BRAIN TYPE II CALCIUM AND CALMODULIN-DEPENDENT PROTEIN KINASE: CHARACTERIZATION OF A BRAIN-REGION SPECIFIC ISOZYME AND REGULATION BY AUTOPHOSPHORYLATION

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Abstract

A variety of molecular approaches have been used to investigate the structural and enzymatic properties of rat brain type II Ca^{2+} and calmodulin-dependent protein kinase (type II CaM kinase). This thesis describes the isolation and biochemical characterization of a brain-region specific isozyme of the kinase and also the regulation the kinase activity by autophosphorylation.

The cerebellar isozyme of the type II CaM kinase was purified and its biochemical properties were compared to the forebrain isozyme. The cerebellar isozyme is a large (500-kDa) multimeric enzyme composed of multiple copies of 50-kDa α subunits and 60/58-kDa β/β′ subunits. The holoenzyme contains approximately 2 α subunits and 8 β subunits. This contrasts to the forebrain isozyme, which is also composed of α and β/β′ subunits, but they are assembled into a holoenzyme of approximately 9 α subunits and 3 β/β′ subunits. The biochemical and enzymatic properties of the two isozymes are similar. The two isozymes differ in their association with subcellular structures. Approximately 85% of the cerebellar isozyme, but only 50% of the forebrain isozyme, remains associated with the particulate fraction after homogenization under standard conditions. Postsynaptic densities purified from forebrain contain the forebrain isozyme, and the kinase subunits make up about 16% of their total protein. Postsynaptic densities purified from cerebellum contain the cerebellar isozyme, but the kinase subunits make up only 1-2% of their total protein.

The enzymatic activity of both isozymes of the type II CaM kinase is regulated by autophosphorylation in a complex manner. The kinase is initially completely dependent on Ca^{2+}/calmodulin for phosphorylation of exogenous substrates as well as for autophosphorylation. Kinase activity becomes partially Ca^{2+}-independent after autophosphorylation in the presence of Ca^{2+}/calmodulin. Phosphorylation of only a few subunits in the dodecameric holoenzyme is sufficient to cause this change, suggesting an allosteric interaction between subunits. At the same time, autophosphorylation *itself* becomes independent of Ca^{2+}. These observations suggest that the kinase may be able to exist in at least two stable states, which differ in their requirements for Ca^{2+}/calmodulin.

The autophosphorylation sites that are involved in the regulation of kinase activity have been identified within the primary structure of the α and β subunits. We used the method of reverse phase-HPLC tryptic phosphopeptide mapping to isolate individual phosphorylation sites. The phosphopeptides were then sequenced by gas phase microsequencing. Phosphorylation of a single homologous threonine residue in the α and β subunits is correlated with the production of the Ca^{2+}-independent activity state of the kinase. In addition we have identified several sites that are phosphorylated only during autophosphorylation in the absence of Ca^{2+}/calmodulin.

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CHAPTER 1

INTRODUCTION

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1 Summary.

The body of this thesis (chapters 2-5) reports the results of studies on the biochemical structure and enzymatic regulation of the neuronal type II Ca^{2+}/calmodulin-dependent protein kinase. This kinase is activated by the second messenger Ca^{2+} via the ubiquitous Ca^{2+}-binding protein calmodulin. In this chapter I would like to review briefly the concept of second messengers and describe in some detail the structure and regulation of the other known Ca^{2+}/calmodulin-dependent protein kinases. In particular I have focused on the role of phosphorylation in the regulation of these kinases.

Chapter 2 of this thesis is concerned with the association of isozymes of the neuronal type II Ca^{2+}/calmodulin-dependent protein kinase with the postsynaptic density, a subcellular structure unique to the central nervous system. The exact structure and function of the postsynaptic density is poorly understood at this time. I have included a discussion of the current knowledge of this intriguing structure in this introductory material.

2 Second Messengers.

The effects of a wide variety of extracellular signals, such as hormones and neurotransmitters, are mediated by alterations in the concentration of intracellular "second messengers" (Sutherland *et al.*, 1968). The extracellular molecules are in general hydrophilic and therefore incapable of crossing the lipid bilayer of the cell membrane. They alter the concentration of the intracellular second messenger by binding to cell surface receptor molecules and inducing conformational changes that transmit the signal to the cell interior. The translation of receptor activation into a change in second-messenger concentration occurs through a variety of mechanisms. These mechanisms include the activation of enzymatic activities that may either synthesize (e.g., adenylate and guanylate cyclases,) or degrade (e.g., phosphodiesterases) the second messenger (reviewed in Robison *et al.*, 1971; Goldberg and Haddox, 1977); the opening of specific ion channels permeable to the second messenger (Ca^{2+}, reviewed in Carafoli, 1987); and the release of the second messenger (Ca^{2+}) from intracellular stores (Streb *et al.*, 1983; Carafoli, 1987). The most widely studied second messengers to date are adenosine (3′-5′)-cyclic monophosphate (cAMP), guanosine (3′-5′)-cyclic monophosphate (cGMP), 1,2-diacylglycerol (DAG), D-inositol 1,4,5-trisphosphate ($InsP_3$), and Ca^{2+} ion. Recent studies have shown that certain metabolites of arachidonic acid may also act as second messengers (reviewed in Axelrod *et al.*, 1988), and it is likely that other examples will be found in the future.

The cyclic nucleotides, cAMP and cGMP, are formed by the action of adenylate and guanylate cyclases on ATP and GTP (Goldberg and Haddox, 1977; Rall, 1982). In most cells the actions of cAMP and cGMP are mediated by two specific protein kinases: the cAMP-dependent protein kinase and the cGMP-dependent protein kinase. These two kinases are serine/threonine kinases with relatively broad substrate specificities. Their biochemical and enzymatic properties have been extensively investigated (reviewed in Beavo and Mumby, 1982; Goldberg and Haddox, 1977). Until recently cAMP and cGMP were believed to act **exclusively** through the activation of the cAMP- and the cGMP-dependent protein kinases in eukaryotes (Walsh *et al.*, 1968; Kuo and Greengard, 1969; Kuo and Greengard, 1970; Goldberg and Haddox, 1977). This dogma has recently been challenged by the finding that cyclic nucleotides are capable of directly regulating ion channels in both visual and olfactory transduction (Fesenko *et al.*, 1985; Haynes and Yau, 1985; Nakamura and Gold, 1987; Gold and Nakamura, 1987). The second-messenger signals of cAMP and cGMP are terminated by the action of phosphodiesterases, which hydrolyze them to AMP and GMP, respectively (Goldberg and Haddox, 1977; Rall, 1982).

The role of diacylglycerol as a second messenger has recieved significant attention in recent years. DAG and $InsP_3$ are produced by the action of phospholipase C on membrane inositol phospholipids. $InsP_3$ releases Ca^{2+} from intracellular stores while DAG appears to act through the activation of a unique kinase, protein kinase C, by increasing its sensitivity to Ca^{2+} ion (Kishimoto *et al.*, 1980; Takai *et al.*, 1979). Protein kinase C, discussed in more

detail below, is a serine/threonine kinase with a broad substrate specificity that translocates from the cytosol to the membrane during activation. Diacylglycerol therefore resembles the cyclic nucleotides in that its actions are mediated by a specific protein kinase with broad substrate specificity. Diacylglycerol is rapidly removed by reincorporation into membrane phospholipids or further breakdown into arachidonic acid, thereby terminating the signal. $InsP_3$ is primarily eliminated by hydrolysis of the 5-phosphate (Berridge and Irvine, 1984; Berridge, 1987).

In contrast to the cyclic nucleotides and diacylglycerol, Ca^{2+} ion appears to regulate the activities of a number of target proteins. Ca^{2+}-regulated proteins include both adenylate and guanylate cyclases (Brostrom *et al.*, 1975; Cheung *et al.*, 1975; Kakiuchi *et al.*, 1981), a cyclic-nucleotide phosphodiesterase (Teo and Wang, 1973; Kincaid and Vaughan, 1986), a protein phosphatase (Yang *et al.*, 1982; Stewart *et al.*, 1983), a phospholipase (phospholipase A_2, Wong and Cheung, 1979), a protease (Pontremoli and Melloni, 1986), and several protein kinases (Stull *et al.*, 1986; Kennedy *et al.*, 1987). Many of these actions of Ca^{2+} are mediated by the ubiquitous Ca^{2+}-binding protein calmodulin (see below). The Ca^{2+} signal is terminated by the binding of Ca^{2+} by intracelluar proteins and membrane transport either to the extracellular space or to intracellular storage compartments (Carafoli, 1987).

Most, but not all, of the actions of second messengers ultimately involve control of the phosphorylation/dephosphorylation of proteins (Krebs and Beavo, 1979; Cohen, 1980a). In some cases this is the direct result of the

activation/inactivation of the kinases/phosphatases by the second messenger. In other cases the mechanism is indirect, as in the Ca^{2+}-dependent regulation of cyclases and phosphodiesterases, thereby indirectly regulating the activities of the cyclic nucleotide-dependent kinases.

It is clear that second-messenger systems do not operate independently, but rather interact with one another at various levels. Therefore a complete understanding of the responses to any single second messenger must ultimately take into account interactions with each of the other systems. This *synarchic* relationship has been discussed in detail by Rasmussen (1970, 1981) and Berridge (1975) (for Ca^{2+} and cAMP) and Berridge (1983) (for Ca^{2+} and diacylglycerol). Three examples that demonstrate the interaction of the second messenger systems are the phosphorylation and activation of phosphorylase kinase (a calmodulin-dependent kinase) by the cAMP-dependent protein kinase (Krebs *et al.*, 1959; Krebs *et al.*, 1964; DeLange *et al.*, 1968; Walsh *et al.*, 1971); the activation of guanylate cyclase by protein kinase C (Zwiller *et al.*, 1985); and the stimulation of cyclic nucleotide phosphodiesterase by Ca^{2+}/calmodulin (Teo and Wang, 1973). The regulational "repertoire" of a particular cell therefore depends on its specific combination of receptors, effectors (the components that produce/degrade the second messengers: cyclase, phospholipase, ion channels, etc.), second-messenger target proteins (often kinases), and substrates (structural proteins, enzymes).

3 Ca^{2+} Target Proteins.

The concentration of **free** Ca^{2+} ion in the cytosol ($[Ca^{2+}]_i$) is normally maintained at a very low level (10-100n*M*)(Carafoli, 1987). Stimulation of the cell by hormones, neurotransmitters, and other signals (e.g., antigen binding to T-lymphocytes [Oettgen *et al.*, 1985]) can result in increases in $[Ca^{2+}]_i$ to 1μ*M* or higher (Michell *et al.*, 1981; Berridge, 1984). The increase in $[Ca^{2+}]_i$ is generally transient but can be sustained (Kojima *et al.*, 1986; Takuwa *et al.*, 1987) and in some cell types may undergo oscillations lasting for seconds to minutes (Rapp and Berridge, 1981; Schlegel *et al.*, 1987; Wilson *et al.*, 1987; Woods *et al.*, 1987; Ambler *et al.*, 1988). The source of the Ca^{2+} may be extracellular, entering the cell through membrane channels; intracellular, being released from intracellular stores; or a combination of the two. A variety of membrane channels have been described that allow Ca^{2+} to enter the cell when activated. These channels can be gated by membrane voltage, neurotransmitters, second messengers, or a combination of these methods (e.g., NMDA receptors). D-Inositol 1,4,5-trisphosphate ($InsP_3$) appears to be responsible for release of Ca^{2+} from intracellular stores (Berridge, 1987). Although the main store of $InsP_3$-releasable Ca^{2+} has often been localized to the endoplasmic reticulum, recent work (Volpe *et al.*, 1988) suggests the possibility that a specialized organelle (the "calciosome") may be the target of $InsP_3$. Muscle cells seem to represent a special case in which Ca^{2+} is released from an intracellular source (the sarcoplasmic reticulum) by voltage-gated channels.

When the cytosolic concentration rises to sufficient levels, Ca^{2+}-binding proteins complex with Ca^{2+} and remain in this state until $[Ca^{2+}]_i$ returns to resting levels and Ca^{2+} dissociates. Calmodulin appears to be the major Ca^{2+}-binding protein responsible for the known physiological effects of increased cytosolic Ca^{2+} concentrations in eukaryotic cells. A large and diverse group of proteins are regulated by binding the Ca^{2+}/calmodulin complex. Several proteins that are highly related to calmodulin (troponin c, parvalbumin) are responsible for Ca^{2+} regulation in specialized cells (see below). A second major Ca^{2+} effector in eukaryotes is protein kinase C, a serine/threonine kinase regulated by Ca^{2+}, lipid, and diacylglycerol (see below). In addition Ca^{2+} regulates a number of processes either directly (e.g., Ca^{2+}-activated K^+-channels [Meech, 1978]) or through mechanisms that are not yet understood (e.g., the release of neurotransmitters [Reichardt and Kelly, 1983]).

3.1 Calmodulin.

Calmodulin was first described by Cheung (1967) as an activator of the 3′,5′-nucleotide phosphodiesterase from bovine brain. The work of Teo and Wang (1973) later demonstrated that this protein bound Ca^{2+} and that it was the Ca^{2+}-complexed form that activated the phosphodiesterase. It is a single polypeptide chain of M_r 16,700 (Watterson *et al.*, 1980), which is capable of binding four Ca^{2+} ions (Lin *et al.*, 1974; Teo and Wang, 1973). The binding of Ca^{2+} induces a conformational change in the structure of the molecule that allows it to bind reversibly to other molecules (Klee *et al.*, 1980). The Ca^{2+}/calmodulin-enzyme complex is the active form of the enzyme.

Calmodulin is widely distributed; it is found in nearly every eukaryotic species and tissue examined, suggesting a generalized role in the regulation of cellular processes (Klee *et al.*, 1980). The distribution of calmodulin does not match that of any of the known calmodulin-dependent enzymes, which again suggests that it is not specialized to serve any specific cellular function. The primary structure of calmodulin is highly conserved between species (Klee *et al.*, 1980). This may be the result of evolutionary contraints on a protein that must interact specifically with a large group of proteins.

Calmodulin regulates a diverse array of physiological processes by alteration of specific enzymatic activities. Enzymes known to be regulated by calmodulin include a cyclic nucleotide phosphodiesterase (Kakiuchi and Yamazaki, 1970), adenylate and guanylate cyclases (Birnbaumer, 1973; Nagao *et al.*, 1979), several protein kinases (Stull *et al.*, 1986; Kennedy *et al.*, 1987), NAD kinase (Anderson and Cormier, 1978), plasma membrane and sarcoplasmic reticulum Ca^{2+}-ATPase (Larsen and Vincenzi, 1979; Katz and Remtulla, 1978; LePeuch *et al.*, 1979; Sobue *et al.*, 1979), phospholipase A_2 (Wong and Cheung, 1979), and the protein phosphatase calcineurin (Yang *et al.*, 1982). This list emphasizes the high degree of interaction among different second-messenger pathways mentioned above. Ca^{2+}/calmodulin directly regulates the activities of the enzymes that produce and degrade cyclic nucleotides, as well as the enzyme that generates arachidonic acid (phospholipase A_2).

Several proteins that are closely related to calmodulin are responsible for Ca^{2+} regulation in specialized cell types. Troponin C is found in muscle cells and is responsible for the Ca^{2+}-dependence of skeletal muscle contraction. Troponin C shares approximately 50% sequence identity with calmodulin and the similarity rises to approximately 78% if conservative amino acid substitutions are included (Klee *et al.*, 1980). Parvalbumin, another calmodulin-related protein found in muscle, is a soluble Ca^{2+}-binding protein found in the sarcoplasmic reticulum (reviewed in Wnuk *et al.*, 1982). While the exact functional role of parvalbumin is not currently known, it may be involved in increasing the rate of muscle relaxation by sequestering Ca^{2+} (Wnuk *et al.*, 1982).

3.2 Protein Kinase C.

Hokin and Hokin (1953) were the first to note the increased turnover of membrane inositol phospholipids in response to cell surface receptor activation using pancreatic slices treated with acetylcholine. Since that time it has been shown that a number of cell types respond to a variety of signals with an increased rate of breakdown and resynthesis of inositol phospholipids (Irvine *et al.*, 1982). Activation of a variety of cell surface receptors results in the transient activation of phospholipase C, which hydrolyzes membrane phospholipids, specifically inositol phospholipids. Phosphatidylinositol 4,5-bisphosphate (PtdIns-4,5-P_2) appears to be the physiologically relevent inositol phospholipid that is hydrolyzed during signal transduction (Berridge, 1987). Hydrolysis of PtdIns-4,5-P_2 releases D-inositol 1,4,5-trisphosphate ($InsP_3$),

which mediates the release of Ca^{2+} from intracellular stores (Berridge, 1987). The other product of PtdIns-4,5-P_2 breakdown is 1,2-diacylglycerol (DAG), which remains in the membrane and is responsible for activation of protein kinase C (Kikkawa and Nishizuka, 1986). The two second messengers produced by hydrolysis of PtdIns-4,5-P_2, $InsP_3$ and DAG are rapidly removed by separate processes, thus terminating the signal. The dominant mechanism for elimination of $InsP_3$ appears to be hydrolysis of the 5-phosphate by a specific phosphatase (Berridge and Irvine, 1984; Berridge, 1987). Diacylglycerol is rapidly removed by resynthesis into phospholipids (via phosphatidic acid) and also by further degradation to arachidonic acid (which can be further processed to form several active metabolites) (Kikkawa and Nishizuka, 1986; Berridge, 1987).

Protein kinase C was originally described in brain tissue as a serine/threonine kinase which was activated by a Ca^{2+}-dependent protease (Inoue *et al.*, 1977; Kishimoto *et al.*, 1983). The activity of protein kinase C is now known to be regulated by three factors: Ca^{2+}, phospholipids, and diacylglycerol. Phosphatidylserine is an absolute requirement; other phospholipids are unable to activate the enzyme, although they are capable of modulating the activation of protein kinase C (Takai *et al.*, 1979; Kaibuchi *et al.*, 1981). Diacylglycerol acts by dramatically increasing the affinity of the kinase for phospholipid and Ca^{2+} (Kishimoto *et al.*, 1980). When activated by diacylglycerol, the kinase is believed to be active at or near the resting levels of Ca^{2+} found within the cell. The physiological relevance of activation by proteolysis mentioned above is not known at this time. Proteolytic activation of protein kinase C has been

shown to occur *in vivo* after treatment of cells with phospholipase C (Tapley and Murray, 1984). Protein kinase C is also activated by a number of compounds known collectively as phorbol esters (Kikkawa and Nishizuka, 1986). These compounds are tumor promoters, raising the possibility of the involvement of protein kinase C in tumor formation and carcinogenesis (Nishizuka, 1984). Thus, this kinase has been the subject of intensive research.

Protein kinase C has been purified from a number of tissues and is composed of a single polypeptide of M_r 77-83,000 (Kikkawa *et al.*, 1982; Wise *et al.*, 1982; Walsh *et al.*, 1984; Parker *et al.*, 1984; Woodgett and Hunter, 1987; Azhar *et al.*, 1987). The complete primary structure of protein kinase C has been determined by cloning and sequencing cDNAs complementary to rat (Knopf *et al.*, 1986) and bovine (Parker *et al.*, 1986) brain mRNAs. These studies indicate that at least three isozymes of protein kinase C are present in rat and cow. The isozymes appear to originate by transcription of separate genes rather than alternative splicing of mRNA. The physiological significance of multiple isozymes of the kinase is presently unknown.

Protein kinase C exhibits a broad substrate specificity *in vitro*, phosphorylating proteins at both serine and threonine, but not tyrosine, residues. The kinase also autophosphorylates on serine and threonine residues when activated. Autophosphorylation is a common property of protein kinases, often regulating kinase activity, but the function of protein kinase C autophosphorylation (if any) is currently unknown (Kikkawa *et al.*, 1982; Huang *et al.*, 1986; Mochly-Rosen and Koshland, 1987; Newton and Koshland, 1987).

Proteins that have been shown to be substrates for the kinase *in vitro* include membrane proteins, cytoskeletal elements, soluble enzymes, and membrane receptors (reviewed in Kikkawa and Nishizuka, 1986). The physiological relevance of phosphorylation of many of these substrates by protein kinase C *in vivo* remains to be established.

An important aspect of the activation of protein kinase C is its subcellular redistribution during activation. Protein kinase C translocates from the cytosol to a membrane-bound form during activation (Naor *et al.*, 1985) and this is mediated in part by Ca^{2+} (Wolf *et al.*, 1985; May *et al.*, 1985). This translocation may be the critical role of the increase in Ca^{2+} in this pathway. Ca^{2+} also appears to increase the affinity of protein kinase C for diacylglycerol (Kojima *et al.*, 1985; Dougherty *et al.*, 1986). When cells are stimulated by the activation of phospholipase C, the response is generally a transient rise in both Ca^{2+} and diacylglycerol, resulting in a synergistic activation of protein kinase C by the two pathways. Although this may be the common method of protein kinase C activation, in some cells it may be activated solely by the influx of Ca^{2+} through voltage-gated membrane channels (Brocklehurst *et al.*, 1985).

4 Ca^{2+}/Calmodulin-Dependent Protein Kinases.

Five protein kinases that are activated by Ca^{2+} and calmodulin have been well characterized in mammalian systems (Hunter, 1987; Kennedy *et al.*, 1987). In addition a number of other Ca^{2+}/calmodulin-stimulated kinase activities have been described but not fully characterized. In this section the structural and enzymatic properties of the well-characterized Ca^{2+}/calmodulin-stimulated kinases are reviewed. The regulation of these kinases by phosphorylation, both by exogenous kinases and autophosphorylation, is emphasized.

4.1 Phosphorylase Kinase.

Glycogen metabolism in liver and skeletal muscle is regulated by both neuronal and hormonal inputs *via* the second messengers Ca^{2+} and cAMP. Phosphorylase kinase catalyzes the conversion of inactive phosphorylase *b* to its active form, phosphorylase *a*, *via* phosphorylation and thus stimulates glycogenolysis. The activity of phosphorylase kinase in skeletal muscle and liver is regulated by at least two distinct mechanisms. Kinase activity is dependent on an increase in intracellular Ca^{2+}, which can result from either neuronal or hormonal (α-adrenergic) inputs. The catalytic activity of the kinase is also activated (although it still requires Ca^{2+}) by phosphorylation by the cAMP-dependent protein kinase. This pathway is the consequence of hormonal inputs that stimulate adenylate cyclase (i.e., β-adrenergic stimulation). Thus the regulation of phosphorylase kinase activity by both Ca^{2+} and cAMP provides an excellent example of a synarchic relationship between

these two second-messenger systems.

Phosphorylase kinase has been purified from a number of tissue sources including rabbit skeletal muscle (Cohen, 1973; Hayakawa *et al.*, 1973), bovine cardiac muscle (Cooper *et al.*, 1980), rat liver (Chrisman *et al.*, 1982), and dogfish skeletal muscle (Pocinwong *et al.*, 1981). The structural properties of phosphorylase kinase have been most extensively studied in rabbit skeletal muscle. In addition, phosphorylase kinase activity has been described in other tissues and species, although its exact molecular characteristics are not known in every case.

4.1.1 Structure and Activity.

Rabbit skeletal muscle phosphorylase kinase is composed of four subunits, α (M_r 118-145,000), β (M_r 108-128,000), γ (M_r 41-45,000), and δ (M_r 17,000) assembled into a tetramer $(\alpha\beta\gamma\delta)_4$ of M_r 1.2×10^6 (Cohen, 1973; Hayakawa *et al.*, 1973; Carlson *et al.*, 1979). Slow- and fast-twitch skeletal muscles appear to contain different isozymes of phosphorylase kinase. In fast-twitch muscle the predominant enzyme contains an α subunit of M_r 145,000, while in slow-twitch muscle an α′ subunit of M_r 133,000 is found (Jennissen and Heilmeyer, 1974; Burchell *et al.*, 1976). Although the exact functional significance of isozymic forms has not been determined, it was found that they differ in their ability to be activated by exogenous calmodulin (see below)(Sharma *et al.*, 1980; Wang *et al.*, 1981).

The determination of the functional role of each subunit of phosphorylase kinase has been the subject of many investigations. The δ subunit is identical with calmodulin with the exception of two amide substitutions (Grand *et al.*, 1981) and a single phosphate (Crabb and Heilmeyer, 1984). The δ subunit is assumed to be responsible for the Ca^{2+} sensitivity of phosphorylase kinase in the absence of exogenous calmodulin. Phosphorylase kinase differs from most other calmodulin-regulated enzymes in that it contains calmodulin as a tightly bound subunit (δ) in the absence of Ca^{2+}. Phosphorylase kinase activity can also be stimulated by exogenous Ca^{2+}/calmodulin or troponin C (De-Paoli-Roach *et al.*, 1979; Cohen, 1980c), but this is subject to regulation by autophosphorylation (below).

Several lines of evidence suggest that the γ subunit is the catalytic subunit of the enzyme. The initial observation leading to this conclusion was that limited tryptic digestion of the holoenzyme causes activation and degrades the α and β subunits but not the γ subunit (Hayakawa *et al.*, 1973b). Treatment of phosphorylase kinase with LiBr at 0°C allowed the isolation of αγδ and γδ complexes and the isolated γ subunit, all of which retain catalytic activity (Chan and Graves, 1982a, 1982b; Skuster *et al.*, 1980; Kee and Graves, 1986). The isolated γ subunit retains a high rate of catalytic activity in the absence of Ca^{2+} but is stimulated 1-2 fold by the addition of Ca^{2+}/calmodulin (Kee and Graves, 1986). The entire amino acid sequence of the skeletal muscle γ subunit has been determined, and examination shows that it shares extensive sequence similarity with known protein kinases (Reimann *et al.*, 1984; da Cruz e Silva and Cohen, 1987; Bender and Emerson, 1987).

The α and β subunits appear to be involved in the regulation of kinase activity. The catalytic activity of the isolated γ subunit is inhibited by the addition of the αβ complex (Paudel and Carlson, 1987). The α and β subunits both bind exogenous calmodulin (the endogenous δ subunit appears to interact mainly with the γ subunit [Picton *et al.*, 1980]) and this may relieve their inhibitory effect. Phosphorylation of both the α and β subunits by the cAMP-dependent protein kinase or autophosphorylation is involved in regulation of activity (see below). In addition some evidence has been presented that suggests that the β subunit may be catalytic (Fischer *et al.*, 1978; Malencik and Fischer, 1982; Gulyaeva *et al.*, 1972; King and Carlson, 1981; King *et al.*, 1982). Determination of the primary structure of the α and β subunits may help to clarify their role completely.

The activity of skeletal-muscle phosphorylase kinase is subject to regulation by a number of factors. In addition to activation by Ca^{2+} the kinase is activated by phosphorylation by the cAMP-dependent protein kinase (Walsh *et al.*, 1971; Cohen, 1973; below), by autophosphorylation (Krebs *et al.*, 1964; Walsh *et al.*, 1971; below), and by exogenous calmodulin (DePaoli-Roach *et al.*, 1979; Shenolikar *et al.*, 1979). In each case concentrations of Ca^{2+} in the micromolar range is required for activity. A number of substrates other than phosphorylase *b* have been described *in vitro*. These substrates include glycogen synthase (Roach *et al.*, 1987), troponin I (Stull *et al.*, 1972), troponin T (Perry and Stull, 1974), casein (DePaoli-Roach *et al.*, 1981), histone H1 (Tabuchi *et al.*, 1981), myelin basic protein (Grand and Perry, 1979), a component of the sarcoplasmic reticulum that leads to increased Ca^{2+} uptake (Scwhartz *et al.*,

1976), and a component of the cardiac sarcolemma that leads to a stimulation of Na^+/K^+-ATPase activity (St. Louis *et al.*, 1977). The physiological relevance of the ability of phosphorylase kinase to catalyze phosphorylation of these substrates remains to be determined.

4.1.2 Phosphorylation.

Phosphorylase kinase is capable of undergoing reversible activation and deactivation by a mechanism involving phosphorylation and dephosphorylation of the α and β subunits. The kinase is phosphorylated and activated by the cAMP-dependent protein kinase and also undergoes Ca^{2+}-dependent autophosphorylation. Several other protein kinases have been shown to phosphorylate phosphorylase kinase, but the functional significance of these reactions is unknown.

4.1.2.1 Phosphorylation of Phosphorylase Kinase by the cAMP-Dependent Protein Kinase.

Early studies on the activation of phosphorylase kinase by phosphorylation were hampered by the presence of contaminating cAMP-dependent protein kinase. Eventually analysis of the dependence of phosphorylation on Ca^{2+}, cAMP, MgATP, and the heat-stable inhibitor of the cAMP-dependent protein kinase allowed the involvement of autophosphorylation and cAMP-dependent protein kinase catalyzed phosphorylation to be studied independently (Krebs *et al.*, 1959; Krebs *et al.*, 1964; DeLange *et al.*, 1968;

Walsh *et al.*, 1971).

Phosphorylation of phosphorylase kinase by the cAMP-dependent protein kinase results in the rapid incorporation of phosphate into the β subunit followed by slower incorporation into the α subunit (Hayakawa *et al.*, 1973; Cohen, 1973). This phosphorylation results in activation of kinase activity. No phosphorylation of the γ or δ subunits has been reported. In skeletal muscle it has been suggested that significant phosphorylation of the α subunit does not occur until at least 1-2 mol PO_4 has been incorporated into each β subunit (Pickett-Gies and Walsh, 1985).

The maximal stoichiometry of phosphorylation of the β subunit is typically less than four moles per mole of holoenzyme ($(\alpha\beta\gamma\delta)_4$). Maximal β subunit stoichiometries of 2-3 mols PO_4/ mol $(\alpha\beta\gamma\delta)_4$ have typically been reported (Hayakawa *et al.*, 1973; Pickett-Gies and Walsh, 1985; Malencik and Fischer, 1979). Although purified phosphorylase kinase contains endogenous phosphate (Crabb and Heilmeyer, 1984; Kilimann *et al.*, 1984), Pickett-Gies and Walsh (1985) were able to show, using specific protein phosphatases, that the substoichiometric phosphorylation was not the result of endogenous phosphate at these sites. It is most likely to be the result of site-site interactions (i.e., negative allostery) within the holoenzyme. Singh and Wang (1977) found higher stoichiometries of phosphate incorporation if one used either high concentrations of Mg^{2+} (10m*M*) or high concentrations

of the catalytic subunit of cAMP-dependent protein kinase. Phosphorylation of the β subunit under these conditions reaches 2 mols PO_4 / mol β subunit (8 mol / mol $(\alpha\beta\gamma\delta)_4$).

Incorporation of the first mol of phosphate into the β subunit (per mole $(\alpha\beta\gamma\delta)_4$) does not appear to activate the kinase and a significant amount of α subunit phosphorylation does not occur until the β subunit has incorporated 1-2 mol PO_4 (Pickett-Gies and Walsh, 1985). It is possible that phosphorylation of the β subunit to levels greater than 1 mol per holoenzyme leads to a conformational change that both activates the kinase and allows α subunit phosphorylation. The stoichiometry of phosphorylation is typically slightly higher than 1 mol PO_4 / mol α subunit at the concentrations of the cAMP-dependent protein kinase and Mg^{2+} usually employed. At 10m*M* Mg^{2+} or with high concentrations of the cAMP-dependent protein kinase the α subunit can incorporate up to 6 mols PO_4 / mol α subunit (Singh and Wang, 1977). Some of these additional sites may be the same as those modified by autophosphorylation (see below). The single sites on each of the α and β subunits that are phosphorylated by the cAMP-dependent protein kinase at low Mg^{2+} have been sequenced after isolating the phosphopeptides (Yeaman *et al.*, 1977). A single serine is modified in each peptide. These sites appear to be the same as those utilized *in vivo* following epinephrine-stimulated glycogenolysis (Yeaman and Cohen, 1975).

A number of studies have been carried out in an attempt to correlate the activation of phosphorylase kinase with phosphorylation of either the α,

β, or both subunits. Although some studies appear to correlate activation with β subunit phosphorylation (Cohen, 1973; Cohen and Antonia, 1973; Cohen, 1980b) the preponderance of evidence clearly favors a role for both α (or α') and β subunit phosphorylation in the regulation of activity (Singh and Wang, 1977; Cooper *et al.*, 1981; Sul and Walsh, 1982; Sul *et al.*, 1982; Pickett-Gies and Walsh, 1985). As pointed out by Pickett-Gies and Walsh (1985), the situation is complicated by the existence of multiple-site phosphorylation on two subunits of a very complex enzyme. The effect of phosphate incorporation at a particular site is probably dependent on the phosphorylation state of other sites in the molecule. In this regard it is interesting to note that Chan and Graves (1982a, 1982b) found no effect of α subunit phosphorylation on the activity of the isolated αγδ complex, even though the studies mentioned above clearly indicate that it can play a role in activation of the holoenzyme.

Krebs *et al.* (1964) demonstrated that the primary effect of phosphorylation by the cAMP-dependent protein kinase is a lowering of the K_m for phosphorylase *b*, resulting in activation of the enzyme. It is interesting to note that while activation of phosphorylase kinase by phosphorylation lowers the K_m for phosphorylase *b*, activation does not alter the K_m for peptide substrates (Tessmer *et al.*, 1977; Krebs *et al.*, 1964; Kemp and John, 1981). This suggests that one should be cautious in interpreting the regulation of kinase activity when one uses peptide substrates rather than

physiological substrates. These substrate-directed differences may be the result of interactions of the kinase with portions of the substrate outside of the immediate phosphorylatable site.

In addition to a change in the K_m for phosphorylase *b*, several other changes in kinase regulation have been reported to occur after phosphorylation. Cohen (1980) reported the loss of stimulation of activity by exogenous Ca^{2+}/calmodulin after phosphorylation (conversely, Cox and Edstrom (1982) noted that calmodulin binding inhibits phosphorylation by the cAMP-dependent protein kinase). Cohen (1980) also reported an increase in sensitivity of the kinase to Ca^{2+} after phosphorylation and suggested that prior to activation the kinase requires the binding of 3-4 molecules of Ca^{2+} to the δ subunit for activity, while the phosphorylated kinase only requires binding of 2 molecules of Ca^{2+}. Cooper *et al.* (1980), using the cardiac isozyme, reported a change in the K_a for Ca^{2+} from 1.94μ*M* to 1.35μ*M* after phosphorylation. Heilmeyer and colleagues have proposed a complex model for phosphorylase kinase activity which suggests that it contains three separate catalytic activities, A0, A1, and A2 (Killiman and Heilmeyer, 1982a, 1982b). They propose that phosphorylation by the cAMP-dependent protein kinase leads to an increase in V_{max} of A2, while autophosphorylation (below) leads to an increase in the V_{max} of A1.

4.1.2.2 Autophosphorylation of Phosphorylase Kinase.

As noted above, early studies on activation and phosphorylation of phosphorylase kinase indicated that two distinct kinase activities were able to phosphorylate the enzyme. By using the heat-stable inhibitor of the cAMP-dependent protein kinase Walsh *et al.* (1971) were able to demonstrate that phosphorylase kinase was capable of autophosphorylation on the α and β subunits. The autophosphorylation reaction is faster at pH 8.2 than 6.8 (Wang *et al.*, 1976), dependent on Ca^{2+} (Krebs *et al.*, 1964), and requires much higher concentrations of ATP than phosphorylation by the cAMP-dependent protein kinase (Walsh *et al.*, 1971; Hallenbeck and Walsh, 1983; Pickett-Gies and Walsh, 1985). Autophosphorylation can be stimulated by several effectors including glycogen (DeLange *et al.*, 1968), peptide analogs of phosphorylase *b* (Carlson and Graves, 1976), heparin (Erdödi *et al.*, 1984), and organic solvents (Singh and Wang, 1979). Autophosphorylation is inhibited by glucose-6-P, glucose-1-P, fructose-1-P, 2-phosphoglycerate, 2,3-diphosphoglycerate, UDPG, and P_i (Wang *et al.*, 1976; Krebs *et al.*, 1964).

The maximal stoichiometry of phosphate incorporation by autophosphorylation is higher than that obtained with the cAMP-dependent protein kinase. Singh and Wang (1977) found incorporation of up to 6 mol PO_4 / mol α subunit and 2 mol PO_4 / mol β subunit. There is evidence that the sites phosphorylated by the cAMP-dependent protein kinase and autophosphorylation are different. Using sequential phosphorylation by the cAMP-dependent protein kinase and autophosphorylation, Wang *et. al*

(1976) found that prior phosphorylation by the cAMP-dependent protein kinase did not affect autophosphorylation stoichiometry. Conversely, maximal autophosphorylation followed by the cAMP-dependent protein kinase resulted in the addition of 1-2 mol PO_4. Singh *et al.* (1977) noted that additional activation is observed by sequential phosphorylation using the cAMP-dependent protein kinase followed by autophosphorylation but not when the reverse sequence is carried out.

Autophosphorylation is presumably catalyzed by the catalytic γ subunit of phosphorylase kinase (see above). This is supported by the finding that the isolated γ subunit is capable of increasing the rate of autophosphorylation of unactivated kinase, but only under conditions where autophosphorylation itself can occur (Skuster *et al.*, 1980). Chan and Graves (1982a, 1982b) found that the isolated γδ complex is capable of phosphorylating and activating the native kinase in the presence of EGTA and the inhibitor of the cAMP-dependent protein kinase at pH6.8. As discussed above, the β subunit has been proposed to contain a catalytic site, but Chan and Graves (1982a, 1982b) found that the isolated αγδ complex is capable of autophosphorylation (into the α subunit only), therefore the β subunit is not required for autophosphorylation.

Whether autophosphorylation occurs primarily by an inter- or intramolecular reaction is not clear. The importance of the mechanism of autophosphorylation lies in the regulatory properties that it imparts to the system. An intramolecular mechanism would mean that activation by

autophosphorylation would be independent of the local concentration of phosphorylase kinase. Intermolecular autophosphorylation would be dependent on phosphorylase kinase concentration, adding another element to the regulation of its activity. Studies by DeLange *et al.* (1968) and Killiman and Heilmeyer (1982) indicate an intermolecular autophosphorylation (the latter group calculated an apparent K_m of 60n*M* for this reaction), while the results of Hallenbeck and Walsh (1983) favor an intramolecular mechanism for autophosphorylation. The finding by DeLange *et al.* (1968) that addition of activated phosphorylase kinase to nonactivated kinase (substrate kinase) enhanced activation of the substrate kinase and the results discussed above using the isolated γ subunit and the $\gamma\delta$ subunit complex suggest that intermolecular phosphorylation is at least possible.

4.1.2.3

4.1.2.4 Phosphorylation of Phosphorylase Kinase by cAMP-Independent Kinases.

Several protein kinases other than the cAMP-dependent protein kinase are able to phosphorylate and activate phosphorylase kinase. The cGMP-dependent protein kinase has been demonstrated to phosphorylate phosphorylase kinase, the α subunit being phosphorylated more rapidly than the β subunit (Lincoln and Corbin, 1977; Khoo *et al.*, 1977). Although this provided evidence for a role of α subunit phosphorylation in the regulation of kinase activity, the rates of phosphorylation are low, and therefore phosphorylation by the cGMP-dependent protein kinase is unlikely to be of physio-

logical significance. Phosphorylation of phosphorylase kinase has also been demonstrated using glycogen synthase kinase I (Singh *et al.*, 1982) and protein kinase C (Inoue *et al.*, 1977; Kishimoto *et al.*, 1977; Yamamoto *et al.*, 1978). The possible physiological relevance of phosphorylation by these kinases has not been investigated. Phosphorylase kinase has also been reported to be a target for ADP-ribosylation (Tsuchiya *et al.*, 1985).

4.1.2.5 Phosphorylation of Phosphorylase Kinase Isozymes.

Several differences have been noted in the regulation of phosphorylase kinase isozymes by autophosphorylation. The liver isozyme is phosphorylated and activated by the cAMP-dependent protein kinase, but, unlike the skeletal-muscle isozyme, no change in Ca^{2+} sensitivity has been observed (Chrisman *et al.*, 1982). The cardiac- and red-muscle isozyme (which contain the α' subunit rather than the α subunit of skeletal muscle) is only phosphorylated to a maximum stoichiometry of 0.25 mol PO_4 / mol β subunit (1 mol / mol $(\alpha'\beta\gamma\delta)_4$ holoenzyme) by the the cAMP-dependent protein kinase (Sul *et al.*, 1982). This contrasts to the 2-3 mol PO_4 / mol $(\alpha\beta\gamma\delta)_4$ observed in the skeletal muscle isozyme (see above). The maximal activation of the α'-isozyme by the cAMP-dependent protein kinase phosphorylation is less than that observed for the α isozyme (Cooper *et al.*, 1981). Phosphorylase kinase isolated from dogfish skeletal muscle, although structurally similar to the mammalian enzyme, is apparently not subject to phosphorylation by either the cAMP-dependent protein kinase or autophosphorylation (Pocinwong *et al.*, 1981).

4.1.2.6 Physiological Significance of Phosphorylation of Phosphorylase Kinase.

Krebs and colleagues (1966) first proposed a role for the activation of phosphorylase kinase *via* phosphorylation by the cAMP-dependent protein kinase as the biochemical basis for the hormonal control of glycogen degradation. The sites phosphorylated *in vitro* by the cAMP-dependent protein kinase have been demonstrated to undergo phosphorylation *in vivo* in response to hormonal stimulation (Yeaman and Cohen, 1975). The conversion of phosphorylase *b* to phosphorylase *a* also occurs *in vivo* in response to hormonal stimulation and this occurs, at least partially, due to phosphorylation and activation of phosphorylase kinase (Drummond *et al.*, 1969; Stull and Mayer, 1971).

