A STUDY OF THE TYPE II CA^{2+}/CALMODULIN-DEPENDENT PROTEIN KINASE IN HIPPOCAMPAL NEURONS

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This thesis is dedicated to my wife, Sandie.

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Abstract

Characterization of the type II Ca^{2+}/calmodulin-dependent protein (CaM) kinase *in vitro* has revealed several intriguing physical and biochemical properties, including the induction of Ca^{2+}-independent activity by autophosphorylation. This thesis describes our attempts to determine the importance of autophosphorylation to the regulation of the kinase in hippocampal neurons. In order to study the type II CaM kinase in these neurons, we established long-term cultures of rat hippocampal slices. We used these cultures to address several questions regarding the phosphorylation of the CaM kinase in the intact neurons, namely: 1) is the CaM kinase phosphorylated in the cultures under basal conditions, 2) if so, is phosphate incorporated into the sites previously characterized *in vitro*, 3) can phosphorylation of the CaM kinase be modulated in the neurons. Incubation of slice cultures with radiolabeled phosphate *in situ* showed that both the α and β subunits of the kinase incorporate phosphate under basal conditions in intact neurons. Furthermore, HPLC analysis of tryptic fragments derived from α subunit radiolabeled in the cultures *in situ* revealed that the majority of phosphate was incorporated into Thr_{286} (the site which controls Ca^{2+}-independent activity *in vitro*). Measurements of Ca^{2+}-independent activity in homogenates showed that approximately one third of the kinase is autophosphorylated and constitutively active in the cultures. The proportion of Ca^{2+}-independent enzyme in the cultures decreased by 80-90% following removal of external Ca^{2+}. Application of the membrane permeant kinase inhibitors H7 and W7 also caused a substantial decrease in Ca^{2+}-independent kinase

activity, while the phosphatase inhibitor, okadaic acid, increased the proportion of Ca^{2+}-independent kinase. Therefore, the resting level of Ca^{2+}-independent CaM kinase apparently reflects a balance between continual Ca^{2+}-dependent autophosphorylation and dephosphorylation by phosphatases. Homogenates of rat forebrains and hippocampi also had substantial levels of Ca^{2+}-independent CaM kinase. These results suggest that the autophosphorylation mechanism acts to maintain a relatively high proportion of constitutively active kinase under conditions of low resting Ca^{2+} in neurons. This finding is in direct contrast to some models of kinase regulation in hippocampal neurons which predicted that the enzyme would only autophosphorylate following prolonged, synaptically driven increases in intracellular Ca^{2+}. Furthermore, these studies indicate that pharmacological agents could either up or down regulate the level of constitutively active CaM kinase locally, at or near the synapse, by affecting the rate of autophosphorylation or dephosphorylation.

Table of Contents

Chapter 1

Introduction

I. Second Messengers

General Characteristics

Cells are required to respond to a vast array of signals present in their environment. These extracellular signals include hormones and neurotransmitters. The vast majority of such signalling molecules are hydrophilic. This allows the signal molecules to disperse in the extracellular fluid to reach their target cells. Their hydrophilic nature, however, also prevents them from crossing the hydrophobic lipid bilayer to gain access to the cells. Systems have developed within target cells which allow the cells to respond to the presence of signal molecules in the extracellular fluid. These systems translate the presence of signal molecules at the cell membrane to changes in the concentration of intracellular molecules known as "second messengers."

Typically, this transformation occurs by the binding of the extracellular signal molecule to a receptor in the cell membrane. Binding of a signal molecule produces a conformational change in the receptor which then transmits the signal across the membrane. The mechanism by which the activated receptor translates ligand binding into a change in second messenger concentration varies. Mechanisms include changing the rate of second messenger synthesis or degradation, release from internal stores, and activation of channels that allow the second messenger to cross the membrane. The most widely studied second messengers include the cyclic nucleotides adenosine (3'-5')-cyclic monophosphate (cAMP) and guanosine (3'-5')-cyclic monophosphate (cGMP), calcium ion, 1,2-diacylglycerol (DAG), and D-

inositol 1,4,5-trisphosphate ($InsP_3$). Other examples are the metabolites of arachidonic acid (Piomelli and Greengard, 1990), and nitric oxide (Brendt and Synder, 1989 and 1990). It is likely that this list will grow as more sensitive assay techniques are developed.

Cyclic Nucleotides

The cyclic nucleotides, cAMP and cGMP, are formed from ATP and GTP by the action of adenylate and guanylate cyclases (Goldberg and Haddox, 1977; Rall, 1982). Phosphodiesterases hydrolyze the cyclic nucleotides to AMP and GMP. Receptor activated second messenger systems are known to control the level of the cyclic nucleotides by altering the activities of the cyclases and phosphodiesterases (reviewed in Robison *et al.*). These enzymes appear to have basal levels of activity which establish a resting concentration of the second messenger. When the concentration of messenger is up-regulated by activation of the cyclase, continued phosphodiesterase activity will eventually terminate the signal by returning the cyclic nucleotide concentration to its resting level. Changes in the expression of the catabolic or metabolic enzymes could shift the basal concentrations of the cyclic nucleotides. Such prolonged changes in second messenger concentration have been proposed to play a role in the regulation of neuronal activity.

