 65 ° C for 20 min.

Western blot analysis. An adult male rat was euthanized and tissues were harvested. The brain was quickly removed and different regions were dissected. The samples were homogenized in lysis buffer containing 10mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.2 mM orthovanadate, 1% Triton X-100, 0.5% NP-40, 19 μg/ml leupeptin, 10 μg/ml pepstatin, 1 mM benzamidine, and 1mM PMSF. Approximately 50 μg of lysate was then electrophoresed on a 15% SDS PAGE. The gel was transferred onto Immobilon and blotted with the antibody. For preadsorption, 100 μl of a 100 μM solution of the peptide CaM-KIINtide was used to preincubate the antibody for 2hr at 4 °C before addition to the membrane.

GST-precipitations. Samples were incubated with purified recombinant GST/CaM-KIIN and glutathione-Sepharose in the appropriate buffer for 1hr at 4°C, washed extensively and eluted with 10mM glutathione. The eluant was separated by SDS-PAGE and either stained with Coomassie blue or immunoblotted.

Immunoprecipitations. For co-precipitation studies, samples were harvested in lysis buffer with the addition of 5μg/ml calpain inhibitor 1. They were then incubated with either a monoclonal CaM-KIIα antibody (Zymed) or a polyclonal CaM-KII antibody with or without 3mM $CaCl_2$ overnight at 4°C. Immunoprecipitates were washed extensively and eluted with SDS loading buffer, separated by SDS-PAGE and

immunoblotted. Immunoprecipitation of GluR1 was performed as described [42] using Ab from Robert Wenthold (NIH).

Transient transfections. Either HEK 293 or COS-7 cells were transiently transfected using lipofectAMINE (Gibco-BRL protocol). Briefly, either 10μg (for HEK 293) of plasmid per 42μl lipofectAMINE or 1μg (for COS-7) of plasmid per 10μl of lipofectAMINE were transfected into 100mm or 35mm dishes, respectively. Cells were grown for 1-2 days and subsequently harvested.

Immunofluorescence. COS-7 cells were transiently transfected, grown for one day and fixed with 4% paraformaldehyde, 4% sucrose in PBS. Cells, were then permeabilized with 0.025% Triton X-100 in PBS and stained with a phospho-T^{286} specific antibody to CaM-KII (Promega) and visualized with a Leitz DMRB scope.

Kinase Assays. Standard CaM-KII assay conditions contained 0.8 mM $CaCl_2$, 2 μM CaM, 1 mM DTT, 50 mM HEPES, pH 7.5, 10 mM $Mg(OAc)_2$, and 0.2 mM [γ-^{32}P]ATP (1000-2000 cpm/pmol) in 20 to 25 μl reactions. The assays were initiated by adding enzyme diluted in buffer (50 mM HEPES, pH 7.5, 2 mg/ml bovine serum albumin, and 10% ethylene glycol) to the reaction. ^{32}P-incorporation was determined by spotting 10 to 15 μl of the reaction to Whatman P-81 phosphocellulose paper and subsequently washing in 75 mM phosphoric acid as described [84]. For PKC phosphorylations in Fig. 3A, PKC (gift of Dr. John Scott) was activated using 140

μM phosphatidylserine, 3.8 μM diacylglycerol and 0.3 mM $CaCl_2$. PKA catalytic subunit was generously provided by Dr. Richard Maurer.

Orthophosphate labeling. HEK 293 cells were transiently transfected with vectors expressing GluR1 and CaM-KII (His282Arg mutant) and ^{32}P labeled as described [42]. PKC was activated by a 20 min. treatment with 100μM TPA before harvesting.

D. Results and Discussion

To identify proteins that interact with CaM-KII, a yeast two-hybrid screen was performed on a rat brain cDNA library. With the regulatory/association domains of α CaM-KII (residues 282-478) as bait, 7 positive clones were identified. Five of these clones corresponded to the COOH-termini of the various CaM-KII isoforms (data not shown), consistent with the known role of the COOH-terminus as the subunit interaction domain [41]. The remaining 2 clones corresponded to other portions of CaM-KII that might interact with the autoinhibitory domain. We next used as bait the catalytic domain of βCaM-KII (residues 1-269) with Lys43 mutated to Ala to generate an inactive kinase [29]. This was done since CaM-kinase II can stimulate transcription, and, by using an inactive mutant, we might stabilize interactions with unknown substrates. Sequence analyses showed that several positive clones corresponded to an insert size of 1.4 kb (Fig. 1A). This sequence has an initiation

ATG that is consistent with a Kozak consensus sequence [85], a TAG stop codon and a 3' noncoding sequence including a poly (A) tail. The open reading frame of 237 nucleotides encoded a protein of 79 amino acids with a calculated molecular mass of about 8 kD. This interacting protein appeared to be unique as no sequence homology was detected with known proteins using the BLAST program. We have named this protein CaM-KIIN since, as shown below, it is a potent and specific CaM-KII INhibitor.

Tissue distribution of CaM-KIIN was assessed by performing a Northern analysis using a radiolabeled probe. Screening both polyA RNA and total RNA showed that the message was expressed in rat forebrain, hippocampus, midbrain, cerebellum and testis but not in several other tissues examined (Fig. 1B). To compare protein expression with message levels, a polyclonal antibody was generated in rabbits to a COOH-terminal portion of CaM-KIIN (Fig. 1A, underlined sequence). Western blots of different brain regions showed a specific immunoreactive band at approximately 7 kD and a less reactive protein at 18-19 kD, both of which were absent using Ab preadsorbed with antigen, as well as a non-specific band at 50 kD (Fig. 1C). When the CaM-KIIN Ab was used for immunoprecipitation from a brain extract, a Western of the immunoprecipitate showed strong bands at 7 and 19 kDa (not shown), suggesting that the 19 kD band may be another isoform of the inhibitor. Expression of the CaM-KIIN cDNA in COS-7 cells also identified a 7 kD

immunoreactive protein which co-migrated with the 7 kD immunoreactive protein in brain extracts (Fig. 1C, right panel). Although the message for the clone is found within testis, CaM-KIIN protein was not detectable in this tissue. These results indicate an expression of CaM-KIIN that is much more restricted than CaM-KII. Although CaM-KII is 20- to 50-fold higher in brain, it can be detected in most other tissues [6]. The restricted expression of CaM-KIIN suggests it has a unique function in brain.

We next tested whether CaM-KIIN could interact with CaM-KII both *in vitro* and in cells. CaM-KIIN was expressed as a bacterial GST-fusion construct and purified using glutathione-Sepharose. When the GST/CaM-KIIN was incubated with purified recombinant α CaM-KII [25] and then isolated by glutathione-Sepharose, CaM-KII was co-purified only if it had been preactivated in the presence of Ca^{2+}/CaM (Fig. 2A, panel 1). After formation of the complex, subsequent removal of Ca^{2+}/CaM by the addition of excess EGTA reversed the interaction between CaM-KII and CaM-KIIN (Fig. 2A, panel 2). This suggests that the interaction required a portion of the kinase catalytic domain that was masked in the absence of Ca^{2+}/CaM. There may be little isoform specificity for the interaction since the catalytic domain of the β subunit was used as bait, but CaM-KIIN can also interact with the α subunit. However, there is specificity for the CaM-KII family because the GST/CaM-KIIN did not bind to CaM-KIV (60 kD) in the absence or presence of Ca^{2+}/CaM (Fig. 2A, panel 1). This is

significant since the catalytic domains of CaM-KII and CaM-KIV exhibit 45% identity in amino acid sequence [86] and have considerable overlap in substrate specificities. To determine whether CaM-KIIN could interact with endogenous CaM-KII, GST/CaM-KIIN was incubated with a soluble rat brain extract in the absence or presence of Ca^{2+}/CaM. Interacting proteins were isolated by glutathione-Sepharose, washed extensively and separated by SDS-PAGE. Coomassie staining identified a major protein at 50 kD and some fainter bands at about 60 kD that interacted with CaM-KIIN only in the presence of Ca^{2+}/CaM (Fig. 2A, panel 3). These proteins were reactive to polyclonal CaM-KII antibody, confirming that they represent the α and βisoforms (Fig. 2A, panel 4). The absence of other major bands indicates the specificity of this interaction for CaM-KII.

Interaction between endogenous CaM-KII and endogenous CaM-KIIN was investigated using co-immunoprecipitation experiments. Soluble rat brain extract was incubated with a monoclonal CaM-KIIα Ab in the presence or absence of 3mM Ca^{2+}, and the immune complex was separated by SDS-PAGE and immunoblotted with the CaM-KIIN Ab. Only in the presence of Ca^{2+} was the 7 kD CaM-KIIN co-precipitated along with the 19 kD protein (Fig. 2B) that was also seen in the Western blot of forebrain in Fig. 1C. Both of these bands were eliminated with preadsorption of the CaM-KIIN Ab using the antigenic peptide (Fig. 2B, right panel). This 19 kD protein suggests the existence of another isoform of CaM-KIIN, similar to the multiple

isoforms of PKI [47]. Interaction between CaM-KIIN and CaM-KII was further demonstrated in HEK 293 cells transfected with cDNAs for CaM-KIIN and/or for the constitutively active CaM-KII (His282Arg) mutant [22]. Using a polyclonal CaM-KII antibody, there was only co-precipitation of CaM-KIIN from the cell extracts when CaM-KII was co-expressed (Fig. 2C).

Although the previous experiments demonstrate that CaM-KII and CaM-KIIN interact *in vitro*, they do not document their interaction within the cell. This question was investigated using transient transfection experiments in COS-7 cells. CaM-KIIN was fused to green fluorescent protein (Clontech) (GFP-KIIN) and transiently transfected either with wild-type CaM-KII or the constitutively-active His282Arg mutant. Thr^{286} autophosphorylation is indicative of activated CaM-KII [6], and this species was detected using a phospho-T^{286} specific Ab (Fig. 2D). GFP alone (not shown) or co-expressed with active (H292R mutant) CaM-KII (Fig. 2D, panel 4) was localized in both the nucleus and cytosol. GFP-KIIN when expressed alone or with wild-type CaM-KII was also distributed in both the nucleus and cytosol (Fig. 2D, panels 1 and 2). However, when co-expressed with activated CaM-KII (i.e., the His282Arg mutant), GFP-KIIN was excluded from the nucleus (Fig. 2D, compare panels 2 and 3). Since CaM-KIIα is found predominantly in the cytosol [87], this result indicates that the GFP-KIIN bound to the activated CaM-KII in the cytosol and was sequestered from freely diffusing into the nucleus. However, we cannot exclude

the possibility that activated CaM-KII phosphorylated some protein that altered the localization of CaM-KIIN.

What is the functional relevance of the interaction between CaM-KII and CaM-KIIN? *In vitro* kinase assays were performed to test whether CaM-KIIN was a substrate for CaM-KII. A phosphorylation time course using 20 nM CaM-KII showed no significant ^{32}P-incorporation into 4 μM CaM-KIIN after 45 min. at 30 °C (data not shown). In these experiments ^{32}P-autophosphorylation of CaM-KII was observed, but it was significantly suppressed by the presence of CaM-KIIN. This result suggested that CaM-KIIN might be an inhibitor of CaM-KII activity, so a concentration response curve was performed (Fig. 3A). GST/CaM-KIIN exhibited an apparent IC_{50} of 50 nM toward CaM-KII using its peptide substrate Syntide 2 (Fig. 3A) whereas GST alone gave no inhibition up to concentrations of 10μM (not shown). Both the Ca^{2+} independent as well as total activities of autophosphorylated CaM-KII were inhibited (Fig. 3A), and an identical inhibition was obtained for CaM-KII not previously autophosphorylated. COOH-terminal truncations of CaM-KIIN indicated that its inhibitory potency resided largely in 28 residues near its COOH-terminus (data not shown), so a peptide was synthesized corresponding to this sequence (Fig. 1A, underlined sequence). This peptide, CaM-KIINtide (KRPPKLGQIGRSKRVVIEDDRIDDVLK), had a similar IC_{50} of 50 nM for both the total and the Ca^{2+} independent activities of CaM-KII (Fig. 3A). Inhibition of kinase

autophosphorylation was further investigated using 1 or 10 μM of CaM-KIINtide and detecting CaM-KII autophosphorylation by either ^{32}P-incorporation or with an antibody specific for autophosphorylation of Thr^{286} (Fig. 3B). Reactions were performed at 5 °C in an effort to limit autophosphorylation to Thr^{286}. Although there was a dose-dependent inhibition of autophosphorylation, it was not as potent as inhibition of substrate phosphorylation. This higher inhibitory potency towards phosphorylation of an exogenous substrate versus CaM-KII autophosphorylation on Thr^{286} (Fig. 3B) is not surprising since the latter is an intramolecular, intersubunit reaction within the oligomeric protein [28], [29]. Preliminary results suggest that inhibition of CaM-KII by CaM-KIINtide was not competitive towards peptide substrate (data not shown).

Is CaM-KIIN a specific inhibitor of CaM-KII as suggested by the fact that GST/CaM-KIIN did not interact with CaM-KIV (Fig. 2A)? Concentrations of 1 or 10 μM of CaM-KIINtide gave no significant inhibition of PKC, PKA, and CaM-KI and only 30% inhibition of CaM-KIV at 10 μM (Fig. 3A). At 10 μM, GST/CaM-KIIN did not inhibit CaM-KIV (not shown), suggesting that the peptide may have lost some specificity relative to the full-length protein. Furthermore, 10μM of the peptide had no effect on the activation of either CaM-KI and CaM-KIV by CaM-KK (data not shown), indicating no inhibition of CaM-KK. This high specificity of CaM-KIIN and CaM-KIINtide will make it a very useful probe for identifying specific CaM-KII

reactions. One of the major limitations of previous peptide inhibitors of CaM-KII, based on its autoinhibitory domain sequence, is their lack of specificity at higher concentrations [88], [89]. Therefore, CaM-KIINtide was tested for inhibition of CaM-KII activity in extracts of rat forebrain, goldfish brain, and Drosophila. For all three extracts CaM-KIINtide showed similar IC_{50} values ranging from 100nM to 400nM towards phosphorylation of syntide 2 (data not shown)

The *in vitro* data show that CaM-KIIN and CaM-KIINtide are inhibitors of CaM-KII autophosphorylation and activity towards an exogenous peptide substrate. We next investigated its inhibitory properties and specificity using a protein substrate *in vitro* and in transfected cells. It has been previously shown that the AMPA receptor subunit GluR1 ion channel is potentiated through phosphorylation by CaM-KII and by PKC on Ser^{831} in its intracellular COOH-terminus [42], [90], [43]. To determine whether CaM-KIINtide would inhibit the phosphorylation of a protein substrate, the GST-fusion of the COOH-terminus of GluR1 (residues 816-889) was used in *in vitro* kinase assays with either PKC or CaM-KII as catalysts. CaM-KIINtide (1μM) completely inhibited phosphorylation of $GST/GluR1_{816-889}$ by CaM-KII and most of CaM-KII autophosphorylation (Fig. 4A). PKC catalyzed weaker phosphorylation of $GST/GluR1_{816-889}$, and this was not blocked by CaM-KIINtide. Next, HEK 293 cells were transfected with GluR1 without or with co-transfection with CaM-KII (His282Arg mutant) and/or CaM-KIIN. Cells transfected without CaM-KII were

stimulated with phorbol esters to activate PKC as this has been shown to result in the phosphorylation of GluR1 [90], [43]. Cells were ^{32}P-labeled, harvested, immunoprecipitated with GluR1 Ab, and analyzed by SDS-PAGE/autoradiography. As reported previously, the low basal phosphorylation of GluR1 was strongly stimulated by co-expression of the constitutively-active CaM-KII [42], [90], [43], and this phosphorylation was blocked by expression of CaM-KIIN (Fig. 4B, panel 1). Furthermore, activation of PKC by 100μM TPA resulted in phosphorylation of GluR1 [90], [43], but this phosphorylation could not be inhibited by transfection of CaM-KIIN (Fig. 4B panel 2). These results document that CaM-KIIN can act as a specific inhibitor of CaM-KII within the cell.

A number of protein kinases have inhibitory proteins that exert important physiological regulation. For example, the specific inhibitor of PKA [79] has been very useful in identifying PKA-mediated reactions *in vitro* and in transfected cells, and this inhibitor appears to act physiologically to promote nuclear export of PKA [91, 92]. Likewise, the inhibitor proteins of the cyclin-dependent kinases are critical in cell cycle control [80], [81], and the cytosolic inhibitor of JNK functions to retain this kinase in the cytosol [82]. In this study we have identified a potent and specific inhibitor of CaM-KII and have demonstrated its ability to inhibit CaM-KII- but not PKC-mediated phosphorylations *in vitro* and in transfected cells. Further studies will be necessary to assess the physiological role of this inhibitor in CaM-KII actions in

brain. Since one can detect high CaM-KII activity in brain extracts in the presence of Ca^{2+}/CaM, conditions where CaM-KIIN interacts with and inhibits CaM-KII, it is obvious that the levels of expression of CaM-KIIN are much lower than those of CaM-KII. This implies that CaM-KIIN may physiologically regulate a specific pool of CaM-KII, and studies are in progress to examine this hypothesis. It is anticipated that CaM-KIIN and CaM-KIINtide will also be important molecular probes in identifying reactions mediated by CaM-KII. For example, we have shown that intradendritic injections of CaM-KIINtide prevented the induction of activity-dependent synaptic potentiations at mixed synapses on the goldfish Mauthner cell [93].

