AUTOPHOSPHORYLATION SITES ON THE TYPE II Ca^{2+}/CALMODULIN-DEPENDENT PROTEIN KINASE: IDENTIFICATION, REGULATION OF KINASE ACTIVITY, AND SITE-SPECIFIC ANTIBODIES.

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in memoriam

Joan Eisele Mowll Patton

(1937 - 1984)

"I arise in the morning torn between a desire to improve (or save) the world and a desire to enjoy (or savor) the world. This makes it hard to plan the day.

Elwyn Brooks (E. B.) White (1899-1985).

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Abstract

Biochemical and immunological approaches have been developed to study the regulation of the rat neuronal type II Ca^{2+}/calmodulin-dependent protein kinase (type II CaM kinase) by autophosphorylation. This thesis describes the identification of *in vitro* autophosphorylation sites on the CaM kinase and their role in regulating the catalytic activity of the CaM kinase. In addition, this thesis describes the development of antibodies against the type II CaM kinase that specifically recognize either the autophosphorylated kinase or the nonphosphorylated kinase.

The autophosphorylation sites on *in vitro* autophosphorylated type II CaM kinase were identified by tryptic phosphopeptide mapping using reverse phase HPLC to isolate individual autophosphorylation sites. The sequence of the purified phosphopeptides was determined by gas phase microsequencing and compared to the known sequences of the kinase subunits, deduced from the cDNAs encoding them. The rates of site-specific autophosphorylation, or dephosphorylation by protein phosphatases, was compared with the rate of change in the Ca^{2+}/calmodulin-dependence of kinase catalytic activity. In the presence of Ca^{2+} and calmodulin, type II CaM kinase autophosphorylated an homologous residue in the α and β subunits of the type II CaM kinase, Thr^{286} and Thr^{287}, respectively. Phosphorylation of this site correlated with the generation of Ca^{2+}-independent catalytic activity. Removal of free Ca^{2+} ion

from the autophosphorylation reaction resulted in the autophosphorylation of two pairs of homologous residues, Thr^{305} and Ser^{314} in the α subunit and Thr^{306} and Ser^{315} in the β subunit. $Ser^{314/315}$ is resistant to dephosphorylation by purified protein phosphatases 1 and 2A. Selective dephosphorylation of the $Thr^{305/306}$ autophosphorylation site demonstrated that the presence of phosphate on $Thr^{305/306}$ inhibits Ca^{2+}/calmodulin-stimulated catalytic activity. The presence of phosphate on $Ser^{314/315}$ slightly decreases the sensitivity of the kinase to Ca^{2+}/calmodulin.

Antibodies that bind to the type II CaM kinase at the $Thr^{286/287}$ autophosphorylation site were produced in mice and rabbits by immunization with thiophosphorylated and nonphosphorylated peptide haptens. A monoclonal antibody was obtained that specifically recognized the autophosphorylated type II CaM kinase. The monoclonal antibody recognized the $Thr^{286/287}$ autophosphorylation site. A polyclonal antisera was obtained that, when affinity purified, specifically recognized the nonphosphorylated type II CaM kinase. Autophosphorylation of type II CaM kinase on $Thr^{286/287}$ potently inhibited binding of the polyclonal antibodies. The monoclonal antibody and polyclonal antisera recognized type II CaM kinase in immunocytochemical sections and were used to assess the extent and distribution type II CaM kinase autophosphorylation in organotypic cultures of rat brain hippocampal slices. Double immunofluorescence immunocytochemistry with the antibodies specific for phosphorylated and

nonphosphorylated type II CaM kinase indicated that most neurons and dendrites contain a mixture of phosphorylated and nonphosphorylated kinase, in varying proportions. Removal of extracellular Ca^{2+} greatly reduced the immunoreactivity specific for the phosphorylated kinase, implying that the type II CaM kinase phosphorylation state is in dynamic equilibrium in neurons.

Table of Contents

		Page
Acknowledgements		iv
Abstract		vi
Table of Contents		ix
Chapter 1.	Introduction	1
Chapter 2.	Sequences of Autophosphorylation Sites in Neuronal Type II CaM Kinase That Control Ca^{2+}-Independent Activity	65
Chapter 3.	Activation of Type II Calcium/Calmodulin-dependent Protein Kinase by Ca^{2+}/Calmodulin is Inhibited by Autophosphorylation of Threonine within the Calmodulin Binding Domain	78
Chapter 4.	Autophosphorylation-Site Specific Antibodies to the Neuronal Type II Ca^{2+}/Calmodulin-Dependent Protein Kinase	89
Chapter 5.	Immunohistochemical Detection of Phosphorylated and Nonphosphorylated Forms of the Neuronal Type II Ca^{2+}/Calmodulin-Dependent Protein Kinase	165
Appendix	Identification of Functionally Significant Phosphorylation Sites on Neuronal Proteins and Preparation of Antibodies That Recognize Them	195

CHAPTER 1

INTRODUCTION

SUMMARY

The body of this thesis, chapters 2 through 5 and the appendix, examines the enzymatic regulation of the neuronal type II Ca^{2+}/calmodulin-dependent protein kinase by autophosphorylation, and describes the development of new immunological methods for studying protein phosphorylation at higher resolution. The importance of protein phosphorylation in intracellular regulation is generally acknowledged, and a number of excellent, detailed reviews of protein phosphorylation and its role in brain function have appeared. This introductory chapter briefly reviews the control of protein function by protein phosphorylation, the role of protein phosphorylation in second messenger-mediated signal transduction mechanisms in the brain, and the regulation of protein kinases, in particular by autophosphorylation.

In such difficult fields, however, the full truth rarely emerges at one stroke. It is pieced together, little by little, through many trials and corrections.

Santiago Ramon y Cajal, 1852-1943
Recollections of My Life.

...Cajal complained that, whereas other scientists could discuss theories and hypotheses, neuroanatomists could not even agree upon the facts.

paraphrased by Marinesco (1909)

I. From Memories to Molecules.

The animal nervous system is characterized by the great variety of cell types organized in a complex reticulated fashion for the purpose of processing and relaying information. The neuronal cytoarchitecture, consisting of long branched processes terminating with distinctive intercellular contacts, synapses, is specialized for collecting and delivering information at specific sites (Ramon y Cajal, 1890). Nervous information is encoded in the particular pattern of electrical impulses that travel in waves along neuronal processes, and the particular distribution of this electrical activity among domains of the network (Shepherd, 1979; Shepherd, 1983; Kuffler *et al.*, 1984). This description emphasizes the importance of synaptic transmission. The primary mode of synaptic transmission is chemical. Presynaptic depolarization stimulates the release of neurotransmitters into the intercellular fluid; the specific binding of these chemical messengers by protein receptors in the plasma membrane of the target cell triggers an electrical or biochemical postsynaptic response. A major focus of research in the neurosciences has been the biochemistry of synaptic transmission, that is, the molecular mechanisms by which neurons release, and respond to, neurotransmitters. Of particular interest are the mechanisms by which the strength of synaptic transmission is changed, both by specific neurotransmitters, hormones, and neuromodulators, and by previous use (Kaczmarek and Levitan, 1987; Kennedy, 1989). Postsynaptic cells respond in two general ways to chemical transmission. One type of response is an immediate and relatively transient change in membrane potential, produced by the direct regulation of ion channels in the

membrane. The second type of response is slower and prolonged, acting through intracellular second messengers to produce a wide variety of responses, including modulation of neuronal excitability, neurotransmitter synthesis and release, and neuronal growth and differentiation. A large and growing body of work indicates that biochemical second messengers act principally (but not exclusively) through protein phosphorylation, and that reversible protein phosphorylation is an obligatory aspect of normal brain function.

II. Protein Phosphorylation.

Newly synthesized proteins are often posttranslationally modified as a means of controlling their function. Although many covalent modifications, such as proteolysis, are irreversible, a number are reversible. Reversible modifications include acetylation, methylation, nucletidylylation, phosphorylation, sulfation, and ADP-ribosylation. Among these mechanisms of regulating protein function, protein phosphorylation is the most universal, although recognition of its importance developed slowly. Enzymes are required to transfer the modifying group from a donor molecule, and later to remove it from the protein, usually by hydrolysis. In the case of phosphorylation, protein kinases are the enzymes responsible for the transfer of phosphate from ATP, the physiological donor molecule, to proteins. Those catalyzing hydrolysis of the phosphoprotein ester bond are protein phosphatases. Several amino acid residues are known to be phosphorylated *in vivo*, including serine, threonine, tyrosine, histidine, lysine, and hydroxylysine. Serine and threonine are the major phosphorylated residues in cellular protein; phosphotyrosine usually accounts for less than 0.5% of the total (Hunter and Cooper, 1985). The function of histidine and lysine phosphorylation is not known at this time, although specific kinases have been reported (Smith *et al.*, 1974, 1978; Huebner and Mathews, 1985; Urishizaki and Seifter, 1985). In general, protein kinases may be classified as protein serine/threonine kinases (Edelman *et al.*, 1987) or protein tyrosine kinases (Hunter and Cooper, 1985). The amino acid substrate distinction may not be complete since the fission yeast mitotic inhibitor $p107^{wee1}$ was recently shown to intermolecularly autophosphorylate

both serine and tyrosine residues (Featherstone and Russell, 1991). The activities appear to be intrinsic to one kinase since only one ATP-binding site could be identified, and its inactivation resulted in the loss of all kinase activity. Nevertheless, the serine/threonine versus tyrosine residue specificity classification is nearly complete, and is reflected in the relative amino acid sequence similarity of the kinases (Hunter, 1987).

The ability to regulate protein function by protein phosphorylation is derived in part from the high potential energy of the protein-phosphate bond. The free energy of hydrolysis (ΔG°) of the serine-phosphate bond in proteins has been determined to be about -6.5 kcal mol^{-1} (Shizuta *et al.*, 1975; El-Maghrabi *et al.*, 1980). Under similar conditions, ΔG° for free phosphoserine hydrolysis is only -2.9 kcal mol^{-1} (Shizuta *et al.*, 1975). The ΔG° for ATP was taken as -8.4 kcal mol^{-1}. In practical terms, removal of covalently attatched phosphate from proteins releases a large amount of energy not intrinsic to the phospho-serine ester bond. In reverse, this energy is imparted to the protein during the transfer of phosphate to the protein from (higher energy) ATP (phosphorylation), and is then available for straining and distorting the conformation of the protein. Indeed, the high energy of the protein-phosphate bond implies that distortions in the protein structure are required in order to accomodate the highly charged phosphate group. In many cases, this change in protein conformation alters the behavior of the protein.

Burnett and Kennedy made the first observation of kinase catalyzed protein phosphorylation in liver (Burnett and Kennedy, 1954). The first

discovery of a substrate protein functionally controlled by protein phosphorylation was the demonstration that glycogen phosphorylase was converted between an inactive, unphosphorylated form and an active, phosphorylated form (Sutherland and Wosilait, 1955; Fischer and Krebs, 1955). Phosphorylase kinase, the kinase responsible for the activation, was purified and itself found to be activated by phosphorylation (Krebs *et al.*, 1959). This phosphorylase kinase *kinase* (*sic*) was later identified as the cAMP-dependent protein kinase (Walsh *et al.*, 1968; Kuo and Greengard, 1969). Cyclic AMP had been identified by Sutherland and co-workers as the intracellular mediator of adrenalin-stimulated glycogen breakdown (Rall *et al.*, 1957, 1958; Sutherland and Rall, 1958; Robison *et al.*, 1968), work from which was born the concept of second messengers (Sutherland, 1965).

In studying cellular regulation, second messengers and protein phosphorylation have been intertwined ever since, and the story of the hormonal control of glycogen metabolism through second messenger activated protein phosphorylation has become the conceptual theme of signal transduction. There are now countless proteins known to be regulated by phosphorylation, many of them enzymes, quite a few of them protein kinases (Shacter *et al.*, 1986). Protein phosphorylation is, in general, a mechanism for processing extracellular signals such as hormones and neurotransmitters (Greengard, 1978; Krebs and Beavo, 1979; Nestler and Greengard, 1984; Krebs, 1986). Protein phosphorylation systems are, therefore, tightly regulated. Most protein phosphorylation systems are activated by increasing the activity of kinases rather than inhibiting protein phosphatases, although some phosphatases are

regulated by extracellular signals (Cohen, 1982; Ingebritsen and Cohen, 1983a,b). Protein kinases are activated by two principal mechanisms. Some kinases, notably protein tyrosine kinases, are associated with membrane receptors and are activated directly upon binding the extracellular messenger. Examples include the insulin and epidermal growth factor (EGF) receptors (reviewed by Yarden and Ullrich, 1988). Most kinases, however, are activated through chemical second messengers.

III. Second Messengers.

Although there is a limited repertoire of known second messenger molecules, the number is growing. The best understood include the cyclic nucleotides adenosine 3´,5´-monophosphate (cAMP) and guanosine 3´,5´-monophosphate, cytosolic free calcium ions ($[Ca^{2+}]_i$), 1,2-diacylglycerol (DAG), and D-myo-inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$). Evidence indicates that arachidonic acid and its lipoxygenase metabolites may also function as second messengers (Piomelli and Greengard, 1990).

The free radical nitric oxide also appears to function as a cellular messenger (Garthwaite, 1991), although its membrane permeability limits its role in cell-specific regulation. The major action identified in the nervous system so far is the activation of the soluble form of guanylate cyclase (Bredt and Snyder, 1989). Since nitric oxide synthase is a Ca^{2+}/calmodulin-dependent enzyme (Bredt and Snyder, 1990), the action of nitric oxide may be largely coupled to the regulation of this second messenger.

III.1 *Cyclic Nucleotides.*

The cyclic nucleotides, cAMP and cGMP, are produced from ATP and GTP by adenylyl and guanylyl cyclases, respectively. The cyclases are membrane-associated enzymes that are activated in response to binding of extracellular messengers (e.g. hormones) to specific receptors. The intracellu-

lar concentration of cyclic nucleotide remains elevated until the cyclase is inactivated, allowing phosphodiesterases to reduce the cylic nucleotide concentration to basal levels (Goldberg and Haddox, 1977; Rall, 1982). Using photoaffinity analogs of cAMP and cGMP, the principal intracellular targets of cAMP and cGMP have been shown to be the cAMP- and cGMP-dependent protein kinases (Walter *et al.*, 1977; Casnellie *et al.*, 1978). These serine/threonine kinases phosphorylate a wide variety of substrate proteins, thereby regulating many cellular events (reviewed by Beavo and Mumby, 1982; Beebe and Corbin, 1986). Not all effects of cAMP and cGMP are through protein kinase activation. Cyclic nucleotides have also been shown to directly regulate membrane ion channels in photoreceptors (Fesenko *et al.*, 1985; reviewed in Stryer, 1986; Yau and Baylor, 1989) and olfactory receptors (Nakamura and Gold, 1987; Dhallan *et al.*, 1990).

III.2 *Calcium.*

In most cells, $[Ca^{2+}]_i$ is maintained at low resting levels (10 to 100 nM) and regulated by controlling the conductance properties of Ca^{2+}-selective ion channels in the plasma membrane and endoplasmic reticulum (or calciosomes) (reviewed in Tsien and Tsien, 1990; Berridge and Irvine, 1989). $[Ca^{2+}]_i$ may rise as high as 1 μM in response to some signals (Berridge, 1984; Murphy and Miller, 1988). The rise is generally transient, but in some cases may undergo sustained oscillations under complex feedback control mechanisms involving Ca^{2+}-regulated Ca^{2+}-release from intracellular stores (reviewed in Berridge *et al.*, 1988; Berridge and Irvine, 1989; Rink and Jacob, 1989; Tsien and Tsien, 1990). The number and character of Ca^{2+} channels is remarkably varied. Classified by regulatory mechanism, they include voltage-operated, receptor-operated, second messenger-operated, and mechanically-operated channels (VOCs, ROCs, SMOCs, and MOCs, respectively; Tsien and Tsien, 1990; after Meldolesi and Pozzan, 1987).

In excitable cells, Ca^{2+} channels maintain the only link between membrane depolarization and cellular regulation (Hille, 1984). The actions of $[Ca^{2+}]_i$ are mediated by Ca^{2+}-binding proteins, of which calmodulin is the most prominent. Calmodulin is present at high concentration in virtually all eukaryotic cells. A number of related molecules exist for specialized functions, including troponin C, parvalbumin, and the β-subunit of calcineurin, a protein phosphatase. Ca^{2+}/calmodulin regulates a much broader assortment of enzymes than either the cyclic nucleotides (above) or DAG (below). Ca^{2+}/cal-

modulin-regulated enzymes include several protein kinases (Stull *et al.*, 1986; Kennedy *et al.*, 1987), the protein phosphatase calcineurin (Yang *et al.*, 1982), cyclic nucleotide phosphodiesterase (Kakiuchi and Yamazaki, 1970; Teo and Wang, 1973; Kincaid and Vaughan, 1986), adenylyl and guanylyl cyclases (Kakiuchi *et al.*, 1981; Cheung *et al.*, 1975), phospholipase A_2 (Wong and Cheung, 1979), nitric oxide synthase (Bredt and Snyder, 1990), and the intracellular protease calpain (Pontremoli and Melloni, 1986). Thus, in addition to directly regulating protein phosphorylation, Ca^{2+} influences the metabolism of the other known second messengers as well.

III.3 *Diacylglycerol and Inositoltrisphosphate.*

DAG and Ins(1,4,5)P_3 are produced simultaneously by the hydrolysis of membrane-bound inositol phospholipid (reviewed in Berridge and Irvine, 1989). Hydrolysis is initiated by the stimulation of phosphoinositidase C coupled to cell-surface receptors by G proteins. DAG is the physiological activator of protein kinase C, increasing its affinity for Ca^{2+} (Kishimoto *et al.*, 1980; Nishizuka, 1986). While kinase C is soluble, DAG is strongly hydrophobic and remains in the plasma membrane where it is generated. Therefore, activation of the soluble protein kinase C by DAG requires the translocation of the enzyme from the cytosol to the plasma membrane, localizing its activity. Protein kinase C is similar to the cyclic nucleotide-dependent protein kinases in its broad specificity for substrate proteins, and its serine/threonine specificity. The appearance of DAG in the membrane is transient. DAG is removed within seconds to minutes by conversion to phosphatidic acid (Majerus *et al.*, 1986; Majerus *et al.*, 1988), and by degradation to arachidonic acid (Holub *et al.*, 1970). Ins(1,4,5)P_3 releases Ca^{2+} from non-mitochondrial intracellular stores. In conjunction with other inositol phosphate metabolites, such as Ins(1,3,4,5)P_4, Ins(1,4,5)P_3 has also been implicated in the stimulation of slow Ca^{2+} entry through the plasma membrane (Berridge and Irvine, 1989). Thus, DAG and Ins(1,4,5)P_3 act in concert to regulate a wide array of cellular processes (Berridge and Irvine, 1989; Nishizuka, 1988).

III.4 *Arachidonic Acid and Metabolites.*

The production of arachidonic acid and its eicosenoid metabolites is stimulated in neuronal tissue by a variety of neurotransmitters, including glutamate, norepinephrine (α-adrenergic receptors), histamine, and serotonin (reviewed in Piomelli and Greengard, 1990). The metabolism of arachidonic acid is complex, and the identity of the active eicosenoids is unknown in most cases, as is their mechanism of action. Nevertheless, a number of studies have demonstrated their role as second messengers. In some neuronal preparations, eicosenoids appear to reduce neuronal excitability through the activation of K^+ channels (Piomelli *et al.*, 1987a, 1989; Schweitzer *et al.*, 1990). For example, in the marine mollusc *Aplysia*, it has been suggested that K^+ channel activation by an unidentified eicosenoid (the 12-lipoxygenase-derived eicosenoid 12-HPETE is a precursor) underlies the presynaptic inhibition of neurotransmitter release caused by histamine in the neural ganglia (Piomelli *et al.*, 1987a, 1987b, 1989). In this case, the evidence suggests that protein phosphorylation is not required, nor are G proteins involved (Belardetti *et al.*, 1989). However, micromolar concentrations of arachidonic acid have been found to activate protein kinase C (γ isozyme) by a mechanism that does not depend on Ca^{2+}, phospholipid, or DAG (Naor *et al.*, 1988). In contrast, 12-HPETE was recently found to be a potent inhibitor of the type II Ca^{2+}/calmodulin-dependent protein kinase (IC_{50}, 0.7 uM) *in vitro* and in intact synaptic terminals; other protein kinases were not inhibited (Piomelli *et al.*, 1989). There is no direct evidence to date indicating that eicosenoid regulation of protein kinase activity affects neuronal transmission. In contrast with the

results in invertebrates, arachidonic acid has been proposed as an extracellular *retrograde* synaptic messenger in mammalian brain, capable of enhancing synaptic transmission (Williams *et al.*, 1989). Reconciliation of these disparate functions of arachidonic acid and its metabolites will require more information about the biochemical mechanisms underlying the responses. The enzyme that catalyzes the production of arachidonic acid, phospholipase A_2, is regulated by Ca^{2+} (see above), further illustrating the interactions between the various second messenger systems, and the complexity involved in the regulation of cell function.

IV. Structure and Regulation of Protein Kinases.

Souls have complexions, too: what will suit one will not suit another.

George Eliot

Middlemarch (1872)

The number of identified and characterized protein kinases has risen dramatically over the last several years to more than 50. An even greater number of putative protein kinase sequences have been identified by molecular cloning of homologous sequences and by matching viral oncogene products to cellular protein tyrosine kinases (Hunter, 1987). This wealth of data relating kinase sequence and function has revealed some general principles about kinase domain organization and regulation of kinase catalytic activity. Known protein kinases have similar molecular topology composed of a ligand binding regulatory domain and a catalytic domain. In keeping with their role in signal transduction, the majority of protein kinases so far discovered are catalytically inactive when purified to homogeneity, as well as in unstimulated cells. In some cases, catalytic activity is apparently inhibited by an internal sequence resembling exogenous substrates (Corbin *et al.*, 1978; House and Kemp, 1987; Soderling, 1990). Protein kinases are activated by binding specific agonists, such as hormones or the intracellular second messengers described above. In the case of autoinhibited kinases, ligand-binding acts to displace the inhibitory

sequence, thereby permitting substrates access to the catalytic active site. Some kinases autophosphorylate the inhibitory sequence. Such a mechanism appears to activate the type II Ca^{2+}/calmodulin (CaM) dependent protein kinase, and the insulin receptor protein tyrosine kinase *in vitro* and *in vivo*. The cAMP-dependent protein kinase and protein kinase C are also positively regulated, in vitro, by autophosphorylation in the inhibitory domain, although the effects are more moderate and may not be physiologically important.

IV.1 *Distinct Regulatory and Catalytic Subunits: Phosphorylase Kinase and cAMP-Dependent Protein Kinase.*

In some cases, regulatory and catalytic activities reside in separate protein kinase subunits. Phosphorylase kinase and the cyclic AMP-dependent protein kinases are the best studied examples.

Phosphorylase kinase from rabbit skeletal muscle is composed of four subunits, α (M_r 118-145,000), β (M_r 108-128,000), γ (M_r 44,673), and δ (M_r 16,680), assembled into a large oligomeric structure $(\alpha\beta\gamma\delta)_4$ of M_r 1.3×10^6 (Cohen, 1973; Hayakawa *et al.*, 1973a, 1973b). Catalytic activity resides in the γ subunit (Kee and Graves, 1986). The β subunit may also be catalytically active (Fischer *et al.*, 1976; Gulyaeva *et al.*, 1977a, 1977b). The α and β subunits inhibit γ subunit activity. This inhibition can be relieved in two ways. First, Ca^{2+}-dependent binding of troponin C or calmodulin to α and/or β subunits (Picton *et al.*, 1980) activates the enzyme by increasing the $V_{max,app}$ 2- to 10-fold (Cohen, 1980; Burger *et al.*, 1983). Troponin C may be the physiological activator (Cohen, 1980; Flockhart and Corbin, 1982). Second, in the absence of troponin C or calmodulin, stoichiometric phosphorylation of the β subunit by the cAMP-dependent protein kinase activates the enzyme by decreasing the K_M for substrate (phosphorylase b) 25-fold (Yeaman and Cohen, 1975; Pickett-Geis and Walsh, 1985, 1986). The phosphorylation site has been identified (Cohen, 1985). Autophosphorylation of the same site on the β

subunit activates the enzyme in a similar fashion (Wang *et al.*, 1976; Hallenbeck and Walsh, 1983; King *et al.*, 1983). The α subunit is also phosphorylated by cAMP-dependent protein kinase, although only a modest increase in activity is associated with this phosphorylation (Hayakawa *et al.*, 1973). The δ subunit is a tightly bound calmodulin and is most closely associated with the γ subunit (Cohen *et al.*, 1978). Binding of Ca^{2+} to the δ subunit activates the holoenzyme at μM concentrations of Ca^{2+}. This Ca^{2+}/δ subunit activation is further augmented by phosphorylation of the α and β subunits (Cohen, 1980). Thus, phosphorylation of the α and β subunits both increases the sensitivity to Ca^{2+} and lowers the K_M for substrate at the active site.

The cAMP-dependent protein kinase (reviewed in Taylor, 1989) is composed of two subunit types, a catalytic subunit C (M_r 39-42,000) and one of two types of regulatory R subunit, R_I (M_r 49,000) or R_{II} (M_r 54-56,000), in a tetrameric assembly, R_2C_2. Each regulatory subunit binds two molecules of cAMP. Binding of cAMP decreases the affinity of the regulatory subunits for the catalytic subunits dramatically; at cellular concentrations of holoenzyme (approximately 0.2 to 0.7 μM), the subunits dissociate (Flockhart and Corbin, 1982). Autophosphorylation of R_{II} on Ser^{95} increases the dissociation constant for the R_2C_2 complex and slows the rate of reassociation (Rangel-Aldao, 1976a, 1976b). Ser^{95} is part of a substrate recognition sequence (Arg-Arg-X-Ser) internal to the R_{II} subunit that is essential for potent C subunit inhibition

(Kuret *et al.*, 1988). Thus, its phosphorylation may decrease the inhibitory interaction of this domain with the catalytic active site of the C subunit. Although Ser^{95} is phosphorylated in intact tissues by interventions that change cAMP levels (Scott and Mumby, 1985), a physiological role has not been established. R_I does not have a phosphorylatable residue at the corresponding position and is not autophosphorylated. By analogy with the R_{II} subunit, the R_I sequence (Arg^{94}-Arg-Gly-Ala^{97}) is thought to act as a "pseudosubstrate" that competetively blocks the active site of the C subunit in the holoenzyme complex. Interestingly, a nearby residue, Ser^{99} (Hashimoto *et al.*, 1981; Titani *et al.*, 1984) is phosphorylated by the cGMP-dependent protein kinase *in vitro*. Phosphorylation at this site results in a reduction of R_I inhibitory activity towards the C subunit, but phosphorylation of this site *in vivo* is uncertain (Geahlen and Krebs, 1981).

IV.2 *Activity and Regulation in Kinases with a Single Subunit Type: cGMP-Dependent Protein Kinase and Protein Kinase C.*

In many kinases, the catalytic and regulatory domains are contained within a single polypeptide. The cyclic GMP-dependent protein kinase and protein kinase C are examples.

The cytosolic form of cGMP-dependent protein kinase is composed of two identical subunits (M_r 76,331, bovine heart and lung) (Takio *et al.*, 1984) linked by disulfide bonds (Gill *et al.*, 1977). A distinct membrane-bound form has been described from intestinal brush border (de Jonge, 1981). Several regions of the cGMP-dependent protein kinase show high sequence homology with the cAMP-dependent protein kinase, including the cyclic nucleotide-binding and catalytic domains (Wernet *et al.*, 1989). The two subunits of cGMP-dependent protein kinase are thought to be arranged in antiparallel fashion with the regulatory N-terminal domain of one subunit inhibiting the C-teminal catalytic domain of the other (Gill, 1977; Lincoln *et al.*, 1978; Monken and Gill, 1980). The cGMP-dependent protein kinase is activated by nanomolar cGMP as well as micromolar cAMP and cIMP (Lincoln and Corbin, 1983). Unlike the cAMP-dependent protein kinase, the subunits do not dissociate upon activation. Although the cGMP-dependent protein kinase (α type) autophosphorylates stoichiometrically *in vitro*, the rate is quite slow (Foster *et al.*, 1981). cGMP does not stimulate autophosphorylation, but cAMP does so at micromolar

concentrations. The maximal turnover number is still quite low ($k = 0.07$/min; Foster *et al.*, 1981), belying a physiological role. The major site of autophosphorylation *in vitro* is Thr^{58} in the regulatory domain of the enzyme (Takio *et al.*, 1983, 1984; Aitken *et al.*, 1984) although cAMP stimulates the substoichiometric phosphorylation of as many as five additional sites (Aitken *et al.*, 1984). Thr^{58} appears to be part of an autoinhibitory sequence, Pro^{55}-Arg-Thr-Thr^{58}. Autophosphorylation causes an approximately 10-fold reduction in the $K_{a,app}$ for cAMP, to about 0.17 µM (Landgraf *et al.*, 1986). Thus, if autophosphorylation of the cGMP-dependent protein kinase does occur *in vivo*, this might allow activation by cAMP as well as cGMP.

Protein kinase C purified from brain, where it is highly concentrated, is composed of a single subunit species (Kikkawa *et al.*, 1982). Several highly homologous isozymes (Mr 76,800 to 83,500) have been identified by molecular cloning (reviewed in Kikkawa *et al.*, 1989). One form (γ) is apparently expressed only in the central nervous system. Protein kinase C is reversibly activated by Ca^{2+}-dependent association with phospholipid and sn-1,2-diacylglycerol (DAG) (Takai *et al.*, 1979; Kishimoto *et al.*, 1980). DAG is capable of activating the enzyme at basal $[Ca^{2+}]_i$, but increases in $[Ca^{2+}]_i$ may also contribute to kinase C activation. The various purified isozymes show subtle differences in catalytic activity and sensitivity to Ca^{2+} (Kikkawa *et al.*, 1989).

Kinase C was originally discovered as a proteolytically activated "proenzyme" (Inoue *et al.*, 1977). Limited digestion with trypsin or Ca^{2+}-dependent neutral protease (calpain) splits the kinases into a M_r 51,000 carboxy-terminal fragment possessing full catalytic activity and complete independence from Ca^{2+}, phospholipid, and diacylglycerol, and an amino-terminal M_r 30,000 fragment that still binds the activators (Huang and Huang, 1986). Similar proteolytic activation has been reported for several other protein kinases *in vitro*, including phosphorylase kinase (Pickett-Gies and Walsh, 1986), the type II CaM kinase (LeVine and Sahyoun, 1987; Kwiatkowski and King, 1989; Colbran *et al.*, 1989), myosin light chain kinase (MLCK) (Edelman *et al.*, 1985; Stull *et al.*, 1986), and growth factor receptor protein tyrosine kinases (Yarden and Ullrich, 1988). Because of the moderately high concentrations of Ca^{2+} required to activate calpain digestion of protein kinase C, and the rapid digestion of the active fragment in cellular systems, the physiological relevance of proteolytic activation of kinase C has not been convincingly established (Kikkawa *et al.*, 1989). Nevertheless, activation by proteolysis indicates the presence of a regulatory domain responsible for inhibition of catalytic activity in the full size enzyme. Accordingly, binding of Ca^{2+}, DAG and phospholipid to the amino-terminal regulatory domain relieves the inhibitory action of this domain on the catalytic center of the kinase.

Based upon similarities between particular internal protein kinase C amino acid sequences and sequences surrounding phosphorylation sites in

protein kinase C substrates, House and Kemp (1987) proposed that a pseudosubstrate autoinhibitory domain resided between residues 19 and 31 of the α isozyme. This sequence feature is conserved in all kinase C isozymes. In addition, these investigators found that a synthetic peptide with the proposed autoinhibitory sequence (residues 19 to 36) was a potent competetive inhibitor of kinase C phosphorylation of other synthetic peptide substrates.

Kinase C autophosphorylates following ligand-dependent activation. Autophosphorylation appears to modestly increase kinase C sensitivity to Ca^{2+} and DAG (Huang *et al.*, 1986), and to increase its sensitivity to proteolysis (Ohno *et al.*, 1990). Six prominent autophosphorylation sites have been identified in the βII isozyme (Flint *et al.*, 1990). Not all six are autophosphorylated in a single enzyme molecule. They are located in three widely separated regions of the primary sequence. One set of sites is near the amino-terminus (immediately before the proposed autoinhibitory domain), one near the proteolytic cleavage site, and the third near the carboxy-terminus. Since autophosphorylation of protein kinase C appears to be an intramolecular reaction (within one molecule) (Huang *et al.*, 1986), these three regions are likely to be arranged near each other and the active site, in the folded polypeptide (Flint *et al.*, 1990).

IV.3 *Ca^{2+}/Calmodulin-Dependent Protein Kinases: MLCK and the Type II CaM kinase.*

The best-studied CaM-dependent kinases are myosin light chain kinase (MLCK) and the neuronal type II CaM kinase (also known as multifunctional CaM-dependent protein kinase). The CaM kinases represent a sort of medley with respect to their domain structure. In addition to the active site, the catalytic subunit(s) contains a calmodulin binding domain and an inhibitory sequence. Calmodulin itself may be considered a dissociable subunit, shared among all Ca^{2+}/calmodulin regulated enzymes. Exogenous calmodulin has been referred to as the δ' (delta prime) subunit of phosphorylase kinase (Pickett-Gies and Walsh, 1986). Thus, part of the regulatory structure of CaM kinase is contained within the catalytic polypeptides, while another part (the Ca^{2+}-binding domain) associates with the holoenzyme only in the presence of the activating ligand, Ca^{2+}.

Myosin light chain kinase is a single polypeptide chain. A number of isozymes (M_r 68-150,000) have been characterized from various muscle and non-muscle tissues. The complete amino acid sequence of the skeletal muscle isozyme is known (Mayr and Heilmeyer, 1983a, 1983b), as is the carboxy-terminal sequence of the smooth muscle isozyme (Guerriero *et al.*, 1986). The amino-terminal half of the kinase is proline-rich and is thought to form a rod-like extension that may localize MLCK to myosin filaments. The carboxy-half

of the kinase contains the conserved kinase catalytic sequence (Hanks *et al.*, 1988) and the calmodulin binding domain. All MLCK isozymes absolutely require Ca^{2+}/calmodulin for catalytic activity.

Comparison of the MLCK CaM-binding domain sequence with the sequences of the phosphorylation sites of myosin P-light chains (Perrie *et al.*, 1973; Pearson *et al.*, 1984) reveals significant similarities in the number and distribution of basic amino acid residues. In the myosin light chains, the basic residues are important MLCK substrate specificity determinants (Kemp and Pearson, 1985; Kemp *et al.*, 1987). Kennelly *et al.* (1987) proposed that the MLCK internal substrate-like sequences might bind the active site and inhibit substrate binding. Synthetic peptides based on the myosin light chain sequence were competitive inhibitors of substrate binding (K_i = 3 µM). Proteolytically activated MLCK is inhibited more potently (Ki = 40 nM) by the peptide (Ikebe *et al.*, 1988). One interpretaion is that the endogenous inhibitory sequence has been removed allowing the inhibitory peptide to bind in a more "native" orientation.

Autophosphorylation of several MLCK isozymes has been reported (Walsh *et al.*, 1980; Wolf and Hofmann, 1980; Rappoport and Adelstein, 1980; Nishikawa *et al.*, 1984). However, the slow rate of autophosphorylation and the lack of any effect on catalysis or activation by calmodulin indicate that this reaction may not be biologically important. On the other hand, the cAMP-

dependent protein kinase phosphorylates two residues on smooth muscle MLCK stoichiometrically and rapidly *in vitro* (Conti and Adelstein, 1981). The phosphorylation of one residue, Ser^{512} (Lukas *et al.*, 1986), is inhibited by bound Ca^{2+}/calmodulin (Conti and Adelstein, 1981). Phosphorylation of this site also increases the K_{act} for calmodulin 10- to 20-fold, an effect that is reversible by protein phosphatase catalyzed dephosphorylation (Conti and Adelstein, 1981). Ser^{512} is located at the carboxy-terminal end of the calmodulin binding domain.

Adelstein and colleagues proposed that cAMP may mediate β-adrenergic stimulation of smooth muscle relaxation by phosphorylating MLCK and reducing its sensitivity to Ca^{2+}/calmodulin. However, a number of studies have found no effect of adrenergic agonists on MLCK activity or phosphorylation state (reviewed by Stull *et al.*, 1986). Although protein kinase C also phosphorylates Ser^{512} *in vitro* (Nishikawa *et al.*, 1985), a physiological role for this event has not been established either.

The type II CaM kinase is most highly concentrated in mammalian brain tissue, comprising as much as 1% of total protein (Erondu and Kennedy, 1985). Isozymes purified and cloned from brain are the best characterized (Fukunaga *et al.*, 1982; Goldenring *et al.*, 1983; Bennett *et al.*, 1983; Yamauchi and Fujisawa, 1983; Kuret and Schulman, 1984; McGuiness *et al.*, 1985). They are large oligomeric holoenzymes (M_r 300-700,000) comprised of multiple M_r

50,000 to 60,000 subunits. The forebrain isozyme contains approximately 9 α subunits (M_r 54,000) and 3 β (M_r 60,000) or β' (M_r 58,000) subunits. The sequences for the α (Hanley *et al.*, 1987; Lin *et al.*, 1987; Bulleit *et al.*, 1988), β (Bennett and Kennedy, 1987), and β' (Bulleit *et al.*, 1988) subunits have been deduced from cDNA clones and are highly homologous. The β' subunit is derived from the β subunit mRNA transcript by alternative splicing. The sequences also confirm that each subunit is catalytic, containing consensus domain sequences for ATP binding, calmodulin binding, and kinase catalytic activity (Hanks *et al.*, 1988). The ATP-binding and catalytic domains of the kinase reside in the amino-terminal half of the sequence. The carboxy-terminal one third of the kinase has no known function or homology. Its function has been variously hypothesized to mediate subunit interactions, substrate binding, and subcellular localization. The calmodulin-binding domain is centrally located, approximately residues 290 to 314 (α subunit, corresponding to residues 291 to 315 of the β subunit).

Although there is little amino acid sequence homology among the calmodulin-binding domains of Ca^{2+}/calmodulin-binding proteins, the amino acid sequences of the CaM kinase subunits are identical to one another in this region. The sequence is also identical in the mouse and fly α subunits (Hanley *et al.*, 1989; Cho *et al.*, 1991), implying that considerable interaction between this domain and the catalytic domain of the kinase has constrained evolutionary variation of this sequence. (The overall sequence identity between fly and

rat α subunits was 70%, and 90% with conservative substitutions; Cho *et al.*, 1991.) The ability of various synthetic peptides corresponding to the CaM-binding domain sequence to compete with the kinase for calmodulin indicates that residues 296 to 309 (α subunit sequence) contribute most to the binding of Ca^{2+}/calmodulin (Payne *et al.*, 1988). A stretch of five hydrophobic residues, Thr^{305}-Thr-Met-Leu-Ala^{309}, have been identified as essential for high-affinity binding of calmodulin to the type II CaM kinase (Hanley *et al.*, 1987).

The calmodulin binding domain of the type II CaM kinase, and sequence immediately amino-terminal to it, inhibit the kinase catalytic activity. The synthetic peptide corresponding roughly to the calmodulin binding domain of the type II CaM kinase (α residues 290 to 309) competitively inhibits (K_i = 25 μM) a Ca^{2+}/calmodulin-***independent*** form of the kinase (Payne *et al.*, 1988) generated either by limited proteolysis (LeVine and Sahyoun, 1987; Kwiatkowski and King, 1989) or by autophosphorylation (described below). A slightly longer version of the peptide, extended in the amino-terminal direction (α residues 281 to 309), was far more potent (K_i = 0.2 μM). This peptide contains a consensus substrate phosphorylation sequence (Arg-X-X-Thr/Ser; Pearson *et al.*, 1985), but since the kinase is inhibited, the peptide is not phosphorylated (but see below). The first three residues appear to be important for inhibitory activity since the synthetic peptide containing residues 284 to 309 is a much less potent inhibitor of the kinase (Kelly *et al.*, 1988). The inhibitory ability of peptides that do not contain part of calmodulin-binding domain have not

been reported. Nevertheless, a CaM kinase autoinhibitory sequence appears to be located just amino-terminal to the calmodulin-binding domain; these two domains may partially overlap. This supposition is supported by studies of CaM kinase autophosphorylation.

Autophosphorylation of the type II CaM kinase regulates the kinase catalytic activity in a complex manner. The purified CaM kinase is completely dependent on Ca^{2+}/calmodulin. Binding of Ca^{2+}/calmodulin stimulates rapid autophosphorylation of Thr^{286} on the α subunit (and Thr^{287} in the β subunit), located near the calmodulin binding domain, as described above (Miller *et al.*, 1988; Schworer *et al.*, 1988; Theil *et al.*, 1988). Autophosphorylation of $Thr^{286/287(\alpha/\beta)}$ generates a Ca^{2+}/calmodulin-independent activity (Miller and Kennedy, 1986; Lai *et al.*, 1986; Schworer *et al.*, 1986; Lou *et al.*, 1986). In other words, after autophosphorylation, CaM kinase is active in the absence of Ca^{2+}. Activation occurs through an increase of the V_{max} of the kinase in the absence of Ca^{2+}, consistent with an increase in the number of catalytic sites; no significant changes in the K_{act} for Ca^{2+}/calmodulin, or K_M for ATP or substrate were observed. The level of autophosphorylation required to maximally stimulate Ca^{2+}-independent activity is only about 3 mol phosphate per mol holoenzyme (Miller and Kennedy, 1986). Since holoenzymes contain approximately 12 subunits (Bennett *et al.*, 1983), activation by autophosphorylation is highly cooperative. Activation is reversed by dephosphorylation of Thr^{286} by protein phosphatases (Miller and Kennedy, 1986; Lai *et al.*, 1986;

Miller *et al.*, 1988). The autophosphorylation data is consistent with the autoinhibitory substrate sequence model described above. Binding of Ca^{2+}/calmodulin alters the interaction of the substrate domain with the active site and activates the kinase. Autophosphorylation of Thr^{286} partially stabilizes or mimics this altered conformation, resulting in an active kinase after the dissociation of Ca^{2+}/calmodulin. In further support, inhibition of the proteolytically activated (Ca^{2+}-independent) kinase by the synthetic peptide described above (α residues 281-309, containing the calmodulin-binding and inhibitory domains, as well as the Thr^{286} autophosphorylation site) is reduced 10-fold by first phosphorylating the peptide at Thr^{286}. Although the proteolyzed kinase is active towards exogenous substrates in the absence of Ca^{2+}, phosphorylation of the α(281-290) peptide required Ca^{2+}/calmodulin.

The level of Ca^{2+}-independent activity stimulated by autophosphorylation is only 20% to 80% of the activity stimulated by Ca^{2+}/calmodulin (Miller and Kennedy, 1986; Lai *et al.*, 1986; Schworer *et al.*, 1986; Lou *et al.*, 1986). The variability appears to be due to differences in substrate protein preparations (Patton *et al.*, 1990). Autophosphorylation also continues in the absence of Ca^{2+} (Miller and Kennedy, 1986). The ability of the CaM kinase to maintain itself in a Ca^{2+}-independent state has important implications for its potential role in neuronal function (Miller and Kennedy, 1986) (see below). Recent evidence has demonstrated that the CaM kinase is autophosphorylated on $Thr^{286/287}$ in living neurons and that the basal activity level of the CaM kinase

is regulated by autophosphorylation at this site (Molloy and Kennedy, 1991; Chapter 5).

A second site on the β subunit (βThr^{382}) is autophosphorylated rapidly. Although autophosphorylation at this site has no detectable effect on CaM kinase catalytic activity, it is located in an insertion/deletion domain that is not present in either the α subunit or the β' subunit, suggesting a role in regulating the association of the CaM kinase with other cellular structures.

Continued CaM kinase autophosphophorylation in the absence of Ca^{2+} further regulates CaM kinase activity (Hashimoto *et al.*, 1987; Patton *et al.*, 1990). Several additional autophosphorylation sites have been identified (Patton *et al.*, 1990), including α subunit Thr^{305} and Ser^{314} (Thr^{306} and Ser^{315} in β subunit). Autophosphorylation of these sites has two prerequisites: 1) the kinase must first be made Ca^{2+}-independent by autophosphorylation of $Thr^{286/287}$, and 2) the free Ca^{2+} concentration must be reduced to allow calmodulin to dissociate from the holoenzyme. Autophosphorylation of $Thr^{305/306}$ inhibits Ca^{2+}-stimulated kinase activity, but has no effect on the Ca^{2+}-independent activity previously established (Patton *et al.*, 1990). Ca^{2+}-dependent activity is recovered by selective dephosphorylation of $Thr^{305/306}$, catalyzed by protein phosphatase. The $Thr^{305/306}$ residues are located in the calmodulin-binding domain of the CaM kinase, within the sequence of five hydrophobic residues, $Thr^{306/306}$-Thr-Met-Leu-$Ala^{309/310}$, that are essential for

high-affinity binding of calmodulin (Hanley *et al.*, 1988). Therefore, phosphorylation of these residues would be expected to strongly inhibit calmodulin binding, possibly by direct steric interference. The inhibition of activity associated with autophosphorylation of $Thr^{305/306}$ is produced by reducing the V_{max}, with no observed effect on K_{act} for calmodulin.

CaM kinase autophosphorylation of the other Ca^{2+}-independent autophosphorylation site, $Ser^{314/315}$, has a slightly increased K_a for Ca^{2+}/calmodulin. The location of this site within the calmodulin binding domain is homologous to the location of Ser^{512} in smooth muscle MLCK (Guerriero *et al.*, 1986; Lukas *et al.*, 1986). As described above, cAMP-dependent protein kinase phosphorylation of MLCK Ser^{512} increases the K_{act} for calmodulin 10- to 20-fold. The differences in effect of phosphorylation of homologous residues in the two enzymes may be partially explained by the lower affinity for calmodulin displayed by the type II CaM kinase (K_{act} = 30-125 nM; Kennedy *et al.*, 1987; Colbran *et al.*, 1989) relative to MLCK (K_{act} = 1-6 nM; Kennedy *et al.*, 1987; Conti and Adelstein, 1981). Thus, the CaM kinase calmodulin-binding domain already has a somewhat lower affinity for calmodulin even before autophosphorylation of $Ser^{314/315}$.

IV.5 *Variation on a Theme: Receptor Tyrosine Kinases.*

Growth factor receptors with protein tyrosine kinase activity, or receptor tyrosine kinases, present an interesting variation on the theme of regulatory and catalytic domains. Several excellent reviews have presented comprehensive information regarding their structure and role in cellular regulation (Carpenter, 1987; Yarden and Ullrich, 1988; Williams, 1989; Ullrich and Schlessinger, 1990). Rather than rely on intracellular second messengers, receptor tyrosine kinases transduce the binding of an extracellular hormone ligand into an intracellular protein phosphorylation response. Receptor tyrosine kinases are integral membrane proteins with an extracellular ligand binding domain and an intracellular catalytic kinase domain. Three subclasses have been defined. The receptors for EGF, PDGF, FGF, and CSF-1 are single polypeptides and their respective catalytic and ligand binding domains are linked through a single hydrophobic transmembrane domain of approximately 22 to 26 amino acids. The EGF receptor contains an extracellular sequence rich in cysteine residues that is repeated twice; it represents receptor tyrosine kinase subclass I. The PDGF, FGF, and CSF-1 receptors lack the repeated cysteine-rich sequence in their extracellular domain, but conserve, instead, an extracellular immunoglobulin-like repeated structure of cysteine residues and specific flanking sequences; they represent receptor tyrosine kinase subclass III (this subclass has been further divided by the number of repeated immunoglobulin-like sequences). Receptor tyrosine subclass II is represented

by the insulin receptor (InsRec), which is composed of two distinct subunits in a heterotetrameric structure, $\alpha_2\beta_2$. The heterodimeric ($\alpha\beta$) form displays reduced ligand affinity (Boni-Schnetzler *et al.*, 1986). The α subunits (calculated molecular weight 83,700, Mr 135,000 by SDS-PAGE) are extracellular and contain the insulin binding sites. They are linked by disulfide bonds to the β subunits (69,700 calculated molecular weight, Mr 95,000 by SDS-PAGE), which transverse the membrane and contain the catalytic kinase domain. The discrepancy between calculated and apparent molecular weights is due to the large amount of N-linked carbohydrate present on the extracellular domains (Carpenter, 1987).

For all receptor tyrosine kinase types, the arrangement of effector-binding and catalytic domains straddling the plasma membrane presents a potential difficulty if signal transduction occurs through an intramolecular interaction between ligand-binding and catalytic domains. Some studies have considered the possibility that altered conformations of the extracellular and intracellular domains could be coupled by obligatory changes in the transmembrane domain conformation (Kashles *et al.*, 1988; Escobedo *et al.*, 1988; Williams, 1989; Riedel *et al.*, 1989; Lammers *et al.*, 1989; Lee *et al.*, 1989). Although alterations in the transmembrane region can affect ligand-dependent kinase activation (Williams, 1989), experiments with mutant and chimeric receptors in which transmembrane domains have been swapped between various receptors indicate that large changes in sequence may be accepted with

no infuence on the ligand binding, signal transduction or the resulting kinase activity (Ullrich and Schlessinger, 1990). The last word on this subject may not yet be written.

Some evidence favors an intermolecular receptor tyrosine kinase activation mechanism. Receptors must first form dimers (or higher-order oligomers), and this process is initially dependent on binding the cognate hormone ligand. Aggregation of receptors has been detected in preparations of purified receptors, in isolated cell membranes, and in living cells (Yarden and Schlessinger, 1987a; Cochet *et al.*, 1988). Monomeric ligands such as EGF apparently induce conformational changes in the extracellular domain which favor receptor dimerization (Greenfield *et al.*, 1989). Bivalent ligands such as PDGF and CSF-1 promote dimerization by binding to two receptors. Antibodies against the EGF receptor also stimulate tyrosine kinase activity of the receptor (Schreiber *et al.*, 1981, 1983; Fernandez-Pol, 1985). The activation required multivalent antibodies, but Fab fragments worked if secondary antibodies were included. Similarly, bivalent antibodies against the insulin receptor are capable of kinase stimulation (Zick *et al.*, 1984; Heffetz and Zick, 1986). Both oligomeric EGF receptors and tetrameric insulin receptors have a higher affinity for their cognate ligands compared with the dissociated forms (100, 101, 119, 121). Chimeric receptors combining heterologous extracellular and intracellular domains of the Insulin and EGF receptors are fully functional, indicating that these two receptors share a common transduction

mechanism. Stronger evidence has recently been presented by Accili *et al.* (1991). A mutation in the human insulin receptor associated with impaired transport of the receptor to the plasma membrane, $Phe^{382} \rightarrow Val^{382}$, impairs the ability of insulin to activate receptor autophosphorylation. Insulin receptor autophosphorylation precedes and is required for activity toward substrates (discussed below). However, mixing the Val^{382} mutant receptors with Phe^{382} wild-type receptors (prephosphorylated) resulted in both phosphorylation of the Val^{382} mutant receptor and activation of its tyrosine kinase activity.

All receptor tyrosine kinases so far characterized have been shown to autophosphorylate in response to ligand activation. In the case of the insulin receptor, the effect of autophosphorylation is known. In others, autophosphorylation appears to have little or no effect. Binding of insulin to the insulin receptor *in vitro* (Kasuga *et al.*, 1982a; Avruch *et al.*, 1982; Petruzelli *et al.*, 1982) and in intact cells (Kasuga *et al.*, 1982b) results in the autophosphorylation of several tyrosine residues in the β subunit cytoplasmic domain. Autophosphorylation precedes substrate phosphorylation (Rosen, 1987), and kinase activity towards substrate is dependent on the presence of phosphate on the autophosphorylated residues (Yu and Czech, 1984). Kinetic evidence indicates that Tyr^{1150}, Tyr^{1154}, and Tyr^{1155} (human sequence) are the autophosphorylated residues responsible for kinase activation (Flores-Riveros *et al.*, 1989). Mutation of Tyr^{1150} markedly decreases the kinase activity of the insulin receptor (Ellis *et al.*, 1986). Activation of substrate phosphorylation

occurs primarily through an increase in V_{max} (Rosen *et al.*, 1983). Although the heterotetrameric insulin receptor contains two catalytic β subunits, there is no strong evidence to suggest that autophosphorylation occurs either between subunits or within a single subunit.

Activation of the EGF receptor by EGF also appears primarily as an increase in V_{max} of the kinase activity (Erneaux *et al.*, 1983; Downward *et al.*, 1985). Only small changes in K_M for ATP and substrates have been reported. The EGF receptor also autophosphorylates upon activation. Autophosphorylation is not, however, required for kinase activity toward substrate proteins. Direct evidence for an effect of EGF receptor kinase autophosphorylation on kinase activity is conflicting (Carpenter, 1987). Some investigators have reported an increase in ligand-stimulated activity (Bertics and Gill, 1985), while others found no effect (Downward *et al.*, 1985). More recently, site directed mutagenesis of the three known autophosphorylation sites to the non-phosphorylatable residue phenylalanine showed no major effect on kinase activity, on internalization and degradation, and on EGF-dependent mitogenesis of cells expressing mutant receptors (Honegger *et al.*, 1988a, 1988b). The EGF receptor is negatively regulated by protein kinase C. Phosphorylation of Thr^{654}, located near the transmembrane domain, occurs *in vitro* and *in vivo* and inhibits kinase activity (Cochet *et al.*, 1984; Friedman *et al.*, 1984; Davis, 1988). The physiological role of kinase C phosphorylation of the EGF receptor is not known. The Thr^{654} residue is not conserved in the insulin receptor.

A unifying model for growth factor receptor tyrosine kinase regulation may eventually resemble the following. The receptors have evolved physically separated ligand binding and inhibitory domains, in contrast with the serine/threonine protein kinases activated by second mesengers. In the absence of agonist, protein tyrosine kinase activity is inhibited. Agonist-induced dimerization of the receptors brings the intracellular kinase domains into association, inducing a conformational change that relieves the inhibition of the catalytic domain. Activated insulin receptor may autophosphorylate an internal inhibitory substrate sequence, thereby maintaining the tyrosine kinase in an active state after the insulin signal has dissipated, somewhat analogous to type II CaM kinase activation by autophosphorylation.

V. Intramolecular versus Intermolecular Autophosphorylation.

Protein kinases are often activated by autophosphorylation (self-phosphorylation). Occasionally, kinase autophosphorylation acts to negatively regulate kinase activity. As described above, almost all protein kinases are capable of autophosphorylation *in vitro* (Flockhart and Corbin, 1982; Krebs, 1986), including the cAMP-dependent protein kinase (types I and II), the cGMP-dependent protein kinase, phosphorylase kinase, MLCK, protein kinase C, glycogen synthase kinase 3, and a number of the hormone receptor-linked tyrosine kinases including the insulin receptor, epidermal growth factor receptor, fibroblast growth factor receptor, and several oncogene protein tyrosine kinases.