In most cases, the effects of cAMP and cGMP are thought to be mediated by activation of the cAMP-dependent and cGMP-dependent protein kinases. Both of these kinases have broad substrate specificities and phosphorylate proteins at serine and/or threonine residues (reviewed in Beavo and Mumby, 1982; Goldberg and Haddox, 1977). Recently, studies of isolated ion channels from the visual and

olfactory transduction systems showed that cyclic nucleotides are capable of directly regulating these proteins (Fesenko *et al.*, 1985; Nakamura and Gold, 1987; Gold and Nakamura, 1987). Therefore, contrary to what was originally believed (Walsh *et al.*, 1968; Kuo and Greengard, 1969; Kuo and Greengard, 1970), not all effects of the cyclic nucleotides are mediated through protein phosphorylation. Activation of the cAMP-dependent or cGMP-dependent protein kinases, however, still appear to be the most common pathways for these second messenger systems.

Diacylglycerol (DAG) and inositol trisphosphate ($InsP_3$) are formed from the degradation of membrane inositol phospholipids by phospholipase C. As with the cyclic nucleotides, activated membrane receptors are capable of enhancing the production of the second messenger by stimulating the activity of specific enzymes, phospholipase C in this instance. The concentration of DAG is reduced in the cell by its reincorporation into newly synthesized phospholipids or its breakdown into arachidonic acid. The $InsP_3$ signal is terminated by hydrolysis of the 5'-phosphate to from $InsP_2$, which is generally believed to be inactive (Berridge and Irvine, 1984; Berridge, 1987). DAG acts via the stimulation of protein kinase C. When DAG binds to protein kinase C, it increases the sensitivity of the enzyme to Ca^{2+}, thereby facilitating its activation (Kishimoto *et al*, 1980; Takai *et al.*, 1979). Protein kinase C is similar to the cyclic nucleotide-dependent kinases in its ability to phosphorylate a broad range of substrate proteins (see below). $InsP_3$ appears to act by stimulating the release of Ca^{2+} from internal stores. This released calcium may act synergistically with the DAG to activate protein kinase C as well as other calcium dependent enzymes (see below).

The action of cyclic nucleotides, DAG, and $InsP_3$ through protein kinases demonstrates a common theme among second messenger systems. Many of the known actions of these systems ultimately rely on their ability to stimulate the phosphorylation of a critical substrate, thereby altering its activity. This cascade of regulatory events will be described in more detail in the discussions of the individual kinases. The ability of $InsP_3$ to stimulate Ca^{2+} release demonstrates that these second messenger systems are often interactive at various levels. This second common characteristic will also become more apparent in the descriptions of kinase substrates.

Calcium as a Second Messenger

Ca^{2+} is distinct from the previously described second messengers in that it is an ion that is always present inside and outside the cell. The level of Ca^{2+} clearly cannot be regulated by changes in its production or degradation, as occurs with other second messengers. Instead, the resting level of free Ca^{2+} in the cytosol is maintained at a very low level (10-100 nM) by systems that actively exclude or sequester the ion (Carafoli, 1987). Ca^{2+} entry into cells is restricted by the functional selectivity filters present in most membrane channels (Hille, 1971). The Ca^{2+} that does enter is either pumped back across the membrane by ion exchange mechanisms or sequestered within mitochondria, endoplasmic reticulum, and possibly organelles called calciosomes (Volpe *et al.*, 1988). In addition, muscle cells sequester Ca^{2+} in a specialized structure called sarcoplasmic reticulum.

There are several mechanisms by which extracellular signals such as hormones and neurotransmitters increase the concentration of free Ca^{2+} in the cytosol

(reviewed in Mayer and Miller, 1990). Voltage and agonist gated Ca^{2+} channels are common in neurons (reviewed in Miller, 1987). $InsP_3$ releases Ca^{2+} from some internal stores (endoplasmic reticulum and perhaps calciosomes) while there appears to be Ca^{2+} stimulated release from mitochondria. Muscle cells release Ca^{2+} from the sarcoplasmic reticulum via voltage gated channels. Activation of these Ca^{2+} release mechanisms can raise the cytosolic concentration of free Ca^{2+} to 1 μM or more (Mitchell *et al.*, 1981; Berridge, 1984; Murphy and Miller, 1988). These increases in cytosolic free Ca^{2+} are generally brief. They can, however, be maintained in some instances (Kojima *et al.*, 1986; Takuwa *et al.*, 1987). The dynamic nature of the mechanisms that control the level of cytosolic Ca^{2+} is apparent in some cell types that undergo oscillations in intracellular Ca^{2+} concentration lasting seconds to minutes (Rapp and Berridge, 1981; Schegel *et al.*, 1987; Wilson *et al.*, 1987; Woods *et al.*, 1987; Ambler *et al.*, 1988; Berridge *et al.*, 1988; Meyer and Stryer, 1988).