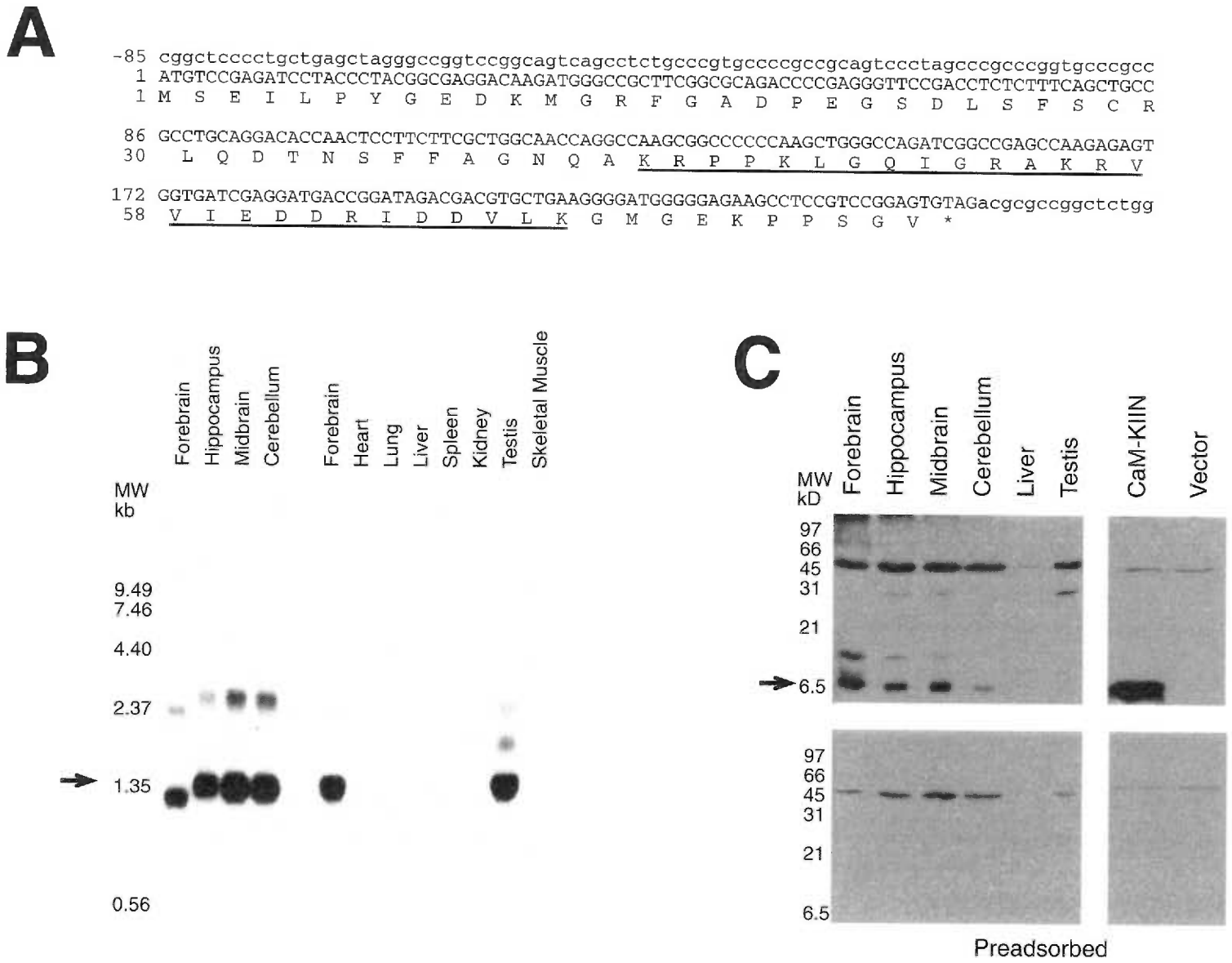
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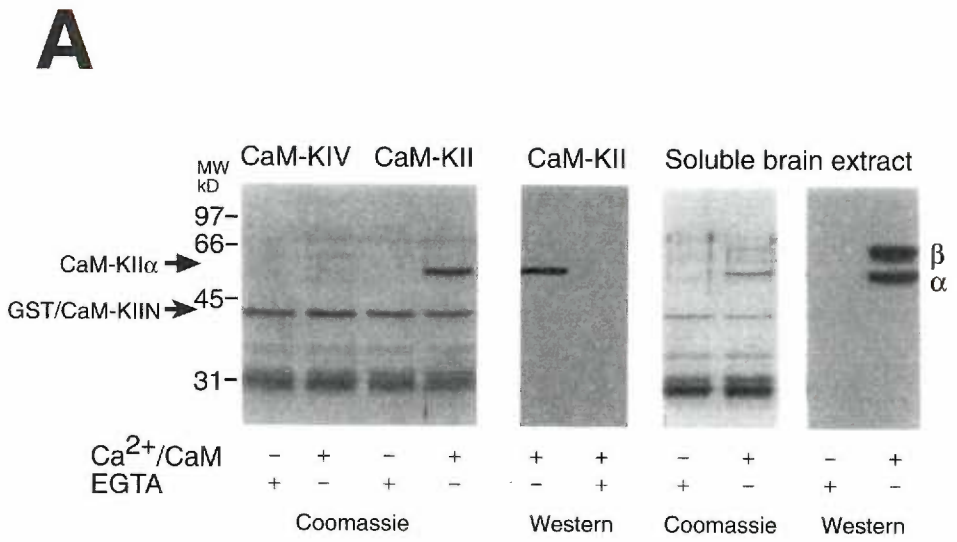
Figure 1. Cloning and expression of CaM-KIIN. (**A**) DNA sequence of CaM-KIIN and deduced amino acid sequence. The underlined region denotes the inhibitory domain. Only part of the 3' non-coding sequence is shown. (**B**) Tissue expression of CaM-KIIN message. A Northern blot was performed on polyA RNA (left panel) and on total RNA (right panel). The estimated size of the band is 1.4 kb (arrow). (**C**) Protein expression of CaM-KIIN. Western blot was performed using a polyclonal antibody produced to a peptide within the underlined region of CaM-KIIN. Left Top Panel: rat tissues were harvested, and 50 μg of total protein was run per lane. Right Top Panel: COS-7 cells were transiently transfected with CaM-KIIN or vector alone, harvested and 20 μg of soluble protein was run per lane. Bottom Panels: same as top panels except the antibody was preadsorbed by preincubation with 100 μM of peptide antigen.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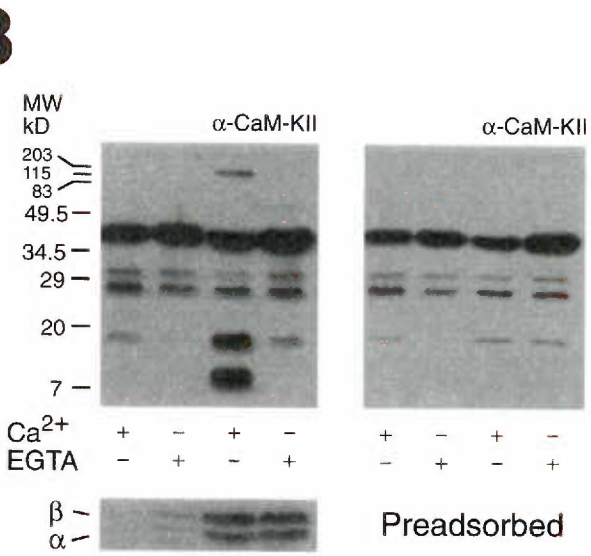
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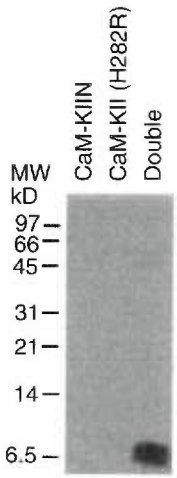
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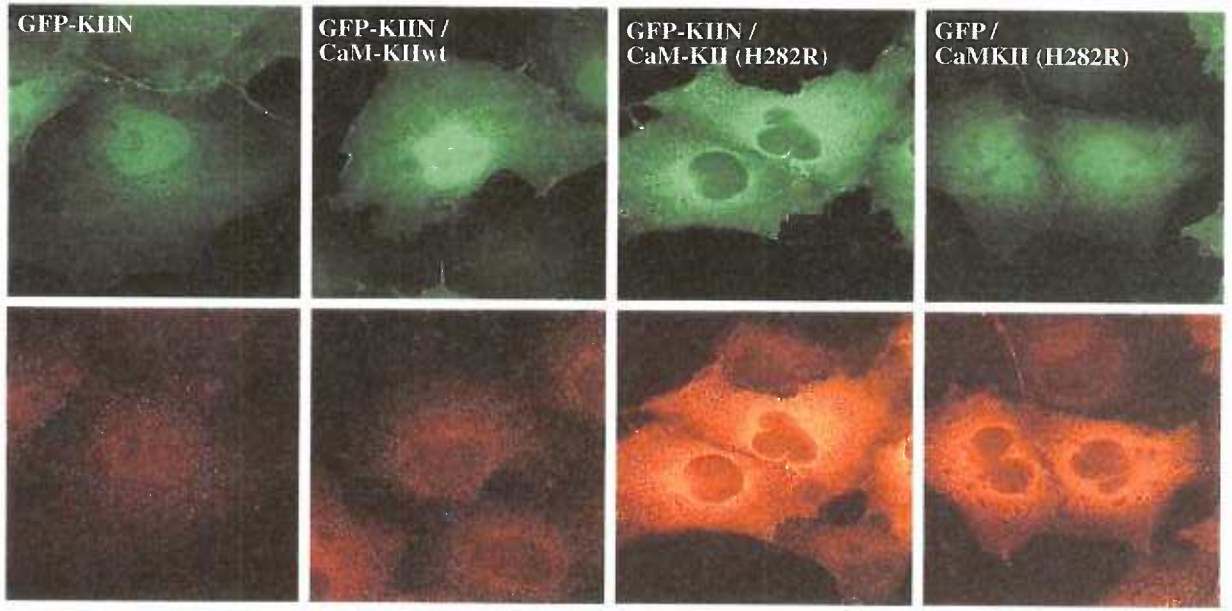
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Figure 2. Binding of CaM-KII to CaM-KIIN. (**A**) GST/CaM-KIIN precipitation of CaM-KII. Either recombinant CaM-KIV, CaM-KII, or soluble brain extract was incubated with GST/CaM-KIIN in the presence of Ca^{2+}/CaM or EGTA, precipitated with glutathione-Sepharose, washed and separated by SDS-PAGE. Samples were then visualized by Coomassie staining or by Western blot with anti-CaM-KII Ab. The positions of CaM-KII (upper arrow) and GST/CaM-KIIN (lower arrow) are indicated. The Western of the soluble brain extract precipitation (panel 4) visualized both the α and β subunits of CaM-KII. (**B**) Co-immunoprecipitation of CaM-KII and CaM-KIIN. Rat forebrain supernatant was split into 200μl aliquots and incubated with either protein A sepharose alone (first two lanes) or with a monoclonal CaM-KIIα Ab in the presence of 3mM $CaCl_2$ or 1mM EGTA. After centrifugation the immune complex was separated by SDS-PAGE and immunoblotted with the CaM-KIIN Ab (panel 1) or with preadsorption with the antigenic peptide (panel 2). The membrane was then immunoblotted with the polyclonal CaM-KII Ab showing both the α and β isoforms (lower panel). (**C**) Co-immunoprecipitation of CaM-KIIN with CaM-KII from transfected cells. HEK 293 cells were transiently transfected with CaM-KIIN, CaM-KII (His282Arg), or together and subsequently lysed. CaM-KII was immunoprecipitated from the soluble cell extract with 3 mM Ca^{2+} and separated by SDS-PAGE. The samples were then immunoblotted with anti-CaM-KIIN. (**D**) Colocalization of active CaM-KII and CaM-KIIN with transfected cells.

COS-7 cells were transiently transfected with the indicated combinations of green fluorescent protein (GFP), GFP/CaM-KIIN fusion protein (GFP-KIIN), wild-type CaM-KII or activated CaM-KII (H282R). Cells were visualized for GFP (top panels) or for activated CaM-KII using a Thr^{286} phosphospecific Ab (lower panels).

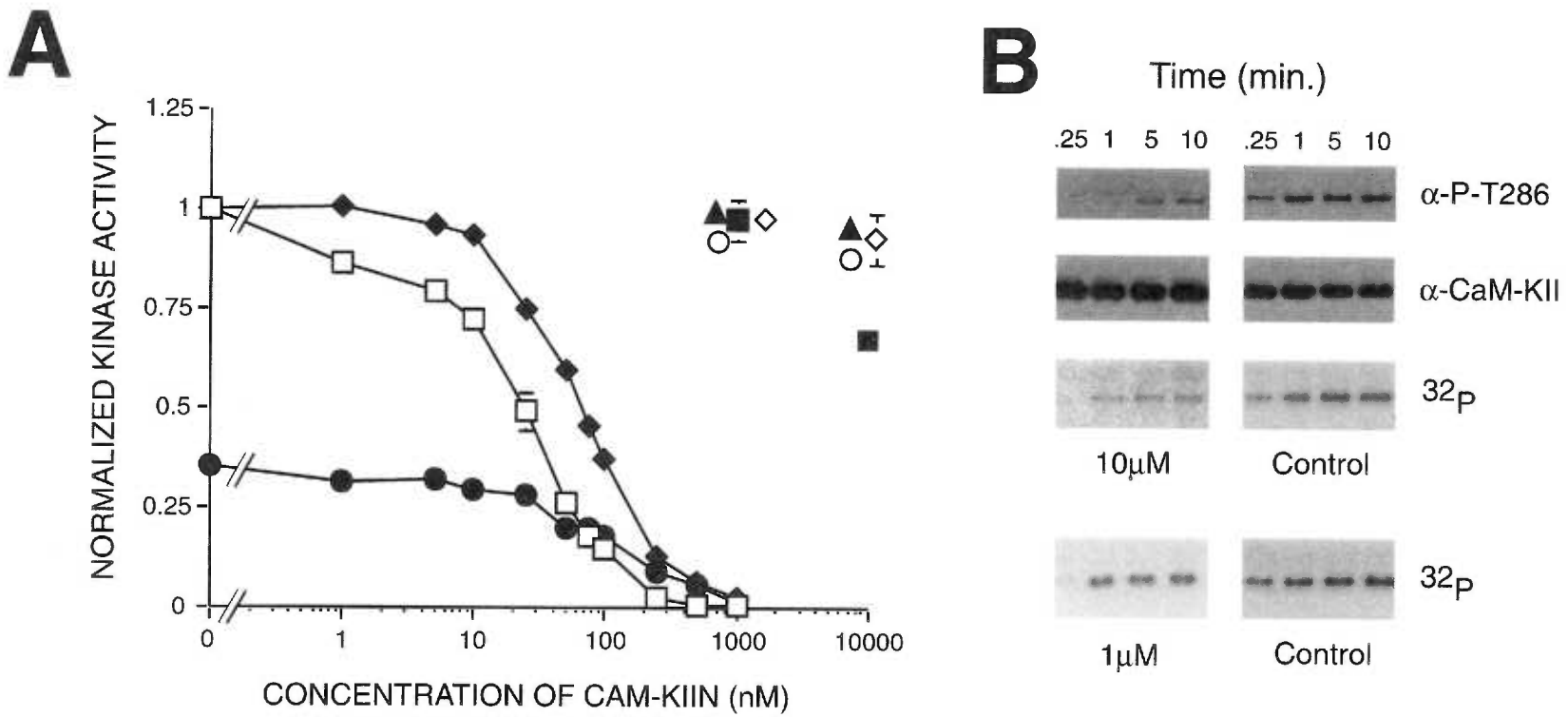

A
NORMALIZED KINASE ACTIVITY
1.25
1
0.75
0.5
0.25
0
0
1
10
100
1000
10000
CONCENTRATION OF CAM-KIIN (nM)
B
Time (min.)
.25 1 5 10
.25 1 5 10
α-P-T286
α-CaM-KII
32P
10μM
Control
32P
1μM
Control

Figure 3. Inhibition of CaM-KII by CaM-KIIN. (**A**) Concentration response curves of inhibition of kinase activity by CaM-KIIN and CaM-KIINtide. Autophosphorylated CaM-KII was assayed in the presence of Ca^{2+}/CaM and varying concentrations of GST/CaM-KIIN (filled diamonds) or CaM-KIINtide (open squares) for its ability to phosphorylate the peptide substrate Syntide 2. Ca^{2+} independent activity (normalized to total activity) was also assayed in the presence of EGTA and CaM-KIINtide (closed circles). Other kinases were assayed with their substrates using either 0, 1 or 10 μM CaM-KIINtide and normalized to 0 μM CaM-KIINtide: PKC (1 nM, solid triangles), EGF receptor Peptide (Sigma); PKA (100 nM, open circles), RII peptide; CaM-KI (20 nM, open diamond) and CaM-KIV (40 nM, filled square) were activated by CaM-KK and then assayed for their abilities to phosphorylate Syntide 2. (**B**) Effect of CaM-KIINtide on autophosphorylation of CaM-KII. Autophosphorylation reactions were performed at 5 °C with 0 (Control), 1 or 10μM CaM-KIINtide containing either cold ATP or [γ^{32}P]-ATP, stopped at the indicated times and blotted with either anti-phosphospecific Thr^{286} antibody (gift from Dr. L. Griffith, Brandeis Univ.) or polyclonal CaM-KII antibody, or visualized by ^{32}P incorporation, respectively.

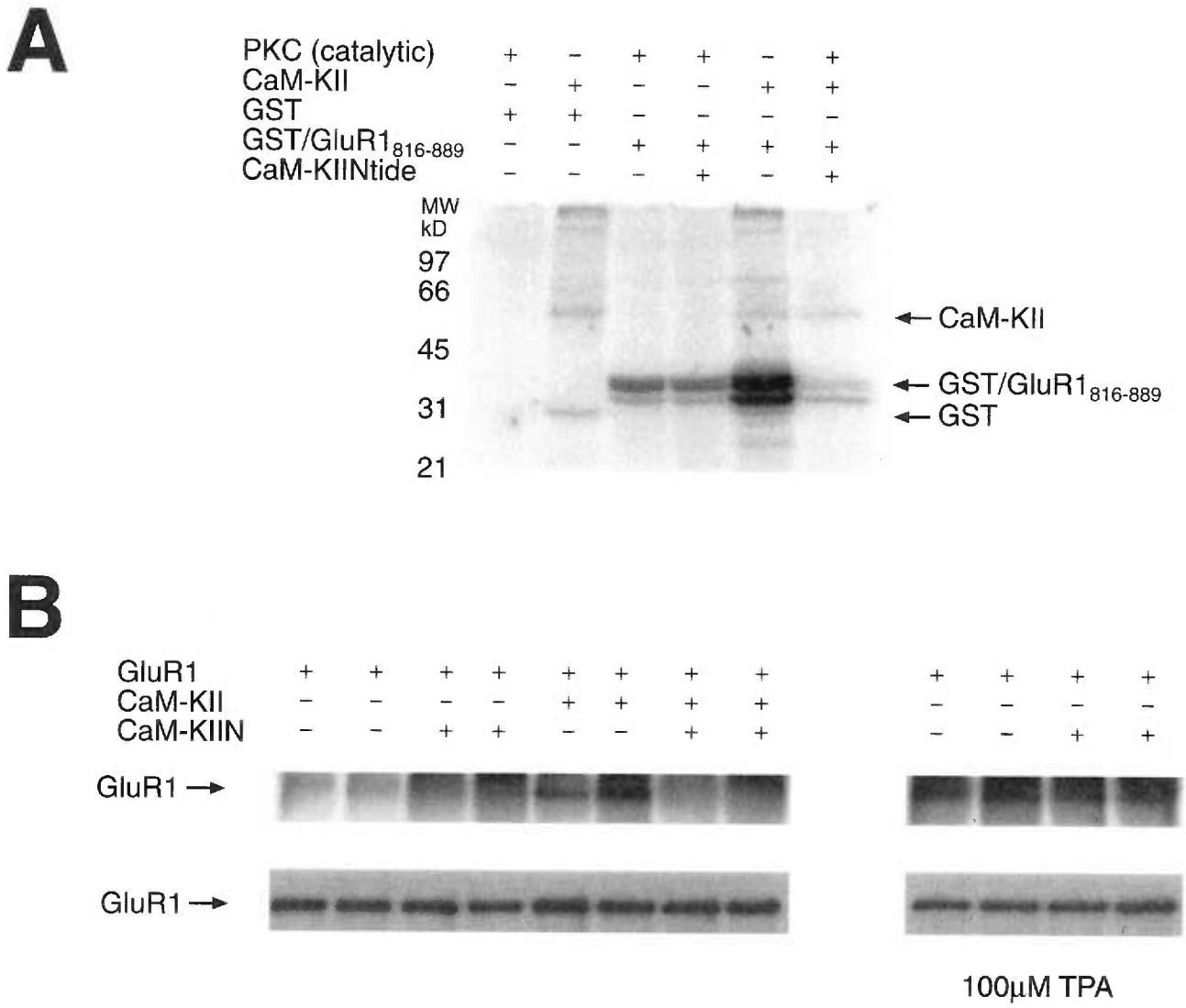

A
PKC (catalytic) + − + + − +
CaM-KII − + − − + +
GST + + − − − −
GST/GluR1816-889 − − + + + +
CaM-KIINtide − − − + − +
MW
kD
97
66
45
31
21
← CaM-KII
← GST/GluR1816-889
← GST
B
GluR1 + + + + + + + + + + + +
CaM-KII − − − − + + + + − − − −
CaM-KIIN − − + + − − + + − − + +
GluR1 →
GluR1 →
100μM TPA

Figure 4. Effect of CaM-KIIN on GluR1 phosphorylation by CaM-KII and PKC. (**A**) Kinase reactions, performed at 30 °C for 20 min. with the indicated mixtures of 0.2 mM [γ^{32}P] -ATP, 20 nM PKC catalytic fragment (PKM, Calbiochem) or 20nM CaM-KII, 4 μM GST or 4 μM GST/GluR1 $_{816\text{-}889}$ with or without 1μM CaM-KIINtide, were separated by SDS/PAGE and visualized by autoradiography. GST/GluR1$_{816\text{-}889}$ migrated as a doublet. (**B**) HEK 293 cells, transfected with GluR1 without or with co-transfected CaM-KII (H282R), were ^{32}P-labeled. Some cells without CaM-KII transfection were stimulated with TPA to activate PKC for 20 min. Cells were harvested, and GluR1 was immunoprecipitated, analyzed by SDS PAGE/autoradiography (upper panel) and subjected to GluR1 Western blot (lower panel).