For several protein kinases autophosphorylation occurs by an intramolecular reaction, within one holoenzyme, rather than an intermolecular reaction (between enzymes). The experimental evidence for an intramolecular mechanism is the concentration-independence of the reaction with respect to kinase. This has been demonstrated for type II cAMP-dependent protein kinase phosphorylation of the regulatory (R_{II}) subunit (Rangel-Aldao and Rosen, 1976), cGMP-dependent protein kinase (Lincoln *et al.*, 1978), protein kinase C (Huang *et al.*, 1986; Newton and Koshland, 1987), type II CaM kinase (Miller and Kennedy, 1986; Lai *et al.*, 1986), the Ins-Rec tyrosine kinase (Shia

et al., 1983; Petruzzelli *et al.*, 1984; White *et al.*, 1984), and the EGF-Rec tyrosine kinase (Biswas *et al.*, 1985). Since most kinase holoenzymes contain as multiple catalytic subunits, autophosphoryaltion may actually occur between the subunits of a holoenzyme complex. Some evidence indicates that protein kinase C, which is composed of a single subunit type, may be capable of autophosphorylating several residues while in monomeric association with mixed lipid/detergent micelles (Hannun *et al.*, 1985; Newton and Koshland, 1987; Flint *et al.*, 1990). However, the monomeric nature of the kinase in these studies has not been independently confirmed. The issue of intersubunit versus intrasubunit autophosphorylation may be of interest primarily for the implications on protein conformational dynamics (Newton and Koshland, 1987). Although similar results were reported for Ins-Rec autophosphorylation (Shia *et al.*, 1983; Petruzzelli *et al.*, 1984; White *et al.*, 1984) and for the EGF-Rec (Weber *et al.*, 1984; Biswas *et al.*, 1985), more recent evidence suggests that ligand-induced receptor dimerization initiates the receptor autophosphorylation reactions, which occur between the subunits (Schlessinger, 1988; Ullrich and Schlessinger, 1990). The reaction has therefore been referred to as intermolecular. However, activated receptor dimers apparently do not rapidly dissociate into active monomers. Thus, assignment of an intermolecular or intramolecular designation to the autophosphorylation reaction is largely a matter of reference point; although the subunits are initially separated, a stable complex is formed such that monomers do not catalyze the phosphorylation of many

other kinase monomers. In this case, autophosphorylation is effectively intramolecular.

In all cases, autophosphorylation follows ligand-dependent activation. Intramolecular autophosphorylation may permit a tighter (more rapid and regular) coupling between ligand activation and autophosphorylation, independent of local enzyme concentration, than that resulting from an intermolecular mechanism. A further, and possibly more important consequence of an intramolecular autophosphorylation mechanism directly coupled to ligand-dependent activation, is that only activated enzymes will autophosphorylate; that is, phosphorylation will not propagate to non-activated enzymes. Where autophosphorylation serves to activate the kinase, an intermolecular autophosphorylation mechanism could act as a self-regenerative, activating mechanism. Such a ligand-triggered "switch" has been proposed as a biochemical memory storage mechanism (Lisman, 1985; Lisman and Goldring, 1988).

References

Accili, D., Mosthaf, L., Ullrich, A., and Taylor, S. I. (1991). A mutation in the extracellular domain of the insulin receptor impairs the ability of insulin to stimulate receptor autophosphorylation. *J. Biol. Chem.* **266**, 434-439.

Aitken, A., Hemmings, B. A., Hofmann, F. (1984). Identification of the residues on cyclic GMP-dependent protein kinase that are autophosphorylated in the presence of cyclic AMP and cyclic GMP. *Biochim. Biophys. Acta* **790**, 219-225.

Avruch, J., Nemenoff, R. A., Blackshear, P. J., Pierce, M. W., and Osathanondh, R. (1982). Insulin-stimulated tyrosine phosphorylation of the insulin receptor in detergent extracts of human placental membranes: comparison to epidermal growth factor-stimulated phosphorylation. *J. Biol. Chem.* **257**, 15162-15166.

Beavo, J. A., and Mumby, M. C. (1982). Cyclic AMP-dependent protein phosphorylation. *In* "Cyclic Nucleotides," Vol. 1 (Nathanson and Kebabian, eds.), pp. 363. Springer-Verlag, Berlin.

Beebe, S. J., and Corbin, J. D. (1986). Cyclic nucleotide-dependent protein kinases. *In* "The Enzymes," Vol. XVII (Boyer and Krebs, eds.), pp. 43-111. Academic Press, Orlando.

Belardetti, F., Campbell, W. B., Falck, J. R., Demontis, G., and Rosolowsky, M. (1989). Products of heme-catalyzed transformation of the arachidonate derivative 12-HPETE open S-type K^+ channels in *Aplysia*. *Neuron* **3**, 497-505.

Bennett, M. K., Erondu, N. E., and Kennedy, M. B. (1983). Purification and characterization of a calmodulin-dependent protein kinase that is highly concentrated in brain. *J. Biol. Chem.* **258**, 12735-12744.

Bennett, M. K., and Kennedy, M. B. (1987). Deduced primary structure of the β subunit of brain type II Ca^{2+}/calmodulin-dependent determined by molecular cloning. *Proc. Natl. Acad. Sci. (USA)* **84**, 1794-1798.

Berridge, M. J., Cobbold, P. H., and Cuthbertson, K. S. R. (1988). Spatial and temporal aspects of cell signalling. *Philos. Trans. R. Soc. London Ser. B* **320**, 325-343.

Berridge, M. J., and Irvine, R. F. (1989). Inositol phosphates and cell signalling. *Nature (Lond.)* **341**, 197-205.

Berridge, M. J. (1984). Inositol Trisphosphate and diacylglycerol as second messengers. *Biochem J.* **220**, 345-360.

Bertics, P. J., and Gill, G. N. (1985). Self-phosphorylation enhances the protein-tyrosine kinase activity of the epidermal growth factor receptor. *J. Biol. Chem.* **260**, 14642-14647.

Biswas, R., Basu, M., Sen-Majumbar, A., and Das, M. (1985). Intrapeptide autophosphorylation of the epidermal growth factor receptor: regulation of kinase catalytic function by receptor dimerization. *Biochemistry* **24**, 3795-3802.

Bredt, D. S., and Snyder, S. H. (1989). Nitric Oxide mediates glutamate-linked enhancement of cGMP levels in the cerebellum. *Proc. Natl. Acad. Sci. (USA)* **86**, 9030-9033.

Bredt, D. S., and Snyder, S. H. (1990). Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. *Proc. Natl. Acad. Sci. (USA)* **87**, 682-685.

Bulleit, R. F., Bennett, M. K., Molloy, S. S., Hurley, J. B., and Kennedy, M. B. (1988). Conserved and variable regions in the subunits of brain type II Ca^{2+}/calmodulin-dependent protein kinase. *Neuron* **1**, 63-72.

Burger, D., Stein, E. A., and Cox, J. A. (1983). *J. Biol. Chem.* **258**, 14733-14739.

Burnette, G., and Kennedy, E. P. (1954). The enzymatic phosphorylation of proteins. *J. Biol. Chem.* **211**, 969.

Carpenter, G. (1987). Receptors for epidermal growth factor and other polypeptide mitogens. *Ann. Rev. Biochem.* **56**, 881-914.

Casnellie, J. E., Schlichter, D. J., Walter, U., and Greengard, P. (1978). Photoaffinity labeling of a guanosine 3':5'-monophosphate-dependent protein kinase from vascular smooth muscle. *J. Biol. Chem.* **253**, 4771-4776.

Cheug, W. Y., Bradham, L. S., Lynch, T. J., Lin, Y. M., and Tallant, E. A. (1975). Protein activator of cyclic 3':5'-nucleotide phosphodiesterase of bovine

or rat brain also activates its adenylate cyclase. *Biochem. Biophys. Res. Commun.* **66**, 1055-1062.

Cho, K.-O., Wall, J. B., Pugh, P. C., Ito, M., Mueller, S., and Kennedy, M. B. (1991). The α subunit of type II Ca^{2+}/calmodulin-dependent protein kinase is highly conserved in Drosophila. *Neuron* (submitted for publication).

Cochet, C., Kashles, O., Chambaz, E. M., Borrello, I., King, C. R., and Sclessinger, J. (1988). Demonstration of epidermal growth factor-induced dimerization in living cells using a chemical covalent cross-linking agent. *J. Biol. Chem.* **263**, 3290-3295.

Cohen, P. (1973). The subunit structure of rabbit skeletal muscle phosphorylase kinase, and the molecular basis of its activation reactions. Eur. *J. Biol. Chem.* **34**, 1-14.

Cohen, P. (1980). The role of calcium ions, calmodulin and troponin in the regulation of phosphorylase kinase from rabbit skeletal muscle. *Eur. J. Biochem.* **111**, 563-574.

Cohen, P. (1982). Th role of protein phosphorylation in neural and hormonal control of cellular activity. *Nature (Lond.)* **296**, 613-620.

Cohen, P. (1985). *Eur. J. Biochem.* **151**, 439-448.

Cohen, P., Burchell, A., Foulkes, J. G., Cohen, P. T. W., Vanaman, T. C., and Nairn, A. C. (1978). Identification of the Ca^{2+}-modulator protein as the fourth subunit of rabbit skeletal muscle phosphorylase kinase. *FEBS Lett.* **92**, 287-293.

Colbran, R., Schworer, C., Hashimoto, Y., Fong, Y.-L., Rich, D., Smith, M., and Soderling, T. R. (1989). Calcium/calmodulin-dependent protein kinase II. *Biochem J.* **258**, 313-325.

Corbin, J. D., Sugden, P. H., West, L., Flockhart, D. A., Lincoln, T. M. and McCarthy, D. (1978). Studies on the properties and mode of action of the purified regulatory subunit of bovine heart adenosine 3':5'-monophosphate-dependent protein kinase. *J. Biol. Chem.* **253**, 3997-4003.

Davis, R. J. (1988). Independent mechanisms account for the regulation by protein kinase-C of the EGF-receptor affinity and tyrosine-protein kinase activity. *J. Biol. Chem.* **263**, 9462-9469.

de Jonge, H. R. (1981). *Adv. Cyclic Nucleotide Res.* **14**, 315-333.

Dhallan, R. S., Yau, K.-W., Schrader, K. A., Reed, R. R. (1990). Primary structure and functional expression of a cyclic nucleotide-activated channel from olfactory neuron. *Nature (Lond.)* **347**, 184-187.

Downward, J., Waterfield, M. D., and Parker, P. J. (1985). Autophosphorylation and protein kinase C phosphorylation of the epidermal growth factor receptor: effect on tyrosine kinase activity and ligand binding affinity. *J. Biol. Chem.* **260**, 14538-14546.

Edelman, A. M., Takio, K., Blumenthal, D. K., Hansen, R. S., Walsh, K. A., Titani, K., and Krebs, E. G. (1985). Characterization of the calmodulin-binding and catalytic domains in skeletal muscle myosin light chain kinase. *J. Biol. Chem.* **260**, 11275-11285.

Edelman, A. M., Blumenthal, D. K., and Krebs, E. G. (1987). Protein serine/threonine kinases. *Ann. Rev. Biochem.* **56**, 567-613.

El-Maghrabi, M. R., Haston, W. S., Flockhardt, D. A., Claus, T. H., and Pilkis, S. J. (1980). Studies on the phosphorylation and dephosphorylation of L-type pyruvate kinase by the catalytic subunit of cyclic AMP-dependent protein kinase. *J. Biol. Chem.* **255**, 668-675.

Ellis, L., Clauser, E., Morgan, D. O., Edery, M., Roth, R. A., and Rutter, W. T. (1986). Replacement of insulin receptor residues 1162 and 1163 compromises insulin-stimulated kinase activity and uptake of 2-deoxyglucose. *Cell* **45**, 721-732.

Erneaux, C., Cohen, S., and Garbers, G. L. (1983). *J. Biol. Chem.* **258**, 4137-4142.

Escobedo, J. A., Keating, M. T., Ives, H. E., and Williams, L. T. (1988). Platelet-derived growth factor receptors expressed by cDNA transfection couple to a diverse group of cellular responses associated with cell proliferation. *J. Biol. Chem.* **263**, 1482.

Featherstone, C., and Russell, P. (1991). Fission yeast p107^{wee1} mitotic inhibitor is a tyrosine/serine kinase. *Nature (Lond.)* **349**, 808-811.

Fernandez-Pol, J. A. (1985). Epidermal growth factor receptor of A431 cells: characterization of a monoclonal anti-receptor antibody noncompetitive agonist of epidermal growth factor action. *J. Biol. Chem.* **260**, 5003-5011.

Fesenko, E. E., Kolesnikov, S. S., and Lyubarsky, A. L. (1985). Induction by cyclic GMP of cationic conductance in plasma membrane of retinal rod outer segment. *Nature (Lond.)* **313**, 310-313.

Fischer, E. H., and Krebs, E. G. (1955). Conversion of phosphorylase b to phosphorylase a in muscle extracts. *J. Biol. Chem.* **216**, 121-132.

Fischer, E. H., Blum, H. E., Byers, B., Heizman, C., Kerrick, G. W., Lehky, P., Malencik, D. A., and Pocinwong, S. (1976). *In* "Metabolic Interconversion of Enzymes." (Shaltiel, E., Ed.), pp. 1-. Springer-Verlag, New York.

Flint, A. J., Paladini, R. D., and Koshland, D. E. (1990). Autophosphorylation of protein kinase C at three separated regions of its primary sequence. *Science* **249**, 408-411.

Flores-Riveros, J. R., Sibley, E., Kastelic, T., and Lane, M. D. (1989). Substrate phosphorylation catalyzed by the insulin receptor tyrosine kinase: kinetic correlation to autophosphorylation of specific sites in the β subunit. *J. Biol. Chem.* **264**, 21557-21572.

Foster, J. L., Guttmann, J., and Rosen, O. M. (1981). Autophosphorylation of cGMP-dependent protein kinase. *J. Biol. Chem.* **256**, 5029-5036.

Fukunaga, K., Yamamoto, H., Matsui, K., Higashi, K., and Miyamoto, E. (1982). *J. Neurochem.* **39**, 1607-1617.

Garthwaite, J. (1991). Glutamate, nitric oxide and cell-cell signalling in the nervous system. *Trends in Neurosciences* **14**, 60-67.

Gaehlen, R. L., Allen, S. M., and Krebs, E. G. (1981). Effect of phosphorylation on the regulatory subunit of the type I cAMP-dependent protein kinase. *J. Biol. Chem.* **256**, 4536-4540.

Gill, G. N. (1977). A hypothesis concerning the structure of cAMP- and cGMP-dependent protein kinase. *J. Cyclic Nucleotide Res.* **3**, 153-162.

Gill, G. N., Walton, G. M., Sperry, P. J. (1977). Guanosine 3':5'-monophosphate-dependent protein kinase from bovine lung. *J. Biol. Chem.* **252**, 6443-6449.

Gilman, A. G. (1987). G proteins: transducers of receptor-generated signals. *Ann. Rev. Biochem.* **56**, 615-649.

Goldberg, N. D., and Haddox, M. K. (1977). Cyclic GMP metabolism and involvment in biological regulation. *Ann Rev. Biochem.* **46**, 823-896.

Goldenring, J. R., Gonzalez, B., McGuire, J. S., Jr., and DeLorenzo, R. J. (1983). *J. Biol. Chem.* **258**, 12632-12640.

Greenfield, C., Hils, I., Waterfield, M. D., Federwisch, W., Wollmer, A., Blundell, T. L., and McDonald, N. (1989). EGF binding induces a conformational change in the external domain of its receptor. *EMBO J.* **8**, 4115-4124.

Greengard, P. (1978). Phosphorylated Proteins as Physiological Effectors. *Science* **199**, 146-152.

Guerriero, V., Russo, M. A., Olson, N. J., Putkey, J. A., and Means, A. R. (1986). Domain organization of chicken gizzard myosin light chain kinase deduced from a cloned cDNA. *Biochemistry* **25**, 8372-8381.

Gulyaeva, N. B., Pul'fson, P. L., and Severin, E. S. (1977b). The role of different ATP analogs on the catalytic function of phosphorylase kinase. *Biochemistry* **43**, 299-306.

Gulyaeva, N. B., Guskova, R. A., Baranova, L. A., Gulyaev, N. N., Val'fson, P. L., and Severin, E. S. (1977a). *Dokl. Akad. Nauk. S.S.S.R.* **235**, 696.

Hallenbeck, P. C., and Walsh, D. A. (1983). Autophosphorylation of phosphorylase kinase. *J. Biol. Chem.* **258**, 13493-13501.

Hanks, S. K., Quinn, A. M., and Hunter, T. (1988). *Science* **241**, 42-52.

Hanley, R. M., Payne, M. E., Cruzalegui, F., Christenson, M. A., and Means, A. R. (1989). Sequence of the cDNA for the alpha subunit of calmodulin kinase II from mouse brain. *Nucl. Acids Res.* **17**, 3992.

Hanley, R. M., Means, A. R., Ono, T., Kemp, B. E., Burgin, K. E., Waxham, N., and Kelly, P. T. (1987). *Science* **237**, 293-297.

Hanley, R. M., Means, A. R., Kemp, B. E., and Shenolikar, S. (1988). Mapping of calmodulin-binding domain of Ca^{2+}/calmodulin-dependent protein kinase II from rat brain. *Biochem. Biophys. Res. Commun.* **152**, 122-128.

Hannun, Y. A., Loomis, C. R., and Bell, R. M. (1985). Activation of protein kinase C by triton X-100 mixed micelles containing diacylglycerol and phosphatidylserine. *J. Biol. Chem.* **260**, 10039-10043.

Hashimoto, E., Takio, K., and Krebs, E. G. (1981). Studies on the site in the regulatory subunit of type I cAMP-dependent protein kinase phosphorylated by cGMP-dependent protein kinase. *J. Biol. Chem.* **256**, 5604-5607.

Hayakawa, T., Perkins, J. P., Walsh, D. A., and Krebs, E. G. (1973a). Physicochemical properties of rabbit skeletal muscle phosphorylase kinase. *Biochemistry* **12**, 567-573.

Hayakawa, T., Perkins, J. P., Walsh, D. A., and Krebs, E. G. (1973b). Studies on the subunit structure of rabbit skeletal muscle phosphorylase kinase. *Biochemistry* **12**, 574-580.

Heffetz, D., and Zick, Y. (1986). Receptor activation is necessary for activation of the soluble insulin receptor kinase. *J. Biol. Chem.* **261**, 889-894.

Hille, B. (1984). "Ionic Channels of Excitable Membranes." Sinaur, Sunderland, Massachussetts.

Holub, B. J., Kuksis, A., Thompson, W. (1970). Molecular species of mono-, di-, and triphosphoinositides of bovine brain. *J. Lipid Res.* **11**, 558.

Honegger, A., Dull, T. J., Bellot, F., Van Obberghen, E., Szapary, D., Schmidt, A., Ullrich, A., and Sclessinger, J. (1988a). Biological activities of EGF-receptor mutants with individually altered autophosphorylation sites. *EMBO J.* **7**, 3045-3052.

Honegger, A., Dull, T. J., Szapary, D., Komoriya, A., Kris, R., Ullrich, A., and Sclessinger, J. (1988b). Kinetic parameters of the protein kinase activity of EGF-receptor mutants with individually altered autophosphorylation sites. *EMBO J.* **7**, 3053-3060.

House, C., and Kemp, B. E. (1987). Protein kinase C contains a pseudosubstrate prototype in its regulatory domain. *Science* **238**, 1726-1728.

Huang, K. P., and Huang, F. L. (1986). Conversion of protein kianse C from a Ca^{2+}-dependent to an independent of phorbol ester-binding protein by digestion with trypsin. *Biochem. Biophys. Res. Commun.* **139**, 320-326.

Huang, K.-P., Chan, K.-F. J., Singh, T. J., Nakabayashi, H., and Huang, F. L. (1986). Autophosphorylation of rat brain Ca^{2+}-activated and phospholipid-dependent protein kinase. *J. Biol. Chem.* **261**, 12134-12140.

Hunter, T. (1987). A thousand and one protein kinases. *Cell* **50**, 823-829.

Hunter, T., and Cooper, J. A. (1985). Protein-Tyrosine Kinases. *Ann. Rev. Biochem.* **54**, 897-930.

Ikebe, M., Kemp, B. E., and Hartshorne, D. J., (1988). *Biophys. J.* **53**, 182.

Ingebritsen, T. S., and Cohen, P. (1983a). Protein phosphatases: properties and role in cellular regulation. *Science* **221**, 331.

Ingebritsen, T. S., and Cohen, P. (1983b). The protein phosphatases involved in cellular regulation. 1. Classification and substrate specificities. *Eur. J. Biochem.* 132, 255-261.

Inoue, M., Kishimoto, A., Takai, Y., and Nishizuka, Y. (1977). Studies on a cyclic nucleotide-dependent protein kinase and its proenzyme in mammalian tissues. *J. Biol. Chem.* **252**, 7610-7616.

Kaczmarek, L. K., and Levitan, I. B. (1987). "*Neuromodulation.*" Oxford Univ. Press, Oxford and New York.

Kakiuchi, S., and Yamazaki, R. (1970). Calcium-dependent phosphodiesterase activity and its activating factor (PAF) from brain. *Biochem. Biophys. Res. Commun.* **41**, 1104-1110.

Kakiuchi, S., Sobue, K., Yamazaki, R., Nagao, S., Umeki, S., Nozawa, Y., Yazawa, M., and Yagi, K. (1981). Ca^{2+}-dependent modulator proteins from Tetrahymena pyrifomis, sea anemone, and scallop and guanylate cyclase activation. *J. Biol. Chem.* **256**, 19-22.

Kashles, O., Szapary, D., Bellot, F., Ullrich, A., Schlessinger, J., and Schmidt, A. (1988). Ligand-induced stimulation of EGF-receptor mutants witrh altered transmembrane regions. *Proc. Natl. Acad. Sci. (USA)* **85**, 9567-9571.

Kasuga, M., Zick, Y., Blithe, D. C., Crettaz, M., and Kahn, C. R. (1982a). Insulin stimulates tyrosine phosphorylation of the insulin receptor in a cell-free system. *Nature* **298**, 667-669.

Kasuga, M., Zick, Y., Bith, D. L., Karlsson, F. A., Häring, H. U., and Kahn, C. R. (1982b). Insulin stimulation of phosphorylation of the β subunit of the insulin receptor: formation of both phosphoserine and phosphotyrosine. *J. Biol. Chem.* **257**, 9891-9894.

Kee, S. M., and Graves, D. J. (1986). Isolation and properties of the active γ subunit of phosphorylase kinase. *J. Biol. Chem.* **261**, 4732-4737.

Kelly, P. T., Weinberger, R. P., and Waxham, M. N. (1988). Active site-directed inhibition of Ca^{2+}/calmodulin-dependent protein kinase type II by a bifunctional calmodulin-binding peptide. *Proc. Natl. Acad. Sci. (USA)* **85**, 4991-4995.

Kemp, B. E., and Pearson, R. B. (1985). Spatial requirement for location of basic residues in peptide dubstrates for smooth muscle myosin light chain kinase. *J. Biol. Chem.* **260**, 3355-3359.

Kemp, B. E., Pearson, R. B., Guerriero, V., Jr., Bagchi, I. C., and Means, A. R. (1987). The calmodulin binding domain of chicken smooth muscle myosin light chain kinase contains a pseudosubstrate sequence. *J. Biol. Chem.* **262**, 2542-2548.

Kennedy, M. B. (1989). Regulation of Synaptic Transmission in the Central Nervous System: Long-Term Potentiation. *Cell* **59**, 777-787.

Kennedy, M. B., Bennett, M. K., Erondu, N. E., and Miller, S. G. (1987). Calcium/calmodulin-dependent protein kinases. In "*Calcium and Cell Function.*" Vol. 7 (Cheung, ed.), pp. 61-107. Academic Press, Orlando.

Kennelly, P. J., Edelman, A. M., Blumenthal, D. K., and Krebs, E. G. (1987). Rabbit skeletal muscle myosin light chain kinase: the calmodulin binding domain as a potential active-site directed inhibitory domain. *J. Biol. Chem.* **262**, 11958-11963.

Kikkawa, U., Takai, Y., Minakuchi, R., Inohara, S., and Nishizuka, Y. (1982). Ca^{2+}-activated, phospholipid-dependent protein kinase from rat brain. *J. Biol. Chem.* **257**, 13341-13348.

Kincaid, R. L., and Vaughan, M. (1986). Molecular and regulatory properties of calmodulin-dependent phosphodiesterase from brain. In "*Calcium and Cell Function.*" Vol. 6 (Cheung, ed.), pp. 43-70. Academic Press, New York.

King, M. M., Fitzgerald, T. J., and Carlson, G. M. (1983). Characterization of initial autophosphorylation events in rabbit skeletal muscle phosphorylase kinase. *J. Biol. Chem.* **258**, 9925-9930.

Kishimoto, A., Takai, Y., Mori, T., Kikkawa, U., and Nishizuka, Y. (1980). Activation of calcium and phospholipid-dependent protein kinase by diacylglycerol, its possible relation to phosphatidylinositol turnover. *J. Biol. Chem.* 255, 2273-2276.

Krebs, E. G. (1986). *In* "The Enzymes," Vol. XVII (Boyer, P. D., and Krebs, E. G., eds.), pp. 3-20. Academic Press, Orlando.

Krebs, E. G., and Beavo, J. A. (1979). Phosphorylation-dephosphorylation of enzymes. *Ann. Rev. Biochem.* **48**, 923-959.

Krebs, E. G., Graves, D. J., and Fischer, E. H. (1959). Factors affecting the activity of muscle phosphorylase b kinase. *J. Biol. Chem.* **234**, 2867-2873.

Kuffler, S. W., Nicholls, J. G., and Martin, A. R. (1984). "*From Neuron to Brain.*" Sinnaur, Sunderland, Massachusetts.

Kuo, J. F., and Greengard, P. (1969). An adenosine 3':5'-monophosphate-dependent protein kinase from *Escherichia coli*. *J. Biol. Chem.* **244**, 3417-3419.

Kuret, J., and Schulman, H. (1984). Purification and characterization of a Ca^{2+}/calmodulin-dependent protein kinase from rat brain. *Biochemistry* **23**, 5495-5504.

Kuret, J., Johnson, K. E., Nicolette, C., and Zoller, M. J. (1988). Mutagenesis of the regulatory subunit of yeast cAMP-dependent protein kianse: isolation of site-directed mutants with altered binding affinity for catalytic subunit. *J. Biol. Chem.* **263**, 9149-9154.

Kwiatkowski, A. P., and King, M. M. (1989). Autophosphorylation of the type II calmodulin-dependent protein kinase is essential for formation of a proteolytic fragment with catalytic activity. Implications for long term synaptic potentiation. *Biochemistry* **28**, 5380-5385.

Lai, Y., Nairn, A. C., and Greengard, P. (1986). Autophosphorylation reversibly regulates the Ca^{2+}/calmodulin-dependence of Ca^{2+}/calmodulin-dependent protein kinase II. *Proc. Natl. Acad. Sci. (USA)* **83**, 4253-4257.

Lammers, R., Gray, A., Schlessinger, J., and Ullrich, A. (1989). Differential signalling potential of insulin- and IGF-1-receptor cytoplasmic domains. *EMBO J.* **8**, 1369-1375.

Landgraf, W., Hullin, R., Göbel, C., and Hofmann, F. (1986). Phosphorylation of cGMP-dependent protein kinase increases the affinity for cAMP. *Eur. J. Biochem.* **154**, 113-117.

Lee, J., Dull, T. J., Lax, I., Schlessinger, J., and Ullrich, A. (1989). HER2 cytoplasmic domain generates normal mitogenic and transforming signals in a chimeric receptor. *EMBO J.* **8**, 167-173.

LeVine, H., and Sahyoun, N. (1987). Characterization of a soluble Mr-30,000 catalytic fragment of the neuronal calmodulin-dependent protein kinase II. *Eur. J. Biochem.* **168**, 481-486.

Lin, C. R., Kapiloff, M. S., Durgerian, S., Tatemoto, K., Russo, A. F., Hanson, P., Schulman, H., and Rosenfeld, M. G. (1987). *Proc. Natl. Acad. Sci. (USA)* **84**, 5962-5966.

Lincoln, T. M., Flockhart, D. A., and Corbin, J. D. (1978). Studies on the structure and mechanism of activation of the guanosine 3':5'-monophosphate-dependent protein kinase. *J. Biol. Chem.* **253**, 6002-6009.

Lincoln, T. M., and Corbin, J. D. (1983). *Adv. Cyclic Nucleotide Res.* **15**, 139-192.

Lisman, J. E. (1985). A mechanism for memory storage insensitive to molecular turnover: a bistable autophosphorylating kinase. *Proc. Natl. Acad. Sci. (USA)* **82**, 3055-3057.

Lisman, J. E., and Goldring, M. A. (1988). The feasibility of long-term storage of graded information by the Ca^{2+}/calmodulin-dependent kinase molecules of the post-synaptic density. *Proc. Natl. Acad. Sci. (USA)* **85**, 5320-5324.

Lou, L. L., Lloyd, S. J., and Schulman, H. (1986). Activation of the multifunctional Ca^{2+}/calmodulin-dependent protein kinase by autophosphorylation: ATP modulates production of an autonomous enzyme. *Proc. Natl. Acad. Sci. (USA)* **83**, 9497-9501.

Majerus, P. W., Connolly, T. E., Deckmyn, H., Ross, T., Bross, T. E. *et al.* (1986). The metabolism of phosphoinositide-derived messenger molecules. *Science* **234**, 1519-1527.

Majerus, P. W., Connolly, T. E., Bansal, V. S., Inhorn, R. C., Ross, T. S., and Lips, D. L. (1988). Inositol phosphates: synthesis and degradation. *J. Biol. Chem.* **263**, 3051-3054.

Mayr, G. W., and Heilmeyer, L. M. G. (1983a). Skeletal muscle light chain kinase. *FEBS Lett.* **157**, 225-231.

Mayr, G. W., and Heilmeyer, L. M. G. (1983b). Shape and substructure of skeletal muscle myosin light chain kinase. *Biochemistry* **22**, 4316-4326.

McGuiness, T. L., Lai, Y., and Greengard, P. (1985). Ca^{2+}/calmodulin-dependent protein kinase II. Isozymic forms from rat forebrian and cerebellum. *J. Biol. Chem.* **260**, 1696-1704.

Meldolesi, J., and Pozzan, T. (1987). Pathways of Ca^{2+} influx at the plasma membrane: voltage-, receptor-, and second messenger-operated channels. *Exp. Cell Res.* **171**, 271-283.

Miller, S. G., and Kennedy, M. B. (1986). Regulation of brain type II Ca^{2+}/calmodulin-dependent protein kinase by autophosphorylation: a Ca^{2+}-triggered molecular switch. *Cell* **44**, 861-870.

Miller, S. G., Patton, B. L., and Kennedy, M. B. (1988). Sequences of autophosphorylation sites in neuronal type II CaM kinase that control Ca^{2+}-independent activity. *Neuron* **1**, 593-604.

Monken, C. E., Gill, G. N. (1980). Structural analysis of cGMP-dependent protein kinase using limited proteolysis. *J. Biol. Chem.* **255**, 7067-7070.

Murphy, S., and Miller, R. (1988). A glutamate receptor regulates Ca^{2+} mobilization in hippocampal neurons. *Proc. Natl. Acad. Sci. (USA)* **85**, 8737-8741.

Nakamura, T., and Gold, G. H. (1987). A cyclic nucleotide-gated conductance in olfactory receptor cilia. *Nature (Lond.)* **325**, 442-444.

Naor, Z., Shearman, M. S., Kishimoto, A., Nishizuka, Y. (1988). *Mol. Endocrinol.* **2**, 1043-1048.

Nestler, E. J., Greengard, P. (1984). "*Protein Phosphorylation In The Nervous System.*" Wiley, New York.

Newton, A. C., and Koshland, D. E. (1987). Protein kinase C autophosphorylates by an Intrapeptide reaction. *J. Biol. Chem.* **262**, 10185-10188.

Nishikawa, M., deLanerolle, P., Lincoln, T. M., and Adelstein, R. S. (1984). Phosphorylation of mammalian light chain kinase by the catalytic subunit of cyclic AMP-dependent protein kinase and by cyclic GMP-dependent protein kinase. *J. Biol. Chem.* **259**, 8429-8436.

Nishikawa, M., Shirakawa, S., and Adelstein, R. S. (1985). Phosphorylation of smooth muscle myosin light chain kinase by protein kinase C. *J. Biol. Chem.* **260**, 8978-8983.

Nishizuka, Y. (1986). Studies and perpectives of protein kinase C. *Science* **233**, 305-312.

Nishizuka, Y. (1988). The molecular heterogeneity of protein kinase C and its implications for cellular regulation. *Nature* **334**, 661-665.

Ohno, S., Konno, Y., Akita, Y., Yano, A., Suzuki, K. (1990). A point mutation at the putative ATP-binding site of protein kinase Cα abolishes the kinase

activity and renders it down-regulation-insensitive: a molecular link between autophosphorylation and down-regulation. *J. Biol. Chem.* **265**, 6296-6300.

Patton, B. L., Miller, S. G., and Kennedy, M. B. (1990). Activation of type II calcium/calmolulin-dependent protein kinase by Ca^{2+}/calmodulin is inhibited by autophosphorylation of threonine within the calmodulin-binding domain. *J. Biol. Chem.* **265**, 11204-11212.

Payne, M. E., Fong, Y.-L., Ono, T., Colbran, R. J., Kemp, B. E., Soderling, T. R., and Means, A. R. (1988). Calcium/calmodulin-dependent protein kinase II: characterization of distinct calmodulin-binding and inhibitory domains. *J. Biol. Chem.* **263**, 7190-7195.

Pearson, R. B., Jakes, R., John, M., Kendrick-Jones, J., and Kemp, B. E. (1984). Phosphorylation site sequence of smooth muscle myosin light chain (M_r = 20,000). *FEBS Lett.* **168**, 108-112.

Pearson, R. B., Woodgett, J. R., Cohen, P., and Kemp, B. E. (1985). Substrate specificity of a multifunctional calmodulin-dependent protein kinase. *J. Biol. Chem.* **260**, 14471-14476.

Perrie, W. T., Smillie, L. B., and Perry, S. V. (1973). A phosphorylated light-chain component of myosin from skeletal muscle. *Biochem. J.* **135**, 151-164.

Petruzzelli, L. M., Ganguly, S., Smith, C. J., Cobb, M. H., Rubin, C. S., and Rosen, O. M. (1982). Insulin activates a tyrosine-specific protein kinase in extracts of 3T3-L1 adipocytes and human placenta. *Proc. Natl. Acad. Sci. (USA)* **79**, 6792-6796.

Petruzzelli, L., Herrera, R., and Rosen, O. M. (1984). Insulin receptor is an insulin-dependent tyrosine protein kinase: copurification of insulin-binding activity and protein kinase activity to homogeneity from human placenta. *Proc. Natl. Acad. Sci. (USA)* **81**, 3327-3331.

Pickett-Gies, C. A., and Walsh, D. A. (1985). Subunit phosphorylation and activation of skeletal muscle phosphorylase kinase by the cAMP-dependent protein kinase. *J. Biol. Chem.* **260**, 2046-2056.

Pickett-Gies, C. A., and Walsh, D. A. (1986). Phosphorylase kinase. *The Enzymes*, Vol. XVII. (Boyer, P. D., and Krebs, E. G., eds.), pp. 395-459. Academic Press, Orlando.

Picton, C., Klee, C. B., and Cohen, P. (1980). Phosphorylase kinase from rabbit skeletal muscle: identification of the calmodulin-binding subunits. *Eur. J. Biochem.* **111**, 553-561.

Piomelli, D., Volterra, A., Dale, N., Siegelbaum, S. A., Kandel, E. R., Schwartz, J. H., and Belardetti, F. (1987a). Lipoxygenase metabolites of arachidonic acid as second messengers for presynaptic inhibition of *Aplysia* sensory cells. *Nature (Lond.)* **328**, 38-43.

Piomelli, D., Shapiro, E., Feinmark, S. J., and Schwartz, J. H. (1987b). Metabolites of arachidonic acid in the nervous system of *Aplysia*: possible mediators of synaptic modulation. *J. Neurosci.* **7**, 3675-3686.

Piomelli, D., Wang, J. K. T., Sihra, T. S., Nairn, A. C., Czeernik, A. J., and Greengard, P. (1989). Inhibition of Ca^{2+}/calmodulin-dependent protein kinase II by arachidonic acid and its metabolites. *Proc. Natl. Acad. Sci. (USA)* **86**, 8550-8554.

Piomelli, D., and Greengard, P. (1990). Lipoxygenase metabolites of arachidonic acid in neuronal transmembrane signalling. *Trends in Pharm. Sci.* **11**, 367-373.

Pontremoli, S., and Melloni, E. (1986). Regulation of Ca^{2+}-dependent proteinase of human erythrocytes. *In* "Calcium and Cell Function." Vol. 6 (Cheung, ed.), pp. 159-183. Academic Press, New York.

Rall, T. W. (1982) Function and degradation of cyclic nucleotides: an overview. *In* "Cyclic Nucleotides." Vol. 1 (Nathanson and Kebabian, eds.), Springer-Verlag, Berlin.

Rall, T. W., Sutherland, E. W., Berthet, J. (1957). The relationship of epinephrine and glucagon to liver phosphorylase. *J. Biol. Chem.* **224**, 463-475.

Rall, T. W., and Sutherland, E. W. (1958). Formation of a cyclic adenine ribonucleotide by tissue particles. *J. Biol. Chem.* **232**, 1065-1076.

Ramon y Cajal, S. (1890). Sur l'origine et les ramifications des fibres nerveuses de la moelle embryonnaire. *Anat. Ang.* **5**, 609-613.

Rangel-Aldao, R., and Rosen, O. M. (1976a). Mechanism of self-phosphorylation of adenosine 3':5'-monophosphate-dependent protein kinase from bovine cardiac muscle. *J. Biol. Chem.* **251**, 7526-7529.

Rangel-Aldao, R., and Rosen, O. M. (1976b). Dissociation and reassociation of the phosphorylated and nonphosphorylated forms of adenosine 3':5'-monophosphate-dependent protein kinase from bovine cardiac muscle. *J. Biol. Chem.* **251**, 3375-3380.

Rappoport, L., and Adelstein, R. S. (1980). Phosphorylation of canine cardiac muscle myosin light chain kinase. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **2293**, (Abstract).

Riedel, H., Dull, T. J., Honegger, A. M., Schlessinger, J., and Ullrich, A. (1989). Cytoplasmic domains determine signal specificity, cellular routing characteristics and influence ligand binding of epidermal growth factor and insulin receptors. *EMBO J.* **8**, 2943-2954.

Rink, T. J., and Jacob, R. (1989). Calcium oscillations in non-excitable cells. *Trends in Neurosci.* **12**, 43-46.

Robison, G. A., Butcher, R. W., and Sutherland, E. W. (1968). Cyclic AMP. *Ann Rev. Biochem.* **37**, 149-174.

Rosen, O. M. (1987). After insulin binds. *Science* **237**, 1452-1458.

Rosen, O. M., Herrera, R., Olowe, Y., Petruzzelli, L. M., and Cobb, M. H. (1983). Phosphorylation activates the insulin receptor tyrosine kinase. *Proc. Natl. Acad. Sci. (USA)* **80**, 3237-3240.

Schlessinger, J. (1988). Signal transduction by allosteric receptor oligomerization. T*rends in Biochem. Sci.* **13**, 443-447.

Schreiber, A. B., Libermann, T. A., Lax, I., Yarden, Y., and Schlessinger, J. (1983). Biological role of epidermal growth factor-receptor clustering: investigation with monoclonal anti-receptor antibodies. *J. Biol. Chem.* **258**, 846-853.

Schreiber, A. B., Lax, I., Yarden, Y., Eshhar, Z., and Schlessinger, J. (1981). Monoclonal antibodies against receptor for epidermal growth factor induce early and delayed effects of epidermal growth factor. *Proc. Natl. Acad. Sci. (USA)* **78**, 7535-7539.

Schweitzer, P., Madamba, S., and Siggins, G. R. (1990). Arachidonic acid metabolites as mediators of somatostatin-induced increase of neuronal M-current. *Nature (Lond.)* **346**, 464-467.

Schworer, C. M., Colbran, R. J., and Soderling, T. R. (1986). Reversible generation of a Ca^{2+}-independent form of Ca^{2+}(calmodulin)-dependent protein kianse II by an autophosphorylation mechanism. *J. Biol. Chem.* **261**, 8581-8584.

Schworer, C. M., Colbran, R. J., Keefer, J. R., and Soderling, T. R. (1988). Ca^{2+}/calmodulin-dependent protein kianse II. *J. Biol. Chem.* **263**, 13486-13489.

Scott, C. W., and Mumby, M. C. (1985). Phosphorylation of type II regulatory subunit of type I cAMP-dependent protein kinase in intact smooth. *J. Biol. Chem.* **260**, 2274-2280.

Shacter, E., Chock, P. B., Rhee, S. G., Stadtman, E. R. (1986). *In* "The Enzymes." Vol. XVII (Boyer, P. D., and Krebs, E. G., eds.), pp. 21-42. Academic Press, Orlando.

Shepherd, G. M. (1979). "*The Synaptic Organization of the Brain.*" Oxford Univ. Press, London and New York.

Shepherd, G. M. (1983). "*Neurobiology.*" Oxford Univ. Press, London and New York.

Shia, M. A., Rubin, J. B., and Pilch, P. F. (1983). The insulin receptor protein kinase. *J. Biol. Chem.* **258**, 14450-14455.

Shizuta, Y., Beavo, J. A., Bechtel, P. J., and Krebs, E. G. (1975). Reversibility of cAMP-dependent protein kinase reactions. *J. Biol. Chem.* **250**, 6891-6896.

Soderling, T. R. (1990). Protein kinases: regulation by autoinhibitory domains. *J. Biol. Chem.* **265**, 1823-1826.

Stryer, L. (1986). Cyclic GMP cascade of vision. *Ann. Rev. Neurosci.* **9**, 87-119.

Stull, J. T., Nunnally, M. H., and Michnoff, C. H. (1986). Calmodulin-dependent protein kinases. *In* "The Enzymes." Vol. XVII (Boyer, P. D. and Krebs, E. G., eds.), pp. 113-166. Academic Press, Orlando.

Sutherland, E. W., Oye, I., and Butcher, R. W. (1965). The action of epinephrine and the role of the adenyl cyclase system in hormone action. *Recent Progr. Hormone Res.* **21**, 623-646.

Sutherland, E. W., and Wosilait, W. D. (1955). Inactivation and activation of liver phosphorylase. *Nature (London)* **175**, 169-171.

Sutherland, E. W., and Rall, T. W. (1958). Fractionation and characterization of a cyclic adenine ribonucleotide formed by tissue particles. *J. Biol. Chem.* **232**, 1077-1091.

Takai, Y., Kishimoto, A., Kikkawa, U., Mori, T., and Nishizuka, Y. (1979). Unsaturated diacylglycerol is a possible messenger for the activation of calcium-activated, phospholipid-dependent protein kinase system. *Biochem. Biophys. Res. Commun.* **91**, 1218-1224.

Takio, K., Smith, S. B., Walsh, K. A., Krebs, E. G., and Titani, K. (1983). Amino acid sequence around a "hinge" region and its "autophosphorylation" site in bovine lung cGMP-dependent protein kinase. *J. Biol. Chem.* **258**, 5531-5536.

Takio, K., Wade, R. D., Smith, S. B., Krebs, E. G., Walsh, K. A., Titani, K. (1984). Guanosine cyclic 3':5"-phosphate dependent protein kinase, a chimeric protein homologous with two separate protein families. *Biochemistry* **23**, 4207-4218.

Taylor, S. S. (1989). cAMP-dependent protein kinase: model for an enzyme family. *J. Biol. Chem.* **264**, 8443-8446.

Teo, T. S., and Wang, J. H. (1973). Mechanism of activation of a cyclic adenosine 3′,5′-monophosphate phosphodiesterase from heart by calcium ions: Identification of the protein activator as a Ca^{2+}-binding protein. *J. Biol. Chem.* **248**, 5950-5955.

Theil, G., Czernik, A. J., Gorelik, F., Nairn, A. C., and Greengard, P. (1988). Ca^{2+}/calmodulin-dependent protein kianse II: Identification of threonine-286 as the autophosphorylation site in the α subunit associated with the generation of Ca^{2+}-independnet activity. *Proc. Natl. Acad. Sci. (USA)* **85**, 6337-6341.

Titani, K., Sasagawa, T., Ericsson, L. H., Kumar, S., Smith, S. B. *et al.* (1984). Amino acid sequence of the regulatory subunit of bovine type I adenosine cyclic 3':5'-monophosphate-dependent protein kinase. *Biochemistry* **23**, 4193-4199.

Tsien, R. W., and Tsien, R. Y. (1990). Calcium channels, stores, and oscillations. *Ann. Rev. Neurosci.* **13**, 715-760.

Ullrich, A., and Schlessinger, J. (1990). Signal transduction by receptors with tyrosine kinase activity. *Cell*, **61**, 203-212.

Walsh, D. A., Perkins, J. P., Krebs, E. G. (1968). An adenosine 3':5'-monophosphate-dependent protein kinase from rabbit skeletal muscle. *J. Biol. Chem.* **243**, 3763-3765.

Walsh, M. P., Cavadore, J., Vallet, B., and Demaille, J. G. (1980). Calmodulin-dependent myosin light chain kinases from cardiac and smooth muscle: a comparative study. *Can. J. Biochem.* **58**, 299-308.

Walter, U., Uno, I., Liu, A. Y.-C., Greengard, P. (1977). Identification, characterization, and quantitative measurement of cyclic AMP receptor proteins in cytosol of various tissues using a photaffinity ligand. *J. Biol. Chem.* **252**, 6494-6500.

Wang, J. H., Stull, J. T., Huang, T. S., and Krebs, E. G. (1976). A study on the autoactivation of rabbit muscle phosphorylase kinase. *J. Biol. Chem.* **251**, 4521-4527.

Weber, W., Bertics, P. J., and Gill, G. N. (1984). Immunoaffinity purification of the epidermal growth factor receptor. *J. Biol. Chem.* **259**, 14631-14636.

Wernet, W., Flockerzi, V., and Hofmann, F. (1989). The cDNA of the two isoforms of bovine cGMP-dependent protein kinase. *FEBS Lett.* **251**, 191.

White, M. F., Haring, H.-U., Kasuga, M., and Kahn, C. R. (1984). Kinetic properties and sites of autophosphorylation of the partially purified insulin receptor from hepatoma cells. *J. Biol. Chem.* **259**, 255-264.

Williams, L. T. (1989). Signal transduction by the platelet-derived growth factor receptor. *Science* **243**, 1564-1570.

Williams, J. H., Errington, M. L., Lynch, M. A., and Bliss, T. V. P. (1989). Arachidonic acid induces a long-term activity-dependent enhancement of synaptic transmission on the hippocampus. *Nature (Lond.)* **341**, 739-742.

Wolf, H., and Hofmann, F. (1980). Purification of myosin light chain kinase from bovine cardiac muscle. *Proc. Natl. Acad. Sci. (USA)* **77**, 5852-5855.

Wong, P. Y. K., and Cheung, W. Y. (1979). Calmodulin stimulates human platelet phospholipase A_2. *Biochem. Biophys. Res. Commun.* **90**, 473-480.

Yamauchi, T., and Fujisawa, H. (1983). Purification and characterization of the brain calmodulin-dependent protein kinase (Kinase II) which is involved in the activation of tryptophan 5-monooxygenase. *Eur. J. Biochem.* **132**, 15-21.

Yang, S-.D., Tallant, E. A., and Cheung, W. Y. (1982). Calcineurin is a calmodulin-dependent protein phosphatase. *Biochem. Biophys. Res. Commun.* **106**, 1419-1425.

Yarden, Y., and Schlessinger, J. (1985). *Ciba Found. Symp.* **116**, 23-45.

Yarden, Y., and Ullrich, A. (1988). Growth Factor Receptor Tyrosine Kinases. *Ann. Rev. Biochem.* **57**, 443-478.

Yarden, Y., and Schlessinger, J. (1987a). Epidermal growth factor induces rapid, reversible aggregation of the purified epidermal growth factor receptor. *Biochemistry* **26**, 1443-1451.

Yau, K.-W., and Baylor, D. A. (1989). Cyclic GMP-activated conductance of retinal photoreceptor cells. *Ann. Rev. Neurosci.* **12**, 289-327.

Yeaman, S. J., and Cohen, P. (1975). The hormonal control of activity of skeletal muscle phosphorylase kinase, phosphorylation of the enzyme at two sites *in vivo* in response to adrenelin. *Eur. J. Biochem.* **51**, 93-104.

Yu, K.-T., and Czech, M. P. (1984). Tyrosine phosphorylation of the insulin receptor β subunit activates the receptor-associated tyrosine kinase activity. *J. Biol. Chem.* **259**, 5277-5286.

Zick, Y., Rees-Jones, R. W., Taylor, S. I., Gordon, P., and Roth, J. (1984). The role of antireceptor antibodies in stimulating phosphorylation of the insulin receptor. *J. Biol. Chem.* **259**, 4396-4400.

CHAPTER 2

SEQUENCES OF AUTOPHOSPHORYLATION SITES IN NEURONAL TYPE II CaM KINASE THAT CONTROL Ca^{2+}-INDEPENDENT ACTIVITY*.

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Sequences of Autophosphorylation Sites in Neuronal Type II CaM Kinase That Control Ca^{2+}-Independent Activity

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Summary

After initial activation by Ca^{2+}, the catalytic activity of type II Ca^{2+}/calmodulin-dependent protein kinase rapidly becomes partially independent of Ca^{2+}. The transition is caused by autophosphorylation of a few subunits in the dodecameric holoenzyme, which is composed of varying proportions of two homologous types of subunits, α (50 kd) and β (58–60 kd). We have identified one site in the α subunit (Thr_{286}) and two in the β subunit (Thr_{287} and Thr_{382}) that are rapidly autophosphorylated. We show that phosphorylation of α-Thr_{286} and β-Thr_{287}, which are located immediately adjacent to the calmodulin binding domain, controls Ca^{2+}-independent activity. In contrast, phosphorylation of β-Thr_{382} is not required to maintain Ca^{2+} independence. It is absent in the α subunit and is selectively removed from the minor β′ subunit, apparently by alternative splicing. Regulation of the presence of β-Thr_{382} in the holoenzyme by both differential gene expression and alternative splicing suggests that it may have an important but highly specialized function.

Introduction

Considerable attention has been given to understanding the functions of type II Ca^{2+}/calmodulin-dependent protein kinase (type II CaM kinase) in the brain. It is the most abundant of the known brain Ca^{2+}-regulated protein kinases (Hunter, 1987), constituting about 1% of total brain protein (Erondu and Kennedy, 1985). It is expressed primarily in neurons and has been found in several subcellular pools including cytosolic, membrane, cytoskeletal, and nuclear fractions (reviewed in Stull et al., 1986; Kennedy et al., 1987). In solution, it is a large (M_r 600,000–700,000) holoenzyme containing varying proportions of two highly homologous subunits, α (50 kd) and β (60 kd), as well as a minor β′ (58 kd) subunit (Bennett and Kennedy, 1983; McGuinness et al., 1985; Miller and Kennedy, 1985). When activated, it phosphorylates a broad range of substrate proteins including synapsin I, microtubule-associated protein 2, tyrosine hydroxylase, smooth muscle myosin light chain, and glycogen synthase (Stull et al., 1986; Kennedy et al., 1987). Thus, it may perform many regulatory functions.

Several of its properties are particularly intriguing and may offer important clues about its functions. The subunits of the kinase are expressed in different proportions in different brain regions (Erondu and Kennedy, 1985; McGuinness et al., 1985; Miller and Kennedy, 1985; Bulleit et al., 1988). For example, the α subunit predominates in the forebrain (Erondu and Kennedy, 1985; Bulleit et al., 1988), while the β and β′ subunits predominate in the cerebellum where the overall expression of the kinase is lower (Erondu and Kennedy, 1985; McGuinness et al., 1985; Miller and Kennedy, 1985). Within the forebrain, but not in the cerebellum, the kinase appears to be concentrated in postsynaptic densities, where it constitutes 20%–30% of the total protein (Kennedy et al., 1983; Kelly et al., 1984; Miller and Kennedy, 1985). Finally, the kinase acquires a partially Ca^{2+}-independent activity when it phosphorylates itself (reviewed in Kennedy et al., 1987). This activation is highly cooperative, requiring phosphorylation of only 2 to 3 subunits per dodecameric holoenzyme. Therefore, once the holoenzyme is activated, it will continue to phosphorylate itself after Ca^{2+} has been removed. We have postulated that this mechanism may allow the kinase to resist dephosphorylation by cellular phosphatases and remain active within the cell for a period of time after an initial activating Ca^{2+} signal has fallen (Miller and Kennedy, 1986; see also Lisman, 1985).

In addition to these facts about the location and regulation of the kinase, a great deal has been learned recently about its structure. The amino acid sequences of the subunits have been deduced from the sequences of cDNAs encoding them. The β and β′ subunits appear to be generated by alternative splicing of the transcript of a single β gene (Bennett and Kennedy, 1987; Bulleit et al., 1988), while the α subunit is encoded by a second gene (Hanley et al., 1987; Lin et al., 1987; Bulleit et al., 1988). The amino acid sequences have revealed several functional domains that are common to all of the subunits (Bennett and Kennedy, 1987; Hanley et al., 1987; Lin et al., 1987; Bulleit et al., 1988; see Figure 7). The 270 amino acids at the amino terminus constitute a "conserved kinase catalytic domain" homologous to that found in the sequences of all known protein kinases (Barker and Dayhoff, 1982; Hunter, 1987). At the carboxy-terminal end of this domain, all of the subunits contain an identical 20 amino acid calmodulin binding domain (Bennett and Kennedy, 1987; Hanley et al., 1987; 1988; Payne et al., 1988). Just beyond the calmodulin binding domain, the β subunit contains three small segments that are deleted from the β′ and/or α subunits. The final 150 amino acids are similar in all of the subunits but are not homologous to any other known protein (Bulleit et al., 1988). This segment has been proposed to encode binding or regulatory domains that are unique to the type II CaM kinase (Bennett and Kennedy, 1987; Lin et al., 1987; Bulleit et al., 1988).

In this study, we have taken advantage of the recently acquired structural data to probe more deeply into the mechanism by which autophosphorylation regulates type II CaM kinase activity. We have identified the rap-

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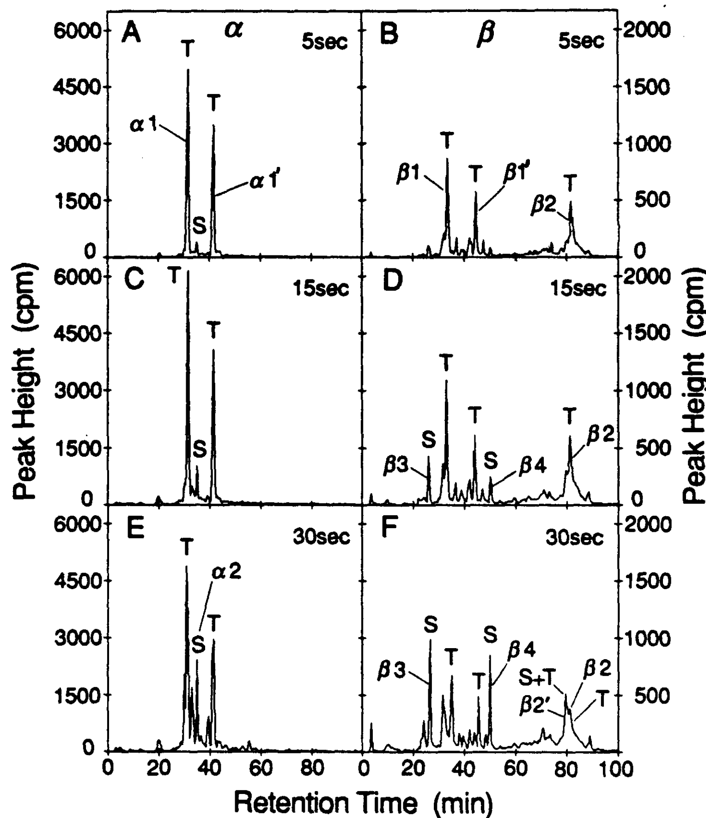

Figure 1. Time Course of Autophosphorylation of Sites on the α and β Subunits

Rat forebrain type II CaM kinase (10 μg) was autophosphorylated for 5 s (A and B), 15 s (C and D), or 30 s (E and F) in the presence of [γ-^{32}P]ATP, Ca^{2+}, and calmodulin. The holoenzyme was reduced and alkylated, and the α and β subunits were separated by SDS-PAGE and digested with trypsin. The resulting tryptic phosphopeptides were fractionated by reverse-phase HPLC on a C4 column (see Experimental Procedures). The identities of phosphorylated residues in each major peak were determined after partial acid hydrolysis as described in Experimental Procedures. Peaks generated from the α subunit are shown in (A), (C), and (E); those from the β subunit are shown in (B), (D), and (F). The scale for the β subunit peaks has been magnified 3-fold to compensate for the 3:1 ratio of α to β subunits in the forebrain holoenzyme. Thus, the peak heights reflect the approximate proportions of the sites per subunit. T, phosphothreonine-containing peak; S, phosphoserine-containing peak.

idly autophosphorylated sites that mediate the transition to a partially Ca^{2+}-independent state. A single homologous site in both the α and the β subunits controls Ca^{2+}-independent activity. It is located at the boundary between the catalytic and calmodulin binding domains and appears to be an "internal substrate" that is phosphorylated as soon as the kinase is activated by calmodulin. Phosphorylation of a few of these sites in a holoenzyme can apparently relieve inhibition by the calmodulin binding domains and maintain the kinase in a conformation that is active in the absence of bound calmodulin. We have also identified a second rapidly autophosphorylated site in the β subunit. Its function is unknown, but its location is extremely interesting. It is contained in a domain that is not present in the α subunit and is specifically deleted from the β' subunit, apparently by alternative splicing (Bulleit et al., 1988). Regulation of the presence of this site by both differential gene expression (as in the forebrain versus the cerebellum) and alternative splicing suggests that it may have an important function that is unique to the β subunit.