While only a few target proteins have been identified for the cyclic nucleotides and DAG, Ca^{2+} interacts with a host of regulatory molecules. These include the adenylate and guanylate cyclases (Brostrom *et al.*, 1975; Cheung *et al.*, 1975; Kakiuchi *et al.*, 1981) as well as a cyclic-nucleotide phosphodiesterase (Teo and Wang, 1973; Kincaid and Vaughan, 1986). Other proteins regulated by Ca^{2+} are phospholipase A_2 (Wong and Cheung, 1979), protein phosphatase 2B (Yang *et al.*, 1982; Stewart *et al.*, 1983) and the protease calpain (Pontremoli and Melloni, 1986). Like the other second messengers, Ca^{2+} also activates protein kinases (see below). As with the cyclic nucleotides, Ca^{2+} is capable of directly affecting the gating of ion channels (Meech, 1978). This list demonstrates both the interdependence of the

various second messenger systems and their importance in controlling the phosphorylation and dephosphorylation of cellular proteins.

Many of the actions of calcium are mediated through calmodulin. Although originally described as an activator of a phosphodiesterase from bovine brain (Cheung, 1967), calmodulin was later found to be a Ca^{2+} binding protein (Teo and Wang, 1973). It is a single polypeptide of 16.7 kD that binds 4 Ca^{2+} ions in its fully complexed form (Watterson *et al.*, 1980; Lin *et al.*, 1974; Wang, 1976). The wide distribution of calmodulin in almost all tissues and eukaryotic organisms, combined with a highly conserved primary structure, indicates its importance in many Ca^{2+}-dependent regulatory processes (Klee *et al.*, 1980). The binding of Ca^{2+} induces a conformational change in calmodulin which reveals a binding site for other molecules (Klee *et al.*, 1980). It is the association with this complexed form of calmodulin that alters the activity of many Ca^{2+}-regulated enzymes.

Calmodulin regulates the activity of a broad array of enzymes involved in numerous physiological processes. Such calmodulin-regulated enzymes include both cyclases (Birnbaumer, 1973; Nagao *et al.*, 1979) and a phosphodiesterase (Kakiuchi and Yamazaki, 1979) responsible for controlling the levels of cAMP and cGMP. Calmodulin also regulates phospholipase A_2 (Wong and Cheung, 1979), thereby affecting the production of arachidonic acid related second messengers.

Calmodulin not only regulates enzymes involved in the control of second messenger concentrations, it can also activate enzymes that phosphorylate and dephosphorylate proteins. Such proteins include calcineurin (phosphatase 2B; Yang *et al.*, 1982), and several kinases that are discussed below.

A second Ca^{2+} effector is protein kinase C. Protein kinase C is a polypeptide of 77-83 kD that has been purified from a number of tissues (Kikkawa *et al.*, 1982; Wise *et al.*, 1982; Walsh *et al.*, 1984; Woodgett and Hunter, 1987; Azhar *et al.*, 1987). The cloning and sequencing of complementary cDNAs from rat (Knopf *et al.*, 1986) and bovine (Parker *et al.*, 1986) mRNA libraries revealed the presence of several forms of the kinase. These original studies identified three isozymes of the enzyme, all of which were the products of different genes. The reason for the multiple forms of protein kinase C is not understood. However, immunocytochemical studies using monoclonal antibodies suggest that there may be regional differences in their expression within the brain (Mochly-Rosen *et al.*, 1987).

The activity of protein kinase C is controlled by three regulatory molecules: phospholipid, DAG, and Ca^{2+}. Phosphatidyl serine is the only phospholipid capable of activating the enzyme, although other lipids can modulate its activity (Takai *et al.*,1979; Kaibuchi *et al.*, 1981). The kinase can be activated *in vitro* by high concentrations of Ca^{2+} in the presence of phosphatidyl serine. DAG increases the affinity of the kinase for Ca^{2+}, allowing its activation at physiologic concentrations of the ion (Kishimoto *et al.*, 1980). It has been reported that fatty acids such as oleic and arachidonic acid can activate protein kinase C in the absence of Ca^{2+} and phospholipid (Murakami *et al.*, 1986). Also, the kinase can be activated by proteolysis, which produces a Ca^{2+}-independent form of the enzyme (Inoue *et al.*, 1977; Kishimoto *et al.*, 1983). It is not clear how these alternative mechanisms of activation function *in vivo*, however, a calpain mediated activation of protein kinase C by proteolysis has been reported in intact, stimulated neutrophils (Melloni *et al.*,

1986). Interestingly, this latter mechanism of activation still requires Ca^{2+} to stimulate the protease. The tumor promoting phorbol esters are also capable of directly activating the kinase, both *in vitro* and *in vivo* (Kikkawa and Nishizuka, 1986). This suggests a possible role for the enzyme in tumor formation (Nishizuka, 1984).