CHAPTER III

RESULTS

CAM-KINASE II INHIBITOR PROTEIN: LOCALIZATION OF ISOFORMS IN RAT BRAIN AND CONSTRUCTION OF A CELL-PERMEABLE PROBE

Bill H. Chang, Sucheta Mukherji, Eric Guire and Thomas R. Soderling

A. Abstract

A second isoform of CaM-kinase II inhibitor protein (CaM-KIINα) has been identified using the yeast two-hybrid screen. The 1.8kb message encodes CaM-KIINα that is 65% identical in its putative open reading frame and 95% identical in its inhibitory domain to CaM-KIINβ. CaM-KIINα exhibits non-competitive inhibitory properties towards CaM-kinase II indistinguishable from CaM-KIINβ. The 26 amino acid inhibitory peptide has the ability to inhibit CaM-kinase II activity from multiple organisms including Drosophila and goldfish. Northern analysis of various tissues indicates that CaM-KIINα is specific to brain whereas CaM-KIINβ is also present in testis. *In situ* hybridization shows a general distribution of both isoforms in rat brain with stronger localization of CaM-KIINβ in cerebellum and hindbrain and CaM-KIINα in frontal cortex, hippocampus and inferior colliculus. An antibody that recognizes both isoforms shows a distribution of CaM-KIIN in rat brain that correlates with immunoreactivity of CaM-kinase II. In cultured mature hippocampal neurons, CaM-KIIN is present in cell bodies and dendrites but, unlike CaM-kinase II, does not display punctate straining at postsynaptic densities.

To facilitate the use of CaM-KIIN as a probe for identifying physiological functions of CaM-KII, we have engineered fusion constructs of Antennepedia peptide to CaM-

KIIN and its inhibitor peptide (CaM-KIINtide) that allow them entry into cells where they may inhibit CaM-kinase II.

B. Introduction

Calcium/calmodulin-dependent protein kinase II (CaM-kinase II) comprises a protein kinase family that phosphorylates a variety of substrates (for review see [94]). The holoenzyme is comprised of 10-12 subunits. Each subunit is approximately 50 to 60kD and contains an N-terminal catalytic domain, an autoinhibitory domain (AID) and an overlapping Ca^{2+}/calmodulin binding domain and a C-terminal subunit association domain. The AID interacts with the catalytic domain and maintains the kinase in an inactive conformation. Binding of Ca^{2+}/CaM adjacent to the AID disrupts its interaction with the catalytic domain and results in activation of the kinase. Upon activation by binding of Ca^{2+}/CaM, the kinase exhibits intersubunit autophosphorylation on Thr^{286} within the AID. When transient elevation of intracellular Ca^{2+} returns to basal values, the kinase remains in a partially active form (autonomous activity) because the autophosphorylation on Thr^{286} prevents the AID from completely suppressing catalytic activity.

Although the enzyme is found in a variety of organisms and tissue types, the α and βisoforms are highly enriched in the brain. CaM-kinase II comprises almost 0.3% of the total protein in rat brain [95], up to 2% of protein within the hippocampus

and 1.3% within the cortex [49]. The ratio between α and β isoforms differs from region to region within the brain. The concentration of the α isoform is higher within the cortex, while the β isoform is higher in the cerebellum [96].

CaM-kinase II is found throughout neurons including the cell body, dendrites, axons, pre-synaptic boutons, and post-synaptic densities within dendritic spines. In the presynaptic terminus CaM-kinase II phosphorylates synapsins to enhance transmitter release [51]. At the postsynaptic spines CaM-kinase II can phosphorylate and modulate numerous proteins including the AMPA-type glutamate receptor [42], [43], a small GTPase activating protein (SynGAP) [44], and a scaffold protein (DLG) in Drosophila [45]. Phosphorylation of AMPA-type receptors enhances their single channel conductance [62] that contributes to early-phase long-term potentiation (LTP) [97]. There is also evidence that mRNA for CaM-kinase II localizes adjacent to post-synaptic sites [33], and that tetanic stimulation may increase synthesis of CaM-kinase II at the dendrites [98]. Recent studies have begun to address how CaM-kinase II localizes to different subcellular sites. For example, the β isoform appears to bind to cytoskeletal elements [41]. Upon activation, CaM-kinase II appears to translocate to post-synaptic sites, in part through interaction with the NMDA-type glutamate receptor [40] [39].

A potent inhibitor of CaM-kinase II was recently cloned from a cDNA library of a rat brain [99]. The mRNA for this novel protein, CaM-KIINβ, is expressed in rat brain

and testis. In the present study, we again used the yeast two-hybrid screen with the catalytic domain of CaM-kinase II to identify another cDNA encoding for an inhibitor to CaM-KII (CaM-KIINα).

The message for CaM-KIINα is specific for the brain. These data would suggest there may be a family of inhibitory proteins for CaM-KII similar to the inhibitors of PKA [79], [47]. The highly specific PKA inhibitors have been very useful tools for dissecting physiological roles of PKA, and microinjected CaM-KIIN has been used to similar advantage to establish a role of CaM-KII in synaptic plasticity at the Mauthner cell synapse in goldfish [93]. However, engineering a construct of CaM-KIIN that is cell permeable would be highly advantageous, and we report that a fusion construct of CaM-KIIN with Antennapedia peptide allows CaM-KIIN access into cultured cells to inhibit CaM-KII. This new construct should be highly useful to test the physiological roles of CaM-kinase II in various cellular systems.

C. Methods

Yeast 2-hybrid Screen. The screen was performed as described [83], [99]. The DNA sequence (1-830 bp) corresponding to the catalytic domain (amino acids 1-269) of the inactive β CaM-KII mutant (K43A) was inserted into the pBTM116 vector in frame with the LexA-DNA binding domain. The rat brain matchmaker cDNA library (Clontech) was constructed in a pGAD10, GAL4 activation domain vector.

Interaction with the bait protein was monitored by prototrophy for histidine and/or β-galatosidase activity.

Northern Analysis. This was performed as previously described [99]. cDNA insert was isolated from the pGAD10 vector and labeled with [α-^{32}P]dCTP by random priming. Total RNA from rat tissues was isolated with Trizol (Gibco-BRL), and poly A RNA was further purified using Oligotex mRNA mini kit (QIAGEN). Either 2μg of poly A RNA or 20μg of total RNA were subjected to electrophoresis on a formaldehyde-containing 1.2% agarose gel and then blotted onto Hybond-N (Amersham). The blot was hybridized in Rapid-H4B hybridization buffer (Amersham) with the radioprobe at 65° C for 2 hr and was washed once in 2xSSC, 0.1% SDS at 25 °C for 15 min. and then twice in 1xSSC, 0.1% SDS at 65 ° C for 20 min.

Expression of proteins. GST-fusion constructs were expressed in pGEX-2T (Pharmacia). Histidine fusion constructs were expressed in pET-16B (Novagen). The following recombinant proteins and peptides were constructed:

GST-CaM-KIINα; GST-CaM-KIINβ[99]; GST-KIINα$_{1\text{-}41}$; GST-KIINα$_{1\text{-}53}$; GST-KIINα$_{1\text{-}68}$; GST-CaM-KIIN2δ; H_6-CaM-KIIN; Ant-CaM-KIINα; Ant-KIINtide. These were bacterially expressed in BL21 (DE3) cells and purified with either glutathione-Sepharose or nickel Sepharose. Purified proteins were then dialyzed to a 100mM HEPES, pH 7.5, 1mM DTT solution. Synthetic peptides were produced by

Peptide Express.

GST-precipitations. Samples were incubated with purified recombinant GST-fusions and glutathione-Sepharose in the appropriate buffer for 1hr at 4°C, washed extensively and eluted with 10mM glutathione. The eluant was separated by SDS-PAGE and immunoblotted with the appropriate antibody.

Immunoblots. Appropriate samples were separated by a 15% SDS-PAGE and transferred to immobilon. The blots were blocked in TBST (Tris HCl pH 7.5, 150mM NaCl, 0.05% Tween-20) and 5% nonfat milk. Primary antibody was used at a concentration of 1/1000. Secondary antibody was linked to horseradish peroxidase and developed with enhanced chemiluminescence.

Kinase Assays. Standard CaM-KII assay conditions contained 0.8 mM $CaCl_2$, 2 μM CaM, 1 mM DTT, 50 mM HEPES, pH 7.5, 10 mM $Mg(OAc)_2$, 40 μM syntide-2 and 0.2 mM [γ-^{32}P]ATP (1000-2000 cpm/pmol) in 20-25 μl reactions. The assays were initiated by adding enzyme diluted in buffer (50 mM HEPES, pH 7.5, 2 mg/ml bovine serum albumin, and 10% ethylene glycol) to the reaction. ^{32}P- incorporation was determined by spotting 10 to 15 μl of the reaction to Whatman P-81 phosphocellulose paper and subsequently washing in 75 mM phosphoric acid as described [84].

Extract Assays. Goldfish brain (gift from Dr. Pereda) were harvested in 2mls of CaM-KII lysis buffer (10mM Tris, pH 7.5, 1mM EDTA, 1mM EGTA, 1mM DTT, 10μg/ml leupeptin, 10μg/ml soy bean trypsin inhibitor, 10μg/ml aprotinin, 1mM

benzamidine, 1mM PMSF). 0.5µl of the supernatant (~1µg protein) was used in a standard CaM-KII assay. Also, one rat brain was harvested in 5mls of CaM-KII lysis buffer. 1ml of *Drosophila* heads were separated through a wire mesh (gift from Dr. M. Forte) and homogenized in 2mls CaM-KII lysis buffer. All the samples were then centrifuged in a microcentrifuge for 30min.

***In situ* Hybridization.** The assay was performed as previously described [100]. Briefly, 2 µm parasaggital slices were obtained (Clontech). Slices were then fixed in 4% paraformaldehyde for 15min, permeabilized with 0.001% proteinase K treatment, and treated with triethanolamine and acetic anhydride prior to hybridization. Probes were constructed by inserting CaM-KIINα and CaM-KIINβ into pBluescript (SK). Plasmids were then digested 3' to either the T3 or T7 promoter to make both the anti-sense probe and the sense control. Probes were labeled using ^{35}S-UTP labeling (Promega riboprobe synthesis system) and hybridized 24 hrs at 55°C. Samples were then washed with decreasing concentrations of SSC, dehydrated with increasing ethanol and exposed to sheetfilm overnight.

Immunohistochemistry. Adult male rats were anesthetized with ketamine/xylazine solution and fixed with 4% paraformaldehyde, sodium borate solution by transcardiac perfusion. The brain was harvested and incubated in 10% sucrose in PBS at 4°C overnight. Samples were blocked and frozen. 15µm parasaggital slices were cut with a cryostat and then dried. Samples were blocked with 10% horse serum in PBS, and

then incubated with 1° antibodies overnight at 4°C. Samples were incubated with 2° antibody-HRP and visualized with DAB precipitation (Vectastain system).

Primary hippocampal neurons. Cultured hippocampal neurons were generously provided by Dr. Gary Banker [101]. Briefly, hippocampi from 18 day rat embryos were dissociated with trypsin (0.25% for 15min at 37°C), triturated and plated on poly-L-lysine coated glass cover slips in minimal essential medium (MEM) with 10% horse serum. After 3-4 hours to attach, the cover slips were suspended above an astroglial monolayer. Cells were grown in serum-free MEM with N2 supplements, 0.1% ovalbumin, and 0.1mM pyruvate. After 3days, the cells were treated with 5 μM cytosine arabinoside to inhibit nonneuronal proliferation. The cells were cultured for >3wks, fixed in 4% paraformaldehyde/4% sucrose in PBS pH7.4 and permeabilized in 0.25% triton X-100. They were blocked with PBS pH7.4 with 10% horse serum and incubated with 1° antibody overnight at 4°C and visualized by confocal microscopy.

Fusion protein uptake. Ant-CaM-KIINtide was synthesized with the following sequence (RQIKIWFQNRRMKWKKGKRPPKLGQIGRSKRVVIEDDRIDDVLK) (Peptide Express). COS-7 cells in 35 mm plates were transiently transfected with CaM-KII(H282R) (1 μg plasmid), using 8 μl lipofectAMINE (Gibco-BRL). Cells were grown for two days. 5μM of CaM-KIIN, CaM-KIINtide, Ant-KIIN, Ant-

KIINtide were layered onto the cells in DMEM for 2 hours at 37°C. Cells were then fixed and visualized as above for confocal microscopy.

D. Results

Brain expresses two distinct clones of CaM-KIIN.

A yeast two-hybrid screen of a rat brain cDNA library, using as bait an inactive catalytic domain of βCaM-kinase II, identified a unique 79 amino acid protein (CaM-KIIN) that is a highly specific and potent inhibitor of CaM-kinase II [99]. Sequence analysis of another clone from this screen has identified an additional isoform of CaM-KIIN (Fig. 1A). The putative open reading frame encodes a 78 amino acid protein that is highly homologous to CaM-KIIN, especially in its inhibitory domain (underlined) which is 95% identical (Fig. 1B). Numerous attempts at 5' RACE and cDNA lambda library screens identified additional in-frame 5' sequences encoding up to another 143 amino acids, but the sequences varied from clone to clone and no upstream methionine was identified. Thus, we believe that the sequence shown in Fig. 1 represents CaM-KIINα, but we cannot exclude the possibility of additional N-terminal sequence. This new isoform will be referred to as CaM-KIINα and the original isoform as CaM-KIINβ since their regional expressions in rat brain (see below) are most similar to CaM-kinase IIα and CaM-kinase IIβ, respectively.

Biochemical analysis of CaM-KIINα

A GST-fusion protein, using the coding sequence of CaM-KIINα in Fig. 1, was constructed, expressed in bacteria and purified. The recombinant protein was tested as an inhibitor of αCaM-kinase II - it showed an IC_{50} of 50nM (Fig. 2A) which is similar to CaM-KIINβ [99]. Also like CaM-KIINβ, inhibition of CaM-kinase II by CaM-KIINα appears to be non-competitive with the synthetic peptide substrate syntide-2 (Fig. 2B). This is important since both CaM-KIIN isoforms contain a potential pseudosubstrate sequence, RVVIED where the underlined residues are consensus recognition motifs in most substrates of CaM-kinase II [102], [103] and the Ile would be replaced in substrates by phosphorylatable Ser or Thr. The GST-fusion of CaM-KIINα (1 μM) was not phosphorylated *in vitro* by CaM-KII (20 nM, 20 min reaction), and a mutant in which RVVIED was changed to a consensus CaM-kinase II sequence, RVVSED, was only slightly phosphorylated (data not shown). The fact that both CaM-KIIN isoforms inhibit CaM-kinase II non-competitively with regard to substrate is important since the inhibitory potential, both of endogenous CaM-KIIN as well as recombinant CaM-KIIN introduced into cells to probe CaM-kinase II functions, will be independent of substrate concentration.

The inhibitory domain of CaM-KIINα was determined using truncation analysis. Truncated recombinant GST-fusion proteins (Fig. 2C) were expressed,

purified and tested for their abilities to both bind CaM-kinase II and inhibit its activity. To assess binding, the GST-fusion proteins were incubated with CaM-kinase II and glutathione-Sepaharose followed by centrifugation and washing. Proteins eluted by 10 mM glutathione were analyzed by SDS/PAGE and Western analysis for bound CaM-kinase II (Fig. 2D). Truncation at residue 68 did not influence either binding to CaM-kinase II (Fig. 2D) or inhibition of CaM-kinase II (Fig. 2E) compared to full length CaM-KIINα. Further truncation at residue 53 reduced inhibitory potency by 60-70% as well as ability to bind CaM-kinase II, and truncation at residue 43 showed no significant inhibition of CaM-kinase II. These results identify the highly conserved residues 43-68 as the inhibitory domain, consistent with our previous result that a synthetic peptide corresponding to this sequence retains both the inhibitory potency and specificity for CaM-kinase II inhibition [99]. In an effort to make a mutant (CaM-KIIN2δ) of CaM-KIIN which has reduced inhibitory potency, several residues in this inhibitory sequence in the full-length GST fusion were mutated (**R**PPKLGQIGRSKRVV**IEDD**RIDDVLK to **P**PPKLGQIGRSKRVV**IKLA**RIDDVLK). As shown in Fig. 2A, this CaM-KIIN2δ mutant had at least a 100-fold reduction in inhibitory potency.

As is true for CaM-KIINβ, both binding to (Fig. 2D) and inhibition of CaM-kinase II by CaM-KIINα requires the presence of Ca^{2+}/CaM. However, one

difference in terms of binding to CaM-kinase II was detected between the two isoforms. When CaM-KIINβ binds CaM-kinase II in the presence of Ca^{2+}/CaM and EGTA is subsequently added, interaction between the two proteins is lost, but this is not true for CaM-KIINα (Fig. 2D, bottom panel). This suggests some difference in the interaction of the two isoforms with CaM-kinase II, presumably by residues N-terminal of the inhibitory domain. Consistent with this interpretation, the mutant CaM-KIINα truncated at residue 43 displays no inhibition of CaM-kinase II (Fig. 2E) but still exhibits slight binding of CaM-kinase II (Fig. 2D, top panel).

CaM-KIIN is expressed in the brain of multiple organisms and can inhibit CaM-kinase II acitivity within their extracts.

Northern analysis of different rat tissues showed hybridization to a 1.8kb message for CaM-KIINα that was limited to brain (Fig. 3A). Forebrain and hippocampus express much higher message levels of CaM-KIINα than midbrain or cerebellum. Immunoblotting brain extract from both the rat using the anti-CaM-KIIN antibody showed that the 7 and 19KD proteins are predominantly within the soluble fraction (fig. 3B, left panel). Also, this antibody appears to recognize these proteins within goldfish extract. Consistent with this data the 7 and 19kD species are not seen within fractions that purify the PSD's (fig. 3B, right panel).

It would appear that CaM-KIIN may be expressed within multiple species. It is known that CaM-kinase II is expressed within a multitude of organisms. Therefore, the inhibitory potency for CaM-KIINtide on CaM-kinase II activity from brain extract was tested from several organisms. Both goldfish and *Drosophila* CaM-kinase II activity was inhibited by CaM-KIINtide (fig. 3C). The IC_{50} was 300nM to 500nM, respectively.

mRNA for CaM-KIINα and CaM-KIINβ are differentially expressed within the brain

The mRNA for CaM-KIINα shows some differential expression within regions of the brain. This is in contrast to the broader expression of CaM-KIINβ in brain [99]. *In situ* hybridization by probes specific for CaM-KIINα and CaM-KIINβ also show differences in the intensity of message expression in specific regions of the brain (Fig. 4). In the cortex, CaM-KIINα and CaM-KIINβ are expressed more in laminae II and III versus laminae IV-VI. Both clones are expressed within the pyramidal layer of the hippocampus and granular layer of the dentate gyrus as well as in the olfactory bulb. CaM-KIINβ is diffusely expressed within the caudate-putamen and globus pallidus whereas CaM-KIINα is stronger within the caudate-putamen. CaM-KIINβ is diffusely expressed throughout the regions of the midbrain. In contrast, there is very little CaM-KIINα expression within the midbrain regions

except for the thalamus and inferior colliculus. In cerebellum, CaM-KIINβ is expressed within the Purkinje and granular layer. In general CaM-KIINα shows stronger expression in the cortex whereas CaM-KIINβ is more intense in cerebellum, distributions that are similar to αCaM-kinase II and βCaM-kinase II, respectively. Neither of the sense controls for CaM-KIINα or CaM-KIINβ showed specific hybridization.

CaM-KIIN immunoreactivity in the brain

The antibody previously generated to the highly conserved inhibitory domain of CaM-KIINβ [99] reacts with both CaM-KIIN isoforms (data not shown). This antibody specifically recognizes 7 and 19 kD proteins by Western blot analysis of rat forebrain. The 7 kD protein presumably represents the α and β isoforms of CaM-KIIN. The 19 kD protein may be an additional isoform or the α isoform if additional coding sequence is present. Western blot analysis of supernatant and particulate fractions from hippocampus demonstrated that both the 7 and 19 kD proteins are in the soluble fraction (fig. 3B). Fractionation of hippocampal extracts to yield synaptosomes and the postsynaptic density fraction did not support association of CaM-KIIN with the PSD although CaM-KII was clearly enriched (fig. 3B).