Results

Rapid and Slow Autophosphorylation Sites

We first examined the time course of autophosphorylation of individual sites on the type II CaM kinase in the presence of Ca^{2+} and calmodulin by digesting autophosphorylated kinase with trypsin (Figure 1).

After 5 s, two phosphothreonine-containing peptides were generated from the α subunit and three from the β subunit (Figures 1A and 1B). The two threonine-containing peaks in the α subunit (α1 and α1') are derived from the same phosphorylation site (see next section). Similarly, two of the phosphothreonine-containing peaks in the β subunit (β1 and β1') are derived from a single phosphorylation site. The third phosphothreonine-containing peak in β (β2) is derived from a distinct site. These three sites, α1, β1, and β2, are phosphorylated at a rapid rate (complete within 10–15 s) and to a high stoichiometry.

At later times, phosphoserine-containing peaks appear in both subunits; one in α (α2) and two in β (β3 and β4) (Figures 1D–1F). In addition, phosphoserine begins to appear within the β2 tryptic peptide, slightly shifting the mobility of the peptide (Figure 1F). We call the shifted peptide β2'. The precursor-product relationship between β2 and β2' was confirmed by a pulse-chase experiment in which the kinase was labeled for 5 s in the presence of [^{32}P]ATP, then chased in the presence of unlabeled ATP. The mobility of peak β2 shifted to that of β2' during the chase (data not shown).

All of the serine sites are autophosphorylated more slowly than the rapid threonine sites (complete within

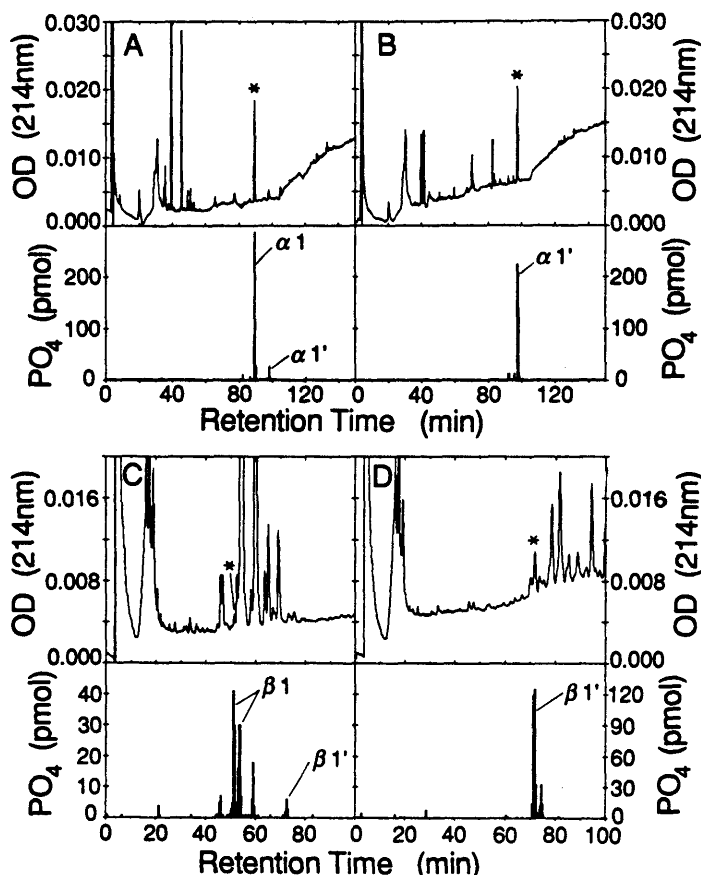

Figure 2. Purification of Peptides α1, α1', β1, and β1' by C18 Column Chromatography

(A and B) Forebrain kinase (1 mg) was phosphorylated for 15 s in the presence of [γ-^{32}P] ATP, Ca^{2+}, and calmodulin. It was reduced and alkylated, and the α subunit was purified and digested with trypsin as described in Experimental Procedures. The resulting phosphopeptides were first fractionated by chromatography on a C4 reverse-phase HPLC column. Fractions corresponding to peptides α1 and α1' (Figure 1) were concentrated and then fractionated on a C18 column with a shallow, highly resolving gradient (see Experimental Procedures). Most of the fractionation occurred between 0% and 10.5% acetonitrile. Starred peaks were sequenced. (A) Peptide α1; 89% of the counts loaded onto the C18 column were recovered. (B) Peptide α1'; 72% of the counts loaded onto the C18 column were recovered.

(C and D) Kinase holoenzyme containing a ratio of approximately 1 β subunit to 1 α subunit (1.5 mg; see Experimental Procedures) was autophosphorylated for 15 s in the presence of [γ-^{32}P]ATP, Ca^{2+}, and calmodulin. Tryptic phosphopeptides were generated and fractionated by C4 column chromatography as described for (A) and (B). Fractions corresponding to peptides β1 and β1' (Figure 1) were concentrated and fractionated by chromatography on a C18 column as described in Experimental Procedures. Most of the fractionation occurred between 5% and 10.5% acetonitrile. Starred peaks were sequenced. (C) Peptide β1; 84% of the counts loaded onto the C18 column were recovered. (D) Peptide β1'; 92% of the counts loaded onto the C18 column were recovered.

30–60 s) and, in some cases, to a lower stoichiometry. Rapid autophosphorylation of threonine sites and slower autophosphorylation of serine sites on the type II CaM kinase was also reported by Lai et al. (1987).

Sequence of the Rapid α Site

From the outset, the chromatographic behavior of peptides α1 and α1' suggested that they were related. Peak α1 was partially converted to α1' when it was isolated and chromatographed a second time. The conversion appeared to be irreversible since the opposite conversion, from α1' to α1, did not occur. We also noted that all of the α1 and β1 peaks were occasionally resolved into closely spaced doublets (Figure 1B, 1D, and 1F; Figures 2C and 2D). This is a reversible interconversion since both halves of each doublet could be regenerated from either isolated half (data not shown).

To determine the sequence of the autophosphorylation site within peptides α1 and α1' we scaled up the method for preparing them. Preparative amounts of the phosphopeptides were resolved by a chromatographic method similar to that illustrated in Figure 1. The peaks were further purified by chromatography on a more hydrophobic C18 column employing a highly resolving gradient (see Experimental Procedures).

Peptide α1 eluted from the C18 column primarily as a single peak, although a small amount of radioactivity appeared at the position of α1' (Figure 2A). The sequence of peptide α1 was determined twice by gas-phase sequencing and was found to be QE_VD_LK (Table 1). This corresponds to the predicted tryptic peptide QETVDCLK containing residues Gln_{284} to Lys_{291} of the α subunit (see Figure 6). Two blank cycles occurred during the sequencing, one at the third position, a predicted threonine, and the other at the sixth position, a predicted cysteine. Since phenylthiohydantoin (PTH)-phosphothreonine is not recovered during gas-phase sequencing (Bengur et al., 1987; Annan et al., 1982), autophosphorylation of Thr_{286} probably accounts for the blank at position 3. Cysteines were modified by carboxamidomethylation before the preparation of the tryptic peptides. PTH-carboxamidomethyl cysteine elutes near PTH-glutamate and dimethyl phenylthiourea (DMPTU) (a biproduct of the Edman reaction) and is often not well separated from them. In these sequencing runs, no distinct peak was resolved from DMPTU.

Peptide α1' eluted as a single peak (Figure 2B). Two attempts to sequence it (once with 200 pmol and again with 700 pmol) yielded no sequence, so it appeared to be blocked at the N-terminal. We determined its amino

Table 1. Sequences of Phosphopeptides α1 and β1

Sequence of α1				Sequence of β1			
Cycle	Residue	Yield[a] (pmol)	Yield[b] (pmol)	Cycle	Residue	Yield[c] (pmol)	Yield[d] (pmol)
1	Gln	51	21	1	Gln	18	16
2	Glu	37	18	2	Glu	17	15
3	-	-	-	3	-	-	-
4	Val	18	19	4	Val	10	7
5	Asp	19	37	5	Glu	9	8
6	-	-	-	6	[Glu]	[9]	[7]
7	Leu	10	24	7	Leu	6	5
8	Lys	-	5	8	Lys	3	2

All sequencing was done as described in Experimental Procedures.
[a] Approximately 100 pmol was submitted for sequencing.
[b] Approximately 40 pmol was submitted for sequencing.
[c] Approximately 40 pmol was submitted for sequencing.
[d] Approximately 30 pmol was submitted for sequencing.

acid composition, which was identical to that of peptide α1 (data not shown). Since there is no other predicted tryptic peptide with this amino acid composition in the α subunit (Bulleit et al., 1988), we conclude that α1 and α1′ contain the same sequence. α1 is apparently converted to α1′ by deamidation and cyclization of the N-terminal glutamine to form pyroglutamate. This is an irreversible reaction that occurs readily at N-terminal glutamine residues (Podell and Abraham, 1978). The cyclization explains the N-terminal blockage of α1′. Thus, both peptide α1 and peptide α1′ contain the same site, α1, which is identified as Thr_{286}.

Sequence of the Rapid β Site, β1

Phosphopeptides β1 and β1′ were purified by the method described above. Figures 2C and 2D illustrate their elution profiles after the C18 chromatography. Peptide β1 eluted as three major peaks and a few minor peaks. Most of the radioactivity (71%) was contained in one closely spaced doublet with the same retention time as the β1 doublet in smaller scale analytical fractionations (data not shown). As was the case for α1, a small amount of β1 eluted at the position of β1′. The peak eluting at about 59 min was not present in smaller scale separations and was not analyzed further.

The major β1 peaks eluted near a large unlabeled peptide peak (Figure 2C). Therefore, only the left portion of the doublet (starred in Figure 2C) was pure enough to yield a clear sequence. Its sequence was determined twice and was QE_VE(E)LK (Table 1). This corresponds best to the predicted tryptic peptide QETVECLK encompassing residues Gln_{285} to Lys_{292} in the β subunit. As in α1, the single phosphorylation site, Thr_{287}, at the third position was not recovered. The reason for the apparent recovery of Glu at the sixth position may again be the presence of PTH-carboxamidomethyl cysteine, which is not well resolved from PTH-glutamate and DMPTU.

Peptide β1′ eluted as one major peak (Figure 2D). Because of the relationship between α1/α1′ and β1/β1′, we assumed that β1′ would contain pyroglutamate at the N-terminal. Therefore, we did not attempt to sequence it, but instead subjected it to amino acid analysis. Its composition was consistent with that of peptide β1 (data not shown). We conclude that peptides β1 and β1′ contain the same site, β1, which is identified as Thr_{287}.

Sites α1 and β1 are homologous. Within their respective subunits, they are both located immediately N-terminal to the calmodulin binding domain (see Figure 6) 3 residues downstream from an arginine. Therefore they are contained within the consensus sequence (R-X-Y-Thr/Ser) described by Pearson et al. (1985) for substrates of the type II CaM kinase.

Sequence of the Rapid β Site, β2

The two tryptic peptides, β2 and β2′, were also purified as described above. On C4 and C18 columns, they both comigrated with a large peak containing an autolytic peptide derived from trypsin (data not shown). To generate smaller peptides that could be separated from the contaminating trypsin fragment, β2 and β2′ were further cleaved by digestion with thermolysin (which hydrolyzes peptide bonds on the N-terminal side of hydrophobic residues). The resulting peptides were resolved by chromatography on a C18 column (Figure 3). A single major thermolytic peptide containing phosphothreonine and a minor peptide containing phosphoserine were produced from β2 (Figure 3C). The same phosphothreonine-containing peptide was generated from β2′ along with a larger amount of the phosphoserine-containing peptide and a second phosphothreonine-containing peptide (Figure 3D). The sequences of the three phosphothreonine-containing peptides were nearly identical (Table 2). They all contain the sequence encompassing residues Leu_{377} to Pro_{387} in the β subunit (LEPQTT-VIHNP). Peptide β2-C is an alternative thermolytic peptide that elutes later from the C18 column. The blank cycle identifies Thr_{382} as the phosphorylated residue (Annan et al., 1982; Bengur et al., 1987). We conclude that site β2 is Thr_{382}. It is located in one of two regions of the β subunit that are not present in the α subunit (Bulleit et al., 1988).

The phosphoserine-containing thermolytic peptide

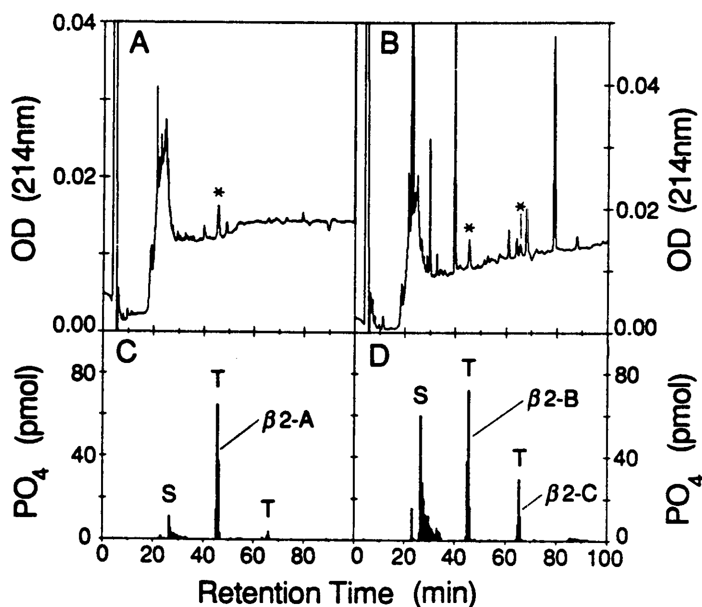

Figure 3. Purification of Peptides β2 and β2′ by C18 Column Chromatography

Kinase holoenzyme with a 1:1 ratio of α to β subunits (0.6 mg) was autophosphorylated for 120 s in the presence of [γ-^{32}P]ATP, Ca^{2+}, and calmodulin, then for an additional 60 s after 100 μl of 22 mM EGTA was added to chelate Ca^{2+}. The β subunit was reduced, alkylated, purified, and digested with trypsin. The resulting phosphopeptides were fractionated by chromatography on a C4 column. Fractions corresponding to phosphopeptides β2 and β2′ (Figure 1) were concentrated and digested with thermolysin. The resulting thermolytic peptides were fractionated on a C18 column (see Experimental Procedures). Starred peaks were sequenced. (A) Peptide β2; 83% of the counts applied to the column were recovered. (B) Peptide β2′; 82% of the counts applied to the column were recovered.

generated from peptide β2′ (Figures 3C and 3D) apparently contains a slow serine autophosphorylation site. It was not pure enough to sequence; however, we have deduced the identity of the site. Both the slow serine site and Thr_{382} are contained within the parent peptide β2′. From the sequence of the β subunit (Bennett and Kennedy, 1987), we predict β2′ contains residues Gly_{370} to Lys_{392} (see Figure 6). This sequence contains only 1 serine residue, Ser_{371}, which must be the slow serine site (β5). Like β2, site β5 is not present in the α subunit.

Site β2 Is Removed from the β′ Subunit

We recently found that one message for the 58,000 dalton β′ subunit appears to be identical to that for the β subunit except for the deletion of a segment encoding amino acid residues 378 to 392 (Bulleit et al., 1988). It seems most likely that this β′ message is generated by alternative splicing of the β gene transcript. The deleted 15 amino acid segment, 378 to 392, contains site β2. We tested whether site β2 was in fact absent in the β′ subunit. β and β′ were separated by SDS-PAGE after brief autophosphorylation. Tryptic phosphopeptides from the two subunits were resolved as in Figure 1. The amount of site β2 was much reduced in β′ compared with β (Figure 4). There are two possible explanations for the presence of a small amount of site β2 in the β′ subunit. It could result from contamination by the more abundant β subunit, which is often not completely resolved from β′ by SDS-PAGE. It could also be an indication that there are two forms of alternatively spliced β′ subunits, one of which contains site β2. There is already suggestive evidence for two distinct alternative splicing events that can generate different β′ subunits (Bulleit et al., 1988).

Table 2. Sequences of Thermolytic Peptides Derived from Peaks β2 and β2′

Cycle	Residue	β2-A Yield[a]	β2-B Yield[b]	β2-C Yield[c]
1	Leu	111	93	43
2	Glu	82	72	35
3	Pro	74	56	32
4	Gln	65	54	39
5	Thr	24	17	11
6	–	–	–	–
7	Val	43	18	14
8	Ile	32	18	14
9	His	13	5	4
10	Asn	23	12	6
11	Pro	11	5	7
12	Val	–	–	5

All sequencing was done as described in Experimental Procedures.
[a] Approximately 120 pmol was submitted for sequencing.
[b] Approximately 135 pmol was submitted for sequencing.
[c] Approximately 55 pmol was submitted for sequencing.

The α1 and β1 Sites Control Ca^{2+}-Independent Kinase Activity

Having identified the sites that are rapidly autophosphorylated in the presence of Ca^{2+}, we wished to determine which of them control the appearance of Ca^{2+}-independent activity. In our original study we showed that an average of about 3 phosphates per holoenzyme was sufficient to produce the full Ca^{2+}-independent kinase activity (Miller and Kennedy, 1986). Thus, activation of the kinase by autophosphorylation involves cooperative interactions among the subunits. At the earliest time shown in Figure 1 (5 s), the kinase has acquired an average of 8 phosphates per dodecameric holoenzyme and has also acquired its full rate of Ca^{2+}-independent kinase activity. At this time, 80% of the phosphate is lo-

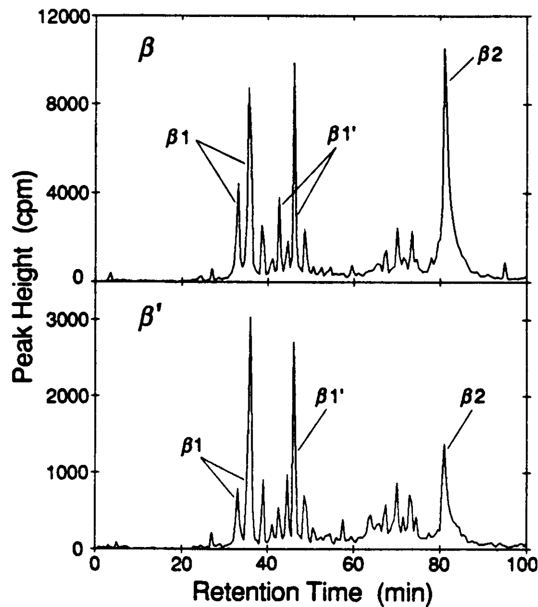

Figure 4. Comparison of Tryptic Phosphopeptides from β and β′ Subunits after Brief Autophosphorylation.

Kinase holoenzyme with a 1:1 ratio of α to β subunits (10 μg) was phosphorylated for 5 s in the presence of [γ-^{32}P]ATP, Ca^{2+}, and calmodulin as in Figure 1 (see Experimental Procedures). The β and β′ subunits were separated by gel electrophoresis and digested with trypsin. The resulting tryptic phosphopeptides were fractionated on a C4 column as in Figure 1.

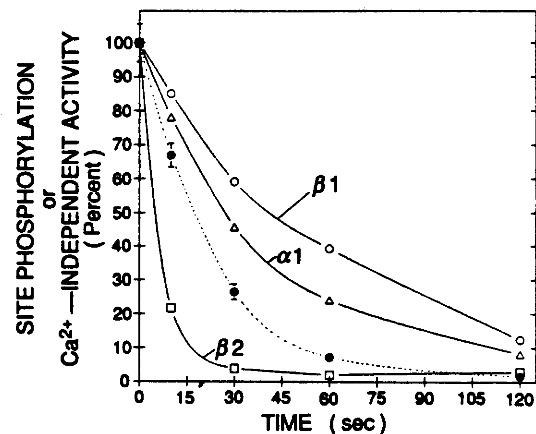

Figure 5. Effect of Dephosphorylation of Sites α1, β1, and β2 on Ca^{2+}-Independent Kinase Activity

Forebrain kinase was autophosphorylated for 5 s in the presence of Ca^{2+} and calmodulin (see Figure 1), then dephosphorylated for the indicated times as described in Experimental Procedures. In three experiments, kinase activity in the absence of Ca^{2+} was measured at each time point. At time zero it was 1.6 μmol/min per mg (100% in the figure). In another set of experiments, the phosphate present on sites α1, β1, and β2 was measured at each time point as described in Experimental Procedures. The amounts of phosphate on sites α1, β1 and β2 before dephosphorylation were 3.2, 1.5, and 0.6 mol per mol of holoenzyme, respectively. At 10 s, the corresponding values were 2.5, 1.3, and 0.1. At 30 s, they were 1.5, 0.9, and 0. (●) Percent Ca^{2+}-independent activity; (Δ) percent phosphate in α1; (O) Percent phosphate in β1; (□) percent phosphate in β2.

cated on sites α1, β1 or β2. Per forebrain holoenzyme, an average of 4–5 phosphates are on site α1 or site β1 and an average of 0.5–1 phosphates are on site β2. Because the initial rates of autophosphorylation at sites α1, β1, and β2 are similar, we cannot distinguish on this basis which of these sites is responsible for activation. However, we found that we could distinguish among the sites when we dephosphorylated the kinase and measured the rate of consequent decay of Ca^{2+}-independent activity (Figure 5). Protein phosphatase 2A dephosphorylated site β2 faster than sites α1 or β1. The decay of Ca^{2+}-independent activity was slower than the rate of dephosphorylation of site β2 and was consistent with the hypothesis that phosphorylation of about 3 α1/β1 sites per holoenzyme is required for Ca^{2+}-independent activity. The data do not rigorously exclude a role for site β2 in the generation of Ca^{2+}-independent activity, but they indicate that its phosphorylation is not required to maintain Ca^{2+}-independent activity.

Table 3. Sequences of Slow Peptides α2, β4, and β3

	α2		β4		β3	
Cycle	Residue	Yield[a]	Residue	Yield[b]	Residue	Yield[c]
1	Ser	117	Ser	47	Ser[d]	452
2	Thr	42	Thr	20	Leu	342
3	Val	59	Val	32	Leu	390
4	Ala	66	Ala	34	Asn	142
5	–	–	–	–	Lys	104
6	[Glu]	65	Met	15		
7	Met	34	Met	18		

All sequencing was done as described in Experimental Procedures.
[a] Approximately 300 pmol of α2 was submitted for sequencing.
[b] Approximately 150 pmol of β4 was submitted for sequencing.
[c] Approximately 600 pmol of β3 was submitted for sequencing.
[d] In this sequencing run phosphoserine was recovered predominantly (90%) as the hydrolysis product dehydroalanine.

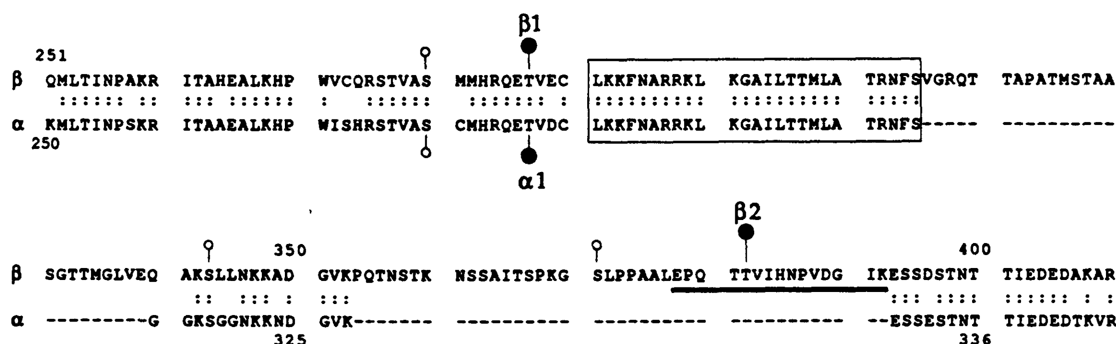

Figure 6. Location of Autophosphorylation Sites in the Sequences of the α and β Subunits of Neuronal Type II CaM Kinase

The portions of the amino acid sequences of α and β subunits that contain rapid autophosphorylation sites are shown. Amino acid identities between the subunits are indicated by colons. The region that is specifically deleted in the β' subunit is underlined. The boxed region contains the putative CaM binding domains. Rapid autophosphorylation sites are indicated by filled circles, and slow autophosphorylation sites are indicated by small open circles. α2 is α-Ser_{279}; β4 is β-Ser_{280}; β3 is β-Ser_{343}; β5 is β-Ser_{371}.

Slow Autophosphorylation Sites in the α and β Subunits

Peaks α2, β3, and β4 labeled in Figures 1D–1F each contain discrete serine sites that are autophosphorylated at a slower rate than the rapid threonine sites. We purified phosphopeptides from each of these peaks by the methods described above. They yielded the sequences shown in Table 3. The autophosphorylation sites contained in peptides α2 and β4 are homologous; they are α-Ser_{279} and β-Ser_{280}. Peptide β3 contains residues Ser_{343} to Lys_{347} in β. Thus site β3 is β-Ser_{343}.

Discussion

In its basal state, type II CaM kinase is inactive in the absence of either Ca^{2+} or calmodulin. When it is activated by the Ca^{2+}/calmodulin complex it phosphorylates itself, as well as exogenous substrates. The autophosphorylated kinase remains active even after the initial Ca^{2+} signal is removed (Miller and Kennedy, 1986). The production of Ca^{2+}-independent activity by autophosphorylation is highly cooperative; it is maximal after autophosphorylation of only a few subunits in a holoenzyme (Miller and Kennedy, 1986; Lai et al., 1987; Kwiatkowski et al., 1988). We have postulated that the kinase may be able to prolong its Ca^{2+}-independent state in the presence of cellular protein phosphatases by continuing to autophosphorylate itself, thus maintaining the critical number of phosphorylated sites. There are several sites that can be autophosphorylated (~2 mol PO_4 per mol of α subunit and ~3 mol PO_4 per mol of β subunit). In order to test the above hypothesis more rigorously with purified kinase and to assess whether Ca^{2+}-independent activity occurs and is maintained in vivo, it is important to know which of the several autophosphorylation sites control generation and maintenance of Ca^{2+}-independent activity.

In this study, we have identified three sites that are rapidly autophosphorylated when the kinase is activated by Ca^{2+} and calmodulin. We have shown definitively that two homologous sites, Thr_{286} in the α subunit and Thr_{287} in the β subunit, regulate Ca^{2+}-independent activity. These sites are located between the catalytic and calmodulin binding domains in each of their respective subunits (Figure 6; Figure 7). Because they are contained within the substrate consensus sequence Arg-X-Y-Thr (Pearson et al., 1985) and are immediately adjacent to the calmodulin binding sequences, we and others have previously speculated that they or other similarly located sites might be involved in the generation of Ca^{2+}-independent activity by autophosphorylation (Bennett and Kennedy, 1987; Hanley et al., 1987; Lai et al., 1987; Lin et al., 1987; Bulleit et al., 1988; Payne et al., 1988).

Two lines of evidence now establish the central role of these sites. First, we have shown that they are rapidly autophosphorylated upon activation of the kinase in the presence of Ca^{2+} and calmodulin. The kinase attains its maximum Ca^{2+}-independent activity at a time when most of the phosphate present in the enzyme is on these sites (Figure 1). The remaining phosphate is primarily on the third rapidly autophosphorylated site, β-Thr_{382}. Second, we have shown that holoenzymes phosphorylated almost exclusively on α-Thr_{286} and β-Thr_{287} retain nearly maximum Ca^{2+}-independent activity (Figure 5). This phosphorylation state was produced by selective dephosphorylation of partially autophosphorylated kinase. As these two sites are gradually dephosphorylated, Ca^{2+}-independent activity begins to drop. The results are quantitatively consistent with the observation, first reported by us (Miller and Kennedy, 1986) and later confirmed by others (Lai et al., 1987; Kwiatkowski et al., 1988), that autophosphorylation of as few as 2 or 3 sites per holoenzyme is sufficient to produce maximum Ca^{2+}-independent activity.

A more specific model for regulation of Ca^{2+}-independent activity by $Thr_{286/287}$ can be proposed in light of recent experiments by Hanley et al. (1988) and Payne et al. (1988). By examining the binding and inhibitory effects of synthetic peptides, they have divided the calmodulin

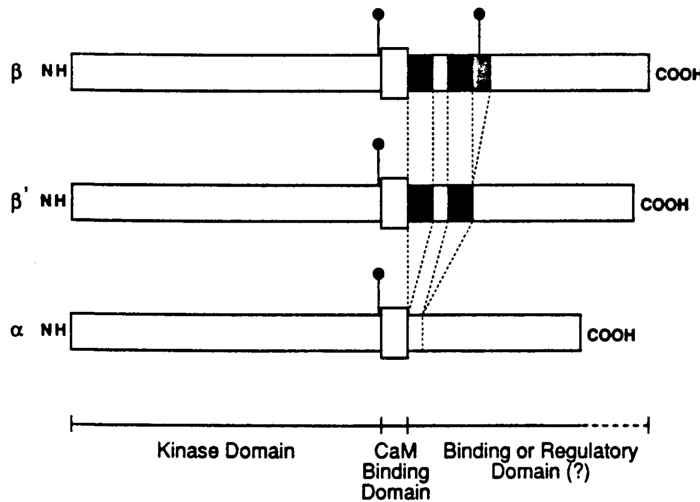

Figure 7. Schematic Representation of Rapid Autophosphorylation Sites and Functional Domains in the α, β, and β' Subunits

The primary structures of the α, β, and β' subunits are shown schematically. The thick segment represents the putative CaM binding domain. The filled and shaded regions shown in the β subunit are absent in the α subunit. The shaded region is absent in the β' subunit. All other regions are identical in the β and β' subunits and are similar but not identical in the α subunit. The locations of the rapid autophosphorylation sites identified in this study are indicated by a ball and stick.

binding domain of the α subunit (see Figure 6) into two subdomains. The sequence LKKFNA (residues 290 through 295) is an inhibitory domain that suppresses phosphorylation of peptide substrates but has low affinity for calmodulin. In contrast, the sequence TTMLA (residues 305 through 309) is required for high affinity binding of calmodulin but does not mediate potent substrate-directed inhibition. Payne et al. postulate that binding of calmodulin to the kinase may alleviate the inhibition produced by the association of residues 290 to 295 with the active site. In addition, they postulate that phosphorylation of the nearby Thr_{286} (Figure 6; Figure 7) may also relieve inhibition. Our results provide direct evidence that phosphorylation of this "internal substrate" does indeed produce a partially Ca^{2+}-independent activity.

The function of the third rapidly autophosphorylated site, β-Thr_{382}, is not known. Its phosphorylation does not seem to be required to maintain Ca^{2+}-independent activity (Figure 5). However, its location is extremely interesting. It is not contained within an Arg-X-Y-Thr consensus sequence and so was not a predicted autophosphorylation site. It is absent in the α subunit and is specifically removed from the β' subunit, apparently by alternative splicing (Figure 6; Figure 7; Bulleit et al., 1988). Although its role is unknown, regulation of its presence within the holoenzyme by both differential gene expression (α versus β) and alternative splicing (β') suggests that it has a highly specialized function. It is located on the carboxyl side of the calmodulin binding domain, in a region that may control binding to the cytoskeleton or to subcellular organelles (Figure 7; Miller and Kennedy, 1985; Lin et al., 1987; Bulleit et al., 1988). Thus, it may regulate the subcellular location of the kinase.

When autophosphorylation proceeds for several minutes in the presence of Ca^{2+} and calmodulin, a number of additional serine sites become phosphorylated with little additional effect on kinase activity (Lai et al., 1987; data not shown). Because the autophosphorylated form of the kinase is more labile (Lai et al., 1986), the specific activity of the phosphokinase falls appreciably during long incubations compared with that of nonphosphorylated kinase. Less than 5% of the lost activity is recovered when the enzyme is subsequently dephosphorylated (data not shown). Thus, most of the loss is not a reversible effect of autophosphorylation and may not be significant in vivo. In the course of this study, we have identified several of the slower autophosphorylation sites (Table 3; Figure 6). α-Ser_{279} (α2) and β-Ser_{280} (β4) are 7 residues upstream of $Thr_{286,287}$, while β-Ser_{371} is 11 residues upstream of the rapid site β-Thr_{382}. β-Ser_{343} (β3) is midway between the two rapid β autophosphorylation sites. We have not yet determined whether any of the slow sites can substitute for $Thr_{286,287}$ in maintaining Ca^{2+}-independent activity.

An interesting generalization that emerges from this study concerns the consensus sequence that is recognized by the active site of type II CaM kinase. Pearson et al. (1985) previously defined a minimum consensus substrate sequence as Arg-X-Y-Ser/Thr. A comparison of the sequences surrounding the most rapidly autophosphorylated sites on the kinase with sequences surrounding phosphorylation sites on several of its substrates suggests that additional residues may contribute to substrate recognition (Table 4). Two of the rapid sites, α1 and β1, share the common sequence Arg-Gln-X-Thr-Val. The third rapid site, β2, shares part of this sequence, but the canonical arginine is replaced by proline (Pro-Gln-X-Thr-Val). This suggests that Arg at the −3 position is not an absolute requirement, at least for an internal substrate, and that Gln at −2 and Val at +1 can enhance the rate of phosphorylation. The exogenous substrate sites also fit this general pattern. They all contain Arg at the −3 position. Two contain Gln at the −2 position, and 4 contain Val at +1. Thus, we propose the more complete sequence,

Table 4. Consensus Sequences Surrounding Rapid Autophosphorylation Sites and Exogenous Substrate Sites

Substrate	Phosphorylation Site
Type II CaM kinase (α1)	–Met-His -Arg-Gln-Glu- Thr -Val -Asp-Cys -Leu -Lys–
Type II CaM kinase (β1)	–Met-His -Arg-Gln-Glu- Thr -Val -Glu-Cys -Leu -Lys–
Type II CaM kinase (β2)	–Leu-Glu-Pro-Gln-Thr- Thr -Val -Ile -His -Asn -Pro–
Glycogen synthase (site 2)[a]	–Leu -Ser -Arg-Thr -Leu- Ser -Val -Ser -Ser -Leu -Pro–
Glycogen synthase (site 1b)[a,b]	–Ser -Lys -Arg-Ser -Asn- Ser -Val -Asp-Thr -Ser -Ser–
Synapsin I (site 2)[c]	–Ala -Thr -Arg-Gln-Thr- Ser -Val -Ser -Gly -Gln -Ala–
Synapsin I (site 3)[c]	–Pro -Thr -Arg-Gln-Ala- Ser -Gln-Ala -Gly -Pro -Met–
Tryosine hydroxylase (site A)[d]	–Ile -Gly -Arg-Arg-Gln- Ser -Leu-Ile -Glu-Asp-Ala–
Tyrosine hydroxylase (site C)[d]	–Phe-Arg -Arg-Ala -Val- Ser -Glu-Gln-Asp-Ala -Lys–
MAP_2[e](?)	–Val -Ala -Arg-Arg-Lys- Ser -Val -Pro -Ser -Glu -Thr–

[a] From Rylatt and Cohen (1979).
[b] From Parker et al. (1981).
[c] From Czernik et al. (1987) (bovine sites).
[d] From Campbell et al. (1986).
[e] Proposed site in microtubule-associated protein 2; from Kosik et al. (1988).

Arg-Gln-X-Thr/Ser-Val, for optimal phosphorylation by type II CaM kinase. Arg at −3 appears to be most important, but Gln at −2 and Val at +1 may contribute to the likelihood that the site will be phosphorylated at a high rate. These findings should permit more precise identification of possible phosphorylation sites for type II CaM kinase within the sequences of potential substrates.

Identification of the rapidly autophosphorylated sites that control generation of Ca^{2+}-independent activity allows us to refine the original model of the type II CaM kinase as a Ca^{2+}-triggered switch (Miller and Kennedy, 1986). We now know that the initial transition to a partially Ca^{2+}-independent state occurs through rapid autophosphorylation of a specific threonine located near the calmodulin binding domain in each of the subunits. In the continued presence of Ca^{2+}, several additional serine sites are autophosphorylated at a slower rate. It is possible that these additional phosphorylated sites can prolong the Ca^{2+}-independent state by acting as competitive substrates for protein phosphatases; however, we have not yet determined whether they alone can support Ca^{2+}-independent activity. When Ca^{2+}/calmodulin is removed from the phosphokinase, at least 1 additional site becomes autophosphorylated and the kinase can no longer be stimulated by Ca^{2+}/calmodulin (Hashimoto et al., 1987; our own unpublished data). It will be interesting to determine the location of this site.

Two important questions remain. Does activation of type II CaM kinase by autophosphorylation occur in vivo, and if it does, what are the physiological roles of the Ca^{2+}-independent form? The work reported here will enable us to measure phosphorylation of the functionally important autophosphorylation sites in intact neurons under different physiological conditions. This will be a first step toward understanding the functions of the kinase in vivo.

Experimental Procedures

Materials

Acetonitrile (HPLC/UV Grade) was purchased from Burdick and Jackson (Muskegon, MI), trifluoroacetic acid (TFA, Sequenal grade) was from Pierce Biochemical, and dithiothreitol (DTT) was from Schwarz/Mann Biotech. Trypsin treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone (trypsin-TPCK) was purchased from Cooper Biomedical, and thermolysin was from Boehringer Mannheim Biochemicals. Iodoacetamide, caffeine, phosphorylase b, and phosphorylase kinase were purchased from Sigma, and [γ-^{32}P]ATP was from ICN Nutritional Biochemicals. C4 and C18 reverse-phase HPLC columns (4.5 × 250 mm) were obtained from Vydac.

Calmodulin was purified from bovine brain by the method of Watterson et al. (1976). The forebrain and cerebellar holoenzymes of the type II CaM kinase were purified from rat as described previously (Miller and Kennedy, 1985). A third holoenzyme variant contains approximately equimolar amounts of the α and β subunits. This ratio corresponds to the ratio of subunits observed in homogenates of lower brain regions such as the pons and medulla (Erondu and Kennedy, 1985; Walaas et al., 1983a, 1983b). This variant was eluted from the CaM-Sepharose affinity column when the column was washed with buffer containing 0.2 M NaCl and 2 mM EGTA after elution of the forebrain isozyme in 2 mM EGTA. The catalytic subunit of protein phosphatase 2A was purified from rabbit skeletal muscle by the method of Tung et al. (1984), omitting the final HPLC step. Loss of activity during storage was reversed by incubation at 30°C for 1 hr in the presence of 50 mM Tris-HCl (pH 7.0), 2 mM $MnCl_2$, 50 mM DTT, 5 mM caffeine.

Autophosphorylation of Type II CaM Kinase

For analytical experiments, kinase was autophosphorylated in a mixture (final volume 50 µl) containing 10 µg of kinase, 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 10 µg of calmodulin, 10 mM DTT, 0.6 mM EGTA, 0.9 mM $CaCl_2$, and 200 µM [γ-^{32}P]ATP (2.0–3.0 × 10^3 cpm/pmol). The mixtures were warmed for 30 s at 30°C, and reactions were initiated by the addition of kinase. After the indicated times (see figure legends), the reactions were terminated by the addition of 50 µl of ice-cold 0.2 M EDTA (pH 8.0) followed by immediate cooling in an ice water bath. In general, the quenched reaction mixtures were divided into two 50 µl aliquots. One was used to determine the stoichiometry of phosphate incorporation into the α and β subunits after their

separation by SDS-PAGE as described previously (Miller and Kennedy, 1986), and the other was used for tryptic phosphopeptide mapping by reverse-phase HPLC as described below.

Reversal of Autophosphorylation by Dephosphorylation with Protein Phosphatase

The kinase was autophosphorylated essentially as described above in a final volume of 30 µl with unlabeled ATP. Bovine serum albumin (0.5 mg/ml) was included. Autophosphorylation was terminated after 5 s by the addition of 15 µl of 0.4 M EDTA, 25 mM Tris-HCl (pH 7.0), 1 mg/ml BSA (stop solution) at 30°C. Controls were incubated in the absence of ATP, and ATP was added immediately after the stop solution. Dephosphorylation (30°C) was initiated 10 s after terminating autophosphorylation by the addition of 0.4 µg of protein phosphatase 2A (7.0 µmol of PO_4 removed from phosphorylase a per min per mg) to a final volume of 60 µl. Phosphatase dilution buffer (50 mM Tris-HCl [pH 7.0], 30 mM DTT, 20 mM caffeine, 1 mg/ml bovine serum albumin) was added to controls. At the indicated times (see Figure 5), aliquots were diluted 50-fold into ice-cold 40 mM Tris-HCl (pH 8.0), 1 mg/ml bovine serum albumin. Kinase activity was assayed immediately in the presence or absence of Ca^{2+}. Ten microliters of diluted kinase was added to reaction mixtures containing 0.2 mg/ml synapsin, 50 µg/ml calmodulin, 0.4 mM EGTA, 0.9 mM $CaCl_2$, and 200 µM [γ-^{32}P]ATP (0.6–1.4 × 10^3 cpm/pmol). After 15 s, reactions were stopped by the addition of SDS-stop solution. Incorporation of phosphate into synapsin I was measured after gel electrophoresis as previously described (Kennedy et al., 1983). The small amount of phosphatase carried over from the kinase dephosphorylation had no detectable effect on the level of kinase activity.

For measurement of dephosphorylation of specific sites, autophosphorylation and subsequent dephosphorylation were performed as described above but with [γ-^{32}P]ATP. Autophosphorylation was performed in a final volume of 165 µl containing 33 µg of kinase and was terminated after 5 s by addition of 82.5 µl of 0.4 M EDTA stop solution. One aliquot containing 6 µg of kinase was mixed with an equal volume of 6% (w/v) SDS, 10% (w/v) glycerol, 4% (v/v) 2-mercaptoethanol, 120 mM Tris-HCl (pH 6.7), and a trace of bromphenol blue (2× gel sample buffer). Dephosphorylation of the remaining kinase was initiated by the addition of 1.9 µg of protein phosphatase 2A (final volume 270 µl). It was terminated at the indicated times (see Figure 5) by the addition of 2× gel sample buffer to aliquots containing 6 µg of kinase. Two aliquots from each SDS-quenched reaction, each containing 0.5 µg of kinase, were removed, and the subunits were separated by SDS-PAGE as described previously. The amount of labeled phosphate incorporated into α and β subunits was determined. The remaining kinase (5 µg) was used for tryptic phosphopeptide mapping by reverse-phase HPLC as described below. We calculated the area under each phosphopeptide peak using an integration program. The fraction of radioactivity in individual sites was calculated by summing the areas under all peaks that contained the site and then dividing by the total area under the elution profile. Moles of phosphate incorporated into each site per mole subunit were calculated by multiplying the total moles of phosphate per mole subunit by the fraction of radioactivity in the site.

Reduction and Alkylation of Autophosphorylated Kinase

One milliliter of ice-cold 10% (w/v) TCA was added to a 50 µl aliquot of the quenched autophosphorylation mixture, which was then cooled in ice water for 15 min. Precipitated protein was pelleted by centrifugation at 12,000 × g for 15 min. The supernatant was removed by aspiration, and the pellet was washed once with 1 ml of ice-cold ether. Pellets from SDS-quenched dephosphorylation reactions were washed two more times with 1 ml of −20°C acetone to remove SDS. Protein was denatured under N_2 for 30 min at 50°C in 400 µl of 6 M guanidine-HCl, 0.5 M Tris-HCl (pH 8.1), 2 mM EDTA. Fifty microliters of a fresh solution of 5 mM DTT was added, and the protein was reduced under N_2 for 4 hr at 50°C. The tube was then cooled to room temperature, and 50 µl of fresh 20 mM iodoacetamide was added. Alkylation was allowed to proceed for 30 min in the dark at room temperature or for 60 min in the dark at 0°C. The reaction was quenched by the addition of 50 µl of 0.1 M DTT and was immediately dialyzed against 4 l of 50 mM NH_4HCO_3 in the dark for 10–12 hr at 4°C with one change of buffer. The dialyzed protein was lyophilized and resuspended in 70 µl of 1% SDS, 0.67% (v/v) 2-mercaptoethanol, 1.67% (w/v) glycerol, 20 mM Tris-HCl (pH 6.7), and a trace of bromphenol blue. The sample was heated to 100°C for 2 min, and the kinase subunits were separated by SDS-PAGE. Autophosphorylated subunits were located in dried gels by autoradiography.

Tryptic Digestion of Subunits

Pieces containing the α or β subunits were cut from the dried gels and washed twice for 1 hr each in 50 ml of 20% (v/v) isopropanol, then twice for 1 hr each in 50 ml of 10% (v/v) methanol. The washed gel pieces were lyophilized, 100 µl of 50 mM NH_4HCO_3 (pH 8.1) containing 50 µg of trypsin (20-fold excess of trypsin by weight) was added to each piece, and the mixture was incubated for 4 hr at 37°C. One milliliter of 50 mM NH_4HCO_3 (pH 8.1) containing an additional 50 µg of trypsin was added, and the incubation was continued for 12 hr at 37°C. The solutions, which contained most of the tryptic peptides were removed and filtered through 0.2 µm filters. The gel pieces were washed with 0.4 ml of distilled H_2O, and the wash was filtered and combined with the first supernatant. Radioactivity in the filtrates and gel pieces was determined by measuring Cerenkov radiation. The filtrates were lyophilized. From the α subunit, 78% ± 9% of [^{32}P]phosphate was recovered in the supernatants and from the β subunit, 82% ± 8% was recovered. Recovery of ^{32}P could be increased to 95%–100% by re-extracting the gel pieces with 1 ml of 50 mM NH_4HCO_3 (pH 8.1) for 8–12 hr, but this made no qualitative difference in the phosphopeptide maps.

Reverse-Phase HPLC Fractionation of Phosphopeptides

Lyophilized peptides were resuspended in 100 µl of buffer A (0.07% [v/v] TFA [pH 2.3]) and loaded onto a C4 reverse-phase HPLC column equilibrated in buffer A. Buffer B consisted of 0.07% (v/v) TFA, 70% acetonitrile, 30% H_2O. The column was developed at a flow rate of 1 ml/min with the following gradient: 0–5 min, 0% buffer B; 5–95 min, 0%–35% buffer B; 95–100 min, 35%–100% buffer B. Thus, most of the fractionation occurred between 0% and 24.5% acetonitrile. Labeled peptides were detected in fractions by measuring Cerenkov radiation (48% efficiency). The concentration of acetonitrile in the samples had no significant effect on counting efficiency. Recovery of [^{32}P]phosphate from C4 columns was 80%–95% for both α and β subunits.

Phospho-Amino Acid Analysis

Fractions containing phosphopeptides of interest were pooled and dried in a Speedvac vacuum concentrator. The lyophilized phosphopeptides were partially hydrolyzed in 6 N HCl for 2 hr at 110°C in vacuo. Hydrolyzed phosphopeptides were dissolved in 5 µl of electrophoresis buffer (acetic acid:formic acid:H_2O [78:25:897], pH 1.9) and separated by electrophoresis for 2 hr at 750 V on 20 × 20 cm thin layer cellulose sheets in the same buffer. Marker phosphoamino acids (phosphoserine, phosphothreonine, and phosphotyrosine) were located with ninhydrin, and labeled unknown phospho-amino acids were detected by autoradiography.

Proteolysis of Phosphopeptides with Thermolysin

Fractions containing phosphopeptides were pooled and reduced in volume to approximately 25–50 µl in a Speedvac. Four hundred microliters of 100 mM NH_4HCO_3 (pH 8.0) containing 1 µg of thermolysin was added, and the mixture was incubated for 4 hr at 37°C. The digest was fractionated on a C18 reverse-phase HPLC column developed with the following gradient: 0–10 min, 0% buffer B; 10–20 min, 0%–15% buffer B; 20–90 min, 15%–25% buffer B; 90–100 min, 25%–100% buffer B. Fractions (0.5 ml) were collected by hand, and absorbance of the eluent was monitored at 214 nm.

Large Scale Autophosphorylation and Tryptic Digestion

To obtain nanomolar quantities of phosphopeptides, batches of pure type II CaM kinase were autophosphorylated in a reaction mixture (1 ml) containing 200 µg of kinase, 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 100 µg of calmodulin, 10 mM DTT, 0.6 mM EGTA, 0.9 mM $CaCl_2$, and 200 µM [γ-^{32}P] ATP (20–40 cpm/pmol). The mixtures were warmed for 60 s at 30°C,

and reactions were initiated by the addition of kinase. After the indicated times (see figure legends), the reactions were quenched by the addition of 120 µl of ice-cold 100% TCA and cooled in an ice water bath. Reduction, alkylation, SDS-PAGE, and tryptic digestion were carried out as described above. To improve recovery of peptides, the gel pieces were extracted a second time with 1 ml of 50 mM NH_4HCO_3 for 8-12 hr at 37°C. The second supernatant was filtered and combined with the first. Chromatography on a C4 column was performed as described above, and absorbance of the eluent was monitored at 214 nm. In general, several reactions, each containing peptides from α and β subunits derived from 200 µg of kinase, were combined and fractionated in a single C4 column run. In one case, separate C4 column fractionations were performed on peptides derived from each 200 µg reaction.

Fractions containing individual peaks were pooled and reduced to 50-100 µl in a Speedvac. After addition of 300 µl of buffer A, the peaks were purified on a C18 column equilibrated with buffer A. In each case, the column was developed with a gradient of increasing buffer B that optimized fractionation of the desired phosphopeptide. Peptides α1 and α1′ (Figures 2A and 2B) were eluted with this gradient: 0-10 min, 0% buffer B; 10-100 min, 0%-15% buffer B; 100-145 min, 15%-40% buffer B; 145-155 min, 40%-100% buffer B; 155-165 min, 100% buffer B. Peptides β1 and β1′ (Figures 2C and 2D) were eluted with this gradient: 0-5 min, 0% buffer B; 5-10 min, 0%-7.5% buffer B; 95-100 min, 15%-100% buffer B. Thermolytic peptides generated from β2 and β2′ (Figure 3) were eluted with this gradient: 0-10 min, 0% buffer B; 10-20 min, 0%-15% buffer B; 20-90 min, 15%-25% buffer B; 90-100 min, 25%-100% buffer B. Fractions (0.5 ml) were collected by hand while monitoring absorbance of the eluent at 214 nm. Pure phosphopeptides were sequenced or subjected to amino acid analysis.

Other Procedures

Automated gas-phase sequencing was performed at the Comprehensive Cancer Center Microchemical Facility of the University of Southern California; at the Vollum Institute for Advanced Biomedical Research, Oregon Health Sciences University, Portland; or at the Applied Microsequencing Facility at Caltech. For amino acid analysis, total hydrolysis was performed in 6 N HCl for 1 hr at 165°C in vacuo. Amino acids were converted to their phenylthiocarbamyl derivatives by an Applied Biosystems 420A Derivatizer, then identified and quantified on an Applied Biosystems 130A Separation System.

Protein concentrations were measured by the method of Peterson (1977) with bovine serum albumin as standard. SDS-PAGE was performed by the method of Laemmli (1970). Stacking gels contained 3.5% acrylamide, 0.09% bisacrylamide; running gels contained 8% acrylamide, 0.27% bisacrylamide.

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References

Annan, W. D., Manson, W., and Nimmo, J. A. (1982). The identification of phosphoseryl residues during the determination of amino acid sequences in proteins. Anal. Biochem. *121*, 62-68.

Barker, W. C. and Dayhoff, M. O. (1982). Viral *src* gene products are related to the catalytic chain of mammalian cAMP-dependent protein kinase. Proc. Natl. Acad. Sci. USA *79*, 2836-2839.

Bengur, A. R., Robinson, E. A., Apella, E., and Sellers, J. R. (1987). Sequence of the sites phosphorylated by protein kinase C in the smooth muscle myosin light chain. J. Biol. Chem. *262*, 7613-7617.

Bennett, M. K., and Kennedy, M. B. (1987). Deduced primary structure of the β subunit of brain type II Ca^{2+}/calmodulin-dependent protein kinase determined by molecular cloning. Proc. Natl. Acad. Sci. USA *84*, 1794-1798.

Bulleit, R. F., Bennett, M. K., Molloy, S. S., Hurley, J. B., and Kennedy, M. B. (1988). Conserved and variable regions in the subunits of brain type II Ca^{2+}/calmodulin-dependent protein kinase. Neuron *1*, 63-72.

Campbell, D. G., Hardie, D. G., and Vulliet, P. R. (1986). Identification of four phosphorylation sites in the N-terminal region of tyrosine hydroxylase. J. Biol. Chem. *261*, 10489-10492.

Czernick, A. J., Pang, D. T., and Greengard, P. (1987). Amino acid sequences surrounding the cAMP-dependent and calcium/calmodulin-dependent sites in rat and bovine synapsin I. Proc. Natl. Acad. Sci. USA *84*, 7518-7522.

Erondu, N. E., and Kennedy, M. B. (1985). Regional distribution of type II Ca^{2+}/calmodulin-dependent protein kinase in rat brain. J. Neurosci. *5*, 3270-3277.

Hanley, R. M., Means, A. R., Ono, T., Kemp, B. E., Burgin, K. E., Waxman, N., and Kelly, P. T. (1987). Functional analysis of a complementary DNA for the 50-kilodalton subunit of calmodulin kinase II. Science *237*, 293-297.

Hanley, R. M., Means, A. R., Kemp, B. E., and Shenolikar, S. (1988). Mapping of calmodulin-binding domain of Ca^{2+}/calmodulin-dependent protein kinase II from rat brain. Biochem. Biophys. Res. Comm. *152*, 122-128.

Hashimoto, Y., Schworer, C. M., Colbran, R. J., and Soderling, T. R. (1987). Autophosphorylation of Ca^{2+}/calmodulin-dependent protein kinase. Effects on total and Ca^{2+}-independent activities and kinetic parameters. J. Biol. Chem. *262*, 8051-8055.

Hunter, T. (1987). A thousand and one protein kinases. Cell *50*, 823-829.

Kelly, P. T., McGuinness, T. L., and Greengard, P. (1984). Evidence that the major postsynaptic density protein is a component of a calcium-calmodulin-dependent protein kinase. Proc. Natl. Acad. Sci. USA *81*, 945-949.

Kennedy, M. B., Bennett, M. K., and Erondu, N. E. (1983). Biochemical and immunochemical evidence that the "major postsynaptic density protein" is a subunit of a calmodulin-dependent protein kinase. Proc. Natl. Acad. Sci. USA *80*, 7357-7361.

Kennedy, M. B., Bennett, M. K., Erondu, N. E., and Miller, S. G. (1987). Calcium-calmodulin-dependent protein kinases. In Calcium and Cell Function, Vol. VII, W. Y. Cheung, ed. (New York: Academic Press, Inc.), pp. 62-107.