Cohen (1980a, 1982) has proposed that phosphorylation by the cAMP-dependent protein kinase may change the regulatory properties of phosphorylase kinase. He suggests that *in vivo* troponin-C and Ca^{2+} may be the major regulators of phosphorylase kinase in its dephosphorylated form. Upon phosphorylation, however, interaction with exogenous Ca^{2+}/calmodulin or troponin-C has little effect on enzyme activity. The phosphorylated form would therefore be regulated solely by Ca^{2+} *via* the δ subunit.

Regulation of phosphorylase kinase by autophosphorylation has not been demonstrated *in vivo*. The observed properties of phosphorylase kinase autophosphorylation *in vitro* suggest that such regulation might occur *in vivo* when subjected to a sufficiently prolonged Ca^{2+} stimulus. One

difficulty in establishing the occurence of *in vivo* autophosphorylation is that the cAMP-dependent protein kinase and autophosphorylation both phosphorylate the same primary site (King *et al.*, 1983). It is therefore difficult to distinguish the two mechanisms by phosphorylation site mapping.

4.2 Myosin Light Chain Kinase.

The coupling of excitation and contraction in muscle cells is dependent on a rise in intracellular Ca^{2+} concentration. The primary action of Ca^{2+} is mediated by troponin C, which binds Ca^{2+} and thus allows the interaction of actin and myosin. A secondary action of Ca^{2+} is activation of myosin light chain kinase *via* the Ca^{2+}/calmodulin complex. Vertebrate myosins contain associated regulatory light chains (M_r 20,000), which bind Ca^{2+} and are capable of being phosphorylated ("P"-light chains). Reversible phosphorylation/dephosphorylation of the P-light chains was first demonstrated in fast-twitch skeletal muscle (Perrie *et al.*, 1973) and later in platelets (Adelstein *et al.*, 1973), cardiac muscle (Frearson and Perry, 1975), slow-twitch muscle (Frearson and Perry, 1975), and smooth muscle (Frearson *et al.*, 1976). Phosphorylation of the P-light chains by myosin light chain kinase is *required* for actin activation of Mg^{2+}-dependent myosin ATPase activity in smooth muscle and nonmuscle cells (Adelstein and Eisenberg, 1980). The phosphorylation of the P-light chains is not required for contraction in skeletal and cardiac muscle, where it may play a secondary modulatory role. Myosin light chain kinase may be

important in secretion and motility in other tissues (Korn, 1978). The exact role of phosphorylation in contraction has been reviewed by Kamm and Stull (1985) and Marston (1982).

4.2.1 Structure and Activity.

Myosin light chain kinases have been identified in a number of vertebrate muscle and nonmuscle tissues. Myosin light chain kinase has been purified from mammalian skeletal muscle (Pires and Perry, 1977; Yazawa and Yagi, 1978; Edelman and Krebs, 1982; Mayr and Heilmeyer, 1983), avian skeletal muscle (Nunnally *et al.*, 1985), mammalian smooth muscle (Vallet *et al.*, 1981; Nishikawa *et al.*, 1984; Walsh *et al.*, 1982; Higashi *et al.*, 1983), avian smooth muscle (Dabrowska *et al.*, 1977; Adelstein and Klee, 1981; Uchiwa *et al.*, 1982; Walsh *et al.*, 1982), bovine brain (Hathaway *et al.*, 1981; Dabrowska and Hartshorne, 1978), mammalian cardiac muscle (Walsh *et al.*, 1979; Wolf and Hofmann, 1980), platelets (Dabrowska and Hartshorne, 1978; Hathaway and Adelstein, 1979), *Limulus* striated muscle (Sellers and Harvey, 1984) and a mammalian tissue culture cell line (*BHK*) (Yerna *et al.*, 1979). The determination of the exact physical properties of myosin light chain kinase has been hampered by its apparent sensitivity to limited proteolysis.

Myosin light chain kinase is a single polypeptide chain which is capable of binding Ca^{2+}/calmodulin. The apparent molecular weight of the protein varies between tissues and species. The avian smooth-muscle kinase has been reported to be a M_r 136,000 protein (Dabrowska *et al.*, 1977; Adelstein and

Klee, 1981; Uchiwa *et al.*, 1982; Walsh *et al.*, 1982), while the mammalian smooth-muscle kinase appears somewhat larger (estimates from different groups and using several species range from 130-160kDa [Vallet *et al.*, 1981; Nishikawa *et al.*, 1984; Walsh *et al.*, 1982; Higashi *et al.*, 1983]). The mammalian skeletal-muscle enzyme is smaller than the smooth-muscle enzymes (estimates range from 77-94 kDa [Pires and Perry, 1977; Yazawa and Yagi, 1978; Edelman and Krebs, 1982; Mayr and Heilmeyer, 1983]). The mammalian cardiac enzyme was reported to be 85 kDa (Walsh *et al.*, 1979) and 94 kDa (Wolf and Hofmann, 1980) by separate groups. In nonmuscle cells the enzyme has been reported to have a molecular weight of 105-130 kDa (Dabrowska and Hartshorne, 1978; Hathaway and Adelstein, 1979; Hathaway *et al.*, 1981).

The discrepency in the apparent molecular weight of myosin light chain kinase isolated from different tissues and species may originate from several sources. The kinase is known to be highly susceptible to proteolytic cleavage during preparation (Walsh and Guilleux, 1981; Guerriero *et al.*, 1981). The apparent size of the purified enzyme may therefore reflect the precautions taken to prevent proteolysis. On the other hand, the size disparity may reflect the presence of biochemically distinct isozymes in different tissues and species. Several studies have been carried out employing specific antibodies to determine the native size of the myosin light chain kinase from a number of sources (Stull *et al.*, 1986; Kennedy *et al.*, 1987). The results of these studies suggest that the observed variability is *primarily* due to the existence of discrete isozymes of myosin light chain kinase in different tissues and species.

Sellers and Harvey (1984) purified a myosin light chain kinase from *Limulus* striated muscle. Although it has a similar substrate specifity and dependence on Ca^{2+}/calmodulin to the mammalian enzyme, its structure is quite different. It appears to be composed of two peptides of M_r 37,000 and M_r 39,000, both of which bind calmodulin and possess catalytic activity. Unlike the vertebrate kinase the *Limulus* kinase is apparently not phosphorylated by the cAMP-dependent protein kinase (see below). Further work will be necessary to determine the exact relationship between this kinase and the vertebrate kinases.

All of the isozymes of myosin light chain kinase require Ca^{2+}/calmodulin for activity, forming a 1:1 complex during activation (Stull *et al.*, 1986). It is interesting to note that the catalytic and calmodulin-binding domains are readily separated by limited proteolysis to yield a calmodulin-independent catalytically active fragment (Stull *et al.*, 1986). The activity of myosin light chain kinase is characterized by a very limited substrate specificity. A number of substrates have been tested, but only myosin light chains are phosphorylated at a significant rate (Stull *et al.*, 1986).

4.2.2 Phosphorylation.

Myosin light chain kinase is phosphorylated by the cAMP-dependent protein kinase, leading to a reduction in affinity for Ca^{2+}/calmodulin. Phosphorylation of myosin light chain kinase by protein kinase C, the cGMP-de-

pendent protein kinase and autophosphorylation have recently been described, although the physiological consequences of these events is not yet clear.

4.2.2.1 hosphorylation of Myosin Light Chain Kinase by the cAMP-dependent Protein Kinase.

Phosphorylation of myosin light chain kinase was initially characterized by Adelstein and colleagues (Adelstein *et al.*, 1978; Conti and Adelstein, 1981) using the turkey gizzard (smooth muscle) enzyme. The catalytic subunit of the cAMP-dependent protein kinase incorporates up to 1 mol PO_4/mol kinase in the presence of Ca^{2+}/calmodulin with no effect on activity. In the absence of Ca^{2+}/calmodulin, up to 2 mol PO_4/mol kinase is incorporated, which results in a decrease in the activity of myosin light chain kinase. Proteolytic phosphopeptide mapping revealed two phosphorylation sites: one site (site 1) is phosphorylated in the presence or absence of bound Ca^{2+}/calmodulin, the other site (site 2) was only phosphorylated in the absence of bound Ca^{2+}/calmodulin (Conti and Adelstein, 1981). Phosphorylation of site 2 is therefore correlated with the 10-20-fold reduction in affinity for Ca^{2+}/calmodulin observed when myosin light chain kinase is phosphorylated in the absence of Ca^{2+}/calmodulin. The reduction in affinity for Ca^{2+}/calmodulin caused by phosphorylation is reversed *in vitro* by treatment with a protein phosphatase purified from turkey gizzard (Conti and Adelstein, 1981), suggesting that regulation of myosin light chain kinase activity by phosphorylation/dephosphorylation may occur *in vivo*.

Both of the sites phosphorylated by the cAMP-dependent protein kinase are contained on a M_r 20,000 peptide released by limited tryptic digestion (Conti and Adelstein, 1981). The peptide corresponds to the N-terminal 20,000 daltons of the kinase (Walsh *et al.*, 1982; Walsh, 1985). The remaining M_r 110,000 protein retains both catalytic activity and Ca^{2+}/calmodulin sensitivity (Walsh *et al.*, 1982). Therefore, although the phosphorylation of site 2 is sensitive to the binding of calmodulin and its phosphorylation affects calmodulin binding, the calmodulin-binding site and the phosphorylation site do not overlap in the primary structure of the enzyme. Walsh *et al.* (1982a) suggested that the phosphorylation sites are located on a flexible portion of the molecule which moves over the calmodulin-binding site and masks it when site 2 is occupied. However, Walsh (1985) and Malencik *et al.* (1982) found no evidence for large conformational changes in the kinase after autophosphorylation. Therefore the interaction of the calmodulin-binding site and the phosphorylation sites may involve more subtle changes in kinase tertiary structure.

The phosphorylation of myosin light chain kinase by the cAMP dependent protein kinase has been studied in other smooth-muscle preparations: bovine trachea (Nishikawa *et al.*, 1984), bovine stomach (Walsh *et al.*, 1982), bovine carotid artery (Bhalla *et al.*, 1982), bovine aorta (Vallet *et al.*, 1981), and porcine myometrium (Higashi *et al.*, 1983). These results suggest that all smooth-muscle myosin light chain kinases are regulated in a manner identical to that described above for avian gizzard smooth muscle. Two

sites are phosphorylated in the absence and one in the presence of bound Ca^{2+}/calmodulin. In each case phosphorylation at site 2 leads to a decrease in the affinity for Ca^{2+}/calmodulin with no change in V_{max}.

Studies on the phosphorylation and regulation of the cardiac form of myosin light chain kinase have produced conflicting results. Initially Walsh *et al.* (1979, 1980) reported no phosphorylation of purified bovine or canine cardiac kinase by the catalytic subunit of the cAMP-dependent protein kinase. Walsh and Guilleux (1981) later reported that cardiac myosin light chain kinase was phosphorylated by the cAMP-dependent protein kinase, resulting in a 3-4-fold reduction in its affinity for Ca^{2+}/calmodulin. Wolf and Hofman (1980) isolated a bovine cardiac myosin light chain kinase with high specific activity, which was phosphorylated to 1 mol PO_4/mol kinase with either the catalytic subunit of the cAMP-dependent protein kinase or in the presence of Ca^{2+}/calmodulin (suggesting a possible autophosphorylation). The presence of both Ca^{2+}/calmodulin and the catalytic subunit increased the rate but not the extent of phosphorylation. They observed no changes in kinase activity or calmodulin-binding properties after autophosphorylation. In a preliminary report Rappaport and Adelstein (1980) indicated that canine cardiac myosin light chain kinase was not subject to phosphorylation by the cAMP-dependent protein kinase. In the presence of Ca^{2+}/calmodulin, however, cardiac myosin light chain kinase rapidly incorporated 2 mol PO_4/mol kinase.

Edelman and Krebs (1982) described phosphorylation of rabbit skeletal-muscle myosin light chain kinase by the cAMP-dependent protein kinase. Approximately 1mol PO_4/mol kinase was rapidly incorporated with up to 1.9 mol PO_4/mol kinase observed after prolonged incubations at high concentrations of the cAMP-dependent protein kinase. They could not detect any change in V_{max} or calmodulin affinity after phosphorylation. Hathaway *et al.* (1981) reported that myosin light chain kinase from human platelets was phosphorylated at 1-2 sites with a 3-fold increase in the K_a for calmodulin as well as a 3-fold decrease in V_{max} at saturating concentrations of calmodulin. A protein phosphatase isolated from turkey gizzard was able to reverse these effects. A report by Nishikawa *et al.* (1984) found that the platelet kinase was regulated in a manner identical to that described above for avian smooth muscle. They attributed the difference between their results and those of Hathaway *et al.* (1981) to improved purification procedures. Myosin light chain kinase purified from *Limulus* striated muscle by Sellers and Harvey (1984) was not a substrate for the cAMP-dependent protein kinase. Phosphorylation of brain myosin light chain kinase (Dabrowska and Hartshorne, 1978; Hathaway *et al.*, 1981) has not been reported.

4.2.2.2 Phosphorylation of Myosin Light Chain Kinase by Protein Kinase C.

Two groups have reported the phosphorylation of turkey gizzard smooth-muscle myosin light chain kinase by the Ca^{2+}-activated, phospholipid-dependent protein kinase (protein kinase C). Ikebe *et al.* (1985) found

that protein kinase C incorporates 2 mol PO_4/mol kinase in the absence of bound calmodulin and little or none in its presence. The apparent K_a for calmodulin increased 10-fold in the diphosphorylated enzyme. Protein kinase C appeared to recognize two sites on myosin light chain kinase. These sites appear to be different than those phosphorylated by the cAMP-dependent protein kinase, since 4 mol PO_4/mol myosin light chain kinase could be incorporated by sequential phosphorylation by the two kinases. Nishikawa *et al.* (1985) observed a somewhat different pattern of phosphorylation. In the presence of bound calmodulin they found incorporation of 1 mol PO_4/mol kinase with no change in activity. In the absence of bound calmodulin the stoichiometry was 2 mol PO_4/ mol kinase accompanied by a 3-4-fold increase in the apparent K_a for calmodulin. They concluded that the site phosphorylated in the presence of bound calmodulin (site 3) was different than that phosphorylated by the catalytic subunit of the cAMP-dependent protein kinase (site 1). Protein kinase C and the cAMP-dependent protein kinase both phosphorylate site 2 when calmodulin is not bound. By performing sequential phosphorylation with these two kinases, they concluded that site 1 may play a role in the modulation of calmodulin affinity, but that this modulation is observed only when site 2 is also phosphorylated.

4.2.2.3 Phosphorylation of Myosin Light Chain Kinase by the cGMP-Dependent Protein Kinase.

Nishikawa *et al.* (1984) have reported that myosin light chain kinases isolated from bovine tracheal smooth muscle and human platelets are both substrates for the cGMP-dependent protein kinase. The tracheal smooth muscle myosin light chain kinase was subject to phosphorylation only in the absence of bound calmodulin. The phosphorylation occurred at a single site and did not alter catalytic activity. The site appears to be the same as the one phosphorylated by the cAMP-dependent protein kinase when calmodulin is bound (site 1). The platelet myosin light chain kinase was phosphorylated at two sites in the absence and one site in the presence of bound Ca^{2+}/calmodulin. Phosphorylation at **both** sites in the platelet myosin light chain kinase reduced calmodulin affinity, but to a lesser degree than when the cAMP-dependent protein kinase was used. The second site phosphorylated by the cGMP-dependent protein kinase in the absence of bound Ca^{2+}/calmodulin differs from the corresponding site phosphorylated by the cAMP-dependent protein kinase (site 2). The relationship between the phosphorylation sites recognized by protein kinase C and the cGMP-dependent protein kinase has not been established.

4.2.2.4 Autophosphorylation of Myosin Light Chain Kinase.

Several groups have reported the apparent autophosphorylation of myosin light chain kinase isolated from turkey gizzard (Adelstein *et al.*,

1978; Walsh *et al.*, 1980), tracheal smooth muscle, and human platelets (Nishikawa *et al.*, 1984). The rates and stoichiometries of autophosphorylation reported in each case were low. Two groups have reported stoichiometrically significant autophosphorylation of the bovine and canine cardiac isozymes of myosin light chain kinase (Wolf and Hofmann, 1980; Rappaport and Adelstein, 1980). The two groups reported different stoichiometries. One mol PO_4/mol kinase was incorporated by autophosphorylation of the bovine cardiac kinase (Wolf and Hofmann, 1980) while the canine cardiac kinase incorporated 2 mol PO_4/mol kinase (Rappaport and Adelstein, 1980). Wolf and Hofmann (1980) reported that autophosphorylation of the bovine cardiac enzyme required Ca^{2+}/calmodulin but did not modify enzymatic activity.

Foyt and Means (1985) reported an apparent autophosphorylation of chicken-gizzard smooth-muscle myosin light chain kinase at two sites. The rate of autophosphorylation was inhibited approximately 75% by bound Ca^{2+}/calmodulin. Whereas phosphorylation by the cAMP-dependent protein kinase occurs primarily at serine residues (Edelman and Krebs, 1982; Foyt and Means, 1985) the autophosphorylation reaction incorporated phosphate into both serine and threonine residues. They did not determine whether this autophosphorylation resulted in a change in catalytic activity. Foyt and Means (1985) provided evidence for the existence of a combined total of four phosphorylation sites for autophosphorylation and the

cAMP-dependent protein kinase. The physiological significance of this reaction, although stoichiometrically significant, is doubtful since incorporation approaching 2 mol PO_4/mol kinase requires up to **six hours** *in vitro*.

4.2.2.5 Physiological Significance of Myosin Light Chain Kinase Phosphorylation.

The physiological significance of myosin light chain kinase phosphorylation has not been firmly established. The role of myosin light chain phosphorylation in the regulation of the contractile apparatus in smooth-muscle cells and nonmuscle cells was mentioned above. It is clear from many studies that light chain phosphorylation is a primary regulatory event in these two contractile systems (Marston, 1982; Kamm and Stull, 1985; Daniel *et al.*, 1981; Daniel *et al.*, 1984; Barany and Barany, 1981; Hartshorne and Siemenkowski, 1981; Adelstein *et al.*, 1981). The role of myosin light chain phosphorylation in cardiac and skeletal muscle is at present less clear, but it has been proposed to regulate contractile events in these muscle types (Adelstein *et al.*, 1981; Barany and Barany, 1981; Stull *et al.*, 1981).

Myosin light chain kinase has been shown to be a phosphoprotein *in vivo* in certain nonmuscle cell types (Bourguignon *et al.*, 1982). Phosphorylation of myosin light chain kinase by the cAMP-dependent protein kinase at sites 1 and 2, leading to a decrease in the affinity for Ca^{2+}/calmodulin, has been proposed to play a role in modulating the *relaxation* of smooth muscle induced by β-adrenergic receptor stimulation (Conti and Adelstein, 1981).

deLanerolle *et al.* (1984) have reported that myosin light chain kinase is phosphorylated *in vivo* in response to forskolin-stimulated increases in intracellular cAMP in smooth muscle. The results of a number of studies have led to the conclusion that phosphorylation of myosin light chain kinase in response to increases in intracellular cAMP concentration is not a **primary** event in smooth muscle relaxation, but it may play a secondary role in regulating contractile events (Adelstein *et al.*, 1981; Marston, 1982; Miller *et al.*, 1983; Kamm and Stull, 1985). Identifying any potential role for myosin light chain kinase autophosphorylation (see above) will require further work.

4.3 Neuronal Ca^{2+}/Calmodulin-Dependent Protein Kinase I.

Several protein kinase activities have been identified in brain by their ability to phosphorylate the synaptic vesicle-associated protein synapsin I (Kennedy and Greengard, 1981). They phosphorylate unique sites on synapsin I and have been classified by this property. Ca^{2+}/calmodulin-dependent protein kinase I phosphorylates site I on Synapsin I, the same site phosphorylated by the cAMP-dependent protein kinase, while the broad-specificity Ca^{2+}/calmodulin-dependent protein kinases (type II CaM kinases, below) phosphorylate site II. Ca^{2+}/calmodulin-dependent protein kinase I has been described in two preliminary reports (Nairn and Greengard, 1983; Nairn *et al.*, 1985), and very recently the complete purification and characterization of this kinase from bovine brain was reported (Nairn and Greengard, 1987).

4.3.1 Structure and Activity.

Ca^{2+}/calmodulin-dependent protein kinase I purified from bovine brain is composed of two major polypeptides of M_r 37,000 and 39,000 with a minor component of M_r 42,000. All three of the subunits bind Ca^{2+}/calmodulin, are photoaffinity labeled by 8-azido-ATP, and undergo autophosphorylation (Nairn and Greengard, 1987). The hydrodynamic properties of this kinase indicate a monomeric enzyme. Phosphopeptide maps of the three peptides are similar, suggesting that the two smaller peptides may be generated from the M_r 42,000 peptide by limited proteolysis during purification. An alternative hypothesis is that they represent isozymic forms of the enzyme. The kinase exhibits a narrow substrate specificity. Synapsin I is the best substrate of those examined. Synapsin I is phosphorylated at site I (the same site as that recognized by the cAMP-dependent protein kinase) and not at site II (which is recognized by the type II CaM kinase, see below). Protein III, a brain-specific protein phosphorylated by the cAMP-dependent protein kinase (Browning *et al.*, 1987), and smooth muscle myosin light chain were the only other susbstrates phosphorylated to a significant extent.

Nairn and Greengard (1987) examined the tissue and subcellular distribution of Ca^{2+}/CaM kinase I in rat. The kinase is mainly found in the cytosolic fraction and is most highly concentrated in brain, with lower levels in pancreas, lung, adrenal gland, and spleen. Heart, skeletal muscle, liver, and kidney had very low levels of this kinase compared to brain. Preliminary

studies by these authors suggest that the Ca^{2+}/calmodulin-dependent protein kinase I in bovine heart and rat pancreas is very similar to the purified brain enzyme.

4.3.2 Phosphorylation.

All three of the purified Ca^{2+}/calmodulin-dependent protein kinase I polypeptides undergo Ca^{2+}/calmodulin-stimulated autophosphorylation on threonine residues (Nairn and Greengard, 1987). The authors did not investigate possible changes in kinase activity resulting from autophosphorylation in this report. Phosphorylation of Ca^{2+}/calmodulin-dependent protein kinase I by other protein kinases has not been reported.

4.4 Neuronal Ca^{2+}/Calmodulin-Dependent Protein Kinase III.

A Ca^{2+}/calmodulin-dependent protein kinase, Ca^{2+}/calmodulin-dependent protein kinase III, was recently identified and partially purified on the basis of its ability to phosphorylate a M_r 100,000 substrate (Nairn *et al.*, 1985). The M_r 100,000 substrate has recently been identified as elongation factor 2 (EF-2) (Ryazanov, 1987; Nairn and Palfrey, 1987). Ca^{2+}/calmodulin-dependent protein kinase III appears to have a very limited substrate specificity and a widespread tissue distribution.

4.4.1 Structure and Activity.

Ca^{2+}/calmodulin-dependent protein kinase III was partially purified, using EF-2 as a substrate, from rat pancreas (Nairn *et al.*, 1985). The final fraction exhibited a M_r of 140,000 by gel filtration but contained a number of proteins; therefore the exact polypeptide composition of this kinase has not been determined. The kinase is completely dependent on Ca^{2+}/calmodulin for activity. When a wide variety of substrates were tested only EF-2 was phosphorylated at a significant rate. A single threonine was phosphorylated on EF-2.

4.4.2 Phosphorylation.

There have been no reports in the literature concerning autophosphorylation of the Ca^{2+}/calmodulin-dependent protein kinase III.

4.5 Broad-Specificity Ca^{2+}/Calmodulin-Dependent Protein Kinases.

The Ca^{2+}/calmodulin-dependent protein kinases described above phosphorylate a relatively narrow range of substrates at appreciable rates, and it is believed that this reflects a similar specialization *in vivo*. In contrast, a family of related kinases have been described in a number of tissues that exhibit broad substrate specificity. The members of this family of kinases share several biochemical properties in addition to having broad substrate specificities (reviewed in Stull *et al.*, 1986; Kennedy *et al.*, 1987). They exist as large holoenzymes (M_r 300-700,000) consisting of multiple copies of structurally related M_r 50,000-60,000 subunits. The subunits all bind calmodulin, autophosphorylate, and appear to be catalytically active. Members of this family of kinases are referred to by several names, including broad-specificity calmodulin-dependent protein kinase, multifunctional calmodulin-dependent protein kinase, type II calmodulin-dependent protein kinase, and calmodulin-dependent kinase II.

4.5.1 Structure and Activity.

Broad-specificity calmodulin-dependent kinases have been purified from mammalian brain, liver, and skeletal muscle. In addition this class of kinase has been described in several other tissues and in other species.

4.5.1.1 Neuronal Type II CaM Kinase.

Several groups have purified a Ca^{2+}/calmodulin-dependent kinase with broad substrate specificity from mammalian brain (Fukunaga *et al.*, 1982; Bennett *et al.*, 1983; Goldenring *et al.*, 1983; McGuinness *et al.*, 1983; Yamauchi and Fujisawa, 1983; Kuret and Schulman, 1984; Sahyoun *et al.*, 1985). The holoenzyme is composed of α (Mr 50,000) and β/β′ (Mr 60/58,000) subunits in an approximately 3:1 molar ratio (α:β/β′). Holoenzyme molecular weights have been reported in the range of 460-650,000 and the holoenzyme is therefore composed of 9α and 3β subunits (Bennett *et al.*, 1983). The α and β/β′ subunits are homologous proteins encoded by separate genes (see below). It has been suggested that the β and β′ subunits are related by proteolysis occurring during purification (McGuinness *et al.*, 1985), but recent studies indicate that they may be the products of alternative splicing of the β gene transcript (Bulleit *et al.*, 1988, and below). The α and β/β′ subunits of the kinase both bind calmodulin (Bennett *et al.*, 1983; Goldenring *et al.*, 1983; Kuret and Schulman, 1984) and autophosphorylate in the presence of Ca^{2+}/calmodulin (Bennett *et al.*, 1983; Goldenring *et al.*, 1983; Kuret and Schulman, 1984).

The kinase exists in both soluble and particulate fractions in mammalian brain, and the relative amounts in these two pools vary between brain regions. The soluble and particulate enzymes appear to have identical physical and enzymatic properties. Extraction of the particulate kinase by low ionic strength and comparison to the soluble kinase revealed no

differences in catalytic properties (Kennedy *et al.*, 1983). Specific isozymes of the kinase appear to exist in different regions of rat brain. In each case the kinase is composed of α and β/β′ subunits, but they are assembled in different ratios in each isozyme. The major isozyme in brain (described above) is found in the forebrain and constitutes up to 1% of total protein in this tissue (Erondu and Kennedy, 1985). The forebrain isozyme is composed of α and β/β′ subunits in a 3:1 (α:β/β′) ratio. The cerebellar isozyme is also composed of α and β/β′ subunits, but they are present in a ratio of 1:4 (α:β/β′) (McGuinness *et al.*, 1985; Miller and Kennedy, 1985). The cerebellar isozyme is present in lower amounts than in forebrain, and a greater proportion of it exists in the particulate fraction than the forebrain enzyme. An additional isozyme of the brain kinase, composed of α and β/β′ subunits in a 1:1 (α:β/β′) ratio, has been purified in this laboratory (M.K. Bennett, unpublished observations). This is the ratio of α:β/β′ subunits that would be expected of the kinase isozyme found in the pons and medulla (Erondu and Kennedy, 1985).

The functional significance of brain-region-specific isozymes of the type II kinase is not known at the present time. The observation that the proportion of kinase found in the particulate fraction differs between brain regions (50% particulate in forebrain vs. 80-85% particulate in cerebellum; McGuinness *et al.*, 1985; Miller and Kennedy, 1985) suggests that the isozymes may exhibit different affinities for membranes or cytoskeletal structures. The kinase is the major component of the postsynaptic density (PSD, described below) in the forebrain, but in the cerebellum the amount of

kinase found in the PSD is highly reduced (Miller and Kennedy, 1985). Again, this suggests a possible role of kinase isozymes in regulating subcellular localization. Recent work describing the autophosphorylation sites of the kinase suggests another possiblity, that of differential regulation of enzymatic activity (due to autophosphorylation) dependent on the relative amounts of α and β/β′ subunits (chapter 4 of this thesis).

The complete primary structures of the α and β/β′ subunits have now been determined by molecular cloning (Bennett and Kennedy, 1987; Hanley *et al.*, 1987; Lin *et al.*, 1987; Bulleit *et al.*, 1988). The α and β/β′ subunits are homologous, presumably derived from a common ancestral gene. Each subunit is composed of an amino-terminal domain, which shares homology with other protein kinases; a calmodulin-binding domain; and a carboxy-terminal domain, which is not homologous to any known protein (Bulleit *et al.*, 1988). The α and β/β′ subunits share extensive sequence identity at the amino acid level, 91% in the amino-terminal region and 76% in the carboxy-terminal region (including "conservative" substitutions, Bulleit *et al.*, 1988). The major difference between the α and β/β′ subunits is the deletion of two amino acid segments from the α subunit compared to the β subunit (Bulleit *et al.*, 1988). It was also reported that the β′ subunit appears to be related to the β subunit by alternative splicing of the β subunit mRNA (Bulleit *et al.*, 1988).

The neuronal type II CaM kinase exhibits a broad substrate specificity *in vitro*. Some of the better substrates phosphorylated by the kinase include

synapsin I (Bennett *et al.*, 1983; McGuinness *et al.*, 1983; Miller and Kennedy, 1985), tryptophan hydroxylase (Yamauchi and Fujisawa, 1983), tubulin (Goldenring *et al.*, 1983), casein (Kuret and Schulman, 1984), microtubule-associated protein 2 (Bennett *et al.*, 1983; McGuinness *et al.*, 1983; Schulman, 1984), smooth-muscle myosin light chain (Fukunaga *et al.*, 1982; Bennett *et al.*, 1983; McGuinness *et al.*, 1983), and myelin basic protein (Goldenring *et al.*, 1983). In addition a large number of proteins have been shown to be phosphorylated by the kinase at lower rates (reviewed in Stull *et al.*, 1986; see also Pearson *et al.*, 1985). It seems unlikely that all of the substrates phosphorylated by the kinase *in vitro* are phosphorylated *in vivo* to a significant extent. It is also possible that many of the physiologically relevant substrates are proteins whose identity and function are currently unknown.

4.5.1.2 Glycogen Synthase Kinase.

Glycogen synthase is phosphorylated at multiple sites by a diverse group of protein kinases (Cohen, 1982; Soderling and Khatra, 1983). One of these kinases is a Ca^{2+}/calmodulin-dependent protein kinase with a broad substrate specificity (Payne and Soderling, 1980). Phosphorylation of glycogen synthase by the Ca^{2+}/calmodulin-dependent glycogen synthase kinase decreases its activity and thus acts in concert with the activation of phosphorylase by phosphorylase kinase to increase the net breakdown of

glycogen. The Ca^{2+}/calmodulin-dependent glycogen synthase kinase has been purified to homogeneity from two sources, rabbit liver (Ahmad *et al.*, 1982; Payne *et al.*, 1983) and rabbit skeletal muscle (Woodgett *et al.*, 1983).

Glycogen synthase kinase purified from liver is composed of approximately equal molar amounts of two types of subunits (M_r 50,000 and M_r 53,000) assembled into a multimeric holoenzyme of M_r 300,000 (Ahmad *et al.*, 1982; Payne *et al.*, 1983). Both subunits of this kinase undergo autophosphorylation to a level of approximately 4 mol PO_4 / mol of the two subunits (Ahmad *et al.*, 1982). The glycogen synthase kinase purified from skeletal muscle is composed of two types of subunits (M_r 54,000 and M_r 58,000) assembled into a multimeric holoenzyme of M_r 700,000 (Woodgett *et al.*, 1983). The subunits are present in a ratio of approximately 1:4 of M_r 54,000 to M_r 58,000 subunits (Woodgett *et al.*, 1983), and both subunits undergo autophosphorylation. Both subunits of the skeletal-muscle glycogen synthase kinase are recognized by antibodies raised against the neuronal type II CaM kinase (McGuinness *et al.*, 1983). The liver and skeletal-muscle forms of glycogen synthase kinase phosphorylate two sites on glycogen synthase, 1b and 2. Phosphorylation at site 2 is associated with a decrease in enzymatic activity of glycogen synthase, while the function of site 1b is unknown.

4.5.1.3 Broad Specificity Kinases in Other Tissues and Species.

Ca^{2+}/calmodulin-dependent protein kinases with many of the properties of the broad substrate specificity kinases described above have been described in other tissues and species. Some of these kinases have been purified to homogeneity, while others have only been studied in crude or partially purified preparations. In addition, antibodies directed against the neuronal type II CaM kinase have been used to probe for similar enzymes in a number of tissues and species.

A broad-specificity calmodulin-dependent kinase has been purified from rat pancreas that is composed of a single M_r 51,000 subunit assembled into a large (M_r 600,000) holoenzyme (Gorelick *et al.*, 1983; Cohn *et al.*, 1984). This kinase is capable of undergoing autophosphorylation. Palfrey and colleagues have partially purified broad-specificity calmodulin-dependent kinases from several tissues. Mammalian heart contains a kinase composed of M_r 55,000 and M_r 75,000 subunits (Palfrey, 1984), both of which undergo autophosphorylation. The kinase found in avian erythrocytes (Palfrey *et al.*, 1984) contains a major M_r 58,000 subunit and minor subunits of M_r 50,000 and M_r 54,000. All of the subunits undergo autophosphorylation and bind calmodulin. Sato *et al.* (1988) have purified a broad-specificity kinase from mammalian skeletal muscle which is composed of a single M_r 58,000 subunit. This kinase was found in the cytosol as well as in the sarcoplasmic reticulum and sarcolemma and phosphorylates troponin I and troponin T.

Torpedo electric organ contains a broad-specificity calmodulin-dependent kinase that appears to have subunits of M_r 52,000 and M_r 54,000 (Palfrey *et al.*, 1983).

Several groups have investigated the properties of a Ca^{2+}/calmodulin-dependent kinase in the neuronal tissues of *Aplysia californica*. DeRiemer *et al.* (1984) described a M_r 51,000 protein in *Aplysia* neural tissue that cross-reacts with antibodies raised against the mammalian neuronal type II CaM kinase. This protein undergoes phosphorylation, binds calmodulin, and has a phosphopeptide map similar to the mammalian brain enzyme. Saitoh and Schwartz (1985) have also described a similar enzyme in *Aplysia* neuronal tissues. Several endogenous substrates for this protein (other than autophosphorylation) have been reported (Novak-Hofer and Levitan, 1983; DeRiemer *et al.*, 1984). A Ca^{2+}/calmodulin-dependent kinase has also been studied in neuronal tissues in squid (Bass *et al.*, 1987). This enzyme consists of subunits of M_r 54,000 and M_r 58-60,000 and undergoes autophosphorylation.

Work in this laboratory has demonstrated the presence of a broad-specificity calmodulin-dependent protein kinase in *Drosophila* (Leonard *et al.*, 1987). The activity of this kinase is higher in *Drosophila* heads than in bodies. Monoclonal antibodies raised against mammalian neuronal type II CaM kinase immunoprecipitate three phosphoproteins (M_r 52-, 58-,

and 60,000) from *Drosophila* head homogenates. The activity of the *Drosophila* head Ca^{2+}/calmodulin-dependent kinase is also inhibited by monoclonal antibodies raised against the mammalian kinase.

4.5.2 Phosphorylation.

Rapid Ca^{2+}/calmodulin-dependent autophosphorylation is one of the distinguishing features of the broad-specificity calmodulin-dependent protein kinases (Ahmad *et al.*, 1982; Bennett *et al.*, 1983; Goldenring *et al.*, 1983; McGuinness *et al.*, 1983; Woodgett *et al.*, 1983). Several of the initial reports regarding purification of the kinase reported no effects of autophosphorylation on kinase activity (Bennett *et al.*, 1983; McGuinness *et al.*, 1983; Woodgett *et al.*, 1983). Recent studies, however, have found that the neuronal type II CaM kinase is regulated in a complex fashion by autophosphorylation (Saitoh and Schwartz, 1985; Miller and Kennedy, 1986; Lai *et al.*, 1986; Schworer *et al.*, 1986). There have been no reports of phosphorylation of broad-specificity calmodulin-dependent protein kinases by exogenous kinases.

4.5.2.1 Biochemistry of Autophosphorylation.

Autophosphorylation of the neuronal type II CaM kinase has been studied in more detail than other members of the broad-specificity calmodulin-dependent kinase family and will be the focus of this discussion. In crude brain homogenates the α and β/β' subunits of the type II kinase are the major endogenous Ca^{2+}/calmodulin-stimulated phosphoproteins ob-

served (Kennedy *et al.*, 1983b; Bennett *et al.*, 1983; Kelly *et al.*, 1984; McGuinness *et al.*, 1985; Miller and Kennedy, 1985). This reflects the high abundance of the kinase, the type II kinase constitutes approximately 1% of total protein in crude brain homogenates (Erondu and Kennedy, 1985), and the high stoichiometry of autophosphorylation. The rate of incorporation of phosphate into the α and β/β′ subunits of the kinase is rapid *in vitro*, reaching 50% of its maximal level in <30sec (Miller and Kennedy, 1986). The stoichiometry of autophosphorylation is approximately 2 mol PO_4 / mol α subunit and 3 mol PO_4 / mol β subunit, or 30 mol PO_4 / mol $\alpha_3\beta_3$ forebrain isozyme (Miller and Kennedy, 1986). The rate of phosphate incorporation into the β subunit is faster than the α subunit (Kuret and Schulman, 1985; Miller and Kennedy, 1986).

The initial rate of autophosphorylation of the kinase is constant over a wide range of kinase concentrations (Kuret and Schulman, 1985; Miller and Kennedy, 1986). This is consistent with an intramolecular mechanism for autophosphorylation and imparts specific properties on the regulatory behaviour of the kinase, as discussed above for phosphorylase kinase. Although the α and β subunits are independently capable of autophosphorylation (Kuret and Schulman, 1985; R. Bulleit, personal communication), it is not known whether this is the primary mechanism in the intact holoenzyme.

Autophosphorylation of the kinase occurs on serine and threonine residues; no phosphorylation of tyrosine has been observed. The initial

autophosphorylation occurs on threonine residues, followed by slower incorporation into serine residues (Lai *et al.*, 1987; chapter 4, submitted for publication). The α and β subunits share a common initial phosphorylation site, while the β subunit contains an additional unique site (Lai *et al.*, 1987; chapter 4, submitted for publication). Some of the slower phosphorylation sites are common to the two subunits, while others are found only in the β subunit (chapter 4, submitted for publication). Comparison of phosphopeptide maps generated from the autophosphorylated kinase isolated from brain, skeletal muscle, and *Aplysia* suggests that the sites of autophosphorylation may be conserved between tissues and species (McGuinness et al, 1983; DeRiemer *et al.*, 1984). Identification of the sites of autophosphorylation in the neuronal type II CaM kinase is the subject of Chapters 4 and 5 of this thesis.

Autophosphorylation of the neuronal type II CaM kinase no longer requires Ca^{2+}/calmodulin after a brief Ca^{2+}/calmodulin-dependent autophosphorylation (Miller and Kennedy, 1986). When EGTA is added to chelate Ca^{2+} during Ca^{2+}/calmodulin-dependent autophosphorylation, the α and β subunits continue to incorporate PO_4 at the same or higher rates (Miller and Kennedy, 1986). The sites phosphorylated in the absence of Ca^{2+} during this paradigm have been characterized and are the subject of chapter 5 of this thesis. Several sites are phosphorylated in both the α and β subunits during Ca^{2+}-independent autophosphorylation that are not ob-

served to be phosphorylated in the presence of Ca^{2+}/calmodulin. In addition the rate and extent of phosphorylation of the sites phosphorylated in the continuous presence of Ca^{2+}/calmodulin are inhibited.

4.5.2.2 Regulation of Kinase Activity by Autophosphorylation.

The initial reports of the effects of autophosphorylation on type II CaM kinase activity were contradictory and often used crude preparations rather than purified kinase. Shields *et al.* (1984) and LeVine *et al.* (1985), using cytoskeletal preparations, reported that autophosphorylation resulted in an *increase* of 30-70% in apparent calmodulin binding to the kinase subunits on gel overlays. The latter authors also measured calmodulin-binding in solution and found a 2-fold *decrease* in the affinity and number of calmodulin binding sites as well as an increase in the apparent K_a for Ca^{2+} from 90n*M* to 200n*M* after autophosphorylation (LeVine *et al.*, 1985). They suggested that the increase in ^{125}I-calmodulin binding observed using gel overlays was due to greater access to the subunits resulting from broadening of the subunit bands that occurs after autophosphorylation. LeVine *et al.* (1985) therefore concluded that autophosphorylation rendered the kinase less sensitive to stimulation by Ca^{2+}/calmodulin. In direct contrast to these results, Shields *et al.* (1984) found that the activity of the phosphorylated kinase was as much as 3-fold higher than that of nonphosphorylated kinase when measured at low calmodulin concentrations (15n*M*). There was no apparent difference in kinase activity at saturating concentrations of calmodulin.