Regional distribution of CaM-KIIN was determined by immunohistochemistry in parasaggital sections of adult rat brains (Fig. 5). Staining

was specific, except for the nucleus, in that preadsorption of the antibody with antigen strongly reduced immunoreactivity (Figs. 5 and 6). For purposes of comparison, adjacent slices were stained with an antibody that was specific for CaM-KIIα or a polyclonal CaM-KII antibody that reacts to both the alpha and beta isoforms. Consistent with the *in situ* mRNA analysis (Fig.4), there was immunoreactivity for CaM-KIIN throughout the brain. The frontal cortex, hippocampus, olfactory bulb, caudate-putamen, and cerebellum exhibit the highest levels of CaM-KIIN (Fig. 5). It is interesting that the inferior colliculus does not have a corresponding increase in staining as compared to the mRNA. Within the frontal cortex, staining is seen throughout all laminae (Fig. 6A). Furthermore, there appears to be dendritic processes from laminae II-IV are labeled. There seems to be high immunoreactivity within laminae I-III. CaM-KIIN is present thoughout the hippocampus (Fig. 6B), especially within the stratum oriens, stratum pyramidale, stratum radiatum, and the ectal and endal limbs of the dentate gyrus. The apparent strong straining in the cell body layers with preabsorbed antigen is probably an artifact of the nonspecific staining in the nuclei. Within the cerebellum, there appears to be specific staining within the molecular level of the cerebellar cortex and within Purkinje cells (Fig. 6C). Most of this immunoreactivity, except for the cell bodies, is blocked by preadsorption. Therefore, it is difficult to comment on specific cell body staining (however, see Fig. 7), but it is clear that the dendritic staining is specific

within these regions. For comparison, the CaM-KII shows similar immunostaining patterns, consistent with prior published data [32], [49], [104].

CaM-KIIN immunocytochemistry in primary hippocampal cultures.

Three week old primary hippocampal cultures were triple-labeled with anti-CaM-KIIN, anti-CaM-KII, and anti-MAP2B and visualized by confocal microscopy (Fig. 7). Although there is coincidence of straining between CaM-KIIN and CaM-KII in cell bodies and the central portions of dendrites, the dendritic projections show puntate CaM-KII but no CaM-KIIN (Figs. 7E and F). This is consistent with the lack of CaM-KIIN immunoreactivity in purified synaptosomes and PSDs as mentioned earlier. The dendrites show strong coincidence of staining between CaM-KIIN and MAP2B (Fig. 7C). Furthermore, there are many thin processes stained for CaM-KII only, suggesting axonal phenotype (Fig. 7E). Therefore, CaM-KIIN seems to be evenly distributed throughout the cell body and dendrites. Preadsorption of the anti-CaM-KIIN antibody removes most of the CaM-KIIN staining and has no effect on MAP2B and CaM-KII staining (Fig. 7J, K, L)

Molecular engineering of CaM-KIIN to make it cell permeable

It has been shown that the transcription factor Antennapedia has the ability to enter cells, and this cell permeability appears to be due to a 16 amino acid sequence [105], [106]. Fusion of this Antennapedia sequence with peptides or small proteins allows

them to gain access into cells [107]. A fusion protein between CaM-KIIN and the 16 amino acids from Antennapedia was constructed using either the entire open reading frame of CaM-KIINα or the 27 residue inhibitory domain. The fusion peptide has the ability to inhibit CaM-KII *in vitro* with similar potencies (IC_{50} = 50 nM, Fig. 2A). These fusion constructs (5μM) were incubated with cultured COS-7 cells that had been transfected with CaM-KII, and cells were visualized by immunocytochemistry (Fig. 8). His_6-CaM-KIINβ by itself and the inhibitory peptide CaM-KIINtide show peripheral punctate staining, consistent with binding to the plasma membrane (figure 8B). In contrast, the Antennapedia fusion products (Ant-KIIN, Ant-KIINtide) exhibit distribution throughout the cell including the nucleus (Fig. 8C, D). Interestingly, the fusion peptide (Ant-KIINtide) seems to be able to localize within the nucleolus.

E. Discussion

CaM-kinase II is a rather ubiquitously expressed kinase that is particularly abundant in brain. It is subject to complex regulatory mechanisms including activation by Ca^{2+}/CaM, generation of autonomous activity by autophosphorylation, and subcellular interactions. We have now identified another level of regulation in brain. There are at least two different 7kD gene products, CaM-KIINα and CaM-KIINβ, expressed in brain that specifically inhibit CaM-kinase II. A 19 kD protein

which is specifically recognized by the antibody may represent another isoform, but this protein has not been cloned and its inhibitory properties have not been confirmed. Importantly, CaM-KIIN only interacts with activated CaM-kinase II, and it inhibits both total and autonomous activities. Presumably the interaction sites in CaM-KII are conformationally unavailable in the absence of bound Ca^{2+}/CaM. Upon binding of CaM-KIIN in the presence of Ca^{2+}/CaM, removal of the Ca^{2+}/CaM by addition of EGTA results in rapid dissociation of CaM-KIINβ but not of CaM-KIINα. This suggests that the off-rates of the two inhibitors with CaM-kinase II are different which may be physiologically interesting. Furthermore, although the inhibitor binds to the catalytic domain of CaM-kinase II, inhibition is non-competitive with substrate. This is in contrast to other kinase inhibitors such as the PKA inhibitor [79], [47] which contains a pseudosubstrate domain that is the key inhibitory motif. Although CaM-KIIN contains a potential pseudosubstrate domain, it does not appear to be important in the inhibitory mechanism since 1) inhibition is non-competitive with regards to peptide substrate, and 2) mutation of the pseudosubstrate sequence (RVVIED) into a consensus CaM-kinase II phosphorylation site (RVVSED) did not reduce its inhibitory potential.

The various isoforms of CaM-kinase II are differentially expressed in mammalian tissues [108], [5] with α and β CaM-kinase II restricted to neural tissues

[49]. CaM-kinase IIα is most prominent in forebrain and hippocampus whereas CaM-kinase IIβ is enriched in cerebellum [49], and CaM-KIINα and β exhibit similar distributions. However, even within regions of the brain where both isoforms of CaM-kinase II are expressed, they may have different roles dictated by their subcellular localizations. CaM-kinase IIβ appears to interact with cytoskeletal elements whereas CaM-kinase IIα is largely cytosolic [41] except in dendritic spines where it concentrated in the PSD. CaM-KIIN immunostaining in cultured hippocampal neurons appears to be very similar to MAP2B, suggesting it may have a localized inhibitory function. In particular, CaM-KIIN was not detected in dendritic spines or in purified PSDs which contain high concentrations of CaM-kinase II. This suggests that these proteins have a specific role in inhibiting certain pools of CaM-kinase II within the brain.

Unfortunately, the antibody used for the immunostaining and immunoblotting showed some nonspecific staining that could not be removed. There were multiple attempts at producing other, more specific antibodies, which were unsuccessful. Yet, from the preadsorption data it would be safe to argue that the 7kD and 19kD bands are specific to this antibody. Furthermore, if you preadsorb the antibody prior to immunohistochemistry, the staining within the dendritic regions is removed . This

would suggest that the dendritic staining is specific for the CaM-KIIN antibody. The remaining stain is most likely the nonspecific bands seen in the immunoblot.

The overall levels of CaM-KIIN in rat brain are relatively low compared to levels of CaM-kinase II since one can measure very robust CaM-kinase II activity in brain extracts in the presence of Ca^{2+}/CaM, conditions that favor interaction of CaM-KIIN with CaM-kinase II. Since CaM-KIIN shows a fairly general distribution throughout the cell body and central portion of dendrites, only a small fraction of total CaM-kinase II could be potentially inhibited by CaM-KIIN. However, it is not clear how much CaM-kinase II gets activated physiologically, especially in terms of autonomous activity. For example, LTP induction only generates about 10-15% autonomous CaM-kinase II activity whereas CaM-kinase II autophosphorylation *in vitro* can produce up to 50-70% autonomous activity. If other physiological stimuli similarly activate only a small fraction of CaM-kinase II, the levels of CaM-KIIN may be sufficient to attenuate autonomous CaM-kinase II. Alternatively, it is possible that levels of CaM-KIIN could be dramatically altered through transcriptional regulation. Neurotoxic events such as ischemia result in dramatic reductions of CaM-KII activity without measureable changes in CaM-KII immunoreactivity [73]. This inhibition of CaM-kinase II activity does not appear to be a consequence of hyperphosphorylation since it cannot be reversed by protein phosphatases [73]. A speculative suggestion is that perhaps levels of CaM-KIIN

might be upregulated under these conditions. Further investigation is required to understand the physiological functions of CaM-KIIN.

CaM-KIIN and its inhibitory domain peptide (CaM-KIINtide) are useful tools for dissecting cellular functions of CaM-kinase II. Previous studies have used either peptides modeled after the autoinhibitory domain (AID) of CaM-kinase II or the cell permeable compound KN-62. AID peptides are not specific in that they also inhibit other CaM-kinases as well as PKC [88], [89], and KN-62 inhibits other CaM-kinases (e.g., CaM-KIV) [109] and in some cells appears to nonspecifically block voltage-gated potassium channels [110]. In contrast, CaM-KIIN is highly specific for inhibition of CaM-kinase II [99]. Furthermore, CaM-KIINtide has the ability to inhibit CaM-kinase II activity from many different organisms. Therefore, the peptide could be used to dissect CaM-kinase II function in many animal models including *Drosophila* and goldfish. However, prior studies were limited by the fact that cells had to be either transfected or microinjected with CaM-KIIN or CaM-KIINtide. For example, microinjection of CaM-KIINtide into the Mauthner cell synapse from goldfish, which contains high concentrations of CaM-kinase II, blocks activity-induced synaptic potentiation [93]. We have now engineered a fusion construct between the Antennapedia peptide and CaM-KIIN and CaM-KIINtide that facilitate their entry into cultured cells. Incubation of COS-7 cells with Antennapedia/CaM-KIIN (Ant-KIIN) results in uptake of the inhibitor into the cell (Fig. 7). This

approach also appears to work in hippocampal slices since LTP can be blocked by incubation of the slice with Ant-KIIN prior to electrical induction (Guire, E., Chang, B., and Soderling, unpublished results). It is anticipated that CaM-KIIN and CaM-KIINtide will be key reagents in establishing cellular functions of CaM-kinase II.

A

```
   1  GACGCGACCATGTCGGAGGTGCTGCCCTACGGCGACGAGAAGCTGAGCCCCTACGGCGAC   60
              M  S  E  V  L  P  Y  G  D  E  K  L  S  P  Y  G  D      17

  61  GGCGGCGACGTGGGCCAGATCTTCTCGTGCCGCCTGCAGGACACCAACAACTTCTTCGGC  120
  18  G  G  D  V  G  Q  I  F  S  C  R  L  Q  D  T  N  N  F  F  G     37

 121  GCCGGGCAGAGCAAGCGGCCGCCCAAGCTGGGCCAGATCGGCCGGAGCAAGCGCGTTGTT  180
  38  A  G  Q  S  K  R  P  P  K  L  G  Q  I  G  R  S  K  R  V  V     57

 181  ATTGAAGATGATAGGATCGATGACGTGCTGAAAACCATGACCGACAAGGCACCTCCTGGT  240
  58  I  E  D  D  R  I  D  D  V  L  K  T  M  T  D  K  A  P  P  G     77

 241  GTCTAACTGCCCCCAAAGACAATGTGTTGAGGGAAGGAATAAGAAGAGTGGCGGGCTGTG  300
  78  V  *

 301  ACAGTTACTGACAAAAAGCATGAGGAGGAGAAAGCACTTTGGAATTTATTATTAGCTTGC  360
 361  TACCTACGATGAAATCGACAACCTGTGTCTCAAGTCAGGCCGGGAGACA             409
```

B

```
CaM-KIINβ      1 MSEILPYGEDKMGRFGADPEGSDLSFSCRLQDTNSFFAGNQAKRPPKLGQ 50
                 |||:||||::|:  :|   :   : ||||||||||.||   |.|||||||
CaM-KIINα      1 MSEVLPYGDEKLSPYGDGGDVGQI.FSCRLQDTNNFFGAGQSKRPPKLGQ 49
                   .         .
              51 IGRAKRVVIEDDRIDDVLKGMGEKPPSGV 79
                 |||.|||||||||||||||| | :| | ||
              50 IGRSKRVVIEDDRIDDVLKTMTDKAPPGV 78
```

Figure 1. Sequence of CaM-KIINα. (**A**) DNA sequence of CaM-KIINα and deduced amino acid sequence. (**B**) Amino acid sequence comparison between CaM-KIINα and CaM-KIINβ. The two proteins show 80% similarity with 70% identity. The underlined sequence is the inhibitory region against which an antibody was generated.

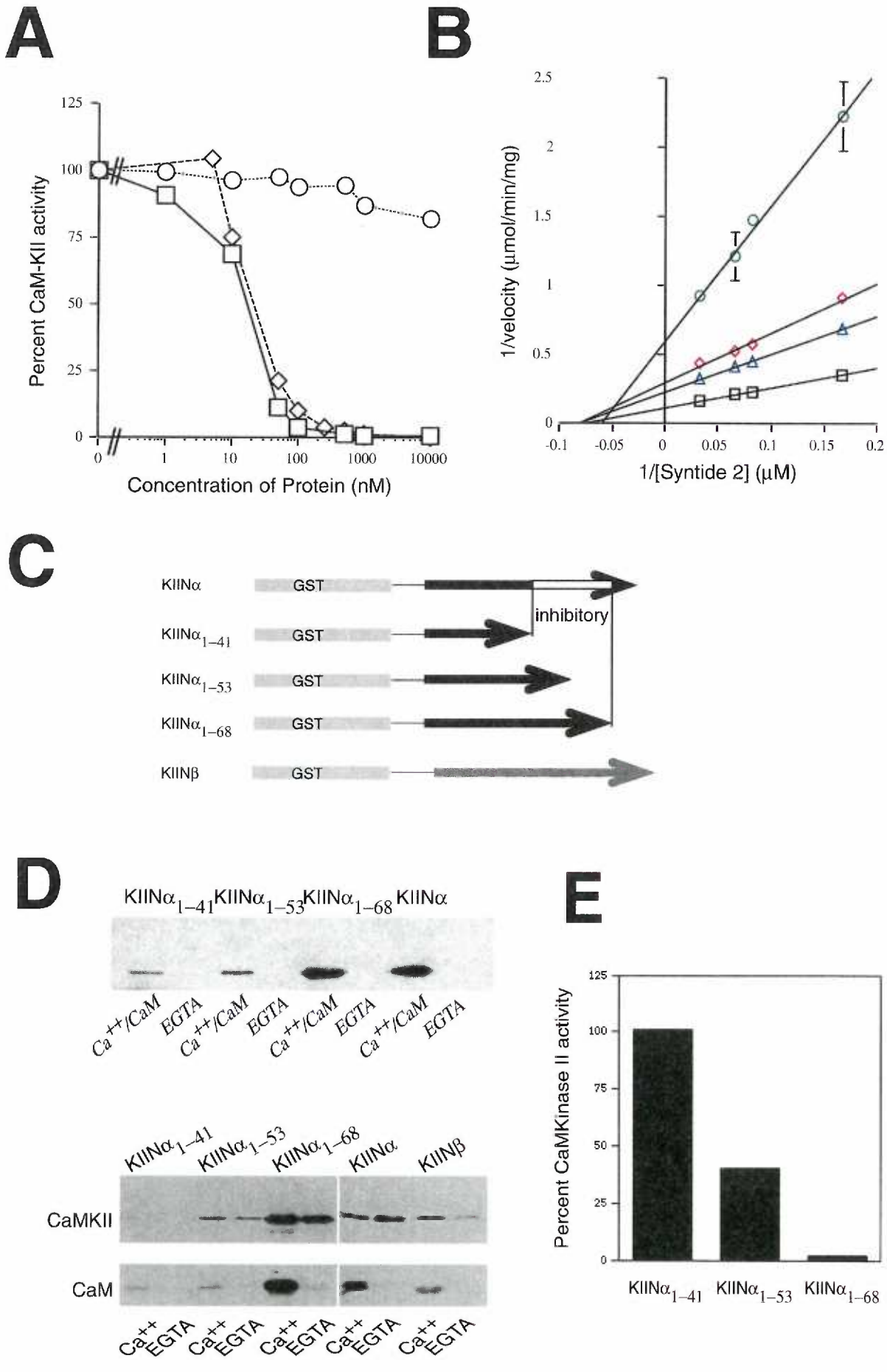

A
Percent CaM-KII activity
125
100
75
50
25
0
0
1
10
100
1000
10000
Concentration of Protein (nM)
B
1/velocity (µmol/min/mg)
2.5
2
1.5
1
0.5
0
-0.1
-0.05
0
0.05
0.1
0.15
0.2
1/[Syntide 2] (µM)
C
KIINα
GST
KIINα1–41
GST
KIINα1–53
GST
KIINα1–68
GST
KIINβ
GST
inhibitory
D
KIINα1–41 KIINα1–53 KIINα1–68 KIINα
Ca++/CaM
EGTA
Ca++/CaM
EGTA
Ca++/CaM
EGTA
Ca++/CaM
EGTA
KIINα1–41
KIINα1–53
KIINα1–68
KIINα
KIINβ
CaMKII
CaM
Ca++
EGTA
Ca++
EGTA
Ca++
EGTA
Ca++
EGTA
Ca++
EGTA
E
Percent CaMKinase II activity
125
100
75
50
25
0
KIINα1–41
KIINα1–53
KIINα1–68

Figure 2. Biochemical characteristics of CaM-KIINα. (**A**) Inhibition of CaM-kinase II activity by CaM-KIINα. Recombinant CaM-kinase II (5 nM) was used to phosphorylate 40μM Syntide-2 for 5min. in the presence of the indicated concentrations of GST-CaM-KIINα (squares), GST-CaM-KIIN2δ (circles) or Ant-KIINtide (triangles). The mutant contains mutations in the inhibitory domain as described in the text. **(B)** Inhibition is non-competitive with syntide-2. CaM-kinase II (10 nM) was assayed (1 min) as in panel A with variable concentrations of CaM-KIINtide [0 nM (square), 20 nM (triangle), 30 nM (diamond), 40 nM (circle)] or varying amounts of Syntide 2 (6 μM, 12 μM, 15 μM, and 30 μM). Recombinant CaM-kinase II (10 nM) was incubated for 1min with the cocktail (see Methods). (**C**) Diagramatic representation of truncations of GST-CaM-KIINβ. The inhibitory domain (residues identified in Fig. 1B) is highlighted and the residue of trunction is indicated. (**D**) GST-fusions (1μg) were incubated with 1μg of CaM-kinase II for 1h at 4°C either with Ca^{2+}/CaM or EGTA, precipitated with GSH-Sepharose, and immunoblotted with anti-CaM-kinase II. In the lower panels, after the 1 hr incubation with Ca^{2+}/CaM, excess of EGTA was added to one-half of the sample and incubation was continued for another hour. Samples were then precipitated with GSH-Sepharose and immunblotted with anti-CaM-kinase II and anti-CaM. (**E**)

Recombinant CaM-kinase II (5 nM) was assayed as in panel A with 1μM of the GST-fusion indicated. CaM-kinase II activity was normalized to samples containing only GST.