Kosik, K. S., Orecchio, L. D., Bakalis, S., Duffy, L., and Neve, R. L. (1988). Partial sequence of MAP2 in the region of a shared epitope with Alzheimer's neurofibrillary tangles. J. Neurochem., in press.

Kwiatkowski, A. P., Shell, D. J., and King, M. M. (1988). The role of autophosphorylation in activation of the type II calmodulin-dependent protein kinase. J. Biol. Chem. *263*, 6484-6486.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680-685.

Lai, Y., Nairn, A. C., and Greengard, P. (1986). Autophosphorylation reversibly regulates the Ca^{2+}/calmodulin-dependence of Ca^{2+}/calmodulin-dependent protein kinase II. Proc. Natl. Acad. Sci. USA *83*, 4253-4257.

Lai, Y., Nairn, A. C., Gorelick, F., and Greengard, P. (1987). Ca^{2+}/calmodulin-dependent protein kinase II: identification of autophosphorylation sites responsible for generation of Ca^{2+}/calmodulin-independence. Proc. Natl. Acad. Sci. USA *84*, 5710-5714.

Lin, C. R., Kapiloff, M. S., Durgerain, S., Tatemoto, K., Russo, A. F., Hanson, P., Schulman, H., and Rosenfeld, M. G. (1987). Molecular cloning of a brain-specific calcium/calmodulin-dependent protein kinase. Proc. Natl. Acad. Sci. USA *84*, 5962-5966.

Lisman, J. E. (1985). A mechanism for memory storage insensitive to molecular turnover: a bistable autophosphorylating kinase. Proc. Natl. Acad. Sci. USA *82*, 3055–3057.

McGuinness, T. L., Lai, Y., and Greengard, P. (1985). Ca^{2+}/calmodulin-dependent protein kinase II. Isozymic forms from rat forebrain and cerebellum. J. Biol. Chem. *260*, 1696–1704.

Miller, S. G., and Kennedy, M. B. (1985). Distinct forebrain and cerebellar isozymes of type II Ca^{2+}/calmodulin-dependent protein kinase associate differently with the postsynaptic density fraction. J. Biol. Chem. *260*, 9039–9046.

Miller, S. G., and Kennedy, M. B. (1986). Regulation of brain type II Ca^{2+}/calmodulin-dependent protein kinase by autophosphorylation: a calcium-triggered molecular switch. Cell *44*, 861–870.

Parker, P. J., Aitken, A., Bilham, T., Embie, N., and Cohen, P. (1981). Amino acid sequence of a region in rabbit skeletal muscle glycogen synthase phosphorylated by cyclic AMP-dependent protein kinase. FEBS Lett. *123*, 332–336.

Payne, M. E., Fong, Y.-L., Ono, T., Colbran, R. J., Kemp, B. E., Soderling, T. R., and Means, A. R. (1988). Calcium/calmodulin-dependent protein kinase II. Characterization of distinct calmodulin binding and inhibitory domains. J. Biol. Chem. *263*, 7190–7195.

Pearson, R. B., Woodgett, J. R., Cohen, P., and Kemp, B. E. (1985). Substrate specificity of multifunctional calmodulin-dependent protein kinase. J. Biol. Chem. *260*, 14471–14476.

Peterson, G. L. (1977). A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal. Biochem. *83*, 346–356.

Podell, D. N., and Abraham, G. N. (1978). A technique for the removal of pyroglutamic acid from the amino terminus of proteins using calf liver pyroglutamate amino peptidase. Biochem. Biophys. Res. Commun. *81*, 176–185.

Rylatt, D. B., and Cohen, P. (1979). Amino acid sequence at the site on rabbit skeletal muscle glycogen synthase phosphorylated by the endogenous glycogen synthase kinase-2 activity. FEBS Lett. *98*, 71–75.

Stull, J. T., Nunnally, M. H., and Michnoff, C. H. (1986). Calmodulin-dependent protein kinase. In The Enzymes, Vol. XVII (New York: Academic Press, Inc.), pp. 113–166.

Tung, H. Y. L., Resink, T. J., Hemmings, B. A., Shenolikar, S., and Cohen, P. (1984). The catalytic subunits of protein phosphatase-1 and protein phosphatase 2A are distinct gene products. Eur. J. Biochem. *138*, 635–641.

Walaas, S. I., Nairn, A. C., and Greengard, P. (1983a). Regional distribution of calcium and cAMP-regulated protein phosphorylation systems in mammalian brain. I. Particulate systems. J. Neurosci. *3*, 291–301.

Walaas, S. I., Nairn, A. C., and Greengard, P. (1983b). Regional distribution of calcium and cAMP-regulated protein phosphorylation systems in mammalian brain. II. Soluble systems. J. Neurosci. *3*, 302–311.

Watterson, D. M., Harrelson, W. G., Keller, P. M., Sharief, F., and Vanaman, T. C. (1976). Structural similarities between the Ca^{2+}-dependent regulatory proteins of 3':5'-cyclic nucleotide phosphodiesterase and actomyosin ATPase. J. Biol. Chem. *251*, 4501–4513.

CHAPTER 3

ACTIVATION OF TYPE II CALCIUM/CALMODULIN-DEPENDENT PROTEIN KINASE BY Ca^{2+}/CALMODUIN IS INHIBITED BY AUTOPHOSPHORYLATION OF THREONINE WITHIN THE CALMODULIN-BINDING DOMAIN*.

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Activation of Type II Calcium/Calmodulin-dependent Protein Kinase by Ca^{2+}/Calmodulin Is Inhibited by Autophosphorylation of Threonine within the Calmodulin-binding Domain*

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It is now well established that autophosphorylation of a threonine residue located next to each calmodulin-binding domain in the subunits of type II Ca^{2+}/calmodulin-dependent protein kinase causes the kinase to remain active, although at a reduced rate, after Ca^{2+} is removed from the reaction. This autophosphorylated form of the kinase is still sensitive to Ca^{2+}/calmodulin, which is required for a maximum catalytic rate. After removal of Ca^{2+}, new sites are autophosphorylated by the partially active kinase. Autophosphorylation of these sites abolishes sensitivity of the kinase to Ca^{2+}/calmodulin (Hashimoto, Y., Schworer, C. M., Colbran, R. J., and Soderling, T. R. (1987) *J. Biol. Chem.* 262, 8051–8055). We have identified two pairs of homologous residues, Thr^{305} and Ser^{314} in the α subunit and Thr^{306} and Ser^{315} in the β subunit, that are autophosphorylated only after removal of Ca^{2+} from an autophosphorylation reaction. The sites were identified by direct sequencing of labeled tryptic phosphopeptides isolated by reverse-phase high pressure liquid chromatography. $Thr^{305-306}$ is rapidly dephosphorylated by purified protein phosphatases 1 and 2A, whereas $Ser^{314-315}$ is resistant to dephosphorylation. We have shown by selective dephosphorylation that the presence of phosphate on $Thr^{305-306}$ blocks sensitivity of the kinase to Ca^{2+}/calmodulin. In contrast, the presence of phosphate on $Ser^{314-315}$ is associated with an increase in the K_{act} for Ca^{2+}/calmodulin of only about 2-fold, producing a relatively small decrease in sensitivity to Ca^{2+}/calmodulin.

Type II Ca^{2+}/calmodulin-dependent protein kinases (CaM kinases)[1] are a family of closely related enzymes that phosphorylate several substrate proteins in different tissues (1–4). The brain CaM kinase is a hetero-oligomer of molecular weight 600,000 to 700,000 (5–8). It is expressed at high concentrations in many forebrain neurons and constitutes approximately 1% of total brain protein (9). The isozyme purified from the forebrain is composed of structurally similar α (54 kDa) and β (58–60 kDa) subunits in an average ratio of approximately 9 α to 3 β (5). Both subunits are catalytic, and can be autophosphorylated at several sites (5, 6).

The CaM kinase is regulated in a complex way by autophosphorylation. When purified kinase is activated in the presence of CaM, a threonine residue adjacent to the calmodulin-binding domain (Thr^{286} in α and Thr^{287} in β) is rapidly autophosphorylated (10–12). The presence of phosphate at this site allows the kinase to remain active, at a reduced rate, when free Ca^{2+} is removed by chelation with EGTA. This mechanism may allow the CaM kinase to remain active *in vivo* after an initial activating calcium transient is over (13). The rate of calcium-independent phosphorylation of exogenous substrates by kinase autophosphorylated at $Thr^{286-287}$ is 20–80% of the fully calcium-stimulated rate of nonphosphorylated kinase. The activity can be stimulated to the maximum rate by the readdition of CaM (13–16).

Upon removal of Ca^{2+}, new sites become accessible for autophosphorylation by the partially active form of the kinase (13, 17–19). Autophosphorylation of these new sites inhibits stimulation of kinase activity by CaM (17, 19). We report here the identification of two of these new sites. One, Thr^{305} in α and Thr^{306} in β, is located within the calmodulin-binding domain in a five-amino acid segment found previously to be essential for high affinity binding of CaM (20). The other, Ser^{314} in α and Ser^{315} in β, is located at the carboxyl end of the calmodulin-binding domain. By selective dephosphorylation, we have found that only autophosphorylation of $Thr^{305-306}$ correlates closely with the loss of sensitivity to CaM. When this site is maximally phosphorylated, stimulation of kinase activity is inhibited even at concentrations of CaM 30 times higher than the K_{act} of nonphosphorylated kinase. The inhibition is reversed when this site is dephosphorylated with protein phosphatases 1 or 2A (44). In contrast, the sensitivity of the kinase to CaM is reduced only about 2-fold by the presence of phosphate on $Ser^{314-315}$.

EXPERIMENTAL PROCEDURES[2]

RESULTS

New Sites Autophosphorylated after Removal of Ca^{2+} from the Autophosphorylation Reaction—To identify new sites that are autophosphorylated in type II CaM kinase after removal of Ca^{2+} from the autophosphorylation reaction, purified kinase was autophosphorylated with [γ-^{32}P]ATP for 5 and 35 s in the presence of Ca^{2+} or for 5 s in the presence of Ca^{2+}

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[1] The abbreviations used are: CaM, Ca^{2+}/calmodulin; CaM kinase, type II CaM-dependent protein kinase; SDS, sodium dodecyl sulfate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; MLCK, myosin light chain kinase; PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography; PTH, phenylthiohydantoin; S.E., standard error of the mean.

[2] Portions of this paper (including "Experimental Procedures" and Figs. 2–4) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

followed by 30 s in its absence. Tryptic phosphopeptide maps of kinase labeled under each condition were compared (Fig. 1, *A*, *C*, and *E*). As we reported previously (10), α-Thr[286] (Fig. 1*A*), β-Thr[287], and β-Thr[382] were autophosphorylated after incubation for 5 s in the presence of Ca^{2+} (10–12). One or more additional identified sites in both subunits were autophosphorylated at a slower rate (Fig 1*C*; Ref. 10). Two new sets of phosphopeptides appeared only when autophosphorylation in the presence of Ca^{2+} was followed by addition of EGTA to chelate free Ca^{2+} (Fig. 1*E*). One set eluted early from the HPLC column and contained phosphoserine; the other set eluted later and contained phosphothreonine. The same new phosphopeptides were also observed when EGTA was added after 60 s of autophosphorylation in the presence of Ca^{2+} (data not shown).

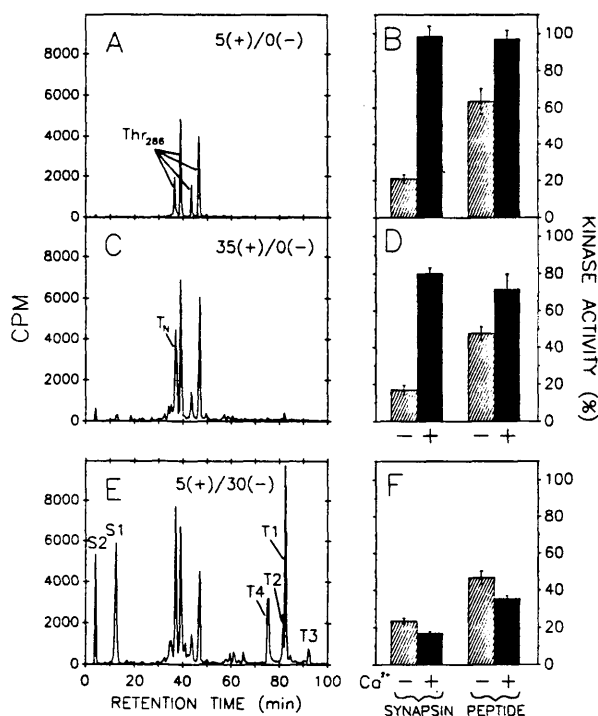

FIG. 1. Autophosphorylation of new sites after removal of Ca^{2+} is associated with loss of sensitivity of type II CaM kinase to CaM. Type II CaM kinase (5 μg) was autophosphorylated for 5 s (*A* and *B*) or 35 s (*C* and *D*) in the presence of Ca^{2+}, calmodulin, and [γ-^{32}P]ATP, or for 5 s in the presence of Ca^{2+} followed by 30 s in the absence of Ca^{2+} (*E* and *F*), as described under "Experimental Procedures." An aliquot (1 μg) was removed and kinase activity was measured in the presence and absence of Ca^{2+} with both a synthetic peptide and synapsin I as substrates (*B*, *D*, and *F*). The activity of unautophosphorylated kinase (100%; not shown) was 9.9 ± 1.1 μmol/min/mg (n = 4) with peptide as substrate and 6.5 ± 0.4 μmol/min/mg (n = 5) with synapsin I as substrate. Values are the average ± S.E. In each experiment, activity was measured in triplicate. Labeled tryptic phosphopeptides were prepared from the remaining 4 μg of kinase and fractionated by chromatography on a C4 reverse-phase HPLC column as described under "Experimental Procedures." Only peptide maps from the α subunit are shown in the figure (*A*, *C*, and *E*). *A* and *C* represent sites autophosphorylated in the presence of Ca^{2+}. The peptides containing Thr[286] were identified previously (10). A peptide containing phosphothreonine that appeared after 35 s of autophosphorylation in the presence of Ca^{2+} (T_N; *C*) has not been described previously. This peptide is resolved from those containing Thr[286] in other HPLC runs. It was blocked at the NH_2 terminus upon Edman sequencing and was identified by amino acid analysis as a tryptic peptide derived from the NH_2 terminus of the α subunit (data not shown). It is not discussed further in this study. *E* represents sites autophosphorylated after the removal of Ca^{2+}. Peptides S1 and T1–T4 also appeared in maps of the β subunit (data not shown). Peptide S1 from the β subunit had a slower mobility than S1 from the α subunit, eluting near the peaks containing Thr[286–287]. Peptides T1–T4 from the β subunit had mobilities identical to those of T1–T4 from the α subunit. The chemical identity of phosphorylated residues in each peak was determined as described under "Experimental Procedures." Maps similar to those in *A* and *E* were obtained in eight separate experiments. Maps similar to that in *C* were obtained in two separate experiments. *S*, peak containing phosphoserine; *T*, peak containing phosphothreonine.

Autophosphorylation of Thr[286–287] produced a significant Ca^{2+}-independent kinase activity toward exogenous substrates (Fig. 1*B*; Refs. 10 and 13–16). When the substrate was a synthetic peptide, the Ca^{2+}-independent activity was 64% of the activity of unphosphorylated kinase in the presence of calcium (control activity). When the substrate was synapsin I, Ca^{2+}-independent activity was 21% of control activity. Thus, the rate of Ca^{2+}-independent activity relative to control is highly dependent on the substrate protein. Activity declined slightly upon further autophosphorylation in the presence of Ca^{2+}, apparently due to thermal instability (14); however, the Ca^{2+}-independent activity remained approximately 67% (peptide substrate) and 21% (synapsin I) of the activity in the presence of Ca^{2+} (Fig. 1*D*). When autophosphorylation was allowed to continue in the absence of Ca^{2+} after 5 s of autophosphorylation in the presence of Ca^{2+}, sensitivity of the kinase to CaM was abolished (Ref. 17, Fig. 1*F*). This was evident when kinase activity was measured with either of the exogenous substrates. Calcium-independent activity remained unchanged. Loss of sensitivity to CaM could not be overcome by addition of concentrations of CaM as high as 4.5 μM, 30 times higher than the K_{act} for CaM of nonphosphorylated kinase (data not shown). However, the sensitivity rapidly reappeared when the kinase was dephosphorylated with protein phosphatase 2A (Fig. 8) or with protein phosphatase 1 (Ref. 17; data not shown).

Identification of Sites Autophosphorylated at Serine after Removal of Calcium—Material from the peak labeled *S1* in Fig. 1*E* was purified in sufficient quantity for automated gas-phase sequencing, as described under "Experimental Procedures." After the second step of purification, C18 reverse-phase chromatography, the peptide was obtained as a single symmetrical peak containing 560 pmol of radioactive phosphate (Fig. 2*A*). The major sequence obtained from this peak, Asn-Phe-Ser-Gly-Gly-Lys (Table I), corresponds to the predicted sequence of a tryptic peptide in the α subunit containing residues Asn[312] to Lys[317] (43, Fig. 9). The recovery of 512 pmol of PTH-Asn in the first cycle is consistent with a stoichiometry of 1 mol of phosphate/mol of peptide. Fewer

TABLE I

Sequences of α-S1 and β-S1 peptides

Recoveries of PTH-derivatives were consistent with the moles of PO_4 submitted, assuming a stoichiometry of 1 mol of PO_4/mol of peptide.

Cycle	α-S1		β-S1		
	Residue	Yield[a]	Residue	Yield[b]	Yield[c]
1	Asn	512	Asn	46	74
2	Phe	347	Phe	37	26
3	Ser	390[d]	Ser		38[d]
4	Gly	183	Val	9	22
5	Gly	116	Gly	30	48
6	Lys	57			

[a] Sample contained 560 pmol of PO_4.
[b] Sample contained 200 pmol of PO_4.
[c] Sample contained 150 pmol of PO_4.
[d] >90% recovered as PTH-dehydroalanine.

than 5 pmol of any other PTH-derivative was recovered in each cycle. Nearly all of the serine (90%) at the third position was recovered as PTH-dehydroalanine, as expected if it were phosphorylated (28, 29). During sequencing on the gas-phase sequencer, one-third to one-half of unmodified serine is recovered as PTH-serine (10), the rest as PTH-dehydroalanine. Therefore, if the serine in the major sequence were unmodified, we would have expected 130–195 pmol of PTH-serine. We conclude that the phosphorylated serine in peak S1 is α-Ser^{314}.

The other phosphoserine-containing peak generated from the α subunit, labeled *S2*, eluted in the void volume of both C4 and C18 reverse-phase columns. Electrophoresis of material from this peak on cellulose sheets revealed that over 90% of the radioactivity was associated with a peptide, rather than with free phosphoserine. The S2 peptide has not been sufficiently purified to determine its sequence. Its rate of autophosphorylation and its rate of dephosphorylation by protein phosphatase (data not shown) were essentially identical to that of peptide S1 (Figs. 5*A* and 7*A*). It may be an alternatively cleaved tryptic peptide containing Ser^{314} or a peptide containing phosphorylated α-Ser^{318} (10).

Tryptic peptide maps of the β subunit autophosphorylated under the same set of conditions revealed only a single new phosphoserine-containing peak that eluted near the peaks containing Thr^{287} (β-S1; data not shown). Material from this peak was purified after two separate large scale autophosphorylation reactions. In both cases, the radioactive peak eluted from the C18 column was associated with one major peptide peak (Fig. 2*B*). The NH_2-terminal sequence of this peptide was determined. The major sequence, Asn-Phe-Ser-Val-Gly (Table I), corresponds to the predicted sequence of a tryptic peptide in the β subunit containing residues Asn^{313} to Arg^{318} (Fig. 9). This peptide contains only one potential phosphorylation site, Ser^{315}. During the sequencing of both peptides, minor PTH-amino acid derivatives were recovered in one cycle, but the identity and position of these amino acids differed for the two peptides. Neither of the alternative sequences that included these amino acids was found in the sequence of the CaM kinase. In each case, the recovery of PTH-derivatives was consistent with a stoichiometry of 1 mol radioactive phosphate/mol of peptide (Table I). Again, the failure to recover any PTH-serine in the third cycle is consistent with the presence of phosphorylated serine at this position. We conclude that the phosphorylated serine in peak β-S1 is Ser^{315}. The autophosphorylation sites containing α-Ser^{314} and β-Ser^{315} are homologous and are located at the carboxyl-terminal end of the calmodulin-binding domain (Ref. 43 and Fig. 9).

Identification of Sites Autophosphorylated at Threonine after Removal of Calcium—Four new tryptic peptides containing phosphothreonine appeared in maps of both the α and β subunits after removal of Ca^{2+} from the autophosphorylation reaction (T1–T4, Fig. 1*E*; data not shown for the β subunit). Peptide T1 was the major phosphothreonine containing peptide and was the only one of the four autophosphorylated at a rate that correlated closely with the rate of loss of sensitivity to CaM (Fig. 5*A*).

Peak T1 from the β subunit was purified twice from large scale autophosphorylation reactions as described under "Experimental Procedures." In both cases, the purified radioactive peak was associated with one major peptide peak (Fig. 3*A*). The NH_2-terminal sequence of this peptide was determined. The major sequence, Gly-Ala-Ile-Leu-*X*-Thr-Met-Leu-Ala (Table II), corresponds to that of the predicted tryptic peptide containing residues Gly^{302} to Arg^{312} in the β subunit (Fig. 9). During the sequencing of one peptide, there were no other contaminating PTH-derivatives. During the sequencing of the second peptide, there were some contaminating PTH-amino acid derivatives at each cycle. However, none of the alternative sequences that included these amino acids was found within the sequence of the CaM kinase. For both of these peptides, the recovery of PTH-derivatives was consistent with a stoichiometry of 1 mol of radioactive phosphate/mol of peptide (Table II). There are two predicted tryptic peptides within the β subunit that contain threonine residues and might be blocked at the NH_2 terminus; Met^{1} to Arg^{9} and Gln^{319} to Lys^{342} (Gln can cyclize to pyroglutamate). It was possible that these peptides could be present as contaminants of the major peptide in peak T1 and could be the source of the phosphothreonine. Therefore, in a separate experiment, the T1 peptide was purified by one cycle of chromatography on a C4 reverse-phase HPLC column and then digested with thermolysin as described under "Experimental Procedures." Thermolysin hydrolyzes peptide bonds on the NH_2-terminal side of hydrophobic residues. Therefore it would be expected to remove three to four amino acids, including Thr^{311}, from the carboxyl terminus of peptide T1. The major thermolytic phosphopeptide was purified by C18 reverse-phase chromatography. The purified radioactive peak was associated with one major peptide peak (Fig. 3*B*), which eluted earlier than the parent peptide and contained 90 pmol of radioactive phosphate. The NH_2-terminal sequence of this peptide was determined and the major sequence was Gly-Ala-Ile-Leu-*X*-*X*-Met (Table II). During the sequencing, small amounts of contaminating PTH-amino acid derivatives were recovered in the first and fourth through sixth cycles. However, none of the alternative sequences that included these amino acids were found in the sequence of the CaM kinase. The sequence of the thermolytic peptide confirms the sequence of the parent tryptic phosphopeptide, T1, and indicates that the phosphothreonine was in fact contained in that sequence.

Although peptide T1 contains three potential threonine autophosphorylation sites, we have concluded that the phosphorylated threonine is most likely Thr^{306} (homologous to Thr^{305} in the α subunit) for the following reasons. No PTH-threonine and only traces of PTH-dehydrothreonine (30) were

TABLE II

Sequence of peptides containing β-Thr$^{306(307)}$

Recoveries of PTH-derivatives were consistent with the moles of PO_4 submitted, assuming a stoichiometry of 1 mol of PO_4/mol of peptide.

Cycle	Tryptic peptides			Thermolytic peptide	
	Residue	β-T1 yield[a]	β-T1 yield[b]	Residue	Yield[c]
1	Gly	30	50	Gly	43
2	Ala	27	24	Ala	21
3	Ile	21	15	Ile	11
4	Leu	15	31	Leu	14
5		—[d]	—		—
6	Thr	2[e]	2.5		—
7	Met	3[e]	2	Met	<2
8	Leu	4[e]			
9	Ala	4[e]			
10		—			
11	Arg	<0.5			

[a] Sample contained 85 pmol of PO_4.
[b] Sample contained 80 pmol of PO_4.
[c] Sample contained 91 pmol of PO_4.
[d] Trace quantities of dehydrothreonine recovered.
[e] Approximately equal recovery in the following cycle.

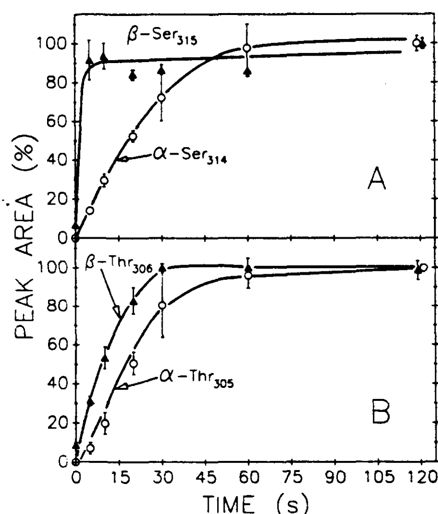

FIG. 5. **Time course of autophosphorylation of Ser$^{314-315}$ and Thr$^{305-306}$.** Kinase (5 μg) was autophosphorylated for 5 s in the presence of Ca^{2+}, calmodulin and [γ-^{32}P]ATP, then for the indicated times in the absence of Ca^{2+} as described under "Experimental Procedures." Labeled phosphopeptides were prepared from 3.6 μg of kinase from each reaction and fractionated by HPLC chromatography as described under "Experimental Procedures." Radioactivity recovered in peaks containing phosphorylated α-Ser^{314} (O) and β-Ser^{315} (▲), (*A*), and in those containing phosphorylated α-Thr^{305} (O) and β-Thr^{306} (▲) (*B*), was determined at each time point and normalized as described under "Experimental Procedures." Values are the average, ± the range, of the results of two separate experiments.

recovered from the tryptic peptides in the fifth cycle, corresponding to Thr^{306}. In contrast, PTH-threonine was recovered in significant amounts in the sixth cycle, corresponding to Thr^{307} (Table II). Finally, removal of Thr^{311} from the peptide by thermolytic digestion did not remove the radioactive phosphate.

Purified peptide T1 from the α subunit (α-T1) was not recovered in sufficient quantity for sequencing. However, several lines of evidence indicate that it is identical to T1 from the β subunit (β-T1), as expected from the sequences of α and β (Fig. 9). First, α-T1 coeluted with β-T1 during reverse-phase HPLC chromatography (Fig. 4, *A-C*). Second, when either α-T1 or β-T1 were chromatographed a second time under the same conditions, the original single peak split into three peaks (Fig. 4, *D* and *E*). The retention times of the new peaks were identical for T1 derived from either subunit. Third, if dithiothreitol was added to α-T1 or β-T1 immediately following the first chromatographic separation, recovery of the parent peak during the second separation increased to 70%. Dithiothreitol may stabilize the parent peptide by inhibiting oxidation of Met^{307} in α and Met^{308} in β. Finally, proteolysis of α-T1 and β-T1 with thermolysin produced identical new peptide peaks upon fractionation by HPLC (data not shown).

Functional Effects of Autophosphorylation of the New Sites Exposed after Removal of Ca^{2+}—Thr$^{305-306}$ is located near the center of the CaM binding domain, and Ser$^{314-315}$ is located at its carboxyl-terminal end. Therefore, autophosphorylation of either of these sites might be expected to influence binding of CaM to the kinase. We examined the functional effects of autophosphorylation of these sites by comparing the kinetics of their autophosphorylation with the kinetics of loss of sensitivity to CaM. The rates of autophosphorylation of α-Ser^{314} (Fig. 5*A*), α-Thr^{305}, and β-Thr^{306} (Fig. 5*B*) were all similar to the rate of loss of sensitivity to CaM (Fig. 6). However, we found that the rates of dephosphorylation of these sites by protein phosphatase 2A were quite different from each other (Fig. 7). α-Thr^{305} and β-Thr^{306} were dephosphorylated rapidly, whereas α-Ser^{314} and β-Ser^{315} were relatively resistant to dephosphorylation. S2, the serine-containing peptide from the α-subunit that eluted at the void volume during reverse-phase HPLC (Fig. 1*C*), was similarly resistant to dephosphorylation (data not shown).

The rate of dephosphorylation of Thr$^{305-306}$ (Fig. 7*B*) was similar to the rate of recovery of sensitivity to CaM (Fig. 8*C*). In contrast, at the end of 3 min, long after the kinase had again become fully responsive to CaM, less than half of the phosphate on Ser$^{314-315}$ had been removed (Fig. 7*A*). Similar results were obtained with protein phosphatase 1 (data not shown). To test whether the presence of phosphate on Ser$^{314-315}$ had any affect on the sensitivity of the kinase to CaM, the apparent K_{act} for CaM of unphosphorylated kinase was compared with that of kinase phosphorylated only at Ser$^{314-315}$. The latter was prepared by dephosphorylating autophosphorylated kinase for 60 s as described in the legend to Fig. 7. The apparent K_{act} for CaM of unphosphorylated kinase was 127 ± 5.7 nM ($n = 3$) and for kinase phosphorylated only at Ser$^{314-315}$ was 206 ± 16 nM ($n = 2$). Thus, we conclude that autophosphorylation of Thr$^{305-306}$ inhibits activation of CaM kinase by CaM completely; whereas autophosphorylation of Ser$^{314-315}$ alone produces only a 1.6-fold increase in the apparent K_{act} for CaM. Slower autophosphorylation of other threonine residues represented by peptides T2–T4 may contribute to loss of sensitivity to CaM (see "Discussion").

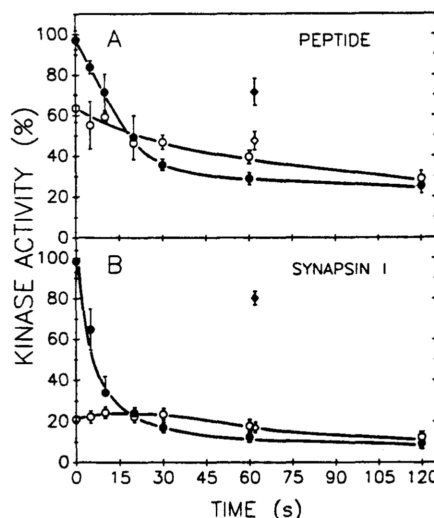

FIG. 6. **Time course of changes in kinase activity produced by autophosphorylation in the absence of Ca^{2+}.** Kinase (5 μg) was autophosphorylated for 5 s in the presence of Ca^{2+} and then for the indicated times in the absence of Ca^{2+} as described under "Experimental Procedures." Aliquots (1 μg) were removed at the indicated times and kinase activity was measured with either peptide (*A*) or synapsin I (*B*) as substrate as described under "Experimental Procedures." Values are plotted as percent of the activity of unphosphorylated kinase in the presence of Ca^{2+}, which was 6.4 ± 0.7 μmol/min/mg with synapsin I as substrate and 9.2 ± 1.7 μmol/min/mg with peptide as substrate. In a separate set of control incubations (♦, ◇) EDTA was added after 5 s of autophosphorylation in the presence of Ca^{2+}, and the incubation was continued for an additional 60 s. Values for each substrate are the average ± S.E. of the results of three separate experiments in which activity was measured in triplicate. ●, ♦, activity in the presence of Ca^{2+}; O, ◇, activity in the absence of Ca^{2+}.

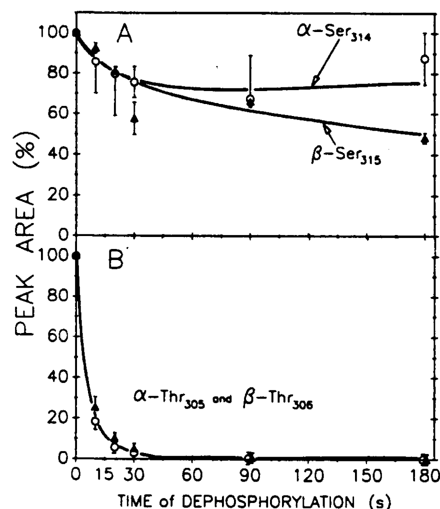

FIG. 7. Time course of dephosphorylation of α-Ser[314], β-Ser[315], α-Thr[305], and β-Thr[306]. Kinase (5 μg) was autophosphorylated with [γ-^{32}P]ATP for 5 s in the presence of Ca^{2+} and then for 30 s in the absence of Ca^{2+}, as described in the legend to Fig. 5. Dephosphorylation was initiated by addition of protein phosphatase 2A as described under "Experimental Procedures" and continued for the indicated times. Labeled phosphopeptides were prepared from 3.6 μg of kinase from each time point and fractionated by HPLC chromatography as described under "Experimental Procedures." Radioactivity recovered in peaks containing α-Ser[314] (○) and β-Ser[315] (▲) (*A*) and in those containing α-Thr[305] (○) and β-Thr[306] (▲) (*B*) was determined at each time point and normalized as described under "Experimental Procedures." The amount of radioactive phosphate recovered in each site before dephosphorylation was taken as 100%. Values are the average ± range of the results from two separate experiments.

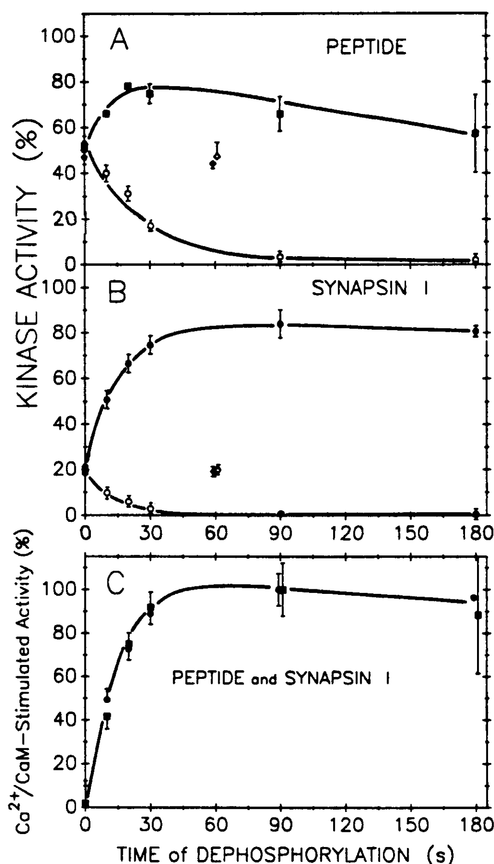

FIG. 8. Time course of reversal of insensitivity to CaM by dephosphorylation. Kinase (5 μg) was autophosphorylated for 5 s in the presence of Ca^{2+} and then for 30 s in the absence of Ca^{2+} as described in the legend to Fig. 5. Dephosphorylation was initiated by addition of protein phosphatase 2A as described in the legend to Fig. 7. Aliquots (1 μg) were removed at the indicated times and kinase activity was measured with either peptide (■, ○; *A* and *C*) or synapsin I (●, ○; *B* and *C*) as substrate as described under "Experimental Procedures." Values in *A* and *B* are plotted as the percent of the activity of unphosphorylated kinase in the presence of Ca^{2+}, which was 5.4 ± 0.9 μmol/min/mg ($n = 3$) with synapsin I as substrate and 10.8 ± 2.6 μmol/min/mg ($n = 2$) with peptide as substrate. To control for thermal instability, autophosphorylated kinase was incubated for 60 s, at 30 °C, in the absence of protein phosphatase 2A (♦, ◇). In *C*, the difference between activity in the presence of Ca^{2+} and in its absence is plotted for both substrates as a percent of the maximum difference, which is set at 100%. ■, ●, ♦, activity in the presence of Ca^{2+} and calmodulin; ○, ◇, activity in the absence of Ca^{2+} and calmodulin. Values are the average ± S.E. In each experiment, activity was measured in triplicate.

DISCUSSION

Activation of type II CaM kinase in the presence of CaM is accompanied by rapid autophosphorylation of a pair of homologous residues, Thr[286] in the α subunit and Thr[287] in the β subunit, that are adjacent to the calmodulin binding domain in each subunit (10–12). Autophosphorylation of two to three of these sites in a dodecameric holoenzyme switches it to a new state in which all the subunits continue to phosphorylate themselves as well as exogenous substrates, at a reduced rate, after Ca^{2+} is removed from the reaction (10, 13–16). When the kinase is in this state, it is still sensitive to CaM which is required for the maximum catalytic rate. However, 15–30 s after removal of Ca^{2+} from the autophosphorylation reaction additional sites become autophosphorylated that cause the kinase to become insensitive to stimulation by CaM (17, 19).

In this study we have identified autophosphorylation sites that regulate sensitivity to CaM. Two homologous sites in each of the kinase subunits are autophosphorylated only after removal of calcium from an ongoing autophosphorylation reaction. They are Thr[305] and Ser[314] in the α-subunit and Thr[306] and Ser[315] in the β-subunit (Fig. 9). All of these sites are located within the calmodulin-binding domain (32–35). We have shown by selective dephosphorylation that autophosphorylation of Thr[305–306] abolishes sensitivity of the kinase to CaM even at concentrations of CaM 30 times higher than the K_{act} of nonphosphorylated kinase. Autophosphorylation of Ser[314–315] alone has a much smaller effect on the requirement for CaM, increasing the apparent K_{act} for CaM only about 2-fold.

Lou and Schulman (19) reported that calcium-independent phosphorylation of protein substrates was inhibited after autophosphorylation in the absence of Ca^{2+}. However, in our experiments, calcium-independent autophosphorylation of synapsin I and a synthetic peptide substrate were not inhibited after autophosphorylation in the absence of Ca^{2+}, in agreement with the results of Hashimoto *et al.* (17). Autophosphorylation of Ser[314–315] may account for the enhanced incorporation of phosphate into serine-containing tryptic peptides after removal of Ca^{2+} from the autophosphorylation reaction reported by Lickteig *et al.* (18). These workers did not observe phosphopeptides T1 through T4, perhaps because the kinase subunits were not alkylated prior to trypsinization. We have observed that alkylation dramatically alters the

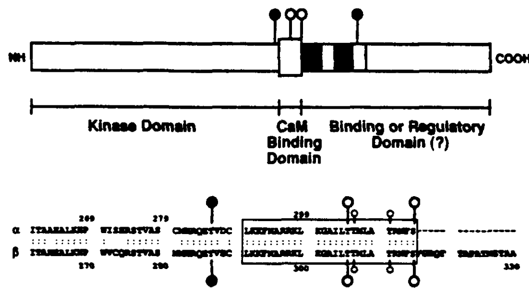

FIG. 9. Summary of new sites autophosphorylated after removal of Ca^{2+}. *A*, schematic representation of sites autophosphorylated in the absence of Ca^{2+} and functional domains in the CaM kinase subunits. The largest subunit (shown) is the β subunit. The *shaded region* is removed from the β' subunit by alternative splicing (43). The *filled* and *shaded* regions are not present in the α subunit. All other regions are identical in the β and β' subunits and similar, but not identical in the α subunit (43). The locations of sites autophosphorylated rapidly in the presence of Ca^{2+} are marked by *black ball and stick symbols*. The locations of sites autophosphorylated only after Ca^{2+} is removed from an autophosphorylation reaction are marked by *open ball and stick symbols*. B, specific location of these sites in the sequences of the α and β subunits. (The full sequences are reported in Ref. 43.) The calmodulin binding domain (32–35) is enclosed in a *box*. The *small open ball and stick symbols* mark the location of additional threonine residues in the calmodulin binding domain that may be autophosphorylated at a slower rate than $Thr^{305-306}$ or $Ser^{314-315}$ (see "Discussion").

pattern of cleavage of the subunits by trypsin (data not shown).

We were a bit surprised to observe autophosphorylation of $Thr^{305-306}$, because it is not preceded by the sequence Arg-*X*-*X*, which is a minimal requirement for phosphorylation of serines and threonines in exogenous proteins by type II CaM kinase (36). However, this is the second example of an autophosphorylation site in the CaM kinase that is not contained within a consensus sequence. The first to be identified was Thr^{382} in the β subunit, which is autophosphorylated rapidly in the presence of Ca^{2+} (10). The rapid autophosphorylation of these "nonconsensus" sites implies that the three-dimensional structure of the kinase places them near the active site. $Thr^{305-306}$ is located within a sequence of 5 hydrophobic residues, $Thr^{305-306}$-Thr-Met-Leu-$Ala^{309-310}$, that are essential for high-affinity binding of calmodulin to type II CaM-kinase (34). Autophosphorylation of this threonine would therefore be expected to inhibit calmodulin binding perhaps by disrupting an important hydrophobic interaction or by changing the secondary structure of the calmodulin binding domain in a critical region.

In contrast to $Thr^{305-306}$, $Ser^{314-315}$ is situated within a substrate consensus sequence, $Arg^{311-312}$-Asn-Phe-$Ser^{314-315}$. The location of this site within the calmodulin binding domain is homologous to the location of Ser^{512} in smooth muscle myosin light chain kinase (MLCK; Refs. 37 and 38). In the absence of bound CaM, Ser^{512} in MLCK can be phosphorylated by the cAMP-dependent protein kinase (38, 39). This phosphorylation produces a 10–20-fold increase in the apparent K_{act} for CaM, thus dramatically reducing the sensitivity of MLCK to stimulation by CaM (39). The homology between $Ser^{314-315}$ in type II CaM kinase and Ser^{512} in MLCK caused several labs to propose that autophosphorylation of $Ser^{314-315}$ controls the sensitivity of type II CaM kinase to CaM (19, 40, 41). However, we find that autophosphorylation of $Ser^{314-315}$ has only a small effect on affinity for CaM, whereas autophosphorylation of $Thr^{305-306}$ appears to block calmodulin binding completely. The diminished effect of autophosphorylation of $Ser^{314-315}$ compared with Ser^{512} in MLCK may be explained by differences in the sequences of the calmodulin binding domains of the two kinases that could produce different orientations of the phosphoserine residues. It may also be related to the observation that nonphosphorylated type II CaM kinase has a lower affinity for CaM (K_{act} of 30–125 nM; Refs. 2, 4, and 7) than does nonphosphorylated MLCK (K_{act} of 1–6 nM; Refs. 2 and 39). Thus, the conformation of the calmodulin binding domain of type II CaM kinase may produce a relatively low affinity for CaM even before $Ser^{314-315}$ becomes autophosphorylated.

The peptide represented by peak T1 contains 2 threonine residues in addition to $Thr^{305-306}$ (Fig. 9; $Thr^{306-307}$ and $Thr^{310-311}$). Our data suggest that autophosphorylation of $Thr^{306-307}$ and/or $Thr^{310-311}$ may also contribute to the inhibition of sensitivity to CaM. In addition to peak T1, three smaller unidentified phosphothreonine-containing peaks (T2–T4) appear in tryptic digests of kinase when autophosphorylation occurs in the absence of calcium (Fig. 1*C*). They all have a mobility similar to T1, but they appear at a significantly slower rate. We have not obtained sufficient quantities of peaks T2–T4 for sequencing; however, we believe they may contain the same tryptic peptide as T1, each phosphorylated at one or more of the other threonines. Peaks T2 and T3 appear slowly, then decrease in size after about 20 s. By 60 s they are nearly gone (data not shown). Peak T4 rises after a brief lag and is maximal by 60 s. This behavior is consistent with a model in which the peptide in peak T2 is autophosphorylated at $Thr^{306-307}$, the peptide in peak T3 is autophosphorylated at $Thr^{310-311}$, and the peptide in peak T4 is autophosphorylated at both. This model predicts that when the sites are dephosphorylated (Fig. 7), peak T4 will give rise to peaks T2 and T3, which will then slowly disappear. This predicted relationship was observed (data not shown). Furthermore, the rate of autophosphorylation of $Thr^{305-306}$ is consistently slightly slower than the rate of loss of sensitivity to CaM (Figs. 5*B* and 6) and the rate of dephosphorylation of this site is slightly faster than the rate of recovery of sensitivity to CaM (Figs. 7*B* and 8). When peaks T2–T4 are summed with peak T1 the summed rate of autophosphorylation correlates more closely with the rate of loss of sensitivity to CaM. Similarly, the summed rate of dephosphorylation correlates more closely with the rate of recovery of sensitivity to CaM. These observations suggest that slow autophosphorylation of the $Thr^{306-307}$ or $Thr^{310-311}$ may contribute to loss of sensitivity to CaM.

We have proposed previously that autophosphorylation of α-Thr^{286} and β-Thr^{287}, which switches type II CaM kinase to a partially calcium-independent state, may play an important physiological function by prolonging the effects of elevated intracellular Ca^{2+} that accompanies neuronal activity (13). A second stage of regulation of the kinase by autophosphorylation is the suppression of sensitivity to CaM that occurs after removal of Ca^{2+} from an autophosphorylation reaction (17). We show here that autophosphorylation of one or more threonine residues within the calmodulin binding domain occurs only after Ca^{2+} is removed and causes this suppression of sensitivity to CaM. The importance, in intact neurons, of these two stages of regulation will depend upon the abundance, specificity, and subcellular location of neuronal protein phosphatases. The catalytic subunits of protein phosphatase 1 and phosphatase 2A can rapidly dephosphorylate all autophosphorylation sites on the kinase except $Ser^{314-315}$. Shields *et al.* (42) have shown that these two classes of phosphatase appear to contribute most of the phosphatase activity towards the CaM kinase in synaptosomes. It will be interesting to

learn whether there are additional highly specialized phosphatases that participate in regulation of the kinase. It will also be important to study the steady state level of phosphorylation of Thr[286-287], Thr[305-306], and Ser[314-315] in living neurons under different physiological conditions.

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REFERENCES

1. Stull, J. T., Nunnally, M. H., and Michnoff, C. H. (1986) *The Enzymes* (Boyer, P. D., and Krebs, E. G., eds) Vol. 17, pp. 113–165, Academic Press, Orlando, FL
2. Kennedy, M. B., Bennett, M. K., Erondu, N. E., and Miller, S. G. (1987) in *Calcium and Cell Function* (Cheung, W. Y., ed) Vol. VII, pp. 61–107, Academic Press, Orlando, FL
3. Edelman, A. M., Blumenthal, D. K., and Krebs, E. G. (1987) *Annu. Rev. Biochem.* **56,** 567–613
4. Colbran, R. J., Schworer, C. M., Hashimoto, Y., Fong, Y.-L., Rich, D. P., Smith, M. K., and Soderling, T. R. (1989) *Biochem. J.* **258,** 313–325
5. Bennett, M. K., Erondu, N. E., and Kennedy, M. B. (1983) *J. Biol. Chem.* **258,** 12735–12744
6. Goldenring, J. R., Gonzalez, B., McGuire, J. S., Jr., and DeLorenzo, R. J. (1983) *J. Biol. Chem.* **258,** 12632–12640
7. Miller, S. G., and Kennedy, M. B. (1985) *J. Biol. Chem.* **260,** 9039–9046
8. McGuiness, T., Lai, Y., and Geengard, P. (1985) *J. Biol. Chem.* **260,** 1696–1704
9. Erondu, N. E., and Kennedy, M. B. (1985) *J. Neurosci.* **5,** 3270–3277
10. Miller, S. G., Patton, B. L., and Kennedy, M. B. (1988) *Neuron* **1,** 593–604
11. Schworer, C. M., Colbran, R. J., Keefer, J. R., and Soderling, T. R. (1988) *J. Biol. Chem.* **263,** 13486–13489
12. Theil, G., Czernik, A. J., Gorelick, F., Nairn, A. C., and Greengard, P. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 6337–6341
13. Miller, S. G., and Kennedy, M. B. (1986) *Cell* **44,** 861–870
14. Lai, Y., Nairn, A. C., and Greengard, P. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 4253–4257
15. Schworer, C. M., Colbran, R. J., and Soderling, T. R. (1986) *J. Biol. Chem.* **261,** 8581–8584
16. Lou, L. L., Lloyd, S. J., and Schulman, H. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 9497–9501
17. Hashimoto, Y., Schworer, C. M., Colbran, R. J., and Soderling, T. R. (1987) *J. Biol. Chem.* **262,** 8051–8055
18. Lickteig, R., Shenolikar, S., Denner, L., and Kelly, P. T. (1988) *J. Biol. Chem.* **263,** 19232–19239
19. Lou, L. L., and Schulman, H. (1989) *J. Neurosci.* **9,** 2020–2032
20. Hanley, R. M., Means, A. R., Kemp, B. E., and Shenolikar, S. (1988) *Biochem. Biophys. Res. Commun.* **152,** 122–128
21. Rylatt, D. B., Aitken, A., Bilham, T., Condon, G. D., Embi, N., and Cohen, P. (1980) *Eur. J. Biochem.* **107,** 529–537
22. Watterson, D. M., Harrelson, W. G., Jr., Keller, P. M., Sharief, F., and Vanaman, T. C. (1976) *J. Biol. Chem.* **251,** 4501–4513
23. Ueda, T., and Greengard, P. (1977) *J. Biol. Chem.* **252,** 5155–5163
24. Tung, H. Y. L., Resink, T. J., Hemmings, B. A., Shenolikar, S., and Cohen, P. (1984) *Eur. J. Biochem.* **138,** 635–641
25. Kennedy, M. B., McGuiness, T. L., and Greengard, P. (1983) *J. Neurosci.* **3,** 818–831
26. Roskowski, R. (1985) *Methods Enzymol.* **99,** 3–6
27. Darbre, A. (1986) in *Practical Protein Chemistry* (Darbre, A., ed) pp. 252–255, John Wiley & Sons, New York
28. Proud, C. G., Rylatt, D. B., Yeaman, S. J., and Cohen, P. (1977) *FEBS Lett.* **80,** 435–442
29. Murray, K. J., El-Maghrabi, M. R., Kountz, P. D., Lukas, T. J., Soderling, T. R., and Pilkis, S. J. (1984) *J. Biol. Chem.* **259,** 7673–7681
30. Bengur, A. R., Robinson, E. A., Apella, E., and Sellers, J. R. (1987) *J. Biol. Chem.* **262,** 7613–7617
31. Peterson, G. L. (1977) *Anal. Biochem.* **83,** 346–356
32. Bennett, M. K., and Kennedy, M. B. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 1794–1798
33. Hanley, R. M., Means, A. R., Ono, T., Kemp, B. E., Burgin, K. E., Waxham, N., and Kelly, P. T. (1987) *Science* **237,** 293–297
34. Hanley, R. M., Means, A. R., Kemp, B. E., and Shenolikar, S. (1988) *Biochem. Biophys. Res. Commun.* **152,** 122–128
35. Payne, M. E., Fong, Y.-L., Ono, T., Colbran, R. J., Kemp, B. E., Soderling, T. R., and Means, A. R. (1988) *J. Biol. Chem.* **263,** 7190–7195
36. Pearson, R. B., Woodgett, J. R., Cohen, P., and Kemp, B. E. (1985) *J. Biol. Chem.* **260,** 14471–14476.
37. Guerriero, V., Jr., Russo, M. A., Olson, N. J., Putkey, J. A., and Means, A. R. (1986) *Biochemistry* **25,** 8372–8380
38. Lukas, T. J., Burgess, W. H., Prendergast, F. G., Lau, W., and Watterson, D. M. (1986) *Biochemistry* **25,** 1458–1464
39. Conti, M. A., and Adelstein, R. S. (1981) *J. Biol. Chem.* **256,** 3178–3181
40. Lin, C. R., Kapiloff, M. S., Durgerian, S., Tatemoto, K., Russo, A. F., Hanson, P., Schulman, H., and Rosenfeld, M. G. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 5962–5966
41. Colbran, R. J., Fong, Y.-L., Schworer, C. M., and Soderling, T. R. (1988) *J. Biol. Chem.* **263,** 18145–18151
42. Shields, S. M., Ingebritsen, T. S., Kelly, P. T. (1985) *J. Neurosci.* **5,** 3414–3422
43. Bulleit, R. F., Bennett, M. K., Molloy, S. S., Hurley, J. B., and Kennedy, M. B. (1988) *Neuron* **1,** 63–72
44. Patton, B. L., Miller, S. G., and Kennedy, M. B. (1988) *Soc. Neurosci. Abstr.* **14,** 107

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Supplemental Material To

Activation of Type II CaM Kinase by Ca^{2+}/Calmodulin is Inhibited By Autophosphorylation of Threonine Within The Calmodulin Binding Domain

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EXPERIMENTAL PROCEDURES

Materials - Iodoacetamide, caffeine, bovine serum albumin, and phosphorylase b, were purchased from Sigma and dithiothreitol was purchased from Schwarz/Mann Biotech. SDS was purchased from Biorad and [γ-^{32}P]ATP was purchased from ICN Biochemicals. The synthetic peptide substrate for CaM kinase (Calmodulin-dependent protein kinase substrate analog) was purchased from Peninsula Laboratories. This substrate is a 10 residue synthetic peptide, Pro-Leu-Arg-Arg-Thr-Leu-Ser-Val-Ala-Ala, derived from phosphorylation site-2 of rabbit skeletal muscle glycogen synthase (21). It was dissolved at a concentration of 0.5 mM in 40 mM Tris-HCl (pH 7.0) and stored in aliquots at -80°C. Trypsin treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone was purchased from Worthington and thermolysin from Boehringer-Mannheim Biomedical. C4 and C18 reverse-phase HPLC columns (0.46 x 25 cm) were purchased from Vydac and acetonitrile (HPLC/UV Grade, Burdick and Jackson) from Baxter Healthcare. Trifluoroacetic acid (sequenal grade) and hydrochloric acid (constant boiling, sequenal grade) were purchases from Pierce Biochemical and thin layer cellulose sheets from J. T. Baker. Type II CaM kinase was purified from rat brain as described previously (7, 10). Calmodulin was purified from bovine brain by the method of Watterson *et al.* (22) and synapsin I was prepared by a modification of the method of Ueda and Greengard (23) as described previously (5). The catalytic subunit of protein phosphatase-2A was purified from rabbit skeletal muscle by the method of Tung, *et al.* (24) and was activated as described previously (10). Purified protein phosphatase-2A had a specific activity of 7.0 µmol PO_4 released from phosphorylase a per min per mg enzyme.

Autophosphorylation - Autophosphorylation was performed in a mixture (typically in a final volume of 25 µl) containing 5 µg kinase, 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 5 µg calmodulin, 5 mM 2-mercaptoethanol, 25 mM dithiothreitol, 100 µM [γ-^{32}P]ATP (3.0-6.0 x 10^3 cpm/pmol), 0.6 mM EGTA, and 0.9 mM $CaCl_2$. Reaction mixtures were pre-warmed at 30°C for 60 s and autophosphorylation was initiated by the addition of kinase. Autophosphorylation in the presence of Ca^{2+} was terminated by the addition of 12.5 µl of ice-cold 0.4 M EDTA, 25 mM Tris-HCl (pH 7.0) (EDTA Stop-Buffer) followed by 2.5 µl of 33 mM EGTA. When autophosphorylation in the presence of Ca^{2+} was followed by autophosphorylation in the absence of Ca^{2+}, 2.5 µl of 33 mM EGTA was added after 5 s of autophosphorylation, and the reaction was allowed to continue. It was terminated by addition of 12.5 µl of ice-cold EDTA Stop-Buffer. Ten µl of H_2O or buffer (next section) was added to bring the volume to 50 µl. A 10 µl aliquot of each reaction containing 1 µg of kinase was immediately diluted 50-fold into ice-cold 40 mM Tris-HCl (pH 8.0), 1 mg/ml bovine serum albumin, and its activity was measured with exogenous substrate in the absence and presence of CaM. Forty µl of 6% (w/v) SDS, 10% (w/v) glycerol, 4% (v/v) 2-mercaptoethanol, 120 mM Tris-HCl (pH 6.7), and a trace of bromphenol blue (2X gel sample buffer) were added to the remaining quenched autophosphorylation reaction in preparation for gel electrophoresis, tryptic digestion and separation of phosphopeptides by reverse-phase HPLC.