Once activated, protein kinase C phosphorylates a variety of proteins at serine and/or threonine residues. Substrates for the kinase *in vitro* include membrane proteins, membrane receptors, cytoskeletal elements and soluble enzymes (reviewed in Kikkawa and Nishizuka, 1986). Membrane proteins and receptors may be important substrates *in vivo* since the soluble enzyme translocates to the plasma membrane upon activation (Wolf *et al.*, 1985; May *et al.*, 1985). Like many kinases, protein kinase C will autophosphorylate following activation, however, the function of this autophosphorylation is not known (Kikkawa *et al.*, 1982; Huang *et al.*, 1986; Mochly-Rosen and Koshland, 1987; Newton and Koshland, 1987).

Ca^{2+}/Calmodulin-Regulated Kinases

The Ca^{2+}/calmodulin-regulated kinases have a variety of forms. Some of these appear to serve specific functional roles in specialized cell types, while others may be involved in a host of regulatory processes. Unlike the cAMP-dependent protein kinase, the Ca^{2+}-regulated kinases often have restricted patterns of expression. This tissue specific-expression, combined with their importance to the physiology of the cells in which they are expressed, justifies a detailed description of these enzymes.

Phosphorylase Kinase

Phosphorylase kinase has been purified from both muscle (Cohen, 1973;

Hayakawa *et al.*, 1973; Cooper *et al.*, 1980; Pocinwong *et al.*, 1981) and liver (Chrisman *et al.*, 1982) of several species, however, it has been most thoroughly studied in rabbit skeletal muscle. In this tissue, phosphorylase kinase is composed of four subunits (α, β, δ and γ) assembled into a tetramer of 1.2×10^6 kD (Cohen, 1973; Hayakawa *et al.*, 1973; Carlson *et al.*, 1979). There appears to be muscle fiber specific forms of the holoenzyme, since fast-twitch and slow-twitch muscles express α subunits of distinct molecular weights (Jennissen and Heilmeyer, 1974; Burchell *et al.*, 1976).

The principle role of phosphorylase kinase in muscle and liver is the regulation of glycogen metabolism. The kinase stimulates glycogenolysis by converting phosphorylase b to its active, phosphorylated form, phosphorylase a. Phosphorylase kinase is activated in cells by two mechanisms. Stimulation by hormonal (α-adrenergic) or neuronal inputs causes an increase in intracellular Ca^{2+} which will activate the enzyme. Alternatively, β-adrenergic inputs will stimulate adenylate cyclase, increase the level of cAMP and thereby enhance cAMP-dependent kinase activity. The cAMP-dependent kinase is able to activate phosphorylase kinase by phosphorylation. This dual regulation emphasizes two common themes in this discussion of second messenger systems, namely the interdependence of the various systems, and the regulation of kinase activities via phosphorylation of the enzymes themselves.

A considerable effort has been made to determine the functions of the individual phosphorylase kinase subunits. There is strong evidence that indicates that γ is the catalytic subunit while the others fill regulatory roles. Isolated γ

subunit retains a substantial amount of catalytic activity (Kee and Graves, 1986). Also, sequence analysis reveals extensive homology with other known protein kinases (Reimann *et al.*, 1984; da Cruz e Silva and Cohen, 1987; Bender and Emerson, 1987). The sequence of the δ subunit is identical to calmodulin (Grand *et al.*, 1981; Crabb and Heilmeyer, 1984) which may explain the activation of the kinase by Ca^{2+} in the absence of exogenous calmodulin. Phosphorylase kinase can also be activated by added Ca^{2+}/calmodulin or troponin C (DePaoli-Roach *et al.*, 1979; Cohen, 1980c). This regulation may occur by the binding of calmodulin to the α and β subunits, thereby relieving their inhibition of the catalytic δ subunit (Paudel and Carlson, 1987).

Substrates. Once activated, the kinase will phosphorylate a number of proteins besides phosphorylase *in vitro*. These substrates include glycogen synthase (Roach *et al.*, 1978), troponin T (Perry and Stull, 1974), troponin I (Stull *et al.*, 1972), histone H1 (Tabuchi *et al.*, 1981), myelin basic protein (Grand and Perry, 1979), and components of the sarcoplasmic reticulum and cardiac sarcolemma (Schwartz *et al.*, 1976,; St. Louis *et al.*, 1977). The importance of most of these proteins as substrates *in vivo* is not known.

Phosphorylation. An important aspect of the regulation of phosphorylase kinase activity is modulation by phosphorylation. The skeletal muscle form of the enzyme is phosphorylated by several kinases, including the cAMP-dependent protein kinase, the cGMP-dependent protein kinase (Lincoln and Corbin, 1977; Khoo *et al.*, 1977), glycogen synthase kinase I (Singh *et al.*, 1982) and protein kinase C (Inoue *et al.*, 1977; Kishimoto *et al.*, 1977; Yamamoto *et al.*, 1978). In addition, phosphorylase

kinase is capable of Ca^{2+}-dependent autophosphorylation. To date, only autophosphorylation and phosphorylation by the cAMP-dependent protein kinase have apparent physiological roles.