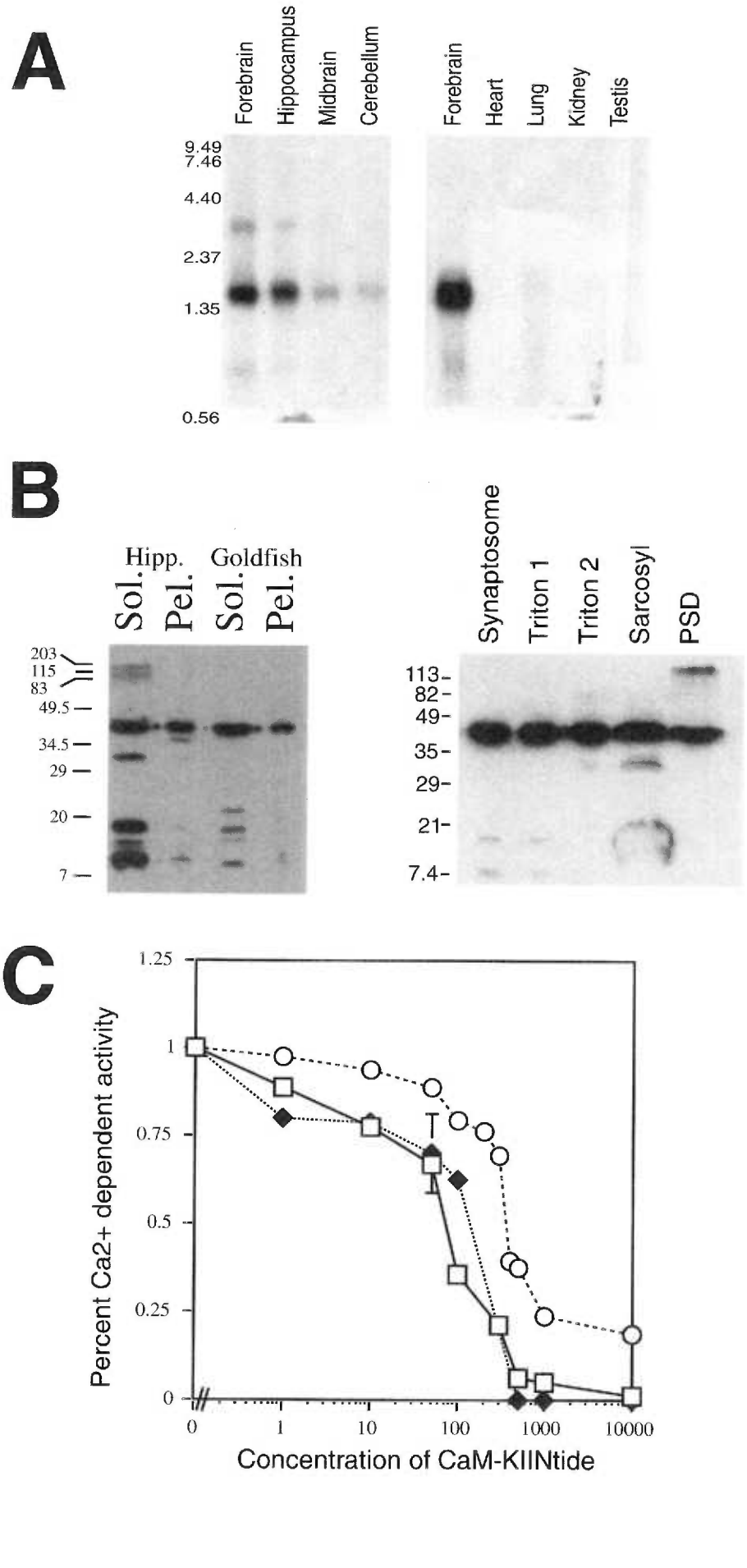

A
Forebrain
Hippocampus
Midbrain
Cerebellum
Forebrain
Heart
Lung
Kidney
Testis
9.49
7.46
4.40
2.37
1.35
0.56
B
Hipp.
Goldfish
Sol.
Pel.
Sol.
Pel.
203
115
83
49.5
34.5
29
20
7
Synaptosome
Triton 1
Triton 2
Sarcosyl
PSD
113
82
49
35
29
21
7.4
C
Percent Ca2+ dependent activity
1.25
1
0.75
0.5
0.25
0
0
1
10
100
1000
10000
Concentration of CaM-KIINtide

Figure 3. Expression and inhibition within different species. (**A**) Northern analysis of CaM-KIINα. Rat brain polyA RNA (2 μg, left panel) or total RNA (20 μg, right panel) from indicated rat tissues were probed (see Methods). The estimated size of the message is 1.8kb. (**B**) Western blot analysis of CaM-KIIN. Left panel. Hippocampi from the rat and whole brain from goldfish were harvested and and centrifuged at 14K rpm for 30min and the supernatant and pellet were separated. Each sample was separated and immunoblotted. Right panel. PSD's were purified (a gift from Maree Faux) and separated in a 15% SDS PAGE and immunoblotted with anti-CaM-KIIN. (**C**) Dose-Response of CaM-KIINtide from several species. The Ca^{2+} dependent activity from tissue extract of rat brain (open squares), goldfish brain (closed diamonds), and *Drosophila* heads (open cricles) was assayed for 1min. with varying concentrations of CaM-KIINtide.

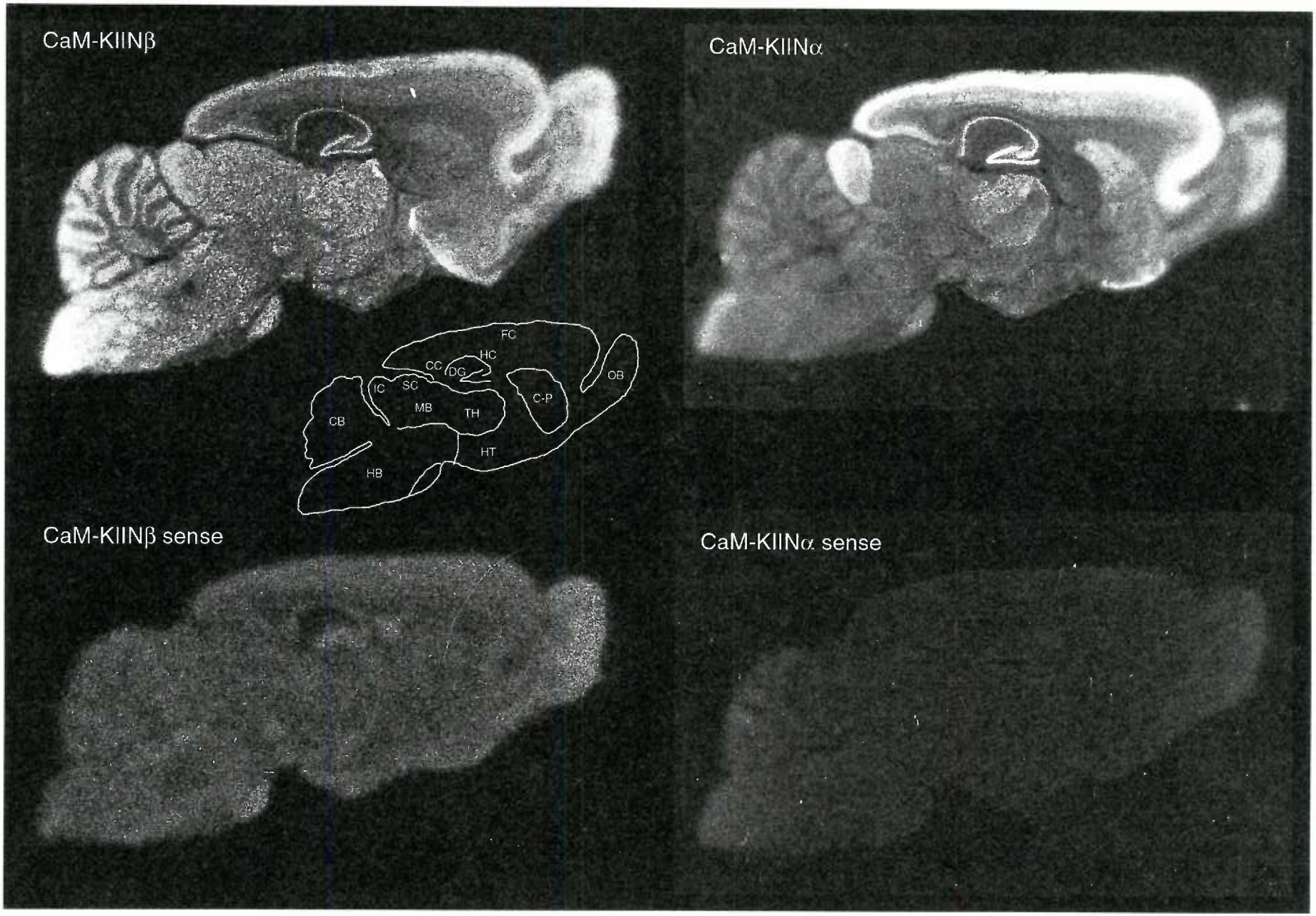
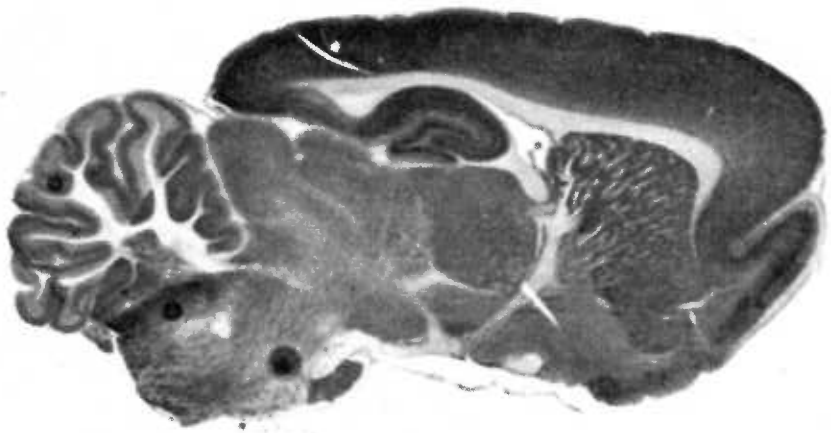
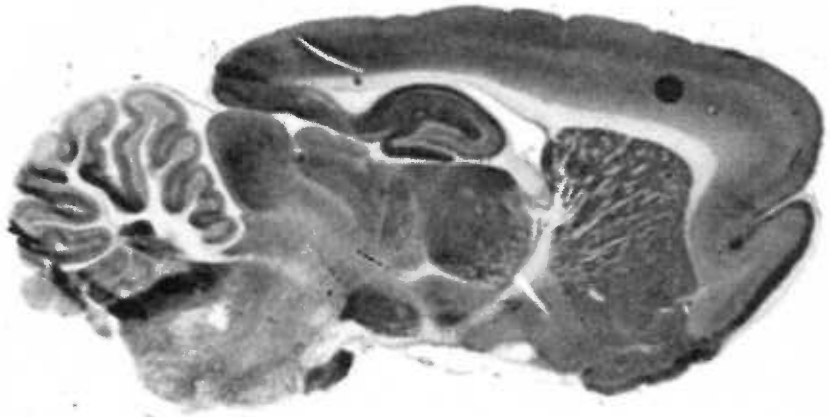

CaM-KIINβ
CaM-KIINα
FC
HC
CC
DG
IC
SC
MB
TH
C-P
OB
CB
HT
HB
CaM-KIINβ sense
CaM-KIINα sense

Figure 4. *In situ* hybridization. Anti-sense and sense probes were generated with ^{35}S-dUTP. Parasaggital sections were labeled and exposed to autoradiography. Films were scanned and inverted. Inset legend: (FC) frontal cortex, (CC) corpus callosum, (DG) dentate gyrus, (HC) hippocampus, (C-P) caudate-putamen, (OB) olfactory bulb, (HT) hypothalamus, (TH) thalamus, (MD) midbrain, (SC) superior colliculus, (IC) inferior colliculus, (CB) cerebellum, (HB) hindbrain.

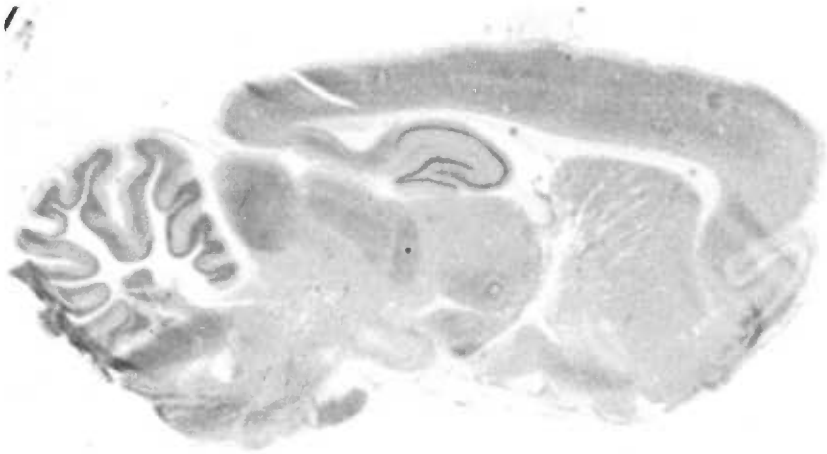
CaM-KIIN



CaM-KIIα



Preadsorbed



CaM-KII(poly)

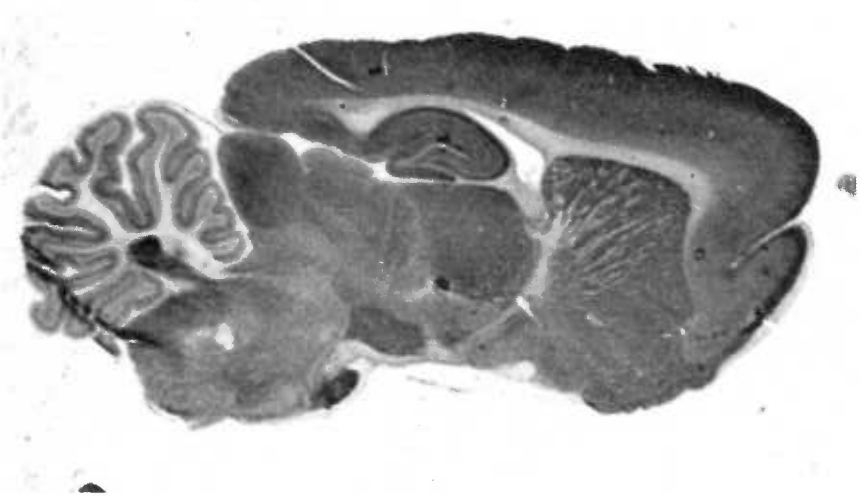


Figure 5. Immunohistochemistry of CaM-KIIN in rat brain. Antibodies for CaM-KIIN, CaM-KIIα (monoclonal), and CaM-KII (polyclonal) were used to stain parasaggital slices of adult male rat brains. Preadsorbed antibody, used to assess non-specific interactions, was incubated for 2 hrs at 4 °C with 1mM of KIINtide prior to incubating the slices.

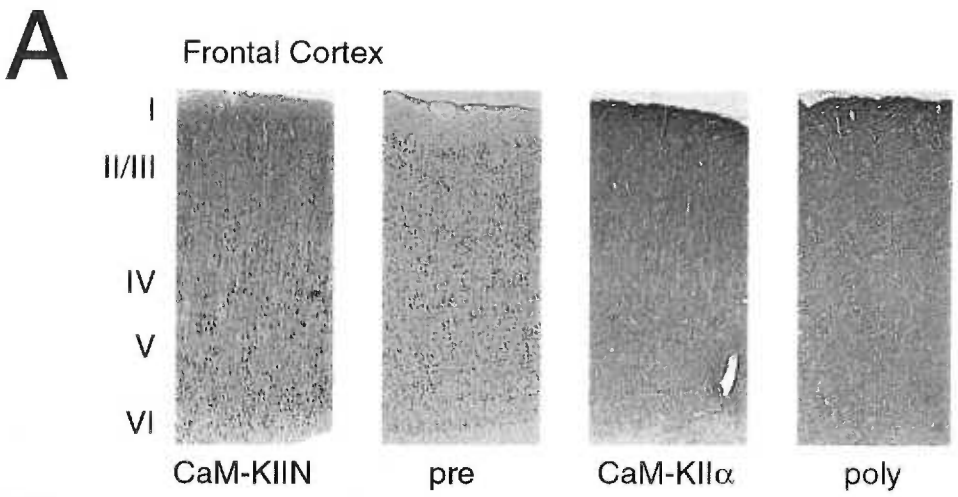

A
Frontal Cortex
I
II/III
IV
V
VI
CaM-KIIN
pre
CaM-KIIα
poly
B
Hippocampus
so
sp
sr
sc
en
CaM-KIIN
CaM-KIIα
pre
poly
C
Cerebellum
G
P
M
CaM-KIIN
CaM-KIIα
pre
poly

Figure 6. Immunohistochemistry of magnified regions of parasaggital slices of adult rat brain. (**A**) Frontal cortex. Laminae I-VI. (**B**) Hippocampus. (so) stratum oriens, (sp) stratum pyramidale, (sr) stratum radiatum, (ec) ectal and (en) endal limbs of the dentate gyrus. (**C**) Cerebellum. (G) granular, (P) pyramidal, (M) molecular layers.

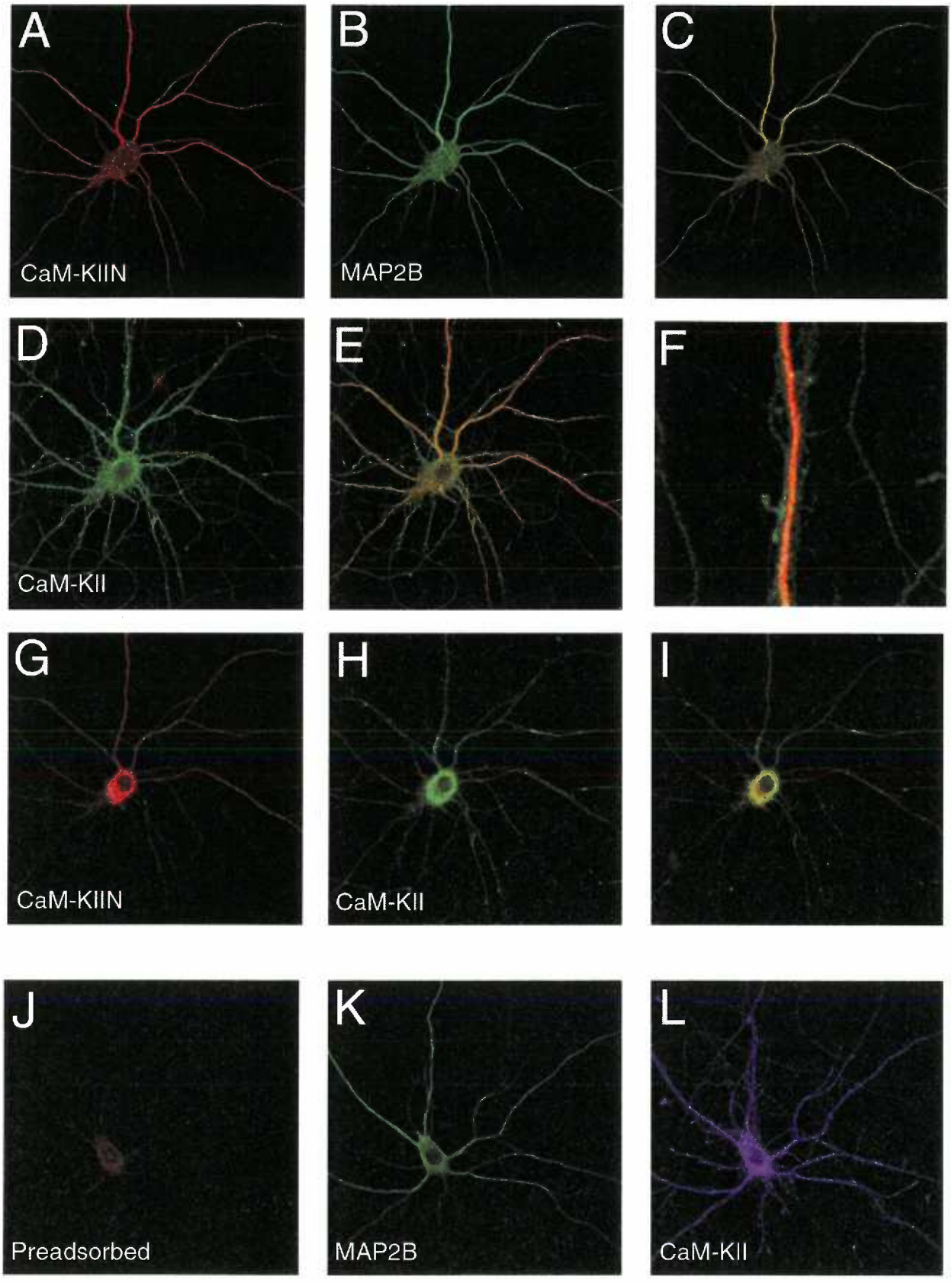

A
CaM-KIIN
B
MAP2B
C
D
CaM-KII
E
F
G
CaM-KIIN
H
CaM-KII
I
J
Preadsorbed
K
MAP2B
L
CaM-KII

Figure 7. Immunocytochemical staining of 3 wk old primary hippocampal cultures. Cells were triple-labeled with the anti-CaM-KIIN (rabbit), anti-MAP2B (mouse), and anti-CaM-KII (goat). Cultures were counterstained with anti-rabbit Texas red, anti-mouse-FITC, and anti-goat-CY5 and visualized by confocal microscopy. Samples were given artificial colors. (**A**) CaM-KIIN (**B**) MAP2B (**C**) Overlay of A and B (**D**) CaM-KII (**E**) Overlay of A and D (**F**) Magnification (10X) of a dendritic process from E; (**G-I**) Different Y-values scanned showing the cell body for staining of CaM-KIIN (**G**), CaM-KII (**H**), and overlay of G and H (**I**). (**J**) Anti-CaM-KIIN preadsorbed; same neuron as in J labeled with MAP2B (**K**) or CaM-KII (**L**).