Dephosphorylation of Autophosphorylated Kinase - Protein phosphatase-2A was diluted with phosphatase buffer (50 mM Tris-HCl [pH 7.0], 30 mM dithiothreitol, 5 mM caffeine, 1 mg/ml bovine serum albumin). Type II CaM kinase was autophosphorylated as described above, and reactions were terminated with EDTA stop-buffer (30°C). Dephosphorylation was initiated 5 s later by the addition of 0.33 µg protein phosphatase-2A in 10µl phosphatase buffer. Control incubations were diluted with 10µl of phosphatase buffer. At the times indicated in figure legends, 10 µl aliquots were diluted 50-fold into ice-cold 50 mM Tris-HCl (pH 8.0), 1 mg/ml bovine serum albumin. Kinase activity was measured immediately with exogenous substrate in the presence and absence of CaM. Dephosphorylation reactions were terminated, at the same times, by the addition of 50 µl of 2X gel sample buffer, in preparation for gel electrophoresis, tryptic digestion and phosphopeptide mapping.

Assays of Kinase Activity - Kinase activity was measured in a reaction mixture (final volume 50 µl) containing 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 10 mM dithiothreitol, 0.2 mM [γ-^{32}P]-ATP (1.5-3.0 x 10^3 cpm/pmol), either 10 µg synapsin I (2.5 µM), or 2.16 µg CaM kinase peptide substrate (40 µM), and either 0.4 mM EGTA (minus calcium) or 0.4 mM EGTA/0.9 mM $CaCl_2$ (plus calcium). Assays in the presence of Ca^{2+} also contained 2.5 µg calmodulin. Reactions were initiated by addition of 5 µl of diluted kinase (10 ng) to pre-warmed (30°C) reaction mixtures and incubated for 15 sec. Reactions were terminated by the addition of 2X SDS gel sample buffer. Incorporation of phosphate into substrates was determined after separation of ^{32}P-labeled product from [γ-^{32}P]ATP by SDS-PAGE, as described previously (25). ^{32}P-labeled synapsin I was separated in 8% polyacrylamide gels and peptide substrate in 21% polyacrylamide gels. Separation of peptide product by gel electrophoresis produced a higher signal to noise ratio than separation of peptide product by binding to phosphocellulose paper (26). The 21% acrylamide gels often polymerized unevenly at the surface, which caused streaking of the peptide during electrophoresis. This problem was prevented by adding a 2 cm layer of 13% acrylamide while the 21% acrylamide was polymerizing. Mixing at the boundary was prevented by the addition of 5% glycerol (v/v) to the 21% acrylamide solution.

Tryptic Peptide Mapping of Phosphokinase - Duplicate aliquots (0.2 µg kinase) of each quenched autophosphorylation and dephosphorylation reaction were subjected to SDS-PAGE and incorporation of labeled phosphate into the subunits was measured as described previously (10, 13). Fifty µl of 0.15% (w/v) deoxycholic acid, and 400 µl of ice-cold 12% (w/v) trichloroacetic acid were added to the remaining phosphokinase (3.6 µg), and the mixture was incubated for 15 min on ice. Precipitated protein was sedimented by centrifugation at 13,000 x g for 15 min, and the supernatants removed by aspiration. SDS was removed from the pellets by two washes with 1 ml of -20°C acetone. Protein was then reduced with dithiothreitol, and carboxamidomethylated by treatment with iodoacetamide as described previously (10), except that reduction was performed in 10 mM dithiothreitol, and carboxamidomethylation proceeded for 90 min in the dark at 4°C. The subunits were then separated by SDS-PAGE on 10% polyacrylamide gels, and digested with trypsin as described previously (10).

After elution from the gel pieces, tryptic peptides were filtered through 0.2 µm filters, lyophilized and resuspended in 250-300 µl of 0.07% (v/v) TFA (pH 2.3). In the elution step, the recovery of radioactivity from the gel pieces was 86% ± 8% for the α-subunit, and 76% ± 8% for the β-subunit. The peptides were fractionated on a C4 reverse-phase column (0.46 x 25 cm) at 1.0 ml/min, as described previously (10). Briefly, the sample was injected onto a column equilibrated for 30-40 min with Buffer A (0.07% (v/v) trifluoroacetic acid in H_2O). The column was developed with the following gradient of Buffer B (70% (v/v) acetonitrile, 30% (v/v) H_2O, 0.07% (v/v) trifluoroacetic acid): 0-5 min, 0% Buffer B; 5-95 min, 0 to 35% Buffer B; 95-100 min, 35% to 100% Buffer B. Column effluent was collected in 200 fractions of 0.5 ml. Radioactivity was detected as Cerenkov radiation (49% efficiency). The total recovery of labeled peptides from the column was 63-85%. An additional 9-18% eluted in a broad peak when the column was washed with 100% buffer B.

Time Courses of Phosphorylation and Dephosphorylation of Sites - The time courses of phosphorylation and dephosphorylation of particular sites were plotted in the following way. For each time point, the areas under peaks of radioactivity were calculated with an integration program. Then, the fraction of radioactivity in each peak was calculated by dividing the area of the peak by the total area of all radioactive peaks. The amount of radioactivity in peptides containing a particular site was calculated by multiplying the fraction of radioactivity in those peptides by the total radioactivity in the kinase subunit before digestion with trypsin (measured for each time point in the gel piece after gel electrophoresis). To plot the time course of autophosphorylation of a particular site, the radioactivity in that site at each time point was plotted as a percent of the radioactivity in the site at the longest time point. To plot the time course of dephosphorylation of a site, the radioactivity in the site at each time point was plotted as a percent of the radioactivity in the site at time zero. The molar amount of phosphate incorporated into each site was estimated by multiplying the fraction of radioactivity in the site by the total moles of phosphate per mole of subunit (measured independently, as described above). The calculated maximal molar amount of phosphate in a given site was variable among different experiments, even under apparently identical conditions. For example, the maximum incorporation of phosphate into some sites, calculated in this way, varied from 0.1 moles per mole subunit to 0.7 moles per mole subunit. The determination of total moles phosphate per mole subunit was the source of the largest variance. This appeared to result from variable recovery of protein subunits during gel electrophoresis, and we were unable to correct for it. Chemical instability of certain peptides may have been an additional source of variance. Nevertheless, the specific catalytic activity of the kinase, the time courses of phosphorylation of each site, and the time to reach maximum phosphorylation were highly reproducible from experiment to experiment (Figs. 1, 3, 4 and 5).

Phosphoamino Acid Analysis - Several labeled phosphopeptides were subjected to partial acid hydrolysis in 6 N HCl for 2 hr at 110°C *in vacuo*. The hydrolysates were lyophilized and resuspended in electrophoresis buffer (acetic acid/formic acid/H_2O [78:25:897], pH 1.9). Phosphoamino acid standards (phosphoserine, phosphothreonine, and phosphotyrosine) were added and the hydrolysates were electrophoresed on thin layer cellulose sheets for 2 hr at 750 V as described by Darbre (27). The phosphoamino acid standards were visualized with ninhydrin and the positions of ^{32}P-labeled phosphoamino acids were determined by autoradiography.

Preparation of Phosphopeptides for Sequencing - To obtain quantities of phosphopeptides sufficient for sequencing, autophosphorylation reactions were scaled up. Reactions were performed in 1 ml batches, each containing 200 µg kinase, as previously described (10). Autophosphorylation was performed for 30 to 120 sec in the presence of calcium before addition of 100 µl of 22 mM EGTA. The reactions continued after the removal of calcium for an additional 60 sec and were terminated by addition of 100 µl of 100% (w/v) trichloroacetic acid. Reduction, carboxamidomethylation, SDS-PAGE, tryptic digestion, and recovery of peptides were performed as described above, except that 100 µg of kinase was fractionated per lane in 10% polyacrylamide gels and the separated subunits from each lane were digested with a total of 50 µg of trypsin. Peptides from all 1 ml reactions were pooled (typically 3 to 5 1 ml reactions) and fractionated by HPLC on a C4 reverse-phase column in two or three batches, as described above, to avoid overloading the column. Fractions containing the peptides to be sequenced were pooled and the volume was reduced to approximately 100 µl in a Speed-Vac concentrator. Final purification of each peak was achieved by chromatography on a C18 reverse-phase column equilibrated in Buffer A. Immediately prior to injection, each sample was brought to 300 µl by addition of Buffer A. The peptide containing α-Ser$_{314}$ was eluted with the gradient: 0-10 min, 0% buffer B; 10-90 min, 0% to 10% buffer B; 90-100 min, 10% to 100% buffer B. The peptide containing β-Ser$_{[illegible]}$ was eluted with the gradient: 0-10 min, 0% buffer B; 10-15 min, 0% to 10% buffer B; 15-95 min, 10% to 15% buffer B; 95-100 min, 15% to 100% buffer B. The peptide containing β-Thr$_{[illegible]}$ was eluted with the gradient: 0-10 min, 0% buffer B; 10-15 min, 0% to 18% buffer B; 15-120 min, 18% to 25% buffer B; 120-150 min, 25% to 100% buffer B. Peptide peaks were detected by monitoring absorbance of the effluent at 214 nm, and 0.5 ml fractions were collected by hand. The pure phosphopeptides were sequenced on an Applied Biosystems automated gas-phase sequencer, or subjected to total hydrolysis and amino acid analysis, as previously described (10).

In some instances, peptide T1 recovered from the C4 column was proteolysed with thermolysin. Fractions containing the peak were concentrated to 25-50 µl. Thermolysin (1µg) in 400 µl of 0.1 M NH_4HCO_3 (pH 8.0) was added and digestion continued for 4 hrs at 37° C. The digest was applied to a C18 reverse-phase HPLC column equilibrated in buffer A. Peptides were eluted with the following gradient of Buffer B: 0-10 min, 0% buffer B; 10-90 min, 0 to 15% buffer B; 90-100 min, 15 to 100% buffer B. Fractions containing radioactive phosphopeptides were identified by counting Cerenkov radiation.

Other Methods - Protein was measured by the method of Peterson (31) with bovine serum albumin as standard.

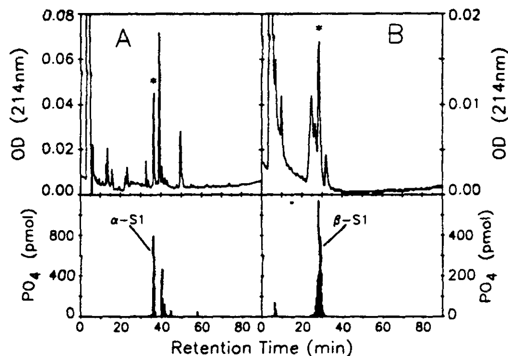

Figure 2. Purification of Peptides α-S1 and β-S1 by HPLC C18 Column Chromatography.
Kinase holoenzyme with a 1:1 ratio of α to β subunits (3.2 mg) was autophosphorylated for 120 s in the presence of Ca^{2+}, calmodulin, and [γ-^{32}P]ATP, then for 60 s after the addition of EGTA to chelate free Ca^{2+}, as described in the legend to Fig. 1. The α and β subunits were reduced, alkylated, purified, and digested with trypsin as described under Experimental Procedures. The resulting phosphopeptides were fractionated by chromatography on a C4 column. Fractions containing phosphopeptides α-S1 (Figure 1) and β-S1 were concentrated and fractionated on a C18 column as described under Experimental Procedures. Absorbance of the eluate was monitored at 214 nm and fractions containing radioactivity were identified by measuring Cerenkov radiation. Fractions corresponding to peaks marked by asterisks were concentrated and submitted for N-terminal sequence analysis (Table 1). (A) Peptide α-S1; The fraction eluting at 36 min was submitted for sequencing. (B) Peptide β-S1; The central fraction eluting at 28 min was submitted for sequencing.

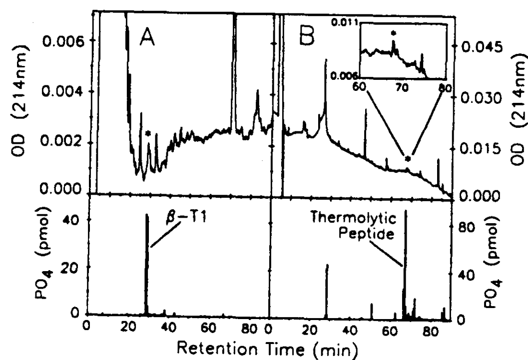

Figure 3. Purification of Peptide β-T1 and a Thermolytic Peptide Derived from β-T1 by HPLC on a C18 Column.
Kinase holoenzyme with a 1:1 ratio of α to β subunits (600 μg) was autophosphorylated for (A) 10 s in the presence of Ca^{2+}, calmodulin and [γ-^{32}P]ATP, then for 90 s in the absence of Ca^{2+} or for (B) 120 s in the presence of Ca^{2+}, calmodulin and [γ-^{32}P]ATP and 60 s in the absence of Ca^{2+} as described in the legend to Fig. 1. In each case, kinase was reduced and alkylated, and the β subunit was purified and digested with trypsin as described under Experimental Procedures. The resulting phosphopeptides were fractionated by chromatography on a C4 reverse-phase HPLC column. (A) Fractions containing peptide β-T1 were concentrated and fractionated by chromatography on a C18 column (see Experimental Procedures). Absorbance of the eluate was monitored at 214 nm, and fractions containing radioactivity were identified by measuring Cerenkov radiation. The two fractions eluting at 28.5 min, each of which contained approximately equal amounts of radioactivity, were pooled, concentrated and submitted for N-terminal sequence analysis (Table 2).
(B) In a separate experiment, fractions from the C4 column, containing the β-T1 peptide, were concentrated, then further proteolysed with thermolysin (1 μg) as described under Experimental Procedures. The digest was fractionated on a C18 column. The ratio of the two major thermolytic peptides varied with the hydrolysis conditions, suggesting that they are alternative thermolytic products of the same peptide. The digestion conditions were optimized to produce one major peak which was sequenced. The fraction eluting at 67 min and containing most of the radioactivity in the peak was concentrated and submitted for N-terminal sequence analysis (Table 2).

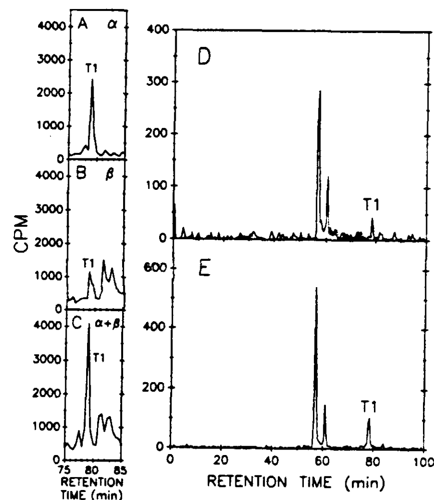

Figure 4. T1 peptides from the α and β subunits are identical.
Type II CaM kinase (7.5 μg) was autophosphorylated for 10 s in the presence of Ca^{2+}, calmodulin, and [γ-^{32}P]ATP, then for 50 s in the absence of Ca^{2+}. Tryptic phosphopeptides were prepared and aliquots containing 7 x 10^4 cpm of labeled peptides from each subunit were fractionated by HPLC chromatography as described in Fig. 1. (A) Peptide T1 from the α subunit. (B) Peptide T1 from the β subunit. (The peaks that eluted later than T1 contain β-Ser[illegible] and β-Thr[illegible], respectively (10).) (C) Peptide T1 from a sample in which equal amounts of phosphopeptides from the α and β subunits were mixed prior to application to the HPLC column. In panels A-E the retention time of the T1 peptide was 79 min.
In a separate experiment, fractions containing the T1 peptide from the α or β subunits were reduced in volume after elution from a C4 HPLC column, and then chromatographed a second time on the same column. (D) T1 peptide originally prepared from the α subunit. (E) T1 peptide originally prepared from the β subunit.

CHAPTER 4

AUTOPHOSPHORYLATION-SITE SPECIFIC ANTIBODIES TO THE NEURONAL TYPE II Ca^{2+}/CALMODULIN-DEPENDENT PROTEIN KINASE.

SUMMARY

Antibodies have been produced that bind specifically to the neuronal type II Ca^{2+}/calmodulin-dependent protein kinase (CaM kinase) at the autophosphorylation site, Thr^{286}, that controls Ca^{2+}-independent activity. Monoclonal antibodies and rabbit polyclonal antisera specific for autophosphorylated kinase were produced by immunization with thiophosphorylated peptides. Phosphokinase-specific monoclonal antibodies were also produced with thiophosphorylated kinase holoenzyme by an immunization strategy in which immunosuppression of the response to nonphosphorylated kinase preceded immunization with thiophosphorylated kinase. Serum with greater specificity for phosphorylated kinase was produced by immunization with thiophosphorylated peptides than by the kinase holoenzyme immunosuppression/immunization method. One phosphokinase-specific monoclonal antibody, 22B1, bound only to the phosphorylated CaM kinase on Western blots of total brain protein. Binding of 22B1 to CaM kinase correlated with autophosphorylation at Thr^{286} using immunoblot and ELISA methods. In addition to the phosphokinase-specific antibodies, rabbit polyclonal antibodies specific for the nonphosphorylated CaM kinase have been produced by immunization with nonphosphorylated peptides. Polyclonal antibodies specific for the nonphosphorylated kinase were purified by affinity chromatography. Binding of these antibodies to the CaM kinase was blocked by CaM kinase autophosphorylation at Thr^{286}.

INTRODUCTION

The neuronal type II Ca^{2+}/calmodulin-dependent protein kinase (CaM kinase) is the most abundant of the Ca^{2+}-regulated protein kinases in the brain. It comprises approximately 1% of total cerebral protein and about 2% of total hippocampal protein. The kinase is associated with neuronal membrane and cytoskeletal structures, and is present in the cytosol. It constitutes 30 to 50% of the postsynaptic density protein. The purified kinase is a hetero-oligomer (M_r 600-700 kDal) composed of two homologous subunits, α (M_r 54 kDal), and β (M_r 60 kDal). A third subunit restricted to brain, β' (M_r 58 kDal), is derived by alternative splicing of the β gene transcript. Two other minor homologous subunits of the CaM kinase have been reported, γ and δ; they are expressed by distinct genes and are not restricted to the brain. The complete amino acid sequence of each subunit has been determined from cDNA clones. All subunits appear to be catalytic. The relative amounts of these subunits in the kinase holoenzyme varies among brain regions.

The CaM kinase phosphorylates a wide variety of substrate proteins, including the synaptic vesicle-associated protein synapsin I, microtubule associated protein (MAP)-2 (Fukunaga *et al.*, 1982; Goldenring *et al.*, 1983; Bennett *et al.*, 1983), tau protein (Baudier and Cole, 1987), tubulin, and tyrosine hydroxylase (Yamauchi and Fujisawa, 1981; Schworer and Soderling, 1983; Vulliet *et al.*, 1984). Phosphorylation of MAP-2 and tau by the CaM

kinase inhibits microtubule assembly stimulated by these proteins *in vitro* (Yamamoto *et al.*, 1983; Lindwall and Cole, 1984). Several studies suggest that site-specific phosphorylation of synapsin I by the CaM kinase facilitates the exocytosis of synaptic vescicles (Llinas *et al.*, 1985; Nichols *et al.*, 1990). Site-specific phosphorylation of tyrosine hydroxylase by the CaM kinase has been correlated with a two- to three-fold activation of tyrosine hydroxylase *in situ* (Griffith and Schulman, 1988; Waymire *et al.*, 1988). In addition, peptides that specifically inhibit the CaM kinase block the induction of long term potentiation (LTP) in hippocampal slice preparations, although the kinase substrates involved are not known (Malinow *et al.*, 1989). These studies suggest that the CaM kinase may modify neuronal structure and the strength of synaptic transmission. Thus, factors that regulate CaM kinase activity may also play a critical role in the regulation of synaptic plasticity. One mechanism by which CaM kinase activity is regulated is autophosphorylation.

Autophosphorylation of the purified holoenzyme requires Ca^{2+}/calmodulin, is intramolecular, and incorporates up to 2 mol phosphate per mol α subunit and 3 mol phosphate per mol β subunit (Bennett *et al.*, 1983). The sites of autophosphorylation have been identified by a variety of methods, most directly by proteolysis of the autophoshorylated kinase and sequencing of the resulting phosphopeptides (Miller *et al.*, 1988; Patton *et al.*, 1990). Autophosphorylation of the most prominent site, $Thr^{286/287}$ (Thr^{286} in α; Thr^{287} in β and β'), activates the kinase such that Ca^{2+}/calmodulin is no longer required for

activation of either substrate phosphorylation or autophosphorylation (Miller and Kennedy, 1986; Lai et al., 1986; Schworer et al., 1986). This "calcium-independent" activity of the CaM kinase is 20% to 80% of the catalytic rate observed when calcium/calmodulin is present, the variation being dependent on the substrate used (Patton *et al.*, 1990). Activation is reversible; protein phosphatase catalyzed dephosphorylation of $Thr^{286/287}$ correlates with loss of Ca^{2+}-independent activity (Miller *et al*, 1988). The mechanism of activation by autophosphorylation at $Thr^{286/287}$ has been partially inferred. The CaM kinase contains an internal pseudosubstrate sequence. In peptide form, this sequence inhibits catalysis by the CaM kinase (Colbran et al., 1989); by extension, the internal sequence is thought to inhibit the kinase as well. Ca^{2+}/calmodulin activates the kinase by binding to a domain located at the carboxyl-terminal end of the inhibitory domain, presumably by inducing a conformational change in the kinase which relieves the psuedosubstrate inhibition (Soderling, 1990). The $Thr^{286/287}$ autophosphorylation site is located near the amino-terminal end of the inhibitory domain. Addition of phosphate to this site is thought to induce or stabilize the change in conformation elicited by the binding of Ca^{2+}/calmodulin. The dependence of Ca^{2+}-independent kinase activity on autophosphorylation is highly cooperative; maximal activation is observed after autophosphorylation of only 3 to 5 subunits per 12-subunit holoenzyme (Miller and Kennedy, 1986).

Recent evidence has demonstrated that this CaM kinase autoregulatory mechanism operates in living neurons. Fukanaga *et al.* (1989) found that depolarization of cultured cerebellar granule neurons with high extracellular K^+ transiently increased the level of Ca^{2+}-independent CaM kinase activity from a basal level of between 4% and 7% to about 13% of its maximal Ca^{2+}-independent value. Molloy and Kennedy (1991) found that approximately 30% of the CaM kinase was Ca^{2+}-independent in organotypic cultures of rat hippocampal slices. Further, they found that Ca^{2+}-independent CaM kinase activity could be reproducibly regulated by treatment of the cultured hippocampal slices with CaM kinase inhibitors (decreased activity), protein phosphatase inhibitors (increased activity), and removal of extracellular Ca^{2+} (decreased activity). They also demonstrated directly that phosphate was incorporated into CaM kinase α subunit Thr^{286} in these cultures. It has not yet been shown that modulation of the Ca^{2+}-independent activity of the CaM kinase also modulates synaptic transmission. Nor is there evidence that kinase autophosphorylation is itself affected by neurotransmission. Nevertheless, these remain attractive hypotheses.

Detection of protein phosphorylation by serine/threonine kinases usually involves following the incorporation of radioactive phosphate into identified proteins or protein fragments. Additionally, the activity of specific kinases can be inferred from the effects of specific kinase activators or inhibitors on secondary cellular events. These methods converge in the case of CaM kinase

autophosphorylation. Although highly sensitive, in general these methods lack the ability to detect phosphorylation at the cellular or subcellular level. Both Fukunaga et al. (1989) and Molloy and Kennedy (1991) measured the overall changes in the autophosphorylation state of the kinase in large populations of neurons, either a single neuronal cell type in dissociated culture, or an organotypically structured but heterogeneous neuronal population. Given the high level of CaM kinase in the cell soma, changes in kinase autophosphorylation that occur only at a subset of cells or synaptic sites may be undetectable due to the high "background" activity in assays of autophosphorylation or kinase activity. Further, since the CaM kinase autophosphorylates at numerous sites, the measurement of total phosphate incorporated into kinase subunits does not necessarily correlate with autophosphorylation at $Thr^{286/287}$, the site that regulates Ca^{2+}-independent activity. In order to more directly observe the phosphorylation state of the CaM kinase, we have developed antibodies that bind specifically either to the autophosphorylated or to the nonphosphorylated form of the CaM kinase.

Two strategies were used in order to increase the chances of obtaining antibodies with high selectivity and affinity. Synthetic peptides corresponding in sequence to the $Thr^{286/287}$ CaM kinase autophosphorylation site were used to immunize both mice and rabbits. In order to prevent dephosphorylation of the phosphopeptide immunogen by serum phosphatases, thiophosphorylated peptide was used to generate phosphokinase-specific antibodies. A second

strategy used autothiophosphorylated kinase holoenzyme as immunogen to produce mouse monoclonal antibodies. Because autophosphorylation is not likely to modify the the majority of the holoenzyme surface, nonphosphorylated epitopes should dominate the immune response. Thus, to enhance a phospho--specific response, the nonphosphokinase immune response was first immunosuppressed with cyclophosphamide prior to immunization with thiophosphorylated kinase. This strategy was originally used to enhance the immune response to rare components in a mixture of antigens (Matthew and Patterson, 1983) and seemed likely to enhance the production of antibodies specific for the phosphorylated form of the CaM kinase. A brief report of some preliminary results has appeared previously (Patton *et al.*, 1991).

EXPERIMENTAL PROCEDURES

Materials.

[γ-^{32}P]ATP was purchased from ICN (Irvine, CA), and nitrocellulose membranes (BA85, 0.45 µm pore diameter) were purchased from Schleicher and Schuell Inc. (Keene, NH). ATP, bovine serum albumin, EDTA, EGTA, keyhole limpet hemocyanin (KLH), p-Nitrophenylphosphate, phenylmethylsulfonyl fluoride (PMSF), and Tris base, were from Sigma Chemical Co. (St. Louis, MO). Ammonium sulfate and dithiothreitol were obtained from Schwarz/Mann Biotech. HL-1 murine myeloma cells were from Ventrex (Portland, ME). Normal rabbit serum and goat serum were purchased from Gibco. 5-Bromo 4-chloro 3-indoyl phosphate ρ-toluidine salt (BCIP), ρ-nitro blue tetrazolium chloride (NBT), and Tween-20 were purchased from Bio-Rad Laboratories (Richmond, CA). "Gentle Elution Buffer," KLH activated with maleimide, (Succinimidyl 4-(N-maleimidomethyl)cyclohexane 1 carboxylate (SMCC), and SulfoLink activated agarose-CL were purchased from Pierce Chemical Co. (Rockford, IL). Reverse-phase high pressure liquid chromatography (HPLC) columns were obtained from Vydac. Adenosine 5'-O(3-thiotriphosphate) (ATPγS), alkaline phosphatase-conjugated goat anti-mouse antibodies and alkaline phosphatase-conjugated goat anti-rabbit antibodies were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). New Zealand female rabbits were purchased from ABC Rabbitry (Chino, CA).

BALB/c mice were purchased from Simonsen (Gilroy, CA). The forebrain and pons/medullary isozymes of the type II CaM kinase was purified from rat brain as previously described (Bennett *et al.*, 1983; Miller and Kennedy., 1985; Miller *et al.*, 1988). Calmodulin was purified from bovine brain by the method of Watterson *et al.* (1976).

Methods.

Preparation of Peptide Immunogens. Synthetic peptides with the sequence surrounding the CaM kinase α subunit Thr^{286} autophosphorylation site were prepared by the Merrifield solid-phase procedure (Merrifield, 1986) on an Applied Biosystems model 430A peptide synthesizer (Kent, 1988). Crude peptide was desalted through Dowex 1-X2 resin and lyopholized to dryness. Amino acid analysis and amino-terminal sequencing confirmed peptide identity. Crude preparations of peptide were 80% to 95% pure. α-9mer(281) had the sequence Met-His-Arg-Gln-Glu-Thr-Val-Asp-Cys, corresponding to residues 281-290 in the α subunit. α-14mer(281) had the sequence Met-His-Arg-Gln-Glu-Thr-Val-Asp-Cys-Leu-Lys-Lys-Phe-Asn, corresponding to residues 281-294 in the α subunit. For use as nonphosphopeptide immunogen, peptides were purified by reverse-phase HPLC to greater than 95% purity by absorbance at 214 nm. Peptide (10 mg) was then coupled to KLH (10 mg) with SMCC (Yoshitake *et al.*, 1982). Reactions were carried out at room temperature for three hours in a solution containing 5 mg/ml peptide, 5 mg/ml KLH, 4 M guanidine chloride, 1 mM KH_2PO_4, 10mM Na_2HPO_4, 154 mM NaCl, and 1 mM $MgCl_2$ (pH 7.5). Conjugated peptide was aliquoted and stored at -80°C. Antisera specific for phosphorylated CaM kinase were raised against peptides phosphorylated with ATPγS (thiophosphorylated). Thiophosphorylated proteins are resistant to dephosphorylation by protein phosphatases (Gratecos and Fischer, 1974; Cassel and Glaser, 1982). Crude α-9mer(281) (30 mg) was

enzymatically thiophosphorylated in a reaction mixture containing 50 mM Tris (pH 8.0), 10 mM $MgCl_2$, 0.4 mM EGTA, 0.7 mM $CaCl_2$, 20 mM DTT, 10 mg/ml peptide (5.7 mM), 0.1 mg/ml CaM kinase, 0.1 mg/ml calmodulin, and 7.0 mM ATPγS. Crude α-9mer(281) (30 mg) was thiophosphorylated in a similar reaction mixture containing 1.45 mg/ml peptide (1.3 mM), 0.03 mg/ml CaM kinase, 0.1 mg/ml calmodulin, and 2.3 mM ATPγS. Reactions were carried out at 30°C for one to five hours and terminated by injection onto an HPLC column. Thiophosphorylated peptide was purified from reactants by HPLC on semi-preparative reverse-phase columns, as previously described (Patton *et al.*, 1991). The overall percent molar yield of pure thiophosphorylated peptide varied between 33% and 44%. Thiophosphorylated peptides were coupled to KLH derivatized with SMCC, available commercially (Pierce Chemical Co., Rockford, IL), as previously described (Patton *et al.*, 1991). Conjugated peptides (5 mg/ml) were aliquoted and stored at -80°C.

Production of Polyclonal Antibodies. Methods for immunizing rabbits have been detailed previously (Patton *et al.*, 1991). Briefly, New Zealand White female rabbits were injected at multiple subcutaneous and intramuscular sites with a mixture of 0.6 mg KLH conjugated with peptide (either nonphosphorylated or thiophosphorylated, as described above) and 0.6 mg free peptide emulsified in Ribi adjuvant (RIBI ImmunoChem Research, Inc., Hamilton, MT). Successive boosts were made at six- to ten-week intervals. Sera were

tested for antibodies that selectively recognized either nonphosphorylated or phosphorylated CaM kinase subunits on immunoblots, as described below. Rabbits that produced antibodies of interest were bled starting nine days after an injection.

Affinity Purification of Nonphosphokinase-Specific Polyclonal Antibodies. Nonphosphokinase-specific polyclonal antibodies were purified from rabbit immune serum selective for nonphosphorylated CaM kinase. First, serum albumin was removed by ammonium sulfate precipitation. Antiserum was brought to 50% saturation by slow addition of a saturated ammonium sulfate solution at 4°C. Precipitated antibody was collected by centrifugation at 10,000 g for 30 minutes at 4°C, and then resuspended in one-half the original serum volume of 20 mM sodium phosphate (pH 7.2), 9% (w/v) NaCl (PBS).

An affinity resin was produced by covalently coupling α-9mer(281) peptide to SulfoLink agarose-CL beads (Pierce) according to the manufacturer's instructions. Coupling occurred through the thiol group of the carboxyl terminus cysteine of α-9mer(281). One milligram of peptide was coupled per ml of wet resin. Higher coupling ratios proved less efficient in releasing bound antibodies. Loss of free peptide during the reaction was assayed by HPLC of reaction aliquots and detection of column effluent at 214 nm. Coupling

efficiency was 100%. Affinity resin was preabsorbed with normal rabbit serum prior to use. Each milliliter of resin was incubated with 4 ml of serum diluted five-fold with 16 ml of 50 mM Tris (pH 7.5), 5 mM EDTA (binding buffer). The mixture was stirred gently for 1 hour at 4°C, and then poured into a column and washed with 10 column volumes of each of the following solutions: binding buffer, antibody elution buffer (see below), and 0.1 M triethylamine (TEA, pH 11.5). The column was re-equilibrated with binding buffer containing 0.02% (w/v) sodium azide and stored at 4°C.

Four milliliters of nonphosphokinase-selective polyclonal antisera, precipitated with ammonium sulfate and resuspended as described above, was diluted five-fold with binding buffer and mixed with 1 ml of α-9mer(281)-affinity resin. After stirring gently for three hours at 4°C, the mixture was poured into a column. Unbound antibodies were collected and the column washed with binding buffer until the absorbance of the effluent at 280 nm returned to background levels. Bound antibodies were eluted with a commercial elution buffer ("Gentle Elution Buffer," Pierce), or with 0.1 M TEA (pH 11.5). Column effluent was collected as 0.5 ml fractions and absorbance monitored at 280 nm. Fractions containing protein were dialyzed against 20 mM sodium phosphate (pH 7.4) at 4°C. Unbound and eluted antibodies were assayed by ELISA and immunoblot for immunoreactivity specific for the CaM kinase in its nonphosphorylated form.

Production of Monoclonal Antibodies with Thiophosphorylated Peptide. BALB/c ByJ mice (6 weeks of age) were primed and boosted twice, at three-week intervals, by intraperitoneal injection of a mixture of KLH conjugated with thiophosphorylated α-14mer(281) and free thiophosphorylated peptide emulsified in Ribi adjuvant, as described previously (Patton *et al.*, 1991). After a final series of three boosts on successive days without adjuvant (Stähli *et al.*, 1980), one of the mice was sacrificed, its spleen removed, and the splenocytes fused with HL-1 murine myeloma cells (Khöler and Milstein, 1975). Efficiency of fusion was typically 1 per 100,000. Hybridomas were plated to a density of approximately three clones per well of in 96-well culture plates. Media collected from hybridoma cultures that had grown to 75% to 85% confluency were tested for anti-phosphokinase antibodies by enzyme-linked immunosorbent assay (ELISA), performed as described below.

Production of Monoclonal Antibodies with Thiophosphorylated CaM Kinase. Purified CaM kinase was thiophosphorylated for 90 s at 30°C in a solution containing 50 mM Tris (pH 8.0), 10 mM $MgCl_2$, 25 mM dithiothreitol, 0.7 mM EGTA, 1.0 mM $CaCl_2$, 0.5 mM ATPγS, 0.4 mg/ml calmodulin, and 0.4 mg/ml kinase. The reaction was terminated by the addition of EDTA to a final concentration of 0.1 M, and cooled in an ice bath. To remove calmodulin and other reagents, the solution was brought to 60% saturation with ammonium sulfate and incubated overnight at 4°C. Immediately prior to immunization,

the precipitated kinase was collected by centrifugation at 13,000 g for 30 minutes at 4°C and resuspended to a concentration of 1 mg/ml in phosphate buffered saline (PBS, 50 mM sodium phosphate (pH 7.3), 9% (w/v) NaCl). Nonphosphorylated kinase was prepared for use as immunogen in an identical fashion; ATPγS was omitted from the reaction.

Immunosuppression of the immune response to nonphosphorylated kinase holoenzyme preceded immunization with thiophosphorylated kinase. BALB/c ByJ mice were injected intraperitoneally with 100 μg of nonphosphorylated kinase emulsified in Ribi adjuvant. Immunosuppression was induced by co-injection of 10 μg cyclophosphamide per gm body weight. Cyclophosphamide injections were repeated on the following two days. Mice were immunized two weeks later with 100 μg thiophosphorylated kinase in Ribi adjuvant. Immunosuppression followed by immunization constituted a suppression/immunization cycle that was repeated on a monthly basis (four weeks). Control mice were immunized with either nonphosphorylated kinase or thiophosphorylated kinase every four weeks; they were not immunosuppressed. Sera taken seven days after each injection was tested by differential ELISA, as described below. On each of the three days prior to sacrifice, mice were boosted with thiophosphorylated kinase without adjuvant (Stähli *et al.*, 1980). Hybridomas selected after screening by ELISA and immunoblot were immediately subcloned. Ascites was produced by intraperitoneal injection of 10 X 10^6 hybridoma cells into pristane-primed BALB/c mice.

Antibodies were partially purified from ascitic fluid by ammonium sulfate precipitation and resuspended in PBS to 30 mg/ml.

ELISA Using Nonphosphorylated and Phosphorylated CaM Kinase Substrate. Monoclonal antibodies were screened by ELISA, using as antigen substrate a CaM kinase isozyme that is composed of an equal ratio of α and β/β′ subunits (Miller *et al.*, 1988). Autophosphorylated CaM kinase was prepared by incubation for 60 s at 30°C as described above, except the reaction mixture contained 0.2 mg/ml kinase, 0.2 mg/ml calmodulin, and 0.2 mM ATP. Reactions were quenched by the addition of 2 volumes of 0.2 M EDTA and cooled to 0°C in an ice bath. Nonphosphorylated kinase was treated identically, except ATP was omitted from the reaction; calmodulin was included. Kinase was diluted to 0.01 mg/ml with ice-cold PBS and immediately plated into 96-well polystyrene culture plates (Falcon, #3072). As previously described (Patton *et al.*, 1991), 30 µl of diluted kinase (0.3 µg kinase, 5.5 pmol subunit) was added to each well, with nonphosphorylated and phosphorylated kinase distributed in alternate rows. After binding overnight at 4°C, wells were washed briefly with PBS containing 0.05% Tween 20 (Tween-PBS) at room temperature. Serum or solutions containing monoclonal antibody were added to a pair of wells, one each containing nonphosphokinase or phosphorylated kinase. Serum and ascites were diluted in Tween-PBS containing 1% normal goat serum (NGS); hybridoma culture supernatants were not diluted.

After two to four hours incubation at room temperature, or overnight at 4°C, unbound antibodies were removed with several brief washes of Tween-PBS. Bound antibodies were detected with alkaline phosphatase-conjugated secondary antibodies (Goat anti-mouse IgG and IgM) and the alkaline phosphatase substrate ρ-nitrophenyl phosphate. The alkaline phosphatase reaction product, ρ-nitrophenol, was quantitated by reading absorbance at 405 nm in a microtiter plate reader. The reaction was linear for several hours, or up to about 1.5 absorbance units.

Immunoblotting CaM kinase and Rat Brain Homogenate. Pure CaM kinase was autophosphorylated at 30°C in the reaction mixture described above for ELISA antigen preparation, in the presence of [γ-^{32}P]ATP, Ca^{2+}, and calmodulin, for the times indicated in the figure legends. Autophosphorylation was stopped by the addition of an equal volume of gel sample buffer containing 2% (w/v) SDS and boiled for two minutes. Nonphosphorylated kinase was prepared in parallel reactions from which ATP was omitted.

Rat brain homogenate was freshly prepared as described previously (Miller and Kennedy, 1985), and kept on ice. Homogenate was centrifuged at 2000Xg for 20 min at 4°C, and the low speed supernatant collected. Endogenous phosphorylation of hogenate protein was carried out for five minutes at 30°C in a reaction containing 3 mg/ml homogenate protein, 80 mM Tris (pH

8.0), 1.2 mM EDTA, 1.0 mM EGTA, 0.2 mM [γ-^{32}P]ATP, and 10 mM DTT. Phosphorylation in the presence or absence of Ca^{2+} was carried out in the presence or absence of 2.5 mM $CaCl_2$ and 50 ug/ml calmodulin, respectively. Phosphorylation was terminated with gel sample buffer, as described above for kinase autophosphorylation.

Aliquots containing 0.2 μg kinase holoenzyme, or 20 μg brain homogenate protein, each phosphorylated or not phosphorylated as described above, were loaded into the appropriate lanes of minigels (5 X 9 X 0.08 cm), as indicated in the figure legends, and the kinase subunits or proteins separated by SDS-PAGE. Gels were washed for 20 minutes in H_2O, then electrophoretically transferred to nitrocellulose membranes in 25 mM sodium borate at a constant current of 350 mA for the times indicated in the figure legends. Nonspecific binding to membranes was blocked with 5% (w/v) nonfat dry milk in TBS-Tween (50 mM Tris [pH 7.4], 0.5 M NaCl, 0.05% (v/v) Tween 20) for five to 12 hours. Blots were incubated for 12 hours at 4°C in 4 ml of solution containing primary antibodies diluted 250-fold in TBS-Tween (50 mM Tris [pH 7.5], 0.5 M NaCl, 0.05% (v/v) Tween 20) supplemented with 2% (v/v) normal goat serum (NGS). In general, control incubations were performed with a combination of Annette polyclonal antibodies and the monoclonal antibody 6G9. Annette was immunized several times with purified CaM kinase holoenzyme; 6G9 antibody was partially purified from ascitic fluid by ammonium sulfate precipitation, and specifically recognizes the CaM kinase α subunit (Erondu

and Kennedy, 1985). Unbound primary antibodies were washed from the blots with TBS-Tween for one hour with three buffer changes. Separate immunoblots in a single experiment were collected into one container, and bound antibodies detected with goat anti-mouse or anti-rabbit IgG+IgM secondary antibodies conjugated with alkaline phosphatase by incubation for two hours at room temperature in TBS-Tween solution containing 2% (v/v) NGS. Unbound secondary antibodies were removed with three 15-minute washes with TBS-Tween, followed by three 15-minute washes with TBS. The blot was incubated for 10 minutes in 0.1 M sodium bicarbonate (pH 9.5), 1 mM $MgCl_2$, and bound secondary antibodies visualized with BCIP and NBT alkaline phosphatase substrates dissolved in the same buffer.

<u>Other Procedures.</u>

Protein concentrations were measured by the method of Peterson (12) with bovine serum albumin as standard. SDS-PAGE was performed by the method of Laemmli (13). Running gels contained 10% acrylamide, 0.34% bisacrylamide. Immunoglobulin class was determined by the Ouchterlony double-diffusion method (17).

Abbreviations used in this paper are: CaM, Ca^{2+}/calmodulin; DTT, dithiothreitol; HPLC, high pressure liquid chromatography; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; CaM kinase, type

II Ca^{2+}/calmodulin-dependent protein kinase; ELISA, enzyme-linked immunosorbent assay.

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RESULTS

Polyclonal Antisera Specifically Recognizing Nonphosphorylated and Autophosphorylated CaM Kinase.

In an attempt to produce antisera that would bind to the CaM kinase in either its nonphosphorylated or autophosphorylated form, four rabbits were immunized with nonphosphorylated peptide haptens and six rabbits were immunized with thiophosphorylated peptide haptens, respectively. Immunoreactivity that appears completely specific for an antigen will subsequently be referred to as "specific"; immunoreactivity that appears only partially specific, having some cross-reactivity, will be referred to as "selective."

Four rabbits were immunized with nonphosphorylated peptides corresponding in sequence to the CaM kinase α subunit Thr^{286} autophosphorylation site, two were immunized with α-9mer(281), and two with α-14mer(281). One of the rabbits immunized with α-14mer(281), Sylvia, produced antisera against the CaM kinase which specifically recognized purified, nonphosphorylated CaM kinase on immunoblots (Figure 1, lanes 1 and 2; ref 6). Autophosphorylation of the purified kinase for two minutes almost completely abolished binding of Sylvia antisera. Slight residual binding to kinase in the sample may be due either to incomplete kinase autophosphorylation, to incomplete specificity for the nonphosphorylated form, or to

both. Preimmune Sylvia serum contained no anti-CaM kinase immunoreactivity on immunoblots of purified kinase (data not shown). There were no antibodies in the other peptide immune sera that bound to CaM kinase in either nonphosphorylated or autophosphorylated forms (data not shown).

Of the six rabbits immunized with thiophosphorylated peptide haptens, four were immunized with α-14mer(281) and two were immunized with α-9mer(281). Antisera showing some selectivity for autophosphorylated CaM kinase were produced in five out of the six rabbits. Antisera bound to kinase autophosphorylated with ATP as substrate, despite immunization with thiophosphorylated peptides (ATPγS as substrate). Three anti-phosphokinase antisera were obtained from four rabbits immunized with thiophosphorylated α-14mer(281): Valerie, Wendy, and Xena (Figure 1, lanes 3 to 8). The fourth rabbit showed no response. Wendy antisera was particularly strong and specifically recognized both major subunits of the kinase, α and β, in an autophosphorylation-dependent manner. Phosphorylation of proteins often results in a shift to higher apparent molecular weights during SDS-PAGE. Autophosphorylation of CaM kinase is well known to cause a series of these shifts in the mobility of the α and β subunits, probably due to the autophosphorylation of several residues on each subunit. The band of Wendy immunoreactivity seen immediately above the main α subunit band is also α subunit, autophosphorylated at additional sites; it is apparent in lanes containing autophosphorylated kinase and blotted with control antibody

(Figure 1, lane 14). The band of immunoreactivity observed immediately below the β subunit may represent binding to autophosphorylated β', gamma, and/or delta subunits; proper control antibodies are not available to confirm these possibilities. Xena immunoreactivity was moderate in strength and selective for the α subunit. The binding of Xena antibodies to kinase β subunit appeared to be insensitive to autophosphorylation, though the shift to lower mobility (higher apparent M_r) of the subunit due to autophosphorylation is readily observed (Figure 1, lane 8). Valerie anti-phosphokinase immunoreactivity was barely detectable, although it was much more visible at higher concentrations of serum (data not shown).

The serum response of both of the rabbits immunized with thiophosphorylated α-9mer(281) was robust. On immunoblots, antisera from Zelda and Alice reacted primarily with the α subunit (Figure 1, lanes 9 to 12). Zelda antisera was quite specific for the autophosphorylated form. Alice antisera was highly selective, but not specific, for the autophosphorylated form of the α subunit. Preimmune sera did not bind to purified CaM kinase on immunoblots (data not shown).

Protein Specificity of Polyclonal Antisera and the Effects of Endogenous Calcium-Dependent Phosphorylation.

The six antisera which bound phosphorylated or nonphosphorylated CaM kinase on immunoblots were tested for their protein specificity on immunoblots of rat brain homogenate (Figure 2A). Although all six antisera contained a significant amount of cross-reacting antibodies to other brain proteins, each recognized the CaM kinase in rat brain homogenates in a phosphorylation-sensitive fashion. Binding of Sylvia antisera to the CaM kinase α and β subunits was reduced significantly by endogenous Ca^{2+}-dependent phosphorylation for two minutes, while binding of Valerie, Wendy, Xena, Zelda, and Alice to kinase subunits was increased. Binding of control antibody was unaffected, indicating that proteolysis of CaM kinase was not a factor (Figure 2A).

Although the reduction in Sylvia binding to CaM kinase alpha subunit caused by endogenous Ca^{2+}/calmodulin-dependent phosphorylation (Figure 2A) is consistent with the effect of CaM kinase autophosphorylation on Sylvia antiserum binding to purified CaM kinase (Figure 1), the effect was not nearly as pronounced. A number of factors may account for this inconsistency. Protein phosphatases active in homogenized tissue against phosphorylated CaM kinase (Shields *et al.*, 1985; Miller *et al.*, 1988; Patton *et al.*, 1990) may compete with kinase autophosphorylation to reduce the overall level of phosphate incorporated into kinase subunits. In addition, a fraction of the

CaM kinase in brain homogenates may be phosphorylated at Thr^{305} (Molloy and Kennedy, 1991); this threonine residue is located within the calmodulin binding domain of the kinase and its phosphorylation blocks activation of the CaM kinase by Ca^{2+}/calmodulin (Patton *et al.*, 1990). Therefore, some fraction of the CaM kinase in homogenates may not be stimulated to autophosphorylate by the addition of Ca^{2+}/calmodulin. Crude brain homogenates also contain unidentified CaM kinase inhibitory factors, possibly further reducing the rate of kinase autophosphorylation (Bennett *et al.*, 1983). Thus, the rate and extent of autophosphorylation at $Thr_{286/287}$ may be reduced in homogenates compared with *in vitro* autophosphorylation.

Immunization of Sylvia with nonphosphorylated α-9mer(281) peptide resulted in the appearance of several new bands of immunoreactivity on immunoblots of brain homogenate, compared with preimmune sera (Figure 2, A and B; lanes 1, 2). Since Sylvia antisera was the only nonphosphokinase specific antibody reagent, it was affinity purified (below).

All of the phosphokinase-selective antisera bound homogenate kinase after Ca^{2+}calmodulin-dependent phosphorylation. In addition, Xena bound to a series of proteins of higher apparent molecular weight in a Ca^{2+}-stimulated phosphorylation-dependent fashion (Figure 2A, lane 7 and 8). Binding to these proteins is not detected in preimmune Xena serum (Figure 2B, lanes 7 and 8). The significance of this binding is unknown, but it is interesting that Ca^{2+}-

stimulated phosphorylation also increased the binding of control anti-CaM kinase antibodies to proteins in this molecular weight region. Binding of proteins other than CaM kinase subunits by the other phosphokinase selective antisera was not affected by endogenous Ca^{2+}-dependent phosphorylation. Valerie immune sera bound to several brain proteins not observed in blots of preimmune sera, but only slightly to phosphorylated CaM kinase α-subunits at these serum dilutions (lanes 3 and 4). Xena immune sera also bound a number of protein besides the CaM kinase. Thus, of the several phosphokinase selective antisera, Wendy and Alice antisera appeared the most specific for the CaM kinase. In these two antisera, the vast majority of the immunoreactivity to blotted rat brain proteins was against the phosphorylated form of the CaM kinase (lanes 5, 6, 11, 12). Comparisons of preimmune and immune antisera indicate that thiophosphopeptide immunization produced little additional immunoreactivity against brain proteins other than the CaM kinase in these two rabbits, especially Wendy. Wendy and Alice antisera detected purified CaM kinase on immunoblots at dilutions as high as 10,000 fold (data not shown). Thus, it should be possible to affinity purify the anti-phosphokinase specific antibodies in these antisera by chromatography on a column matrix coupled with thiophosphorylated peptide. These possibilities have not been pursued in favor of the phosphokinase specific monoclonal antibodies discussed below.

Affinity Purification of Nonphosphokinase-Specific Antibodies from Sylvia Antisera.

Antibodies specific for the nonphosphorylated form of the CaM kinase were purified from Sylvia antisera by affinity chromatography on an α-9mer(281)-column, as described in Experimental Procedures. Although Sylvia was immunized with the α-14mer(281) peptide, preliminary experiments with the antisera showed that both the α-9mer(281) and α-14mer(281) peptides blocked serum binding to nonphosphorylated kinase subunits on immunoblots, but that α-14mer(218) also blocked binding to two other protein bands, of apparent molecular weight 40 kDal and 72 kDal (data not shown). α-9mer(218) blocked binding to only one additional protein band, that of apparent molecular weight 72 kDal (data not shown). Thus, it seemed likely that a more specific population of antibodies could be purified on an α-9mer(281) resin. Antibodies eluted from the column were highly specific for both the CaM kinase α subunit, and for its nonphosphorylated form (Figure 3, lane 5). Some antibodies reacting with the 72 kDal protein were co-eluted. This is not surprising in light of the peptide inhibition experiments mentioned above. The ratio of 72 kDal immunoreactivity to kinase α-subunit reactivity remained the same during several separate purifications, when elution conditions were changed, upon dilution, and during peptide inhibition, suggesting that a single group of antibodies cross-reacts with both proteins

(data not shown). Binding to this protein is not affected by endogenous Ca^{2+}-dependent phosphorylation (Figures 2A and 3).

Autophosphorylation Site Specificity of Purified Sylvia Antisera.

Nonphosphokinase-specific polyclonal antibodies, affinity purified from Sylvia antisera, were tested by the immunoblot method against purified CaM kinase that had been allowed to autophosphorylate for various lengths of time. Autophosphorylation during the first five seconds dramatically decreased binding of Sylvia antibodies (figure 4). Continued autophosphorylation for several minutes further decreased antibody binding. This time course correlates well with autophosphorylation of Thr^{286} on the kinase α subunit. Over 90% of the phosphate incorporated into the α subunit during the first five seconds of autophosphorylation at 30°C is at Thr^{286} (Miller *et al.*, 1988, Chapter 2, Figure 1A). Although the kinase continues to autophosphorylate at this site for over a minute, most of the Thr^{286} sites are phosphorylated during the first few seconds *in vitro* (cp. Miller *et al.*, 1988, Figures 1A, 1C, and 1E therein); the exact time course of prolonged autophosphorylation at this site has not been determined. Thus the time course of autophosphorylation of Thr^{286} accounts for the observed time course of decrease in binding of Sylvia antibody. A similar sensitivity and time course was obtained by ELISA methods (data not shown). Therefore we conclude that the antibodies purified from Sylvia

antisera bind to, and are largely specific for, the nonphosphorylated Thr^{286} autophosphorylation site on the CaM kinase α subunit. Although it is possible that Sylvia antibodies bind weakly to other CaM kinase autophosphorylation sites, this would be surprising since the primary sequence surrounding the other significant Ca^{2+}-dependent autophosphorylation sites on the α subunit (a threonine near the amino terminus, and Ser^{279}) is not similar to the sequence surrounding Thr^{286} (See Chapters 2 and 3). The requirement for sequence close to that of α-14mer(281) is probably quite strict; Sylvia antibodies do not bind to CaM kinase β subunit nearly as well as to α subunit, while the difference in sequence between the two subunits in this 14 residue region is only the conservative substitition of Glu for Asp^{288}. A similar subunit specificity is observed for the phosphokinase specific antisera produced by immunization with the nine-residue peptide, described above, and for several monoclonal antibodies produced by immunization with 14-residue peptide, described below.

Phosphokinase-Specific Monoclonal Antibodies - Thiophosphopeptide Immunization.

One strategy to generate monoclonal antibodies that specifically bind CaM kinase autophospohorylated at Thr^{286} involved immunization of mice with thiophosporylated α-14mer(281). Three mice were immunized, each with a different amount (125 μg, 100 μg, or 75 μg) of a mixture of free and KLH-conjugated peptide, as described in Experimental Procedures. An ELISA was used to evaluate the serum response to immunization (Experimental Procedures). After a single boost injection, serum antibodies that specifically bound CaM kinase autophosphorylated with ATP were at their highest level in the mouse immunized with the largest dose of immunogen, 125 μg, (Figure 5A). However, a second boost injection accelerated the serum response in the second mouse, immunized with 100 μg of immunogen, relative to the other mice (Figure 5B). Specific serum immunoreactivity increased against both phosphorylated and nonphosphorylated CaM kinase during the series of boost injections (FIgure 5C). The mouse immunized with 75 μg of immunogen produced few anti-phosphokinase antibodies. The number of mice immunized was too few to determine whether the variability among the immune responses reflected differences in the immune systems of the mice, or if, in fact, the variability resulted from the differences in immunogen dosage.

Since the mouse immunized with 100 µg of thiophosphorylated peptide had the highest phosphospecific antibody titer, it was chosen for monoclonal antibody production. Hybridomas produced from the splenocytes were screened by ELISA for secreted antibodies that bound to nonphosphorylated and autophosphorylated CaM kinase (described in Experimental Procedures). The cumulative results from screening the 1,248 primary hybridoma cultures containing visible clones are shown in Figure 6. The overall distribution of clonal reactivities was graded in strength, with many hybridoma not producing significant amounts of anti-kinase antibodies. The overall selectivity of the population of clones was for the phosphorylated form of the kinase (above the diagonal line). Most of the clones that secreted strong anti-kinase reactivity (greater than 1.0 in this normalized pool of data) were nonselective or slightly selective for the phosphorylated form of the CaM kinase. However, a number of clones produced antibodies that were highly selective or specific for the autophosphorylated CaM kinase, as well as strongly reactive. Twenty-four clones reacted more than 10 times as strongly with the autophosphorylated kinase than with nonphosphorylated kinase. This is 2% of the total number tested. After further characterization by immunoblot methods of their specificity for phosphorylated forms of the α and β subunits of the CaM kinase, and for the CaM kinase subunits among brain homogenate proteins, five of these clones were selected for subcloning and ascites production. The characteristics of three of them are described below.

Phosphokinase Specific Monoclonal Antibodies - Thiophosphokinase Holoenzyme Immunization.