Studies by several groups using the purified soluble type II CaM kinase suggested that kinase activity was dramatically reduced after autophosphorylation. Yamauchi and Fujisawa (1985), using both rat brain and rabbit skeletal-muscle broad-specificity kinases, found inhibition of kinase activity after autophosphorylation. In light of the results of Shields *et al.* (1984, discussed above), they assayed activity across a broad range of calmodulin concentrations and observed inactivation at all concentrations. Kuret and Schulman (1985) also observed a reduction in kinase activity of about 70% after autophosphorylation.

Saitoh and Schwartz (1985), using a cytoskeletal preparation derived from *Aplysia* neurons, found that incubation under conditions that allow autophosphorylation released a fraction of the kinase from the cytoskeleton. The activity of the released kinase was independent of added Ca^{2+} (*"autonomous"*). In this study it was not clear whether the Ca^{2+}-independence was the direct result of autophosphorylation or whether release from the cytoskeleton was the key event.

Recent studies appear to reconcile some of the differences observed in the reports mentioned above. In a detailed investigation of regulation by autophosphorylation, we used the purified neuronal type II CaM kinase (Miller and Kennedy, 1986). Two major changes in enzymatic activity were observed to occur after phosphorylation of only a few (3-12) of the possible thirty phosphorylation sites in the holoenzyme. The rate of phosphorylation of two exogenous substrates (synapsin I and microtubule-associated protein

2) was found to be reduced by approximately 60% when assayed in the presence of Ca^{2+} and calmodulin. This effect was similar to that reported by Kuret and Schulman (1985) and Yamauchi and Fujisawa (1985). In addition the phosphorylated kinase retained a significant activity in the absence of Ca^{2+} and calmodulin, in agreement with the initial observations of Saitoh and Schwartz (1985) in the crude *Aplysia* system. The activity of the autophosphorylated kinase in the absence of Ca^{2+} was found to be about 20% of that of the nonphosphorylated kinase measured in the presence of Ca^{2+}. As noted above, autophosphorylation itself becomes independent of Ca^{2+} after a brief autophosphorylation in the presence of calmodulin (Miller and Kennedy, 1986).

Lai *et al.* (1986) and Schworer *et al.* (1986) reported findings similar to those of the above study. Lai *et al.* (1986), however, found that the decrease in total kinase activity in the presence of Ca^{2+}/calmodulin was attributable to an increased thermal instability of the phosphorylated kinase. The thermal lability of the phosphorylated kinase has been confirmed by work in our laboratory (B.L. Patton, unpublished data). The physiological significance of this lability remains to be established. It should be noted that in several other systems an increased degradation of phosphorylated proteins has been suggested to be a regulatory mechanism (Hemmings, 1980; Arebalo *et al.*, 1981; Müller and Holzer, 1981; Mazón *et al.*, 1982; Pontremoli *et al.*, 1987). It is interesting to note, in this context, a study that examined the effects of monocular deprivation on the immunoreactivity of the neuronal type II kinase in primary visual cortex. In primates, it was shown

that monocular deprivation results in a relative increase in kinase immunoreactivity in primary visual cortex in those cells recieving input from the deprived eye (Hendry and Kennedy, 1986). The reason for this change has not yet been determined, but it could result from a decrease in the turnover rate of the kinase.

As mentioned above, autophosphorylation continues in the absence of Ca^{2+} after a brief Ca^{2+}-dependent autophosphorylation (Miller and Kennedy, 1986). Hashimoto *et al.* (1987) measured the activity of the kinase after such autophosphorylation. They found that the activity of kinase phosphorylated sequentially in the presence, then absence, of Ca^{2+} was no longer stimulated by Ca^{2+}/calmodulin -- kinase activity became completely Ca^{2+}-independent. The authors suggested that separate sites are autophosphorylated in the absence of Ca^{2+}, resulting in the loss of stimulation by Ca^{2+}/calmodulin. The concept of a second class of autophosphorylation sites was first proposed by Saitoh and Schwartz (1985) to explain observations made in the *Aplysia* cytoskeletal preparation. Work in our laboratory has confirmed the loss of Ca^{2+}/calmodulin stimulation of kinase activity after sequential phosphorylation in the presence, then absence, of Ca^{2+} (B.L. Patton, unpublished observations). Chapter 5 of this thesis reports the characterization of this class of phosphorylation sites.

The effects of autophosphorylation on kinase activity are reversed by dephosphorylation by protein phosphatases. Protein phosphatases purified from skeletal muscle (Lai *et al.*, 1986; Schworer and Soderling, 1986; chapter

4 of this thesis; and B.L. Patton, unpublished observations) as well as endogenous brain protein phosphatases (Shields *et al.*, 1985; Miller and Kennedy, 1986) are capable of dephosphorylating the kinase. Work in our laboratory suggests that the phosphorylation sites of the kinase exhibit differential sensitivity to dephosphorylation (chapter 4 of this thesis and B.L. Patton, unpublished observations). This may be helpful in determining the role of individual phosphorylation sites in the regulation of kinase activity. It also suggests that the substrate specifity and regulatory properties of brain phosphatases may be important in the regulation of kinase activity *in vivo*.

The regulation of the broad-specificity kinases by autophosphorylation appears to be highly conserved between tissues and species. The liver glycogen synthase kinase is regulated in the same fashion as the neuronal type II CaM kinase (Schworer *et al.*, 1985). Regulation of the *Aplysia* enzyme by autophosphorylation has already been discussed (Saitoh and Schwartz, 1985). In a recent report (Bass *et al.*, 1987) the broad-specificity kinase found in squid synaptosomes was also found to become Ca^{2+}-independent after autophosphorylation. The broad-specificity kinase found in *Drosophila* heads is also regulated by autophosphorylation, becoming partially Ca^{2+}-independent after autophosphorylation (Leonard *et al.*, 1987). The conservation of kinase regulation by autophosphorylation across such a broad range of species suggests that it may be a critical feature in the function of the kinase.

4.5.2.3 Physiological Significance of Regulation by Autophosphorylation.

Autophosphorylation of the broad-specificity kinases has not yet been demonstrated to occur *in vivo*. The properties of the autophosphorylation reaction described above suggest that the kinase would become autophosphorylated after a sufficiently prolonged increase in internal Ca^{2+} concentration. The properties of regulation by autophosphorylation suggest that the kinase has many of the properties of a molecular switch such as the one described by Lisman (1985). In the resting cell the kinase is envisioned to exist in the nonphosphorylated state (State 1). Transient increases in intracellular Ca^{2+} concentration would briefly activate the kinase, allowing phosphorylation of exogenous substrates as well as autophosphorylation. If the increase in intracellular Ca^{2+} was sufficiently prolonged (or a burst of such transients occurred sufficiently close together in time) autophosphorylation would proceed to the threshold level required for production of Ca^{2+}-independent kinase activity (State 2). In this state the kinase would still be stimulated to some extent by further Ca^{2+} transients but would retain a high basal level of activity even when Ca^{2+} remains at resting levels. A return to the resting state (State 1) would require removal of phosphate by protein phosphatases, but this would be opposed by the ability of the kinase to autophosphorylate in the absence of Ca^{2+}. Continued autophosphorylation at resting levels of Ca^{2+} would convert the kinase into a state (State 3) in which activity was completely Ca^{2+}-independent and insensitive to further

Ca^{2+} transients. The kinase could theoretically remain in this state for the lifetime of the molecule *if* the rate of dephosphorylation by protein phosphatases is lower than the rate of autophosphorylation.

Further experiments will be required to determine whether the broad-specificity kinases undergo autophosphorylation in response to physiologically relevant stimulation *in vivo.* An understanding of the specificity and regulation of the protein phosphatases that dephosphorylate the kinase *in vivo* is also crucial in determining the potential of autophosphorylation as a regulatory mechanism. In contrast to protein kinases, relatively little is currently known about the structure, distribution, and regulation of brain protein phosphatases.

5 Postsynaptic Densities.

Postsynaptic densities (PSDs) are ultrastructural elements unique to the nervous system. When examined by transmission electron microscopy they appear as electron-dense thickenings immediately underlying the postsynaptic membrane of many central nervous system synapses (Gray, 1959). The exact role of PSDS in nervous system function remains unclear, but their location and composition suggest that they may play an important part in synaptic function and modulation. The PSD is a biochemically complex structure (see below) that contains various cytoskeletal elements, kinases, kinase substrates, and neurotransmitter receptors. The type II CaM kinase appears to be a major component of a subset of PSDS in the central nervous system (Kennedy *et al.*, 1983; Kelly *et al.*, 1984; Goldenring *et al.*, 1984; and chapter 1 of this thesis) therefore I have included here a discussion of the structure and composition of postsynaptic densities.

5.1 PSD Structure, Isolation, and Composition.

As mentioned above the PSD is an electron dense element located just beneath the postsynaptic membrane. PSDS vary in both shape and size; some are thick and cover the entire postsynaptic surface (Type I, asymmetric), while others are thinner and sometimes appear discontinuous (Type II, symmetric). It has been suggested that there may be a correlation between the type of PSD (I or II) and specific types of synapses (excitatory or inhibitory). Type I PSDS are correlated with the presence of excitatory synapses while type II PSDs are

correlated with inhibitory synapses (Landis and Reese, 1974; Sheperd, 1974; Uchizono, 1968; Siekevitz, 1981; Carlin *et al.*, 1980). In forebrain (cerebral cortex and hippocampus) the PSD often appears as a disk of 300-500nm in diameter with a thickness of 50-60nm (Cohen *et al.*, 1977; Blomberg *et al.*, 1977). A central perforation is often observed when the section cuts the PSD obliquely (Peters and Kaiserman-Abramof, 1969; Cohen and Siekevitz, 1978), and fine filaments are often seen extended intracellularly from the PSD (Cohen and Siekevitz, 1978; Gulley and Reese, 1981).

Methods have been developed for the isolation of fractions from mammalian brain which, at the level of electron microscopy, appear to be enriched in PSDs. These PSD-enriched fractions are prepared by lysing synaptosomes derived from crude brain homogenates and recovering the membranes (and adherant PSDs) by centrifugation. The membrane is removed by treatment with detergent and PSDs further enriched by sucrose density gradient sedimentation. PSDs isolated by these methods appear very similar to the structures seen in fixed brain tissue at the electron microscopic level (Fiszer and deRobertis, 1967; Cotman and Taylor, 1972; Cohen *et al.*, 1977; Matus and Taff-Jones, 1978). Negative staining of purified PSD fractions with uranyl acetate reveals the existence a PSD substructure consisting of a planar array of 18-20nm electron dense subunits containing one or more areas of fine granular material (Matus, 1981). Further extraction of PSD fractions with the detergent deoxycholate leaves a lattice of short, branching fibers enclosing small spaces approximately the size of the subunits described above (Blomberg *et al.*, 1977; Matus and Taff-Jones, 1978). It is interesting to note that the major protein

constituent of cerebral PSDs, the type II CaM kinase (see below), has a predicted solution diameter (estimated from its hydrodynamic properties) of approximately 20nm (Bennett *et al.*, 1983; Kennedy *et al.*, 1983) and may therefore make up the PSD subunit structure.

The PSD-enriched fractions described above have been extensively characterized biochemically. Cerebral PSD fractions contain 20-30 major proteins when examined by SDS-PAGE. The most abundant in cerebral PSDs is a M_r 50,000 protein originally termed the "major postsynatpic density protein" (mPSDp). It has since been demonstrated that this protein is identical to the α subunit of the type II CaM kinase (Kennedy *et al.*, 1983; Kelly *et al.*, 1984; Goldenring *et al.*, 1984). Cytoskeletal components are also found in the PSD, including actin (Blomberg *et al.*, 1977; Kelly and Cotman, 1978; Matus and Taff-Jones, 1978), fodrin (brain spectrin) (Carlin *et al.*, 1983; Groswald and Kelly, 1984), and probably tubulin (Blomberg *et al.*, 1977; Kelly and Cotman, 1978; Matus and Taff-Jones, 1978; Walters and Matus, 1975; Feit *et al.*, 1977; Rostas *et al.*, 1979; Carlin *et al.*, 1982). These cytoskeletal proteins have been implicated in the tight binding of the PSD to the postsynaptic membrane and possibly in the tight connection between the pre- and postsynaptic membranes across the synaptic cleft (Ratner and Mahler, 1983; Siekevitz, 1985). A number of glycoproteins have also been found in PSDs (Gurd, 1977; Gurd, 1980; Kelly and Cotman, 1977; Gordon-Weeks and Harding, 1983), suggesting the possibility of interaction with the presynaptic membrane or elements of the synaptic cleft. Various receptors and ion channels have also been detected in PSD fractions, including receptors for γ-aminobutyric acid (GABA) and

flunitrazepam (Wong and Hornig, 1977; Lester *et al.*, 1981; Matus *et al.*, 1981; Carlin and Siekevitz, 1984), glutamate (Fagg and Matus, 1984), and Ca^{2+}-activated K^+-channels (Wu *et al.*, 1985). The fractions are also enriched in proteins that are elements in the cAMP and CaM-dependent protein phosphorylation systems including calmodulin (Grab *et al.*, 1981; Wood *et al.*, 1980), calmodulin-activated cyclic nucleotide phosphodiesterase (Florendo *et al.*, 1971; Grab *et al.*, 1979; Grab *et al.*, 1981), and both cAMP- (Ueda *et al.*, 1979; Bloom *et al.*, 1979; Ng and Matus, 1979; DeBlas *et al.*, 1979) and Ca^{2+}/calmodulin-activated (Carlin *et al.*, 1980; Grab *et al.*, 1981) protein kinase activities (see below). The calmodulin-activated protein phosphatase, calicneurin, is also found in PSD fractions (Wood *et al.*, 1980; Stewart *et al.*, 1982), completing all of the elements for the regulation of substrate proteins by phosphorylation/dephosphorylation .

The major postsynaptic density protein has been widely accepted as a marker for PSDs (Cotman *et al.*, 1974; Kelly and Cotman, 1978; Kelly and Montgomery, 1982) and was thought to be exclusively localized in PSDs (Kelly and Montgomery, 1982; Rostas *et al.*, 1983). After purification and characterization of the type II CaM kinase, several groups investigated the relationship of its α subunit with the mPSDp. The motivation for this was the similarity of a number of properties of the two proteins including molecular size, Ca^{2+}/calmodulin-dependent phosphorylation and ability to bind calmodulin. The groups reported the identity of the mPSDp with the α subunit of the type II CaM kinase based on a number of biochemical and immunochemical criteria (Kennedy *et al.*, 1983; Kelly *et al.*, 1984; Goldenring *et al.*, 1984). Work reported

in chapter 2 of this thesis (Miller and Kennedy, 1985) using quantitative radioimmunoassays demonstrated that the type II CaM kinase makes up 16% of the mass of forebrain and 1-2% of the mass of cerebellar PSDs.

5.2 The Function of the Postsynaptic Density.

The possible functional role of the postsynaptic density is intriguing, but no evidence exists to support the hypotheses that have been presented (e.g., Matus, 1981; Siekevitz, 1985). A common role attributed to the PSD is that of anchoring synaptic membrane proteins at the synapse. When one considers the biochemical composition of the PSD (described above) it appears likely that it may also be involved in dynamic processes occurring at the synapse, i.e., synaptic regulation (discussed in Siekevitz, 1985). The PSD contains of all of the necessary components for regulation of proteins by phosphorylation/dephosphorylation. Despite the ease with which one can postulate functional roles for the postsynaptic density, it is very difficult to formulate experiments that test these ideas. The problem lies in the absence of a suitable *in vitro* experimental system in which the necessary physiology and biochemistry can be carried out. Elucidation of the actual functional role of the postsynaptic density remains a major problem confronting biochemists studying synaptic transmission.

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CHAPTER 2

DISTINCT FOREBRAIN AND CEREBELLAR ISOZYMES OF TYPE II CA^{2+}/CALMODULIN-DEPENDENT PROTEIN KINASE ASSOCIATE DIFFERENTLY WITH THE POSTSYNAPTIC DENSITY FRACTION*

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Distinct Forebrain and Cerebellar Isozymes of Type II Ca^{2+}/Calmodulin-dependent Protein Kinase Associate Differently with the Postsynaptic Density Fraction*

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Forebrain and cerebellar Type II Ca^{2+}/calmodulin-dependent protein kinases have different subunit compositions. The forebrain holoenzyme, characterized in our laboratory, is a 650-kDa holoenzyme composed of 50-kDa α-subunits and 60-kDa β-subunits assembled in approximately a 3:1 ratio (Bennett, M. K., Erondu, N. E., and Kennedy, M. B. (1983) *J. Biol. Chem.* 258, 12735–12744). The cerebellar isozyme is a 500-kDa holoenzyme composed of α-subunits and β-subunits assembled in almost the converse ratio, approximately four β-subunits for each α-subunit. When compared by tryptic peptide mapping and by immunochemical techniques, the β-subunits from the two brain regions are indistinguishable and the α-subunits appear closely related.

The specific activities, substrate specificities, and catalytic constants of the cerebellar and forebrain isozymes are similar, suggesting that the α- and β-subunits contain similar catalytic sites. However, two differences in the properties of the isozymes may result in functional differences between them *in vivo*. First, the apparent affinity of the cerebellar kinase for Ca^{2+}/calmodulin is 2-fold higher than that of the forebrain kinase. Second, the two isozymes appear to associate differently with subcellular structures. Approximately 85% of the cerebellar kinase and 50% of the forebrain kinase remain in the particulate fraction after homogenization under standard conditions. However, they are present in different amounts in postsynaptic density fractions. Postsynaptic densities prepared from forebrain contain the forebrain isozyme. Immunochemical measurements show that it comprises ~16% of their total protein. In contrast, postsynaptic densities prepared from cerebellum contain the cerebellar isozyme, but it comprises only ~1–2% of their total protein. Thus, the α-subunit may play a role in anchoring Type II Ca^{2+}/calmodulin-dependent protein kinase to postsynaptic densities.

Many agents regulate the functions of target cells by altering the concentration of an intracellular second messenger such as a cyclic nucleotide, Ca^{2+} ion, or diacylglycerol (1–4). The cyclic nucleotides and diacylglycerol are believed to act primarily through the activation of specific protein kinases (5–7). The actions of Ca^{2+} as a second messenger are more diverse and often require the Ca^{2+}-binding protein calmodulin (8). Among the enzymes regulated by Ca^{2+}/calmodulin are a family of protein kinases. Ca^{2+}/calmodulin-dependent protein kinase activities have been described in mammalian brain (9, 10), pituitary (11), liver (12), pancreas (13, 14), skeletal and smooth muscle (15–18), avian erythrocytes (19), *Torpedo* electric organ (20), and *Aplysia* ganglia (21, 22). Recent structural characterization of some of these kinases suggests that there are at least four and perhaps more distinct Ca^{2+}/calmodulin-dependent protein kinases (15–18, 23–32). The diversity of these kinases may underlie, in part, the diversity of cellular responses to Ca^{2+}.

We recently reported the purification and characterization of a Ca^{2+}/calmodulin-dependent protein kinase with a broad substrate specificity that is far more highly expressed in brain than in other tissues (27). Brain kinases that are structurally similar and may be identical with this one have been described by other groups (28, 29, 31, 32). Two of the groups have referred to the kinase as "Ca^{2+}/calmodulin-dependent kinase II" (29, 31). Because this kinase now appears to occur as a family of homologous but distinct forms in different tissues as well as brain regions (29, 33, 34, and this report), we refer to them as "Type II" Ca^{2+}/calmodulin-dependent protein kinases. These kinases have several features in common. They are multimeric proteins of high molecular mass (300–700 kDa), composed of structurally related 50–60-kDa subunits. They exhibit similar substrate specificities. All the kinase subunits bind calmodulin and undergo a Ca^{2+}/calmodulin-dependent autophosphorylation. The kinases differ in the exact molecular weights and ratios of their subunits.

The brain Type II CaM kinase[1] previously described by this lab (27) is a 650-kDa holoenzyme composed of ~9 α (50 kDa)- and ~3 β/β' (60/58 kDa)-subunits. This isozyme of the kinase is concentrated in the forebrain (cortex and hippocampus) and makes up about 75% of the total brain Type II CaM kinase.[2] In this paper we report the purification of a distinct isozyme of the kinase from cerebellum. It is composed of subunits similar to those of the forebrain kinase, but they are assembled in a different ratio. We compare its structural and enzymatic properties to those of the forebrain kinase.

We and others have shown that the Type II CaM kinase is a major constituent of brain postsynaptic density fractions (36–38). The postsynaptic density is a specialization of the submembranous cytoskeleton that occurs beneath postsyn-

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[1] For brevity, we will frequently refer to the brain Type II Ca^{2+}/calmodulin-dependent protein kinases as Type II CaM kinases. They are not related to the Type II cAMP-dependent protein kinase.

[2] N. Erondu, M. Bennett, and M. Kennedy, unpublished observations.

aptic membranes in CNS neurons. We show here that forebrain PSD[3] fractions contain the forebrain isozyme as a major component while cerebellar PSD fractions contain the cerebellar isozyme, but in greatly reduced amounts. A preliminary report of part of this work has appeared (33). After this manuscript was submitted, another paper describing the physical properties of the cerebellar isozyme was published (34).

EXPERIMENTAL PROCEDURES

Materials—Alcohol dehydrogenase, ATP, bovine serum albumin, carbonic anhydrase, casein, chloramine-T, EDTA, EGTA, fast green, fibrinogen, hemoglobin (Type II), histones (Type VIII-S), imidazole, ovalbumin, phosvitin, PMSF, polyinosinic-polycytidylic acid, and soybean trypsin inhibitor were purchased from Sigma. Aldolase, blue dextran 2000, catalase, ferritin, protein A, Sepharose 4B, and thyroglobulin were purchased from Pharmacia. DEAE-Bio-Gel A, SDS, acrylamide, and bisacrylamide were purchased from Bio-Rad. Nitrocellulose membranes (BA85, 0.45 μm) were purchased from Schleicher and Schuell, cellulose-coated thin layer chromatography plates from Eastman, trypsin treated with 1-tosylamido-2-phenylethyl chloromethyl ketone from Worthington, ultrapure sucrose from Bethesda Research Laboratories, and Coomassie Brilliant Blue from Searle Diagnostic. Sodium perchlorate was purchased from G. Frederick Smith Chemical Co., dithiothreitol from Boehringer Mannheim, leupeptin from Peninsula Laboratories, $Na^{125}I$ (carrier-free) from Amersham, and [γ-^{32}P]ATP from ICN Nutritional Biochemicals. Simonsen albino rats (140–160 g, male) were purchased from Simonsen Laboratories (Gilroy, CA). Immunoprecipitin was purchased from Bethesda Research Laboratories and washed as described by Kessler (39).

Rabbit anti-mouse IgG antiserum was prepared as described previously (36). ^{125}I-labeled protein A (2–3 Ci/mmol) was prepared by the chloramine-T method (40). Synapsin I was prepared by a modification of the method of Ueda and Greengard (41) as described previously (27). Calmodulin was purified from bovine brain by the method of Watterson *et al.* (42). Microtubule protein was purified by the method of Shelanski *et al.* (43). MAP_2, purified from microtubule protein by the method of Kim *et al.* (44), was the kind gift of James Soha of the California Institute of Technology. Calmodulin-Sepharose was prepared by the method of March *et al.* (45).

Purification of the Cerebellar Type II Ca^{2+}/Calmodulin-dependent Protein Kinase—The purification was carried out as previously described (27) with several modifications. All operations were carried out at 4 °C.

Brains were removed from 90 rats after stunning and decapitation. The cerebellar lobes, weighing approximately 0.23 g each, were removed and immediately homogenized by 12 up and down strokes in a Teflon/glass homogenizer driven at 900 rpm in 10 volumes of Buffer A (40 mM Tris (pH 7.5), 1 mM imidazole, 150 mM Na^+-perchlorate, 250 mM sucrose, 1 mM EGTA, 5 mM EDTA, 2 mM dithiothreitol, 0.1 mM PMSF, 1 mg/l of leupeptin, and 25 mg/l of soybean trypsin inhibitor). The crude homogenate was centrifuged at 2000 × *g* for 20 min and the resulting supernatant was centrifuged at 170,000 × *g* for 1 h.

The 170,000 × *g* supernatant was diluted 5-fold into Buffer B (20 mM Tris (pH 7.5), 1 mM imidazole, 2 mM EGTA, 2 mM dithiothreitol, 0.1 mM PMSF, and 0.02% NaN_3), mixed with 50 ml of DEAE-agarose previously equilibrated with Buffer B, and stirred slowly for 90 min. The gel slurry was poured into a Buchner funnel and excess supernatant was drawn off by gentle suction. The resulting gel slurry was poured into a glass column (1.5 × 30 cm) and packed at 80–100 ml/h. The column was washed with 100 ml of Buffer B containing 0.01 M NaCl. Enzyme activity was eluted with a linear gradient of 0.01–0.30 M NaCl in Buffer B (total volume of 500 ml) at 80 ml/h while collecting 6-ml fractions. Kinase activity peaked at 0.08 M NaCl. Peak fractions were pooled, adjusted to 0.1 M Tris (pH 7.5), 1 mM dithiothreitol, 0.1 mM PMSF, and brought to 50% saturation by the slow addition of solid ammonium sulfate over 1 h. After 3–12 h, precipitated protein was collected by centrifugation and redissolved in 5 ml of Buffer C (40 mM Tris (pH 7.5), 2 mM EGTA, 2 mM dithiothreitol, 0.1 mM PMSF, and 0.02% NaN_3).

Equal volumes of the redissolved ammonium sulfate precipitate were layered onto each of four 37-ml linear gradients of 5–20% sucrose in Buffer C. The gradients were centrifuged for 24 h at 27,000 × *g* in a Beckman SW 27Ti rotor. One-ml fractions were collected with an Isco Model 184 density gradient fractionater while monitoring absorbance at 280 nm. Kinase activity appeared approximately midway through the gradient.

For determination of the Stokes radius, gel filtration through Sepharose 4B replaced the sucrose density gradient centrifugation step. The ammonium sulfate precipitate was redissolved in 4 ml of Buffer C, adjusted to 20% (v/v) glycerol, and loaded on a Sepharose 4B column (1.25 × 100 cm) equilibrated with Buffer C plus 20% glycerol. The column was run at a flow rate of 3.2 ml/h while collecting 0.6-ml fractions. Kinase activity eluted as a single peak at approximately 80 ml.

The sucrose gradient (or Sepharose 4B gel filtration) fractions containing kinase activity were pooled and adjusted to 0.1 M Tris (pH 7.5), 0.2 M NaCl, 2 mM dithiothreitol, 0.1 mM PMSF, 2.5 mg/l of leupeptin, and 0.4 mM free Ca^{2+}. This pool was then applied to a calmodulin-Sepharose affinity column (0.9 × 2.5 cm) equilibrated with Buffer D (40 mM Tris (pH 7.5), 0.4 mM $CaCl_2$, 2 mM dithiothreitol, 0.1 mM PMSF, and 0.02% NaN_3) containing 0.2 M NaCl. The column was washed with 4 column volumes of Buffer D containing 2.0 M NaCl, 5 column volumes of Buffer D, and 3 column volumes of Buffer C. Enzyme activity was then eluted with a linear gradient (12 ml total volume) of 0–0.2 M NaCl in Buffer C while collecting 0.5-ml fractions. Kinase activity eluted at approximately 0.08 M NaCl.

The forebrain Type II CaM kinase was purified from whole brain or from forebrain as described by Bennett *et al.* (27) except that homogenization and batch DEAE-absorption were performed as described above. This resulted in a higher recovery of forebrain kinase (3–5 mg/120 g wet weight of brain) than previously reported.

Preparation of the Postsynaptic Density Fraction—Postsynaptic density fractions were prepared using Triton X-100 as described by Carlin *et al.* (46). Briefly, brains were removed from 25 rats and the cortices and cerebella were separated and homogenized in isotonic sucrose. A mixed synaptosomal/mitochondrial pellet was obtained by differential centrifugation and then fractionated by centrifugation through a discontinuous sucrose gradient. The synaptosomal fraction obtained from the 1.0:1.3 M sucrose interface was adjusted to 0.5% Triton X-100, 6 mM Tris, pH 8.1, and insoluble material was pelleted by centrifugation. This material was resuspended and centrifuged through a second discontinuous sucrose gradient. The material at the interface of the 1.5 and 2.0 M sucrose steps, which is enriched in PSDs, was resuspended in 0.5% Triton X-100, 75 mM KCl and then pelleted by centrifugation. The final pellet was resuspended in 40 mM Tris, pH 8.0, and used for further characterization. Yields were approximately 0.6–0.7 mg/10 g wet tissue from cerebellum and 2–3 mg/10 g wet tissue from forebrain.

Protein Phosphorylation Assays—Kinase activity was assayed as described (27) except that 10 mM dithiothreitol was included in the final reaction mixture. Autophosphorylation of kinase subunits and endogenous phosphorylation of PSDs was performed as described (27) except that [γ-^{32}P]ATP was used at a lower concentration (5–10 μM) and higher specific activity (2.0–5.0 × 10^4 cpm/pmol) and an additional 6 μl of β-mercaptoethanol was added to the stopped reactions just prior to gel electrophoresis.

Determination of Catalytic Constants—Kinetic parameters were measured as described above except that the amount of Synapsin I was increased to 20 μg, except when it was the variable, and the reaction time was decreased to 10–15 s to ensure measurement of initial rates. Each tube contained 20–50 ng of purified enzyme (4–10 nM subunits). Utilization of substrates (ATP and Synapsin I) was less than 10% in each assay. The apparent K_m values of various substrates and the apparent K_a for Ca^{2+}/calmodulin were determined by fitting the data to the Michaelis-Menton equation with a weighted nonlinear least squares computer program adapted from Cleland (47). The apparent K_m for ATP and the apparent K_a values for Ca^{2+}/calmodulin were compared in duplicate experiments using the same reagents.

Preparation of Antibodies—Monoclonal antibodies 6G9 and 4A4 were produced and selected as described (35, 36). These antibodies were purified from ascites fluids by precipitation with 50% ammonium sulfate followed by chromatography on Protein A-Sepharose or by two successive 50% ammonium sulfate precipitations. Polyclonal

[3] The abbreviations used are: PSD, postsynaptic density fraction; PMSF, phenylmethylsulfonyl fluoride; MAP_2, microtubule-associated protein 2; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

rabbit antisera were produced by multiple subcutaneous injections of purified forebrain Type II kinase or of electrophoretically purified subunits in phosphate-buffered saline containing an equal weight of polyinosinic-polycytidylic acid. The antisera were the kind gifts of Ngozi Erondu and Mark Bennett of this laboratory.

Immunoblots and Radioimmunoassays—Proteins were separated by SDS-PAGE and transferred to nitrocellulose paper as described by Towbin *et al.* (48). The nitrocellulose sheets containing transferred protein were labeled with antibodies as previously described (36). Radioimmunoassay was carried out by a quantitative immunoblot method, as described by Erondu and Kennedy (35) using either monoclonal antibody 6G9 for detection of the α-subunit or a rabbit polyclonal antisera for detection of the β-subunit. Briefly, standard amounts of purified forebrain Type II Ca^{2+}/calmodulin-dependent protein kinase and unknown samples were separated by SDS-PAGE and transferred to nitrocellulose paper as above. The nitrocellulose sheets were labeled with appropriate antibodies and subjected to autoradiography after drying. The labeled bands were located on the nitrocellulose sheet using the autoradiograph, cut out, and counted in a γ counter. A standard curve was constructed from the counts bound to standard amounts of forebrain Type II CaM kinase (after subtracting background). Linear standard curves were obtained on log/log plots for 20–500 ng of α-subunit with 6G9 and for 80–500 ng of β-subunit for the rabbit antisera.

Other Procedures—Protein concentrations were measured by the method of Lowry *et al.* (49) as modified by Peterson (50). SDS-PAGE was performed by the method of Laemmli (51). Stacking gels (2.5 × 16 cm × 1.5 mm) contained 3.5% acrylamide, 0.09% bisacrylamide and the running gels (14 × 16 cm × 1.5 mm) contained 10% acrylamide, 0.27% bisacrylamide. Molecular weight standards for SDS-PAGE were: MAP_2 (300,000), phosphorylase *b* (94,000), bovine serum albumin (68,000), ovalbumin (43,000), alcohol dehydrogenase (41,000), carbonic anhydrase (29,000), chymotrypsinogen (25,000), and soybean trypsin inhibitor (21,500). The sedimentation coefficient ($s_{20,w}$) of the kinase was determined by sucrose density gradient centrifugation as described by Martin and Ames (52) with ovalbumin ($s_{20,w}$ = 3.5 S), fibrinogen ($s_{20,w}$ = 7.9 S), catalase ($s_{20,w}$ = 11.3 S), and thyroglobulin ($s_{20,w}$ = 19.2 S) as standards. The Stokes radius (Å) of the kinase was determined by gel filtration through Sepharose 4B as described by Porath (53), with catalase (52 Å), ferritin (61 Å), thyroglobulin (85 Å), and fibrinogen (107 Å) as standards. Iodinated tryptic maps were prepared by the method of Elder *et al.* (54).

RESULTS

Purification of Cerebellar Type II Ca^{2+}/Calmodulin-dependent Protein Kinase

Purification—The homogenization conditions described in our initial report on the purification of brain Type II CaM kinase (27) have been altered to optimize recovery of the cerebellar kinase. When the cerebellum was homogenized in the original homogenization buffer, 80–90% of the Type II CaM kinase activity was recovered in the particulate fraction after centrifugation. The addition of the chaotropic salt, sodium perchlorate (0.15 M), to the homogenization buffer released most of the bound kinase resulting in the recovery of 70–80% of it in a soluble form. The cerebellar kinase activity was unstable in the presence of calcium, therefore 0.2 mM $CaCl_2$ was replaced by 1 mM EGTA, 5 mM EDTA. The solubilized enzyme was adsorbed to DEAE-agarose by a batch process because of the large sample volume after dilution of the 170,000 × *g* supernatant to an appropriate ionic strength. The purification of the cerebellar kinase is summarized in Table I.

Different salt concentrations were required to elute the forebrain and cerebellar Type II CaM kinases from calmodulin-Sepharose. The forebrain isozyme was eluted in EGTA buffers without salt (27), while elution of the cerebellar isozyme required both EGTA and ~0.08 M NaCl. This may result from nonspecific ionic interactions of the cerebellar kinase with the column or from specific low affinity binding of the kinase to calmodulin even in the absence of Ca^{2+}. The calmodulin-Sepharose pool was approximately 90% pure as judged by densitometric scans of stained SDS-polyacrylamide gels. It represented a purification of about 400-fold from the crude homogenate with a yield of approximately 5%. The low overall recovery was due largely to low recoveries from DEAE-agarose. The specific activities of the calmodulin-Sepharose pools varied from 1.23 to 2.90 μmol/min/mg under our standard assay conditions and from 3.4 to 8.0 μmol/min/mg at saturating synapsin I concentrations. This compares to an average value for the forebrain kinase of 2.9 μmol/min/mg under standard conditions and 8.0 μmol/min/mg at saturating synapsin I concentrations. The physical properties of the forebrain kinase were not altered by the changes in purification procedure and recoveries were improved, ranging from 6 to 11%.

Subunit Composition—The Type II CaM kinases purified from cerebellum and from forebrain both contain two major subunits, α (50 kDa) and β (60 kDa), but they are present in different ratios (Fig. 1). Minor subunits of 56 and 58 kDa (β') in the cerebellar kinase are more prominent than the 58-kDa β'-subunit in the forebrain kinase. As for the forebrain kinase, the cerebellar β- and β'-subunits have identical peptide maps and occur in a constant ratio to the α-subunits from preparation to preparation. All of the subunits of the cerebellar kinase were precipitated by a monoclonal antibody (4A4) that recognizes only the α-subunit on Western blots (data not shown). Thus, they exist together in a holoenzyme complex. In several immunoprecipitation experiments with antibody 4A4, 6–7% of the initial kinase activity remained in the supernatant after immunoprecipitation with maximal amounts of antibody (data not shown). Thus, a small proportion of the cerebellar kinase holoenzyme molecules may not contain α-subunits.

The physical properties of the cerebellar isozyme were determined by the methods used to characterize the forebrain kinase (27). The molar ratio of the subunits was determined from densitometric scans of gels stained with fast green. The β'-doublet was treated as a single band for this analysis. The ratio of β-subunit alone to α-subunit was 3:1 and the ratio of total β-subunits ($\beta + \beta'$) to α-subunit was 4:1. The approximate molecular weight of the holoenzyme was calculated from its hydrodynamic properties (Table II). A Stokes radius of 88 Å was measured by gel filtration and a sedimentation coefficient ($s_{20,w}$) of 14.0 S was measured by sucrose density gradient centrifugation. A molecular weight of 508,000 (± 48,000) was calculated from these parameters as described in Table II. The cerebellar isozyme consistently appeared smaller than the forebrain isozyme. This difference was observed even in parallel density gradients run at the same time. The subunit structure of the cerebellar holoenzyme that is most consistent with its molecular weight and the ratio of its subunits is a decamer of ~8 β/β'-subunits and ~2 α-subunits. The molecular weight of such a holoenzyme would be 574,000, slightly higher than the range indicated by its hydrodynamic properties.

Comparison of the Subunits of Forebrain and Cerebellar Isozymes

Iodinated Peptide Maps—We compared maps of iodinated peptides of the α- and β-subunits of the cerebellar kinase to those of the forebrain kinase (Fig. 2). Maps of the β-subunits of the two isozymes were identical. Maps of the α-subunits, however, showed a few reproducible differences. Maps of the β'-subunits from both isozymes (not shown) reveal that they are closely related to the β-subunits. As has been noted before (28, 37), peptide maps of the α- and β-subunits of the forebrain

TABLE I

Summary of purification of rat cerebellar Type II Ca^{2+}/calmodulin-dependent protein kinase

Step	Total activity[a]	Total protein[a]	n	Specific activity[b]	Purification	Recovery
	μmol/min	mg		μmol/min/mg	-fold	%
1. Homogenate	13.5 ± 1.5	2418 ± 450	5	0.006	1.0	100
2. 170,000 × g supernatant	10.4 ± 1.9	724 ± 110	5	0.013	2.8	77
3. DEAE-agarose pool	1.98 ± 0.3	175 ± 23	5	0.012	2.2	15
4. $(NH_4)_2SO_4$ ppt.	1.74 ± 0.3	83 ± 13	5	0.021	4.1	13
5. Sucrose gradient	1.38 ± 0.2	16 ± 4	3	0.091	16.8	10
6. Calmodulin-Sepharose pool	0.76 ± 0.2	0.49 ± 0.29	2	2.02	400	6

[a] Values are mean ± S.D. of n experiments.
[b] Average of n preparations.

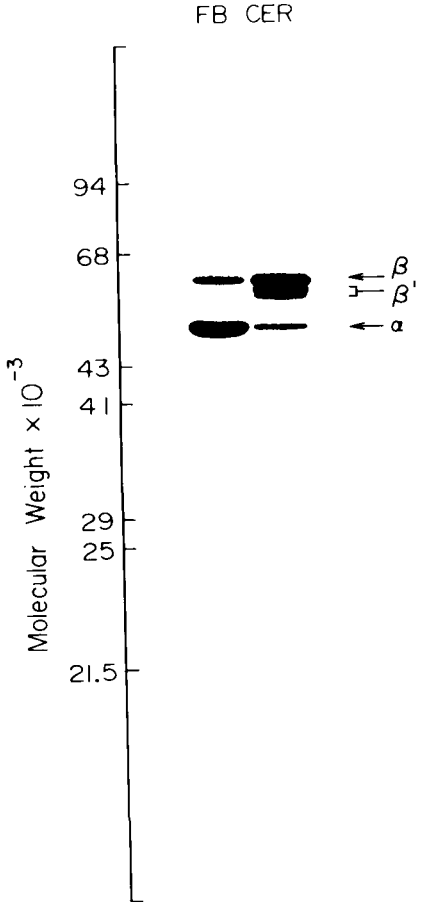

FIG. 1. **Comparison of purified forebrain (*FB*) and cerebellar (*CER*) isozymes of rat brain Type II Ca^{2+}/calmodulin-dependent protein kinase.** Each isozyme (7.5 μg) was subjected to SDS-PAGE on a 12.5% gel, then stained with Coomassie Blue. Positions of molecular weight standards are indicated on the *left*.

kinase contain several peptides in common, indicating that they are structurally related. This is also the case for the cerebellar kinase. However, the structural homology between the α- and β-subunits is not as close as the homology between corresponding subunits in the two holoenzyme forms.

Immunochemical Comparison—Monoclonal and polyclonal antibodies which recognize specific subunits of the forebrain Type II CaM kinase on immunoblots were used to test the immunological relationship between the subunits of the forebrain and cerebellar isozymes. Two polyclonal rabbit antisera that recognize primarily the β-subunit of the forebrain kinase cross-reacted strongly with the cerebellar β/β'-subunits. A monoclonal antibody, 6G9, which binds to the α-subunit of the forebrain isozyme also bound to the α-subunit of the cerebellar isozyme (data not shown). Thus, the denatured subunits of the two kinases were indistinguishable by these immunochemical reagents.