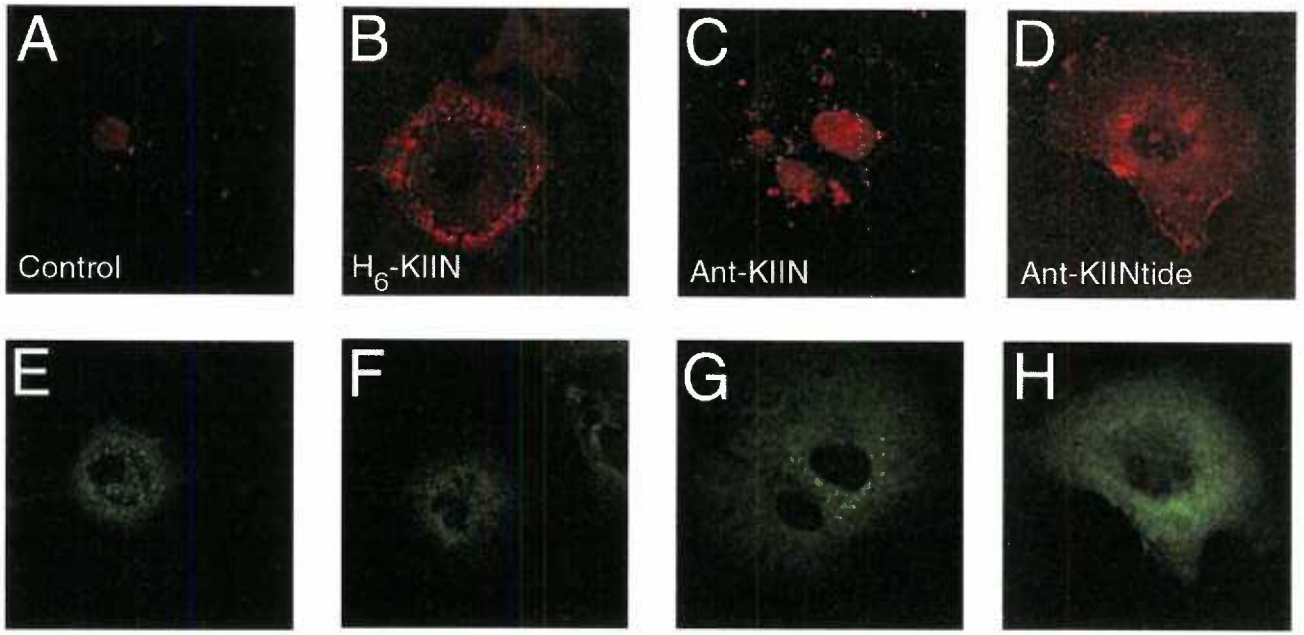

A
Control
B
H_6-KIIN
C
Ant-KIIN
D
Ant-KIINtide
E
F
G
H

Figure 8. Entry of Antennapedia fusions with CaM-KIIN (Ant-KIIN) or CaM-KIINtide (Ant-KIINtide) into COS7 cells. Cells were transfected with 1μg of CaM-KII(H282R) and grown for 1 day. Proteins and peptides (5 μM) were added to the cells in DMEM and incubated for 2hr at 37°C, washed with PBS and fixed, stained with antibodies for CaM-KIIN (A-D) or CaM-KII (E-I), and visualize by confocal microscopy. (**A, E**) No addition control, (**B, F**) His-tagged CaM-KIIN, (**C, G**) Ant-KIIN, (**D, H**) Ant-KIINtide.

CHAPTER IV

RESULTS

CA^{2+}/CALMODULIN-DEPENDENT KINASE II MEDIATES SIMULTANEOUS ENHANCEMENT OF GAP-JUNCTIONAL CONDUCTANCE AND GLUTAMATERGIC TRANSMISSION

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Published in *Proceedings in the National Academy of Sciences, USA*

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A. Abstract

While chemical synapses are very plastic and modifiable by defined activity patterns, gap junctions, which mediate electrical transmission, have been classically perceived as passive intercellular channels. Excitatory transmission between auditory afferents and the goldfish Mauthner cell is mediated by coexisting gap junctions and glutamatergic synapses. Although an increased intracellular Ca^{2+} concentration is expected to reduce gap junctional conductance, both components of the synaptic response were instead enhanced by postsynaptic increases in Ca^{2+} concentration, produced by patterned synaptic activity or intradendritic Ca^{2+} injections. The synaptically induced potentiations were blocked by intradendritic injection of KN-93, a Ca^{2+}/calmodulin-dependent kinase (CaM-K) inhibitor, or CaM-KIINtide, a potent and specific peptide inhibitor of CaM-KII, whereas the responses were potentiated by injection of an activated form of CaM-KII. The striking similarities of the mechanisms reported here with those proposed for long-term potentiation of mammalian glutamatergic synapses suggest that gap junctions are also similarly regulated and indicate a primary role for CaM-KII in shaping and regulating interneuronal communication, regardless of its modality.

B. Introduction

The degree of interneuronal communication via chemical synapses is a dynamic feature of the central nervous system and is mainly determined by the synapses' own activity [111]. In contrast, gap junction-mediated electrical synapses [112] are considered to be largely unmodifiable. Recent studies indicate, however, that these synapses also can undergo activity-dependent potentiation. As with chemical synapses, patterned synaptic activity has been shown to produce short- and long-term modifications of interneuronal coupling [113, 114].

Eighth nerve afferents (see Fig. 1a) terminate on the lateral dendrite of the M-cell as individual "large myelinated club endings" [115]. Stimulation of the posterior branch of the eighth nerve evokes a mixed excitatory synaptic response in the dendrite (Fig. 1b) composed of a fast electrical potential followed by a chemical EPSP mediated by glutamate [116, 117]. Because of the fast membrane time constant of the M-cell (about 400 μs; ref. [118]), these two kinetically distinct components can be easily distinguished and reliably measured (Fig. 1b), providing a unique opportunity to dynamically explore modifications of junctional conductance under different physiological conditions.

As shown previously [113, 114], discontinuous high-frequency stimulation of the eighth nerve quickly induces homosynaptic potentiation (Fig. 1c) of both the electrical and glutamatergic components [113], which usually persist for the

remainder of the experiment (up to 2 hr). However, the changes observed in both components of the synaptic response can be transient [114], lasting only for a period of 3-10 min, suggesting in the case of these gap junctions that junctional conductance is tightly regulated, perhaps involving post-translational modifications of gap junction channels. These short-term potentiations (STP; ref. [114]) are likely to constitute the early phase of the long-lasting potentiations (LTP; ref.[113]), since in both cases the induction depends on the activation of N-methyl-D-aspartate (NMDA) receptors and an increase in postsynaptic intracellular Ca^{2+}. That is, they can be prevented by NMDA receptor antagonists and previous intradendritic injections of the Ca^{2+} chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetate (BAPTA) [113, 114]. The apparent role of Ca^{2+} contradicts the expectation that elevating its intracellular concentration should rather suppress gap junction conductance, given the direct inhibitory action of this cation on gap junction channels [119]. On the other hand, stimulating paradigms known to promote an increase in presynaptic Ca^{2+} concentration (e.g., paired pulses, continuous high-frequency stimulation) were ineffective at inducing changes in junctional conductance [114].

We therefore investigated the role of Ca^{2+} and its molecular mechanism(s) in modulating both the electrical and chemical components of synaptic transmission in the goldfish M-cell. On the basis of this indirect evidence, we postulated that gap junctional conductance at these mixed synapses can be potentiated as a consequence

of post- but not presynaptic elevations in Ca^{2+} concentration. The results presented here show that, in accord with this prediction, activity-dependent modulation of gap junctional conductance and glutamatergic transmission relies on a postsynaptic increase in the intracellular concentration of Ca^{2+}, which in turn leads to activation of Ca^{2+}/calmodulin-dependent kinase II (CaM-KII), an essential step in the induction of the modifications.

C. Material and Methods

Experimental Arrangement. Responses to stimulation of the posterior eighth nerve or to antidromic stimulation of the spinal cord were recorded intracellularly in vivo from the M-cell's lateral dendrite. The intracellular electrode was also used for iontophoretic or pressure injections. Responses were quantified after averaging sets of 20 or more consecutive traces. Student's t test was used to assess statistical significance. Errors are presented as 1 SEM. To obtain activity-dependent potentiation, discontinuous tetanic stimulation of the nerve was used (trains of 4-6 pulses at 500 Hz applied every 2 s during 4 min; strength was sufficient to orthodromically activate the M-cell at least once per train; see refs. [113, 114]). Intraterminal recordings of large myelinated club endings (5-15 μm in diameter) were

obtained at about 20 μm lateral to the initial penetration of the M-cell's lateral dendrite.

Intracellular Injections. For intradendritic injections the following compounds were added to the recording vehicle solutions and pressure injected into the M-cell's lateral dendrite (see ref. [114]): $CaCl_2$ (2-6 mM in 0.5-2.5 M KCl/10 mM Hepes, pH 7.2); EGTA (5 mM in 2.5 M KCl/10 mM Hepes, pH 7.2); or KN-93, a CaM-K inhibitor (Seikagaku America, Rockville, MD; 200-300 μM in 0.5 M KCl/10 mM Hepes, pH 7.2). CaM-KIINtide, a potent and specific peptide inhibitor of CaM-KII: aliquots (10 μl) of 100 μM CaM-KIINtide were diluted to 50-70 μM in the electrode vehicle solution (0.5 M KCl/10 mM Hepes, pH 7.2) just before use and refrigerated. Electrode resistance was about 25 M. The final intradendritic concentration of the peptide was lower than that in the electrode. -CaM-KII: aliquots (5 μl) of 10 μM -CaM-KII or heat-inactivated -CaM-KII were diluted 2-fold in the electrode recording solution (0.5 M KCl/10 mM Hepes, pH 7.2) just before use and refrigerated. It was unnecessary to add phosphatase inhibitors to the recording solution [59]. For intraterminal injections, $CaCl_2$ (2-4 mM) was added to the recording solution (2.5 M KCl/10 mM Hepes, pH 7.2) and Ca^{2+} was injected iontophoretically.

Immunohistochemistry and Immunoblot Analysis. Affinity-purified samples of anti-peptide CaM-KII antibodies directed against sequences in the autoregulatory

domain of the rat brain subunit (G-301) or at the COOH-terminus of the association domain of the /' subunits (RU-16) were used in all experiments [120]. Fish were perfused with 4% paraformaldehyde and brains were stored overnight. Vibratome sections (50 μm) were rinsed with PBS, incubated overnight with either G-301 (dilution 1:1000/5000; n = 7) or RU-16 (dilution 1:200/2000; n = 6), then rinsed in PBS and incubated for 2 hr with secondary antibodies conjugated to fluorescein isothiocyanate (FITC), CY-3, or Texas red, rinsed again with PBS, and mounted on slides. Images obtained with a confocal microscope (Bio-Rad) were processed with NIH IMAGE and CANVAS 3.1. Controls (n = 3) were obtained in the absence of primary antibodies. To assess the specificity of both antibodies in goldfish brain, immunoblots were prepared as described [120], using ECL (enhanced chemiluminescence) reagents (Amersham) for detection. Rat cerebral cortex was rapidly dissected and homogenized in hot (95°C) 1% SDS. Whole goldfish brains were rapidly frozen and pooled prior to homogenization. Protein content was measured by using the BCA assay (Pierce) with BSA as the standard.

Kinase Assays. To measure the specificity of CaM-KIINtide in goldfish brain we compared its effect on the activity of three kinases, CaM-KII, protein kinase A (PKA), and protein kinase C (PKC) (Fig. 3d). One goldfish brain was homogenized in 2 ml of a hypotonic solution (10 mM Tris·HCl, pH 7.5/1 mM EDTA/1 mM EGTA/1 mM DTT and protease inhibitor cocktail) and centrifuged at 4°C, and the supernatant

was collected. Approximately 4 μg of protein was used per assay, which took place in a standard mixture of 50 mM Hepes at pH 7.5 (20 mM for PKC), 1 mM DTT, 10 mM $MgCl_2$, and 0.4 mM [-^{32}P]ATP. For CaM-KII, the activators were 0.8 mM Ca^{2+} and 2μM calmodulin; for PKA, the activator was 1 mM cAMP with 1 mM 3-isobutyl-1-methylxanthine; and for PKC it was 0.3 mM Ca^{2+}, 0.14 mM phosphatidylserine, and 3.8 μM diacylglycerol. The substrates for CaM-KII, PKA, and PKC were 40 μM Syntide 2 (Peptide Express, Fort Collins, CO), 100 μM Kemptide (Peninsula Laboratories), and 100 μM epidermal growth factor receptor (EGFR) peptide, respectively.

D. Results

Effects of Pre- and Postsynaptic Injections of Ca^{2+}. On the basis of indirect evidence, we postulated that gap junctional conductance at these mixed synapses can be potentiated as a consequence of post- but not presynaptic elevations in Ca^{2+} concentration. We directly tested this hypothesis by performing intradendritic and intraterminal recordings and injections close to the synapses themselves, a unique experimental advantage of this system. As predicted, intradendritic pressure injections of Ca^{2+} enhanced both components of the synaptic response. These enhancements grew slowly over a period of 10-15 min, attaining postsynaptic

response magnitudes of approximately 150% of controls (Fig. 1 d-f). The M-cell antidromic spike height, a measure of the cell's input resistance, remained unchanged (Fig. 1f). These Ca^{2+}-induced potentiations occluded those evoked by eighth nerve tetani (n = 3, not shown). Response amplitudes for the electrical and chemical postsynaptic potentials (PSPs) and antidromic spike measured after at least 20 min of recording without Ca^{2+} in the electrode solution were not significantly different from controls (Fig. 1f). Thus, postsynaptic elevations of Ca^{2+} triggered an enhancement of gap junctional conductance.

Intraterminal Ca^{2+} injections (Fig. 2a) did not enhance gap junctional conductance as measured by the electrotonic coupling potential in the terminal due to the passive dendritic depolarization produced by the antidromically evoked M-cell action potential (Fig. 2b Upper). Rather, they actually decreased the amplitude of the coupling potentials, an effect associated with a hyperpolarization of the terminals (Fig. 2b). On average (n = 11), the terminal resting potential was hyperpolarized, from 64.6 ± 1.5 mV to 73.4 ± 2.7 mV, and the coupling potential amplitude was reduced from 4.9 ± 0.4 mV to 3.7 ± 0.4 mV (Fig. 2c). Iontophoretic Ca^{2+} injections of shorter duration (not shown) resulted in reversible changes in both parameters, suggesting that these effects may be due to the activation of a Ca^{2+}-dependent potassium conductance present at many synaptic terminals [121]. Moreover, the decrease in the amplitude of the electrotonic potential can also be attributed to its

previously shown voltage dependence in the terminal [122]. Consistent with this finding, intraaxonal Ca^{2+} injections (afferent recording site in the eighth nerve root), performed while simultaneously recording the lateral dendrite, did not modify the amplitude of the orthodromically evoked unitary coupling potential, which averaged 94.1% ± 3.6% of control (n = 5). Therefore, the Ca^{2+}-dependent process that triggers the modifications in junctional conductance is most likely restricted to the postsynaptic hemichannels.

Synaptically Induced Potentiations: Evidence for the Involvement of CaM-KII. To determine whether induction of the enhancements depends on a localized rather than generalized intradendritic increase of Ca^{2+}, we compared [Addler, 1991 #290] the blocking efficacy of a slower Ca^{2+} buffer, EGTA, with that previously shown for a faster one, BAPTA [114]. EGTA (5 mM) injected intradendritically prior to tetanic stimulation was a significantly less effective blocker of the potentiations than was 5 mM BAPTA. In the presence of BAPTA, tetanic stimulation transiently depressed both components of the synaptic response, whereas a clear potentiation was observed with EGTA (Fig. 3a). To accurately measure the effect of these buffers or other compounds (see above) on the induction of these rapid activity-dependent potentiations, we compared the averages of the last 15-40 traces obtained before and after tetanic stimulation. The dramatic difference between the effects of EGTA and

BAPTA (Fig. 3e) suggests that a localized increase in Ca^{2+} is indeed responsible for the induction process.

Because this localized increase seems to be synaptically mediated through activation of NMDA receptor channels, the Ca^{2+} sensor responsible for the induction should be localized in the postsynaptic cell close to these channels. A likely candidate was multifunctional CaM-KII, whose -subunit represents a major protein in the postsynaptic densities (PSDs; ref. [123]) of vertebrate synapses and has a primary role in modulating neuronal synaptic plasticity [42, 60]. To test for involvement of CaM-KII, we first injected KN-93, a CaM-K inhibitor. As shown in Fig. 3b, KN-93 injection blocked both components of the synaptic response immediately after a tetanus, and this effect was reversible with time, presumably because of washout of the drug (Fig. 3 b and e).

The recent cloning of a CaM-KII inhibitor protein (CaM-KIIN) and a related 28-residue peptide that contains the inhibitory potency and specificity (CaM-KIINtide; see ref. [99]) provided an alternative means to examine the effect of selective inhibition of CaM-KII on the induction of the activity-dependent potentiations. Although postsynaptic injection of other CaM-KII inhibitory peptides has been shown to block induction of long-lasting potentiation in region CA1 of mammalian hippocampus, those peptides have limited CaM-KII specificity and also inhibit several other kinases, thereby complicating interpretation of their effects [58,

88, 89, 124]. In contrast, CaM-KIINtide was shown to be a potent and highly specific inhibitor of CaM-KII activity from several species [99]. The effect of CaM-KIINtide on CaM-KII, PKA, and PKC activities in soluble extracts from goldfish brain homogenates was tested (Fig. 4d). CaM-KIINtide inhibits goldfish CaM-KII with an IC_{50} of 300-400 nM, and it showed no significant inhibition of PKA or PKC at concentrations up to 10 μM. In confirmation of our hypothesis, intradendritic injections of this peptide prevented the activity-dependent potentiations (Fig. 3c). For 12 experiments in which the effect of this peptide was tested, the electrical and chemical components of the synaptic response averaged 100.3% ± 6.3% and 98.8% ± 7.6% of their control amplitudes prior to the tetanus, respectively (Fig. 3e). In addition, the effect of this peptide seems to be specific, since it has been shown that although intradendritic injections of the PKA inhibitory peptide PKI5-24 (see Fig. 3d) blocked the dopamine-mediated potentiations [125], they did not block induction of the activity-dependent enhancements [in a preliminary series, n = 5, PKI5-24 successfully prevented dopamine-evoked potentiations but was unable to block induction of activity-dependent potentiations (S. Kumar and D. S. Faber, personal communication)].

To further investigate the regulatory role of CaM-KII, we next determined whether intradendritic injections of a constitutively active form of CaM-KII could potentiate synaptic responses, as occurs in region CA1 of hippocampus [59]. Indeed,

CaM-KII injection resulted in slow increases in both components of the synaptic response to approximately 150% of control (Fig. 4a-c). On the other hand, injecting a heat-inactivated form of this kinase did not increase the synaptic responses. M-cell antidromic spike did not change significantly in both experimental conditions (Fig. 4c). These enhancements did not saturate or plateau, which prevented us from examining whether the CaM-KII-induced potentiations occluded those evoked by tetanic stimulation.