In an effort to increase the ability of an immune system to mount a response to phosphorylated epitopes on the CaM kinase among the many nonphosphorylated epitopes presumably present on its surface, mice were first injected with nonphosphorylated kinase and the immune response suppressed by administration of cyclophosphamide. Mice were then immunized with autothiophosphorylated CaM kinase, prepared with ATPγS as substrate. Immunosuppression and immunization were alternated through several cycles. Of two mice immunized in this fashion, one was given two cycles of suppression and immunization, while the other recieved three. These procedures are described in Experimental Procedures and the immune responses of the two mice are described in Appendix A. Hybridomas produced from the splenocytes of the two mice were screened by ELISA using nonphosphorylated and autophosphorylated CaM kinase as antigen substrate, as described above for hybridomas derived from the thiophosphopeptide-immunized mouse. The overall selectivity of antibodies from the population of hybridomas was small, with a slight bias for the phosphorylated CaM kinase (Figure 7). This bias appeared greater in the group of hybridoma produced from the mouse that was immunized with three cycles of suppression/immunization (Figure 7A). From the 1,514 hybridomas screened from both mice, only one clone was obtained that secreted antibodies that were strongly selective for the phosphorylated

CaM kinase (Figure 7B). It was successfully subcloned and produced ascites. One other clone was obtained with high specificity, but relatively low reactivity; it was not successfully subcloned. An additional 23 clones produced antibodies with more than three-fold selectivity for the autophosphorylated kinase. Hybridomas were plated at a density of three to five clones per well, increasing the possibility that some phosphokinase-specific clones may have appeared less specific because they were grown in a well that also contains a clone secreting nonselective antikinase antibodies. Thus, it may be possible to obtain additional phosphokinase-specific monoclonal antibodies by subcloning the hybridoma cultures producing merely phosphokinase selective antibodies. The phosphokinase specific monoclonal antibodies obtained through peptide immunizations, described above, dissuaded us from pursuing these possibilities.

Subunit and Protein Specificity of Anti-Phosphokinase Monoclonal Antibodies.

Several monoclonal antibodies that were greater than 10-fold selective for the phosphorylated form of the CaM kinase in ELISA were tested for specificity against purified CaM kinase and brain homogenate protein blotted onto nitrocellulose membranes (Figure 8). Monoclonal antibodies 26G6, 27G10, and 22B1 were derived from a mouse immunized with thiophosphorylated α-

14mer(281), and were of the IgG_1 class. Monoclonal antibody 5B3 was obtained from a mouse immunized with thiophosphorylated kinase holoenzyme, and was of the IgM class. All were found to be completely specific for the autophosphorylated form of the kinase on immunoblots. Monoclonal antibody 5B3 bound almost exclusively to the autophosphorylated β subunit. Antibodies 26G6 and 27G10 bound predominantly to the autophosphorylated α subunit. The antibody 22B1 recognized α, β, and β' subunits, but bound most strongly to α. However, a significant amount of cross-reactivity with other brain proteins was observed on immunoblots (Figure 9) with relatively high concentrations of antibody (legend to Figure 9). Monoclonal antibody 22B1 bound with higher affinity to the kinase alpha subunit than to cross-reactive proteins, and could be used to specifically label alpha subunit with longer washes (Figure 9) or by using higher dilutions of antibody (data not shown). Antibody 22B1, at dilutions of between 40,000- and 2000-fold (antibody concentration of 0.7 to 14 μg/ml, partially purified from ascitic fluid), reproducibly and specifically bound the autophosphorylated CaM kinase presented as antigen on immunoblots in the amounts shown in Figures 9 and 10 (2.7 pmol subunit per lane). Dilution and washing were not completely effective in reducing cross-reactive binding of monoclonal antibodies 5B3, 26G6, 27G10 (Figure 9); background binding with 5B3 was particularly high, in part because it was not strongly reactive with kinase on immunoblots, requiring high antibody concentrations to detect the phosphorylated beta subunit. The most prominent cross-reactive protein in brain homogenate was synapsin I

(Figure 9). Interestingly, anti-phosphokinase monoclonal antibodies bound to synapsin I only after that protein had been phosphorylated in a Ca^{2+}/calmodulin-dependent manner. (Both the type I and type II CaM kinases phosphorylate this substrate, at distinct sites.) We were surprised by this result because there is very little sequence identity between the synapsin I phosphorylation sites and the type II CaM kinase Thr^{286} autophosphorylation site, although there are a few sequence similarities (notably the Arg-Gln-X preceding the phosphorylated residue, Ser in synapsin I). Although there were no significant similarities between the CaM kinase and synapsin I amino acid sequences detected by computer search, similarities in tertiary structure remain a possibility. This, in addition to a strong interaction with the phosphate moiety, may explain the significant cross-reactivity we have observed.

Dependence of Monoclonal Antibody Binding on Time of Autophosphorylation.

Monoclonal antibodies 22B1 and 5B3 were tested by the immunoblot method against purified CaM kinase that had been allowed to autophosphorylate for various lengths of time in the presence of Ca^{2+}/calmodulin (Figures 10 and 11). As described above, neither antibody bound to the nonphosphorylated kinase. However, binding of both antibodies increased

dramatically after only five seconds of autophosphorylation (Figures 10A and 11A). Although the kinase continued to autophosphorylate for several minutes (Figures 10A and 11A), antibody binding increased only slightly (Figures 10B and 11B). This time course correlates well with autophosphorylation of Thr^{286} on the kinase α subunit, and Thr^{287} on the β subunit, as described above for Sylvia polyclonal antibody binding (Miller *et al.*, 1988). A similar dependence of antibody binding on the time of kinase autophosphorylation was observed for both monoclonal antibodies in parallel experiments conducted by ELISA methods. In these experiments, antibody 22B1 binding increased to over 70% of its maximal value during the first five seconds of autophosphorylation (Table I). Binding of 22B1 remained constant for 20 seconds of continued autophosphorylation before increasing with prolonged autophosphorylation. The antibody concentration required for half-maximal binding of 22B1 also decreased over three-fold after prolonged autophosphorylation. Thus, in ELISA also, monoclonal antibody 22B1 binds primarily to Thr^{286}, the only residue autophosphorylated after five seconds *in vitro*. The additional binding observed after prolonged autophosphorylation may represent binding to the several "late" sites that are autophosphorylated at a slower rate (Miller *et al.*, 1988), or to Thr^{286} sites that were initially hidden in folds of the kinase tertiary or quaternary stucture and exposed by prolonged autophosphorylation. The increase in affinity of 22B1 binding may be due to similar changes in holoenzyme conformation, or to the increases in local concentration of phosphorylated residues on the kinase after prolonged autophosphorylation.

It is not known whether the late sites are actually autophosphorylated to significant levels *in situ.*

Monoclonal antibody 5B3 binds in a more simple manner to autophosphorylated CaM kinase in ELISA than does 22B1. Virtually the entire increase in binding of 5B3 to kinase occurred during the first five seconds of autophosphorylation; additional autophosphorylation had no effect on the binding (Table I). Antibody 5B3 binds to the β subunit of the CaM kinase. There are two early autophosphorylation sites on the β subunit, Thr^{287} and Thr^{382}; they are not distinguishable by their rates of autophosphorylation, but may be partly distingished by protein phosphatase catalyzed dephosphorylation kinetics (Miller *et al.*, 1988). Because 5B3 was produced by thiophosphokinase holoenzyme immunization, neither site is *a priori* more likely to be the kinase epitope. Considering the nearly identical sequences surrounding the αThr^{286} and βThr^{287} sites, the essentially absolute preference of antibody 5B3 for the β subunit over the α subunit argues that antibody 5B3 probably binds to the Thr^{382} autophosphorylation site. On the other hand, this argument is weakened by the high concentration of 5B3 that is required to detect autophosphorylated kinase β subunit on immuoblots, relative to that required in ELISA, which suggests that secondary or tertiary structure in the native protein probably contributes to the epitope bound by 5B3. Differences in subunit tertiary structure might then be responsible for the subunit specificity displayed by 5B3. Further work is required to positively identify the early

autophosphorylation site on the β subunit recognized by monoclonal antibody 5B3, as well as those sites contributing to the increase in binding of 22B1 to the α subunit during prolonged autophosphorylation.

DISCUSSION

Protein phosphorylation is the most common and best-studied mechanism by which cells reversibly regulate protein function through covalent modification. Most studies of protein phosphorylation follow the transfer of radiolabeled phosphate from ATP into substrate proteins. Recently, anti-phosphotyrosine antibodies have been developed and used to detect, quantitate, and purify proteins phosphorylated on tyrosine residues. Initial attempts have been made to develop antibodies specific for phosphoserine and phosphothreonine, the major phosphorylated residues in cells, but they have not yet resulted in antibodies that will recognize specific phosphorylated proteins with high affinity (Levine *et al.*, 1989). Antibodies specific for proteins phosphorylated on serine or threonine residues have not been published. This is somewhwat ironic, since the overwhelming majority of phosphorylated residues are serines and threonines, while phosphotyrosine accounts for less than one-half of one percent of the total protein-bound phosphate in cells (Hunter and Cooper, 1985). However, the low abundance of protein tyrosine kinases and their substrates have made anti-phosphotyrosine antibodies all the more useful aids in identifying and characterizing tyrosine phosphorylation.

We have developed monoclonal and polyclonal antibodies that bind to the phosphorylated Thr^{286} autophosphorylation site of the neuronal type II

CaM kinase. The requirement for phosphate on this residue is essentially absolute in conventional ELISA and immunoblotting assays. One monoclonal antibody, 22B1, binds with high affinity to the autophosphorylated α subunit and does not recognize other brain proteins under normal immunoassay conditions. Another monoclonal antibody, 5B3, binds specifically to the autophosphorylated β subunit. The exact early autophosphorylation site that is bound by 5B3 was not determined; it may bind either to βThr^{287} or to βThr^{382}, or to both. We have also developed nonphosphokinase-specific polyclonal antibodies. Antibodies affinity purified from Sylvia antisera bind with high affinity and specificity to purified CaM kinase that has not been autophosphorylated, and lose this capacity as the kinase autophosphorylates at Thr^{286}.

Two immunogens, peptide haptens and kinase holoenzyme, were used to produce these antibodies. A primary concern of early attempts to produce phosphotyrosine-specific antibodies was the potential instability of phosphorylated immunogens to dephosphorylation *in vivo*. Ross *et al.* (1981) used conjugated azobenzylphosphonate as an immunogen, a phosphotyrosine analogue resistant to phosphatase-catalyzed dephosphorylation. In order to reduce dephosphorylation of the immunogen by protein phosphatases *in vivo* and, thus, hopefully increase the specificity of the immune response for the phosphorylated protein, we chose to phosphorylate peptides with ATPγS (thiophosphorylation), rather than with the natural ATP substrate (refs). For

the same reason, kinase holoenzyme was auto*thio*phosphorylated with ATPγS rather than ATP. A potential drawback to this method is that because phosphate and thiophosphate are not chemically identical, antibodies generated with thiophosphorylated immunogen may not bind the phosphorylated antigen with high affinity. Our results demonstrate that antibodies with a high degree of specificity and affinity for antigen phosphorylated on threonine can be produced with thiophosphorylated peptide immunogen. Nevertheless, in practice it may be possible to immunize with phosphorylated peptides or proteins, instead of the thiophosphorylated forms. Immunization with phosphotyrosine haptens has proved successful (Ohtsuka et al., 1980; Ek and Heldin, 1984; Seki et al., 1986, Glenney *et al.*, 1988). In addition, phosphoserine and phosphothreonine amino acids, as haptens, are capable of producing phospho-amino acid-specific antisera of some utility in rabbits (Levine *et al.*, 1989). However, one possible explanation for these successes may be that phosphoamino acids are relatively poor substrates for protein phosphatases; phosphorylated peptides may be more susceptible to dephosphorylation (Titanji *et al.*, 1980). Recently, Nishizawa *et al.* (1991) reported the production of polyclonal antibodies specific for phosphorylated intermediate filament protein using phosphorylated synthetic peptides. Peptides were phosphorylated in the presence of ATP with the catalytic subunit of the cAMP-dependent protein kinase.

The sequences of the peptide haptens used in this study corresponded to the CaM kinase Thr^{286} autophosphorylation site. This sequence included the minimal consensus substrate sequence (Arg-X-X-Ser/Thr) for the CaM kinase phosphorylation, permitting the preparation of phosphorylated analogues by enzymatic thiophosphorylation. Recent advances in methods for *de novo* synthesis of peptides containing phosphoamino acids (Perich and Johns, 1988; Arendt et al., 1989; Lacombe et al., 1990; Kitas et al., 1990) should relieve the kinase substrate requirement and greatly facilitate the development of phosphoprotein-specific antibodies. Presumably, methods for incorporating thiophosphoamino acids into synthetic peptides could be developed based on procedures for incorporating phosphoamino acids.

It is possible that the presence of a thiophosphate group increased the immunogenicity of the peptides in this study. Five of six rabbits immunized with thiophosphorylated peptides produced phosphokinase-specific antibodies, at high levels in three rabbits. Only one of the four rabbits immunized with nonphosphorylated peptides produced nonphosphokinase-specific antibodies. The small number of immunized rabbits, coupled with the inherent variability in the immune systems of outbred populations, prevents any strong conclusions. The thiophosphorylated nine-residue peptide produced polyclonal antisera with a greater specificity for the α subunit (phosphorylated). The sequences of the α and β subunits are almost identical through the corresponding sequences, with one conservative amino acid substitution of Glu in the β

subunit for Asp in the α subunit at position 288/289 (α/β). Thus, the orientation and shape of the acid group in this position in the nine-residue peptide is important to binding most of the antibodies induced by the nine-residue peptide. The addition of five residues to the carboxyl terminus of the peptide may allow for a significant increase in the diversity of the antibody antitopes produced, some of which do not significantly bind to the acid residue, resulting in a polyclonal serum with cross-reacting antibodies. Consistent with this idea, the group of monoclonal antibodies isolated from the mouse immunized with the 14-residue peptide (modeled after the α subunit sequence) displayed a range of subunit specificities, although no antibody was specific for the β subunit.

Immunization of mice with thiophosphorylated peptide hapten was far more successful than immunization with thiophosphorylated kinase holoenzyme in producing phosphokinase-specific monoclonal antibodies. Several factors may have contributed to this result. First, the relative dosage of phosphorylated epitopes was far greater by molar quantity in peptide immunogen than in holoenzyme immunogen. Second, immunosuppression methods used to increase the specificity of the immune response in holoenzyme-immunized mice may compromise the immune system of these mice. Finally, while phosphorylated peptide haptens are exposed on the surface of the carrier proteins, the phosphorylated sites on the kinase holoenzyme may be buried inside or between kinase subunits. Our results indicate that

immunization with thiophosphorylated peptide conjugates is the preferred method. However, the immunosuppression/immunization methods employed with autophosphorylated kinase immunogen were successful in enhancing the anti-phosphokinase immune response (Patton *et al.*, 1991) and produced at least one phosphokinase-specific antibody with high affinity (in ELISA). This antibody, 5B3, recognized the β subunit of the CaM kinase, at a site specifically altered by autophosphorylation at one or both of the two early autophosphorylation sites on this subunit, Thr^{287} and Thr^{382}. Thus, immunization with the thiophosphorylated protein, supported by nonphosphoprotein immunosuppression methods, does offer some chance of success and may complement immunization with peptides in some cases, or substitute in cases where phosphorylation sites connot be identified or guessed, or when no sequence information is available.

We hope to use the phosphokinase-specific monoclonal antibody 22B1, and the nonphosphokinase-specific antibodies from Sylvia, to gather information about the role of CaM kinase autophosphorylation in brain and neuronal function. These antibodies, used separately and in combination, may enable us to study where within the brain CaM kinase is phosphorylated at Thr^{286} and is, therefore, Ca^{2+}-independent, whether subcellular compartments (*e.g.*, PSD, membranes, synapses, cell soma) contain kinase autophosphorylated to different extents, and whether the level of autophosphorylation of Thr^{286} varies in conjunction with particular known neuronal events, such as LTP. We are

interested to know if these antibodies will specifically immunoprecipitate phosphorylated kinase, and whether they will selectively inhibit the activity of the Ca^{2+}-independent form of the kinase, including Ca^{2+}-independent autophosphorylation. If so, Fab′ fragments might then be used to interfere with processes that are dependent on the Ca^{2+}-independent activity of the kinase.

It is also hoped that our results will encourage others to develop antibodies sensitive to the phosphorylation state of other particular proteins, further defining the many protein phosphorylation systems that regulate cell function.

References:

Arendt, A., Palczewski, K., Moore, W. T., Caprioli, R. M., McDowell, J. H., and Hargrave, P. A. (1989). Synthsis of phosphopeptides containing *O*-phosphoserine or *O*-phosphothreonine. *Int. J. Peptide Protein Res.* **33**, 468-476.

Baudier, J., and Cole, R. D. (1987). Phosphorylation of Tau protein to a state like that on Alzheimer's brain is catalyzed by a calcium/calmodulin-dependent kinase and modulated by phospholipids. *J. Biol. Chem.* **262**, 17577-17583.

Bennett, M. K., Erondu, N. E., and Kennedy, M. B. (1983) Purification and characterization of a calmodulin-dependent protein kinase that is highly concentrated in brain. *J. Biol. Chem.* **258**, 12735-12744.

Cassel, D., and Glaser, L. (1982). Resistance to phosphatase of thiophosphorylated epidermal growth factor receptor in A431 membranes. *Proc. Natl. Acad. Sci. (USA)* **79**, 2231.

Colbran, R. J., Schworer, C. M., Hashimoto, Y., Fong, Y.-L., Rich, D. P., Smith, M., and Soderling, T. R. (1989). Calcium/calmodulin-dependent protein kinase II. *Biochem J.* **258**, 313-325.

Ek, B., and Heldin, C.-H. (1984). Use of an antiserum against phosphotyrosine for the identification of phosphorylated components in human fibroblasts stimulated by platelet-derived growth factor. *J. Biol. Chem.* **259**, 11145.

Fukanaga, K., Rich, D. P., and Soderling, T. R. (1989). Generation of the Ca^{2+}-independent form of Ca^{2+}/calmodulin-dependent protein kianse II in cerebellar granule cells. *J. Biol. Chem.* **264**, 21830-21836.

Fukunaga, K., Yamamoto, H., Matsui, K., Higashi, K., and Miyamoto, E. (1982). Purification and characterization of a Ca^{2+}- and calmodulin-dependent protein kinase from rat brain. *J. Neurochem.* **39**, 1607-1617.

Glenney, R. J., Jr., Zokas, L., and Kamps, M. P. (1988). Monoclonal antibodies to phosphotyrosine. *J. Immunol. Methods* **109**, 277.

Goldenring, J. R., Gonzalez, B., McGuire, J. S., Jr., and DeLorenzo, R. J. (1983). Purification and characterization of a calmodulin-dependent kinase

from rat brain cytosol able to phosphorylate tubulin and microtubule associated proteins. *J. Biol. Chem.* **258**, 12632-12640.

Gratecos, D., and Fischer, E. H. (1974). Adenosine 5'-O(3'-thiotriphosphate) in the control of phosphorylase activity. *Biochem. Biophys. Res. Commun.* **58**, 960.

Griffith, L. C., and Schulman, H. (1988). The multifunctional Ca^{2+}/calmodulin-dependent protein kinase mediates Ca^{2+}-dependent phosphorylation of tyrosine hydroxylase. *J. Biol. Chem.* **263**, 9542-9549.

Hodges, R. S. and Merrifield, R. B. (1975) Monitoring of solid phase peptide synthesis by an automated spectrophotometric picrate method. *Anal. Biochem.* **65**, 241-272.

Hunter, T., and Cooper, J. A. (1985). Protein tyrosine kinases. *Ann. Rev. Biochem.* **54**, 897-930.

Kent, S. B. H. (1988). Chemical synthesis of peptides and proteins. *Ann Rev. Biochem.* **57**, 957.

Kitas, E. A., Perich, J. W., Tregear, G. W., and Johns, R. B. (1990). *J. Org. Chem.* **55**, 4181-4187.

Köhler, G., and Milstein, C. (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* **256**, 495.

Lacombe, J. M., Andriamanampisoa, F., and Pavia, A. A. (1990). Solid-phase synthesis of peptides containing phosphoserine using phosphate *tert.*-butyl protecting group. *Int. J. Peptide Protein Res.* **36**, 275-280.

Lai, Y., Nairn, A. C., and Greengard, P. (1986). Autophosphorylation reversibly regulates the Ca^{2+}/calmodulin-dependent protein kinase II. *Proc. Natl. Acad. Sci. (USA)* **83**, 4253-4257.

Leammli, U. K., (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Levine, L., Gjika, H., and Van Vunakis, H. (1989). Antibodies and radioimmunoassays for phosphoserine, phosphothreonine, and phosphotyrosine:

serological specificities and levels of the phosphoamino acids in cytoplasmic fractions of rat tissue. *J. Immunol. Methods* **124**, 239-249.

Lindwall, G., and Cole, R. D. (1984). Phosphorylation affects the ability of Tau protein to promote microtubule assembly. *J. Biol. Chem.* **259**, 5301-5305.

Llinas, R., McGuiness, T. L., Leonard, C. S., Sugimori, M., and Greengard, P. (1985). Intraterminal injection of synapsin I or calcium/calmodulin-dependent protein kinase II alters neurotransmitter release at the squid giant synapse. *Proc. Natl. Acad. Sci. (USA)* **82**, 3035-3039.

Malinow, R., Schulman, H., and Tsien, R. W. (1989). Inhibition of postsynaptic PKC or CaMKII blocks induction but not expresseion of LTP. *Science* **245**, 862-866.

Matthew, W. D., and Patterson, P. H. (1983). The production of a monoclonal antibody that blocks the action of a neurite outgrowth-promoting factor. *Cold Spring Harbor Symp. Quant. Biol.* **48**, 625-631.

Matthew, W. D., and Sandrock, Jr., A. W. J. (1987). Cyclophosphamide treatment used to manipulate the immune response for the production of monoclonal antibodies. *J. Immunol. Methods* **100**, 73-82.

Merrifield, B. (1986). Solid phase synthesis. *Science* **232**, 341-347.

Miller, S. G., Patton, B. L., and Kennedy, M. B. (1988). Sequences of autophosphorylation sites in neuronal type II CaM kinase that control Ca^{2+}-independent activity. *Neuron* **1**, 593-604.

Miller, S. G., and Kennedy, M. B. (1985). Distinct forebrain and cerebellar isozymes of type II Ca2+/calmodulin-dependent protein kinase associate differently with the postsynaptic density fraction. *J. Biol. Chem.* **260**, 9039-9046.

Molloy, S. S., and Kennedy, M. B. (1991). Autophosphorylation of type II Ca^{2+}/calmodulin-dependent protein kinase in organotypic cultures of hippocampal slices. *Proc. Natl. Acad. Sci. (USA)* (*in press*).

Moore, H. H., Fritz, L. C., Raftery, M. A., and Brockes, J. P. (1982). Isolation and characterization of a monoclonal antibody against the saxitoxin-binding

component from the electric organ of the eel *Electrophorus electricus. Proc. Natl. Acad. Sci. (USA)* **79**, 1673-1677.

Nichols, R. A., Sihra, T. S., Czernik, A. J., Nairn, A. C., and Greengard, P. (1990). Calcium/calmodulin-dependent protein kinase II increases glutamate and noradrenaline release from synaptosomes. *Nature* **343**, 647-651.

Ohtsuka, M., Ihara, S., Ogawa, R., Watanabe, T., and Watanabe, Y. (1984). Preparation and characterization of antibodies to O-phosphotyrosine and their use for identification of phosphotyrosine-containing proteins. *Int. J. Cancer* **34**, 855.

Patton, B. L., Miller, S. G., and Kennedy, M. B. (1990). Activation of type II calcium/calmodulin-dependent protein kinase by Ca^{2+}/calmodulin is inhibited by autophosphorylation of threonine within the calmodulin binding domain. *J. Biol. Chem.* **265**, 11204-11212.

Patton, B. L., Miller, S. G., and Kennedy, M. B. (1991). Identification of functionally significant phosphorylation sites on neuronal proteins and preparation of antibodies that recognize them. *Methods in Neuroscience.* (*in press*).

Perich, J. W., and Johns, R. B. (1988). The efficient synthesis of a complex *O*-phosphoseryl-containing peptide Ac-Glu-*P*Ser-Leu-*P*Ser-*P*-Ser-*P*Ser-Glu-Glu-NHMe. *J. Chem. Soc., Chem. Commun.*, pp. 664-666.

Peterson, G. L. (1977). A simplification of the protein assay method of Lowry *et al.* which is more generally applicable. *Anal. Biochem.* **83**, 346-356.

Ross, A. H., Baltimore, D., and Eisen, H. N. (1981). Phosphotyrosine-containing proteins isolated by affinity chromatography with antibodies to a synthetic hapten. *Nature* **294**, 654.

Schworer, C. M., Colbran, R. J., and Soderling, T. R. (1986). Substrate specificity of liver calmodulin-dependent glycogen synthase kinase. *J. Biol. Chem.* **261**, 8581-8584.

Schworer, C. M., and Soderling, T. R. (1983). *Biochem. Biophys. Res. Commun.* **116**, 412-416.

Seki, J. Owade, M. K., Sakato, N., and Fujio, H. (1986). Direct identification of phosphotyrosine-containing proteins in some retrovirus-transformed cells by use of anti-phosphotyrosine antibody. *Cancer Res.* **46**, 907.

Soderling, T. R. (1990). Protein kinases: regulation by autoinhibitory domains. *J. Biol. Chem.* **265**, 1823-1826.

Stähli, C., Staehelin, T., Miggiano, V., Schmidt, J., and Häring, P. (1980). High frequencies of antigen-specific hybridomas: dependence on immunization parameters and prediction by spleen cell analysis. *J. Immunol. Methods* **32**, 297.

Titanji, V. P. K., Ragnarsson, U., Humble, E., and Zeterquist, O. (1980). Phosphopeptide substrates of a phosphoprotein phosphatase from rat liver *J. Biol. Chem.* **255**, 11339-11343.

Vulliet, P. R., Woodgett, J. R., and Cohen, P. (1984). Phosphorylation of tyrosine hydroxylase by calmodulin-dependent multiprotein kinase. *J. Biol. Chem.* **259**, 13680-13683.

Watterson, D. M., Harrelson, W. G., Keller, P. M., Sharief, F., and Vanaman, T. C. (1976). Structural similarities between the Ca^{2+}-dependent regulatory proteins of 3':5'-cyclic nucleotide phosphodiesterase and actomyosin ATPase. *J. Biol. Chem.* **251**, 4501-4513.

Waymire, J. C., Johnston, J. P., Hummer-Lickteig, K., Lloyd, A., Vigny, A., and Craviso, G. L. (1988). Phosphorylation of bovine adrenal chromaffin cell tyrosine hydroxylase. *J. Biol. Chem.* **263**, 12439-12447.

Yamamoto, H., Fukunaga, K., Tanaka, E., and Miyamoto, E. (1983). Ca^{2+}- and calmodulin-dependent phosphorylation of microtubule-associated protein 2 and Tau factor, and inhibition of microtubule assembly. *J. Neurochem.* **41**, 1119-1125.

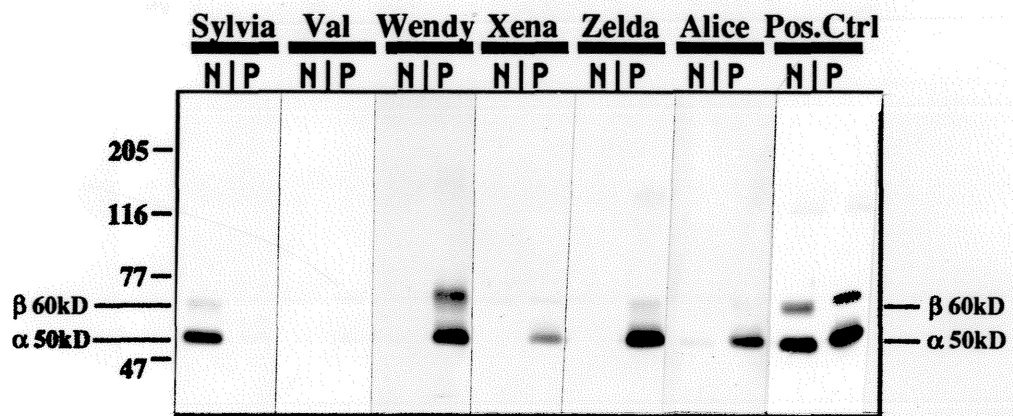
Yamauchi, T., and Fujisawa, H. (1981). Disassembly of microtubules by the action of calmodulin-dependent protein kinase (Kinase II) which occurs only in the brain tissue. *Biochem. Biophys. Res. Commun.* **110**, 287-291.

Yoshitake, S. (1982). Mild and efficient conjugation of rabbit Fab' and horseraddish peroxidase using a maleimide compound and its use for enzyme immunoassay. *J. Biochem.* **92**, 1413-1424.

Figure 1. ***Specificity of Rabbit Antisera for Non-phosphorylated or Autophosphorylated CaM kinase.***

Pure rat forebrain type II CaM kinase was autophosphorylated for two minutes at 30°C in the presence of [γ-^{32}P]ATP, Ca^{2+}, and calmodulin; nonphosphorylated CaM kinase was incubated under identical conditions in the absence of ATP. Incubations were terminated, aliquots containing 0.2 µg of nonphosphorylated (**N**) and autophosphorylated (**P**) kinase were loaded into alternate lanes and the component α (2.7 pmol) and β (0.9 pmol) subunits separated by SDS-PAGE. Protein was transferred to nitrocellulose membrane for two hours, 15 minutes at 350 mA, and nonspecific binding blocked as described in Experimental Procedures. Positions of lanes on the blots were identified by autoradiography and the blots cut into strips containing a pair of lanes (**N** plus **P**). Strips were incubated for 12 hours at 4°C in 4 ml of antisera (**Sylvia, Valerie, Wendy, Xena, Zelda,** or **Alice**) diluted 250-fold in TBS-Tween containing 2% (v/v) normal goat serum (NGS). Antiserum samples were from rabbits immunized as described in Experimental Procedures, as follows: Sylvia, six boosts with nonphosphorylated α-14mer(281); Valerie, Wendy, and Xena, four boosts each with thiophosphorylated α-14mer(281); Zelda and Alice, four boosts each with thiophosphorylated α-9mer(281). Positive control incubations (**Pos.Ctrl**) were performed with a combination of Annette polyclonal antibodies, diluted 500-fold, and the monoclonal antibody 6G9, diluted 2000-fold. Bound antibodies detected with alkaline phosphatase conjugated goat secondary antibodies against rabbit and mouse antibodies. Bound secondary antibodies were

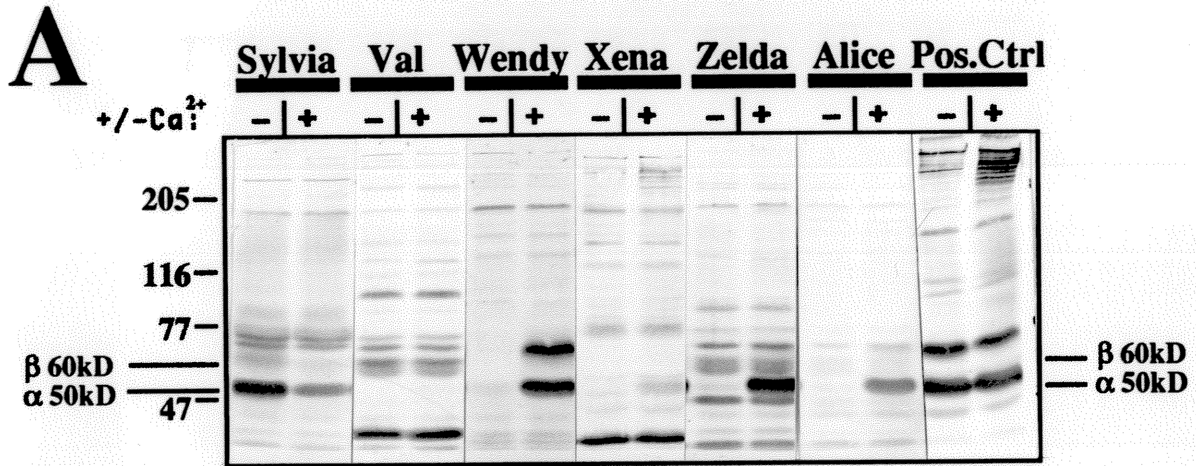
visualized with BCIP and NBT alkaline phosphatase substrates. The positions of protein standards are indicated at the margin by their molecular weight in kDal. The position and apparent molecular weight of the kinase α and β subunits on SDS-polyacrylamide gels is indicated.



Sylvia
Val
Wendy
Xena
Zelda
Alice
Pos.Ctrl
N P
205
116
77
β 60kD
α 50kD
47
β 60kD
α 50kD

Figure 2. ***Specificity of Rabbit Antisera for CaM Kinase in Brain Homogenate.***

Rat brain homogenate was phosphorylated by endogenous kinase activity in the presence of 0.2 mM ATP, and in the presence (+) or absence (-) of added Ca^{2+} and calmodulin, as described in Experimental Procedures. Aliquots of homogenate protein (20 μg) phosphorylated in the presence or absence of Ca^{2+}/calmodulin were loaded into alternate lanes, separated by SDS-PAGE, and electrophoretically blotted onto nitrocellulose membranes, as described in Experimental Procedures. After blocking, blots were cut into strips containing a pair of lanes (+ and -), and incubated with rabbit polyclonal antisera (**A**), or preimmune serum (**B**), as described in the legend to Figure 1. Bound polyclonal antibodies, and the control anti-CaM kinase antibodies Annette and 6G9 (**Pos.Ctrl**), were detected with alkaline phosphatase conjugated secondary antibodies, as decribed in the legend to Figure 1. The positions of the CaM kinase subunits are indicated at the margins (α, β).


A
Sylvia
Val
Wendy
Xena
Zelda
Alice
Pos.Ctrl
+/-Ca2+:
205
116
77
47
β 60kD
α 50kD

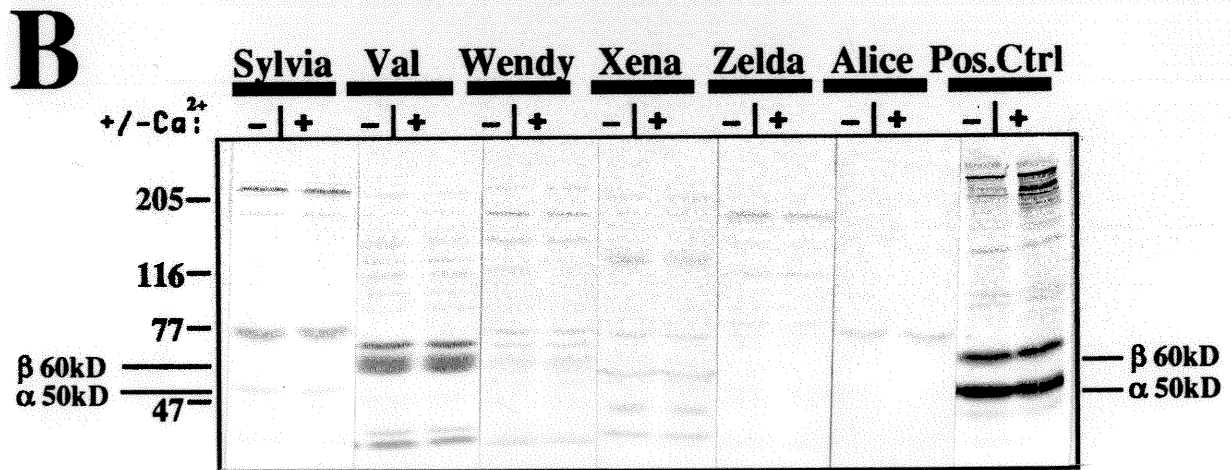

B
Sylvia
Val
Wendy
Xena
Zelda
Alice
Pos.Ctrl
+/-Ca2+:
205
116
77
47
β 60kD
α 50kD

Figure 3. ***Specificity of Affinity Purified Sylvia Antibodies for Nonphosphorylated CaM Kinase.***

Rat brain homogenate was phosphorylated in the absence (N) or presence (P) of Ca^{2+}/calmodulin, as described in Experimental Procedures. Pure CaM kinase forebrain isozyme was autophosphorylated in the presence of Ca^{2+}, calmodulin, and ATP, as described in Experimental Procedures. Nonphosphorylated kinase was incubated in the absence of ATP in an otherwise identical fashion. Aliquots of nonphosphorylated (N) and phosphorylated (P) proteins were separated by SDS-PAGE in alternate lanes. As indicated at the lower margin, homogenate protein (30 µg) was separated in the first six lanes, and CaM kinase (3 µg) was separated in the last two lanes. Proteins were blotted onto nitrocellulose, and the membranes cut into appropriate sections. Blotted protein was incubated with either control anti-kinase antibodies (a combination of Annette and 6G9), Sylvia antisera (taken after 6 boost injections), or Syvia antibodies affinity purified by chromatography on a resin coupled with α-9mer(281) peptide, as described in Experimental Procedures. Annette antiserum was used at 500-fold dilution in 2% NGS, TBS-Tween. Monoclonal antibody 6G9 from partially purified ascites was diluted 2000-fold. Sylvia antiserum partially purified by ammonium sulfate precipitation (50 mg/ml) was diluted 100-fold. Affinity purified antibodies were diluted to 1.7 µg/ml. The positions of molecular weight standards and the CaM kinase subunits (α and β) are indicated at the margins.

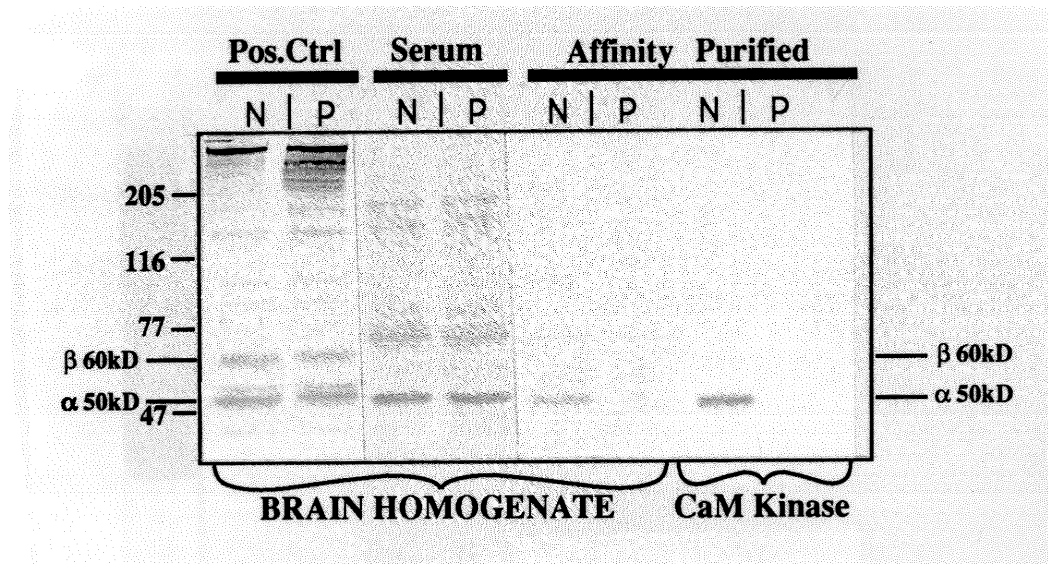

Pos.Ctrl
Serum
Affinity Purified
N P N P N P N P
205
116
77
β 60kD
α 50kD
47
β 60kD
α 50kD
BRAIN HOMOGENATE
CaM Kinase

Figure 4. ***Decrease in Binding of Affinity Purified Sylvia Antibodies to CaM kinase Versus Time Course of Kinase Autophosphorylation.***

CaM kinase forebrain isozyme was autophosphorylated in the presence of ATP, Ca^{2+}, and calmodulin at 30°C for five seconds to 300 seconds. Nonphosphorylated kinase samples were incubated for five seconds at 30°C in the absence of ATP. Aliquots from each reaction time point containing 0.2 µg kinase were separated by SDS-PAGE and transferred to nitrocellulose membranes, as described in Experimental Procedures. The first and last lanes, containing nonphosphorylated kinase and maximally autophosphorylated kinase, respectively, were cut from the rest of the blot and incubated with control anti-kinase antibodies: Annette polyclonal antiserum (1000-fold dilution), and 6G9 (1000-fold dilution). The rest of the membrane was incubated with affinity purified polyclonal antibodies from Sylvia (1.9 µg/ml). Antibodies were diluted in TBS-Tween containing 2% (v/v) NGS, and bound antibodies were detected with alkaline phosphatase-conjugated secondary antibodies, as described in the legend to Figure 1. The positions of the kinase subunits are indicated at the margins. In this experiment, CaM kinase α subunit was autophosphorylated to stoichiometries of 1.01, 1.11, 1.54, 2.11, and 2.38 mol phosphate per mol subunit after 5, 20, 60, 180, and 300 seconds, respectively.

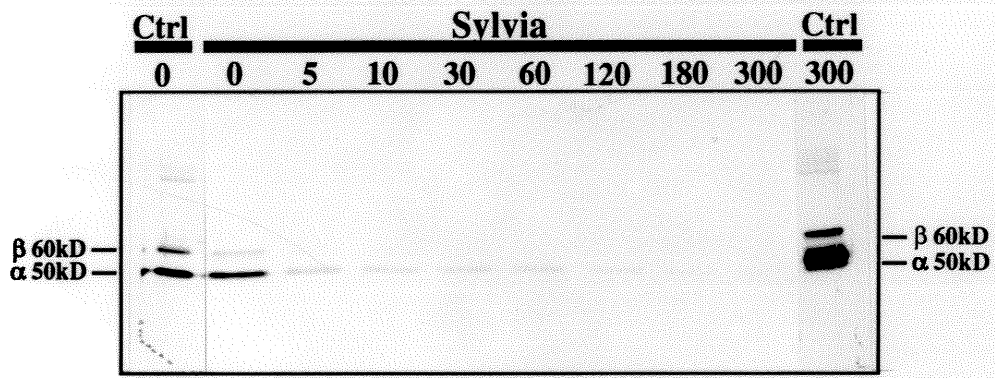
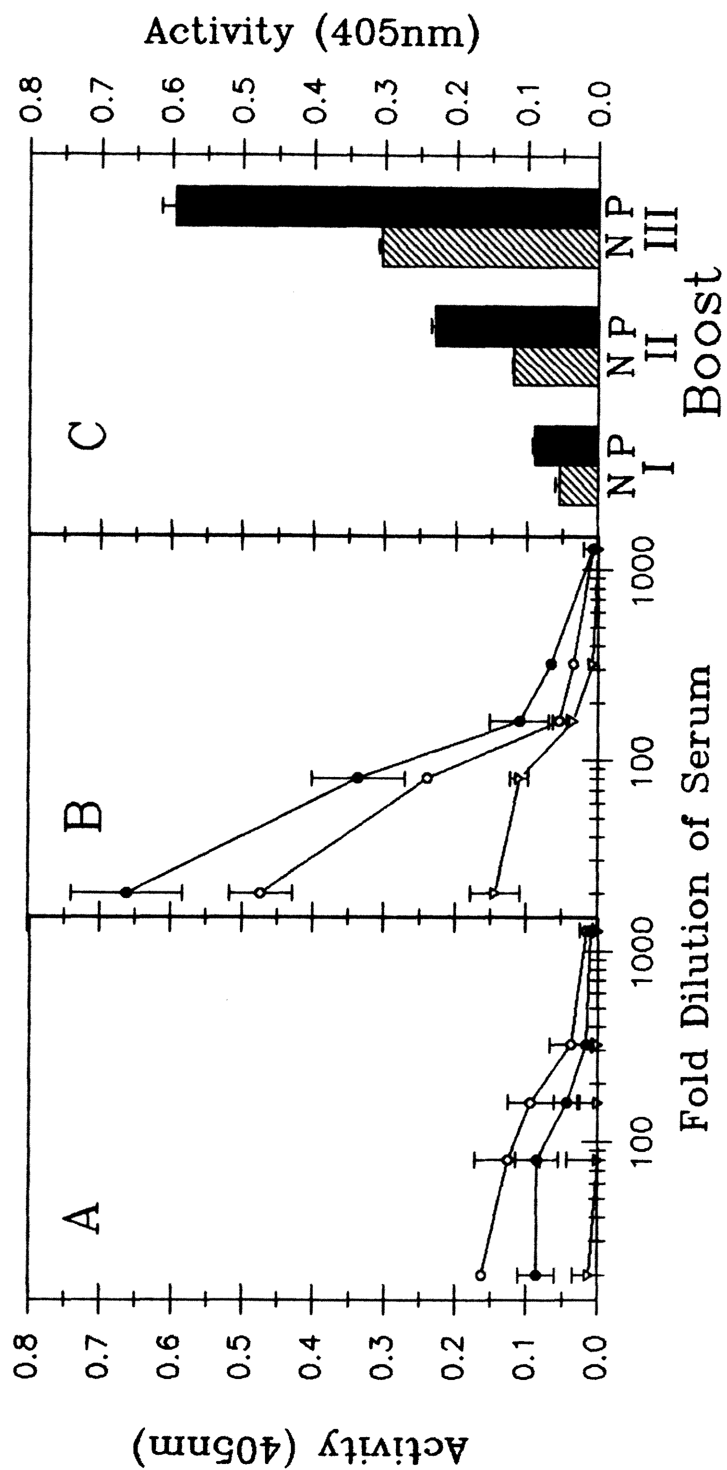

Ctrl
Sylvia
Ctrl
0
0
5
10
30
60
120
180
300
300
β 60kD
α 50kD
β 60kD
α 50kD

Figure 5. ***Anti-phosphokinase Immune Response in Mice Immunized with Thiophosphorylated Peptide.***

Three mice were immunized at four-week intervals with KLH-conjugated thiophosphorylated α-14mer(281), as described in Experimental Procedures. Serum taken seven days after the first boost (**A**) and second boost (**B**) injections was diluted in TBS-Tween containing 2% (v/v) NGS, and assayed by ELISA for antibodies specifically binding to autophosphorylated CaM kinase. (OD), mouse immunized with 75 μg conjugate; (FC), mouse immunized with 100 μg conjugate; (OC), mouse immunized with 125 μg conjugate. Binding with nonphosphorylated kinase as ELISA substrate has been subtracted from that measured with autophosphorylated kinase as ELISA substrate. Kinase antigen preparation and ELISA methods are described in Experimental Procedures.

(**C**) Binding of serum antibodies from the mouse immunized with 100 μg KLH-conjugated peptide to nonphosphorylated (hatched bars) and autophosphorylated (solid bars) CaM kinase, measured by ELISA. Serum was taken seven days after each boost and diluted 320-fold prior to assay. Binding of naive mouse serum to kinase substrate, at equivalent dilution, was taken as background activity and was subtracted.



Activity (405nm)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
A
B
C
100
1000
Fold Dilution of Serum
N P
I
II
III
Boost

Figure 6. ***Phosphokinase/Nonphosphokinase Specificity of Hybridoma Culture Supernatants Derived from Thiophosphopeptide Immunization.*** Hybridomas were produced from a mouse immunized with 100 µg of thiophosphorylated α-14mer(281) and grown in culture, as described in Experimental Procedures. The supernatant from each culture was tested by ELISA for antibodies that bound to nonphosphorylated CaM kinase (ordinate) or autophosphorylated CaM kinase (abscissa). Each hybridoma supernatant is represented once (circles). Antibodies that do not bind to the CaM kinase in ELISA plot near the origin. Antibodies binding equally well to both nonphosphorylated and autophosphorylated kinase plot on the diagonal line. A total of 1248 hybridoma supernatants were tested. Activities of supernatants were normalized to the activity of a common control antibody present in every assay.

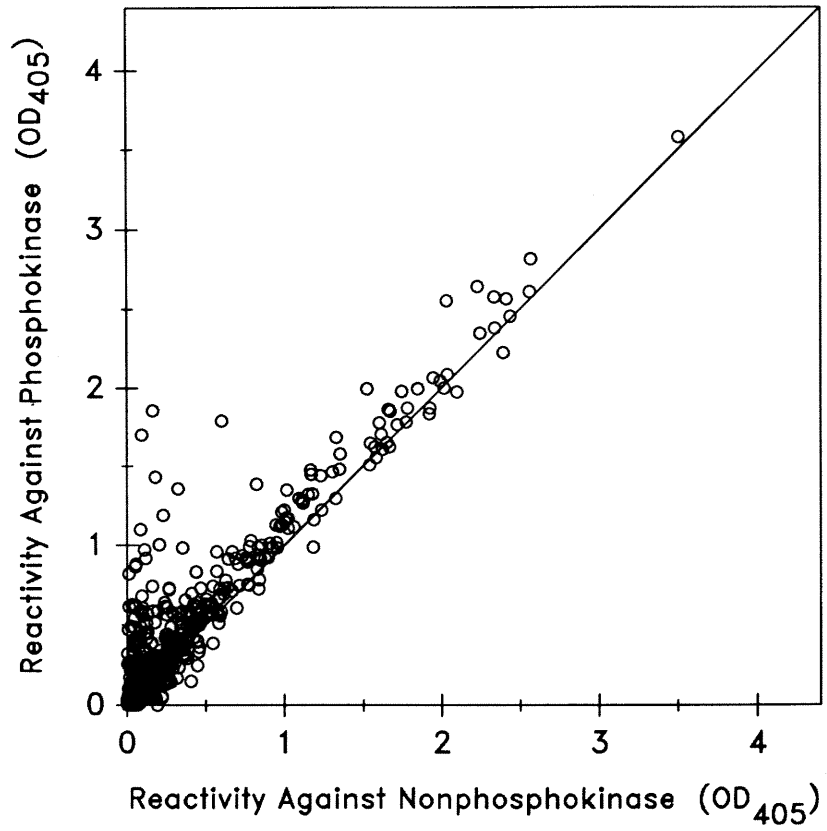
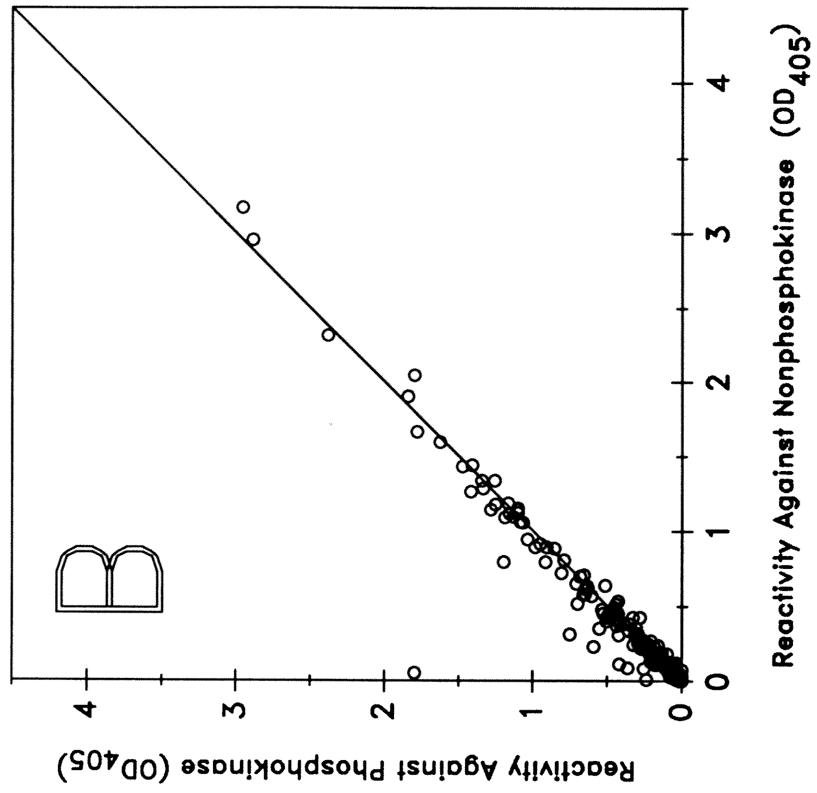

Reactivity Against Phosphokinase (OD_{405})
Reactivity Against Nonphosphokinase (OD_{405})
0
1
2
3
4
0
1
2
3
4

Figure 7. ***Phosphokinase/Nonphosphokinase Specificity of Hybridoma Culture Supernatants Derived from Thiophosphorylated Kinase Holoenzyme Immunization.***

Hybridoma were produced from mice immunized with thiophosphorylated CaM kinase holoenzyme, by the immunosuppression/immunization methods described in Experimental Procedures. The culture supernatants were tested by ELISA for antibodies that bound to nonphosphorylated CaM kinase (ordinate) or autophosphorylated CaM kinase (abscissa). Each hybridoma supernatant is represented once (circles). Antibodies that do not bind to the CaM kinase in ELISA plot near the origin. Antibodies binding equally well to both nonphosphorylated and autophosphorylated kinase plot on the diagonal line. (**A**) Hybridomas derived from a mouse immunized by three cycles of immunosuppression/immunization. A total of 803 supernatants were tested. (**B**) Hybridoma derived from a mouse immunized by two cycles of immunosuppression/immunization. A total of 711 supernatants were tested. Activities of supernatants were normalized to the activity of a common control antibody present in every assay. The control antibody was not the same for (**A**) and (**B**). Thus, the distributions of activities along the diagonals cannot be directly compared. Control antibody for (**A**) was the same as that used in Figure 5.


B
Reactivity Against Phosphokinase (OD405)
Reactivity Against Nonphosphokinase (OD405)
0
1
2
3
4

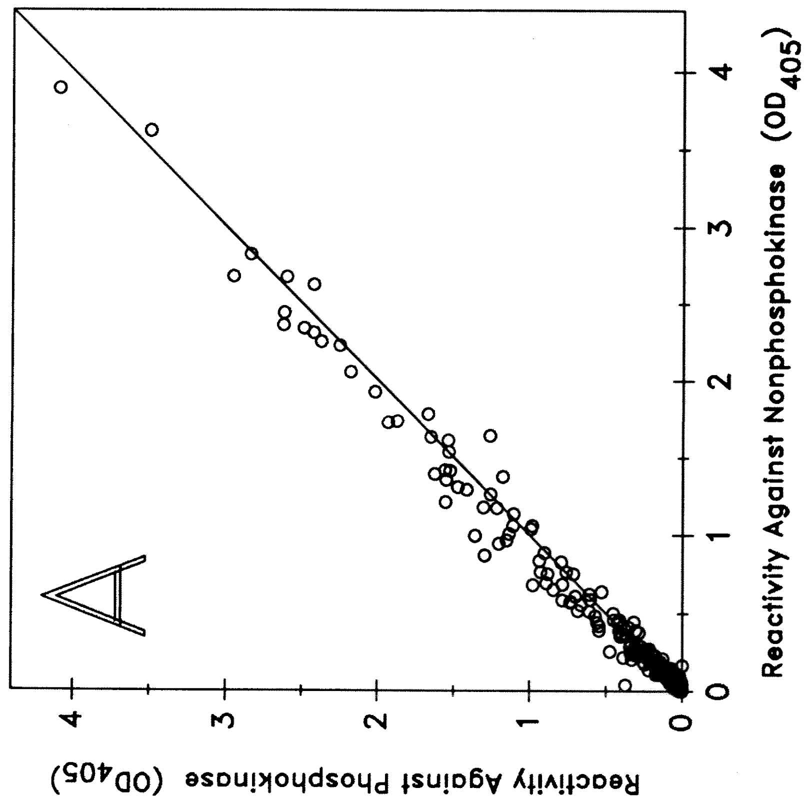

A
Reactivity Against Phosphokinase (OD405)
Reactivity Against Nonphosphokinase (OD405)
0
1
2
3
4

Figure 8. ***Specificity of Monoclonal Antibodies for Autophosphorylated CaM Kinase.***

Pure rat forebrain type II CaM kinase was autophosphorylated for two minutes at 30°C in the presence of [γ-^{32}P]ATP, Ca^{2+}, and calmodulin; nonphosphorylated CaM kinase was incubated under identical conditions in the absence of ATP. Incubations were terminated and 0.2 μg aliquots of nonphosphorylated (**N**) and autophosphorylated (**P**) kinase were separated into their component α (2.7 pmol) and β (0.9 pmol) subunits by SDS-PAGE, as described in the legend to Figure 1. Protein was transferred to nitrocellulose membrane for two hours, 15 minutes, and the resulting blots cut into strips containing nonphosphorylated or autophosphorylated CaM kinase. A pair of strips (**N** plus **P**), were incubated for 12 hours at 4°C in TBS-Tween containing monoclonal antibody and 2% (v/v) normal goat serum (NGS). The monoclonal antibody 6G9 was used as positive control at a 2000-fold dilution. 5B3 was diluted 200-fold. 26G6, 27G10, and 22B1 were diluted 2000-fold. Monoclonal antibodies were prepared from ammonium sulfate-fractionated ascitic fluid, as described. Bound antibodies were detected with alkaline phosphatase conjugated goat secondary antibodies, and visualized with BCIP and NBT alkaline phosphatase substrates. The positions of kinase α and β subunits are indicated at the margin.