The cAMP-dependent protein kinase phosphorylates the α and β subunits of phosphorylase kinase (Hayakawa *et al.*, 1973 a and b; Cohen, 1980b) *in vitro*, resulting in activation of the enzyme. The β subunit is rapidly phosphorylated to a final stoichiometry of 0.5 to 2 moles phosphate/mole subunit, depending on reaction conditions (Hayakawa *et al.*, 1973; Malenick and Fischer, 1979; Pickett-Gies and Walsh, 1985; Singh and Wang, 1977). Phosphorylation of the α subunit lags behind that of β, yet still reaches stoichiometries of 1 mole phosphate/mole subunit.

The majority of studies indicate that the phosphorylation of both α and β subunits within a holoenzyme is important for activation of phosphorylase kinase (Singh and Wang, 1977; Cooper *et al.*, 1981; Sul and Walsh, 1982; Sul *et al.*, 1982; Pickett-Gies and Walsh, 1985). The principle effect of the phosphorylation appears to be a lowered K_m for phosphorylase b. However, other effects such as loss of stimulation by exogenous Ca^{2+}/calmodulin, and an increased K_a for Ca^{2+} have been reported (Cohen, 1980a; Cooper *et al.*, 1980). The initial sites phosphorylated on α and β *in vitro* have been sequenced from isolated phosphopeptides (Yeamen *et al.*, 1977) and appear to be the same serine residue. Previous studies had identified these serines as those phosphorylated *in vivo* during epinepherine-stimulated glycogenolysis (Yeamen and Cohen, 1975). These data suggest that activation of phosphorylase kinase by cAMP-dependent phosphorylation is part of some cellular regulatory pathways.

Autophosphorylation. Phosphorylase kinase is also capable of autophosphorylation on the α and β subunits (Walsh *et al.*, 1971). Although the maximum stoichiometry of autophosphorylation is higher than for cAMP-dependent phosphorylation (6 moles phosphate/mole α, 2 moles phosphate/mole β; Singh and Wang, 1977), it requires much higher concentrations of ATP. The observation that prior phosphorylation with the cAMP-dependent protein kinase did not alter the stoichiometry of autophosphorylation suggests that a different set of sites are involved. However, the work of King *et al.* (1983) indicates that they recognize the same primary site. While autophosphorylation apparently results from the activity of the catalytic γ subunit, it is not clear if this occurs by an inter- or intramolecular mechanism (Chan and Graves, 1982a, 1982b).

Autophosphorylation of phosphorylase kinase can be stimulated *in vitro* by a number of molecules. These include glycogen (DeLange *et al.*, 1968), peptide analogues of phosphorylase b (Carlson and Graves, 1976), heparin (Erdodi *et al.*, 1984) and organic solvents (Singh and Wang, 1979). On the other hand, several glycogen metabolites are capable of inhibiting autophosphorylation (Wang *et al.*, 1976; Krebs *et al.*, 1964). These regulatory properties suggest that autophosphorylation of the kinase may play an important role *in vivo*. However, autophosphorylation in intact cells, in response to a physiologic stimulus, has not been demonstrated.

Myosin Light Chain Kinase

Distribution and Structure. A second Ca^{2+}-dependent kinase, myosin light chain kinase, has been purified from smooth muscle and skeletal muscle in both mammals

(Pires and Perry, 1977; Yazawa and Yagi, 1978; Edelman and Krebs, 1982; Mayr and Heilmeyer, 1983; Vallet *et al.*, 1981; Nishikawa *et al.*, 1984; Walsh *et al.*, 1982a; Higashi *et al.*, 1983) and birds (Nunnally *et al.*, 1985; Dabrowska *et al.*, 1977; Adelstein and Klee, 1981; Uchiwa *et al.*, 1982; Walsh *et al.*, 1982b). This rather ubiquitous enzyme has also been found in bovine brain (Dabrowska and Hartshorne, 1978; Hathaway *et al.*, 1981) and the mammalian cell culture line BHK (Yerna *et al.*, 1979). The presence of myosin light chain kinase in striated muscle of the invertebrate *Limulus* (Sellers and Harvey, 1984) indicates that the enzyme may have originated early in evolution.

Myosin light chain kinase is a single polypeptide the size of which varies between tissues. Size estimates for the mammalian skeletal-muscle form of the kinase range from 77-94 kD (Pires and Perry, 1977; Yazawa and Yagi, 1978; Edelman and Krebs, 1982; Mayr and Heilmeyer, 1983). The smooth-muscle kinase appears to be larger, ranging from 130-160 kD in both mammals and birds (Vallet *et al.*, 1981; Nishikawa *et al.*, 1984; Walsh *et al.*, 1982; Higashi *et al.*, 1983; Dabrowska *et al.*, 1977; Adelstein and Klee, 1981; Uchiwa *et al.*, 1982; Walsh *et al.*, 1982 a and b). In nonmuscle cells the kinase is between 105 and 130 kD (Dabrowska and Hartshorne, 1978; Hathway and Adelstein, 1979; Hathaway *et al.*, 1981). Several studies using antibodies to determine the true molecular weight of the kinase indicate that most of the observed variability results from the presence of different isozymes (reviewed in Stull *et al.*, 1986; Kennedy *et al.*, 1987.