Immunohistochemistry using the G-301 antibody, which exhibits relative selectivity for the -subunit of rat brain CaM-KII (Fig. 5 a-c), demonstrated that CaM-KII was present in the M-cells, particularly in their lateral dendrites (Fig. 5c), where these afferent synapses are localized. Immunoblot analysis of goldfish brain homogenate with G-301 revealed the presence of multiple immunoreactive bands of a size similar to the rat brain -subunit (Fig. 5d). Studies using a second antibody, RU-16, directed against the C terminus of the -subunit of rat CaM-KII, showed comparable results (data not shown).

E. Discusssion

Our data show that a postsynaptic elevation of Ca^{2+} concentration is an essential step for the induction of activity-dependent potentiation of the eighth nerve

mixed synaptic responses. Although there could be other sources, this increase in Ca^{2+} is likely to be synaptically mediated, is probably localized to the postsynaptic densities (possibly forming a NMDA receptor microdomain), and leads to the activation of CaM-KII, which seems to be essential for the induction process (Fig. 5e). Because the changes are relatively rapid, and potentially transient, CaM-KII is also likely to be involved in at least the early phase of the expression of the potentiations. Likely targets of CaM-KII (Fig. 5e) are non-NMDA glutamate receptors [43, 90] and gap junction proteins [126]. Although the mechanism of action of CaM-KII on gap junction channels is presently unknown, it is likely to involve changes in single channel conductance and/or open probability, as is the case for other kinases [127].

Multifunctional CaM-KII has a well established role in modulating chemical synaptic plasticity [42, 60][Lisman, 1993 #292]. The present results show that its modulatory role is not restricted to a specific modality of synaptic transmission, and they suggest a primary role for CaM-KII in shaping and regulating interneuronal communication in general. In the case of the M-cell system, the increased synaptic gain of these eighth nerve synapses will sensitize a vital escape response, lowering its threshold to auditory stimuli.

Finally, in contrast to the generally accepted tenet, elevations of intracellular Ca^{2+} concentration can lead to an increase in gap-junctional conductance. This effect

is synaptically mediated and asymmetrici.e., restricted to only one side of the gap junction plaque. This finding suggests that the hemichannels in the membrane of the postsynaptic M-cell are likely to be different in connexin composition from those in the presynaptic side (heterologous gap junctions; refs. [128, 129]) or that there may be different regulatory mechanisms in operation on the two sides of the connection, namely the M-cell dendrite and the afferent terminals. From the functional point of view, this asymmetric regulatory phenomenon implies that each individual neuron may be capable of exerting autonomous control of the degree of intercellular communication with its neighbors. This strategy could be essential for the rapid and tight regulatory control of these gap junctions in the M-cell system.

The gap junction regulatory mechanism described here may constitute a widespread property of electrical synapses that could apply to these junctions in other tissues. As an example, a synaptically mediated unilateral control mechanism may be relevant during neural development, where both gap-junctional coupling and activity-dependent synaptic plasticity are believed to play essential roles in establishing connections and creating functional compartments [130, 131].

This work was supported by National Institutes of Health Grants DC03186 (to A.P.), NS15335 (to D.S.F), and NS27037 (to T.R.S.).

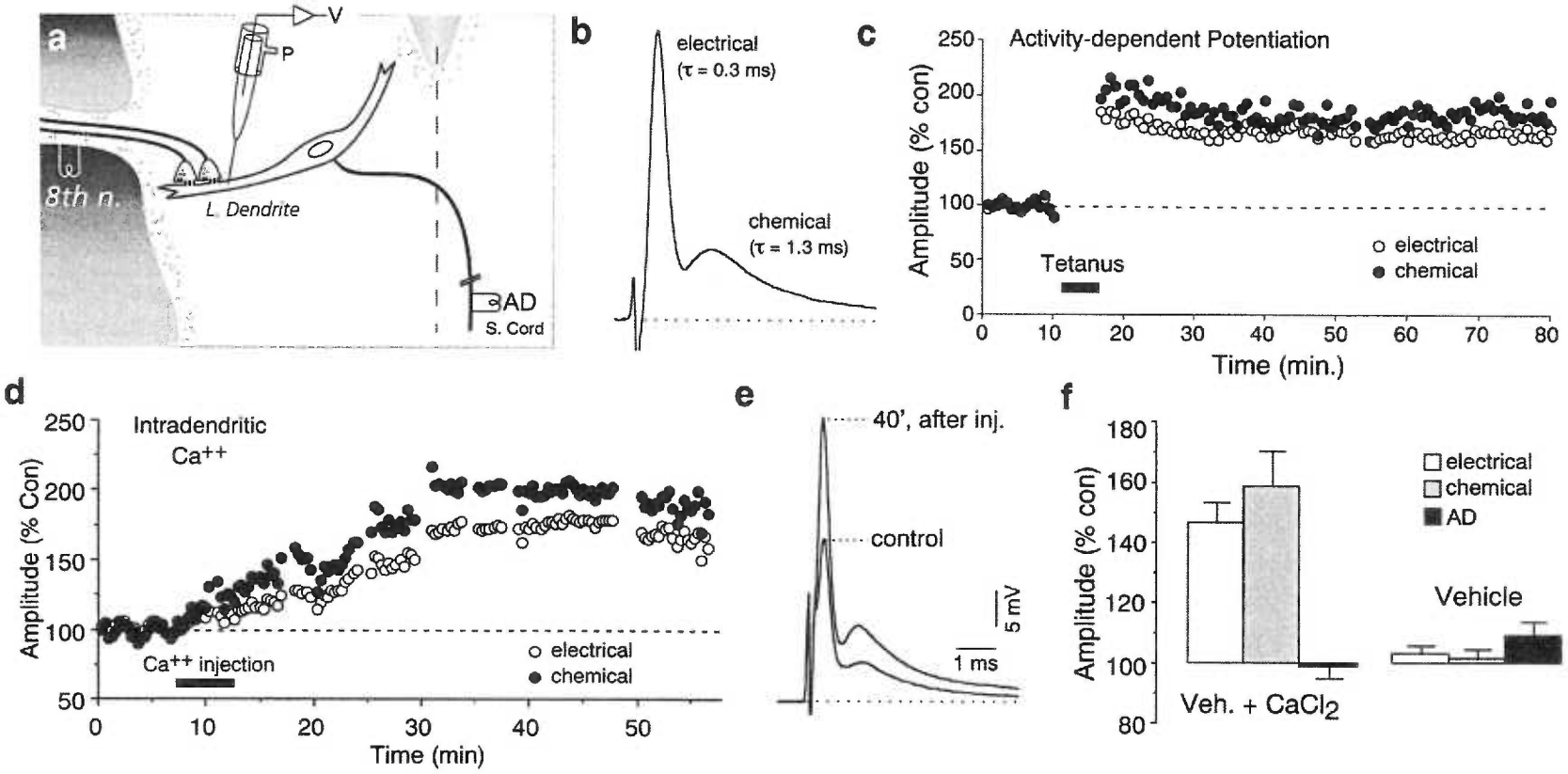

a
V
P
8th n.
L. Dendrite
AD
S. Cord
b
electrical
(τ = 0.3 ms)
chemical
(τ = 1.3 ms)
c
Activity-dependent Potentiation
Amplitude (% con)
250
200
150
100
50
0
Tetanus
electrical
chemical
0
10
20
30
40
50
60
70
80
Time (min.)
d
Intradendritic
Ca++
Amplitude (% Con)
250
200
150
100
50
Ca++ injection
electrical
chemical
0
10
20
30
40
50
Time (min)
e
40', after inj.
control
5 mV
1 ms
f
Amplitude (% con)
180
160
140
120
100
80
electrical
chemical
AD
Vehicle
Veh. + CaCl2

Figure 1. Mixed synapses on the Mauthner cell (M-cell) exhibit activity-dependent potentiations. (a) Experimental arrangement (see Material and Methods). V, voltage; P, pressure; 8th n., eighth nerve; L. dendrite, lateral dendrite; AD, antidromic stimulation of the spinal cord (S. cord). (b) Eighth nerve stimulation evokes a fast electrotonic potential followed by a chemical glutamatergic excitatory postsynaptic potential (EPSP), with the indicated decay time constants. (c) Discontinuous tetanic stimulation of the nerve produces persistent homosynaptic potentiations of both components. Plots here and in subsequent figures illustrate the amplitudes of the electrotonic (open circle) and chemical (closed circle) components versus time (each point represents the average of 20 traces) for one experiment. (d) Intradendritic injections of Ca2+ enhanced both components. (e) Superimposed traces represent the averages of 20 consecutive responses obtained in the control and 40 min after Ca2+ injection, at the maximum level of potentiation. (f) Bar plots represent the amplitudes (% of control) of the electrotonic potential, chemical EPSP, and antidromic spike (AD), all measured once the maximum potentiation had been reached. They averaged 146.4% (±7.2%) and 158.6% (±11.8%) of control for the electrical and chemical components, respectively (n = 5). Antidromic spike height, a measure of the cell's input resistance, remained unchanged (98.8% ± 3.8%). Recordings made with the vehicle solution in the electrode did not affect the amplitudes, as measured at

comparable time intervals, averaging 102.9% ± 2.8%, 101.6% ± 7%, and 109.4% ± 4.6%, for the electrical and chemical postsynaptic potentials and the AD spike, respectively (n = 5). Here and elsewhere, error bars represent 1 SEM.

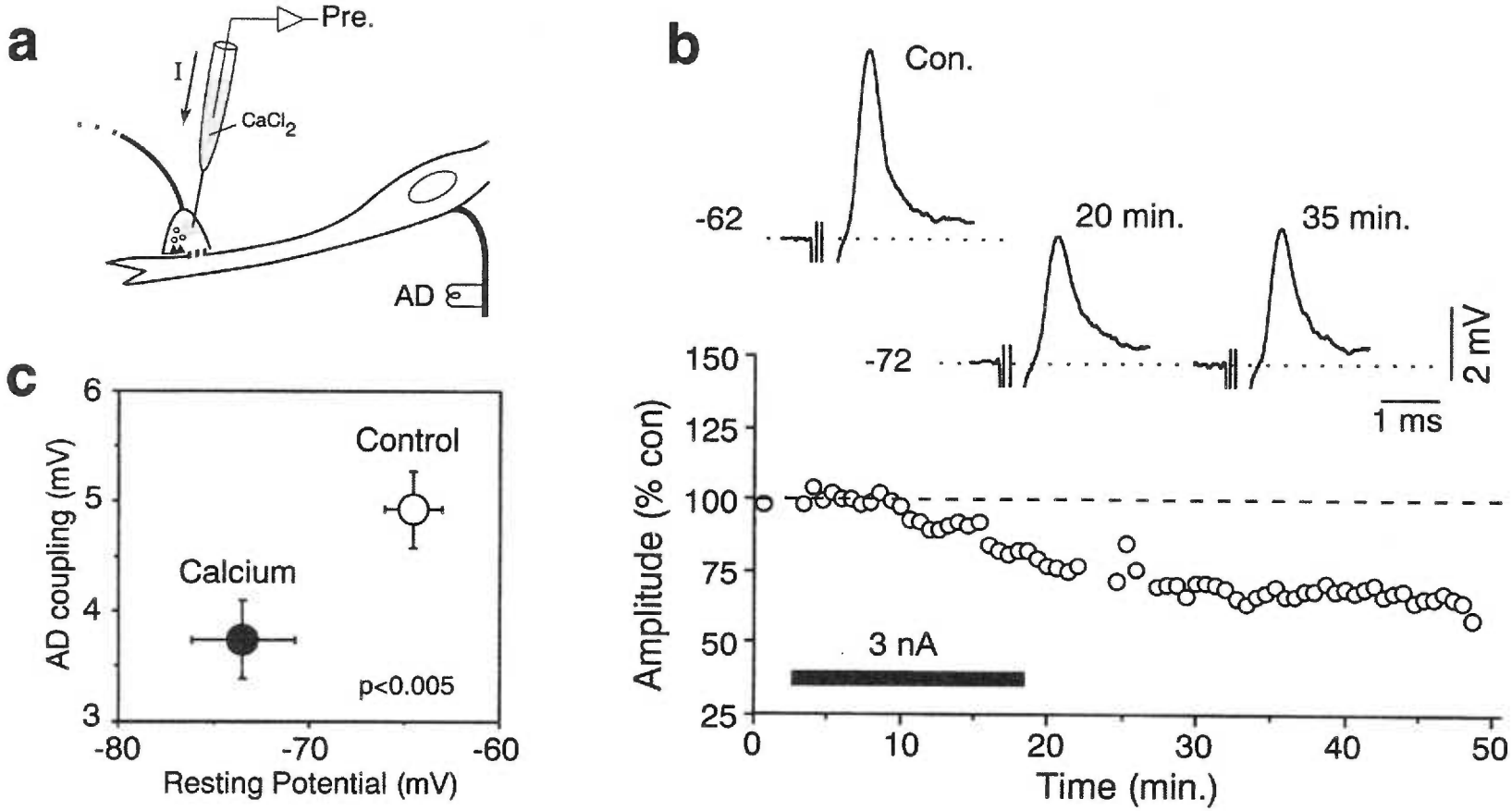

a
Pre.
I
CaCl2
AD
b
Con.
-62
20 min.
35 min.
-72
2 mV
1 ms
150
125
100
75
50
25
Amplitude (% con)
3 nA
0
10
20
30
40
50
Time (min.)
c
6
5
4
3
AD coupling (mV)
Control
Calcium
p<0.005
-80
-70
-60
Resting Potential (mV)

Figure 2. Presynaptic injections of Ca^{2+} do not increase junctional conductance. (a) Intraterminal recordings were obtained from large myelinated club endings, and Ca2+ was injected iontophoretically. (b) After Ca2+ injection the terminal was hyperpolarized from 62 to 72 mV in this case, and the antidromic coupling potential was decreased to approximately 70% of control (Upper). (Lower) Plot of the amplitude of the antidromic coupling potential versus time (each point represents the average of 10 traces) for the same experiment. (c) Diagram summarizing the values of the resting potential and coupling potential amplitudes (AD coupling) obtained for 11 terminals in control (open circle) and after at least 10 min of continuous Ca2+ injection (closed circle). The changes were statistically significant.

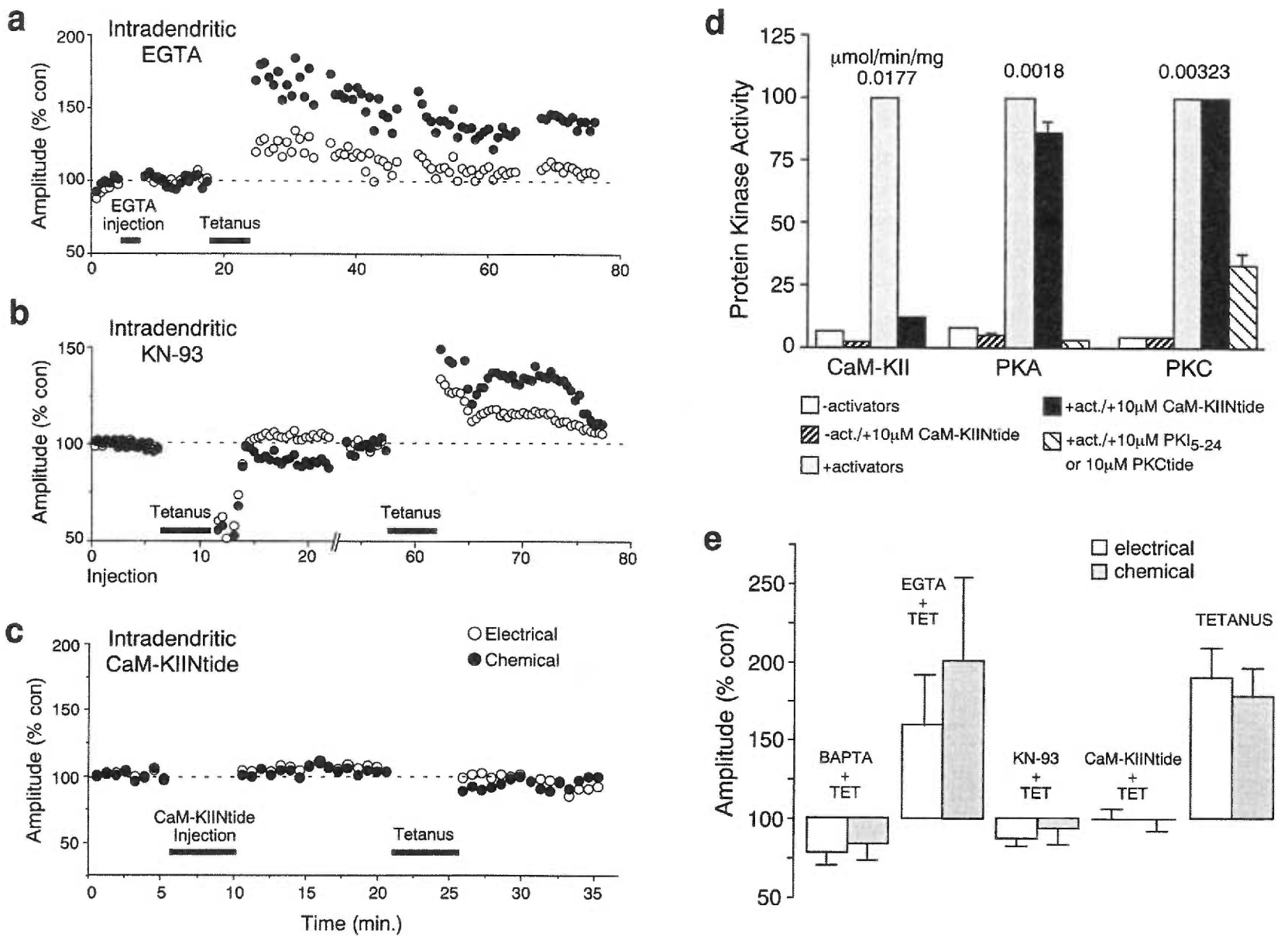

a
Intradendritic
EGTA
Amplitude (% con)
EGTA
injection
Tetanus
b
Intradendritic
KN-93
Amplitude (% con)
Tetanus
Tetanus
Injection
c
Intradendritic
CaM-KIINtide
Electrical
Chemical
Amplitude (% con)
CaM-KIINtide
Injection
Tetanus
Time (min.)
d
Protein Kinase Activity
μmol/min/mg
0.0177
0.0018
0.00323
CaM-KII
PKA
PKC
-activators
-act./+10μM CaM-KIINtide
+activators
+act./+10μM CaM-KIINtide
+act./+10μM PKI5-24
or 10μM PKCtide
e
electrical
chemical
Amplitude (% con)
BAPTA
+
TET
EGTA
+
TET
KN-93
+
TET
CaM-KIINtide
+
TET
TETANUS

Figure 3. Evidence that Ca^{2+} effects are mediated by CaM-KII. (a) Intradendritic EGTA injections did not prevent the induction of potentiations by tetanic eighth nerve stimulation. (b) Intradendritic injection of KN-93 blocked induction. In this experiment KN-93 was pressure injected for 3 min, ending at time 0, and PSP amplitudes postinjection were taken as controls. The antagonist itself had no significant effect. While tetanic stimulation 7-11 min after the injection failed (typically a transient depression followed unsuccessful tetani), a second tetanus 1 hr later successfully triggered potentiations of both components (each point represents the average of 15 traces). A new control was established at 50 min. (c) Intradendritic injections of CaM-KIINtide prevented the induction. (d) Specificity of CaM-KIINtide for CaM-KII in goldfish brain: Bar plots of protein kinase activity in the indicated conditions. CaM-KII was assayed for 1 min, PKA and PKC for 8 min each. Each column represents a mean and standard deviation of three data points. (e) To accurately estimate the effects of intradendritically injected compounds on the induction of these rapid activity-dependent potentiations, averages of the last 15-40 responses obtained before and after tetanic stimulation were compared. Bar plots represent the normalized posttetanus amplitude (% of control) of the electrotonic potential and chemical EPSP. BAPTA and EGTA at 5 mM have significantly different ($P < 0.05$) effects on induction (BAPTA + TET, EGTA + TET). In the presence of BAPTA, tetanic stimulation transiently depressed both PSPs, which

averaged 78.1% (±9.8%, SEM) for the electrical and 83.8% (±7.4%, SEM) for the chemical, of their respective control amplitudes (n = 5; ref. 4). In the presence of EGTA the two components averaged 159.6% (±33.1%) and 202.6% (±53.2%), of their respective control amplitudes (n = 5). Intradendritic injections of KN-93 blocked activity-dependent potentiations (KN-93 + TET). Electrical and chemical responses averaged 86.8% (±4.4%) and 93.7% (±10.5%), of their respective control amplitudes (n = 9). CaM-KIINtide also prevented induction of the potentiations (CaM-KIINtide + TET; n = 12). In contrast, tetanic stimulation in controls (n = 30) produced potentiations that averaged 190.02% (±19.4%) and 178.8% (±18.2%).