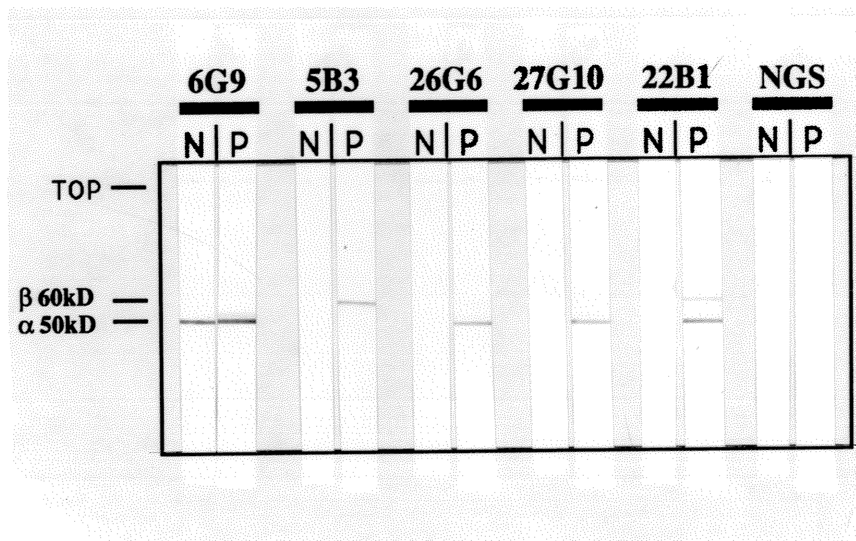

6G9
5B3
26G6
27G10
22B1
NGS
N P
N P
N P
N P
N P
N P
TOP
β 60kD
α 50kD

Figure 9. ***Specificity of Anti-Phosphokinase Monoclonal Antibodies for CaM Kinase in Brain Homogenates.***

Rat brain homogenate proteins were phosphorylated by endogenous kinases in the presence of ATP, Ca^{2+}, and calmodulin, as described in Experimental Procedures. Aliquots containing 20 μg protein were separated by SDS-PAGE and transferred to nitrocellulose membranes. Immunoblotting was performed as described in the legend to Figure 8, with the following changes. 5B3 was used at 40-fold dilution, 26G6 and 27G10 at 50-fold dilution, and 22B1 at 500-fold dilution. In an attempt to reduce nonspecific binding to brain proteins, one strip from each antibody incubation was washed in TBS-Tween for three hours with three changes of buffer, while the other strip was washed for 12 hours, with five changes of buffer. The positions of the CaM kinase α and β subunits are indicated.

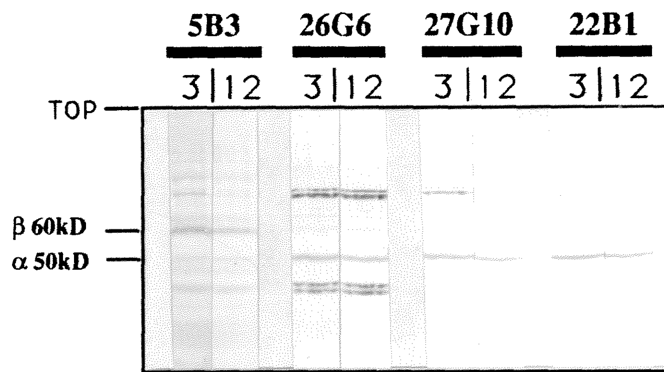
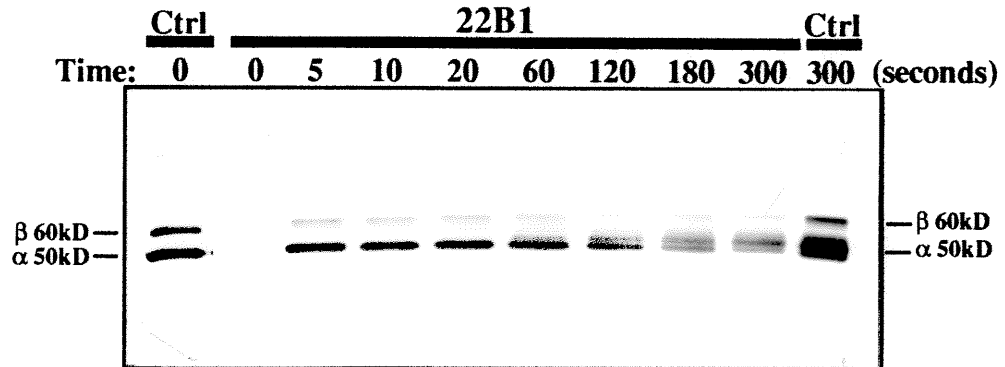

5B3
26G6
27G10
22B1
3 | 12
3 | 12
3 | 12
3 | 12
TOP
β 60kD
α 50kD

Figure 10. ***Increase in Binding of Monoclonal Antibody 22B1 to CaM Kinase Versus the Time Course of Autophosphorylation.***

(**A**) CaM kinase forebrain isozyme was autophosphorylated in the presence of [γ-^{32}P]ATP, Ca^{2+}, and calmodulin for five seconds to 300 seconds. Nonphosphorylated kinase samples were incubated for five seconds at 30°C in the absence of ATP. Aliquots from each reaction time point containing 0.2 µg kinase (2.7 pmol α subunit) were separated by SDS-PAGE and transferred to nitrocellulose membranes, as described in Experimental Procedures. The first and last lanes, containing nonphosphorylated kinase and maximally autophosphorylated kinase, respectively, were cut from the rest of the blot and incubated with the following control anti-kinase antibodies diluted in TBS-Tween containing 2% (v/v) NGS: Annette polyclonal antiserum (1000-fold dilution), and 6G9 (1000-fold dilution). The rest of the membrane was incubated with the monoclonal antibody 22B1, diluted 1000-fold. Bound antibodies were detected with alkaline phosphatase conjugated secondary antibodies, as described in the legend to Figure 1. The positions of the kinase subunits are indicated at the margins. In this experiment, as in figure 4, CaM kinase α subunit was autophosphorylated to stoichiometries of 1.01, 1.11, 1.54, 2.11, and 2.38 mol phosphate per mol subunit after 5, 20, 60, 180, and 300 seconds, respectively.

(**B**) Autoradiogram of the immunoblot shown in (**A**).


Ctrl
22B1
Ctrl
Time: 0 0 5 10 20 60 120 180 300 300 (seconds)
β 60kD
α 50kD
β 60kD
α 50kD

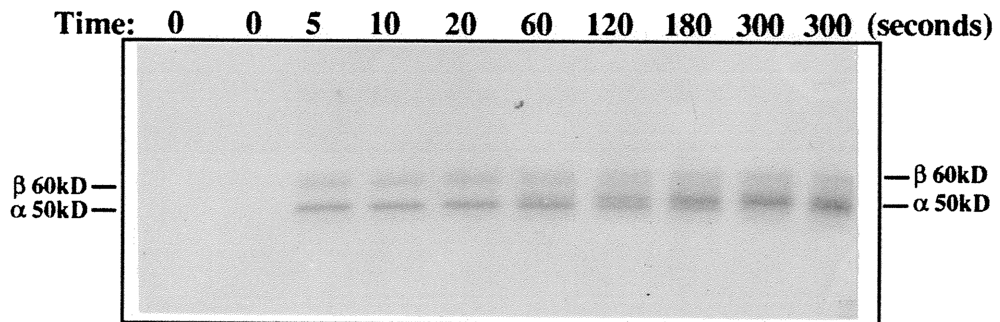
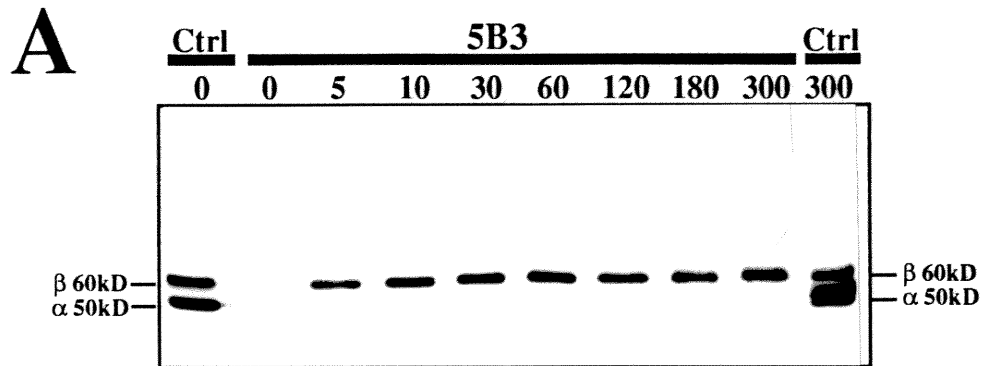
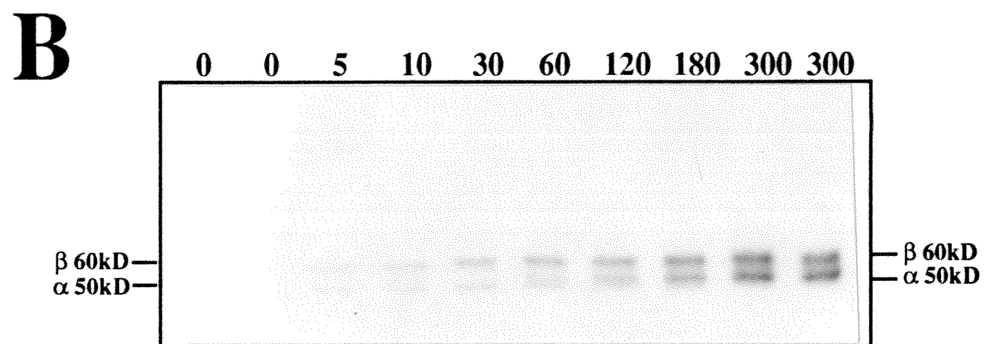

Time: 0 0 5 10 20 60 120 180 300 300 (seconds)
β 60kD
α 50kD
β 60kD
α 50kD

Figure 11. ***Increase in Binding of Monoclonal Antibody 5B3 to CaM Kinase Versus the Time Course of Autophosphorylation.***

CaM kinase pons/medullary isozyme, having an equal ratio of α and β subunits, was autophosphorylated in the presence of [γ-^{32}P]ATP, Ca^{2+}, and calmodulin for five seconds to 300 seconds. Nonphosphorylated kinase samples were incubated for five seconds at 30°C in the absence of ATP. Aliquots from each reaction time point containing 0.2 µg kinase (1.8 pmol β subunit) were separated by SDS-PAGE and transferred to nitrocellulose membranes for 5 hours, as described in Experimental Procedures. The first and last lanes, containing nonphosphorylated kinase and maximally autophosphorylated kinase, respectively, were incubated with control monoclonal antibody 6G9 and Annette polyclonal antiserum, as described in Figure 10. The rest of the membrane was incubated with the monoclonal antibody 5B3, diluted 200-fold. Bound antibodies were detected with alkaline phosphatase-conjugated secondary antibodies, as described in the legend to Figure 1. The alkaline phosphatase reaction was over-developed, emphasizing the specificity for the β subunit in its autophosphorylated form. In this experiment, CaM kinase β subunit was autophosphorylated to stoichiometries of 1.67, 2.2, 2.9, 4.0, and 4.2 mol phosphate per mol subunit after 5, 20, 60, 180, and 300 seconds, respectively.

(B) Autoradiogram of the immunoblot shown in (**A**).


A
Ctrl
5B3
Ctrl
0
0
5
10
30
60
120
180
300
300
β 60kD
α 50kD
β 60kD
α 50kD


B
0
0
5
10
30
60
120
180
300
300
β 60kD
α 50kD
β 60kD
α 50kD

CHAPTER 5

IMMUNOCYTOCHEMICAL DETECTION OF PHOSPHORYLATED AND NONPHOSPHORYLATED FORMS OF THE TYPE II Ca^{2+}/CALMODULIN-DEPENDENT PROTEIN KINASE.

SUMMARY

Autophosphorylation of the type II Ca^{2+}/calmodulin-dependent protein kinase (CaM kinase) on residue Thr^{286} produces Ca^{2+}-independent kinase activity *in vitro*; phosphorylation of Thr^{286} regulates the basal level of kinase activity in organotypically cultured neurons (Molloy and Kennedy, 1991). Recently, we developed antibodies specific for the autophosphorylated and nonphosphorylated forms of the CaM kinase (Chapter 4). In this study, we tested their ability to react with CaM kinase in fixed, plastic-embedded hippocampal slice culture tissue. The results indicate that the CaM kinase is present in both autophosphorylated and nonphosphorylated forms throughout cultured hippocampal slices, in all cell types and synaptic regions. Double immunofluorescence labeling with antibodies specific for nonphosphorylated and phosphorylated kinase revealed considerable variability in the level of phosphorylation of the kinase among cells and dendritic processes. Pre-incubating the cultured hippocampal slice in Ca^{2+}-free medium to inhibit Ca^{2+}-dependent autophosphorylation, decreased the level of phosphokinase-specific immunofluorescence uniformly throughout the slice. These results are consistent with biochemical assays of the level of CaM kinase autophosphorylation in cultured hippocampal slices, which suggested that the CaM kinase phosphorylation state is at dynamic equilibrium in most neurons.

INTRODUCTION

The type II Ca^{2+}/calmodulin-dependent protein kinase is regulated by Ca^{2+}/calmodulin and by autophosphorylation. The enzymatic activity of the nonphosphorylated kinase is completely dependent on Ca^{2+}/calmodulin. Autophosphorylation of $Thr^{286/287}$ relieves the dependence on Ca^{2+}/calmodulin, maintaining the kinase in a partially active state in the absence of Ca^{2+}/calmodulin. This Ca^{2+}-independent kinase activity may be returned to complete Ca^{2+}/calmodulin dependence by protein phosphatase-catalyzed dephosphorylation of $Thr^{286/287}$. In the Ca^{2+}-independent state, the kinase is capable of continued autophosphorylation, as well as phosphorylation of substrate proteins. Autophosphorylation in the absence of Ca^{2+} includes additional sites located within the calmodulin-binding domain that block the ability of Ca^{2+}/calmodulin to activate the kinase until these sites are later dephosphorylated.

Recent studies with cultured neurons have demonstrated that the CaM kinase autophosphorylates at $Thr^{286/287}$ in living cells, maintaining a basal level of Ca^{2+}-independent CaM kinase activity. In hippocampal slice cultures, this level of Ca^{2+}-independent activity is apparently at dynamic equilibrium and can be artificially raised or lowered by experimental methods that raise or lower the level of autophosphorylation at $Thr^{286/287}$. For example, removal of Ca^{2+} from the culture medium with EGTA for half an hour results in the dephos-

phorylation of CaM kinase alpha subunit at Thr^{286} (and presumably Thr^{287} on the beta subunit), and a concomitant reduction in Ca^{2+}-independent CaM kinase activity. Application of okadaic acid, a potent inhibitor of two major protein phosphatase activities known to dephosphorylate the CaM kinase at $Thr^{286/287}$, raised the level of Ca^{2+}-independent activity in the hippocampal cultures. However, it is not possible to determine by biochemical assay of entire hippocampal slices whether the changes in autophosphorylation at $Thr^{286/287}$, and the attendant changes in basal CaM kinase activity, reflect changes in the total pool of kinase throughout the tissue sample or whether the changes are localized to discrete areas of tissue, possibly particular cell types or specific synaptic regions or pathways. In addition, although physiological agents such as neurotransmitters and inhibitors of neuronal activity had no detectable effect on the measured level of basal Ca^{2+}-independent activity in the hippocampal slices, the high basal CaM kinase activity may obscure changes elicited by physiological agents at subsets of synaptic sites.

We were interested in observing what differences in the phosphorylation state of the type II CaM kinase, if any, might exist at the cellular and subcellular level. In addition, we were interested in observing changes in the CaM kinase phosphorylation state elicited by various experimental treatments. We reasoned that maximal sensitivity would be obtained by double immunofluorescence staining of tissue sections with antibodies specific for the autophos-

phorylated kinase and nonphosphorylated kinase. Ideally, a change in the phosphorylation state of the CaM kinase would then be observed as a decrease in fluorescence intensity due to decreased binding of one antibody, combined with an increase in fluorescence intensity due to the increased binding of the other antibody. In Chapter 4, we described antibodies that specifically bind to autophosphorylated and nonphosphorylated CaM kinase. A mouse monoclonal antibody, 22B1, was found to specifically bind to the type II CaM kinase autophosphorylated at Thr^{286}. Rabbit polyclonal antibodies (Sylvia) were developed that specifically bind to the CaM kinase in its nonphosphorylated form; autophosphorylation of Thr^{286} blocked binding of the Sylvia antibodies to the CaM kinase. The species difference in antibody source provided a convenient method of distinguishing between anti-phosphokinase antibodies and anti-nonphosphokinase antibodies in double-stained sections, through the use of species specific anti-IgG secondary antibodies conjugated with either fluorescein (anti-mouse IgG) or rhodamine (anti-rabbit). Long-term hippocampal slice cultures were used as the source of neuronal tissue. The slice cultures offer a number of advantages, including the varied but identified cell types, an organotypic organization of neurons containing active synaptic connections, the ease of pharmacological treatments in culture, and a wealth of biochemical data regarding CaM kinase autophosphorylation in this preparation (Molloy and Kennedy, 1991).

In this chapter the phosphokinase- and nonphosphokinase-specific antibodies have been used to observe the distribution of phosphorylated and nonphosphorylated CaM kinase in the cultured hippocampal slices . Double-immunofluorescence was used to detect variability in the level of CaM kinase phosphorylation. In addition, we have pre-incubated the hippocampal cultures in Ca^{2+}-free medium to experimentally change the phosphorylation state of the kinase before immunocytochemistry with phosphokinase-specific and nonphosphokinase-specific antibodies (Molloy and Kennedy, 1991).

Experimental Procedures

Materials.

Nonphosphokinase-specific polyclonal antibodies were obtained from a rabbit, Sylvia, immunized with a 14 amino acid residue peptide corresponding in sequence to the Thr^{286} autophosphoryation site on the rat CaM kinase alpha subunit (residues 281-295; Met-His-Arg-Gln-Glu-Thr-Val-Asp-Cys-Leu-Lys-Lys-Phe-Asn). The polyclonal antibodies were affinity purified on a peptide-coupled resin, as described previously (Chapter 4). Rabbit antibody concentrations were estimated by absorbance at 280 nm, assuming an extinction coefficient of 15 for a 1% solution. The monoclonal antibody 22B1, produced by immunization of a BALB/c ByJ mouse with the thiophosphorylated form of the 14-residue peptide described above, has been previously described (Chapter 4). The monoclonal antibody 6G9, generated by immunization of a BALB/c ByJ mouse with CaM kinase partially purified from rat brain, has been previously described (Erondu and Kennedy, 1985). Both monoclonal antibodies were partially purified from mouse ascites fluid by ammonium sulfate precipitation. The final protein concentration was approximately 20 mg/ml and 30 mg/ml for 6G9 and 22B1, respectively. Goat anti-rabbit IgG antibodies conjugated with fluorescein-5-isothiocyanate (molar ratio of fluor to protein, 6.06), and goat anti-mouse IgG antibodies conjugated with tetramethyl rhodamine isothio-cyanate (ratio of A_{350} to A_{280}, 0.267), were purchased from Cappel through Organon Teknika (Durham, NC). Purified rabbit IgG was obtained from

Jackson ImmunoResearch. Normal goat serum was from Gibco. Tween 20 was purchased from BioRad. Glutaraldehyde and osmium tetroxide were purchased from Polysciences (Warrington, PA) as 8% (w/v) and 4% (w/v) aqueous solutions, respectively. Dodecenyl succinic anhydride (DDSA), EM bed-812, nadic methyl anhydride (NMA), 2,4,6,-tri(dimethylaminomethyl)phenol (DMP-30), and paraformaldehyde were purchased from Electron Microscopy Sciences (Fort Washington, PA). Triton X-100 was purchased as a 10% aqueous solution from Pierce (Rockford, IL) and stored under nitrogen. Cacodylic acid, 2-amino-5-phosphono-pentanoic acid (AP-5), and ρ-phenyldiamine (PPDA) were from Sigma.

Methods.

Long-term cultures of rat hippocampal slices were established and maintained as previously described (Gahwiler, 1984). For immunohistochemistry, slices were either directly transferred from culture medium to an ice-cold fixative solution (Control), or were first pre-incubated for 30 minutes in Ca^{2+}-free Ringer's solution for 30 minutes at 37°C. Ca^{2+}-free Ringer's solution contained 25 mM HEPES (pH 7.4), 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 125 mM NaCl, 2 mM KCl, 2 mM $MgSO_4$, 10 mM Glucose, and 0.20 mM EGTA.

Hippocampal slice cultures (three to four weeks *in vitro*) were fixed while still attatched to the coverslip on which they were cultured, with 4% (w/v) paraformaldehyde and 0.2% (w/v) glutaraldehyde in 0.1 M cacodylate (pH

7.2), containing 15 mM $MgCl_2$ and 100 µM AP-5 for one hour at 4°C. Fixed cultures were then washed twice for five minutes each with 0.1 M cacodylate (pH 7.2) and post-fixed in 0.04% osmium tetroxide for one hour at 0°C. After rinsing two times for 5 min with 0.1 M sodium phosphate (pH 7.4), the cultures were dehydrated in successive steps with ethanol: two incubations for 5 min each in 70%, 95%, and 100% ethanol. Dehydrated cultures were impregnated with EM bed-812 plastic resin without DMP-30 activator by incubation for three hours at 0°C with two changes of resin. Cultures were scraped off of the cover slips after the first of these incubations. Resin contained 43.2% (v/v) EM bed-812 resin, 41.2% (v/v) DDSA, and 15.5% (v/v) NMA. Cultures were brought to room temperature, added to resin containing 1.5% (v/v) DMP-30 activator and incubated overnight. Two final incubations of 90 minutes followed, one at room temperature and the last at 45°C. Each culture was mounted individually, in resin, onto chucks of the pre-hardened resin, and then flattened against a glass slide with the aid of a small spring-loaded press. While pressed, the resin was hardened by overnight incubation at 65°C. The blocks were cooled slowly to room temperature. Individual blocks were trimmed to expose the face of the slice culture and positively identified by nicking the block face in a distinguishing fashion. The plastic embedded cultures were then sectioned at 2 µM with a glass knife on a Reichardt ultramicrotome and mounted on subbed slides for staining. Since sections from different blocks could be identified by the nicks in their edges, sections from different blocks could be mounted on the same slide, and their positions

recorded. By referral to this record, individual sections could be positively identified after etching away the plastic for staining. In this way, several hippocampal cultures, pre-treated in different ways, could be stained together.

Prior to staining, plastic-embedded sections were etched for four minutes at room temperature with 13.3% (w/v) potassium hydroxide in 33% (v/v) propylene oxide, 67% (v/v) absolute methanol. Reactive aldehyde groups were blocked with 0.1 M glycine (pH 7.2) for 30 minutes. Sections were rinsed briefly with water and reduced further by incubation in 1% (w/v) sodium borohydride for 10 minutes. After brief rinses with water and 20 mM sodium phosphate (pH 7.2), 0.45 M NaCl, 0.1% (v/v) Triton X-100 (PBS-Triton buffer), the sections were blocked for 45 minutes with 5% (v/v) normal goat serum (NGS) in PBS-Triton buffer. Sections were incubated with primary antibody diluted in PBS-Triton buffer containing 5% (v/v) NGS for 12 to 18 hours at 4°C. Antibodies 6G9 and 22B1 were used at final concentrations of 0.08 mg/ml and 0.014 mg/ml, respectively. Affinity purified polyclonal antibodies from Sylvia were used at a final concentration of 0.06 mg/ml. After primary antibody incubation, sections were washed three times for 10 minutes each with PBS-Triton buffer containing 5% (v/v) NGS. Sections were then incubated with fluorescein- or rhodamine-conjugated secondary antibodies against rabbit or mouse IgG, respectively. Secondary antibodies were diluted 100-fold in PBS-Triton buffer containing 5% (v/v) NGS, and incubations were carried out at room temperature for an hour. After washing three times for 20 minutes each

time with PBS-Triton, sections were rinsed with 5 mM sodium phosphate (pH 7.4) and cover slipped in mounting solution containing 10 mg/ml ρ-phenyl-diamine.

Immunofluorescently-stained sections were viewed by reflected fluorescent light using barrier filters for either rhodamine (BP545) or fluorescein (BP490 and EY455), and photographed with Kodak P800/1600 color reversal, push-process film. In order to compare the relative staining intensity among several sections on a slide (stained together, as described above), and among different areas within one section, all sections on the slide were photographed using identical exposure times. An appropriate exposure time was initially determined by the camara light meter while viewing the dentate gyrus of a single section from a control (untreated) culture. Double-stained immunofluorescence images were obtained by double exposure with alternate filter sets. In the experiments reported in this study, antibodies specific for nonphosphorylated kinase and phosphorylated kinase stained with equal intensities in double-stained control (untreated) cultures. Therefore, double-immunofluorescence photomicrographs were recorded using identical exposure times with each filter set. Identical exposure times (for each of the two filter sets) were then used to photograph every tissue section in the experiment, irrespective of fluorescence intensity. In this way, *relative* levels of phosphokinase-specific and nonphosphokinase-specific immunofluorescence could be determined within a section, and among several sections in one experiment.

Immunofluorescence intensity could not be correlated with an absolute level of kinase phosphorylation; therefore, the absolute levels of phosphokinase and nonphosphokinase are not quantitated by this method.

RESULTS

Both monoclonal antibody 22B1 and Sylvia polyclonal antibodies were previously characterized using ELISA and immunoblot methods (Chapter 4). We were first interested in determining whether these antibodies would also bind to the type II CaM kinase in fixed nervous tissue. Hippocampal slice cultures were fixed, embedded in plastic resin, and sectioned at 2 µM. After etching the plastic, separate sections were incubated with either monoclonal antibody 22B1, Sylvia polyclonal antibodies, or a control monoclonal antibody, 6G9, specific for the CaM kinase alpha subunit independent of phosphorylation state, as described in Experimental Procedures (Figure 1). Specific binding of mouse monoclonal antibody 22B1 (phosphokinase specific) and 6G9 (control anti-CaM kinase) was visualized with rhodamine-conjugated goat anti-mouse antibodies (Figures 1A and 1C, respectively). Specific binding of Sylvia (nonphosphokinase-specific) antibodies was visualized with fluorescein-conjugated goat anti-rabbit antibodies (Figure 1B). Both 22B1 and Sylvia antibodies were immunoreactive in fixed, plastic-embedded tissue sections. The overall patterns of phosphokinase-specific immunoreactivity and nonphosphokinase-specific immunoreactivity were similar to each other, and were similar also to the pattern observed with the monoclonal antibody 6G9. Both anti-phosphokinase and anti-nonphosphokinase antibodies reacted throughout the hippocampal cultures, including the CA1, CA3, and dentate regions, essentially as previously observed for 6G9 reactivity in sections

acutely prepared from adult rat hippocampus (Ouimet *et al.*, 1984; Erondu and Kennedy, 1985). Immunoreactivity was strong (bright fluorescence) in the cell soma and proximal dendrites, while nuclei were not strongly stained and appeared dark, in agreement with previous results with 6G9. Most neurons present in the section were immunoreactive. Immunoreactivity was also strong in the molecular layers, regions that contain densely packed dendritic arborizations and synaptic terminals, with no gross differences observed between phosphokinase and nonphosphokinase immunoreactivities. Similar results were obtained in vibratome sections of fixed adult rat brain, visualized with peroxidase-conjugated secondary antibodies (personal communication, C. A. Hunt; data not shown). No immunoreactivity was observed with secondary antibodies alone, or when non-immune control antibodies were substituted for the primary antibody (data not shown).

Despite the overall similarity in distribution of phosphokinase and nonphosphokinase immunoreactivity throughout the hippocampal cultures, there was variability at the cellular level. Differences in phosphokinase immunoreactivity were observed by double-immunofluorescence labeling with Sylvia and 22B1 antibodies (Figure 2). With the aid of appropriate excitation and emission light filters, the immunoreactivity of each antibody, Sylvia (nonphosphokinase-specific) and 22B1 (phosphokinase-specific), was observed separately (Figure 2A and 2B, respectively). Although the separation of the fluorescein and rhodamine emissions is nearly complete, the patterns of

immunoreactivity observed with phosphokinase- and nonphosphokinase-specific antibodies in the same section were relatively similar to each other, as described above for single antibody immunofluorescence in different sections. However, photomicrographs composed of double exposures with the alternate light filters revealed considerable heterogeneity in the relative intensities of phosphokinase- and nonphosphokinase-specific immunofluorescence (Figure 2C). The variability was most apparent between cell soma. Some cells appeared yellow, indicating similar levels of immunofluorescence specific for nonphosphokinase (fluorescein) and phosphokinase (rhodamine). Some cells were notably orange or yellow-green in cast, indicating a predominance of phosphokinase or nonphosphokinase immunoreactivity, respectively. A few proximal dendrites were distingishable, and also varied considerably in the relative levels of phosphokinase immunoreactivity. Immunofluorescence in the molecular layers appeared somewhat more homogeneous, although some dendritic fields were notably shifted in their phosphorylation-specific immunoreactivity relative to others (Figure 3C).

The heterogeneity in kinase phosphorylation state among neurons was observed in all hippocampal regions including CA1, CA3 (Figure 2) and the dentate granule cells. The variation was greatest at the border between the CA1 and CA3 regions (Figure 3A). A number of cells in this region lack immunoreactivity for the phosphorylated CaM kinase, but are strongly reactive for nonphosphokinase-specific antibodies, standing out in striking contrast to

neighboring cells and the underlying molecular layer. Whether the low level of phosphorylated kinase in these neurons, implied by this immunoreactivity pattern, is correlated with a function specific to these neurons, or whether it represents an abnormal state, will require further study.

As a method of testing the fidelity of the phosphokinase-specific immunoreactivity in histological sections, we were interested in changing the CaM kinase phosphorylation state in the cultures prior to immunocytochemistry. We replaced the culture medium with a Ca^{2+}-free Ringer's solution and returned the culture to the incubator for 30 minutes (Molloy and Kennedy, 1991). Cultures were then prepared for immunocytochemistry. Control cultures, not pre-incubated in Ca^{2+}-free Ringer's, were prepared in parallel. Culture sections were assayed for phosphokinase- and nonphosphokinase-specific immunoreactivity by double-immunofluorescence labeling, as described above and in Experimental Procedures (Figure 3). Pre-incubation in Ca^{2+}-free Ringer's caused a general and dramatic decrease in immunoreactivity for the phosphorylated kinase (Figures 3B and 3D), compared with control cultures (Figures 3A and 3C). Thus, the double-immunofluorescence photomicrographs appear mostly green, due to the predominance of nonphosphokinase-specific immunoreactivity labeled with fluorescein-conjugated secondary antibodies. Phosphokinase-specific immunoreactivity was reduced throughout the cultures; the CA1-CA3 border and the dentate gyrus are shown (Figure 3, A and B, and C and D, respectively). Some areas remained phosphokinase-immunoreactive

(Figure 3B). In a separate experiment, the level of phosphokinase immunoreactivity in Ca^{2+}-free Ringer's pre-incubated cultures was also reduced relative to control cultures, although not as extensively as shown in Figure 3 (data not shown). We have not yet attempted to quantitate the changes in immunofluorescence.

Conclusions

Our results demonstrate that monoclonal antibody 22B1 and Sylvia polyclonal antibodies react in fixed tissue with the type II CaM kinase, and indicate that this immunoreactivity is specific for the phosphorylated and nonphosphorylated forms of the CaM kinase, respectively. The use of double-labeled sections and immunofluorescence detection revealed variability in the levels of phosphorylated and nonphosphorylated CaM kinase between cells and dendritic processes with a given region.

The phosphokinase-specific immunoreactivity was robust, supporting the hypothesis that a significant fraction of the CaM kinase in the hippocampal cultures is phosphorylated at any one time. Biochemical assays of Ca^{2+}-independent CaM kinase activity indicated that approximately a third of the kinase holoenzyme molecules were phosphorylated on $Thr^{286/287}$ (Molloy and Kennedy, 1991). Therefore, the immunocytochemical localization of phosphokinase is in general agreement with the biochemical data. It indicates further that the phosphorylated kinase is not localized in discrete pools, but is distributed throughout the culture. The CaM kinase in any one location is a mixture of both phosphorylated and nonphosphorylated kinase, although one form may predominate. Early models proposed that CaM kinase autophosphorylation might be an all or nothing event; the population of kinase molecules in any given location would be kept dephosphorylated by protein

phosphatases until stimulated, by a prolonged rise in intracellular free Ca^{2+}, to autophosphorylate several $Thr^{286/287}$ sites per kinase holoenzyme (Lisman, 1985; Miller and Kennedy, 1986; Lisman and Goldring, 1988). Ca^{2+}-independent autophosphorylation might then occur at a rate sufficient to compete with the dephosphorylation rate in the absence of Ca^{2+}. Thus, the kinase might act as a molecular switch triggered by Ca^{2+}. The models were constructed with the assumption that resting levels of intracellular free Ca^{2+} were not sufficient to significantly stimulate CaM kinase autophosphorylation. Indeed, the intracellular free Ca^{2+} concentration in the cultures has been determined to range between 15 and 40 nM (Molloy and Kennedy, 1991). Thus, the biochemical evidence indicating that much of the kinase was autophosphorylated in unstimulated cultures was surprising (Molloy and Kennedy, 1991). Similar results were obtained with homogenates of freshly dissected rat brains. Although one explanation could be that all the kinase molecules were autophosphorylated in one-third of the cells or dendrites, the immunocytochemical evidence now argues strongly that the CaM kinase everywhere is partially autophosphorylated, to greater or lesser extents.

The elevated level of phosphorylated kinase is partially explained by the observation that removal of extracellular Ca^{2+} substantially reduces the level of phosphorylated kinase, determined by biochemical and immunocytochemical methods. This implies that Ca^{2+}-dependent autophosphorylation of the CaM kinase occurs at resting levels of intracellular free Ca^{2+}. In addition, it implies

that the CaM kinase autophosphorylation state is in dynamic equilibrium; the rate of autophosphorylation and protein phosphatase-catalyzed dephosphorylation are comparable and significant. Although the turnover rate is significant, the overall level of phosphorylated CaM kinase is stable. *In vitro*, autophosphorylation is controlled by Ca^{2+}, and by the prior level of autophosphorylation at $Thr^{286/287}$ (which supports Ca^{2+}-independent autophosphorylation, at a reduced rate relative to the rate supported by Ca^{2+}/calmodulin) (Miller and Kennedy, 1986). In culture, removing extracellular Ca^{2+} should also deplete intracellular stores of Ca^{2+}. The results suggest that Ca^{2+}-stimulated autophosphorylation ceases and protein phosphatase-catalyzed dephosphorylation returns the pool of CaM kinase to a nonphosphorylated state, implying also that the initial rate of dephosphorylation exceeds that of Ca^{2+}-independent autophosphorylation. The phosphokinase and nonphosphokinase immunocytochemistry supports the biochemical evidence that most of the kinase in the cultures is dephosphorylated by protein phosphatases in the absence of Ca^{2+}. Biochemical assays allow for the possibility that CaM kinase in certain compartments (*e.g.*, the postsynaptic density) may not be active in the biochemical activity assays, and, thus, could be resistant to dephosphorylation but not be detectable. However, the immunocytochemical results presented in this study indicate that there is no subcellular pool of CaM kinase that remains phosphorylated for long periods of time in the absence of free Ca^{2+}. These results may further restrict models of the role of CaM kinase autophosphorylation in neuronal function.

References

Erondu, N. E., and Kennedy, M. B. (1985). Regional distribution of type II Ca^{2+}/calmodulin-dependent protein kinase in rat brain. *J. Neurosci.* 5, 3270-3277.

Lisman, J. E. (1985). A mechanism for memory storage insensitive to molecular turnover: a bistable autophosphorylating kinase. *Proc. Natl. Acad. Sci. (USA)* **82**, 3055-3057.

Lisman, J. E., and Goldring, M. A. (1988). Feasibility of long-term storage of graded information by the Ca^{2+}/calmodulin-dependent protein kinase molecules of the postsynaptic density. *Proc. Natl. Acad. Sci. (USA).* **85**, 5320-5324.

Miller, S. G., and Kennedy, M. B. (1986). Regulation of brain type II Ca^{2+}/calmodulin-dependent protein kinase by autophosphorylation: A Ca^{2+}-triggered molecular switch. *Cell* **44**, 861-870.

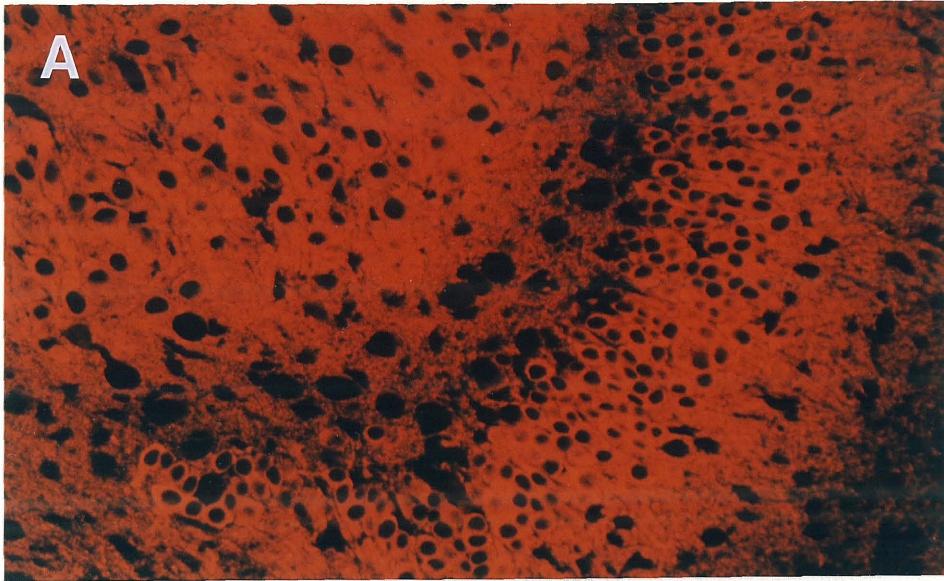
Molloy, S. S., and Kennedy, M. B. (1991). Autophosphorylation of type II Ca^{2+}/calmodulin-dependent protein kinase in organotypic cultures of hippocampal slices. *Proc. Natl. Acad. Sci. (USA) (in press).*

Ouimet, C. C., McGuiness, T. L., and Greengard, P. (1984). Immunocytochemical localization of calcium/calmodulin-dependent protein kinase II in rat brain. *Proc. Natl. Acad. Sci (USA)* **80**, 7357-7361.

Figure 1. ***Immunocytochemical Localization of Nonphosphorylated and Phosphorylated Type II CaM Kinase in Organotypic Hippocampal Cultures.***

Tissue sections (2 µm) were prepared and stained as described in Experimental Procedures. Darkfield fluorescence photomicrographs were recorded on Kodak P1600 color reversal (slide) film, using exciter and barrier filters appropriate for fluorescein or rhodamine fluorescence, as described in Experimental Procedures. Photographic prints were obtained through a color internegative. Photographic prints had considerably less contrast than the original slide film image. **(A)** The section was incubated with a 1:250 dilution of 6G9, a monoclonal antibody that specifically recognizes the α subunit of the type II CaM kinase. Bound antibody was visualized by secondary labeling with rhodamine-conjugated anti-mouse antibodies. The overall pattern of immunoreactivity was similar to that observed in adult hippocampal tissue (Ouimet *et al.*, 1984; Erondu and Kennedy, 1985). The antibody immunoreactivity was present in soma and dendrites of dentate granule cells (shown), as well as pyramidal cells in both CA1 and CA3. Final magnification is 50X. **(B)** The section was incubated with 0.06 mg/ml rabbit polyclonal antibodies that specifically recognize the nonphosphorylated Thr^{286} site of the type II CaM kinase. Polyclonal antibodies were affinity purified from Sylvia antisera as described in Experimental Procedures. Bound antibody was visualized by secondary labeling with fluorescein-conjugated anti-rabbit antibodies. The overall pattern of immunoreactivity was similar to that in A, although

somewhat reduced in intensity. The antibodies were immunoreactive to soma and dendrites of dentate granule cells (shown) as well as the pyramidal cells in CA1 and CA3. (C) The section was incubated with a 1:2000 dilution of 22B1, a monoclonal antibody that specifically recognizes the CaM kinase when autophosphorylated at Thr^{286}. The overall pattern of immunoreactivity was similar to that in A and B; soma and dendrites in CA1, CA3, and the dentate gyrus (shown) were immunoreactive. Final Magnification is 50 X.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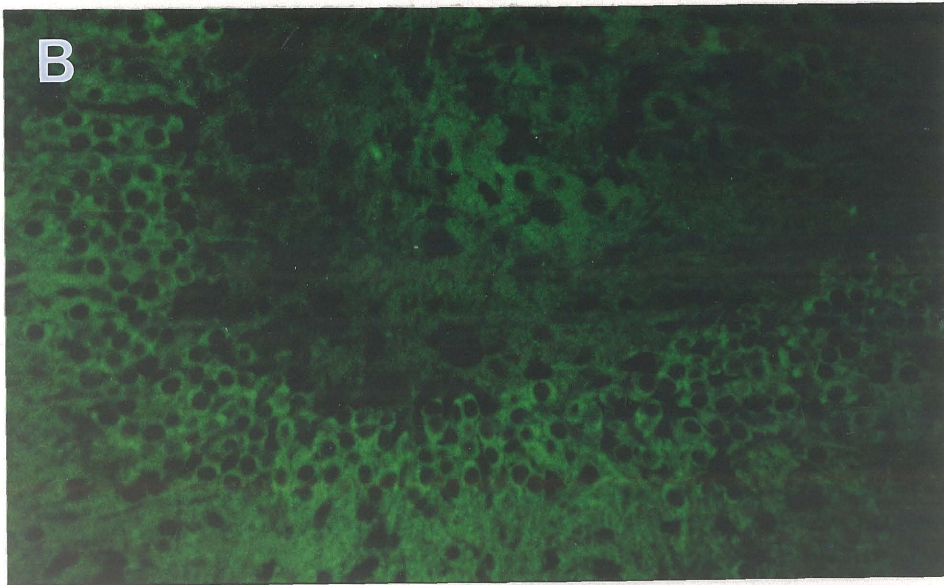
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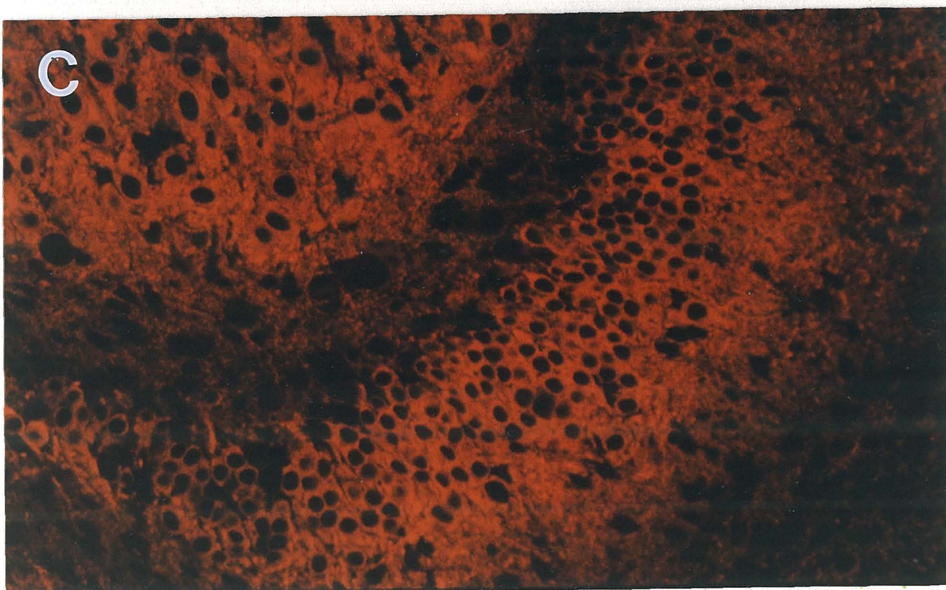
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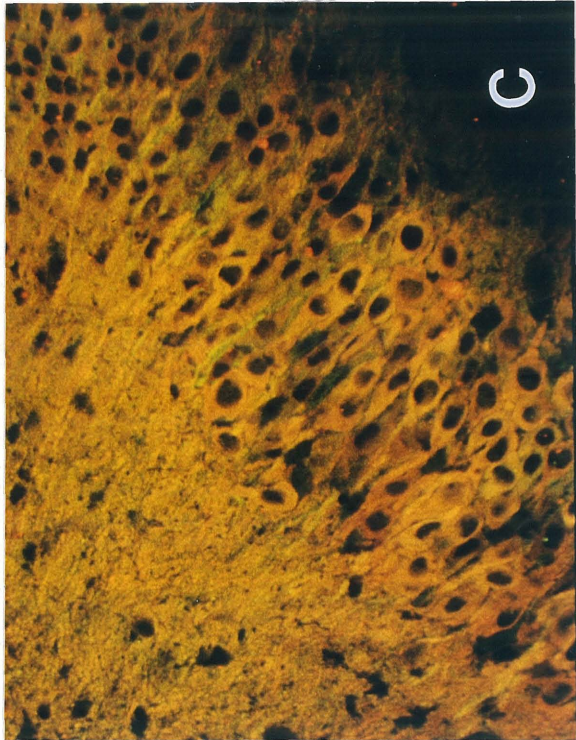
Figure 2. ***Double Immunofluorescence Labeling of Nonphosphorylated and Phosphorylated Type II CaM Kinase in Hippocampal Cultures.*** The tissue section (2 µm) was prepared for immunocytochemistry as described in Experimental Procedures. The section was incubated with a mixture of Sylvia polyclonal antibodies against the nonphosphorylated Thr^{286} site of the Type II CaM kinase and monoclonal antibody 22B1 against the phosphorylated Thr^{286} site of the CaM kinase. Antibody concentrations are as described in Figure 1. Bound antibodies were visualized by secondary labeling with a mixture of fluorescein-conjugated anti-rabbit antibodies and rhodamine-conjugated anti-mouse antibodies. Darkfield photomicrographs and prints were produced as described in Experimental Procedures and Figure 1. (**A**) A photomicrograph of nonphosphokinase immunoreactivity in the CA1 pyramidal cells, obtained by 1.75 minutes exposure of fluorescein immunofluorescence. The photographic print is underexposed compared to the original slide film image. (**B**) A photomicrograph of phosphokinase immunoreactivity obtained by 1.75 minutes exposure of rhodamine immunofluorescence at the identical location shown in A. (**C**) Double immunofluorescence photomicrograph obtained by successive 1.75-minute exposures of fluorescein and rhodamine immunofluorescence at the identical location shown in A and B. Final magnification is 50 X.

B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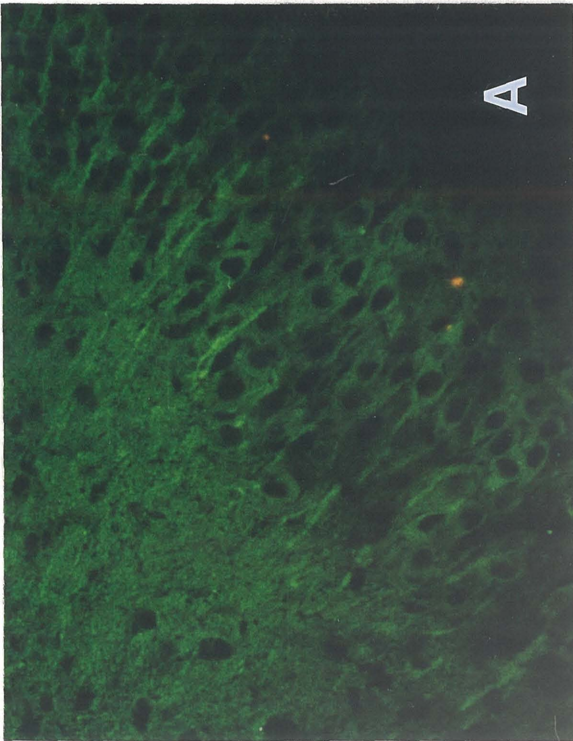
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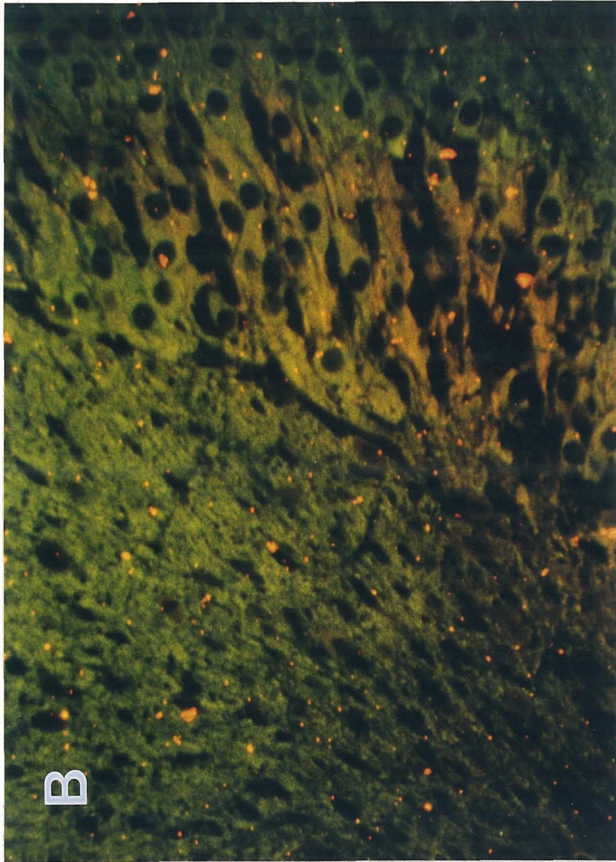
Figure 3. ***Reduction of Immunoreactivity Against Phosphorylated Kinase Caused by Pre-Incubating Hippocampal Cultures in Ca2+-Free Ringer's.***

(**A** and **C**) "Control" hippocampal tissue was transferred directly from culture medium into ice-cold fixative solution. Sections (2 µm) were prepared, and double immunofluorescence labeling performed with antibodies specific for nonphosphorylated (fluorescein-labeled) and phosphorylated (rhodamine-labeled) type II CaM kinase, as described in Experimental Procedures and Figure 2. The cell bodies and dendrites of pyramidal cells in CA1 (A, left side) and CA3 (A, right side) were intensely reactive for both nonphosphokinase and phosphokinase. Nuclei are not immunoreactive. Several oddly shaped cells displayed exclusively nonphosphokinase reactivity (A). Dentate granule cells were also phosphokinase immunoreactive (C). Compared to the cell bodies of the dentate granule cell, a ridge of the molecular layer in the dentate gyrus was relatively nonphosphokinase immunoreactive (C). Some heterogeneity in phosphokinase immunoreactivity is discernable at the borders of this nonphosphokinase-predominant region.

(**B** and **D**) Cultures were pre-incubated in Ca^{2+}-free Ringer's solution for 30 minutes prior to fixing, sectioning, and immunocytochemical localization of nonphosphorylated and phosphorylated CaM kinase, in parallel with the "control" sections shown in A and C. Nonphosphokinase immunoreactivity predominates in both the CA3 (B), and CA1 regions, as well as the dentate gyrus (D).

Double immunofluorescence photomicrographs were recorded and printed as described in Figures 1 and 2. Each image (A-D) is composed of two 1.10-minute exposures, one for each fluorochrome, as in Figure 2. Antibody concentrations are as described in Figure 1. Final magnification is 50 X. Intensely fluorescent puncta are fluorescent debris accidentally introduced by the author during mounting and do not represent synaptic localizations of immunoreactivity.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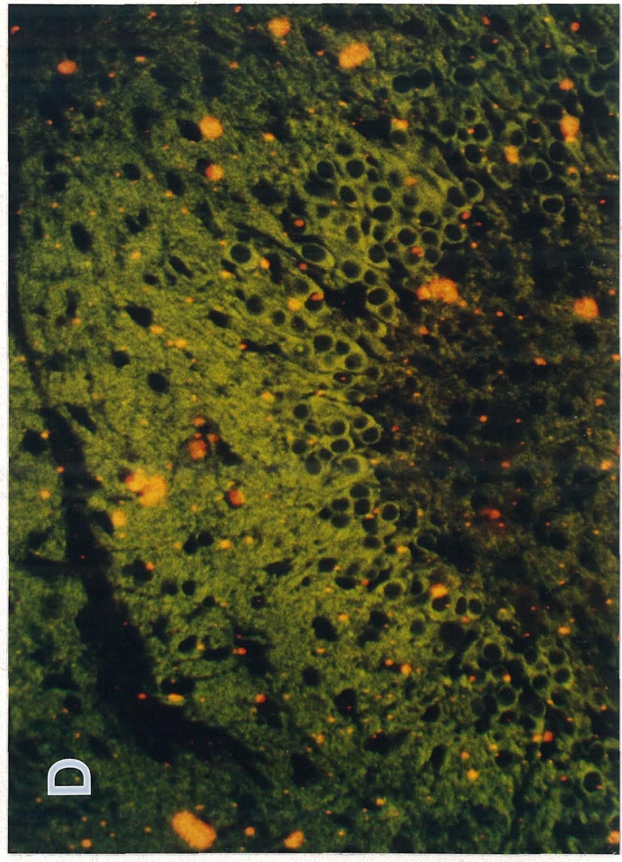
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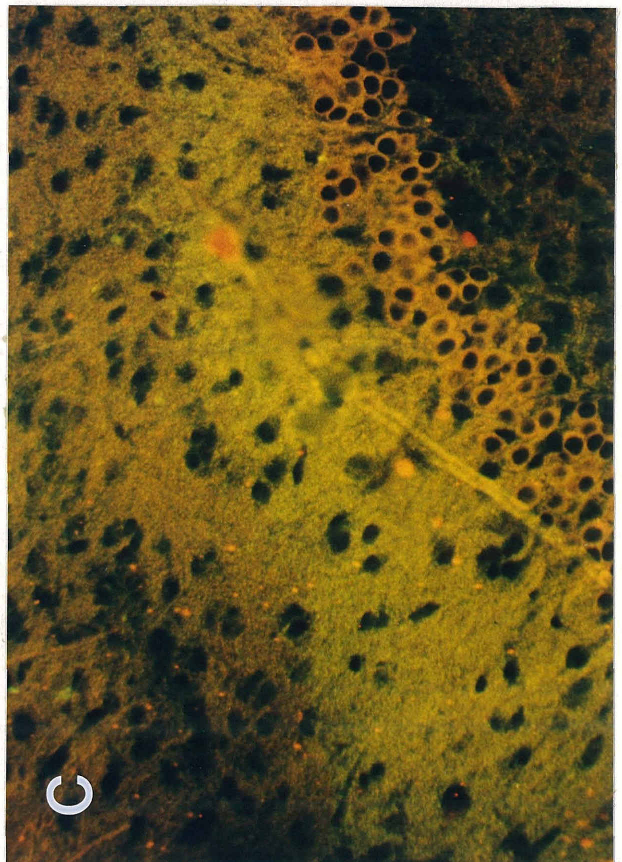
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APPENDIX

IDENTIFICATION OF FUNCTIONALLY SIGNIFICANT PHOSPHORYLATION SITES ON NEURONAL PROTEINS AND PREPARATION OF ANTIBODIES THAT RECOGNIZE THEM*.