TABLE II

Physical properties of the forebrain and cerebellar forms of Type II Ca^{2+}/calmodulin-dependent protein kinase

Property	Forebrain[a]	Cerebellum
Stokes radius (a)	94.7 ± 1.2 Å	88 ± 1.3 Å[b]
Sedimentation coefficient ($s_{20,w}$)	16.4 ± 0.7 S	14.0 ± 1.1 S[c]
Frictional coefficient (f/f_0)	1.67	1.67[d]
Molecular weight (M_r)	650,000	508,000 ± 48,000[d]
Holoenzyme composition	9 α:3 β/β' (654,000 Da)	2 α:8 β/β' (574,000 Da)

[a] Values for the forebrain are those previously published by Bennett *et al.* (27).
[b] Identical values were obtained in two experiments. The range is ±Stokes radii included in one column fraction.
[c] Mean ± S.D. of three experiments.
[d] Calculated according to the equation: $M_r = 6\pi\eta_{20,w}s_{20,w}aN/(1 - \bar{\nu}\rho_{20,w})$ where N = Avogadro's number, $\eta_{20,w}$ = viscosity of water at 20 °C, $\rho_{20,w}$ = density of water at 20 °C, and $\bar{\nu}$ = partial specific volume, assumed to be 0.725 cm^3/g. The frictional coefficient was determined by the formula: $f/f_0 = a(4\pi N/3\bar{\nu}M_r)^{1/6}$.

Comparison of Enzymatic Properties of Forebrain and Cerebellar Kinases—The substrate specificity and catalytic constants of the forebrain and cerebellar isozymes were compared to determine whether their catalytic properties might differ significantly *in vivo*.

Substrate Specificity—The forebrain kinase phosphorylates a number of substrate proteins (27). We measured the ability of the cerebellar kinase to phosphorylate several of these same substrates (Table III). At this level of kinetic resolution, the substrate specificity of the two forms did not differ. Synapsin I and MAP_2 were phosphorylated at the highest rate.

Catalytic Constants—For each of the two kinases, we determined the apparent K_m values for synapsin I, MAP_2, and ATP, as well as the apparent K_a for calmodulin (Table IV). The apparent K_m values for synapsin I and MAP_2 were the same, however the K_m for ATP differed by a factor of ~1.8, the cerebellar kinase having the higher affinity. Since the intracellular ATP concentration is estimated to be about 1–2 mM (55), the difference between the two kinases is not likely to be significant *in vivo*. However, the cerebellar kinase also

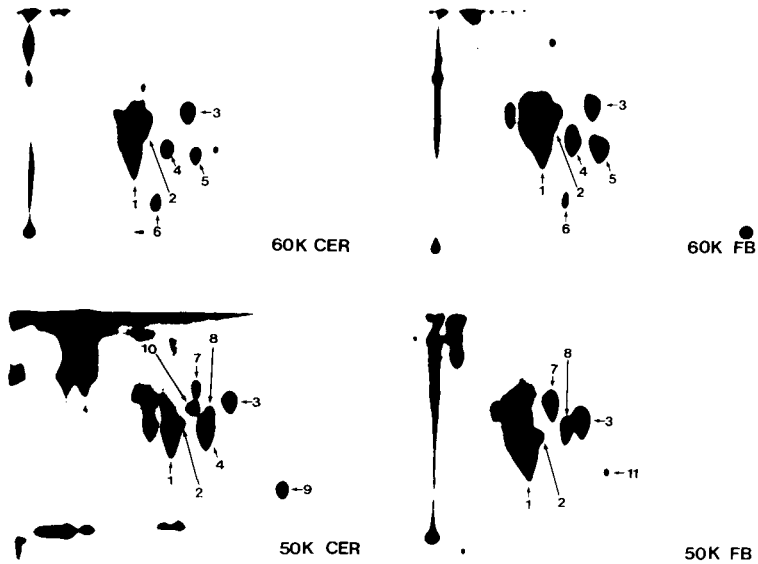

FIG. 2. **Iodinated tryptic peptides of the α- and β-subunits of Type II CaM kinase isolated from forebrain (*FB*) and cerebellum (*CER*).** Five μg of pure kinase isolated from each brain region was subjected to SDS-PAGE and stained with Coomassie Blue. The bands corresponding to the α-, β-, and β′-subunits from each region were cut from the gel. Iodinated tryptic peptide maps were prepared as previously described (54). One μl of the peptide solution was applied to the plate at the *lower left*. For electrophoresis, the anode was to the *left* and the cathode to the *right* for electrophoresis; chromatography was from *bottom* to *top*. The spots labeled *1*, *2*, and *3* were common to both subunits from both brain regions; *4*, *5*, and *6* were common to the β-subunits (*4* was also found in the cerebellar α-subunit); *7* and *8* were common to the α-subunits (although differing in intensity); *9* and *10* were found only in the cerebellar α-subunit; and *11* is found only in the forebrain α-subunit. The identities of the various spots were confirmed by mapping of mixtures of peptides from the different subunits.

TABLE III

Substrate specificity of cerebellar Type II Ca^{2+}/calmodulin-dependent protein kinase

Substrate	Concentration in assay	Rate of Ca^{2+}/calmodulin-stimulated phosphorylation	
		Cerebellum	Forebrain
	mg/ml	%	
Synapsin I[a]	0.1	100	100
MAP_2[b]	0.4	128	141
Histone[c]	0.2	21	14
Phosvitin	0.4	3	8
Casein	0.4	0	0
Phosphorylase *b*	0.4	2	3

[a] The rate of Ca^{2+}-stimulated phosphorylation of synapsin I was 33–37 pmol/min for the cerebellar kinase and 23–30 pmol/min for the forebrain kinase.

[b] The source of MAP_2 was a microtubule protein preparation consisting of approximately 10% MAPs and 90% tubulin.

[c] This sample of arginine-rich histones contained approximately 30% histone H3, which was the only protein phosphorylated. Calmodulin concentration in these assays was increased to 0.2 mg/ml.

TABLE IV

Comparison of K_m values of cerebellar and forebrain Type II CaM kinase isozymes

Substrate	Apparent K_m or K_a	
	Forebrain	Cerebellum
Synapsin I[a] [b]	1.2 μM ± 0.1	1.1 μM ± 0.2
MAP_2[b]	130 nM	114 nM
ATP[a]	16 μM ± 1.3	9 μM ± 1.1
Calmodulin[a]	123 nM ± 6	69 nM ± 1

[a] Values are the mean ± S.D. of at least two experiments.

[b] The K_m of the forebrain kinase for MAP_2 was determined by James Soha of Caltech. The K_m of the cerebellar kinase was determined in one experiment, using the same preparation of purified MAP_2.

had a consistently higher apparent affinity for Ca^{2+}/calmodulin. The difference in affinities occurred in a range over which the concentration of the Ca^{2+}/calmodulin complex would be expected to vary *in vivo*. Thus, this difference may have physiological significance.

Autophosphorylation of Kinase Subunits

The subunits of the Type II CaM kinase undergo rapid and stoichiometrically significant autophosphorylation under conditions in which the kinase is active (27, 28). When crude brain homogenates are phosphorylated under conditions in which the kinase is maximally active, the most prominent endogenous phosphopeptides are the subunits of the kinase itself (27, 56). This probably reflects, at least in part, the relative abundance of the kinase in brain (35). We compared the Ca^{2+}/calmodulin-stimulated endogenous phosphoproteins in forebrain and cerebellar homogenates to the autophosphorylated subunits of the Type II CaM kinase isozymes from each region (Fig. 3). The most prominent endogenous phosphopeptides in both homogenates were the kinase subunits. We confirmed this by comparison of phosphopeptide maps of the endogenous phosphoproteins and the phosphorylated subunits of the pure isozymes (data not shown). The patterns of phosphorylation shown in Fig. 3 were observed in fresh tissue

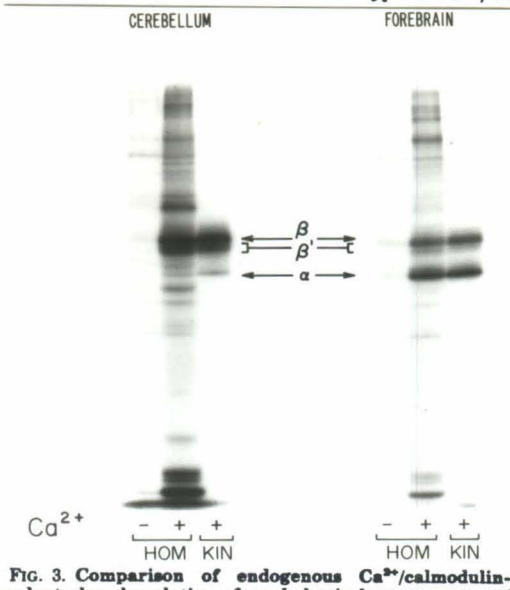

FIG. 3. **Comparison of endogenous Ca^{2+}/calmodulin-dependent phosphorylation of crude brain homogenates and of pure Type II CaM kinases from forebrain and cerebellum.** Samples of crude homogenates (*HOM*, 30 μg of forebrain, 120 μg of cerebellum) and pure kinase (*KIN*, 0.2 μg of forebrain and cerebellum) were incubated under phosphorylating conditions for 10 s with [γ-^{32}P]ATP as described under "Experimental Procedures." The incubations were carried out in the presence or absence of Ca^{2+} as indicated. The samples were then subjected to SDS-PAGE and dried, and labeled bands were detected by autoradiography.

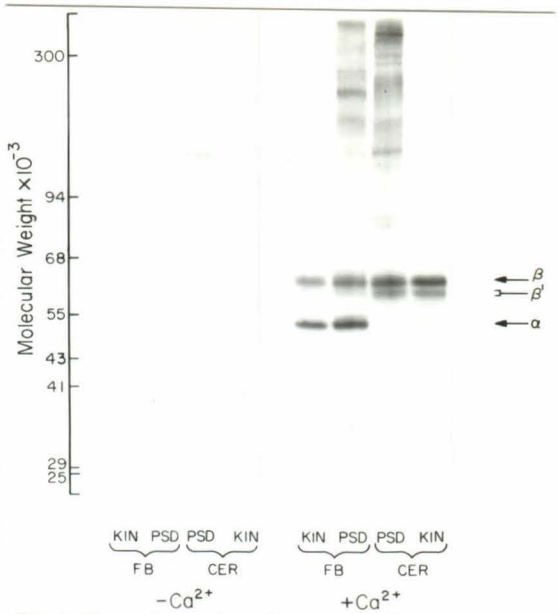

FIG. 4. **Comparison of endogenous Ca^{2+}/calmodulin-dependent phosphorylation of PSDs and purified Type II CaM kinase from forebrain (*FB*) and cerebellum (*CER*).** Samples of PSDs (5 μg from each region) and pure kinase (*KIN*) (0.6 μg from each region) were incubated under phosphorylating conditions for 10 s with [γ-^{32}P]ATP as described under "Experimental Procedures." The incubations were carried out in the presence or absence of Ca^{2+} as indicated. Samples containing 60 ng of each pure kinase, 2.5 μg of the cerebellar PSD, and 0.5 μg of the forebrain PSD were subjected to SDS-PAGE and dried, and the labeled bands were detected by autoradiography.

homogenates, indicating that the different proportions of kinase subunits were not generated artifactually during purification.

Concentration of Cerebellar and Forebrain Isozymes in Postsynaptic Density Fractions

The α-subunit of Type II CaM kinase is a major component of postsynaptic density fractions prepared from whole brain (36–38). However, several groups have reported that it is reduced in concentration or nearly absent in cerebellar PSDs (57–59; in these papers, the α-subunit is referred to as the 51K PSD protein or the major PSD protein). We wondered whether this reduction simply reflected the difference in subunit composition of forebrain and cerebellar Type II CaM kinase isozymes, so we examined the content of α- and β-subunits in PSD fractions from the two brain regions.

Endogenous Phosphorylation of PSDs—Freshly isolated PSDs from forebrain and cerebellum were labeled with ^{32}P by endogenous phosphorylation in the presence of calcium and calmodulin (Fig. 4). Under these conditions, the major endogenous phosphoproteins in the PSDs had the same molecular weights and were present in the same proportions as the subunits of the Type II CaM kinase isozyme from the corresponding region. However, the cerebellar PSD fraction appeared to contain reduced quantities of both subunits (note that the cerebellar PSD lane in Fig. 4 contains 5-fold more protein than the forebrain PSD lane).

Protein Composition of Cerebellar and Forebrain PSDs—We also compared Coomassie Blue-stained protein profiles of cerebellar and forebrain PSDs to the profiles of purified Type II CaM kinases from each region (Fig. 5). As was previously reported, the predominant protein in the forebrain PSDs was the 50-kDa protein that is identical with the α-subunit of Type II CaM kinase. Protein bands corresponding to the β-subunits were also visible. In contrast, the cerebellar PSDs contained much less α-subunit; no increase in bands corresponding to the β-subunits was apparent. We measured the content of each of the subunits in the PSD fractions by a quantitative immunoblot method (35). A polyclonal antisera (Darcy) was used to quantitate the β-subunit and a monoclonal antibody (6G9) was used to quantitate the α-subunit (Table V). Fig. 6 illustrates the reactions of these antibodies with PSD fractions. The forebrain PSDs contained ~3.5 α-subunits for each β-subunit; together, the subunits comprised 16–18% of the total PSD protein. In contrast, the cerebellar PSDs contained ~4 β-subunits for each α-subunit and the subunits comprised only 1–2% of the total PSD protein. Thus, PSD fractions from each region contained the isozyme of the Type II CaM kinase that predominates in that region, but the concentration of the kinase in cerebellar PSDs was only about 8% of the concentration in forebrain PSDs. The kinase holoenzyme is about 1% of total forebrain protein and about 0.3% of total cerebellar protein (35). Therefore, the Type II CaM kinase is 20-fold enriched in forebrain PSDs and only 3–4-fold enriched in cerebellar PSDs.

DISCUSSION

We have shown that brain "Type II" Ca^{2+}/calmodulin-dependent protein kinase exists in a different isomeric form in cerebellum as compared to forebrain. The cerebellar isozyme contains 50- and 60-KDa α- and β-subunits that are

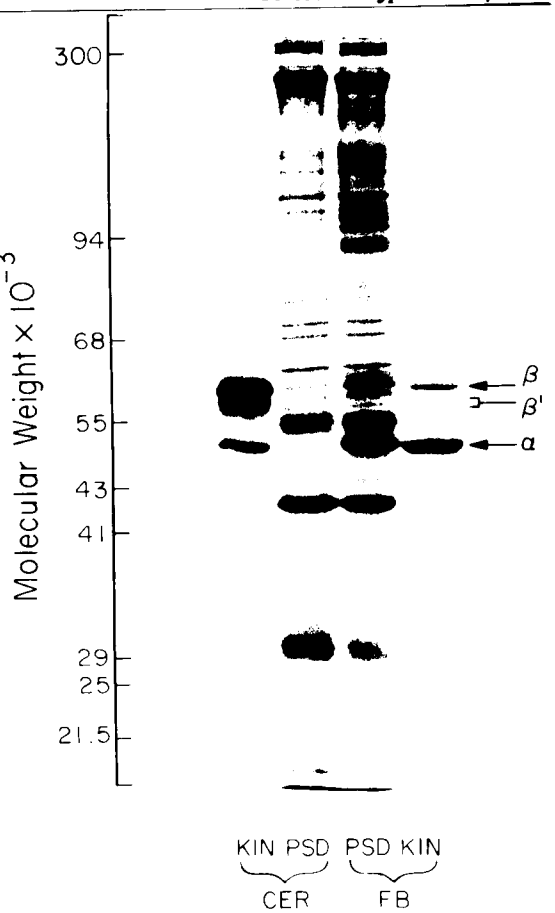

FIG. 5. **Protein composition of PSD and Type II CaM kinase (*KIN*) isolated from either forebrain (*FB*) or cerebellum (*CER*).** Six μg of purified kinase and 40 μg of PSD protein from each region were subjected to SDS-PAGE on a 10% gel then stained with Coomassie Blue. Positions of molecular weight standards are indicated on the *left*.

TABLE V

Concentration of α- and β-subunits of Type II CaM kinase in PSD fractions from forebrain and cerebellum

Region	α-Subunit/PSD protein[a]	β-Subunit/PSD protein[a]	Molar ratio (α:β)	Kinase holoenzyme as % PSD protein[a]
	pg/μg	pg/μg		%
Cerebellum	2.4 ± 0.2 (8)[b]	10.5 ± 1.1 (8)[b]	1.0:3.7	1.3 ± 0.1
Forebrain	129.6 ± 9.6 (35)[b]	44.5 ± 2.7 (12)[b]	3.5:1.0	17.4 ± 1.5

[a] ±S.E.M.
[b] Number of determinations.

closely related to those in the forebrain; however, they are present in a different ratio. The cerebellar α:β ratio is 1:4, whereas the forebrain α:β ratio is 3:1. The resolution of our analytical methods is not sufficient to tell if the kinase holoenzymes from the two brain regions exist as unique oligomers that each contain the same number of α- and β-subunits. It

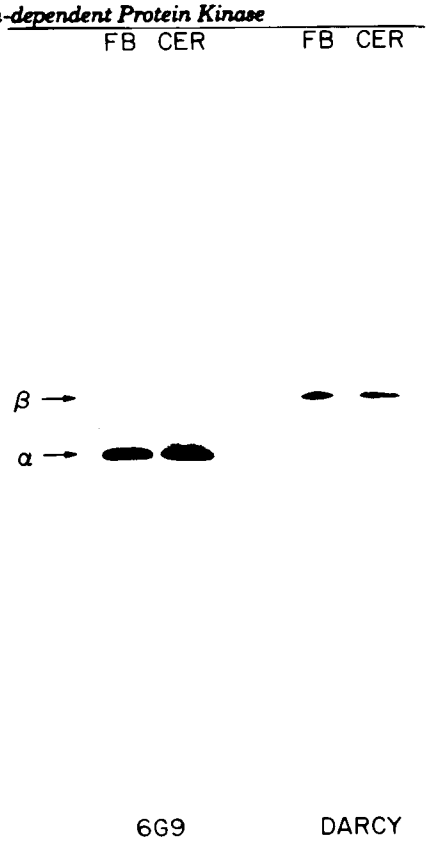

FIG. 6. **Immunoblots of PSDs isolated from forebrain (*FB*) and cerebellum (*CER*).** The *left panel* shows the reaction of a monoclonal antibody (6G9), which is specific for the α-subunit, with 0.43 μg of forebrain PSDs and 15 μg of cerebellar PSDs. The *right panel* shows the reaction of a polyclonal antisera (Darcy), which is specific for the β-subunit, with 2.3 μg of forebrain PSDs and 7 μg of cerebellar PSDs. Immunoreactive bands were detected as described under "Experimental Procedures."

is possible that we have measured an average molecular weight of heterogeneous oligomers that were assembled randomly from newly synthesized subunits. In the latter case, the difference in average holoenzyme composition between the two regions would simply reflect a difference in the ratio of subunits synthesized. Further studies will be necessary to determine whether intermediate isozyme forms exist in other brain regions (35).

The β-subunits from the two brain regions show extensive immunological cross-reactivity and have identical iodinated tryptic peptide maps. The α-subunits also show immunological cross-reactivity and their maps differ by only a few peptides. Studies by our laboratory and others have suggested that the α- and β-subunits are themselves structurally homologous and may both be catalytic (27, 37). The properties of the cerebellar isozyme are consistent with this notion. The specific activity of the purified cerebellar kinase is similar to that of the forebrain kinase, although the ratio of its subunits is dramatically different. In addition, the protein substrate specificities of the two isozymes do not differ, insofar as we have measured them.

What then is the functional significance, if any, of the

different kinase forms? There are several possibilities. In peripheral tissues, closely related isoenzymes sometimes differ in critical kinetic constants or in their association with specific subcellular organelles (60). The cerebellar kinase shows a consistent 2-fold higher apparent affnity for the Ca^{2+}/calmodulin than does the forebrain kinase. The difference is in a concentration range over which calcium-bound calmodulin is likely to vary *in vivo*. Thus, the cerebellar kinase may be more sensitive to increases in calcium concentration than the forebrain kinase.

A second possible functional difference between the α- and β-subunits is in their association with subcellular structures *in vivo*. Protein isozymes are known to have distinct subcellular locations both in peripheral and in neural tissue. This specific organization of the cytoplasm may be important for efficient cellular functioning (60). Recently, isozymes of various neuronal cytoskeletal proteins have been shown to have different subcellular locations. In chick cerebellum, β-spectrin is confined to the plasma membrane of the soma, while γ-spectrin (fodrin) is associated with the membranes of both the soma and processes (61). Similarly, MAP_2 is located in neuronal dendrites and perikarya, while MAP_1 is more evenly distributed throughout the neuron (62, 63). The results presented here suggest that the subcellular locations of the forebrain and cerebellar Type II CaM kinase isozymes differ. Although more of the cerebellar isozyme (85%) than of the forebrain isozyme (50%) is recovered in the particulate fraction of brain homogenates, cerebellar PSDs contain only ~8% as much Type II CaM kinase as do forebrain PSDs. This suggests that the α-subunit is involved in anchoring of the kinase within PSDs, while the β-subunit may have a higher affinity for different subcellular structures. It will be interesting to determine what other particular structures the cerebellar kinase is bound to.

In summary, we have confirmed and extended the observation made by several groups that the major difference in protein composition between cerebellar and forebrain postsynaptic density fractions is their content of a major 51-kDa protein (46, 58, 59). The 50-kDa protein is the α-subunit of the Type II CaM kinase (36–38). We have shown that PSD fractions from cerebellum contain reduced quantities of this protein because the cerebellar kinase contains less α-subunit and more β-subunit than the forebrain kinase and has a reduced affinity for the PSD fraction. This quantitative difference in the concentration of the Type II CaM kinase in PSDs from neurons in the two brain regions may produce different responses in these neurons to postsynaptic changes in calcium flux.

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CHAPTER 3

REGULATION OF BRAIN TYPE II CA^{2+}/CALMODULIN-DEPENDENT PROTEIN KINASE BY AUTOPHOSPHORYLATION: A CA^{2+}-TRIGGERED MOLECULAR SWITCH*

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Regulation of Brain Type II Ca^{2+}/Calmodulin-Dependent Protein Kinase by Autophosphorylation: A Ca^{2+}-Triggered Molecular Switch

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Summary

Calcium/calmodulin-stimulated autophosphorylation of a prominent brain calmodulin-dependent protein kinase (Type II CaM kinase) produces dramatic changes in its enzymatic activity. These changes suggest a mechanism by which the kinase could act as a calcium-triggered molecular switch. Incorporation of 3–12 of a possible total of 30 phosphate groups per holoenzyme causes kinase activity toward exogenous substrates as well as autophosphorylation itself to become independent of calcium. Thus, kinase activity could be prolonged beyond the duration of an initial activating calcium signal. The calcium-independent autophosphorylation could further prolong the active state by opposing dephosphorylation by cellular phosphatases.

Introduction

Type II Ca^{2+}/calmodulin-dependent protein kinase (Type II CaM kinase) is the most abundant protein kinase in brain, comprising approximately 1% of total brain protein (Erondu and Kennedy, 1985). It is most highly concentrated in forebrain neurons (Erondu and Kennedy, 1985), where it is present in presynaptic terminals as well as in cell bodies and dendrites (Ouimet et al., 1984). It also appears to be concentrated in postsynaptic densities (submembranous cytoskeletal structures that occur at many postsynaptic sites in the central nervous system [Kennedy et al., 1983a; Goldenring et al., 1984; Kelly et al., 1984; Miller and Kennedy, 1985]). Thus, it is strategically located to respond to both pre- and postsynaptic calcium signals generated by electrical activity or by extracellular transmitters and hormones.

This kinase is one of a family of homologous kinases, all of which have a similar, relatively broad substrate specificity. They are all large multimeric proteins (300–700 kd), composed of structurally related 50–60 kd subunits that bind calmodulin and are autophosphorylated in the presence of Ca^{2+} and calmodulin. They occur as distinct forms in different tissues (Ahmad et al., 1982; Fukanaga et al., 1982; Bennett et al., 1983; Goldenring et al., 1983; Woodgett et al., 1983; McGuinness et al., 1983; Yamauchi and Fujisawa, 1983; Payne et al., 1983; DeRiemer et al., 1984; Kuret and Schulman, 1984) and brain regions (McGuinness et al., 1985; Miller and Kennedy, 1985). The forms differ in the molecular weights and ratios of their subunits. The major isozyme in brain is an oligomer of ~9 α subunits (50 kd) and ~3 β subunits (60 kd) (Bennett et al., 1983; Goldenring et al., 1983). Both types of subunit appear to be catalytic (Bennett et al., 1983; Goldenring et al., 1983; Kuret and Schulman, 1984, 1985; McGuinness et al., 1985; Miller and Kennedy, 1985) and undergo rapid autophosphorylation in the presence of Ca^{2+} and calmodulin.

The most prominent proteins phosphorylated by the Type II CaM kinase in brain homogenates, in isolated synaptic membranes, and in postsynaptic density fractions are its own subunits (Schulman and Greengard, 1978; Grab et al., 1981; Bennett et al., 1983; Kennedy et al., 1983b; McGuinness et al., 1985; Miller and Kennedy, 1985). Moreover, proteins with molecular weights similar to those of the subunits become phosphorylated in synaptosomes that have been depolarized in the presence of calcium (Krueger et al., 1977; DeLorenzo, 1980; Robinson and Dunkley, 1983; Dunkley and Robinson, 1986). Consequently, several groups have examined potential functional roles for this autophosphorylation. Some studies suggest that kinase activity decreases after autophosphorylation (Levine et al., 1985; Kuret and Schulman, 1985; Yamauchi and Fujisawa, 1985), while others suggest that it increases (Shields et al., 1984) or becomes autonomous (Saitoh and Schwartz, 1985). Here we report detailed studies of the effect of autophosphorylation on the activity of purified forebrain Type II CaM kinase. We present a model that shows how the changes produced by autophosphorylation may cause the kinase to function as a switch, remaining active after the decay of an initial calcium signal. Such switching behavior may provide a mechanism for producing long-lasting changes in neuronal function in response to transient calcium signals. Because the family of protein kinases related to the brain Type II CaM kinase is found in many tissues, and may be present in the nucleus as well as in the cytoplasm (Maizels and Jungmann, 1983; Sahyoun et al., 1984), it is possible that this mechanism is involved in long-term hormonally induced changes in nuclear and cytoplasmic function in many cell types.

Results

Time Course of Autophosphorylation

Activation of Type II CaM kinase by calcium and calmodulin results in autophosphorylation of both α and β subunits (Bennett et al., 1983; Goldenring et al., 1983; Kuret and Schulman, 1984, 1985; McGuinness et al., 1985; Yamauchi and Fujisawa, 1985). The time course of the reaction under the conditions used in this study is shown in Figure 1. After 3 min in the presence of calcium and calmodulin, about 3 mol ^{32}P-phosphate per β subunit and 2 per α subunit were incorporated for a total of about 30 mol ^{32}P-phosphate per mol holoenzyme. The rate of autophosphorylation was constant over a wide range of kinase concentrations (0.4 nM–0.3 μM, data not shown), indicating that the reaction occurs primarily within each holoenzyme (see also Kuret and Schulman, 1985). A low rate of autophosphorylation occurred in the absence of calcium.

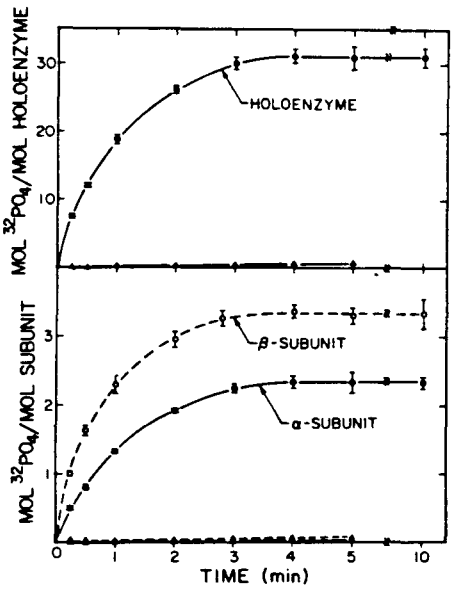

Figure 1. Time Course and Stoichiometry of Autophosphorylation

Purified forebrain Type II CaM kinase was incubated as described in Experimental Procedures, in either 300 μM free Ca^{2+} (plus calcium) or 5 × 10^{-8} M free Ca^{2+} (minus calcium). The stoichiometry of phosphate incorporation into α and β subunits was determined at the indicated times, as described in Experimental Procedures. (Bottom) Average incorporation of ^{32}P-phosphate into the individual subunits; (top) ^{32}P-phosphate incorporation into the holoenzyme, assuming that it contains 9 α and 3 β subunits (Bennett et al., 1983). Values are mean ± SEM for seven separate experiments. (Top) ●, Holoenzyme plus calcium; ▲, holoenzyme minus calcium. (Bottom) ●, ○, α and β subunits, respectively, plus calcium; ▲, △, α and β subunits, respectively, minus calcium.

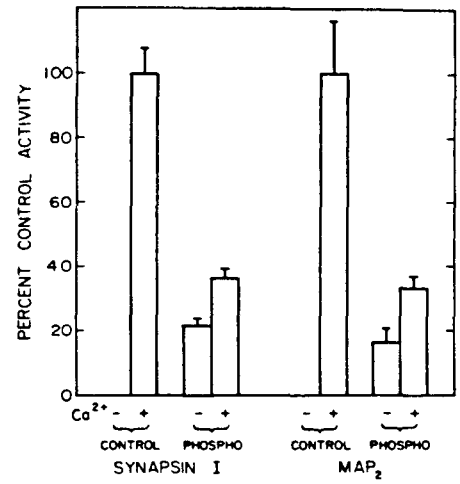

Figure 2. Effect of Autophosphorylation on Kinase Activity

Kinase was prephosphorylated with nonradioactive ATP for 30 sec in the presence of Ca^{2+} and calmodulin, as described in Figure 1. Control kinase was incubated in the absence of ATP. The reactions were stopped with EGTA and cooled (see Experimental Procedures). The kinase was then diluted 500-fold, and the initial rate of phosphorylation of either synapsin I or MAP_2 was determined in the presence and absence of calcium as described in Experimental Procedures. One hundred percent activity (control kinase in the presence of calcium) represented an initial rate of 45 to 100 pmol/min with synapsin I as substrate and 45 to 70 pmol/min with MAP_2 as substrate. The graph summarizes 17 prephosphorylation experiments, including data from 13 TCA and 4 gel assays with synapsin I as substrate, and 4 gel assays with MAP_2 as substrate. Assays of control kinase with the TCA method occasionally showed a small apparent calcium-independent phosphorylation of synapsin I (1%–2% of calcium-stimulated activity). This was not verified in the gel assays and was not included in the graphs. The error bars indicate SEM.

Effect of Autophosphorylation on Kinase Activity toward Exogenous Substrates

Kinase was prephosphorylated for 30 sec at a high concentration, then diluted 500-fold into various solutions for measurement of its ability to phosphorylate synapsin I or microtubule-associated protein 2 (MAP_2) (Figure 2). Control kinase was incubated in the prephosphorylation mixture for the same length of time as experimental kinase but without ATP. In a few cases, controls were incubated with ATP but without Ca^{2+} or calmodulin. Results with each of these controls were identical. Autophosphorylation had two pronounced effects on phosphorylation of exogenous substrates. First, when measured in the presence of calcium, the initial rate of phosphorylation of both synapsin I and MAP_2 was reduced to approximately 35% of the rate of control kinase. Second, a large portion of this activity was independent of calcium, in contrast to the activity of the control kinase, which depended completely on calcium. Thus, autophosphorylation produced a significant new calcium-independent kinase activity. This activity was also independent of added calmodulin (data not shown). Incorporation of more phosphate (20–30 mol/mol holoenzyme) produced by prephosphorylation for 1–3 min caused no further change in activity of phosphokinase compared with control kinase.

The reduced rate of phosphorylation of synapsin I in the presence of Ca^{2+} resulted primarily from a reduced V_{max} (3.1 μmol PO_4 transferred/min/mg before autophosphorylation; 0.72 μmol/min/mg after) rather than an increased K_M for synapsin I (1.0 ± 0.2 μM before autophosphorylation; 1.0 ± 0.3 μM after). We did not detect any significant change in the K_a for calmodulin following autophosphorylation.

To determine the minimum amount of phosphate per holoenzyme that would produce the complete shift in kinase activity, we adjusted the concentration of Mg^{2+} in the prephosphorylation reaction to slow the rate of autophosphorylation. In three sets of experiments at various Mg^{2+} concentrations, the level of incorporation of phosphate into the kinase subunits and the resulting shift in kinase activity were measured (Figure 3). The appearance of Ca^{2+}-independent activity coincided with the reduction in Ca^{2+}-stimulated activity. Incorporation of an average of 3–4 mol of phosphate per mol holoenzyme (less than one

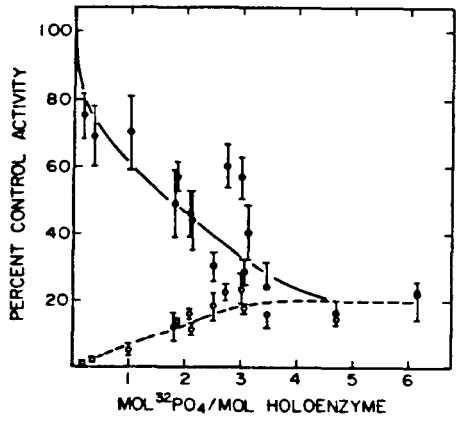

Figure 3. Changes in Kinase Activity Produced by Increasing Levels of Autophosphorylation

Kinase was incubated with Ca^{2+} and calmodulin for 15 sec under the conditions described in Figure 1, except that reduced concentrations of $MgCl_2$ were used to produce low levels of ^{32}P-phosphate incorporation, as described in Experimental Procedures. The reactions were stopped with cold EDTA. Stoichiometry of phosphate incorporation and kinase activity with synapsin I as substrate were then determined, as described in Figure 1 and Figure 2, respectively, and in Experimental Procedures. ●——●, calcium-dependent kinase activity (activity in the presence of calcium minus activity in the absence of calcium); O——O, kinase activity in the absence of calcium. Error bars show the standard deviation of triplicate determinations of kinase activity. Data were pooled from three separate experiments.

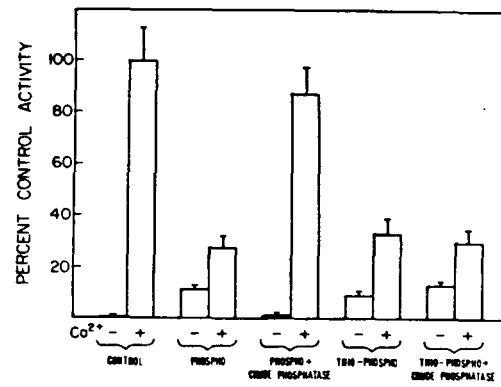

Figure 4. Reversal of Effects of Autophosphorylation by an Endogenous Brain Phosphatase

Kinase was prephosphorylated for 15 sec in the presence of 500 μM Mg^{2+}, and the reactions were stopped as described in Experimental Procedures. Control kinase was incubated in the absence of ATP. Phosphokinase was then incubated for an additional 5 min with either a crude cerebellar fraction in which phosphatase activity had been inactivated by heating at 90°C for 2 min (phospho) or a crude cerebellar fraction containing phosphatase activity (phospho + crude phosphatase). Control kinase was incubated with the crude cerebellar fraction (control). Both control and phosphokinase showed some nonspecific loss of activity during these incubations (see Results). The amount of phosphate in the phosphokinase before dephosphorylation was 3.6 ± 0.2 mol $^{32}PO_4$/mol holoenzyme; after incubation with heat inactivated crude phosphatase, it was 3.3 ± 0.2 mol $^{32}PO_4$/mol holoenzyme. This is the threshold amount of phosphate necessary for the shift in kinase activity (see Figure 3). After the 5 min incubation with crude phosphatase, this kinase contained 0.5 ± 0.1 mol $^{32}PO_4$/mol holoenzyme. Thiophosphorylated kinase (see Experimental Procedures) was treated in the same manner. One hundred percent activity is that of control kinase incubated with crude phosphatase and assayed in the presence of Ca^{2+}. The figure shows the average of three separate experiments. Error bars indicate SEM.

phosphate per subunit) appeared sufficient to produce the complete shift. Since data from several labs suggest that both the α and β subunits are catalytic, it is possible that phosphorylation of a few subunits in each holoenzyme produced an allosteric shift in the conformation of all the subunits. However, since the presence of exogenous substrates does not reduce the rate of autophosphorylation (data not shown), autophosphorylation would have resumed during the assays in which phosphorylation of synapsin I was measured. This means that an average of 1 mol of phosphate per mol of kinase subunit could have been reached during the first 15 sec of these assays. The precision of our measurements of rates of phosphorylation of synapsin I was not high enough to allow us to be certain that the changes in activity were complete before a ratio of 1 mol of PO_4 per mol subunit had been reached.

Reversal by Dephosphorylation

Commercially available phosphatases (alkaline phosphatase and potato acid phosphatase) did not dephosphorylate the kinase under conditions mild enough to preserve its activity (data not shown). However, we found that incubation of phosphorylated kinase with a fraction of a cerebellar homogenate resulted in both dephosphorylation of the kinase and reversal of the effects of prephosphorylation on kinase activity (Figure 4). Thus, after incubation with the cerebellar phosphatase, the catalytic rate of the kinase in the presence of calcium had returned to approximately 80% of that of nonphosphorylated kinase controls, while the calcium-independent activity was reduced to a rate just above the limit of detection. Autophosphorylation of the kinase in the presence of adenosine 5'-0-(3-thiotriphosphate) (ATP-γS) also produced the switch in activity (Sean Molloy, unpublished observations). The change in activity of thiophosphorylated kinase was not reversed by incubation with the cerebellar homogenate. Several other thiophosphorylated proteins are poor substrates for phosphatases (Gratecos and Fischer, 1974; Cassel and Glaser, 1982). Thus, these findings support the interpretation that the switch in activity is caused by autophosphorylation rather than by irreversible denaturation or proteolysis. Furthermore, they indicate that it can be reversed by dephosphorylation by brain phosphatases.

Both control and phosphorylated kinase lost some activity during the incubation at 30°C. The loss was slightly less when they were incubated with crude or heat-inactivated phosphatase than when they were incubated with buffer. After incubation of the phosphokinase with heat-inactivated crude phosphatase for 5 min (Figure 4), its activity in the absence of calcium was ~65%, and in

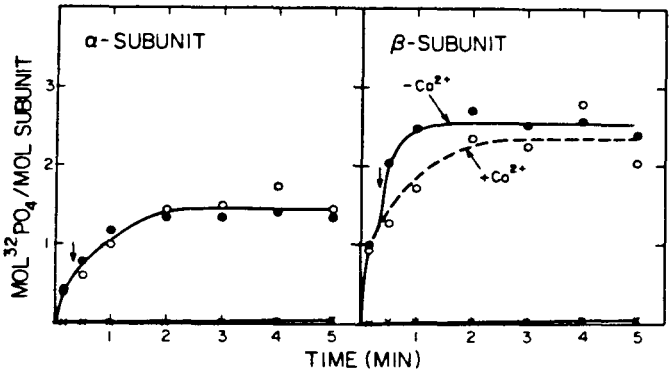

Figure 5. Calcium-Independent Autophosphorylation Triggered by Brief Calcium-Dependent Prephosphorylation

Three test tubes of kinase were incubated with [γ-^{32}P]ATP as described in Figure 1. Tube 1 contained ~5 × 10^{-8} M free Ca^{2+} throughout the incubation. Tubes 2 and 3 contained 300 μM free Ca^{2+}. At 20 sec (arrow), EGTA was added to tube 2 to reduce free Ca^{2+} to approximately 5 × 10^{-8} M. Buffer alone was added to tube 3. At the indicated times, aliquots of the reactions were removed and quenched by addition to SDS-stop solution (see Experimental Procedures). The stoichiometry of ^{32}P-phosphate incorporation into each subunit was determined as described in Figure 1. At 20 sec, the kinase in tubes 2 and 3 contained approximately 8 mol of ^{32}P-phosphate per mol holoenzyme. x———x, tube 1 (control); ●———●, tube 2 (−Ca^{2+}); O———O, tube 3 (+Ca^{2+}). Similar results were obtained in three experiments.