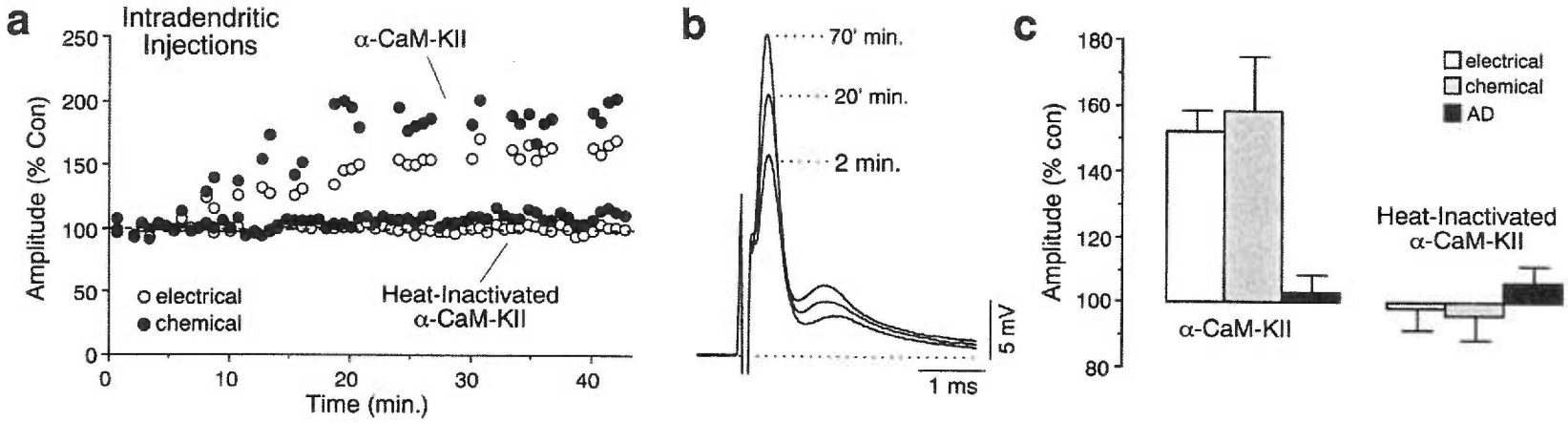
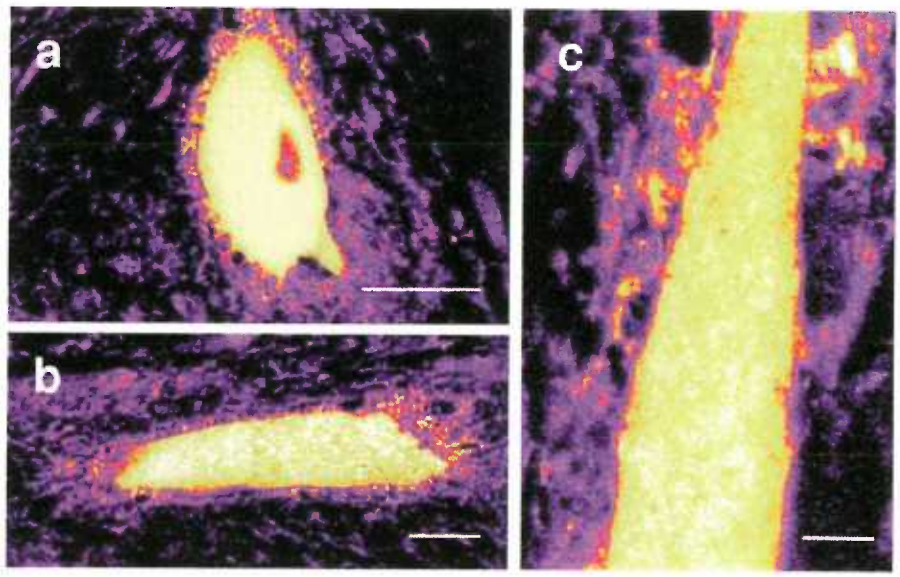
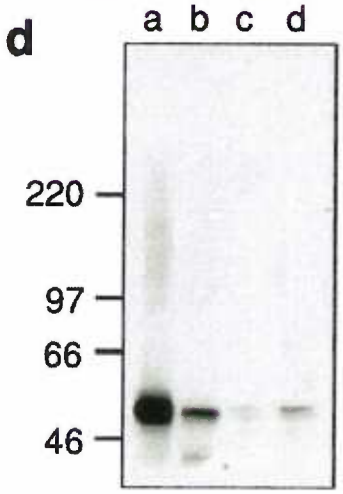

a
Intradendritic Injections
α-CaM-KII
Amplitude (% Con)
250
200
150
100
50
0
0
10
20
30
40
Time (min.)
electrical
chemical
Heat-Inactivated α-CaM-KII
b
70' min.
20' min.
2 min.
5 mV
1 ms
c
Amplitude (% con)
180
160
140
120
100
80
electrical
chemical
AD
α-CaM-KII
Heat-Inactivated α-CaM-KII

Figure 4. Intradendritic injections of a constitutively active form of -CaM-KII enhanced both components of the synaptic response. (a) Time course of two experiments in which recordings were obtained with electrodes containing either the constitutively active or a heat-inactivated form of -CaM-KII. (b) Superimposed average traces (>20) obtained at different intervals after penetrating the dendrite with an electrode containing -CaM-KII. Typically, leakage of -CaM-KII alone caused continuously increasing potentiations. (c) Bar plots represent the normalized amplitudes of the synaptic components and antidromic (AD) spike measured at the end of the recording sessions in experiments with -CaM-KII (30-80 min; electrical and chemical PSPs averaged 152.1% ± 6.5% and 158.3% ± 16.7% of their respective control amplitudes, respectively, n = 7) and heat-inactivated -CaM-KII (40-80 min; corresponding synaptic responses averaged 98.3% ± 6.6% and 96% ± 7.4%, n = 7). M-cell antidromic spike height did not change significantly in either experimental series.


a
b
c


d
a b c d
220
97
66
46

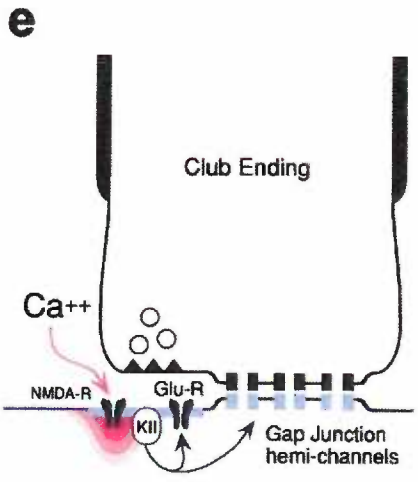

e
Club Ending
Ca++
NMDA-R
Glu-R
KII
Gap Junction
hemi-channels

Figure 5. CaM-KII immunoreactivity is present in goldfish M-cells. (a-c) Immunohistochemical evidence for the presence of CaM-KII in the M-cell. Confocal (pseudocolor; white corresponds to maximum brightness) images obtained with the antibody G-301 (1:1000; Texas red). (a) Soma. (Bar: 50 μm.) (b) Lateral dendrite. (Bar: 30 μm.) (c) Higher magnification view of another stained lateral dendrite. (Bar: 15 μm.) Note the punctate staining surrounding the M-cell soma and lateral dendrite, most likely corresponding to the presynaptic localization of this enzyme. (d) Immunoblot using G-301 (1:200 dilution) and the following: lane a, 2 μg of rat cerebral cortex homogenate; lane b, 2.5 ng of CaM-KII purified from rat forebrain (11); lanes c and d, 30 μg and 90 μg, respectively, of goldfish brain homogenate. Molecular weight markers (? 103) are shown on the left. (e) Schematic representation of the proposed potentiating pathway. KII, CaM-KII; R, receptor.

IV

DISCUSSION AND FUTURE DIRECTIONS

Phosphorylation of proteins is perhaps the most prominent mechanism for regulating their functions in cells, especially in neurons where many protein kinases and phosphatases are particularly abundant. An area of major research interest has been identifying the physiological roles for "multifunctional" protein kinases. This general family of kinases has the ability to phosphorylate many proteins *in vitro*. Identification of physiologically important substrates of CaM-kinase II has been a goal of this laboratory. Other investigators have used techniques such as mutagenesis and transgenic organisms to help identify important substrates. It was our intention to use the yeast two-hybrid system for identifying protein-protein interactions, and an inactive mutant of the catalytic domain was used as bait in hopes of stabilizing substrate interactions. However, the two distinct clones that were isolated are potent inhibitors of activated CaM-kinase II, not substrates.

There are different subcellular pools of CaM-kinase II in cells that have different physiological functions which appears to be a conserved theme throughout signal transduction pathways. For example, the localization of PKA within the cell is important for its function. Briefly, PKA is a kinase made of four subunits; two catalytic domains, and two regulatory domains. Each regulatory domain is bound to the catalytic domain by a competitive pseudosubstrate domain. It also has two sites that can bind the second messenger, cAMP. Once, cAMP binds, the regulatory domains release two catalytic domains that can then phosphorylate a multitude of different proteins.

PKA has been shown to bind to the particulate fraction of the cell such as the PSD by anchoring proteins (AKAP's). This compartmentalization may help coordinate the effector-mediated events that integrate second messenger signals. For example, AKAP's have been shown to facilitate hormone mediated insulin response by correctly localizing the enzyme to respond to the activation [132]. Another AKAP binds PKA to one of its substrates, the L-type Ca^{2+} channel [133]. Furthemore, dissociated catalytic subunits are able to translocate to other subcellular sites to phosphorylate other substrates. Although the holoezyme form of PKA is too large to traverse the nuclear pore complex, upon activation by cAMP the dissociated catalytic

subunits are able to translocate into the nucleus and phoshorylate transcription factors [134].

Another multifunctional kinase that has important subcellular localization is PKC. This family of kinases is activated in response to hormone stimulation. They are dependent of phospholipids, diacyglycerol, and in certain cases Ca^{2+}. It has been shown that upon stimulation the kinase translocates from the cytosol to the plasma membrane. There, it is able to phosphorylate endogenous substrates [135]. Furthermore, there are individual isozymes that are restricted to certain regions of the cell, and that these isozymes are also tethered to their specific substrate [136]. There also appears to be certain anchoring proteins for PKC, known as RACKS that are important for PKC-mediated functions [48]. PKC binds to RACKs after activation through a site distinct from the substrate binding site of PKC. It is thought that upon stimulation, the active PKC can be anchored to a subcellular region by RACKs for mediation of a specific signal. Specificity may be conferred through differential localization by isozyme specific RACKs [137].

Although there has not been any evidence for a scaffolding protein such as AKAP's for CaM-kinase II there is strong evidence that its localization is important for its function. The two major brain isoforms (α and β) of CaM-kinase II bind differently to the cytoskeletal elements, mediated by the β isoform [41]. This may allow for specific distribution of the enzyme depending on the amount of each

isoform within the holoenzyme. Likewise, CaM-kinase II is localized at the presynaptic terminus where it phosphorylates synapsin and may mobilize synaptic vesicles for exocytosis [50]. The mechanism of CaM-kinase II localization here is not clearly defined. CaM-kinase II is also abundant in the PSD of excitatory synapses where it phosphorylates several important regulatory proteins. Recent studies indicate that at least part of CaM-kinase II localization at the PSD is through its interaction with the NMDA-type glutamate receptor [39]. Furthermore, there is an abundant amount of CaM-kinase II within the cell body and dendrites. There is much speculation to the possible role(s) it may play at these sites.

It seems unlikely that CaM-KIIN might serve as an anchoring protein for CaM-kinase II. First, it only binds activated CaM-kinase II, and therefore it would not function analogously to AKAPs for PKA. Although only activated PKC binds RACKS, binding to RACKS does not inhibit PKC activity as binding of CaM-kinase II to CaM-KIIN does. One speculative possibility would be that CaM-KIIN binds and inhibits CaM-kinase II, but some other protein can then compete for the same binding site and release active (i.e., autophosphorylated) CaM-kinase II near strategic substrates. This would be somewhat analogous to the competitive binding of calcineurin and CaM to AKAP79. If when bound to CaM-KIIN, the CaM-kinase II were resistant to dephosphorylation on Thr^{286}, this could be a novel mechanism to store "activated" CaM-kinase II that could be released when intracellular Ca^{2+}/CaM

was low. As mentioned earlier, the microtubule associated proteins are *in vitro* substrates for CaM-kinase II. It is also known that phosphorylation of theses proteins can disrupt the cytoskeleton. Therefore, the cell may have devised the CaM-KIINs to aid in decreasing the amount of activity within these subcellular regions.

Another level of regulation of protein kinases and phosphatases is through small molecular weight proteins that bind and inhibit their catalytic domains. An inhibitor of PKA (PKI) has been known for many years that inhibits PKA by a pseudosubstate interaction [138]. Recent data would suggest that PKI may play a role in exporting PKA out of the nucleus when active [139]. PKI has been used by many investigators to better understand the role(s) of PKA within the cell. Because of its high specificity for PKA, researchers have used PKI to inhibit PKA activity and look at downstream effects. For example, PKI has been used in transient transfection experiments to identify PKA's role in the activation of prolactin gene transcription in GH_3 pituitary tumor cells [140].

CaM-KIIN appears to serve as a cellular inhibitor protein of CaM-KII, but the physiological implications of this regulation are not clear at present. The levels of CaM-KIIN must be very low compared to the amount of CaM-kinase II since one can assay brain homogenates in the presence of Ca^{2+}/CaM, conditions under which CaM-KIIN should interact and inhibit, and measure very robust CaM-kinase II activity. The fairly disperse localization of CaM-KIIN throughout the cell body and dendrites

also does not give clues to its function, although the absence of CaM-KIIN from dendritic spines suggests that CaM-KIIN is not involved in regulating synaptic transmission postsynaptically. One possibility is that the function of CaM-KIIN is to inhibit very low levels of CaM-kinase II activation present at basal levels of Ca^{2+}. For example, microtubule associated proteins are *in vitro* substrates for CaM-kinase II, and phosphorylation of theses proteins can disrupt the cytoskeleton. The presence of CaM-KIIN in the dendrites might suppress microtubule dissociation under basal conditions. Another speculative possibility is that CaM-KIIN could be under transcriptional control, especially in disease states. It is interesting to suggest that enhanced expression of CaM-KIIN could have a protective effect to the cytoskeletal changes that are seen during excitotoxic activation of CaM-kinase II. In mature primary hippocampal cultures, as the neurons begin to die, their processes begin to bleb and take on the appearance of beads on a thread which then further become fragmented [141]. This cytoskeletal change can be enhanced if the cells are treated with excitotoxic concentration of glutamate and glycine. When primary cultures were transiently transfected with GFP tagged CaM-KIIN there appeared to be a qualitative decrease in the amount of GFP labeled neurons with these cytoskeletal changes (Chang, B unpublished data). When these neurons were transfected, the GFP-CaM-KIIN was found throughout the cell, including the cell body, nucleus, dendrites and axons. This is most likely due to the overexpression of the protein. This could

suggest that the protein is limited in its distribution by interacting with other proteins. Unfortunately, this current transfection protocol used has not been very successful. It is possible to increase the efficiency using viral infection protocols. This could increase the amount of transfected cells to be able to quantify any significant differences in the population of cells.

Physiological functions of CaM-KIIN may be revealed through either mouse knockout or overexpression approaches. We have provided our CaM-KIIN cDNA to Dr. Tonegawa's laboratory which is making these mice. CaM-KIIN can also be used as a powerful tool to dissect the role(s) of CaM-kinase II. For example, the inhibitory peptide was used in gold fish Mauthner cells to inhibit CaM-kinase II activity [93]. This showed that CaM-kinase II played a major role in LTP at these sites both for glutamatergic and gap junction-mediated synaptic transmission. Also, there is recent data generated by E. Guire in our lab. using hippocampal slices. He used the Antennaepedia fusion construct of CaM-KIIN to show a blockage of LTP (Guire, E unpublished data).

CaM-kinase II activity has been implicated in many physiological effects. These functions include transcriptional activity of the prolactin gene [142], regulation of Ca^{2+} release through phosphorylation of the cardiac ryanodine receptor [143], inactivation of the M phase promoting factor (MPF) and cytostatic factor (CSF) during fertilization of Xenopus oocytes [144], and activation of chloride channels in

normal and cystic fibrosis airway epithelial cells [145]. Unfortunately, many of these experiments relied on either transfection of constitutively-active recombinant enzyme into the cell or the use of general CaM-kinase inhibitors. Therefore, these prior experiments do not rule out other kinases or a nonspecific effect due to injection of a multifunctional kinase into the cell. CaM-KIIN and its Antennaepedia fusion, since they are so specific for CaM-kinase II could be used to inhibit endogenous CaM-kinase II activity and thereby give much more interpretable results.

V

CONCLUSION

Ca^{2+} plays a pivotal role as a second messenger to activate many intracellular signaling mechanisms. A major downstream effector of this signal in neurons is CaM-kinase II. There have been many studies to show the possible role that this kinase plays in modulating channels, transcription factors and cytoskeletal elements. Furthermore, this kinase has an exquisite mechanism for regulation of the kinase activity. The current project has been able to identify a new class of proteins that further regulate CaM-kinase II activity.

First, a yeast two-hybrid screen, designed to possibly interact with substrates of this enzyme only identified this inhibitory class of proteins. This could suggest that the enzyme substrate interaction is too unstable to be detected by this *in vivo* assay. The interaction between CaM-kinase II and CaM-KIIN is quite potent, with a K_i of close to 10nM. Although the yeast two-hybrid screen was not successful in identifying CaM-kinase II substrates, these inhibitors proteins may well play a pivotal role in establishing physiological substrates.

Second, these inhibitor proteins interact within the catalytic domain of CaM-kinase II only after the autoinhibitory domain is removed by binding of Ca^{2+}/CaM or

by autophosphorylation. The fact that inhibition appears to be largely noncompetitive with substrate is potentially important. Inhibition of endogenous substrate phosphorylation would be independent of its concentration. This is also an important consideration when using CaM-KIIN as a probe (i.e., either by cell transfection or using the Antennapedia fusion construct).

Third, immunoblots suggest that the CaM-KIIN family is only expressed within the brain. Although the message for CaM-KIINβ is expressed in testes, the protein cannot be identified. This does not rule out other possible family members of CaM-KIIN that have not been identified. For example, Western blots of rat brain or hippocampal neurons show specific interaction of the CaM-KIIN antibody with a 19 kD protein. It should be noted that the antibody generated for CaM-KIIN binds to the inhibitory domain. Therefore, the antibody should identify proteins with this domain.

Fourth, CaM-KIIN is found in a specific pool within the neuron. There is overlap with CaM-kinase II within the cell body and dendrite but not in the dendritic spines or in the axon or terminal. This pool of CaM-kinase II may be important for cytoskeletal remodeling. It may be in this region that CaM-KIIN plays some role in modifying the activity of CaM-kinase II. Currently, the role for CaM-KIIN within the cell and organism is an unanswered question.

Finally, a fusion construct has been generated between CaM-KIIN and 16 amino acids from the DNA binding domain of Antennaepedia which has the capability of allowing the protein to enter cells. This has been used to inhibit CaM-kinase II activity in heterologous cells and in slice preparations. This new reagent can now be used to inhibit CaM-kinase II activity *in vivo*. Therefore, it can be used to further understand the roles that CaM-kinase II plays within the cells.

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