The activation of myosin light chain kinase requires the binding of Ca^{2+}/calmodulin in a 1:1 ratio. Unlike phosphorylase kinase, the myosin light chain

kinase does not have separate regulatory and catalytic subunits. However, the catalytic and calmodulin binding domains of the protein can be separated by partial proteolysis (Stull *et al.*, 1986). This produces a form of the enzyme which is constituitively active and independent of calcium, much like the purified γ subunit of phosphorylase kinase. These observations indicate that the myosin light chain kinase incorporates several domains (catalytic, regulatory and calmodulin binding) which are all localized to separate subunits in phosphorylase kinase. The incorporation of these domains into a single large polypeptide can be seen in other Ca^{2+}-dependent kinases (see type II CaM kinase below). Once activated, the kinase will only phosphorylate light chains at a significant rate *in vitro* (Stull *et al.*, 1986).

Phosphorylation. Several other kinases are capable of phosphorylating myosin light chain kinase to appreciable stoichiometries. The light chain kinase from turkey gizzard is phosphorylated at two sites by the cAMP-dependent protein kinase. One site (site 2) is only phosphorylated in the absence of bound Ca^{2+}/calmodulin while the other (site 1) is phosphorylated in the presence or absence of Ca^{2+} (Conti and Adelstein, 1981). Phosphorylation at site 2 results in a 10 to 20-fold reduction in the affinity of the kinase for calmodulin. This reduction in affinity is reversible by treatment with protein phosphatase *in vitro* (Conti and Adelstein, 1981).

Studies using myosin light chain kinase from the smooth muscle of several other species have obtained the same results, suggesting that regulation by cAMP-dependent phosphorylation is a common property of the enzyme (Vallet *et al.*, 1981; Bhalla *et al.*, 1982; Walsh *et al.*, 1982; Higashi *et al.*, 1983; Nishikawa *et al.*, 1984). Experiments using tissues other than smooth muscle are less clear. Myosin

light chain kinase from platelets is reportedly regulated by phosphorylation with the cAMP-dependent protein kinase (Nishikawa *et al.*, 1984), whereas phosphorylation of the enzyme from rabbit skeletal muscle has no apparent effect on activity (Edelman and Krebs, 1982). Conflicting data have been reported regarding the phosphorylation of the cardiac muscle form of the kinase (Walsh and Guilleux, 1981; Rappaport and Adelstein, 1980). The myosin light chain kinase purified from Limulus is apparently not a substrate for the cAMP-dependent protein kinase.

There are reports that myosin light chain kinase is a substrate for protein kinases other than the cAMP-dependent enzyme. Two groups observed the phosphorylation of smooth muscle myosin light chain kinase by activated protein kinase C in the absence of Ca^{2+}/calmodulin (Ikebe *et al.*, 1985; Nishikawa *et al.*, 1985). This phosphorylation produced an increase in the K_a for calmodulin. Both tracheal smooth muscle and human platelet myosin light chain kinase were phosphorylated by cGMP-dependent protein kinase but the K_a for calmodulin was only altered in the platelet enzyme.

Autophosphorylation of myosin light chain kinase has also been observed, however, only the bovine and canine cardiac muscle isozymes incorporate stoichiometrically significant amounts of phosphate at a reasonable rate (Wolf and Hofmann, 1980; Rappaport and Adelstein, 1980). No modification of enzyme activity was reported. These results suggest that despite the similarity of its isoforms, phosphorylation of myosin light chain and its effect on activity is highly tissue specific.

Function. Myosin light chain kinase appears to have a highly defined functional

role *in vivo*. As mentioned above, myosin light chain is the only known substrate of the kinase *in vitro*. This substrate specificity fits with a role for the enzyme in controlling the activation of actin/myosin systems in a variety of cell types. Phosphorylation of light chains by the kinase is an absolute requirement for actin activation of the Mg-dependent myosin ATPase activity in nonmuscle and smooth muscle cells (Adelstein and Eisenberg, 1980). In skeletal and cardiac muscle, however, the phosphorylation of light chains is not a requirement for contraction but may only play a modulatory role (reviewed in Marston, 1982; Kamm and Stull, 1985).

The results of phosphorylation studies suggest that the myosin light chain regulatory system may be modulated by various second messenger pathways acting on the kinase in a tissue specific manner. Myosin light chain kinase is phosphorylated in some nonmuscle cells *in vivo* (Bourguignon *et al.*, 1982), and is reportedly phosphorylated in smooth muscle in response to forskolin induced increases in cAMP. How the phosphorylation of myosin light chain kinase affects the actin/myosin systems in these cells is not known.

Neuronal Ca^{2+}/Calmodulin-Dependent Kinases

Several Ca^{2+}-dependent kinases have been identified in mammalian brain in addition to protein kinase C and myosin light chain kinase. One such kinase, neuronal Ca^{2+}/calmodulin dependent protein kinase III was purified based on its ability to phosphorylate elongation factor 2 (EF-2) (Nairn *et al.*, 1985; Ryazanov, 1987; Nairn and Palfrey, 1987). The subunit composition was not reported in the original study of this enzyme. However, it appears to have a broad tissue

distribution and a very restricted substrate specificity. Only EF-2 was phosphorylated among a wide range of proteins.