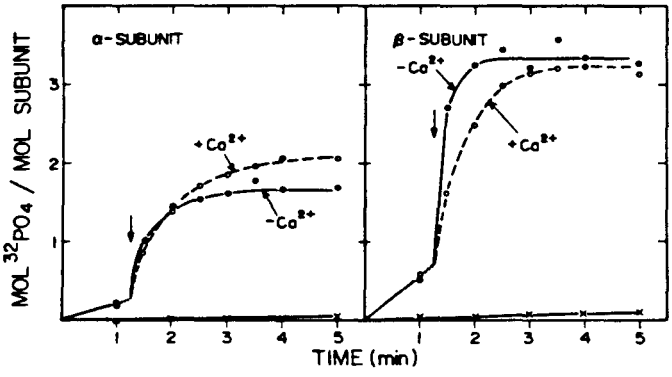

Figure 6. Threshold for Production of Calcium-Independent Autophosphorylation

Three test tubes of kinase were incubated with [γ-^{32}P]ATP as described in Figure 1. Tube 1 contained 10 mM $MgCl_2$ and 5 × 10^{-8} M free Ca^{2+} throughout the incubation. Tubes 2 and 3 contained 300 μM free Ca^{2+} and a reduced concentration of $MgCl_2$ (50 μM). At 75 sec (arrow), $MgCl_2$ was added to tubes 2 and 3 to bring the final concentration to 10.0 mM. At the same time, EGTA was added to tube 2 to reduce the free Ca^{2+} concentration to 5 × 10^{-8} M. At the indicated times, aliquots of the reactions were removed and quenched by addition to SDS-stop solution (see Experimental Procedures). The stoichiometry of ^{32}P-phosphate incorporation into each subunit was determined as described in Figure 1. At 60 sec, the kinase in tubes 2 and 3 contained an average of approximately 4 mol ^{32}P-phosphate per mol holoenzyme. x———x, tube 1 (control); ●———●, tube 2 (−Ca^{2+}); O———O, tube 3 (+Ca^{2+}). Similar results were obtained in several separate experiments in which the prephosphorylated kinase contained from 3–12 mol $^{32}PO_4$ per mol holoenzyme.

the presence of calcium was ~55%, of the corresponding activity measured immediately after the prephosphorylation reaction. The activity of the control kinase in the presence of calcium after incubation with either crude phosphatase or heat-inactivated crude phosphatase was ~90% of its activity before the incubation. Thus the loss of activity was greater for phosphokinase than for control kinase. This loss seems to be due to denaturation, since we saw no evidence for partial proteolysis in autoradiograms of the ^{32}P-phosphokinase subunits after SDS gel electrophoresis.

Calcium-Independent Autophosphorylation

Brief prephosphorylation in the presence of Ca^{2+} also triggered Ca^{2+}-independent autophosphorylation. Figure 5 shows an experiment in which kinase was autophosphorylated for 20 sec in the presence of Ca^{2+} and calmodulin. EGTA was then added to one reaction to chelate free Ca^{2+}, and buffer was added to the other. Autophosphorylation continued at the same rate and to the same extent in both cases. When EGTA was added to a control reaction at zero time, just before addition of ATP, the rate of autophosphorylation was negligible.

The threshold for initiation of Ca^{2+}-independent autophosphorylation was determined in a series of experiments like that shown in Figure 6. Kinase was prephosphorylated in the presence of calcium, calmodulin, and various reduced concentrations of Mg^{2+} to produce low levels of phosphate incorporation (2–12 mol of ^{32}P-phosphate per holoenzyme). Following the prephosphoryla-

tion, the Mg^{2+} concentration was restored to 10 mM, and the rate of autophosphorylation increased to its maximum rate. If, along with the Mg^{2+}, EGTA was added to chelate free Ca^{2+}, autophosphorylation again increased to the same rate and reached the same maximum level as in the presence of calcium. An average of 3–4 mol of ^{32}P-phosphate per mol holoenzyme appeared to be the threshold. More than this produced no further change in the rate or final extent of calcium-independent autophosphorylation, whereas less (1–2 mol ^{32}P-phosphate per mol holoenzyme) produced a lower rate and extent of calcium-independent autophosphorylation, suggesting that not all the holoenzyme molecules had been switched into a calcium-independent state (data not shown).

Sucrose Gradient Sedimentation of Control and Phosphokinase

The kinase was capable of incorporating many more moles of phosphate than required for the shift in activity (Figure 1). We wondered whether the introduction of the full 30 mol phosphate per mol holoenzyme might produce changes in the packing of the subunits leading to their dissociation. To examine this possibility, control and prephosphorylated kinase were sedimented through identical sucrose gradients to test for a change in the sedimentation coefficient. Although the phosphokinase was less stable than the control kinase, their sedimentation rates appeared identical, as determined from the peaks of enzyme activity and from the positions of the enzyme subunits visualized in silver-stained SDS polyacrylamide gels of the gradient fractions (data not shown). Thus, even extensive autophosphorylation does not lead to appreciable dissociation of the kinase subunits.

Intermolecular Autophosphorylation

The constant rate of autophosphorylation over a wide range of kinase concentrations indicates that autophosphorylation is primarily intraholoenzyme. However, even a low rate of interholoenzyme autophosphorylation could result in significant activation in vivo of nonphosphorylated kinase holoenzymes by phosphoholoenzymes. This could transmit a wave of activation of the kinase by a Ca^{2+} transient; for instance, from the membrane toward the interior of the cell. In an attempt to detect any significant rate of interholoenzyme autophosphorylation, nonradioactive phosphorylated kinase and native kinase were mixed in various ratios and at high concentrations (0.2–0.9 μM). We looked for both interholoenzyme autophosphorylation and a shift in the enzyme activity of the native kinase, as described in Experimental Procedures. Interholoenzyme autophosphorylation was barely detectable over background, even after 5 min of incubation, and the rate did not increase with increasing concentrations of native kinase. More significantly, no shift in catalytic activity of the native kinase was apparent. Thus, it seems unlikely that functionally significant interholoenzyme autophosphorylation occurs at a significant rate in vivo. The rates of phosphorylation of exogenous substrates, of intraholoenzyme autophosphorylation, and of interholoenzyme autophosphorylation are summarized in Table 1.

Table 1. Initial Rates of Phosphorylation of Exogenous Substrates and of Autophosphorylation by Type II CaM Kinase

Phosphorylation of Substrate	Native		Phosphokinase	
	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$
Exogenous				
Synapsin I	0	2300–2600	480–540	840–960
MAP_2	0	2000–2300	330–380	660–770
Intraholoenzyme Autophosphorylation				
Holoenzyme	0	29–31	29–31	29–31
Interholoenzyme Autophosphorylation				
Holoenzyme	n.d.		<0.7	n.d.

Turnover numbers for phosphorylation of exogenous substrates defined as mol $^{32}PO_4$ transferred/min/mol holoenzyme, were calculated from reactions performed under the standard conditions described in Experimental Procedures. Those for autophosphorylation of native and phosphorylated kinase were calculated from the data in Figure 1, Figure 4, and Figure 5. To determine the rate of interholoenzyme autophosphorylation, phosphorylated and native kinase were coincubated in the absence of Ca^{2+}, and transfer of phosphate was measured as described in Experimental Procedures. In these latter assays, background incorporation of phosphate into native kinase alone ranged from 0.01 to 0.04 mol $^{32}PO_4$/min/mol holoenzyme; background incorporation into the phosphokinase alone ranged from 0.3 to 1.8. The rates of reactions marked n.d. were not determined.

Discussion

We have shown that the requirements for activation of purified forebrain Type II CaM kinase are dramatically altered after brief autophosphorylation in the presence of calcium and calmodulin. The changes are produced after submaximal incorporation of ^{32}P-phosphate (3–12 mol/mol holoenzyme out of a possible total of 30). There are two coincident changes in phosphorylation of the substrate proteins synapsin I and MAP_2. First, the kinase becomes capable of phosphorylating them in the absence of calcium, although at a reduced rate. In contrast, native (dephospho) kinase activity requires concentrations of calcium ion above about 0.5 μM (Kennedy et al., 1983b; Kuret and Schulman, 1984). Second, the stimulation of phosphorylation by calcium becomes less pronounced, so that activity in the presence of calcium is only 25%–40% of control native kinase. In addition to the changes in phosphorylation of exogenous substrates, autophosphorylation itself is altered, becoming calcium-independent. It proceeds nearly to completion even after calcium ion has been removed.

The effects of incorporation of the threshold level of phosphate into the kinase by autophosphorylation can be reversed by incubation with a cerebellar extract. The extract also catalyzes dephosphorylation of the kinase to below the threshold level of phosphate. Thus, the change in activity produced during autophosphorylation of the kinase is not caused by irreversible calcium and ATP-dependent proteolysis or denaturation but is caused by autophosphorylation itself. We used cerebellar extracts for these experiments because they catalyzed dephosphorylation of endogenous ^{32}P-labeled kinase subunits faster than forebrain extracts (unpublished observation).

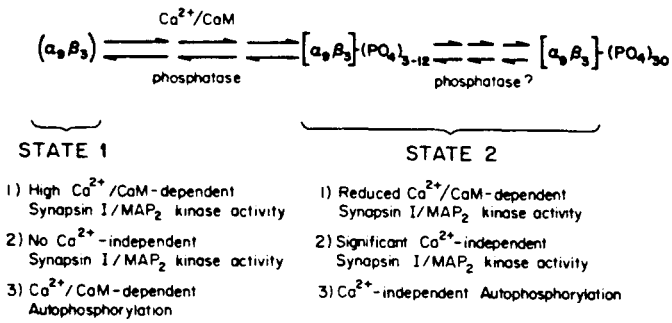

Figure 7. Hypothetical Model of Changes in State of Type II Ca^{2+}/Calmodulin-Dependent Protein Kinase Regulated by Autophosphorylation and Dephosphorylation

Thus, the concentration of phosphatases that can dephosphorylate the kinase may vary in different brain regions. After this study was completed, Shields et al. (1985) reported the identity of the brain phosphatases that can dephosphorylate the kinase subunits. They are phosphatase 1, which is present in both the soluble and the synaptic-junction fractions, and phosphatase 2A, which is primarily soluble.

Our results are consistent with other recent studies and resolve some apparent contradictions among them. While this study was in progress, Kuret and Schulman (1985) and Yamauchi and Fujisawa (1985) reported a reduced rate of Type II CaM kinase activity in the presence of calcium after autophosphorylation. They did not discuss measurements of kinase activity in the absence of calcium following autophosphorylation. In contrast to these studies, Saitoh and Schwartz (1985) reported that phosphorylating conditions in crude homogenates of Aplysia neurons produce an apparent increase in a calcium-independent Type II CaM kinase activity. They did not see the reduction in rate reported by us and other groups, possibly because they did not measure initial rates of synapsin I phosphorylation. We note in addition that they presented evidence that autophosphorylation may cause a translocation of the Aplysia kinase from the membrane–cytoskeleton fraction into the cytosol.

Several observations suggest that autophosphorylation of the Type II CaM kinase can occur in vivo. The kinase subunits themselves are the most prominent proteins phosphorylated in fresh brain homogenates in the presence of calcium and calmodulin (Schulman and Greengard, 1978; Bennett et al., 1983; Kennedy et al., 1983b; McGuinness et al., 1985; Miller and Kennedy, 1985). Furthermore, proteins with molecular weights similar to those of the subunits are phosphorylated in depolarized synaptosomes in the presence of calcium (Krueger et al., 1977; DeLorenzo, 1980; Robinson and Dunkley, 1983; Dunkley and Robinson, 1986). Thus, it is likely that the ability to autophosphorylate is a property of the kinase in vivo. In addition, autophosphorylation is activated in vitro under the same conditions that activate phosphorylation of exogenous substrates such as synapsin I. It is known that calcium-dependent phosphorylation of synapsin I occurs in intact peripheral nerves in response to electrical stimulation (Nestler and Greengard, 1982), as well as in brain slices and in synaptosomes in response to depolarization (Forn and Greengard, 1978; Krueger et al., 1977; Huttner and Greengard, 1979). Therefore, autophosphorylation would be expected to be initiated under these same physiological circumstances.

Figure 7 summarizes a hypothetical model of a Type II CaM kinase/phosphatase system that has some of the properties of a molecular switch described recently in a theoretical paper by Lisman (1985). The switch would operate in the following way. When the kinase is unphosphorylated (state 1), a transient rise in calcium concentration would produce an initial burst of high kinase activity accompanied by autophosphorylation. If the calcium concentration remained elevated long enough to allow autophosphorylation to proceed to the threshold level, it would induce a change to state 2, in which kinase activity would continue after the calcium concentration had returned to the resting level. It would continue at about 20% of the rate of calcium-stimulated native kinase, and would be relatively refractory to further stimulation by calcium. A return to state 1 would require the action of cellular protein phosphatases. Calcium-independent autophosphorylation, which accompanies the other changes in kinase activity, would oppose this dephosphorylation. The effect would be to maintain or prolong the shift in kinase activity. The lifetime of state 2 would be determined by the balance between the rates of calcium-independent autophosphorylation and dephosphorylation by phosphatases. If the rate of autophosphorylation exceeded the rate of dephosphorylation, state 2 could last as long as the lifetime of the holoenzyme itself (see Lisman, 1985). The specific activities of phosphatases in forebrain homogenates measured by Shields et al. (1985) suggest that, at least in hippocampus and cortex, where the Type II CaM kinase is most concentrated, this latter situation could occur.

The threshold level of autophosphorylation necessary to initiate Ca^{2+}-independent autophosphorylation appears to be 3–4 mol ^{32}P-phosphate per mol holoenzyme, clearly less than one per subunit (Figure 6). This suggests that autophosphorylation of a few of the subunits in a holoenzyme may produce an allosteric change in the con-

formation of all of them. The exact threshold for the change in phosphorylation of exogenous substrates was more difficult to determine for technical reasons (see results). However, it also appears to be one mol of ^{32}P-phosphate per subunit or less. Because of the latter ambiguity, we show a threshold of 3–12 in Figure 7.

In order for Ca^{2+}-independent autophosphorylation to play the proposed role in maintaining state 2, the sites phosphorylated must be equivalent to the calcium-dependent sites, the phosphorylation of which initially triggers the shift. We do not as yet know whether the two sets of sites are equivalent. Nor do we know the site specificity or the regional distribution of the brain phosphatases that catalyze dephosphorylation of the kinase. These will be important subjects for future study.

The model in Figure 7 describes a molecular switch that could last until the kinase holoenzyme is destroyed by proteolysis. In contrast, Lisman has pointed out that a protein kinase/phosphatase system could form a bistable switch that would maintain an activated state beyond the lifetime of the individual molecules (Lisman, 1985). The requirements are that the kinase is activated by autophosphorylation and that autophosphorylation is intermolecular. The Type II CaM kinase does not appear to meet the latter requirement, since it undergoes only intraholoenzyme autophosphorylation in vitro. Native kinase appears to be well insulated from autophosphorylation by neighboring phosphokinase (Table 1). However, state 2 might be perpetuated beyond the lifetime of individual holoenzymes, if the subunits are degraded and replaced individually or if the subunits undergo a slow interholoenzyme exchange. Because of the allosteric effect of submaximal autophosphorylation, dephospho-subunits, when added to a preexisting phosphokinase holoenzyme, would be rapidly autophosphorylated (see also Crick, 1984). We cannot rule out the possibility that subunits are replaced individually or undergo a slow exchange, although the association between subunits is strong and is not substantially disrupted by high salt or nonionic detergents (data not shown).

It may be significant in vivo that the rate of autophosphorylation is much slower than the rate of phosphorylation of exogenous substrates (Table 1). In vitro it takes 10–20 sec of autophosphorylation in the presence of saturating calcium to convert all kinase holoenzymes to state 2 (see Figure 1 and Figure 3). Because this rate is independent of kinase concentration, it is likely to be similar in vivo. Therefore, millisecond increases in calcium concentration, such as those produced in neurons by single synaptic depolarizations (Katz and Miledi, 1967; Llinas et al., 1981), would switch only a small portion of the total kinase to state 2. Consequently, the switching mechanism may become significant only after prolonged increases in internal calcium concentration such as may occur in neurons after long or repeated bursts of electrical activity or in neurons and other cells after stimulation of various receptors (Oettgen et al., 1985). Thus, in the nervous system, the switch mechanism could be involved in forms of synaptic plasticity induced by repeated activity, such as long-term potentiation (Bliss and Lomo, 1973), post-tetanic potentiation (Rosenthal, 1969), or "kindling" (Racine et al., 1983). The evidence presented by Llinas et al. (1985) that the Type II CaM kinase is involved in regulating neurotransmitter release is consistent with this possibility. Moreover, the kinase is concentrated in regions of the brain where long-term synaptic plasticity is prominent (Erondu and Kennedy, 1985). Finally, we note that because tissues outside the nervous system contain broad specificity protein kinases that are closely related to the Type II CaM kinase, the switching mechanism may also be involved in more general forms of long-term cellular regulation induced by prolonged increases in intracellular Ca^{2+}. The model presented here provides a framework in which these hypotheses can be tested.

Experimental Procedures

Materials

ATP, EDTA, and EGTA were purchased from Sigma. SDS, acrylamide, and bisacrylamide were purchased from Bio-Rad. Bovine serum albumin (BSA), dithiothreitol (DTT), calf intestine alkaline phosphatase, and adenosine 5'-0-(3-thiotriphosphate) (ATP-γS) were purchased from Boehringer Mannheim, [γ-^{32}P]ATP from ICN Nutritional Biochemicals. Potato acid phosphatase was purchased from Calbiochem. Synapsin I was prepared by a modification of the method of Ueda and Greengard (1977) as described previously (Bennett et al., 1983). Calmodulin was purified from bovine brain by the method of Watterson et al. (1976). Microtubule protein was purified by the method of Shelanski et al. (1973). MAP_2 was purified from microtubule protein by the method of Kim et al. (1979). Rat forebrain Type II CaM kinase was purified by the method of Bennett et al. (1983) with minor modifications as described in Miller and Kennedy (1985).

Time Course and Stoichiometry of Autophosphorylation

Pure forebrain Type II CaM kinase was phosphorylated in a reaction mixture (final volume 50 μl) containing 10 μg kinase, 50 mM Tris HCl (pH 8.0), 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 5 μg calmodulin, 0.4 mM EGTA, 0.7 mM $CaCl_2$, 10 mM DTT, 0.2 mg/ml BSA and 50 μM [γ-^{32}P]ATP (3.0–4.0 × 10^3 cpm/pmol). After preincubation for 30 sec at 30°C, the reaction was initiated by the addition of [γ-^{32}P]ATP. For measurement of the time course of autophosphorylation, aliquots of 5 μl were removed at the indicated times and quenched in 60 μl of a stop solution containing 3% SDS, 2% (w/v) 2-mercaptoethanol, 5% (w/v) glycerol, 62 mM Tris (pH 6.7), and a trace of bromphenol blue (SDS stop solution). The samples were immediately boiled for 2 min. After addition of 5 μl of 2-mercaptoethanol, the proteins were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) in 10% gels as described (Bennett et al., 1983). The gels were dried, and the phosphorylated kinase subunits were detected by autoradiography. The subunits were cut out of the gels, and their radioactivity was measured in a liquid scintillation counter. Areas adjacent to the phosphorylated subunits were also cut from the gels, and their radioactivity was subtracted as background. The stoichiometry of phosphorylation at each point was calculated from the specific activity of the ATP and the amount of each subunit loaded on the gel.

Determination of the Effect of Autophosphorylation on Kinase Activity

Kinase was prephosphorylated as described above but with unlabeled ATP. The entire reaction was quenched after 30 sec by the addition of 5 μl of 22 mM EGTA to bring the final EGTA concentration to 2.4 mM and the final free Ca^{2+} concentration to approximately 5 × 10^{-8} M. The reaction was then cooled immediately in ice water. Parallel reactions were performed with [γ-^{32}P]ATP to measure the incorporation of phosphate into each subunit (as described above). In most experiments, control reactions were carried out in the absence of ATP. ATP was added to these reactions immediately after quenching with EGTA and cooling. In a few experiments, control reactions were performed in the presence of ATP, but in the absence of either calcium or calmodulin. Immediately following the prephosphorylation, the ability of autophosphorylated and control kinases to catalyze phosphoryla-

tion of either synapsin I or MAP_2 was measured. They were first diluted 50-fold into 40 mM Tris HCl (pH 8.0) and 1 mg/ml BSA. They were then diluted an additional 10-fold to measure the initial rate of phosphorylation of synapsin I or MAP_2 in the absence or presence of calcium as previously described (Bennett et al., 1983). These reactions contained 0.2 mg/ml synapsin I (2.5 μM) or 0.14 mg/ml MAP_2 (0.5 μM) and 0.4 μg/ml kinase and were performed at 30°C for 30 sec. Incorporation of phosphate into synapsin I was measured either by a method in which phosphorylated protein was precipitated with trichloroacetic acid (the TCA method), described in Bennett et al. (1983), or after separation of proteins by SDS-PAGE (the gel method) as described in Kennedy et al. (1983b). Incorporation of phosphate into MAP_2 was measured by the gel method.

Determination of the Threshold of Autophosphorylation Required to Produce the Effects on Kinase Activity

Kinase (6 μg) was prephosphorylated in the presence of 50 μM [γ-^{32}P]ATP for 15 sec in a final volume of 30 μl as described above but at reduced concentrations of $MgCl_2$ (10, 25, 50, 75, 100, 150, 250, and 500 μM; 1.0, 2.0, and 10.0 mM). The lower concentrations of Mg^{2+} decreased the initial rates of autophosphorylation. After the prephosphorylation, 30 μl of ice cold 0.2 M EDTA, 40 mM Tris HCl (pH 8.0), and 1 mg/ml BSA was added to each reaction. The EDTA chelated the free Mg^{2+} and stopped the autophosphorylation. For determination of stoichiometry of autophosphorylation, a 20 μl aliquot of each reaction was added to 60 μl of SDS-stop solution, boiled, and subjected to SDS-PAGE. Incorporation of ^{32}P into kinase subunits was then measured as described above. For determination of kinase activity, a 5 μl aliquot of each reaction was diluted 30-fold into cold 40 mM Tris HCl (pH 8.0) and 1 mg/ml BSA. Aliquots of 10 μl were then used to measure kinase activity in the absence and presence of calcium with synapsin I as substrate by the gel assay described above. Autophosphorylation stoichiometry and kinase activity were both determined immediately after each prephosphorylation.

Dephosphorylation of the Kinase by Cerebellar Phosphatase

A cerebellar homogenate was used as a source of phosphatase to dephosphorylate the autophosphorylated kinase. Brains were removed from two rats after stunning and decapitation. The cerebellar lobes were removed and immediately homogenized by 12 up-and-down strokes in a Teflon/glass homogenizer driven at 900 rpm in 5 volumes of ice-cold buffer A (40 mM Tris-HCl; 10 mM imidazole, pH 7.5; 5 mM EDTA; 2 mM EGTA; 1 mM dithiothreitol; 0.1 mM phenylmethylsulfonyl fluoride; 25 mg/l soybean trypsin inhibitor; and 1 mg/l leupeptin). The homogenate was subjected to centrifugation at 10,000 × g for 30 min at 4°C. The supernatant was used immediately as a source of phosphatase.

Kinase was prephosphorylated in 500 μM $MgCl_2$ as described above. After warming for 30 sec at 30°C, reactions were initiated by addition of [γ-^{32}P]ATP or ATP-γS. Reactions with ATP were quenched after 15 sec and those with ATP-γS after 20 sec by the addition of 50 μl of 0.2 M EDTA and 1 mg/ml BSA (pH 7.0). ATP was added to some prephosphorylation reactions after they were quenched to provide controls. When appropriate, three 10 μl aliquots of kinase were removed from the prephosphorylation reactions and added to SDS-stop solution for measurement of ^{32}P incorporated into the kinase as described above. Immediately after removal of these aliquots, 15 μl of each reaction was mixed with either 10 μl of crude phosphatase or 10 μl of crude phosphatase that had been heated for 2 min at 90°C to inactivate the phosphatase. The reactions were incubated for 5 min at 30°C, then quenched by cooling in an ice-cold water bath. When appropriate, three 5 μl aliquots of each dephosphorylation reaction were removed and added to SDS-stop solution for measurement of the amount of ^{32}P remaining in the kinase. The rest of each reaction was immediately diluted 20-fold into 40 mM Tris-HCl (pH 8.0) and 1 mg/ml BSA. Kinase activity in 10 μl aliquots of the diluted reactions was measured in triplicate, in the presence and absence of Ca^{2+}, with synapsin I as substrate, by the gel method described above. Background kinase activity contributed by the crude phosphatase was measured in control reactions that had contained only the crude phosphatase. This activity was subtracted from that measured in reactions that had contained both kinase and phosphatase.

Measurement of Ca^{2+}-Independent Autophosphorylation Following a Brief Prephosphorylation

To measure autophosphorylation in the absence of Ca^{2+} following a brief Ca^{2+}-dependent prephosphorylation, we modified the procedure described above. Kinase was prephosphorylated in two tubes. After 10 sec, a 5 μl aliquot was removed from each tube and was added to 60 μl of SDS-stop solution. Then at 20 sec, 5 μl of 50 mM EGTA was added to one tube to reduce the free calcium concentration to approximately 5×10^{-8} M. Buffer alone was added to the other tube. At the remaining indicated times, aliquots of 5 μl of each of the reactions were withdrawn and added to SDS-stop solution. The stoichiometry of incorporation of ^{32}P-phosphate into the kinase subunits was measured as described above. Control reactions were performed in which EGTA was added 5 sec before the ATP.

To determine the threshold of phosphate incorporation required to produce Ca^{2+}-independent autophosphorylation, we further modified the above procedure. The concentration of $MgCl_2$ in the prephosphorylation reactions was reduced from 10 mM to 50 μM. The reactions were initiated by addition of [γ-^{32}P]ATP, and aliquots of 5 μl were removed and stopped at 60 sec. At 75 sec, 5 μl of 0.1 M $MgCl_2$ was added to one of the tubes to restore the maximum rate of Ca^{2+}-stimulated autophosphorylation; to the other tube, 5 μl of 0.1 M $MgCl_2$/50 mM EGTA was added to restore Mg^{2+} concentration and, at the same time, reduce free calcium concentration to approximately 5×10^{-8} M. Incorporation of ^{32}P-phosphate into the kinase subunits at the indicated times was determined as above. Control incubations were performed in which Mg^{2+} and EGTA were added 5 sec before the ATP.

Sucrose Gradients

Purified forebrain Type II CaM kinase (60 μg) was prephosphorylated for 3 min with nonradioactive ATP as described above. Control kinase was incubated under identical conditions but without ATP. The activity of an aliquot of the phosphorylated kinase was measured to verify that its activity had been altered. Control and phosphorylated kinase (50 μg in a volume of 265 μl) were layered onto two identical 5%–20% linear sucrose gradients (12 ml) prepared in 40 mM Tris HCl (pH 7.5), 2 mM EGTA, 0.2 M NaCl, 10 mM DTT, and 0.1 mM PMSF. The gradients were centrifuged for 16 hr at 40,000 rpm in an SW 40Ti rotor and then were fractionated into fractions of 360 μl. The activity of duplicate 20 μl portions was measured in the presence of calcium with synapsin I as substrate by the TCA method described above. Protein from the remainder of each fraction was precipitated with 10% trichloroacetic acid, resuspended in a small volume, and subjected to SDS-PAGE. The resulting gels were stained by the silver method (Oakley et al., 1980) to determine the positions of the kinase subunits within the gradients.

Measurement of Intermolecular Autophosphorylation of Native Kinase by Phosphorylated Kinase

Phosphorylated kinase was prepared by incubating 10 μg of kinase with nonradioactive ATP for 3 min as described above. The reaction was stopped by bringing the final concentration of EGTA to 2 mM (5×10^{-8} M free Ca^{2+}). Phosphorylation of native kinase by phosphokinase was then measured in a mixture (30 μl) containing 50 mM Tris HCl (pH 8.0), 10 mM $MgCl_2$, 2.5 mM EGTA, 10 mM DTT, 0.2 mg/ml BSA, 50 μM [γ-^{32}P]ATP, and various proportions of phosphokinase to native kinase (1.5:15 μg; 1.5:7.5 μg; and 1.8:1.8 μg). Reactions were performed by warming the reaction mixture containing control kinase to 30°C for 15 sec, then initiating the reaction by addition of phosphokinase. At 30 sec, and 1, 3, and 5 min after starting the reaction, aliquots of 6 μl were removed and mixed with 60 μl of SDS-stop solution. ^{32}P-phosphate incorporation into the kinase subunits was measured as described above. Control reactions were performed with equivalent amounts of either native or phosphokinase alone. Net phosphorylation of native kinase by phosphokinase was taken as the difference between ^{32}P-phosphate incorporated into kinase in the mixed reaction and the sum of incorporation in the two control reactions. To allow measurement of changes in the catalytic activity of native kinase produced during such reactions, they were performed with nonradioactive ATP for 5 min at a 1:1 ratio of phosphokinase to native kinase. The reaction was then diluted 20-fold, and the ability of aliquots of the kinase to phosphorylate synapsin I in the absence and presence of

Ca^{2+} was measured as described above. Control reactions were performed in which native and phosphokinase were incubated alone under the same conditions.

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CHAPTER 4

SEQUENCES OF AUTOPHOSPHORYLATION SITES IN BRAIN TYPE II CA^{2+}/CALMODULIN-DEPENDENT PROTEIN KINASE: REGULATION BY DIFFERENTIAL GENE EXPRESSION AND ALTERNATIVE SPLICING.*

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SUMMARY

Upon activation by calcium/calmodulin, type II calcium/calmodulin-dependent protein kinase undergoes an allosteric transition to a state in which it has a significant catalytic rate in the absence of calcium. The transition occurs when about one-third of the twelve catalytic subunits in a holoenzyme become autophosphorylated. We have sequenced the sites whose autophosphorylation is sufficient to cause the transition. These sites are autophosphorylated rapidly when the kinase is activated by calcium. The 50 kDal α subunit contains one such site, threonine 286; the 60 kDal β subunit contains two, threonine 287 (the homologue of the α site) and threonine 382. Threonines α286 and β287 are located between the kinase domain and the calmodulin-binding site. They may represent an internal "substrate" that must be phosphorylated to free the active site. β threonine 382 is specifically removed from the β′ subunit, apparently by alternative splicing, and is absent in the α subunit. Phosphorylation at β threonine 382 is not required for the generation of Ca^{2+}-independent kinase activity.

INTRODUCTION

The type II Ca^{2+}/calmodulin-dependent protein kinase (type II CaM kinase) is the most abundant of the known brain Ca^{2+}-regulated protein kinases. This kinase represents about 1% of total brain protein and is particularly concentrated in the forebrain, comprising almost 2% of the total protein in the hippocampus (Erondu and Kennedy, 1985). The kinase is found primarily in neurons and exists in several subcellular pools including cytosolic, membrane, cytoskeletal, and nuclear fractions (reviewed in Stull *et al.*, 1986; Kennedy *et al.*, 1987). The brain type II CaM kinase appears to exist as a large (M_r 600-700,000) multimeric holoenzyme containing two related subunits, α (M_r 50,000) and β (M_r 60,000), as well as a minor β′ (M_r 58,000) subunit (Stull *et al.*, 1986; Kennedy *et al.*, 1987). The relative proportion of α and β/β′ subunits as well as the subcellular distribution of the kinase varies during development (Kelly *et al.*, 1985, 1987; Weinberger and Rostas, 1986) and also between brain regions in the mature brain (Walaas et al., 1983a,b; Ouimet et al., 1984; McGuinness et al., 1985; Miller and Kennedy, 1985). The kinase phosphorylates a broad range of substrate proteins including synapsin I, microtubule associated protein 2, tyrosine hydroxylase, tryptophan hydroxylase, smooth-muscle myosin light chains, and glycogen synthase (Stull *et al.*, 1986; Kennedy *et al.*, 1987).

The complete primary structure of the α, β and β′ subunits has now been determined by sequencing cDNA clones derived from brain mRNA (Bennett and Kennedy, 1987; Lin *et al.*, 1987; Hanley *et al.*, 1987; Bulleit *et al.*, 1988). The

α and β/β′ subunits are encoded by separate genes that are expressed primarily in brain. The α and β/β′ subunits share extensive sequence identity throughout their primary structure and differ primarily in the deletion of two segments from the β subunit that are absent in the α subunit. The β and β′ subunits appear to be related to one another by a specific mRNA splicing event which results in the deletion of a fifteen amino acid segment in the β′ subunit. Functional domains responsible for catalytic activity and calmodulin binding have been identified and constitute the N-terminal two-thirds of the kinase primary structure. The function of the C-terminal domain of the α and β/β′ subunits, which is not homologous to any known protein, is not known but may be involved in subunit-subunit interactions and/or interactions with other cellular components.

Previous studies have demonstrated the *in vitro* regulation of the Ca^{2+}/CaM-dependence of type II CaM kinase *via* a Ca^{2+}/CaM-dependent intramolecular autophosphorylation (Saitoh and Schwartz, 1985; Miller and Kennedy, 1986; Lai *et al.*, 1986; Lou *et al.*, 1986; Schworer *et al.*, 1986). The kinase, as isolated from brain, is completely dependent on Ca^{2+}/CaM for activity but becomes partially independent of Ca^{2+}/CaM after Ca^{2+}/CaM-dependent autophosphorylation *in vitro*. Regulation of kinase activity by this mode of autophosphorylation occurs after the phosphorylation of only a few sites on each holoenzyme, suggesting an allosteric interaction between subunits (Miller and Kennedy, 1986; Lai *et al.*, 1986). We have previously shown that brief Ca^{2+}/CaM-dependent autophosphorylation allows further autophosphorylation, as well as exogenous substrate phosphorylation, to become

independent of Ca^{2+}/CaM (Miller and Kennedy, 1986). It has since been demonstrated that this second mode of autophosphorylation results in a loss of stimulation of kinase activity by Ca^{2+}/CaM, producing to a completely Ca^{2+}/CaM-independent form of the kinase (Hashimoto *et al.*, 1987).

Identification of the specific sites of autophosphorylation would be useful for characterization of kinase regulation at the molecular and cellular levels. Several serine and threonine residues that fit the consensus substrate phosphorylation site sequence (Arg-X-Y-Ser/Thr, Pearson *et al.*, 1985) have been suggested as possible sites of autophosphorylation (Bennett and Kennedy, 1987; Lai *et al.*, 1987; Bulleit *et al.*, 1988). In this study we have used tryptic digestion and reverse-phase HPLC to characterize and identify the initial sites phosphorylated in the presence of Ca^{2+}/CaM. We demonstrate that the α subunit is rapidly phosphorylated at a single major site, threonine-286, located between the catalytic domain and the CaM-binding domain. The β subunit is rapidly phosphorylated at a corresponding site, threonine-287, but in addition is rapidly phosphorylated at threonine-382. Rapid phosphorylation of the β subunit at threonine-382 is of interest since it is in a region that is not present in the α subunit and that appears to be specifically deleted in the β′ subunit (Bulleit *et al.*, 1988). Phosphorylation at this site may therefore have functional consequences that are unique to the β subunit, leading to differences in regulatory properties of the type II kinase isozymes found in different brain regions and during different stages of development.

Experimental Procedures

Materials.

Acetonitrile (HPLC/UV Grade) was purchased from Burdick and Jackson (Muskegon, MI), trifluoroacetic acid (TFA, Sequenal grade) from Pierce Biochemical, and dithiothreitol (DTT) from Schwarz/Mann Biotech. Trypsin treated with *N*-tosyl-L-phenylalanine chloromethyl-ketone (trypsin-TPCK) was purchased from Cooper Biomedical, pyro-glutamate amino-peptidase and thermolysin from Boehringer Mannheim Biochemicals. Iodoacetamide, caffeine, phosphorylase *b* and phosphorylase kinase were purchased from Sigma, and [γ-^{32}P]-ATP from ICN Nutritional Biochemicals. Water for buffers and HPLC was double glass distilled. All other reagents were of the highest available purity and obtained from standard sources. C4 (4.5 x 250 mm, Vydac 214TPS) and C18 (4.5 x 250 mm, Vydac 218TP54) reverse-phase HPLC columns were obtained from Vydac.

Calmodulin was purified from bovine brain by the method of Watterson *et al.* (1976). The forebrain isozyme of the type II CaM kinase was purified from rat brain 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 that is observed in homogenates of lower brain regions such as the pons and medulla (Erondu and Kennedy, 1985; Walaas *et al.*, 1983a,b). We found that a significant amount of this variant was eluted from the CaM-sepharose affinity column

when the column was washed with buffer containing 0.2 M NaCl/2 mM EGTA after elution of the forebrain isozyme in buffer containing only 2 mM EGTA. The physical and enzymatic properties of this holoenzyme variant are similar to those of the forebrain and cerebellar holoenzymes (M. K. Bennett, S. G. Miller, B. L. Patton, unpublished observations).

The catalytic subunit of protein phosphatase 2A was purified from rabbit skeletal muscle by the method of Tung *et al.* (1984). The final HPLC step was omitted. The purified enzyme showed a loss of activity during storage, which was completely reversed by incubation at 30°C for one hour in the presence of 50 m*M* Tris-HCl (pH 7.0), 2 m*M* $MnCl_2$, 50 m*M* DTT, and 5 m*M* caffeine. The reactivated enzyme had a specific activity of 7000 Units/mg with phosphorylase *a* as substrate (1 Unit = 1 nmol/min).

Methods.

Autophosphorylation of the Type II CaM Kinase.

For analytical experiments, pure type II CaM kinase was autophosphorylated in a mixture (final volume 50 µl) containing 10 µg kinase, 50 m*M* Tris-HCl (pH 8.0), 10 m*M* $MgCl_2$, 5 m*M* 2-mercaptoethanol, 10 µg calmodulin, 10 m*M* dithiothreitol, 0.6 m*M* EGTA, 0.9 m*M* $CaCl_2$, 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°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 the Effects of Autophosphorylation by Dephosphorylation with Protein Phosphatase.

Type II CaM kinase was autophosphorylated essentially as described above in a final volume of 30 µl with unlabeled ATP. Bovine serum albumin (BSA) was included at 0.5 mg/ml. Autophosphorylation was terminated after 5 sec by the addition of 15 µl of 0.4 *M* EDTA, 25 m*M* Tris-HCl (pH 7.0), 1 mg/ml BSA at 30°C. Controls were incubated in the absence of ATP, and

ATP was added immediately after the stop solution. Dephosphorylation (at 30°C) was initiated 10 sec after terminating the autophosphorylation reaction by the addition of 3 Units of protein phosphatase 2A (total volume of 60 µl). Phosphatase dilution buffer (50 m*M* Tris-HCl (pH 7.0), 30 m*M* DTT, 20 m*M* caffeine, and 1 mg/ml BSA) was added to the controls. At the indicated times (see figure legend) aliquots were diluted 50-fold into ice-cold 40 m*M* Tris-HCl (pH 8.0), 1 mg/ml BSA. Kinase activity was assayed immediately at 30°C in the presence or absence of calcium. Ten µl of diluted kinase was added to prewarmed mixtures (100 µl final volume) containing 0.2 mg/ml synapsin, 50 µg/ml calmodulin, 0.4 m*M* EGTA, 0.2 m*M* EDTA (carryover from the phosphatase reaction), 0.9 m*M* CaCl2, and 200 µ*M* [γ-^{32}P]-ATP (0.6-1.4 x 103 cpm/pmol). After 15 sec the reactions were terminated by the addition of SDS-stop solution. Incorporation of phosphate into synapsin 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 measurement of the level of kinase activity.

For measurement of dephosphorylation of specific sites, kinase phosphorylation and dephosphorylation were performed as described above except with [γ-^{32}P]-ATP. The autophosphorylation reaction was performed in a final volume of 165 µl containing 33 µg kinase. It was terminated after 5 sec 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% (wt/vol) SDS, 10% (wt/vol) glycerol, 4% (wt/vol) 2-mercaptoethanol, 120 m*M* 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 13.5 Units of protein phosphatase 2A (final volume, 270 µl). At the indicated times (see figure legend) dephosphorylation was terminated by the addition of 2x gel sample buffer to aliquots containing 6 µg of kinase. Two aliquots from each SDS-quenched reaction, each containing 0.5 µg kinase, were removed, and the subunits were separated by SDS-PAGE. The stoichiometry of labeled phosphate incorporation into the α and β subunits was determined as described previously (Miller and Kennedy, 1986). The remaining kinase (5 µg) was used for tryptic phosphopeptide mapping by reverse-phase HPLC as described below.

Reduction and Alkylation of Autophosphorylated Kinase.

To remove free [γ-^{32}P]-ATP, the autophosphorylated kinase was first precipitated with trichloroacetic acid (TCA). One ml of ice-cold 10% (wt/vol) TCA was added to a 50 µl aliquot of the quenched autophosphorylation reaction mixture, which was then cooled in ice water for 15 min. Precipitated protein was pelleted by centrifugation at 12,000 rpm 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 1ml of -20°C acetone to remove SDS. Four hundred microliters of 6 *M* guanidine-HCl, 0.5 *M* Tris-HCl (pH 8.1) and 2 m*M* EDTA were added. The tube was flushed with N_2 and incubated for 30 min at 50°C to denature the protein. Fifty µl of a fresh solution of 5 m*M* dithiothreitol was added, the tube flushed with N_2, and the protein reduced for 4 hr at 50°C.

The tube was cooled to room temperature, and 50 µl of fresh 20 m*M* iodoacetamide were added. The reaction was flushed with N_2 and incubated 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 immediately dialyzed against 4 *l* of 50 m*M* NH_4HCO_3 in the dark at 4°C for 10-12 hr with one change of buffer. The dialyzed protein was frozen in liquid nitrogen and lyophilized in a speedvac. It was resuspended in 70 µl of 3% SDS, 2% (w/v) 2-mercaptoethanol, 5% (w/v) glycerol, 60 m*M* Tris-HCl (pH 6.7), and a trace of bromphenol blue. The sample was heated to 100°C for 2 min, then the kinase subunits were separated by SDS-PAGE. The gel was dried, and the autophosphorylated subunits were located by autoradiography.