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Identification of Functionally Significant Phosphorylation Sites on Neuronal Proteins and Preparation of Antibodies That Recognize Them

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Introduction

Many neurotransmitters and neurohormones initiate biochemical changes in postsynaptic cells that modulate important neuronal functions. These modulatory influences allow neurons to maintain homeostasis, to adapt to injury or altered external conditions, and to store information about prior events. A common pathway through which a great many neuronal functions are regulated is protein phosphorylation. Knowledge about the nature, location, and timing of functionally significant protein phosphorylation events is important for understanding adaptive neuronal responses, and for dissecting the complexity of molecular mechanisms underlying learning and memory.

Unfortunately, protein phosphorylation is not easy to study *in situ*. Protein phosphorylation systems are "noisy." Functionally important phosphorylation sites can usually be stoichiometrically phosphorylated at a high rate by the appropriate protein kinase. However, many proteins are also phosphorylated at sites that have little functional significance. Often phosphorylation at these sites is slow and incomplete. The presence of this second class of sites makes quantitative study of the time course and functional significance of phosphorylation and dephosphorylation difficult. Most phosphorylated proteins must be broken into small peptides in order to measure the extent of phosphorylation of particular sites.

In this article, we first describe methods that have been used in our laboratory to identify and sequence functionally important sites of phosphorylation within the brain enzyme, type II Ca^{2+}/calmodulin-dependent protein kinase (type II CaM kinase), an enzyme composed of multiple copies of two major catalytic subunits, α and β, in varying proportions. Then we describe methods that we have used to prepare antibodies that recognize the CaM kinase only when it is

phosphorylated at one of these sites. Such antibodies can be used for sensitive and specific assay of phosphorylation of their cognate site, and possibly for immunocytochemical visualization of the location of the phosphorylation event *in situ*. These methods should prove applicable to the study of a wide variety of functionally significant neuronal protein phosphorylation systems.

Identification of Functionally Significant Autophosphorylation Sites

The strategy is to measure the time courses of phosphorylation (and dephosphorylation) of particular sites on the protein of interest and then to compare these time courses with that of a functional change in the protein, such as a change in enzymatic activity or a change in a binding constant. This method will identify phosphorylation sites that could be responsible for the functional change, and will often unambiguously identify a single site. Such a study can, in theory, be performed with purified protein components or with a protein phosphorylated within a cell. However, in the latter case, the low specific radioactivity of [γ-^{32}P]-ATP that can be achieved within the cell may limit the accuracy with which time courses of phosphorylation can be measured.

After the protein of interest is phosphorylated for different times in the presence of ^{32}P-labeled ATP, the labeled protein is reduced and alkylated, then purified by SDS-gel electrophoresis (Fig. 1). It is digested with trypsin to form tryptic phosphopeptides. These peptides are resolved by high pressure liquid chromatography (HPLC). The areas under each radioactive peptide peak at different times are normalized and plotted to produce a highly reproducible time course of phosphorylation of sites contained within the peptide. To identify the location of the sites, the tryptic phosphopeptides are purified in large quantity and sequenced. We used this strategy to identify two different functionally significant

autophosphorylation sites in brain type II CaM kinase. Autophosphorylation of one of these sites, Threonine-286, produces Ca^{2+}-*independent* kinase activity (1-4). Autophosphorylation of the other, Threonine-305, inhibits activation of the kinase by calmodulin (5, 6).

Autophosphorylation of Type II CaM Kinase.

To measure time courses of phosphorylation of different autophosphorylation sites, pure type II CaM kinase was autophosphorylated for different times in a mixture (final volume 50 µl) containing 10 µg kinase, 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 10 µg calmodulin, 10 mM dithiothreitol, 0.6 mM EGTA, 0.9 mM $CaCl_2$, and 200 µM [γ-^{32}P]-ATP (2.0-3.0 x 10^3 cpm/pmol). The mixtures were warmed for 30 sec at 30°, and reactions were initiated by the addition of kinase. At the appropriate times, the reactions were terminated by the addition of 50 µl of ice-cold 0.2 M EDTA (pH 8.0) followed by immediate cooling in an ice-water bath. In general, the quenched reaction mixtures were divided into two 50 µl aliquots. One aliquot was used to determine the stoichiometry of phosphorylation the individual α and ß subunits after their separation by SDS-PAGE. Each radioactive subunit was cut from the dried gel; the gel pieces were placed into a commercial scintillation fluid and their radioactivity was determined in a β counter. The stoichiometry of phosphorylation was calculated from the specific activity of the ^{32}P-ATP in the autophosphorylation reaction and the amount of kinase loaded onto the gel. The other aliquot was used for tryptic phosphopeptide mapping by reverse-phase HPLC.

To follow the time course of phosphorylation of sites on proteins that are phosphorylated by exogenous kinases, optimum conditions for phosphorylation by the kinase would have to be determined as described below for phosphorylation of

short peptides.

Reduction and Alkylation

This procedure involves the initial reduction of all cystine residues to cysteine, followed by their alkylation with iodoacetamide (7, 8). The alkylation prevents reformation of cystine and retards oxidation of cysteine during later steps. We found that alkylation was necessary to obtain consistent tryptic peptide maps (1).

To remove free [γ-^{32}P]-ATP, one ml of ice-cold 10% (wt/vol) trichloroacetic acid (TCA) and 50 μl of 0.15% deoxycholate was added to a 50 μl aliquot of the quenched autophosphorylation reaction, which was then cooled in ice water for 15 min. Precipitated protein was pelleted by centrifugation at 12,000 g for 15 min. The supernatant was removed by aspiration, and the pellet was washed once with 1 ml of ice-cold ether to extract remaining TCA. Four hundred microliters of 6 M guanidine-HCl, 0.5 M Tris-HCl (pH 8.1) and 2 mM EDTA were added. The tube was flushed with N_2 and incubated for 30 min at 50° to denature the protein. Fifty μl of a fresh solution of 5 mM dithiothreitol (DTT) was added, the tube was flushed again with N_2, and the protein reduced for 4 hr at 50°. The reaction was cooled to room temperature, and 50 μl of fresh 20 mM iodoacetamide was added. The tube was flushed again with N_2 and incubated for 30 min in the dark at room temperature, or for 60 min in the dark at 0°. The reaction was then quenched by the addition of 50 μl of 0.1 M DTT and immediately dialyzed against 4 liters of 50 mM NH_4HCO_3 in the dark at 4° for 10-12 hr with one change of buffer. This dialysis step can be carried out in a microdialysis apparatus. We used an alternative arrangement in which dialysis tubing was stretched over the mouth of a 1.5 ml plastic reaction tube and held in place by rings cut from latex tubing (3/16" i.d.). This method has the advantage that the reaction does not have to be transfered,

however, the dialysis membrane must be checked carefully for leaks. The dialyzed protein was frozen in liquid nitrogen and lyophilized to dryness in a Speedvac. The protein was resuspended in 70 µl of 3% SDS, 2% (w/v) 2-mercaptoethanol, 5% (w/v) glycerol, 60 mM Tris-HCl (pH 6.7), and a trace of bromphenol blue. The sample was heated to 100° for 2 min, then the kinase subunits were separated by SDS-PAGE. The gel was dried, and the autophosphorylated subunits were located by autoradiography with X-ray film.

Tryptic Digestion

Exhaustive trypsinization was carried out in reswollen gels by a modification of a procedure originally described by Axelrod and by Huttner and Greengard (1, 3, 9, 10). Pieces containing the α or ß subunits were cut from the dried gels and washed twice for one hour each in 50 ml of 20% (vol/vol) isopropanol, then twice for 1 hr each in 50 ml of 10% (vol/vol) methanol on a rotating platform at room temperature. The washed gel pieces were transfered to microcentrifuge tubes, frozen, and lyophilized. Each lyophilized gel piece was reswollen by incubation for 4 hr at 37° in 100 µl of 50 mM NH_4HCO_3 (pH 8.1) containing 50 µg of trypsin (20-fold excess of trypsin by weight). One ml of 50 mM NH_4HCO_3 (pH 8.1) containing an additional 50 µg of trypsin was added, and the incubation continued for 12 hr at 37°. Tryptic peptides diffuse from the gel pieces as they are formed, thus the solution contains most of the tryptic peptides. The solutions were removed and filtered through 0.2 µm filters to remove particulates. The gel pieces were briefly vortexed with 0.4 ml of distilled H_2O. This wash was filtered and combined with the first filtrate. Radioactivity in the filtrates and gel pieces was determined by measuring Cerenkov radiation in a β counter (48% efficiency). The filtrates were then frozen and lyophilized. Approximately 80% of ^{32}P-phosphate originally present in the gel

pieces was recovered in the combined filtrates. Recovery could be increased to 95-100% by re-extracting the gel pieces with 1 ml of 50 mM NH_4HCO_3 (pH 8.1) for 8-12 hrs, but this made no qualitative difference in the phosphopeptide maps.

Reverse-phase HPLC Fractionation of Phosphopeptides

There are many useful methods available for fractionating peptides by HPLC and analyzing peptide maps (11, 12). The following method worked best for us. Lyophilized peptides were resuspended in 100 μl of Buffer A [0.07% (vol/vol) trifluoroacetic acid (TFA) in H_2O (pH 2.3)] and loaded onto an 0.46 X 25 cm C4 reverse phase HPLC column equilibrated in Buffer A. The column was developed at a flow rate of 1 ml/min with the following gradient: 0-5 min, 0% B; 5-95 min, 0-35% B; 95-100 min, 35-100% B (Buffer B: 0.07% [vol/vol] TFA, 70% acetonitrile, 30% H_2O). Most of the peptide fractionation occured between 0 and 25% acetonitrile. Two hundred 0.5 ml fractions were collected, and labeled peptides were detected by measuring Cerenkov radiation in a β counter. The resolution of the phosphopeptide maps was limited by the number of fractions collected and counted. Total recovery of ^{32}P-phosphate from the C4 columns was 80-95%.

Calculation of Time Courses of Phosphorylation of Individual Sites

The time course of phosphorylation of a particular site was determined by plotting the fraction of total radioactivity from the intact kinase subunit that was recovered in the particular site at each time point. Different experiments were compared by normalizing the fraction of radioactivity in each peak to the maximum fraction observed for that peak in the experiment.

Data concerning the amount of radioactivity in peaks from the HPLC maps of tryptic phosphopeptides was transfered directly from the scintillation counter into an integration program through a data buffer. For each time point (t), the area under a

single radioactive peak ($A_{p,t}$) and the total area under all radioactive peaks ($A_{tot,t}$) were calculated with the integration program. The fraction of radioactivity in each chosen peak was then calculated by dividing $A_{p,t}$ by $A_{tot,t}$. A normalized amount of radioactivity in peaks containing a particular site was calculated (Eq. 1) by multiplying the fraction of radioactivity in those peaks by the total radioactivity in the kinase subunit before digestion with trypsin ($R_{subunit,t}$; This value was measured in the gel piece before trypsinization).

Eq. 1

$$\frac{A_{p,t}}{A_{tot,t}} \times (R_{subunit,t}) = R_{p,t}$$

For plots of the time courses of autophosphorylation of particular sites, $R_{p,t}$ at each time point was converted to percent phosphorylation by dividing by $R_{p,t}$ for the longest time point ($R_{p,t=max}$; Eq. 2). When the site was contained in more than one radioactive peak, values for each peak were summed.

Eq. 2

$$\frac{R_{p,t}}{R_{p,t=max}} \times 100 = \%\ \text{phosphorylation}$$

In theory, the molar amount of phosphate incorporated into each site could be estimated by multiplying the fraction of radioactivity in the site by the total moles of phosphate per mole of subunit (measured independently, as described above). However, the calculated maximal molar amount of phosphate in a given site often varied among different experiments, even under apparently identical conditions. The determination of total moles phosphate per mole subunit was usually the source of the largest variance. This appeared to result from variable recovery of protein subunits during gel electrophoresis, and we were unable to correct for it. Chemical

instability of certain peptides may have been an additional source of variance. Nevertheless, the time courses of phosphorylation of each site, and the time to reach maximum phosphorylation were highly reproducible from experiment to experiment (1, 5), indicating that this is a valid method for determining rates of phosphorylation of individual sites.

Dephosphorylation with Protein Phosphatase.

To establish which phosphorylation site regulates a particular biological activity, it is often useful to examine the rate of dephosphorylation of the site, as well as the rate of phosphorylation. The catalytic subunits of protein phosphatases 1 and 2A can be purified from rabbit muscle in large quantity, and are responsible for a large fraction of protein phosphatase activity in a variety of tissues (13). For our experiments with CaM kinase, we purified 1.4 to 4.0 mgs of each phosphatase (1) by the methods of Tung et al. (14) and stored them at -80° in aliquots of approximately 1 mg/ml in 0.05 M Tris buffer (pH 7.0), 50% glycerol, 0.1% β-mercaptoethanol. The phosphatases required "activation" before use (1).

To determine the dephosphorylation rate of individual sites on the type II CaM kinase, 33 μg of kinase was autophosphorylated essentially as described above for 5 to 60 sec in a final volume of 165 μl in the presence of γ-^{32}P-ATP. The reaction was terminated by the addition of 82 μl of 0.4 M EDTA, 25 mM Tris-HCl (pH 7.0), 1 mg/ml BSA. An aliquot containing 6 μg of kinase was mixed with an equal volume of 6% (wt/vol) SDS, 10% (wt/vol) glycerol, 4% (wt/vol) 2-mercaptoethanol, 120 mM Tris-HCl (pH 6.7), and a trace of bromphenol blue (2x gel sample buffer). Dephosphorylation of the remaining kinase was initiated by the addition of .014 units (approximately 2 μg) of protein phosphatase 2A, and proceeded at 30° in a final volume of 270 μl. At the desired times, aliquots containing 6 μg of kinase were

removed and added to an equal volume of 2x gel sample buffer to terminate the reaction. Two aliquots, each containing 0.5 μg kinase, were removed from each quenched reaction and the subunits were separated by SDS-PAGE for determination of stoichiometry of labeled phosphate incorporation as described above. The remaining kinase (5 μg) was used for tryptic phosphopeptide mapping by reverse-phase HPLC as described above, except that the TCA pellets were washed two more times with 1 ml acetone at -20° to remove SDS. To plot the time course of dephosphorylation of a site, the radioactivity in the site at each time point ($R_{p,t}$) was plotted as a percent of the radioactivity in the site at time zero.

Large-Scale Autophosphorylation and Tryptic Digestion.

Identification of phosphorylation sites contained within a tryptic peptide requires purification of the peptide in quantities adequate for sequencing on an automated sequencer. As little as 10 pmoles can be sequenced on the most sensitive gas phase sequencers. However, it is wise to plan to purify *several* nmoles because some peptides are recovered in relatively low yield. To obtain nmoles of phosphopeptides from type II CaM kinase, batches of pure kinase were autophosphorylated in a reaction mixture (1 ml) containing 200 μg kinase (approximately 3.6 nmoles subunits), 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 100 μg calmodulin, 10 mM dithiothreitol, 0.6 mM EGTA, 0.9 mM $CaCl_2$, and 200 μM [γ-^{32}P]-ATP (20-40 cpm/pmol). The mixtures were warmed for 60 sec at 30°, and reactions were initiated by the addition of kinase. After the desired times, the reactions were quenched by the addition of 120 μl of ice-cold 100% TCA followed by immediate cooling in an ice-water bath. Reduction, alkylation, SDS-PAGE, and tryptic digestion were carried out as described above. To improve recovery of peptides, the gel pieces were extracted a second time with

monoclonal antibodies were prepared from mice. In the second strategy, monoclonal antibodies were raised against the intact, thiophosphorylated kinase after suppression of the immune response to nonphosphorylated kinase.

Preparation of Peptides

Two peptides with the sequence of the autophosphorylation site surrounding Threonine-286 were synthesized on an Applied Biosystems model 430A peptide synthesizer by the Biopolymer Synthesis Center at Caltech (17). α-9mer had the sequence Met-His-Arg-Gln-Glu-Thr-Val-Asp-Cys; α-14mer had the sequence Met-His-Arg-Gln-Glu-Thr-Val-Asp-Cys-Leu-Lys-Lys-Phe-Asn. After cleavage from the polystyrene, crude peptide was desalted through Dowex 1-X2 resin and lyophilized to dryness for storage. At this stage, the peptides were generally 80 to 95% pure.

The peptides were first tested for their ability to act as substrates for type II CaM kinase. A small quantity of each was dissolved in 40 mM Tris-HCl (pH 7.5), and stored at -80°. Peptides were autophosphorylated in a reaction mixture containing 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 0.4 mM EGTA, 0.7 mM $CaCl_2$, 10 mM dithiothreitol, 50 μg/ml calmodulin, and 100 μM [γ-^{32}P]ATP (0.5-1.0 x 10^3 cpm/pmol). Reaction tubes were warmed to 30°, and phosphorylation initiated by the addition of CaM kinase to a final concentration of 1.0 μg/ml. Reactions were terminated after 3 minutes by the addition of EDTA to a final concentration of 0.1 M. Phosphorylated peptides were separated from ATP by the phosphocellulose filter paper method of Roskowski (18). Radioactivity incorporated into peptides was measured by liquid scintillation counting. Both peptides were substrates for the CaM kinase; the Km for α-9mer was 28 μM and for α-14mer was 85 μM.

Thiophosphorylation

Most protein kinases will use adenosine 5'-O(3-thiotriphosphate), ATPγS, as a

1 ml of 50 mM NH_4HCO_3 for 8-12 hr at 37° and the second supernatant was filtered and combined with the first. Chromatography on a C4 column was performed as described above. The purity of each phosphopeptide was monitored by reading absorbance of the effluent at 214 nm. In general, several reactions, each containing peptides derived from 200 µg of kinase, were combined and fractionated in a single C4 column run. Each peak was pooled for sequencing or for further purification.

Most peptides required a second step of purification prior to sequencing. Fractions containing such individual peaks were pooled and reduced to 50-100 µl in a Speedvac. After addition of 300 µl of Buffer A (see above), the peaks were purified on a C18 column equilibrated with Buffer A (Figure 2). In each case, the gradient of increasing Buffer B that optimized fractionation of the desired peptide was first determined during runs with analytical amounts of peptide. During purification for sequencing, fractions (0.5 ml) were collected by hand in plastic tubes rinsed individually with 1% TFA in 50% acetonitrile while monitoring absorbance of the effluent at 214 nm. Labeled peptides were detected by measuring Cerenkov radiation. Automated gas-phase sequencing was performed on an Applied Biosystems 470A protein sequencer.

Preparation of Antibodies that Recognize Individual Phosphorylated Sites

We have employed two strategies to raise antibodies that specifically recognize type II CaM kinase only when it is phosphorylated at Threonine-286, the site that controls Ca^{2+}-independent activity. In the first strategy, synthetic peptides with the sequence of the autophosphorylation site were enzymatically thiophosphorylated. The thiophosphorylated peptides were purified by semi-preparative reverse-phase HPLC, and coupled to keyhole limpet hemocyanin (KLH) for use as immunogens (15, 16). Polyclonal sera were raised in rabbits, and

substrate for thiophosphorylation of proteins, but thiophosphorylated proteins and peptides are resistant to dephosphorylation by protein phosphatases (19, 20). For this reason, the phosphorylation procedure was optimized for thiophosphorylation. We also determined the minimum proportions of CaM kinase and calmodulin necessary for optimum thiophosphorylation in preparation for large scale reactions. Reactions typically contained 10 to 30 μg of peptide substrate, were terminated by the addition of EDTA to a final concentration of 0.1 M, and used [^{35}S]ATPγS with a specific radioactivity of 0.2-0.4 x 10^3 cpm/pmol.

High pressure liquid chromatography (HPLC) on a C18 reverse-phase column was used to separate thiophosphorylated or phosphorylated peptide product from the non-phosphorylated substrate. Aliquots from reactions containing thiophosphorylated α-9mer were injected onto a C18 reverse-phase HPLC column (0.46 x 25 cm) equilibrated for 30-40 min at 1.0 ml/min with Buffer A (0.1% (v/v) trifluoroacetic acid, pH 2.3). The column was developed with the following gradient of Buffer B (70% (v/v) acetonitrile, 30% (v/v) H_2O, 0.1% (v/v) trifuoroacetic acid): 0-40 min, 0% to 17.5% Buffer B; 40-55 min, 17.5% to 100% Buffer B. Eluent was monitored at 214 nm. Occasionally, peaks of absorbance were collected by hand and radioactivity detected by liquid scintillation spectrometry. Thiophosphorylation of α-9mer reduced its retention time by 2.5 to 3 minutes, while phosphorylation reduced its retention time by about 4 minutes. The phosphorylated and nonphosphorylated forms of the peptide had similar absorbances at 214 nm. Recovery of peptide was typically 75%.

Thiophosphorylated α-14mer could not be separated from the nonphosphorylated peptide on reverse phase columns in acidic buffers. However, a gradient of acetonitrile in neutral phosphate buffer resolved the two cleanly. Completed reactions containing thiophosphorylated α-14mer were injected onto a

C18 column equilibrated at 1.0 ml/min in 95% Buffer C (25 mM sodium phosphate, pH 6.5), 5% Buffer D (50% acetonitrile, 50% 25 mM sodium phosphate, pH 6.5). The column was developed with the following gradient of Buffer D: 0-30 min, 5% to 30%; 30-40 min, 30% to 35%; 40-55 min, 35% to 50%; 55-65 min, 50% to 100%. The effluent was monitored at 214 nm. Both thiophosphorylation and phosphorylation reduced the retention time of α-14mer by 3 minutes.

Preparative Scale Thiophosphorylation

For large scale preparation of antigen, α-14mer was thiophosphorylated in a final volume of 3.0 ml containing 10 mg/ml peptide (30 mg; 5.7 mM), 0.1 mg/ml CaM kinase, 0.1 mg/ml CaM and 7.0 mM [^{35}S]ATPγS (100 cpm/nmol). α-9mer was less soluble than α-14mer and was thiophosphorylated *in* 20.7 ml containing 1.45 mg/ml peptide (1.3 mM), 0.03 mg/ml CaM kinase, 0.1 mg/ml calmodulin, and 2.3 mM [^{35}S]ATPγS. The concentrations of other reagents were as described above for autophosphorylation of the kinase. Reactions were initiated by the addition of kinase, carried out at 30° for 1-5 hours and terminated by injection onto the HPLC column.

Completed reactions (30 mg peptide) were filtered at 0.45 μm and injected through a 5 ml loop onto a semi-preparative C18 reverse-phase HPLC column (1.0 x 25 cm) equilibrated at 3.4 ml/min in the appropriate buffers, described above. After washing until the absorbance of the effluent at 214 nm returned to pre-injection levels, the column was developed with the appropriate gradient of acetonitrile as described above. Fractions were collected by hand. The absorbance of the effluent saturated the U.V. monitor, therefore, aliquots (10 μl) from column fractions that contained radioactivity were removed and chromatographed on an analytical C18 column to monitor purity.

Fractions that contained pure thiophosphorylated α-9mer were pooled and lyophilized to dryness. The overall yield was 10.6 mg (33%). During the 2 hour reaction, 68% of the starting material was thiophosphorylated. The recovery of thiophosphorylated peptide after HPLC purification was 73%, of which 66% was pure.

Fractions that contained pure thiophosphorylated α-14mer were pooled and concentrated to 5 mg/ml under vacuum to remove acetonitrile. Phosphate buffer is not volatile and was not removed, however it is compatible with most hapten-carrier protein coupling methods. The yield was 10.5 mg (33%). During the 5 hour reaction, greater than 95% of the starting material was thiophosphorylated. The recovery of thiophosphorylated peptide after C18 column chromatography was 60%, of which 60% was pure.

Coupling of Peptides to Carrier Protein

Peptides were coupled to keyhole limpet hemocyanin (KLH) with the crosslinking reagent succinimidyl 4-(N-maleimidomethyl) cyclohexane 1-carboxylate (SMCC; ref. 21). SMCC forms an amide bond with lysine residues on the carrier protein and a stable thioether bond with the free sulfhydryl group of cysteine, the ninth residue in both peptides. KLH derivatized with SMCC is available commercially (Pierce Chemical Co). Derivatized KLH (10 mg), supplied as a lyophilized powder containing buffer salts, was resuspended in 1.0 ml H_2O. Thiophosphorylated peptide, dissolved at 5 mg/ml in 0.1 M sodium phosphate (pH 6.5) was added to the resuspended KLH and allowed to react at room temperature for 4 hr. After the reaction, 10% to 15% of the thiophosphorylated peptide was acid precipitable indicating that it was conjugated to KLH. The molar ratio of peptide to crosslinking groups on KLH indicated that the reaction had gone nearly to

completion. Nonphosphorylated peptide was coupled to KLH in a similar manner. We found that it was not necessary to separate conjugated peptide from free peptide for immunization. Therefore, the antigen was stored in aliquots in the reaction buffer at -80°.

Production of Polyclonal Antibodies

New Zealand White female rabbits, aged 5 to 12 months, were immunized with conjugated peptides (0.6 mg KLH conjugated to peptide and 0.6 mg free peptide) emulsified in 1.0 ml of Ribi adjuvant. Half of the antigen was injected at two sites subcutaneously on one side of the back and half was injected at two sites intramuscularly in the opposite thigh. Successive boosts were made on alternate sides of the rabbit at 4 to 6 week intervals, starting at week 4. Test bleeds were taken 7 or 8 days after a boost. Rabbits that produced antibodies of interest were bled at three day intervals starting 9 days after a boost.

One of four rabbits immunized with conjugated nonphosphorylated α-14mer produced antisera that reacts with nonphosphorylated CaM kinase, but not with autophosphorylated kinase on Western blots at dilutions up to 1/5000 (Figure 3). The reaction with kinase is blocked by preabsorption of antiserum with free nonphosphorylated α-14mer. Five of six rabbits immunized with conjugated thiophosphorylated peptide (three with α-14mer, two with α-9mer) produced antisera that react with autophosphorylated CaM kinase on Western blots, but not with nonphosphorylated kinase. Three of these react at dilutions as high as 1/500.

Production of Monoclonal Antibodies

Two different strategies were used to produce monoclonal antibodies with specificity for autophosphorylated CaM kinase. For one method, mice were immunized with conjugated thiophosphorylated peptides. Mice were primed, then

boosted twice at 3 week intervals starting at week 3, by intraperitoneal injection of 100 μg KLH-peptide conjugate and 100 μg free peptide emulsified in 0.2 ml Ribi adjuvant. A final series of boosts were given without adjuvant on the three days preceding removal of the spleen and fusion (22-24). Several of the mice produced antisera with a high degree of selectivity for phosphorylated kinase, measured by enzyme-linked immunosorbent assay (ELISA; see below). Splenocytes from one of these mice was fused with HL-1 murine myeloma cells (Ventrex; Portland, ME) and media from the resulting hybridomas was screened for anti-phosphokinase antibodies by ELISA. Approximately 1200 hybridomas were screened, and 24 that react at least ten times more strongly with autophosphorylated CaM kinase than with nonphosphorylated kinase have been selected for further characterization.

The second strategy involved immunosuppression of the response to nonphosphorylated kinase followed by immunization with autophosphorylated kinase. The immunosuppression technique was originally developed to enhance the immune response to rare components in a mixture of antigens (25, 26). Most of the surface of the kinase holoenzyme remains unchanged by autophosphorylation and thus the majority of kinase epitopes are common to both nonphosphorylated and autophosphorylated kinase. Immunosuppression of the response to the shared epitopes seemed likely to enhance the production of antibodies specific for autophosphorylated kinase. Mice were first injected intraperitoneally with 100 μg of nonphosphorylated CaM kinase, purified from rat forebrain (27), and emulsified in Ribi adjuvant according to the suppliers instructions. The immune response was suppressed by the administration of 10 μg cyclophosphamide per gram body weight on the day of antigen injection and again on the two days following antigen injection. Control mice were immunized with nonphophorylated kinase or thiophosphorylated

kinase and were not immunosuppressed. Suppressed mice were immunized 2 weeks later with 100 μg thiophosphorylated CaM kinase prepared by autophosphorylation for 90 s at 30° in a solution containing 0.5 mM ATPγS, 0.05 mM Tris (pH 8.0), 10 mM $MgCl_2$, 0.7 mM EGTA, 1.0 mM $CaCl_2$, 25 mM dithiothreitol, 0.4 mg/ml kinase and 0.4 mg/ml calmodulin. The reaction was quenched by the addition of EDTA to a final concentration of 0.1 M, and cooled to 0°. The calmodulin concentration was reduced by precipitation of kinase in 60% ammonium sulfate. Precipitated kinase was collected by centrifugation, resuspended in phosphate buffered saline (PBS, 50 mM sodium phosphate (pH 7.3), 9% (w/v) NaCl), and emulsified in Ribi adjuvant. Nonphosphorylated kinase was treated identically in preparation for injection, except that ATPγS was ommitted from the reaction. Sera was taken from immunized mice 7 days after immunization and tested by ELISA as described below. The cycle of immunosuppression followed by immunization was repeated biweekly so that immunosuppression always preceded immunization by 2 weeks. After several cycles, the serum antibody response to nonphosphorylated kinase was reduced, and the response to phosphokinase was enhanced, compared to control mice that were not immunosuppressed (Fig. 4). On each of the three days prior to fusion, mice were boosted with thiophosphorylated kinase without adjuvant (23). Approximately 1500 hybridoma clones were screened from two fusions. Only two produced antibodies that react at least ten times more strongly with autophosphorylated CaM kinase than with nonphosphorylated kinase. An additional 23 showed at least 3 fold specificity. Because many more hybridomas were selected from mice immunized with conjugated thiophosphopeptide, we suspect that, in general, peptide immunization will be superior to the suppression method for preparation of phospho-site specific monoclonal antibodies.

ELISA Screen for Phosphokinase Specific Antibodies

The following differential ELISA was used for monitoring anti-phosphokinase antibody production both in test sera and in hybridoma supernatants. The screen, which was carried out in 96-well microtiter plates, was adapted from previously described procedures (22, 28).

Autophosphorylated and nonphosphorylated kinase was prepared under identical conditions, as described above. Kinase was cooled to 0° and diluted to 0.01 mg/ml with ice-cold phosphate buffered saline (PBS, pH 7.3). Thirty microliters, containing 0.30 μg kinase (5.5 pmol subunit), was added to each well of a 96 well polystyrene tissue culture plate. Phosphokinase and nonphosphokinase were added to alternate rows, and bound to the plates by incubation overnight at 4°. Unbound kinase was removed by three rapid washes with 100 μl PBS containing 0.05% Tween 20 (Tween-PBS). Thirty microliters of serum diluted in Tween-PBS containing 1% (v/v) normal goat serum (NGS), or hybridoma culture supernatant was added to a pair of wells, one containing phosphokinase, the other nonphosphokinase, and incubated 2 to 4 hr at room temperature, or overnight at 4°. Unbound primary antibodies were removed by three washes with Tween-PBS. Thirty μl of alkaline phosphatase-conjugated goat anti-mouse secondary antibody (Boehringer Mannheim Biochemicals), diluted 4000-fold in Tween-PBS plus 1% NGS, was added to each well. After a 1 to 4 hr incubation, unbound secondary antibodies were removed by two washes with Tween-PBS, followed by three washes with PBS. Wells were then washed for 5 min with 100 μl of 0.1 M $NaHCO_3$ (pH 9.8), 5 mM $MgCl_2$ (color development buffer), and 50 μl of 1 mg/ml p-nitrophenyl phosphate in color development buffer was added. Yellow alkaline phosphatase reaction product was quantified after one half hour to several hours of reaction by

measurement of absorbance at 405 nm in a microtiter plate reader. This method is both sensitive and reliable. In addition, it permits screening of as many as 700 hybridoma supernatants in 24 hours.

Conclusions

Immunization with synthetic thiophosphorylated peptides may permit the production of large quantities of antibodies targeted to particular phosphorylated sites on any protein, including proteins that are relatively rare and hard to purify. The only requirements are that the sequence of the protein is known and possible phosphorylation sites have been identified, for example, by their substrate consensus sequence (29). Such antibodies may then be used to identify which sites are functionally significant.

We have obtained phospho-site specific antibodies with the use of nine and fourteen residue thiophosphorylated peptide antigens; the fourteen residue peptide induced antibody production more consistently. It is possible that even longer peptides might be better antigens; however, such peptides can potentially contain many epitopes and the specificity of the immune response may be reduced.

The use of immunosuppression to promote the development of phosphoprotein-selective antibodies after immunization with the entire protein was less successful than immunization with peptides, producing only two highly selective antibodies from screens of approximately 1500 clones. However the strategy may be more successful with other proteins, and it has the advantage that it is not necessary to know the primary sequence of the protein antigen. A disadvantage is that relatively large amounts of protein are required for the immunizations.

Phospho-site specific antibodies produced by methods similar to those described here are likely to be invaluable reagents in the future for revealing the

complex biochemical mechanisms underlying neuronal plasticity.

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References

1. S. G. Miller, B. L. Patton, and M. B. Kennedy, *Neuron* **1**, 593 (1988).

2. P. I. Hanson, M. S. Kapiloff, L. L. Lou, M. G. Rosenfeld, and H. Schulman, *Neuron* **3**, 59 (1989).

3. C. M. Schworer, R. J. Colbran, J. R. Keefer, and T. R. Soderling, *J. Biol. Chem.* **263**, 13486 (1988).

4. G. Theil, A. J. Czernik, F. Gorelik, A. C. Nairn, and P. Greengard, *Proc. Natl. Acad. Sci. U. S. A.*, **85**, 6337 (1988).

5. B. L. Patton, S. G. Miller, and M. B. Kennedy, *J. Biol. Chem.* **265**, 11204 (1990).

6. R. J. Colbran and T. R. Soderling, *J. Biol. Chem.* **265**, 11213 (1990).

7. A. M. Crestfield, S. Moore and W.H. Stein, *J. Biol. Chem.* **238**, 622 (1963).

8. M. J. Waxdal, W. H. Konigsberg, W. L. Henley, and G. M. Edelman, *Biochemistry* **7**, 1959 (1968).

9. N. Axelrod, *Virology* **87**, 366 (1978).

10. W. B. Huttner and P. Greengard, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 5402 (1979).

11. A. Darbre (Ed.), *Practical Protein Chemistry. A Handbook*, John Wiley and Sons, Chichester, New York, (1986).

12. P. T. Matsudaira (Ed.), *A Practical Guide to Protein and Peptide Purification for Microsequencing*, Academic Press, Inc., San Diego, New York (1989).

13. P. Cohen, *Ann. Rev. Biochem.* **58**, 453 (1989).

14. H. Y. L. Tung, T. J. Resink, B. A. Hemmings, S. Shenolikar, and P. Cohen, *Eur. J. Biochem.* **138**, 635 (1984).

15. J. C. Bulinsky and G. G. Gundersen, *Methods in Enzymology* **134**, 453 (1986).

16. M. Zouhair Atassi, *Methods in Enzymology* **121**, 69 (1986).

17. S. B. H. Kent, *Ann. Rev. Biochem.* **57**, 957 (1988).

18. R. Roskowski, *Methods in Enzymol.* **99**, 3 (1985).

19. D. Gratecos and E. H. Fischer, *Biochem. Biophys. Res. Commun.* **58**, 960 (1974).

20. D. Cassel and L. Glaser *Proc. Natl. Acad. Sci. U. S. A.* **79**, 2231 (1982).

21. S. Yoshitake, Y. Yamada, E. Ishikawa, and R Masseyeff, *Eur. J. Biochem.* **101**, 395 (1979).

22. E. Harlow and D. Lane, in *Antibodies: a laboratory manual*, p. 553, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1988).

23. C. Stähli, T. Staehelin, V. Miggiano, J. Schmidt, and P Häring, *J. Immunol. Methods* **32**, 297 (1980).

24. G. Köhler and C. Milstein, *Nature* **256**, 495 (1975).

25. W. D. Matthew and P. H. Patterson, *Cold Spring Harbor Symp. Quant. Biol.* **48**, 625 (1983).

26. W. D. Matthew and A. W. Sandrock, Jr., *J. Immunol. Methods* **100**, 73 (1987).

27. S. G. Miller, and M. B. Kennedy, *J. Biol. Chem.* **260**, 9039 (1985).

28. E. Engvall and P. Perlmann, *J. Immunol.* **109**, 126 (1972).

29. A. M. Edelman, D. K. Blumenthal, E. G. Krebs, *Ann. Rev. Biochem.* **56**, 567 (1987).

Figure Legends

Figure 1. Schematic diagram of strategy for identifying autophosphorylation sites in the sequence of type II CaM kinase.

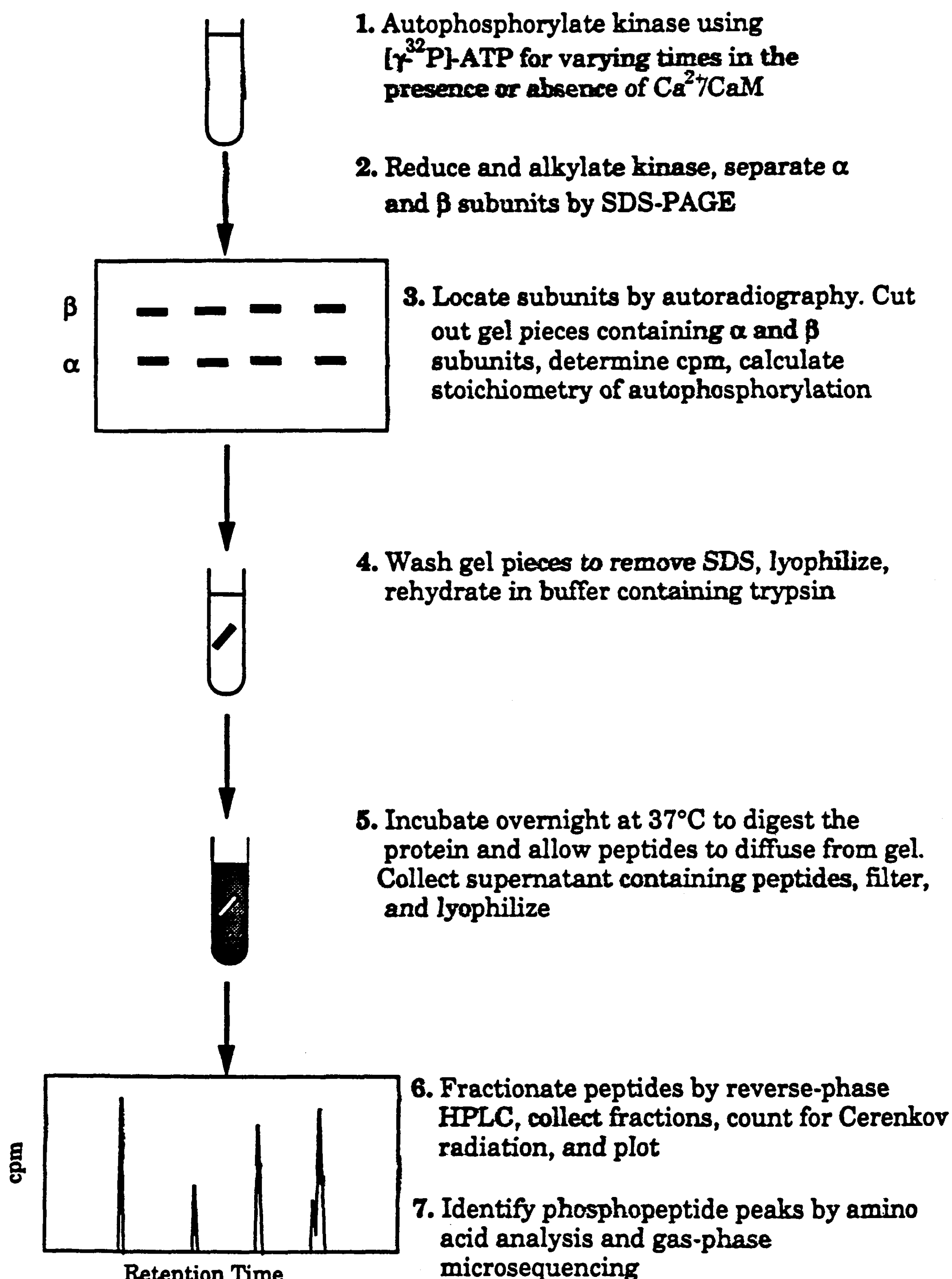
Figure 2. Purification of peptides α1, α1', β1 and β1' from tryptic digest of type II CaM kinase. Purified forebrain CaM kinase was autophosphorylated for 15 s, and the subunits were separated and digested with trypsin as described in the text. The resulting phosphopeptides were first fractionated by chromatography on a C4 reverse-phase column. Fractions containing four individual peptides were concentrated and fractionated again on a C18 column (A-D) as described in the text. The starred peaks were concentrated and sequenced by automatic gas-phase sequencing. [From S. G. Miller and M. B. Kennedy, *Neuron*, 1, 593 (1988), Figure 2., with permission of Cell Press, Cambridge.]

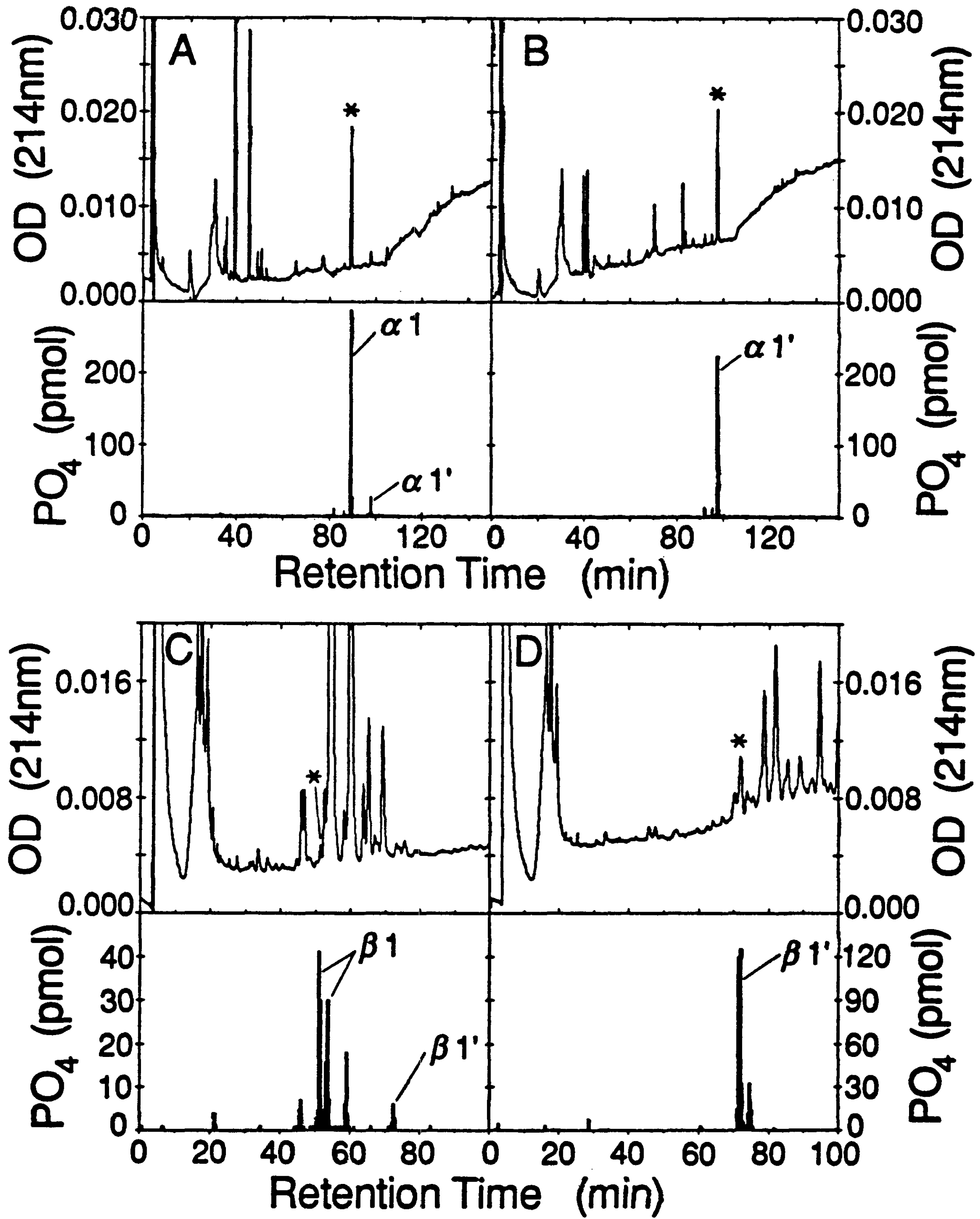
Figure 3. (A) Immunoblot of type II CaM kinase subunits with sera from rabbits immunized with nonphosphorylated α-14mer. Purified forebrain type II CaM kinase was autophosphorylated for 60 s in the presence of [γ-^{32}P]-ATP, as described in the text. An equivalent amount of kinase was incubated under identical conditions in the absence of [γ-^{32}P]-ATP. Aliquots of 0.5 μg of nonphosphorylated (N) or autophosphorylated (P) kinase were loaded into alternate lanes. The subunits (α and β) were then transferred to nitrocellulose membranes. The filter was cut into strips each containing a pair of lanes (N and P). A single strip contained only 0.1 μg of kinase in each lane and was stained for protein (AuroDye Forte, Janssen). The remaining strips were blocked and incubated overnight with 1/200 dilutions of serum from rabbit Annette, pre-immune serum from Sylvia, or antiserum from Sylvia.

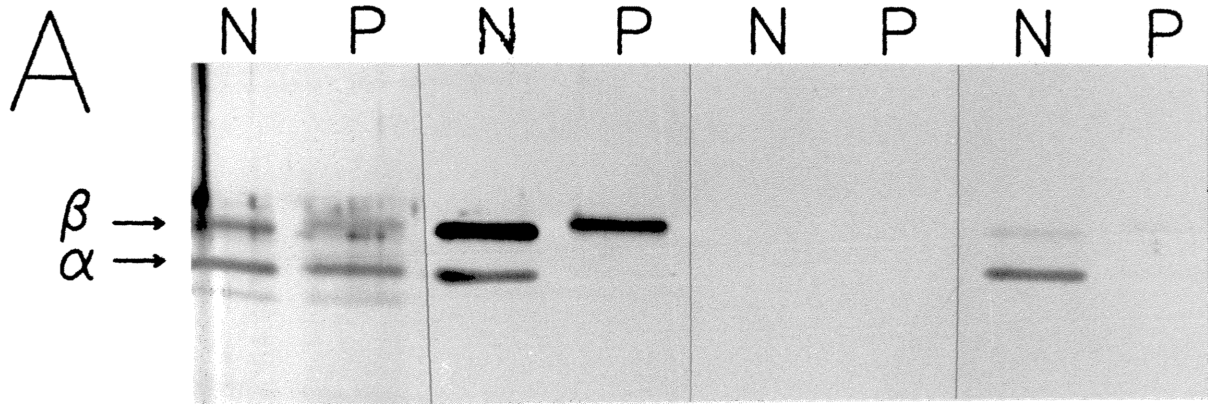
Annette was immunized several times with 200 μg purified CaM kinase holoenzyme in Polyinosine/polycytosine adjuvant (1/1 by weight) injected subcutaneously. Sylvia was immunized with α-14mer as described in the text. Bound antibodies were detected with goat anti-rabbit Ig secondary antibodies conjugated to alkaline phosphatase (Boehringer Mannheim). Alkaline phosphatase was visualized with 5-bromo 4-chloro 3-indolyl phosphate p-toluidine salt (BCIP) and p-nitro blue tetrazolium chloride (NBT) purchased from Biorad.

(B) Autoradiogram of immunoblots shown in (A).

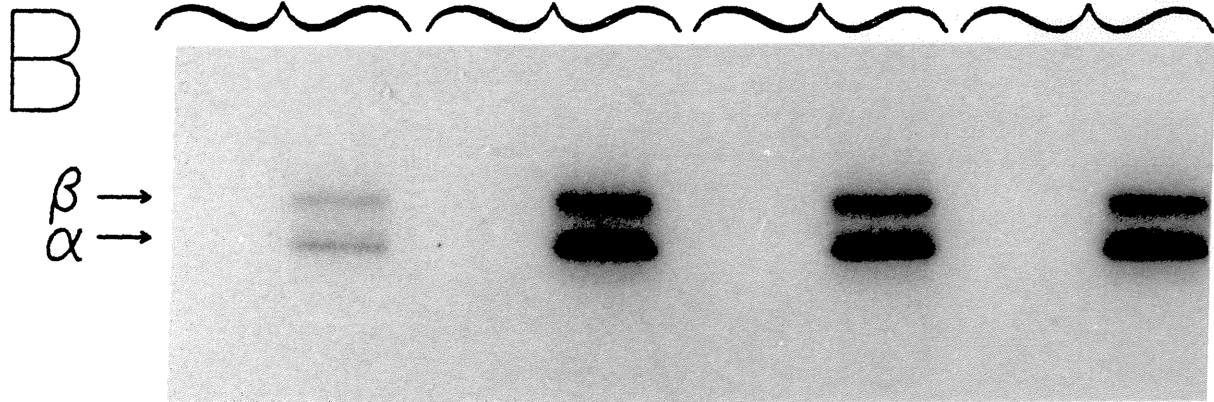
Figure 4. Enhancement of specificity of the serum response to autophosphorylated CaM kinase by immunosuppression methods. The immune response to nonphosphorylated CaM kinase was suppressed by coupling injection of nonphosphorylated CaM kinase with administration of cyclophosphamide into two mice (A and B) as described in the text. Immunosuppression was repeated at four week intervals indicated by (S), alternating with immunizing injections (I) of autothiophosphorylated CaM kinase. Two control mice (C and D) were immunized with autothiophosphorylated CaM kinase at four week intervals without immunosuppression. Sera from the tail, obtained at 7 days after each injection and diluted 1/80, were tested by ELISA for the presence of antibodies recognizing nonphosphorylated (open circles) and autophosphorylated (closed circles) CaM kinase as described in the text. Spleen cells from mouse A were fused in week 12. Of 800 hybridomas screened, 8 bound 3-times more strongly to phosphokinase than to nonphosphokinase in the ELISA. Spleen cells from mouse B were fused in week 8. Of 700 clones screened, 2 bound 10-times more strongly and 15 bound 3-times more strongly to phosphokinase than to nonphosphokinase in the ELISA (see text).

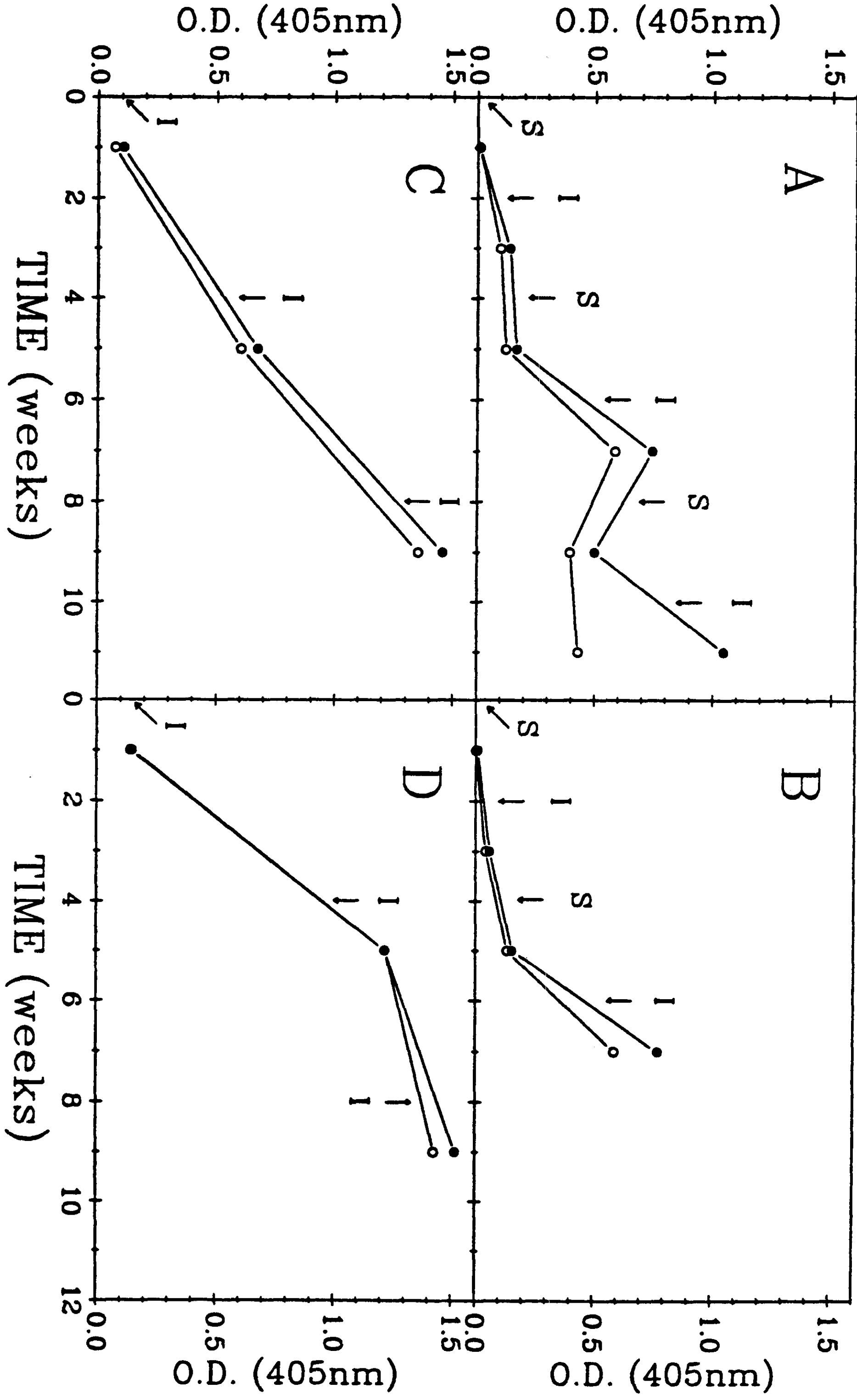

1. Autophosphorylate kinase using [γ-32P]-ATP for varying times in the presence or absence of Ca2+/CaM
2. Reduce and alkylate kinase, separate α and β subunits by SDS-PAGE
β
α
3. Locate subunits by autoradiography. Cut out gel pieces containing α and β subunits, determine cpm, calculate stoichiometry of autophosphorylation
4. Wash gel pieces to remove SDS, lyophilize, rehydrate in buffer containing trypsin
5. Incubate overnight at 37°C to digest the protein and allow peptides to diffuse from gel. Collect supernatant containing peptides, filter, and lyophilize
cpm
Retention Time
6. Fractionate peptides by reverse-phase HPLC, collect fractions, count for Cerenkov radiation, and plot
7. Identify phosphopeptide peaks by amino acid analysis and gas-phase microsequencing


A
B
C
D
OD (214nm)
PO4 (pmol)
Retention Time (min)
α1
α1'
β1
β1'
*



PROTEIN ANNETTE PREIMMUNE SYLVIA SYLVIA




A
B
C
D
O.D. (405nm)
O.D. (405nm)
O.D. (405nm)
O.D. (405nm)
TIME (weeks)
TIME (weeks)
S
I
0.0
0.5
1.0
1.5
0
2
4
6
8
10
12