Neuronal Ca^{2+}/calmodulin-dependent protein kinase I has been purified from bovine brain (Nairn and Greengard, 1987). The enzyme appears to be a monomer, however, the purified kinase is composed of two major and one minor polypeptide of 37, 39, and 42 kD respectively. These peptides all bind ATP (as demonstrated by photoaffinity labeling) and calmodulin, and also autophosphorylate. This suggests that the smaller polypeptides may have been generated by limited proteolysis of the larger protein. The polypeptides could, however, be isoforms of the same enzyme. Although the kinase is most highly concentrated in brain, lower levels were detected in pancreas, spleen, adrenal gland, and lung. Very low levels were detected in skeletal muscle, kidney, heart, and liver.

Neuronal Ca^{2+}/calmodulin-dependent kinase I was originally detected and partially purified based on its ability to phosphorylate the synaptic vesicle-associated protein synapsin I. Synapsin I proved to be the best substrate for the kinase *in vitro* and is phosphorylated at the same site recognized by the cAMP-dependent protein kinase. The enzyme will also phosphorylate smooth muscle myosin light chain and the brain specific protein, protein III (Browning *et al.*, 1987). The phosphorylation of synapsin I by the type I CaM kinase can affect the association of synaptic vesicles with the cytoskeleton. Therefore, the enzyme may be important in transport and/or localization of vesicles to the presynaptic terminal.

Type II CaM Kinase

Distribution and Structure. A third neuronal Ca^{2+}-dependent kinase, the type II

CaM kinase, has received considerably more attention than the others. Type II CaM kinase has been purified from mammalian brain by several groups (Bennett *et al.*, 1983; Goldenring *et al.*, 1983; McGuinness *et al.*, 1983; Yamauchi and Fujisawa, 1983; Kuret and Schulman, 1984; Sahyoun *et al.*, 1985; Vallano, 1988). The kinase makes up as much as 1% of total protein in some brain regions but is undetectable in many other tissues (Erondu and Kennedy, 1985). Several studies show that expression of the mRNA for the α and β subunits of the kinase is restricted to brain tissue (Hanley *et al.*, 1987; Lin *et al.*, 1987; Bulleit *et al.*, 1988; Tobimatsu and Fujisawa, 1989). Analyses of the distribution of the kinase (Erondu and Kennedy, 1985) and its mRNAs (Bulleit *et al.*, 1988; Burgin *et al.*, 1990) within the brain reveal regional differences in expression of the enzyme as a whole, and its component subunits (see below). Immunocytochemical studies indicate that the kinase is localized throughout the cytosol of neurons in several regions of the brain (Ouimet *et al.*, 1984; Erondu and Kennedy, 1985).

The holoenzyme is reportedly between 460 and 650 kD and is composed of α (50 kD) and β/β' (60/58 kD) subunits. The ratio of these subunits found in the holoenzyme complex depends on the brain region in which it is expressed. As isolated from forebrain, the kinase has a 3:1 ratio of α to β/β' subunits (Bennett and Kennedy, 1983) while kinase purified from the cerebellum has a subunit ratio of 1:4 (McGuinness *et al.*, 1985; Miller and Kennedy, 1985). An additional form of the kinase, thought to be from the pons/medulla, has been purified by Mark Bennet in our laboratory and has a subunit ratio of 1:1. The importance of these regional differences in subunit expression is not yet clear. The kinase in the forebrain

appears to be evenly distributed between soluble and particulate fractions (Kennedy *et al.*, 1983b), while 85% of the enzyme is particulate in the cerebellum (McGuinness *et al.*, 1985; Miller and Kennedy, 1985). This disparity in the subcellular distribution of the kinase may reflect differences in the ability of the subunits to associate with cytoskeletal elements (see Ngozi Erondu, Caltech graduate thesis).

The primary sequences of the α, β and β' subunits have been obtained using cDNAs isolated from rat brain libraries (Bennett and Kennedy, 1987; Hanley *et al.*, 1987; Lin *et al.*, 1987; Bulleit *et al.*, 1988). The subunits are highly homologous in the amino-terminal portion of the proteins (91% identity at amino acid level) but less so in the carboxy-terminal half (76%) (Bulleit *et al.*, 1988). The amino-terminal half of each protein contains the kinase domain, and the calmodulin and ATP binding domains (Bulleit *et al.*, 1988). This region also has the highest level of homology to other protein kinases. The primary difference between these subunits is the absence of two amino acid segments from α, and one from β', compared to the β subunit.