Tryptic Digestion of Subunits.

Pieces containing the α or β 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 transferred to microcentrifuge tubes, frozen, and lyophilized. One hundred microliters of 50 m*M* NH_4HCO_3 (pH 8.1) containing 50 µg of trypsin (20-fold excess of trypsin by weight) was added to each lyophilized gel piece, and the mixture was incubated for 4 hr at 37°C. One ml of 50 m*M* NH_4HCO_3 (pH 8.1) containing an additional 50 µg of trypsin was added, and the incubation 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 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. The filtrates were frozen and lyophilized. Seventy-eight ± 9% of ^{32}P-phosphate from the α subunit was recovered in the filtrates and 82 ± 8% from the β subunit. Recovery of ^{32}P could be increased to 95-100% by re-extracting the gel pieces with 1 ml of 50 m*M* 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.

Lyophilized peptides were resuspended in 100 µl of Buffer A [0.07% (vol/vol) TFA in H_2O (pH 2.3)] and loaded onto a 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]. Thus, most of the fractionation occurred between 0 and 24.5% acetonitrile. Two hundred 0.5 ml fractions were collected, and labeled peptides were detected 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. The lyophilized phosphopeptides were subjected to partial hydrolysis 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 x 20 cm thin-layer cellulose sheets in the same buffer. Marker phospho-amino 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 to be digested were pooled and reduced in volume to approximately 25-50 µl in a speedvac. Four hundred µl of 100 m*M* 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% B; 10-20 min, 0-15% B; 20-90 min, 15-25% B; 90-100 min, 25-100% B. Fractions (0.5 ml) were collected by hand as absorbance of the effluent was monitored at 214 nm.

Large-Scale Autophosphorylation and Tryptic Digestion.

To obtain nmol quantities of phosphopeptides, batches of pure type II CaM kinase were autophosphorylated in a reaction mixture (1 ml) containing 200 µg kinase, 50 m*M* Tris-HCl (pH 8.0), 10 m*M* $MgCl_2$, 5 m*M* 2-mercap-

toethanol, 100 μg calmodulin, 10 m*M* dithiothreitol, 0.6 m*M* EGTA, 0.9 m*M* $CaCl_2$, and 200 μ*M* [γ-^{32}P]-ATP (20-40 cpm/pmol). The mixtures were warmed for 60 sec 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 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 1 ml of 50 m*M* 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. Absorbance of the effluent at 214 nm was monitored in order to estimate the purity of each phosphopeptide. 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. Individual peaks were pooled for further purification.

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′ (figure 2) were eluted with this gradient; 0-10 min, 0% B; 10-100 min, 0-15% B; 100-145 min, 15-40% B; 145-155 min, 40-100% B; 155-165 min, 100% B. Peptides β1 and β1′ (figure 2) were eluted with this gradient: 0-5 min, 0% B; 5-10 min, 0-7.5% B;

95-100 min, 15-100% B. Thermolytic peptides generated from β2 and β2′ (figure 3) were eluted with this gradient: 0-10 min, 0% B; 10-20 min, 0-15% B; 20-90 min, 15-25% B; 90-100 min, 25-100% B. Fractions (0.5 ml) were collected by hand while monitoring absorbance of the effluent at 214 nm. Labeled peptides were detected by measuring Cerenkov radiation. Pure phosphopeptides were sequenced or subjected to amino acid analysis.

Other Procedures.

Automated gas-phase sequencing was performed on an Applied Biosystems 470A protein sequencer 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 Sequencing 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 described by Peterson (1977) with bovine serum albumin as standard. Polyacrylamide gel electrophoresis in the presence of SDS was performed by the method of Laemmli (1970). Stacking gels (2.5 x 16 cm x 1.2 mm) contained 3.5% acrylamide, 0.09% bisacrylamide, and running gels (14 x 16 cm x 1.2 mm) contained 8% acrylamide, 0.27% bisacrylamide.

Abbreviations used in this paper are: CaM, calmodulin; DTT, dithiothreitol; RP-HPLC, reverse-phase high-performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; type II CaM kinase, type II Ca^{2+}/calmodulin-dependent protein kinase.

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Results

Early and Late Autophosphorylation Sites.

We first examined the time course of autophosphorylation of individual sites on the type II CaM kinase in the presence of calcium and calmodulin. Purified kinase was autophosphorylated in the presence of calcium, calmodulin, and [γ-^{32}P]-ATP. Reactions were quenched at 5, 15, and 30 sec and the subunits of the kinase were separated by gel electrophoresis. The subunits were digested with trypsin, and tryptic phosphopeptides were separated by reverse-phase HPLC as described in Experimental Procedures (figure 1). The identities of phosphorylated residues in each major peak were determined after partial acid hydrolysis.

After 5 sec, two phosphothreonine-containing peptides were resolved from the α subunit and three from the β subunit (figure 1, A and B). The two threonine-containing peaks in the α subunit are derived from the same phosphorylation site (see next section). Thus we have called them α1 and α1'. Similarly, two of the phosphothreonine-containing peaks in the β subunit are derived from a single phosphorylation site; they are called β1 and β1'. The third phosphothreonine-containing peak in β is derived from a distinct site, and it is called β2. These three sites, α1, β1, and β2, are phosphorylated at a high rate (complete within 10 to 15 sec) and to a high stoichiometry. We have termed them early sites.

At later times, phosphoserine-containing peaks appear in both subunits; one in α and two in β. These are called α2, β3, and β4 (figure 1, C-F). In addition, phosphoserine begins to appear within the β2 tryptic peptide, and its mobility is slightly shifted (figure 1, F). 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 sec in the presence of [γ-^{32}P]-ATP, then "chased" in the presence of unlabeled ATP. Peak β2 appeared after 5 sec, and its mobility slowly shifted to that of β2′ during the chase (data not shown).

All of the serine sites are autophosphorylated more slowly than the early sites (complete within 30 to 60 sec) and sometimes to a lower stoichiometry. We have termed them late sites. Rapid autophosphorylation of threonine sites and slower autophosphorylation of serine sites on the type II CaM kinase were also reported by Lai *et al.* (1987).

Sequence of the Early 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. (This was most pronounced with analytical amounts, data not shown.) The conversion appeared to be irreversible, since the opposite conversion, from α1′ to α1, did not occur. We also noted that both peaks were occasionally resolved into

closely spaced doublets (figures 1, D-F; figure 2, C and D). 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 first scaled up the method for preparing them. The phosphopeptides were then resolved by chromatography on a C4 reverse-phase HPLC column by a method similar to that illustrated in figure 1. Individual peaks were further purified by chromatography on a more hydrophobic C18 column employing a highly resolving gradient (see Experimental Procedures).

Figure 2A illustrates the elution profile of peptide α1 from the C18 column. It eluted primarily as a single peak, although a small amount of radioactivity appeared at the position of α1′. The sequence of peptide α1 was determined twice by gas-phase sequencing and was found to be QE_VD_LK (table 1A). This corresponds to the peptide-containing residues Gln_{284} to Lys_{291} of the α subunit, QETVDCLK (figure 6), which is a predicted tryptic peptide. 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 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 three. Cysteines were modified by carboxyamidomethylation before the preparation of the tryptic peptides. PTH-carboxyamidomethyl cysteine elutes near PTH-glutamate and DMPTU

(a product of a side reaction during Edman degradation) 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 (700pmol and 200pmol) to sequence it yielded no sequence, so it appeared to be blocked at the N-terminal. We determined its amino acid composition, which was identical to that of peptide α1 (Table 1B). Since there is no other predicted tryptic peptide in the α subunit with this amino acid composition (see figure 6), 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, a reaction that is irreversible and occurs readily at N-terminal glutamine residues (Podell and Abraham, 1978). This explains the N-terminal blockage of α1′. Thus, both peptides α1 and α1′ contain the same early site, α1, which is Thr_{286}.

Sequence of the Early Site β1 Phosphopeptides.

β1 and β1′ were purified by the method just described. Figure 2 (C and D) illustrates their elution profiles from the C18 column. 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, upper O.D. trace). Therefore, only the left portion of the doublet (starred) was pure enough to yield a sequence. Its sequence was determined twice and was QE_VE[E]LK (table 1C). This corresponds best to the predicted tryptic peptide QETVECLK encompassing residues Gln_{285} to Lys_{292} in the β subunit (figure 6). 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-carboxyamidomethyl 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 (table 1D) is consistent with its identity with β1, except for unexpectedly high levels of Ser, Gly, and Ala in the ratio 1:2:1. These three amino acids were also found in peptide α1′ at higher than background levels in the same ratio (table 1B). We have traced the source of this variable contamination to the polyethylene microcentrifuge tubes. We conclude that peptides β1 and β1′ contain the same early site, β1, which is Thr_{287}.

The early sites α1 and β1 are homologous. They are both located immediately N-terminal to the calmodulin-binding domain (figure 5) three

residues downstream from an arginine. Therefore they are contained within the "consensus" sequence (Arg-X-Y-Thr/Ser) found by Pearson *et al.* (1985) for exogenous substrates of the type II CaM kinase.

Sequence of the Early 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 generated from peptide β2 (figure 3C). The same phosphothreonine-containing peptide was generated from β2′ together with a larger amount of the phosphoserine-containing peptide and a second phosphothreonine-containing peptide (figure 3D). All three phosphothreonine-containing peptides were sequenced. The sequence of the peptides that eluted at 46 min (β2-A and β2-B) was LEPQT_VIHNP (table 2). This corresponds to the peptide encompassing residues Leu_{377} to Pro_{387} in the β subunit (LEPQTTVIHNP, figure 6). The blank cycle identifies Thr_{382} as the phosphorylated residue (see above). The phosphopeptide that eluted at 65 min (β2-C) yielded the sequence LEPQT_VIHNPV before the signal reached background (table 2). Thus it is an

alternative thermolytic peptide containing the same site, Thr_{382}. We conclude that the early 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 (figures 3C and 3D) that is most prominent in peptide β2′ apparently contains a "late" serine autophosphorylation site. It was not pure enough to sequence; however, it is possible to deduce its identity. Both this site and Thr_{382} are contained within the tryptic peptide β2′. From the sequence of the β subunit (figure 6), we predict that β2′ contains residues Gly_{370} to Lys_{392}. This sequence contains only one serine residue, Ser_{371}, which we call β5. Like β2, site β5 is not present in the α subunit.

Early Site β2 Is Removed from the β′ Subunit.

We have recently shown that at least 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; see figure 6). It seems most likely that this β′ message is generated by alternative splicing of the β gene transcript (Bulleit *et al.*, 1988). The 15-amino-acid segment that is predicted to be removed from β′ contains site β2. We tested whether site β2 was in fact absent in the β′ subunit by separating the β and β′ subunits by SDS-PAGE after a brief autophosphorylation. Tryptic phosphopeptides from the two subunits were then generated and resolved as in figure 1. The proportion of peptide β2 was much less in the β′ subunit than in the β

subunit (figure 4). There are two possible explanations for the presence of a small β2 peak in β′. The peak could result from contamination by the more abundant β subunit, which is often not completely resolved from the β′ subunit. 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).

The α1 and β1 Sites Control Calcium-Independent Kinase Activity.

After 5 sec of autophosphorylation under the conditions described in figure 1, the type II CaM kinase has acquired an average of eight phosphates per dodecameric holoenzyme and has also acquired its full rate of calcium-independent kinase activity (in our experiments, about 20% of the maximal rate in the presence of calcium or about 1 μmol/min/mg kinase, data not shown). The phosphate is distributed almost exclusively among the homologous α1 and β1 sites (an average of 4-5 phosphates per forebrain holoenzyme) and site β2 (an average of 0.5-1.0 sites per forebrain holoenzyme, figures 1, 5). We determined in our original study that an average of 3 to 5 phosphates per holoenzyme was sufficient to produce the full calcium-independent kinase activity (Miller and Kennedy, 1986). This fact indicates that activation of the kinase by autophosphorylation occurs in an allosteric fashion. Because the initial rates of autophosphorylation at sites α1, β1, and β2 are similar, it is difficult to distinguish which of these sites is responsible for activation. In an effort to learn the importance of the different early sites, we dephosphorylated

the kinase after 5 sec of autophosphorylation and measured the rate of consequent decay of calcium-independent activity. This rate was compared to the dephosphorylation rate of each of the early sites (figure 5). Protein phosphatase 2A clearly dephosphorylated site β2 faster than sites α1 or β1. The rate of decay of calcium-independent activity was slower than the rate of dephosphorylation of site β2 and was consistent with the hypothesis that phosphorylation of a threshold number of α1/β1 sites is required for calcium-independent activity. The data do not exclude a role for site β2 in the generation of calcium-independent activity, but they indicate that its role is not obligatory.

Late Autophosphorylation Sites in the α and β Subunits.

Peaks labeled α2, β3, and β4 in figure 1 each contain discrete serine sites that are autophosphorylated at a slower rate than the early threonine sites. We purified phosphopeptides from each of these peaks by the methods described earlier. Peptides α2 and β4 yielded the sequences shown in table 4. The autophosphorylation sites contained within them are homologous and correspond to Ser_{279} in α and Ser_{280} in β. Peptide β3 yielded the sequence shown in table 4 and corresponds to the residues Ser_{343} to Lys_{347} in β (figure 6). Thus site β3 is Ser_{343}.

The "Consensus" Sequences around the Early Autophosphorylation Sites Are Similar to Those around Phosphorylation Sites in Exogenous Substrates.

We compared the sequences surrounding the early autophosphorylation sites with sequences surrounding sites on several exogenous substrates of the type II CaM kinase (table 4). Two of the early sites, α1 and β1, contain the sequence motif Arg-X-Y-Ser/Thr suggested by Pearson *et al.* (1985) to be a minimal requirement for phosphorylation by the type II CaM kinase. In addition they share the common sequence Arg-Gln-X-Thr-Val. The third early site, β2, also shares this sequence except that 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 may also enhance the rate of phosphorylation by type II CaM kinase.

The exogenous substrate sites seem to fit this general pattern. They all contain Arg at the -3 position. In addition, both of the phosphorylation sites on the brain substrate protein, synapsin I, contain Gln at the -2 position. Site 2 in bovine synapsin I (table 4) also contains Val at the +1 position, but rat synapsin I contains Ile at this position (Czernik *et al.*, 1987). The two phosphorylation sites on glycogen synthase and the putative site on MAP_2 contain Val at +1. Thus a more complete consensus sequence is Arg-Gln-X-Thr/Ser-Val. Arg at -3 appears to be most important, but Gln at -2 and Val at +1 may also contribute to the likelihood that a site will be phosphorylated at a high rate.

DISCUSSION.

In this study we identified the *in vitro* autophosphorylation sites of rat brain type II CaM kinase within the primary structure of the α and β subunits. The sites phosphorylated in the presence of Ca^{2+}/calmodulin fall into two general classes based on their rates of autophosphorylation. We have designated them in this paper simply as "early" and "late" autophosphorylation sites. This paper demonstrates that the α subunit contains a single early autophosphorylation site (Thr_{286}), while the β subunit contains two early sites (Thr_{287} and Thr_{382}). Phosphorylation at these sites is correlated with the production of a partially Ca^{2+}/CaM-independent form of the kinase, and dephosphorylation by purified protein phosphatases returns kinase activity to its control state (figure 5).

The autophosphorylation site at α-Thr_{286}/β-Thr_{287} is located between the catalytic domain and the CaM-binding domain of each of the kinase subunits (figures 6, 7). Phosphorylation at this site in only a few of the subunits in the kinase holoenzyme appears to be sufficient for activation of kinase activity in the absence of Ca^{2+}/CaM (figure 5). Phosphorylation at this site may induce a conformational change in the kinase, which allows substrates to bind at the active site. It is interesting to note that phosphorylation of the early sites exposes a previously cryptic chymotryptic cleavage site in this region of the native kinase (M. King, personal communication), suggesting that a conformational change is indeed occurring.

The β subunit contains a unique early autophosphorylation site, β-Thr_{382}, in a region that is not present in the α subunit (figures 6, 7). The functional significance of phosphorylation at this site is not presently known. Purified phosphatase 2A removes phosphate from this site at a significantly higher rate than α-Thr_{286}/β-Thr_{287} (figure 5). These dephosphorylation experiments suggest that phosphorylation of β-Thr_{382} is not *required* for the generation of a Ca^{2+}-independent form of the kinase. The present data does not rule out the possibility that phosphorylation of this site has a modulatory effect on kinase switching. The region containing β-Thr_{382} appears to be deleted by a specific mRNA splicing event in the β′ subunit of the kinase (figures 4, 6, 7). The β′ subunit is found in each of the type II CaM kinase isozymes purified from brain, but the relative amount of this subunit varies among isozymes (Bennett *et al.*, 1983; McGuinness *et al.*, 1985; Miller and Kennedy, 1985; and unpublished observations). Phosphorylation at β-Thr_{382} may therefore be involved in differential regulation of kinase isozymes, dependent on their composition of α, β and β′ subunits.

A recent paper described the characterization of the early autophosphorylation sites of rat brain type II CaM kinase by phospho-amino-acid analysis, two-dimensional phosphopeptide mapping, and reverse-phase HPLC phosphopeptide mapping of thermolytic digests of the α and β subunits (Lai *et al.*, 1987). These authors concluded that the α and β subunits contain a common early phosphorylation site (termed peptide 1/1′) and that the β subunit contains an additional early phosphorylation site not found in the α subunit (termed peptide 2). All of the initial phosphorylation sites in that study

occurred on threonine residues. These results are consistent with the findings presented here. The previous study suggested that peptide 1/1′ may correspond to the phosphorylation of either α-Thr_{286} or α-Thr_{320}. These two residues had previously been suggested as possible autophosphorylation sites based on the consensus substrate specifity proposed by Pearson *et al.* (1987; Bennett and Kennedy, 1987; Hanley *et al.*, 1987). We have shown here that α-Thr_{286}/β-Thr_{287} is the early site common to the α and β subunits and therefore corresponds to peptide 1/1′ of the previous study. The early site unique to the β subunit (β-Thr_{382}) has not previously been suggested as a possible phosphorylation site since it does not conform to the proposed consensus substrate phosphorylation site sequence (Arg-X-Y-Ser/Thr).

In addition to the early sites mentioned above, we have also identified several "late" autophosphorylation sites. We do not know whether phosphorylation of these sites results in any significant changes in the functional state of the kinase. It is possible that they have effects on the association of the kinase with other proteins or have enzymatic effects that have not been apparent in simple assays of exogenous substrate phosphorylation. Autophosphorylation of a third class of sites, which are only phosphorylated when Ca^{2+}/CaM is removed after the phosphorylation of the early sites, results in a major change in kinase activity (Hashimoto *et al.*, 1987), and their characterization is described in chapter 5 of this thesis.

Identification of the autophosphorylation sites responsible for regulation of kinase activity allows us to develop methods for assessing the phosphoryla-

tion state of the type II kinase *in vivo*. The reverse-phase HPLC tryptic phosphopeptide maps of kinase labeled with ^{32}P *in vivo* can be compared to the sites labeled *in vitro*. Site and phosphorylation-state-specific antibodies can be generated and will be useful in determining regional changes in phosphorylation state of the kinase using immunocytochemistry. Site-specific *in vitro* mutagenesis and expression of cDNAs may also provide insight into the structure/function relationships between autophosphorylation at specific sites and kinase activity.

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

Pure rat forebrain type II CaM kinase was autophosphorylated for 5 sec (**A, B**), 15 sec (**C, D**), or 30 sec (**E, F**) in the presence of [γ-^{32}P]-ATP, Ca^{2+}, and calmodulin. The holoenzyme was reduced and alkylated. 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 (Experimental Procedures). The phosphoamino acids present in each major peptide 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 threefold 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. **α1-α2, β1-β4;** names of peaks numbered in approximate order of their appearance. **T,** phosphothreonine-containing peak; **S,** phosphoserine-containing peak.

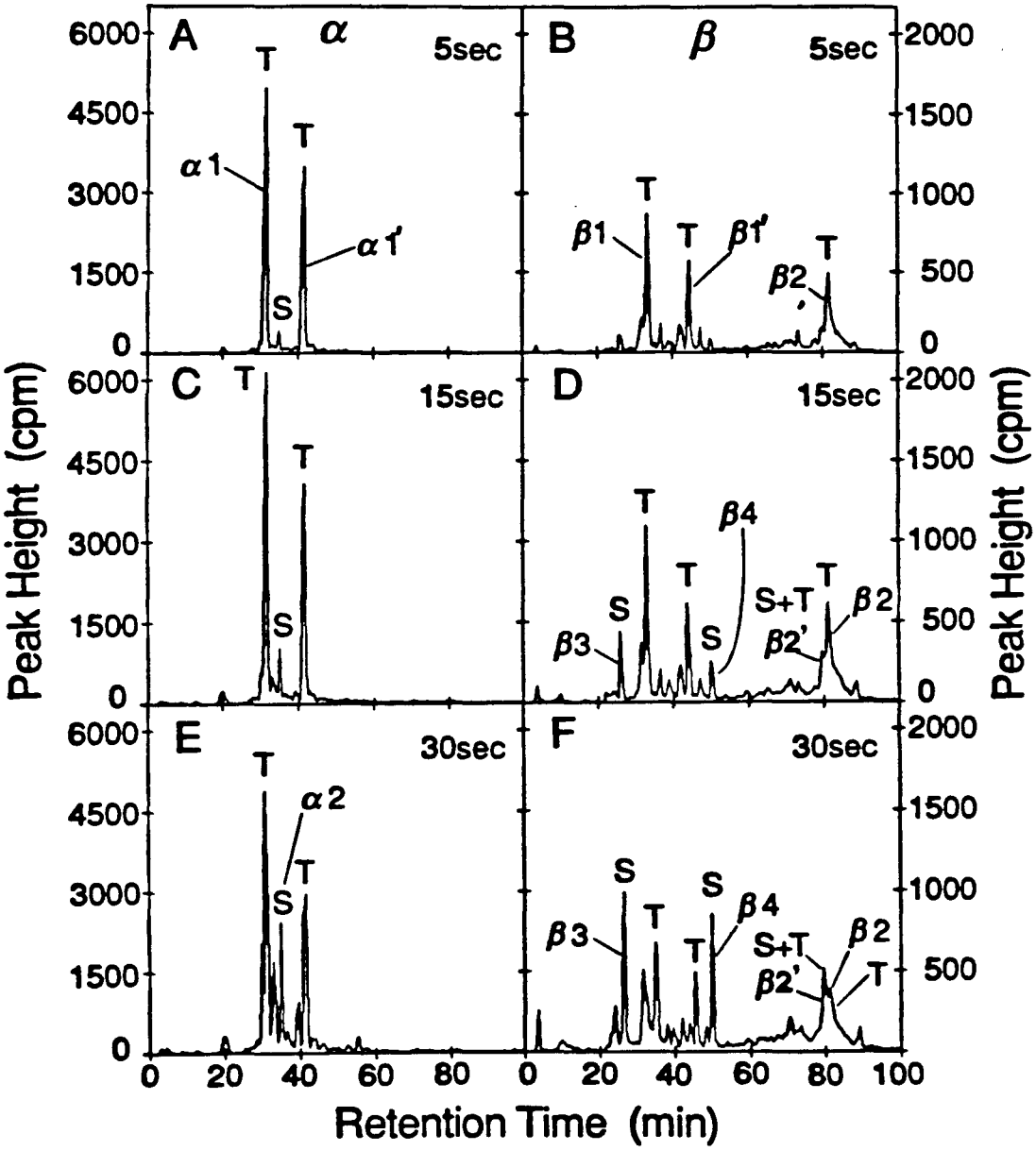
A
α
5sec
B
β
5sec
C
15sec
D
15sec
E
30sec
F
30sec
T
S
α1
α1'
β1
β1'
β2
β2'
β3
β4
S+T
Peak Height (cpm)
Retention Time (min)
6000
4500
3000
1500
0
2000
1500
1000
500
0
20
40
60
80
100

Figure 2. Purification of Peptides α1, α1′, β1, and β1′.

(**A**) and (**B**). Forebrain holoenzyme (1 mg) was phosphorylated for 15 sec 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 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. Peptide peaks were detected by absorbance at 214 nm. Fractions (0.5 ml) were collected by hand, and radioactive peptides were located by measuring Cerenkov radiation. (**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**). The holoenzyme variant containing a ratio of approximately 1 β subunit to 1 α subunit (1.5 mg, see Experimental Procedures) was autophosphorylated for 15 sec in the presence of [γ-^{32}P]-ATP, Ca^{2+}, and calmodulin. Phosphotryptic peptides 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. Labeled and

unlabeled peptides were detected as described for (**A**) and (**B**). (**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.

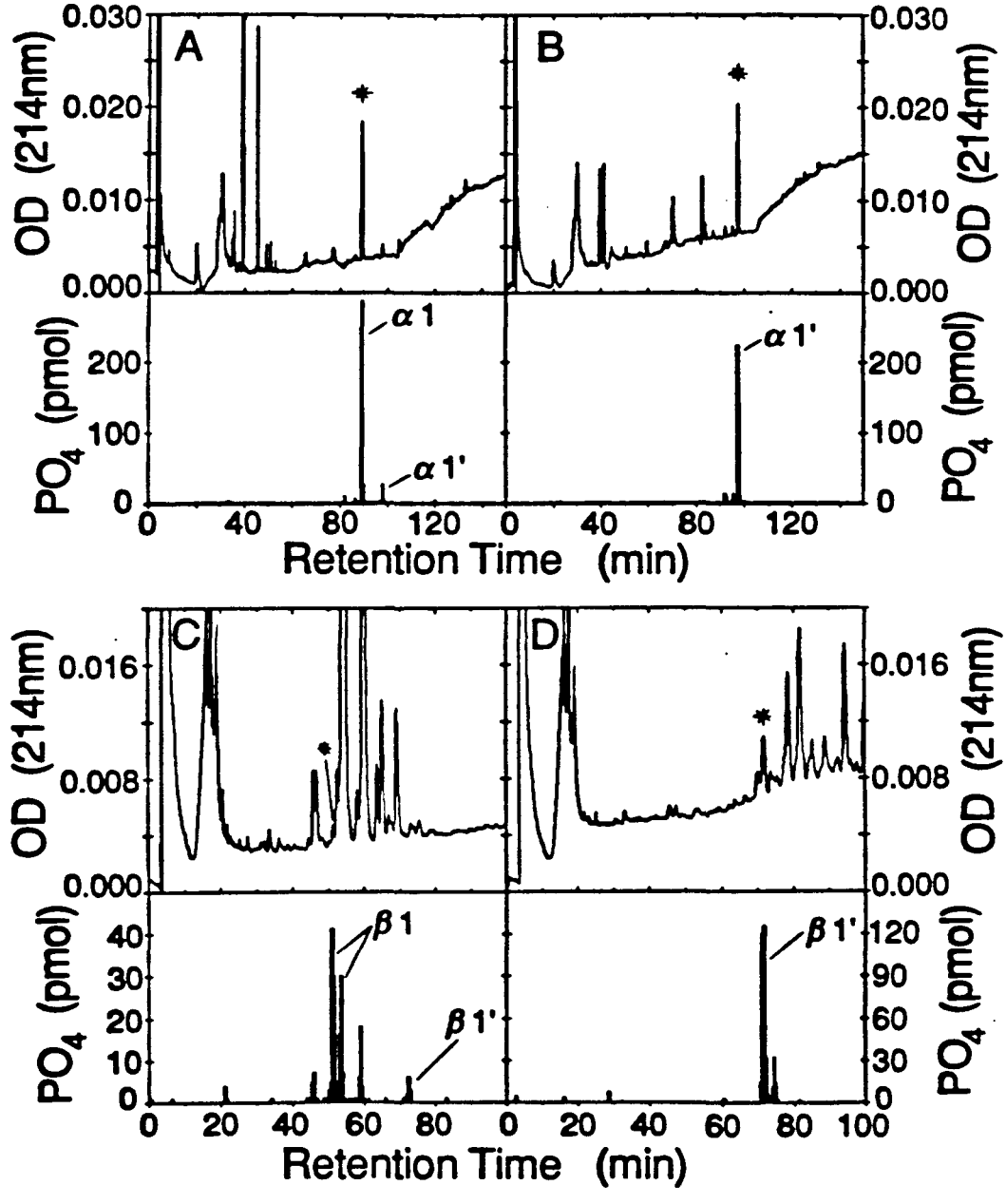
A
B
C
D
OD (214nm)
0.030
0.020
0.010
0.000
0.016
0.008
PO4 (pmol)
α 1
α 1'
β 1
β 1'
200
100
0
40
30
20
10
120
90
60
0 40 80 120
0 20 40 60 80 100
Retention Time (min)

Figure 3. Purification of Peptides β2 and β2′.

Holoenzyme with a 1 to 1 ratio of α and β subunits (0.6 mg) was autophosphorylated for 120 sec in the presence of [γ-^{32}P]-ATP, Ca^{2+} and calmodulin, then for an additional 60 sec in the absence of Ca^{2+} and calmodulin (see Experimental Procedures). The holoenzyme was reduced and alkylated, and the β subunit was 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). Fractions (0.5 ml) were collected by hand while monitoring absorbance at 214nm. Labeled peaks were detected by measuring Cerenkov radiation. (A) Peptide β2. 83% of applied radioactivity was recovered. (B) Peptide β2′. 82% of applied radioactivity was recovered.

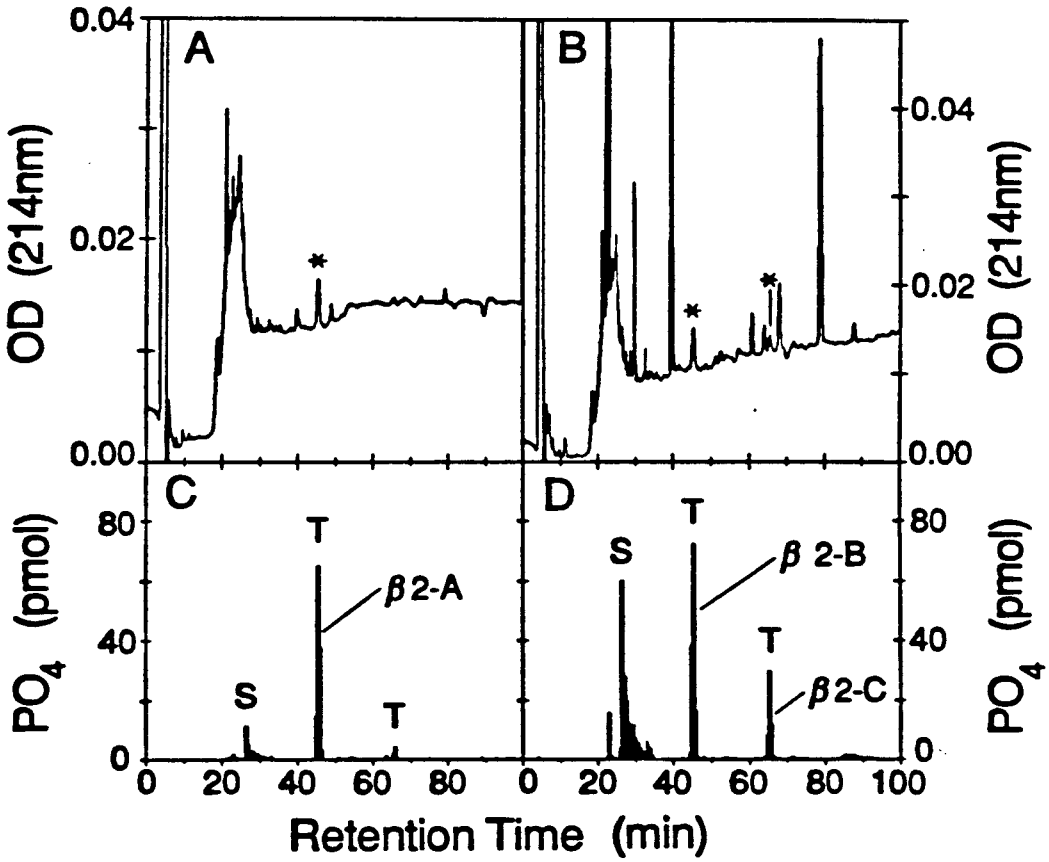
A
B
C
D
0.04
0.02
0.00
OD (214nm)
80
40
0
PO4 (pmol)
S
T
β2-A
β2-B
β2-C
0
20
40
60
80
100
Retention Time (min)

Figure 4. Comparison of Reverse-phase HPLC Tryptic Phosphopeptide Maps of β and β′ Subunits after a 5 sec Autophosphorylation.

Holoenzyme with a 1 to 1 ratio of α and β subunits was phosphorylated with [γ-^{32}P]-ATP for 5 sec in the presence of Ca^{2+} and calmodulin. The β and β′ subunits were separated by gel electrophoresis and digested with trypsin. The resulting phosphopeptides were separated on a C4 column as described in Experimental Procedures.

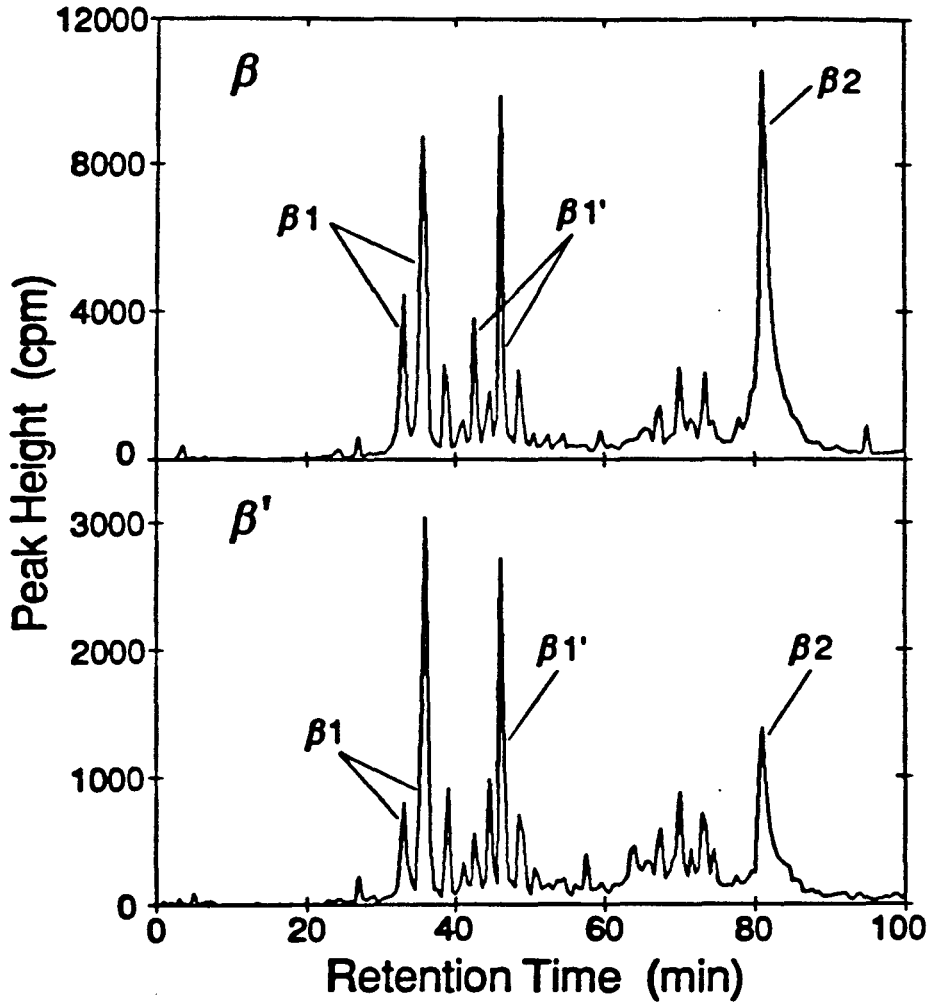
12000
8000
4000
0
β
β2
β1
β1'
Peak Height (cpm)
3000
2000
1000
0
β'
β1'
β2
β1
0
20
40
60
80
100
Retention Time (min)

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

Kinase was autophosphorylated for 5 sec in the presence of Ca^{2+} and calmodulin, then dephosphorylated for the indicated times as described in Experimental Procedures. In one set of experiments kinase activity was measured in the absence of Ca^{2+}, in another set of experiments the amount of phosphate present in sites α1, β1 and β2 was measured as described in Experimental Procedures. Total activity in the presence of Ca^{2+} was 8.9 ± 0.5 μmol/min/mg kinase. Activity in the absence of Ca^{2+} (100% in the figure) was 1.6 ± 0.1 μmol/min/mg kinase (17.8 ± 1% of total activity in the presence of Ca^{2+}). Incorporation of phosphate into α1, β1, and β2 before dephosphorylation was 0.35, 0.51, and 0.19 mol / mol subunit respectively. , percent calcium-independent activity; , percent phosphate in α1; , percent phosphate in β1; , percent phosphate in β2.

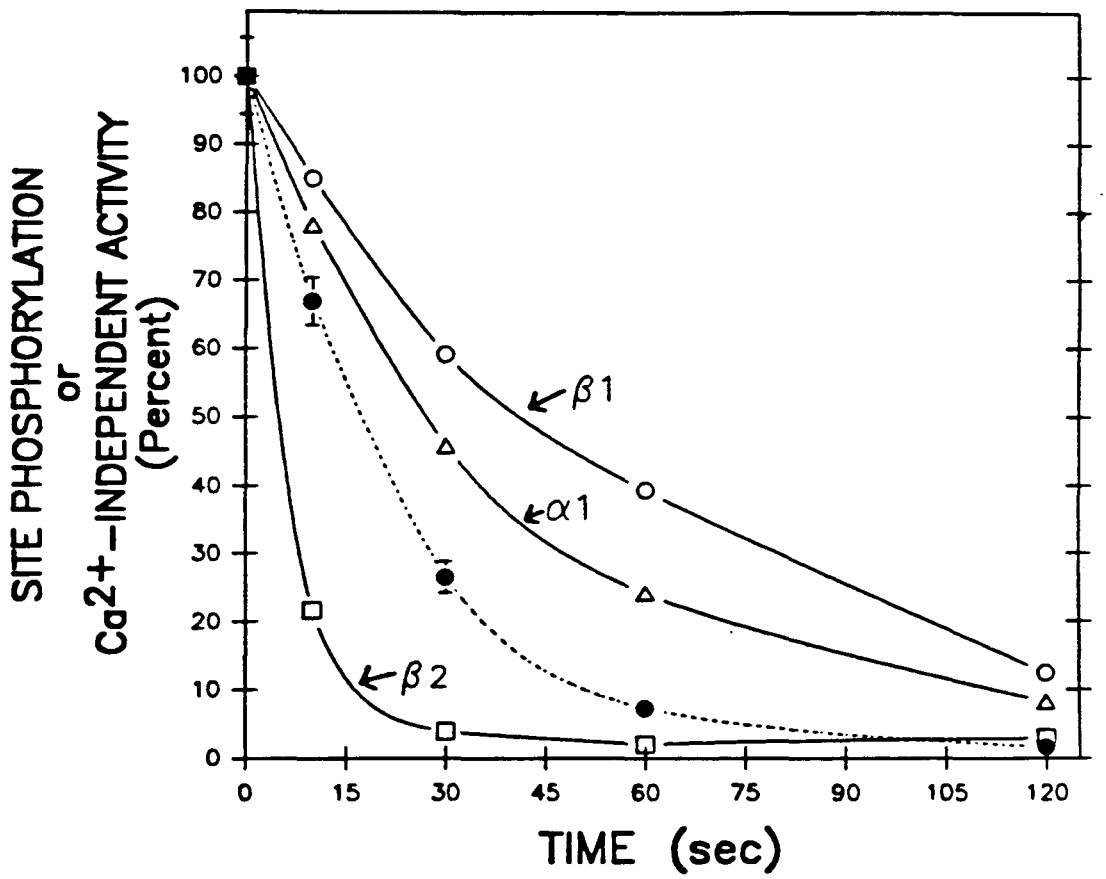
SITE PHOSPHORYLATION
or
Ca2+-INDEPENDENT ACTIVITY
(Percent)
100
90
80
70
60
50
40
30
20
10
0
0
15
30
45
60
75
90
105
120
TIME (sec)
β1
α1
β2

Figure 6. Location of Autophosphorylation Sites within the Amino Acid Sequence of the α and β Subunits of Rat Brain Type II CaM Kinase.

Portions of the amino acid sequence of the α (bottom) and β (top) subunits containing early autophosphorylation sites are shown. Amino acid identities between the α and β subunits are indicated by colons. The region that appears to be specifically deleted by mRNA processing in the β′ subunit is *underlined*. The putative calmodulin binding domain of each subunit is *boxed*. Early autophosphorylation sites are indicated by *filled circles*, and late autophosphorylation sites are indicated by *open circles*.

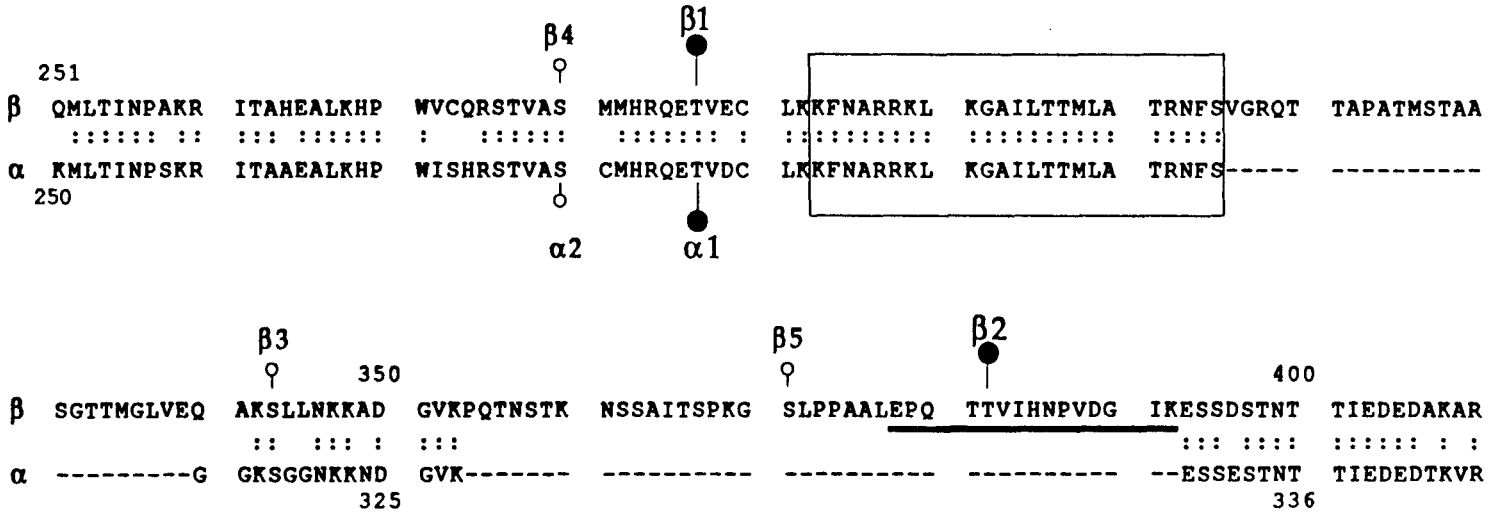
β4
β1
251
β QMLTINPAKR ITAHEALKHP WVCQRSTVAS MMHRQETVEC LKKFNARRKL KGAILTTMLA TRNFSVGRQT TAPATMSTAA
α KMLTINPSKR ITAAEALKHP WISHRSTVAS CMHRQETVDC LKKFNARRKL KGAILTTMLA TRNFS----- ----------
250
α2
α1
β3
β5
β2
350
400
β SGTTMGLVEQ AKSLLNKKAD GVKPQTNSTK NSSAITSPKG SLPPAALEPQ TTVIHNPVDG IKESSDSTNT TIEDEDAKAR
α ---------G GKSGGNKKND GVK------- ---------- ---------- ---------- --ESSESTNT TIEDEDTKVR
325
336

Figure 7. Schematic Representation of Early Autophosphorylation Sites within the Structure of the α, β, and β′ Subunits.

The primary structure of the β (top), β′ (center), and α (bottom) subunits are shown schematically. The thickened segment represents the putative calmodulin-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. The locations of the early autophosphorylation sites identified in this study are indicated by a ball and stick.

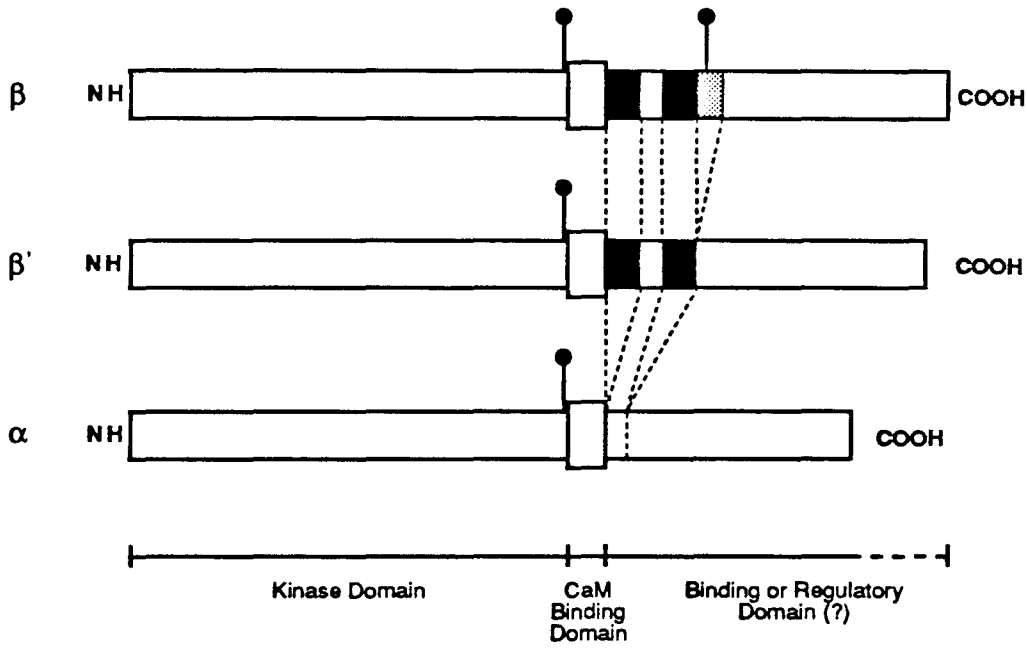
β
NH
COOH
β'
NH
COOH
α
NH
COOH
Kinase Domain
CaM Binding Domain
Binding or Regulatory Domain (?)

Table 1.

Composition of Phosphopeptides α1/α1′ and β1/β1′

A. Sequence of α1

Cycle	Residue	Yield[1]	Residue	Yield[2]
		pmol		*pmol*
1	Gln	51	Gln	21
2	Glu	37	Glu	18
3	-	—	-	—
4	Val	18	Val	19
5	Asp	19	Asp	37
6	-	—	-	—
7	Leu	10	Leu	24
8			Lys	5

[1]Approx. 100pmol of peptide was submitted for sequencing.
[2]Approx. 40pmol of peptide was submitted for sequencing.

B. Amino-Acid Composition of α1′

Residue	Found[3]		Predicted[4]
	mol/mol		*mol/mol*
Asp	0.7	(1)	1
Glu	2.1	(2)	2
Ser	0.4		
Gly	0.8	(1)	
His	0.1		
Arg	0.3		
Thr	0.3		1
Ala	0.4		
Pro	0.1		
Tyr	0.0		
Val	1.0	(1)	1
Met	0.0		
Cys	0.0		
Ile	0.1		
Leu	1.2	(1)	1
Phe	0.0		
Lys	0.7	(1)	

[3]Ave. of 3 determinations (30, 50, and 170pmol).
[4]Predicted from the sequence QETVDCLK.

C. Sequence of β1

Cycle	Residue	Yield[5]	Residue	Yield[6]
		pmol		*pmol*
1	Gln	18	Gln	16
2	Glu	17	Glu	15
3	-	—	-	—
4	Val	10	Val	7
5	Glu	9	Glu	8
6	[Glu]	[9]	[Glu]	[7]
7	Leu	6	Leu	5
8	Lys	3	Lys	2

[5]Approx. 40pmol of peptide was submitted for sequencing.
[6]Approx. 30pmol of peptide was submitted for sequencing.

D. Amino-Acid Composition of β1′

Residue	Found[7]		Predicted[8]
	mol/mol		*mol/mol*
Asp	0.3		
Glu	3.0	(3)	3
Ser	1.3	(1)	
Gly	2.4	(2-3)	
His	0.2		
Arg	0.2		
Thr	0.7	(1)	1
Ala	1.1	(1)	
Pro	0.4		
Tyr	0.2		
Val	1.2	(1)	1
Met	0.1		
Cys	0.0		
Ile	0.3		
Leu	1.6	(1-2)	1
Phe	0.1		
Lys	0.7	(1)	1

[7]Ave. of 2 determinations (30 and80pmol).
[8]Predicted from the sequence QETVECLK.

Table 2

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

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

[1]Approximately 120pmol of peptide was submitted for sequencing.
[2]Approximately 130pmol of peptide was submitted for sequencing.
[3]Approximately 55pmol of peptide was submitted for sequencing.

Table 3

Sequences of "Late" Peptides α2, β4 and β3.

	α2		β4		β3	
Cycle	Residue	Yield[1]	Residue	Yield[2]	Residue	Yield[3]
1	Ser	117	Ser	47	[Ser][4]	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		

[1] Approximately 300pmol of peptide was submitted for sequencing.
[2] Approximately 150pmol of peptide was submitted for sequencing.
[3] Approximately 600pmol of peptide was submitted for sequencing.
[4] Phosphoserine was recovered predominantly (90%) as a breakdown product (see text)

CHAPTER 5

CHARACTERIZATION AND IDENTIFICATION OF CA^{2+}-INDEPENDENT AUTOPHOSPHORYLATION SITES OF NEURONAL TYPE II CA^{2+}/CALMODULIN-DEPENDENT PROTEIN KINASE

SUMMARY.

The enzymatic activity of neuronal type II Ca^{2+}/calmodulin-dependent protein kinase is regulated by autophosphorylation in a complex manner (reviewed in chapter 1). The kinase is initially completely dependent on Ca^{2+}/calmodulin for phosphorylation of exogenous substrates as well as for autophosphorylation. Kinase activity becomes *partially* Ca^{2+}-independent after autophosphorylation in the presence of Ca^{2+}/calmodulin. At the same time autophosphorylation *itself* becomes independent of Ca^{2+}. Sequential autophosphorylation in the presence, then absence, of Ca^{2+} abolishes the Ca^{2+} stimulation of exogenous substrate phosphorylation, resulting in a *completely* Ca^{2+}-independent form of the kinase (Hashimoto *et al.*, 1987). In this study we have used tryptic digestion of the ^{32}P-labeled kinase followed by reverse-phase high-performance liquid chromatography (HPLC) and gas-phase microsequencing to identify the sites that are modified during Ca^{2+}-independent autophosphorylation. Two major Ca^{2+}-independent sites are phosphorylated in the β subunit. Both of these sites are located in or near the C-terminal region of the calmodulin-binding domain. Two sites homologous to the Ca^{2+}-independent β subunit sites were identified as major Ca^{2+}-independent sites in the α subunit. In addition the α subunit contains two additional Ca^{2+}-independent autophosphorylation sites. One of these two sites appears to be located at the extreme N-terminus of the α subunit, and the second has not yet been identified.

INTRODUCTION.

Brief autophosphorylation of neuronal type II CaM kinase in the presence of Ca^{2+} and calmodulin allows autophosphorylation to become independent of Ca^{2+} (Miller and Kennedy, 1986; Hashimoto *et al.*, 1987). Autophosphorylation in the absence of Ca^{2+} abolishes the stimulatory effect of Ca^{2+}/calmodulin on exogenous substrate phosphorylation, leading to a completely Ca^{2+}-independent form of the kinase (Hashimoto *et al.*, 1987; B.L. Patton, unpublished observations). This complex regulatory behaviour may be significant *in vivo* due to the properties of the regulation of intracellular Ca^{2+} concentrations by cellular activation.

The intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$) is believed to be maintained at very low levels (10-100n*M*) in the resting cell, and activation of the cell transiently raises $[Ca^{2+}]_i$ to 1μ*M* or more (see chapter 1 for discussion). If activation of the cell leads to sufficiently prolonged increases in $[Ca^{2+}]_i$, autophosphorylation of the kinase would proceed to the level of 3-5mol PO_4 / mol kinase holoenzyme. This level of kinase phosphorylation is sufficient to allow autophosphorylation to continue in the absence of Ca^{2+} and calmodulin (Miller and Kennedy, 1986). In this event kinase autophosphorylation would continue at a high rate even after $[Ca^{2+}]_i$ returns to resting levels, leading to a completely Ca^{2+}-independent form of the kinase. The kinase would remain in this state until dephosphorylated by phosphatases or degraded. Subsequent cellular activation would therefore be unable to activate the kinase above its new basal activity level.

In the previous chapter (chapter 4) we described the identification of the serine and threonine residues that are modified during autophosphorylation in the *presence* of Ca^{2+} and calmodulin. In this chapter we have used the same methods to begin to identify the residues modified during autophosphorylation in the *absence* of Ca^{2+} and calmodulin. We have found that sites are phosphorylated in both the α and β subunits which were not observed during phosphorylation in the presence of Ca^{2+} and calmodulin. Two of these sites are at homologous positions in the α and β subunits, but one is phosphorylated at a substantially higher rate in the β subunit. In addition, the rate of phosphorylation of the sites described in the previous chapter (chapter 4 of this thesis) is slower in the absence than in the presence of Ca^{2+} and calmodulin.

EXPERIMENTAL PROCEDURES.

MATERIALS.

Acetonitrile (HPLC/UV Grade) was purchased from Burdick and Jackson (Muskegon, MI), trifluoroacetic acid (TFA, Sequenal grade) from Pierce Biochemical, and dithiothreitol (DTT) from Schwarz/Mann Biotech. Trypsin treated with *N*-tosyl-L-phenylalanine chloromethyl-ketone (trypsin-TPCK) was from Cooper Biomedical, and thermolysin was from Boehringer Mannheim Biochemicals. Iodoacetamide was obtained from Sigma, and [γ-^{32}P]-ATP from ICN Nutritional Biochemicals. Water used for buffers and high-performance liquid chromatography (HPLC) was double glass distilled. All other reagents were of the highest available purity and obtained from standard sources. C4 (4.5x250mm, Vydac 214TPS) and C18 (4.5x250mm, Vydac 218TP54) reverse-phase HPLC columns were obtained from Vydac.

Calmodulin was purified from bovine brain by the method of Watterson *et al.* (1976). The forebrain isozyme of the type II CaM kinase was purified from rat as described previously (Miller and Kennedy, 1985). A second isozyme of the type II CaM kinase was used in these studies. This isozyme contains approximately equal molar amounts of the α- and β-subunits. This isozyme of the type II CaM kinase was purified from rat brain using the modified forebrain purification procedure (Miller and Kennedy, 1985). It was eluted from the CaM-sepharose affinity column with 0.2*M* NaCl/2m*M* EGTA after eluting the forebrain isozyme with 0*M* NaCl/2m*M* EGTA (high yields of

both isozymes are obtained from the same preparation). This isozyme was determined to have a molar ratio of α:β subunits of 1:1 and a purity of 80-90% as determined by densitometric scanning of stained sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) using the method described previously (Bennett *et al.*, 1983). The physical and enzymatic properties of this isozyme are similar to those of the forebrain and cerebellar isozymes (M.K. Bennett, S.G. Miller, B.L. Patton, unpublished observations).

METHODS.

Autophosphorylation of the Type II CaM Kinase. Pure type II CaM kinase was autophosphorylated in a reaction mixture (final volume 50 µl) containing 10 µg kinase (0.2 mg/ml), 50 m*M* Tris-HCl (pH 8.0), 10 m*M* $MgCl_2$, 5 m*M* 2-mercaptoethanol, 10 µg calmodulin, 10 m*M* dithiothreitol, 0.6 m*M* EGTA, 0.9 m*M* $CaCl_2$, and 200 µ*M* [γ-^{32}P]-ATP (1.0-2.0 x 10^3 cpm/pmol). After warming for 30 sec at 30°C, the reaction was initiated by the addition of kinase. The reaction was allowed to proceed at 30°C for the indicated time (see Figure Legends), and Ca^{2+} was then chelated by the addition of 5 µl of 22 m*M* EGTA (final 2.55 m*M* EGTA, 0.82 m*M* Ca^{2+}). The reaction was allowed to proceed in the absence of Ca^{2+} for the indicated time (see Figure Legends), and the entire reaction was then terminated by the addition of 55µl of ice-cold 0.2 *M* EDTA (pH8.0) followed by immediate cooling in an ice-water bath. For control reactions (no Ca^{2+}-independent autophosphorylation) the addition of EGTA was omitted, and the reactions were directly quenched by the addition of EDTA (as described above) at the indicated time. The quenched reaction mixture was divided into two 50 µl aliquots. One aliquot was used to determine the stoichiometry of phosphate incorporation into the α- and β-subunits after separation by SDS-PAGE as described previously (Miller and Kennedy, 1986), and the other, for tryptic phosphopeptide mapping by reverse-phase HPLC as described below.

Reduction and Alkylation of Autophosphorylated Kinase. To remove free [γ-^{32}P]-ATP, the autophosphorylated kinase was first precipitated with

trichloroacetic acid (TCA). One ml of 10% (wt/vol) TCA was added to the 50μl aliquot of the quenched autophosphorylation reaction mixture and incubated in an ice-water bath for 15 min. Precipitated protein was pelleted by centrifugation at 12,000 rpm for 15 min, the supernatant removed by aspiration, and the pellet washed once with 1 ml of ice-cold ether. Four hundred microliters of 6 *M* guanadine-HCl, 0.5 *M* Tris-HCl (pH8.1), 2 m*M* EDTA, were added, the tube flushed with N_2, and incubated for 30 min at 50°C to denature the protein. Fifty μl of a fresh solution of 5 m*M* dithiothreitol was added, the tube flushed with N_2, and the protein reduced for 4 hrs at 50°C. The tube was cooled to room temperature, 50 μl of fresh 20 m*M* iodoacetamide was added, the tube flushed with N_2, and incubated for 30 min in the dark at room temperature (alternatively, the alkylation was carried out 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 immediately dialyzed against 4 *l* of 50 m*M* NH_4HCO_3 (pH8.1) in the dark at 4°C for 10-12 hrs with one change of buffer. The tube was frozen in liquid nitrogen and lyophilized in a speedvac. The lyophilized protein was resuspended in 70 μl of 3% SDS, 2% (wt/vol) 2-mercaptoethanol, 5% (wt/vol) glycerol, 60 m*M* Tris-HCl (pH6.7), and a trace of bromphenol blue. The sample was heated to 65°C for 15 min and the kinase subunits separated by SDS-PAGE. The gel was dried, and the autophosphorylated subunits located by autoradiography.

Tryptic Digestion of Kinase Subunits. Gel pieces containing the α or β subunits were cut out of the dried SDS-PAGE gels and washed using two one-hour washes in 50ml of 20% (vol/vol) isopropanol followed by two

one-hour washes with 50ml of 10% (vol/vol) methanol on a rotating platform at room temperature. The washed gel pieces were transferred to microcentrifuge tubes, frozen, and lyophilized. One hundred microliters of 50 m*M* NH_4HCO_3 (pH8.1) containing 50 µg of trypsin-TPCK (approximately 20:1, trypsin:substrate) were added to each lyophilized gel piece and incubated 4 hrs at 37°C. One ml of 50 m*M* NH_4HCO_3 (pH8.1) containing 50 µg of fresh trypsin-TPCK was added and the reaction allowed to continue for 12 hrs at 37°C. The supernatant solution containing the tryptic peptides was removed and filtered through a 0.2-µm filter 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 primary filtrate. The filtrates and gel pieces were counted for Cerenkov radiation to determine the efficiency of recovery. The filtrates were frozen and lyophilized. Recoveries of ^{32}P-phosphate from gel pieces were 78 ± 9% (n=144) for the α subunit and 82 ± 8% (n=165) for the β subunit. Recovery of ^{32}P-phosphate could be increased to 95-100% by re-extracting the gel pieces with 1 ml of 50 m*M* NH_4HCO_3 (pH8.1) for 8-12 hrs but no difference in the reverse-phase HPLC phosphopeptide maps was observed when this was done.

Reverse Phase-HPLC Separation of Kinase Phosphopeptides. The lyophilized peptides were resuspended in 100 µl of Buffer A (0.07% (vol/vol) TFA, pH2.3) and loaded onto a C4 reverse-phase HPLC column that had been equilibrated in Buffer A. The column was developed at a flow rate of 1ml/min using the following gradient: 0-5min, 0% B; 5-95min, 0-35% B; 95-100min, 35-100% B (Buffer B: 0.07% (vol/vol) TFA, 70% acetonitrile, 30% H_2O). Two hundred

fractions (0.5 ml) were collected and counted for Cerenkov radiation (48% efficiency) to detect the eluted phosphopeptides. The concentration of acetonitrile in the samples had no significant effect on counting efficiency. Recovery of ^{32}P-phosphate from C4 reverse-phase HPLC was 80-95% for both the α and β subunits and did not vary with the extent of phosphorylation.

Phospho Amino Acid Analysis. Reverse-phase HPLC fractions containing the phosphopeptides of interest were pooled and dried in a speedvac. The lyophilized phosphopeptides were subjected to partial acid hydrolysis using 6 *N* HCl for 2 hrs at 110°C *in vacuo*. The hydrolyzed phosphopeptides were dissolved in 5 µl of electrophoresis buffer (acetic acid : formic acid : H_2O (78:25:897), pH 1.9) and spotted on 20 X 20cm thin-layer cellulose sheets. PO_4-serine, PO_4-threonine, and PO_4-tyrosine were spotted as markers, and the sheets were electrophoresed for 2 hrs at 750V towards the anode in electrophoresis buffer. The marker phospho-amino acids were located with ninhydrin, and the ^{32}P-labeled phospho-amino acids detected by autoradiography.

Proteolysis of Phosphopeptides with Thermolysin. Fractions containing phosphopeptides to be digested were pooled and reduced in volume to approximately 25-50 µl in a speedvac. Four hundred microliters of 100 m*M* NH_4HCO_3 (pH 8.0) containing 1 µg of thermolysin were added to the tube, and digestion carried out for 4 hrs at 37°C. The entire digest was loaded on a C18 reverse-phase HPLC column and eluted with the gradient described in the Figure Legends. Two hundred fractions (0.5 ml) were collected by hand, and

absorbance of the effluent monitored at 214nm.

Large-Scale Autophosphorylation and Tryptic Digestion for Sequencing. The basic methods described above for analytical-scale reverse-phase HPLC tryptic phosphopeptide mapping were used to obtain sufficient quantities of each phosphopeptide for automated gas-phase sequence analysis. Batches of pure type II CaM kinase were autophosphorylated in a reaction mixture (final volume 1 ml) containing 200 μg kinase (0.2 mg/ml), 50 m*M* Tris-HCl (pH 8.0), 10 m*M* $MgCl_2$, 5 m*M* 2-mercaptoethanol, 100 μg calmodulin, 0.6 mM EGTA, 0.9 mM $CaCl_2$, 10 mM dithiothreitol, and 200 μ*M* [γ-^{32}P]-ATP (20-40 cpm/pmol). After warming for 60sec at 30°C, the reaction was initiated by the addition of kinase. The reaction was allowed to proceed at 30°C for the indicated time, and the entire reaction was quenched by the addition of 120 μgl of ice-cold 100% TCA followed by immediate cooling in an ice-water bath. Reduction, alkylation, SDS-PAGE, and tryptic digestion were carried out exactly as described above. To improve recovery of the tryptic peptides, the gel pieces were subjected to a secondary extraction by adding 1ml of fresh 50 mM NH_4HCO_3 and incubating for 8-12 hrs at 37°C. This secondary supernatant was filtered as before and combined with the primary filtrate. Reverse-phase HPLC on a C4 column was carried out using the buffers and gradient described above. Absorbance of the column effluent was monitored at 214nm in order to estimate the purity of the eluted phosphopeptides.

Fractions containing phosphopeptides to be sequenced were pooled and reduced in volume to 50-100 μl using a speedvac. Three hundred μl of Buffer

A were added, and the entire volume was loaded onto a either a C4 or C18 reverse-phase HPLC column that had been equilibrated in Buffer A. The column was eluted with a gradient of increasing concentration of Buffer B (as described in Figure Legends) while monitoring the absorbance of the effluent at 214nm and manually collecting 0.5ml fractions. Fractions were counted for Cerenkov radiation, and the phosphopeptides of interest were submitted directly for sequencing and/or amino acid analysis. In some cases phosphopeptides were treated with thermolysin and purified by reverse-phase HPLC as described above (see Figure Legends).

Other Procedures. Automated gas-phase sequencing was performed using an Applied Biosystems 470A protein sequencer. Hydrolysis for total amino-acid analysis was carried out using 6 *N* HCl for 1 hr at 165°C *in vacuo.* The resulting amino acids were derivatized using an Applied Biosystems 420A Derivatizer and amino acids identified and quantitated as their phenylthiocarbamyl derivatives using an Applied Biosystems 130A Separation System. Protein concentrations were measured as described by Peterson (1977) using bovine serum albumin as a standard. Polyacrylamide gel electrophoresis in the presence of SDS was performed by the method of Laemmlli (1970). Stacking gels (2.5 X 16 cm X 1.2 mm) contained 3.5% acrylamide, 0.09% bisacrylamide, and the running gels (14 X 16 cm X 1.2 mm) contained 8% acrylamide, 0.27% bisacrylamide.

Abbreviations used in this paper are: CaM, calmodulin; DTT, dithiothreitol; EDTA, ethylenediamine N,N,N′,N′-tetraacetic acid; EGTA,

[ethylenebis-(oxyethylenenitrilo)]tetraacetic acid; reverse-phase HPLC, reverse-phase high-performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; TPCK, *N*-tosyl-L-phenylalanine chloromethyl-ketone; Type II CaM kinase, Type II Ca^{2+}/calmodulin dependent protein kinase;

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RESULTS.

Unique sites are phosphorylated in the α and β subunits during autophosphorylation in the absence of Ca^{2+}.

Autophosphorylation of the type II CaM kinase continues in the absence of Ca^{2+} after a brief Ca^{2+}-dependent autophosphorylation (Miller and Kennedy, 1986; Hashimoto *et al.*, 1987). The sites phosphorylated in the presence of Ca^{2+} have previously been identified (chapter 4 of this thesis, submitted for publication). We were interested in determining the identity of the sites phosphorylated during Ca^{2+}-independent autophosphorylation. Purified kinase was autophosphorylated in the presence of Ca^{2+}, calmodulin, and [γ-^{32}P]-ATP for 5 seconds, and then EGTA was added to chelate Ca^{2+}. The autophosphorylation was allowed to continue in the absence of Ca^{2+} for 5, 15, or 30 seconds. Each reaction was then quenched, and the ^{32}P-labeled α and β subunits separated by gel electrophoresis. The α and β subunits were digested with trypsin, and the tryptic phosphopeptides resolved by reverse-phase HPLC as described in Experimental Procedures (figure 1). The identity of the phosphorylated residue in each major peak was determined after partial acid hydrolysis.

Each of the phosphopeptides present after 5 seconds of autophosphorylation in the presence of Ca^{2+} has been identified (figure 1, A, B; chapter 4 of this thesis). After increasing times of autophosphorylation in the absence of Ca^{2+}, several phosphopeptides were found that were never observed as major peaks

during autophosphorylation in the presence of Ca^{2+} (figure 1, C-H). At the earliest times a single Ca^{2+}-independent phosphopeptide was seen in the β subunit (figure 1, βCI-1) and two Ca^{2+}-independent phosphopeptides were seen in the α subunit (figure 1, αCI-1, αCI-3). At longer times a second prominent phosphopeptide (figure 1, βCI-2) as well as several minor phosphopeptides appeared in the β subunit, while in the α subunit two prominent phosphopeptides appeared (figure 1, αCI-2, αCI-4). These phosphopeptides represent autophosphorylation sites that we have observed as major peaks only when Ca^{2+} is chelated after autophosphorylation in the presence of Ca^{2+}. The same phosphopeptides appear with the same time course when autophosphorylation was allowed to proceed for longer times (60-120 seconds) in the presence of Ca^{2+} before the addition of EGTA (data not shown).

Phospho-amino-acid analysis of the Ca^{2+}-independent phosphopeptides was carried out after partial acid hydrolysis (data not shown). Peptides βCI-1, αCI-1, and αCI-3 contain only phosphoserine. Peptides βCI-2, αCI-2 and αCI-4 contain only phosphothreonine. Phosphopeptides βCI-1 and αCI-1 represent homologous sites on the α and β subunits (see below). Phosphopeptides βCI-2 and αCI-2 also appear to represent homologous sites on the α and β subunits (see below).

Sequence of the β Subunit Peptide βCI-1.

To determine the sequence of the phosphopeptides described above, we scaled up the analytical reverse-phase HPLC tryptic phosphopeptide mapping

methods used in order to obtain sufficient quantities for gas phase sequencing (see Experimental Procedures). Phosphopeptides were first separated by reverse-phase HPLC on a C4 column as shown in figure 1 and then rechromatographed by reverse-phase HPLC on either a C4 column or a more hydrophobic C18 column using highly resolving gradients.

The elution profile for peptide βCI-1, using a highly resolving gradient on a C4 column, is shown for two separate preparations in figure 2 (2A and 2B). In each case peptide βCI-1 eluted as a single peak of radioactivity (90-95% of applied radioactivity). The sequence of each peptide was determined by gas phase sequencing and found to be NF[S]VG (table 1). This sequence corresponds to the residues Asn_{313} to Gly_{317} of the β subunit (figure 6) and begins at a predicted tryptic cleavage site. Peptide βCI-1 is completely resistant to further proteolysis by trypsin and therefore presumably represents the full predicted tryptic peptide NFSVGR. This peptide contains a single potential phosphorylation site (Ser_{315}), which was recovered predominantly as a breakdown product during sequencing (table 1). Phosphoserine is highly unstable during gas-phase sequencing and undergoes β-elimination of phosphate to produce free PO_4 and dehydroalanine, which is recovered as a phenylthiohydantoin-dithiothreitol-dehydroalanine adduct (Kulkarni *et al.*, 1987). We therefore conclude that Ser_{315} is the site phosphorylated in peptide βCI-1.

Sequence of the α Subunit Peptide αCI-1.

Peptide αCI-1 was purified for sequencing as described above except that a C18 column was employed for secondary chromatography. The elution profile of peptide αCI-1 from the C18 column is shown in figure 3. Peptide αCI-1 eluted as two major peaks of radioactivity. The smaller peak, at a retention time of approximately 42 minutes (αCI-1'), was never observed during analytical separations. The sequences of both peaks were determined by gas-phase sequencing (table 2). The peak eluting at approximately 37 minutes (αCI-1, figure 3) corresponds to residues Asn_{311} to Lys_{317} of the α subunit (NFSGGK, figure 6) and is a predicted tryptic cleavage product. The smaller peak, with a retention time of approximately 42 minutes (αCI-1', figure 3), corresponds to residues Lys_{48} to Arg_{52} (KLSAR, figure 6) of the α subunit and is also a predicted tryptic peptide. Each peptide contains a single potential phosphorylation site (Ser_{314} in peptide αCI-1, Ser_{50} in peptide αCI-1'). We therefore conclude that this is the site of phosphorylation in each case. The peptide αCI-1, representing Asn_{311} to Lys_{317} (NFSGGK), is homologous to the peptide βCI-1 (NFSVGR) described above (figure 6).

Sequences of the β Subunit Peptide βCI-2 and α Subunit Peptide αCI-2.

Peptide βCI-2 coeluted with large amounts of a contaminating autolytic peptide derived from trypsin when rechromatographed on C4 or C18 columns (not shown). We therefore digested this peptide with thermolysin, which cleaves at the N-terminal side of hydrophobic amino acids, and purified the

thermolytic digestion products by chromatography on a C18 column. The elution profile for the thermolytic digest of βCI-2 is shown in figure 4. Two major ^{32}P-labeled thermolytic products were observed at retention times of approximately 30 minutes (βCI-2A) and 67 (βCI-2B) minutes. The relative amounts of these two peaks varied during analytical-scale thermolysin digestions (not shown) and probably represent cleavage at alternative sites within the parent peptide. The sequence of the peak labeled βCI-2B (figure 4) was determined by gas-phase sequencing (table 3). The sequence of this peak was found to be GAIL before reaching background levels and corresponds to Gly_{302} to Leu_{305} of the β subunit. This sequence begins at the predicted tryptic cleavage site Lys_{301}-Gly_{302}. Peptide βCI-1 (see above) begins at the cleavage site Arg_{312}-Asn_{313} therefore the parent peptide βCI-2 is predicted to be Gly_{302} to Arg_{312} of the β subunit (GAILTTMLATR, see figure 6). This peptide contains three potential phosphorylation sites (Thr_{306}, Thr_{307}, and Thr_{311}). Thermolysin hydrolyzes peptide bonds on the N-terminal side of hydrophobic residues. Peptides βCI-2A and βCI-2B are both generated by thermolytic cleavage(s), since neither coelutes with the parent peptide, βCI-2 (not shown). From the sequence specificity of thermolysin we predict that βCI-2B is generated by cleavage at either Met_{308}-Leu_{309} or Leu_{309}-Ala_{310} (figure 6). Cleavage at either of these two sites removes βThr_{311}, therefore the phosphorylation site contained in peptide βCI-2 appears to be either Thr_{306} or Thr_{307}. This phosphorylation site is in the proposed calmodulin-binding domain of the kinase (figure 6, Hanley *et al.*, 1988). Further work will be necessary to identify this phosphorylation site conclusively.

Peptide αCI-2 (figure 1) appears to be identical to peptide βCI-2 based on several lines of evidence. Phospho-amino-acid analysis of each peptide shows that they both contain phosphothreonine (not shown). Peptides αCI-2 and βCI-2 exhibit the same retention time during chromatography on C4 columns (figure 1). Thermolytic digests of both peptides followed by chromatography on a C18 column yields the same pattern of thermolytic phosphopeptides (see figure 4, data not shown). Examination of the amino-acid sequences of the α and β subunits (figure 6) predicts that identical tryptic peptides would be generated from both the α and β subunits in the region identified above for βCI-2. This suggests that the peptide αCI-2 represents phosphorylation at either αThr_{305} or αThr_{306}. Unequivocal identification of the phosphorylation sites represented by peptides αCI-2 and βCI-2 will require further work.

Identification of the α Subunit Peptides αCI-3 and αCI-4.

Peptide αCI-3 elutes in the void volume during chromatography on C4 columns (figure 1) as well as on C18 columns using several different buffer systems (not shown), suggesting that it is a very hydrophilic peptide. Due to the presence of large amounts of contaminating material in the nonretained fractions after both C4 and C18 chromatography we were unable to sequence this peptide. Further work using alternative separation strategies (e.g., ion-exchange HPLC) will be required to obtain sufficiently pure peptide for sequence analysis.

Peptide αCI-4 was purified for sequencing by rechromatography on a C18 column (figure 5). A single peak of radioactivity (85-90% of applied radioactivity) was found in two separate experiments. This peptide was found to be completely blocked to Edman degradation during two separate attempts (700pmol and 1600pmol). The amino-acid composition of the peptide αCI-4 was therefore determined (table 4). The best match found for this amino acid composition was the predicted N-terminal tryptic peptide of the α subunit, ATITCTR (figure 6). The N-termini of both the α and β subunits of the kinase are known to be completely blocked to Edman degradation (M.K. Bennett and B. Conti-Tronconi, unpublished observations; LeVine *et al.*, 1987). It was recently suggested that in the mature β subunit the N-terminal methionine is removed, and the penultimate residue (alanine) is acetylated (LeVine *et al.*, 1987). If the N-terminus of the α-subunit is blocked by the same modification, this would be consistent with the lack of methionine in the amino-acid analysis of peptide αCI-4 (table 4). The similarity in amino-acid composition to the N-terminus and the N-terminal blockage suggest that peptide αCI-4 may represent the N-terminus of the α subunit. Positive identification of this phosphorylation site will require further work.

Discussion.

Autophosphorylation of the neuronal type II CaM kinase regulates enzymatic activity by altering its dependence on Ca^{2+}/calmodulin (Miller and Kennedy, 1986; Lai *et al.*, 1986; Schworer *et al.*, 1986; Hashimoto *et al.*, 1987). In the previous chapter of this thesis (chapter 4) we reported the identification of the sites that are modified when autophosphorylation is carried out in the continuous presence of Ca^{2+} and calmodulin. Phosphorylation of a specific threonine residue in the α and β subunits ($\alpha Thr_{286}/\beta Thr_{287}$) is correlated with production of the Ca^{2+}/CaM-independent form of the kinase (chapter 4 of this thesis). Phosphorylation of $\alpha Thr_{286}/\beta Thr_{287}$ also allows autophosphorylation itself to continue in the absence of Ca^{2+} and calmodulin. In this study we have used the technique of reverse-phase HPLC tryptic phosphopeptide mapping to examine the sites modified during autophosphorylatioin in the absence of Ca^{2+}. We have demonstrated that different sites are modified when autophosphorylation occurs in the absence *versus* the presence of Ca^{2+}.

The β subunit contains a Ca^{2+}-independent phosphorylation site that is modified at a much higher rate than any of the other Ca^{2+}-independent sites examined (βCI-1, figure 1). This phosphorylation occurs at serine $_{315}$ of the β subunit and reaches approximately 80% of its maximal stoichiometry within 5 sec of the chelation of Ca^{2+} (figure 1). The α subunit is phosphorylated at a homologous site (αSer_{314}) but at a much lower rate, approximately 40-60 sec are required for this site to reach its maximal stoichiometry (αCI-1, figure 1). These two sites are located near the C-terminal end of the calmodulin binding

domain (figures 6, 7, Hanley *et al.*, 1988). The site occurs at the boundary of a region that is deleted in the α subunit when compared to the β subunit (Bulleit *et al.*, 1988; figures 6, 7).

αSer_{314} and βSer_{315} both share the consensus substrate specifity sequence proposed by Pearson *et al.* (1985), Arg-X-Y-Ser/Thr. The sequences on the C-terminal side of these two sites are highly divergent (figure 6), and this may be responsible for the large difference in their rates of autophosphorylation. In chapter 4 we suggested that the presence of Gln at the -2 position and Val at the +1 position may influence the rate of substrate phosphorylation. It is interesting to note that βSer_{315} is followed by a Val at the +1 position, while αSer_{314} is followed by a Gly at this position (figure 6).

A second site is modified in the β subunit at a slower rate than βSer_{315} (βCI-2, figure 1). This phosphorylation site has been tentatively identified as either βThr_{306} or βThr_{307}. This site is located within the calmodulin-binding domain of the β subunit (figure 6, Hanley *et al.*, 1988). The available evidence suggests that the α subunit phosphopeptide αCI-2 (figure 1) results from phosphorylation at a homologous site in the α subunit (αThr_{305} or αThr_{306}). The phosphorylation sites $\alpha Ser_{314}/\beta Ser_{315}$ and $\alpha Thr_{305/6}/\beta Thr_{306/7}$ therefore occur within the predicted calmodulin-binding domain of the kinase and would be predicted to have an effect on the binding of calmodulin and regulation of kinase activity. The primary effect of Ca^{2+}-independent autophosphorylation is a complete loss of sensitivity of kinase activity to stimulation by Ca^{2+}/calmodulin (Hashimoto *et al.*, 1987; B.L. Patton, unpublished observa-

tions). The Ca^{2+}-independent activity of the kinase is unaffected. It is therefore suggested that phosphorylation of $\alpha Ser_{314}/\beta Ser_{315}$ and $\alpha Thr_{305/6}/\beta Thr_{306/7}$ is involved in the regulation of kinase activity by Ca^{2+}-independent autophosphoryalation.

Three other phosphopeptides were characterized in this study. During purification of phosphopeptide αCI-1 for sequencing, a second peptide was isolated (αCI-1′, figure 3) and sequenced. This peptide corresponds to the phosphorylation site αSer_{50}. We have not observed this phosphopeptide during analytical-scale rechromatography of αCI-1. Autophosphorylation was carried out for 120 sec in the presence of Ca^{2+} followed by 60 sec in the absence of Ca^{2+} when the kinase was prepared for sequencing. It is therefore possible that αSer_{50} is either a *slow* Ca^{2+}-dependent or Ca^{2+}-independent autophosphorylation site. We have tentatively identified the phosphopeptide αCI-4 (figure 1) as the N-terminus of the α subunit. The significance of autophosphorylation in this region of the kinase remains to be determined. A third α subunit phosphopeptide, αCI-3 (figure 1) remains unidentified, and further work will be required to place this site within the primary structure of the α subunit.

It is clear that further work will be required to characterize completely the Ca^{2+}-independent autophosphorylation sites of the the type II CaM kinase. The location of the phosphorylation sites $\alpha Ser_{314}/\beta Ser_{315}$ and $\alpha Thr_{305/6}/\beta Thr_{306/7}$ in or near the calmodulin-binding domain suggests that they play a role in the reduction of Ca^{2+}-stimulation of kinase activity. It will be important to

unequivocally identify each of the Ca^{2+}-independent autophosphorylation sites and correlate their phosphorylation state with alterations in kinase activity. It will also be important to determine whether the type II kinase undergoes regulation by autophosphorylation *in vivo*. Analysis of the autophosphorylation sites of the kinase *in vivo* and comparison to those described in this study and the previous chapter (chapter 4) will help to answer this question.

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Figure 1. Time Course of Ca^{2+}-Independent Autophosphorylation Sites on the α and β Subunits.

Pure rat forebrain type II CaM kinase (10 μg) was autophosphorylated for 5 sec in the presence of [γ-^{32}P]-ATP, Ca^{2+}, and calmodulin (**A,B**) and then EGTA was added to chelate Ca^{2+} (see Experimental Procedures). Autophosphorylation was allowed to continue in the absence of Ca^{2+} for a further 5 sec (**C,D**), 15 sec (**E,F**), or 30 sec (**G,H**). The holoenzyme at each time point were reduced and alkylated, the α and β subunits purified by SDS-PAGE, and digested with trypsin (see Experimental Procedures). The resultant phosphopeptides were separated by reverse-phase HPLC using a C4 column and the gradient described in Experimental Procedures. The ^{32}P elution profiles for the α (**A,C,E,G**) and β (**B,D,F,H**) subunit tryptic phosphopeptides are presented here. Peaks normally phosphorylated during autophosphorylation in the presence of Ca^{2+} are identified by *roman numerals* (I, $\alpha Thr_{286}/\beta Thr_{287}$; II, βThr_{382}; III, βSer_{343}; IV, βSer_{280}: see chapter 4 of this thesis). **S**, peptide containing phosphoserine. **T**, peptide containing phosphothreonine. The threefold difference in scaling of the ordinate reflects the 3:1 ratio of α to β subunits found in the forebrain isozyme of the type II CaM kinase. Thus the peak heights reflect the proportion of phosphate found in each peak.

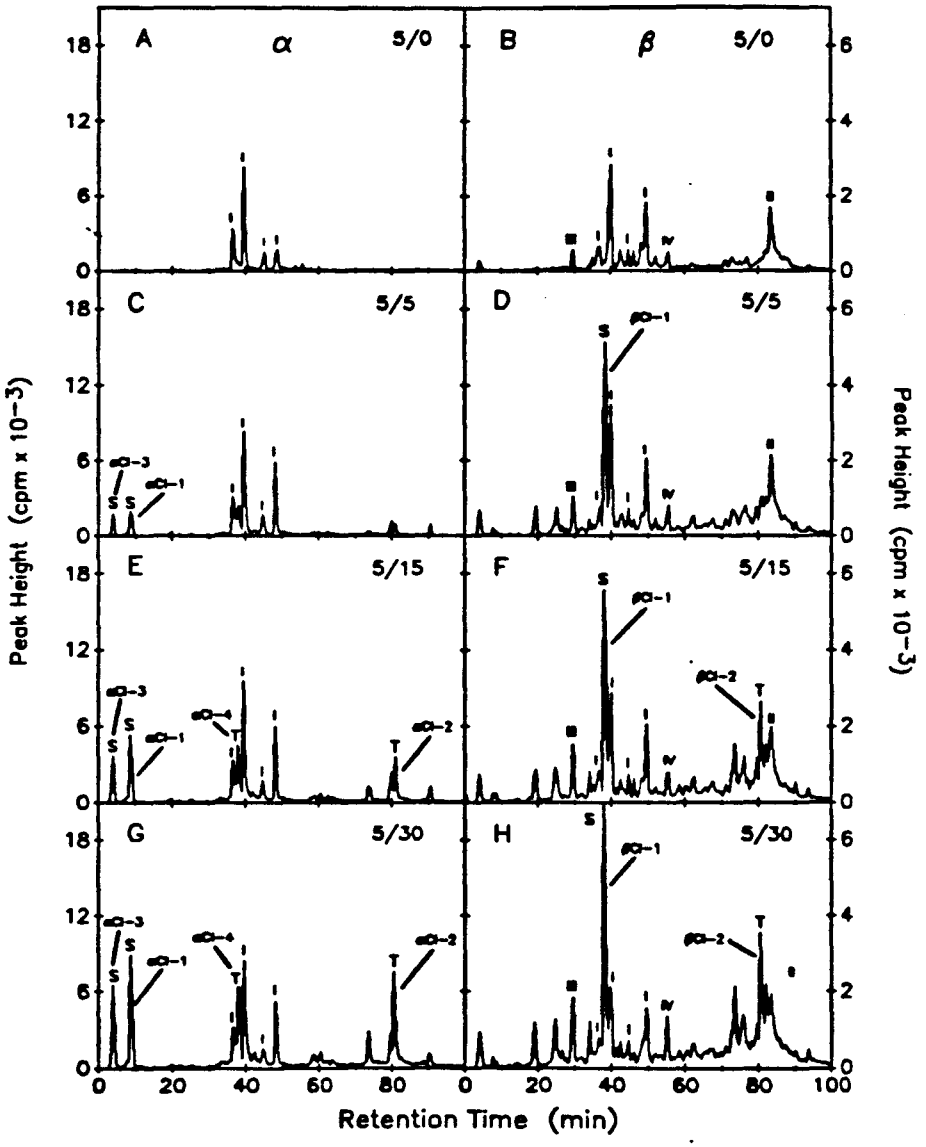
A
α
5/0
B
β
5/0
C
5/5
D
5/5
E
5/15
F
5/15
G
5/30
H
5/30
Peak Height (cpm x 10−3)
Peak Height (cpm x 10−3)
Retention Time (min)

Figure 2. Purification of Peptide βCI-1.

Pure forebrain type II CaM kinase (1mg for **A,C**; 3.8mg for **B,D**) was phosphorylated for 120 sec in the presence of [γ-^{32}P]-ATP, Ca^{2+}, and calmodulin, and then EGTA was added to chelate Ca^{2+}. Autophosphorylation was allowed to continue for 60 sec in the absence of Ca^{2+}. The kinase was reduced and alkylated, the α and β subunits separated by SDS-PAGE, and the β subunit digested with trypsin (see Experimental Procedures). The resultant phosphopeptides were fractionated by reverse-phase HPLC on a C4 column. Fractions corresponding to βCI-1 were pooled, concentrated, and rechromatographed on a C4 column using a highly resolving gradient (shown here). Absorbance was monitored at 214nm (**A,B**), and fractions of 1ml (**C**) or 0.5ml (**D**) were collected by hand and counted for Cerenkov radiation to locate ^{32}P-labeled peptides. The *left* and *right panels* represent two separate phosphorylation reactions and peptide purifications. The gradient used for the *left panels* was : 0-5min, 2.8% acetonitrile; 5-95min, 2.8-8.4% acetonitrile; 95-100min, 8.4-70% acetonitrile. The gradient for the *right panels* was: 0-5min, 3.5% acetonitrile; 5-95min, 3.5-10.5% acetonitrile; 95-100min, 10.5-70% acetonitrile. 95% of applied radioactivity was recovered in **C**, and 92% was recovered in **D**.

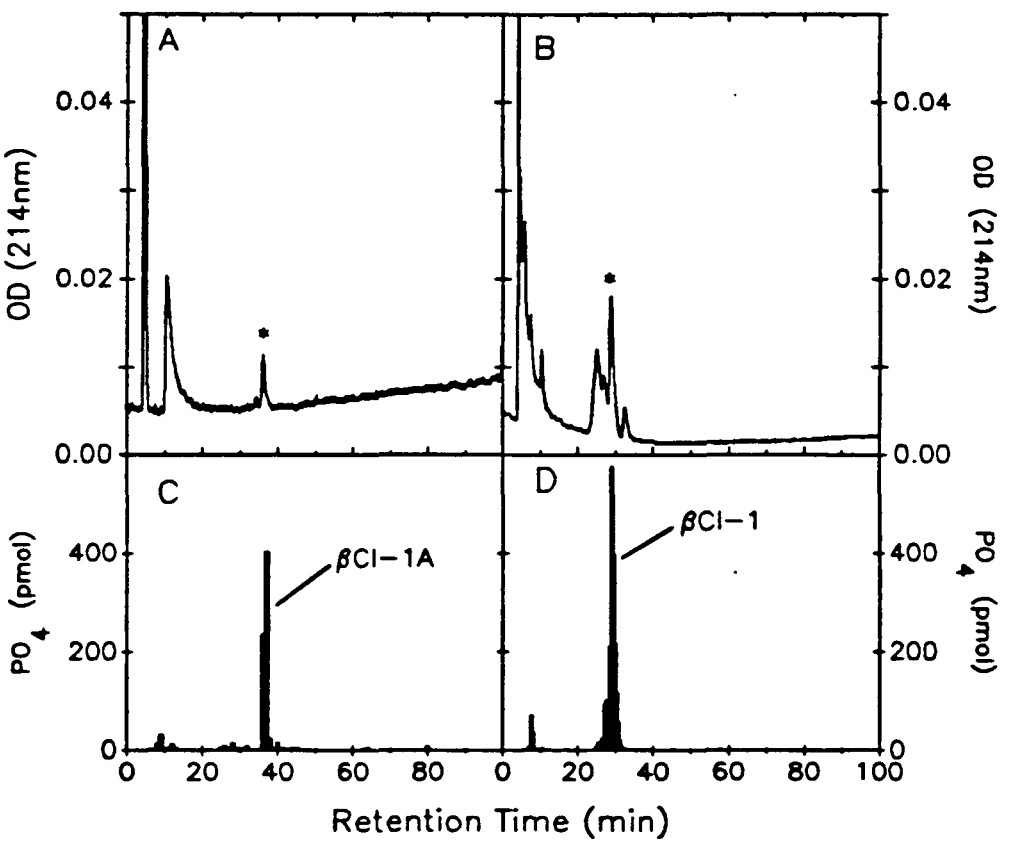
A
B
C
D
0.04
0.02
0.00
OD (214nm)
400
200
0
PO4 (pmol)
βCl−1A
βCl−1
0
20
40
60
80
100
Retention Time (min)

Figure 3. Purification of Peptide αCI-1.

One mg of forebrain type II CaM kinase was phosphorylated, reduced, and alkylated, and the α and β subunits separated as described in Figure 2. The α subunit was digested with trypsin, and the resultant phosphopeptides fractionated by chromatography on a C4 column. Fractions corresponding to peptide αCI-1 were pooled, concentrated, and fractionated on a C18 column using a highly resolving gradient (shown here). Absorbance was monitored at 214nm (**A**), and fractions (0.5ml) were collected by hand and counted for Cerenkov radiation to locate ^{32}P-labeled peptides (**B**). The gradient used was: 0-10min, 0% acetonitrile; 10-90min, 0-10.5% acetonitrile; 90-100min, 10.5-70% acetonitrile. 86% of applied radiactivity was recovered.

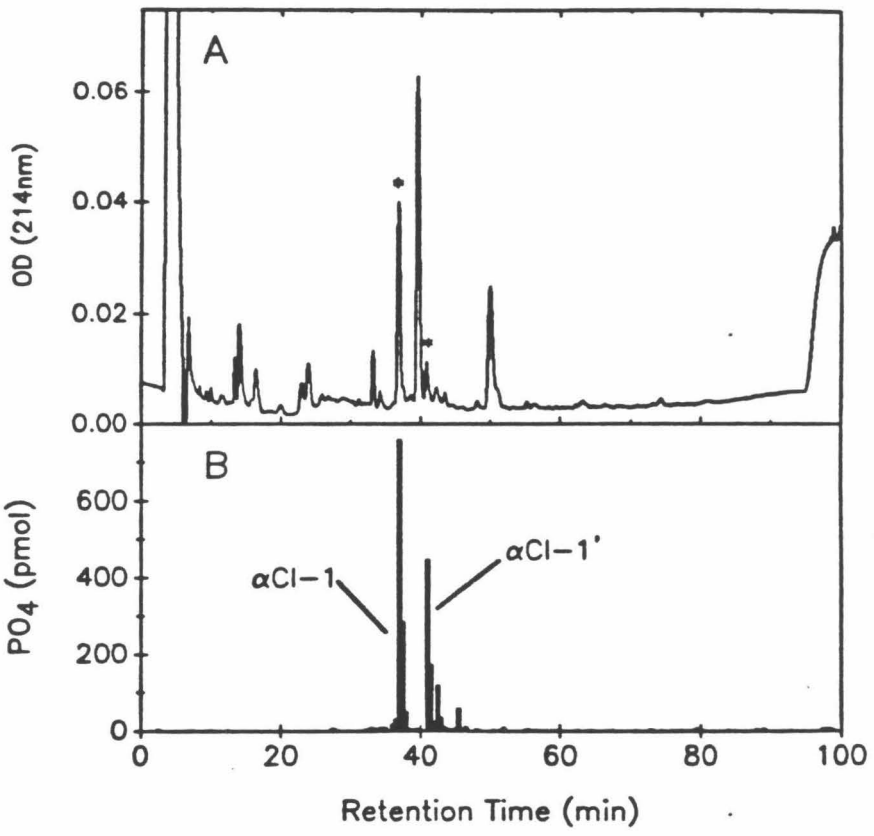
A
0.06
0.04
0.02
0.00
OD (214nm)
B
600
400
200
0
PO_4 (pmol)
αCl−1
αCl−1'
0
20
40
60
80
100
Retention Time (min)

Figure 4. Purification of Peptide βCI-2.

0.6mg of the rat brain type II kinase isozyme containing a 1:1 ratio of α:β subunits was phosphorylated, reduced and alkylated, and the α and β subunits separated as described in Figure 2. The β subunit was digested with trypsin, and the resultant phosphopeptides fractionated by chromatography on a C4 column. Fractions corresponding to peptide βCI-2 were pooled, concentrated, and digested with thermolysin as described in Experimental Procedures. The resultant thermolytic peptides were fractionated on a C18 column using a highly resolving gradient (shown here). Absorbance was monitored at 214nm (**A**), and fractions (0.5ml) were collected by hand and counted for Cerenkov radiation to locate ^{32}P-labeled peptides (**B**). The gradient used was: 0-10 min, 0% acetonitrile; 10-90 min, 0-29% acetonitrile; 90-100 min, 29-70% acetonitrile. 79% of applied radioactivity was recovered.

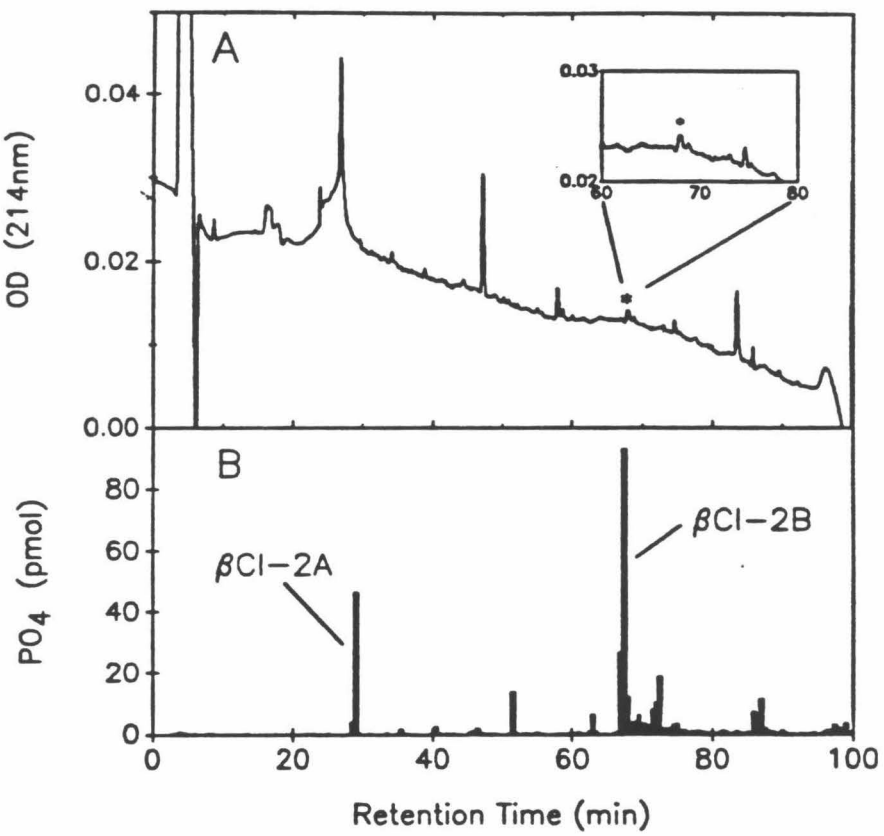
A
B
OD (214nm)
PO4 (pmol)
0.04
0.02
0.00
0.03
0.02
60
70
80
80
60
40
20
0
βCl−2A
βCl−2B
0
20
40
60
80
100
Retention Time (min)

Figure 5. Purification of Peptide αCI-4.

0.6mg of the rat brain type II kinase isozyme containing a 1:1 ratio of α:β subunits was phosphorylated, reduced, and alkylated, and the α and β subunits separated as described in Figure 2. The α subunit was digested with trypsin, and the resultant phosphopeptides fractionated by chromatography on a C4 column. Fractions corresponding to peptide αCI-4 were pooled, concentrated, and fractionated on a C18 column using a highly resolving gradient (shown here). Absorbance was monitored at 214nm (**A**), and fractions (0.5ml) were collected by hand and counted for Cerenkov radiation to locate ^{32}P-labeled peptides (**B**). The gradient used was: 0-10 min, 0% acetonitrile; 10-20 min, 0-5.25% acetonitrile; 20-90 min, 5.25-8.75% acetonitrile; 90-100 min, 8.75-70% acetonitrile. 87% of applied radioactivity was recovered.

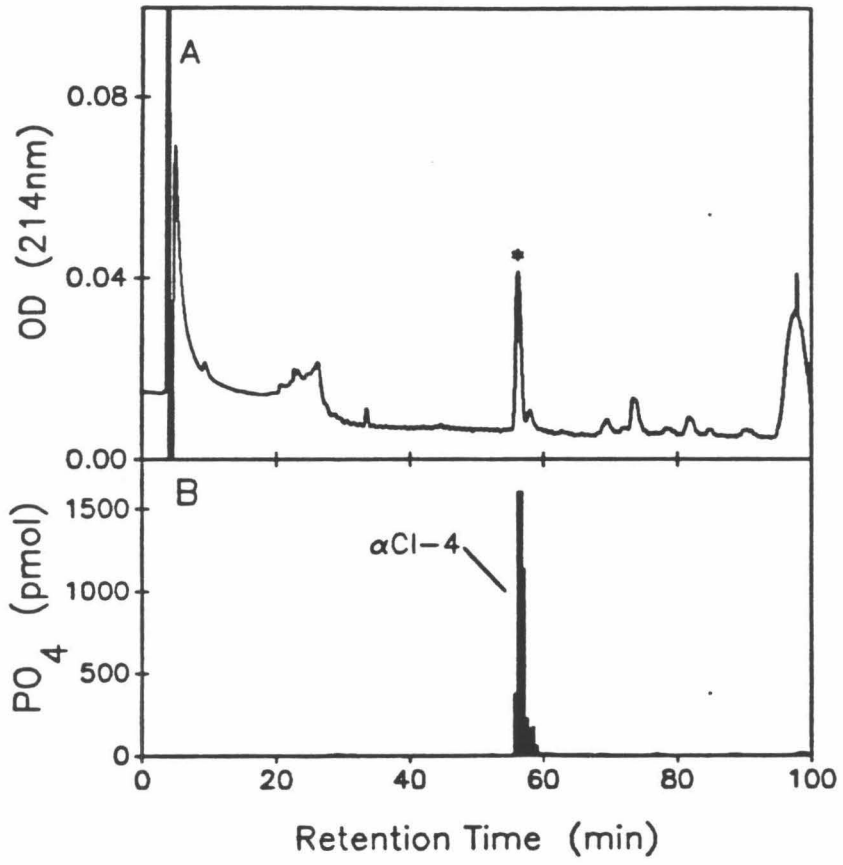
A
0.08
0.04
0.00
OD (214nm)
B
1500
1000
500
0
PO4 (pmol)
αCl-4
0
20
40
60
80
100
Retention Time (min)

Figure 6. Location of Phosphorylation Sites in the Amino-Acid Sequence of the α and β Subunits of Rat Brain Type II CaM Kinase.

The complete deduced amino acid sequence of the α (Bulleit *et al.*, 1988) and β (Bennett *et al.*, 1987) subunits are shown. The autophosphorylation sites identified in this study are indicated by *filled circles* (o). Autophosphorylation sites identified in the previous study (chapter 3) are indicated by *open circles* (o). The nomenclature for the autophosphorylation sites is that used in Chapters 4 and 5 of this thesis. Gaps inserted in the α subunit to optimize alignment are indicated by *dashed lines*. The calmodulin binding domain is *boxed*. The sequence of amino acids deleted in the β′ subunit is *underlined*.

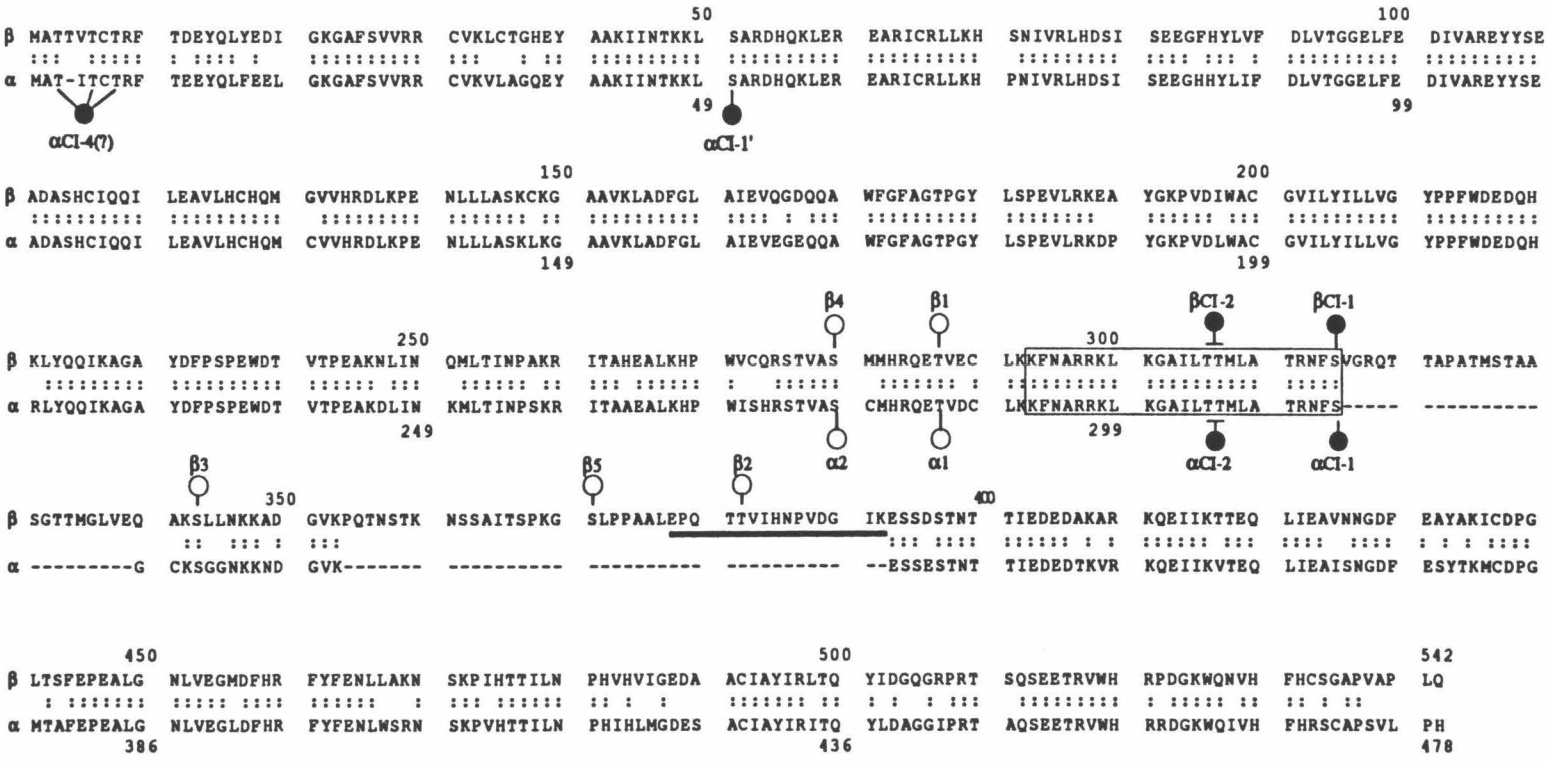
50
100
β MATTVTCTRF TDEYQLYEDI GKGAFSVVRR CVKLCTGHEY AAKIINTKKL SARDHQKLER EARICRLLKH SNIVRLHDSI SEEGFHYLVF DLVTGGELFE DIVAREYYSE
α MAT-ITCTRF TEEYQLFEEL GKGAFSVVRR CVKVLAGQEY AAKIINTKKL SARDHQKLER EARICRLLKH PNIVRLHDSI SEEGHHYLIF DLVTGGELFE DIVAREYYSE
αCI-4(7)
49
αCI-1'
99
150
200
β ADASHCIQQI LEAVLHCHQM GVVHRDLKPE NLLLASKCKG AAVKLADFGL AIEVQGDQQA WFGFAGTPGY LSPEVLRKEA YGKPVDIWAC GVILYILLVG YPPFWDEDQH
α ADASHCIQQI LEAVLHCHQM CVVHRDLKPE NLLLASKLKG AAVKLADFGL AIEVEGEQQA WFGFAGTPGY LSPEVLRKDP YGKPVDLWAC GVILYILLVG YPPFWDEDQH
149
199
β4
β1
βCI-2
βCI-1
250
300
β KLYQQIKAGA YDFPSPEWDT VTPEAKNLIN QMLTINPAKR ITAHEALKHP WVCQRSTVAS MMHRQETVEC LKKFNARRKL KGAILTTMLA TRNFSVGRQT TAPATMSTAA
α RLYQQIKAGA YDFPSPEWDT VTPEAKDLIN KMLTINPSKR ITAAEALKHP WISHRSTVAS CMHRQETVDC LKKFNARRKL KGAILTTMLA TRNFS---------- ----------
249
299
β3
β5
β2
α2
α1
αCI-2
αCI-1
350
400
β SGTTMGLVEQ AKSLLNKKAD GVKPQTNSTK NSSAITSPKG SLPPAALEPQ TTVIHNPVDG IKESSDSTNT TIEDEDAKAR KQEIIKTTEQ LIEAVNNGDF EAYAKICDPG
α ---------G CKSGGNKKND GVK------- ---------- ---------- ---------- --ESSESTNT TIEDEDTKVR KQEIIKVTEQ LIEAISNGDF ESYTKMCDPG
450
500
542
β LTSFEPEALG NLVEGMDFHR FYFENLLAKN SKPIHTTILN PHVHVIGEDA ACIAYIRLTQ YIDGQGRPRT SQSEETRVWH RPDGKWQNVH FHCSGAPVAP LQ
α MTAFEPEALG NLVEGLDFHR FYFENLWSRN SKPVHTTILN PHIHLMGDES ACIAYIRITQ YLDAGGIPRT AQSEETRVWH RRDGKWQIVH FHRSCAPSVL PH
386
436
478

Figure 7. Schematic Representation of Phosphorylation Sites of the α, β′, and β Subunits of Type II CaM Kinase.

The locations of the phosphorylation sites of the type II CaM kinase within the primary structures of the α, β′, and β subunits are shown. Sites phosphorylated in the presence of Ca^{2+}/calmodulin are indicated by *open circles* (o), while those identified in this study are indicated by *filled circles* (o). Only the early Ca^{2+}/calmodulin-dependent autophosphorylation sites are illustrated (see chapter 4).

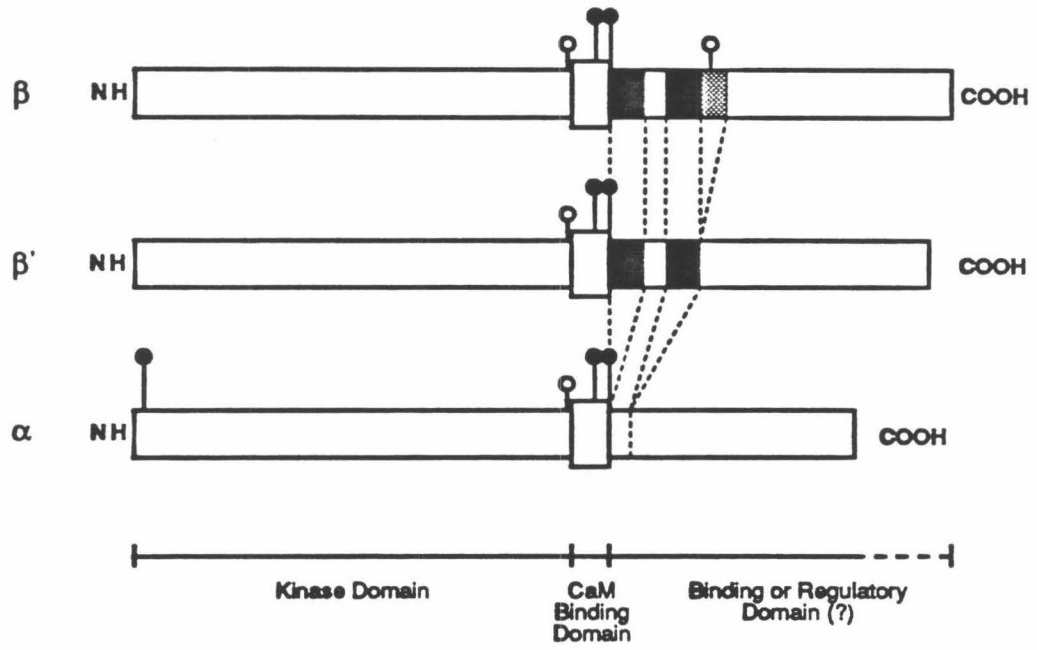
β
NH
COOH
β'
NH
COOH
α
NH
COOH
Kinase Domain
CaM Binding Domain
Binding or Regulatory Domain (?)

Table 1.

Sequence of Phosphopeptide βCI-1

	βCI-1A		βCI-1B	
Cycle	Residue	Yield[1]	Residue	Yield[2]
		pmol		*pmol*
1	Asn	46	Asn	74
2	Phe	37	Phe	26
3	[Ser][3]	[13]	[Ser][3]	[38]
4	Val	9	Val	22
5	Gly	30	Gly	48

[1]Approximately 91pmol of peptide was submitted for sequencing.
[2]Approximately 150pmol of peptide was submitted for sequencing.
[3]Phosphoserine was recovered primarily as a breakdown product (see text).

Table 2.

Sequences of Phosphopeptides αCI-1 and αCI-1′

	αCI-1		αCI-1′	
Cycle	Residue	Yield[1]	Residue	Yield[2]
		pmol		*pmol*
1	Asn	512	Lys	476
2	Phe	347	Leu	453
3	Ser	390	Ser	381
4	Gly	183	Ala	366
5	Gly	116	Arg	76
6	Lys	57		

[1] Approximately 800pmol of this peptide was submitted for sequencing.
[2]Approximately 500pmol of this peptide was submitted for sequencing.

Table 3

Sequence of Phosphopeptide βCI-2B

Cycle	Residue	Yield[1]
		pmol
1	Gly	43
2	Ala	21
3	Ile	11
4	Leu	14

[1]Approximately 90pmol of this peptide was submitted for sequencing.

CHAPTER 6

CONCLUSIONS AND PERSPECTIVES

Functional Significance of Kinase Regulation by Autophosphorylation.

The results presented in this thesis demonstrate that the activity of the neuronal type II Ca^{2+}/calmodulin-dependent protein kinase is regulated by autophosphorylation *in vitro*. This regulation can be correlated with the phosphorylation of specific sites within the primary structure of the kinase (chapters 4 and 5), and is reversed by dephosphorylation of the appropriate sites with protein phosphatases (chapters 3 and 4). The regulation of kinase activity by autophosphorylation has been confirmed by other groups, and extended by further work in this and other laboratories (for references and a complete discussion see chapter 1). Although there continue to be minor discrepencies between the results in different laboratories, these revolve around quantitative rather than qualitative features of the observed regulatory properties. The quantitative differences may arise from the use of different substrates in the various laboratories. An example of the influence of substrate-directed effects on the measurement of kinase activity was mentioned in the discussion of phosphorylase kinase in chapter 1. The major question which remains to be answered is a key one: Is kinase activity regulated by autophosphorylation *in vivo*?

In 1979 Krebs and Beavo suggested four criteria that must be fulfilled in order to demonstrate the physiological significance of the regulation of enzymatic activity by phosphorylation-dephosphorylation (Krebs and Beavo, 1979):

1. Demonstration *in vitro* that the enzyme can be phosphorylated stoichiometrically at a significant rate in a reaction(s) catalyzed by an appropriate protein kinase(s) and dephosphorylated by a phosphoprotein phosphatase(s).

2. Demonstration that functional properties of the enzyme undergo meaningful changes that correlate with the degree of phosphorylation.

3. Demonstration that the enzyme can be phosphorylated and dephosphorylated *in vivo* or in an intact cell system with accompanying functional changes.

4. Correlation of cellular levels of protein kinase and/or phosphoprotein phosphatase effectors and the extent of phosphorylation of the enzyme.

The work described in chapters 3-5 of this thesis appears to fulfill the first and second criteria established by Krebs and Beavo. The third and fourth criteria are more difficult to fulfill, and remain to be demonstrated for the regulation of broad-specificity calmodulin-dependent kinases by autophosphorylation (as well as the majority of phosphorylation/dephosphorylation regulated systems described in the literature to date). In the following sections I would like to address each of the four criteria in relation to the neuronal type II CaM kinase.

Significance of the Rate and Stoichiometry of Autophosphorylation and Dephosphorylation by Protein Phosphatases.

The rate of *in vitro* phosphorylation of the α and β subunits of the forebrain isozyme of the neuronal type II CaM kinase is illustrated in chapter 3 of this thesis (chapter 3, Figure 1). In the nervous system, changes in intracellular Ca^{2+} concentrations are often thought to occur on the time scale of tens of milliseconds to perhaps one second. Analysis of the time scale of kinase autophosphorylation at the gross level illustrated in Figure 1 of chapter 3 might therefore lead one to conclude that stoichiometrically significant incorporation of phosphate is not physiologically relevant. The work presented in chapters 3, 4 and 5 of this thesis as well as several recent findings by other laboratories demonstrate that the overall time course of autophosphorylation is misleading for three reasons:

1. Multiple sites are modified by autophosphorylation in each subunit, but only one site in each subunit is involved in the production of Ca^{2+}-independent kinase activity ($\alpha Thr_{286}/\beta Thr_{287}$).

2. Modification of this critical site reaches approximately 80% of its maximal stoichiometry within the first 5 sec of autophosphorylation in the presence of Ca^{2+}/calmodulin.

3. Only a few (3-5) phosphates in each holoenzyme need to be modified to exhibit the maximal production of Ca^{2+}-independent kinase activity.

The "threshold" level of phosphate incorporation required for the maximal production of Ca^{2+}-independent activity was estimated to be 3-5 phosphates per forebrain holoenzyme in our initial study (Miller and Kennedy, 1986). Lai *et al.* (1987) suggested that modification of as few as two of the twelve subunits may be sufficient. The results described in chapter 4 show that a significant amount of the initial phosphate incorporation is into a site on the β subunit (βThr_{382}) that is not required for the generation of Ca^{2+}-independent kinase activity. This suggests that modification of as few as 2-3 subunits in the holoenzyme may be a reasonable estimate for the threshold level of kinase modification. Given these results, it appears reasonable to conclude that functionally significant autophosphorylation of the kinase can occur in the cell in response to a prolonged elevation in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$).

Recent evidence in non-neuronal cells suggests that $[Ca^{2+}]_i$ may remain elevated for extended periods, or undergo oscillations lasting seconds to minutes after cellular stimulation (discussion and references in chapter 1). In addition, changes in $[Ca^{2+}]_i$ are often non-uniform, and there is evidence that Ca^{2+}-concentrations may remain high near the plasma membrane for extended periods (see Alkon and Rasmussen, 1988). The extension of these types of studies to neuronal cells may provide evidence for the occurrence of similar

calcium regulation in the brain.

Work in our laboratory (chapters 3 and 4), and by other groups, has shown that the regulation of kinase activity by autophosphorylation is reversible by protein phosphatases. We have used purified protein phosphatase 2A to demonstrate this reversal, and to correlate the phosphorylation state of specific sites with changes in kinase activity. We have also shown that endogenous protein phosphatases are present in the brain, and are capable of dephosphorylating the kinase and reversing the effect of phosphorylation on kinase activity. It will be important to characterize the neuronal protein phosphatases and determine their site specificity and regulatory properties.

Correlation of Changes in Kinase Activity with Extent of Phosphorylation.

Chapters 3, 4 and 5 of this thesis discuss in detail the correlation of changes in kinase activity with the phosphorylation of specific sites. The generation of Ca^{2+}-independent kinase activity is the result of Ca^{2+}-dependent autophosphorylation at $\alpha Thr_{286}/\beta Thr_{287}$ (chapter 4). The quantitative relationship between the extent of phosphorylation of these sites and the generation of Ca^{2+}-independent activity suggests an allosteric interaction between subunits within the holoenzyme. Work is still in progress on the correlation of Ca^{2+}-independent autophosphorylation sites and the loss of Ca^{2+}-stimulation of kinase activity.

Demonstration of the *In vivo* Phosphorylation and Regulation of the Type II Kinase.

Satisfaction of this requirement is critical in establishing the physiological significance of kinase regulation by autophosphorylation. Fortunately, there are a number of potentially viable approaches which may be useful in this area. The work reported in chapters 4 and 5 of this thesis provides several useful tools for analyzing autophosphorylation of the kinase *in vivo*. We have identified the sites involved in kinase regulation and the reverse phase-HPLC methods required to separate them have been developed. The ATP pool in intact cells (in tissue culture, acute brain slices, or cultured brain slices) can be labeled with ^{32}P by the addition of ^{32}P-PO_4 to the medium. The cells can then be homogenized directly into denaturing buffers (which will preserve the phosphorylation state of the kinase) and the kinase isolated by immunoprecipitation. Reverse-phase HPLC tryptic phosphopeptide mapping can then be used to compare the *in vitro* autophosphorylation sites with those found *in vivo*. A variety of pharmacological treatments can be applied immediately prior to homogenization to correlate the activity state of the cells with the phosphorylation state of the kinase. This approach has been used successfully to examine the phosphorylation sites of several of phosphoproteins *in vivo* (e.g., insulin receptors [White *et al.*, 1985,1988; Tornqvist *et al.*, 1988] and epidermal growth factor receptors [Downward *et al.*, 1984]).

Identification of the autophosphorylation sites may allow one to investigate the phosphorylation state of the kinase *in vivo* without the need to label

with $^{32}PO_4$. Peptides corresponding to the amino acid sequences surrounding α-Thr_{286} and β-Thr_{287} can be synthesized, phosphorylated *in vitro*, and used to generate antibodies specific for these phosphorylated sites. To be useful the antibodies generated would need to be specific for the phosphorylated state of the site and experience in this (S.S. Molloy, unpublished observations), and other systems suggests that this would be possible (Cohen *et al.*, 1987). These antibodies could then be used to assess the phosphorylation state of **specific** sites *in vivo* by the use of Western blots, well binding assays, or perhaps immunocytochemistry using fixed tissue.

The regulation of kinase **activity** *in vivo* is currently being studied in this laboratory in the hippocampal slice preparation (S.S. Molloy, unpublished results). The experimental approach involves the assay of crude homogenates of the hippocampal slices using exogenous synapsin I as a substrate. The Ca^{2+}-independent phosphorylation of Site II on synapsin I by the neuronal type II kinase is taken as a measure of the *in vivo* regulation of kinase activity. Care must be taken in these experiments to maintain the state of kinase phosphorylation during the homogenization and assays. This can be accomplished by the inclusion of phosphatase inhibitors (e.g., pyrophosphate) in the homogenization buffers. The preliminary results from these studies are very promising.

Correlation of the Extent of Kinase Phosphorylation *in vivo* with the Levels of Ca^{2+}/Calmodulin and Protein Phosphatase(s).

The limiting factor in satisfying this requirement is again our lack of understanding of the specifity and regulation of the physiologically relevant protein phosphatases. Protein phosphatases have not been extensively studied in the nervous system. For many years the perception has been that protein phosphatases are a relatively static component in the regulation of enzyme activity by phosphorylation/dephosphorylation. Regulation of kinase activity has generally been the focus of interest. Although protein phosphatases appear to exist in association with other protein components, only the isolated catalytic subunits are usually studied *in vitro*. Thus, the regulation of protein phosphatases *may* be an important factor *in vivo*.

Conclusion.

The short history of the study of neuronal type II CaM kinase has followed a very typical progression (see chapter 1 for appropriate references). It was originally characterized as an enzymatic activity in crude homogenates which had catalytic and regulatory properties suggesting physiological importance. The kinase was then purified and its biochemical and enzymatic properties were characterized extensively. Several groups have examined its tissue distribution, relative abundance, and subcellular localization using immunocytochemical techniques. The entire primary structure of the kinase has been deduced by molecular cloning techniques and work is in progress to determine its primary structure in *Drosophila*. The work described in this thesis (and by other groups) has determined that kinase activity is regulated in a complex fashion. These results have led to the proposal of intriguing hypotheses regarding a role for the kinase in the long-term regulation of cellular processes. Thus, the *preliminary* characterization of the kinase is largely complete.

Studies regarding the neuronal type II CaM kinase are entering a second phase and are branching into two directions. A detailed understanding of the biochemical basis of catalytic activity and regulation of the kinase is one of the two major goals. The second major goal is the determination of the role of the kinase in neuronal function. Work by Llinas and colleagues suggests that one possible role is the regulation of transmitter release (Llinas *et al.*, 1985). This

type of work is, in many ways, much more difficult than the prelimary characterization, but may be extremely helpful in understanding neuronal function.

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