Southern blot analysis indicates that the α and β subunits originate from two distinct genes. RNAse protection experiments, however, suggest that the β' subunit is derived from β by alternative splicing of mRNA (Bulleit *et al.*, 1988). Molecular cloning by homology has produced cDNA clones for two additional subunits called γ (Tobimatsu *et al.*, 1988) and δ (Tobimatsu and Fujisawa, 1989). Although the γ and δ subunits share considerable sequence homology with α and β/β', they are expressed in a wide range of tissues (Tobimatsu *et al.*, 1989). The mRNAs coding

for the γ and δ subunits are smaller than those for α and β and may have been the cross-reacting bands originally observed in RNA blots probed with α and β cDNA (Bulleit *et al.*, 1988). The importance of these subunits to the neuronal CaM kinase is unclear. They seem likely to form the isozymes present in other tissues.

Isozymes. The various isozymes of the CaM kinase and related enzymes form a family of broad-specificity Ca^{2+}-dependent kinases (reviewed in Stull *et al.*, 1986; Kennedy *et al.*, 1987). These isoforms exist as large holoenzymes consisting of multiple 50-60 kD subunits, all of which contain both catalytic and calmodulin binding domains. Isozymes have been purified from a number of tissues other than brain, including mammalian pancreas (Gorelick *et al.*, 1983; Cohn *et al.*, 1984), heart (Palfrey, 1984), skeletal muscle (Sato *et al.*, 1988), cultured astrocytes (Bronstein *et al.*, 1988a), and retina (Bronstein *et al.*, 1988b). In addition, isozymes have been described in avian erythrocytes (Palfrey *et al.*, 1984), *Aplysia* (De Riemer *et al.*, 1984; Saitoh and Schwartz, 1985), and squid (Bass *et al.*, 1987).

A highly related enzyme, glycogen synthase kinase, is found in liver (Ahmad *et al.*, 1982; Payne *et al.*, 1983) and skeletal muscle (Woodgett *et al.*, 1983). This kinase helps control the rate of glycogen breakdown by phosphorylating, and thus inactivating, glycogen synthase. These observations indicate that forms of the type II CaM kinase are present in a variety of tissues and species where they may have distinct physiological roles.

Substrates. The neuronal type II CaM kinase was first detected and partially purified on the basis of its Ca^{2+}-dependent phosphorylation of synapsin I (Kennedy and Greengard, 1981). The site phosphorylated on synapsin I (site 2) is distinct

from that recognized by the cAMP-dependent and Ca^{2+}-dependent type I kinases (site 1). Several other proteins are good substrates for the CaM kinase *in vitro*. These include tryptophan hydroxylase (Yamauchi and Fujisawa, 1983), smooth-muscle myosin light chain (Fukunaga *et al.*, 1982; Bennett *et al.*, 1983; McGuiness *et al.*, 1983), casein (Kuret and Schulman, 1984), myelin basic protein (Goldenring *et al.*, 1983), and the cytoskeletal proteins tubulin (Goldenring *et al.*, 1983) and microtubule associated protein 2 (MAP-2) (Bennett *et al.*, 1983). CaM kinase is able to phosphorylate several other proteins at lower rates (reviewed in Stull *et al.*, 1986). This list demonstrates one of the distinguishing characteristics of the type II CaM kinase, namely its broad substrate specificity.

Autophosphorylation. In addition to the long list of substrate proteins, the type II CaM kinase will rapidly autophosphorylate when activated by Ca^{2+}/calmodulin. Initially, autophosphorylation was thought to have no effect on enzyme activity (Bennett *et al.*, 1983; McGuiness *et al.*, 1983; Woodgett *et al.*, 1983). More recent studies have shown that autophosphorylation actually regulates kinase activity in a complex manner (Miller and Kennedy, 1986; Lai *et al.*, 1986; Schworer *et al.*, 1986). Autophosphorylation in the presence of Ca^{2+} results in the production of a substantial Ca^{2+}-independent activity following incorporation of approximately 3 phosphates/holoenzyme complex. The measured level of Ca^{2+}-independent activity depends upon the assay conditions and substrate but ranges from 20-80% of the total activity in the presence of Ca^{2+}. Continued autophosphorylation in the absence of Ca^{2+} results in the complete loss of stimulation by Ca^{2+}/calmodulin (Hashimoto *et al.*, 1987). Therefore, autophosphorylation can convert an enzyme that is completely

Ca^{2+}-dependent into one that is completely autonomous. Our attempts to determine the role, if any, of this regulation *in vivo* constitute a major part of this thesis.

This regulation of CaM kinase activity by autophosphorylation has recently been interpreted as an inactivation of an autoinhibitory domain within the enzyme (Shoemaker *et al.*, 1990; reviewed in Solderling, 1990). Inactivation of the autoinhibitory domain normally occurs by virtue of calmodulin binding in these Ca^{2+}-dependent enzymes. Phosphorylase kinase may represent a special case, where the autoinhibitory domain is contained in a separate subunit(s). Such inactivation of an autoinhibitory domain may also occur in the generation of active, Ca^{2+}-independent proteolytic fragments from the CaM kinase (Levine and Sahyon, 1987; Kwiatowski and King, 1989), and protein kinase C.

Several of the serine and threonine sites filled during autophosphorylation were identified by sequence determination of isolated tryptic peptides (Miller *et al.*, 1988; Patton *et al.*, 1990). Kinetic analyses and dephosphorylation studies of these sites indicated that phosphorylation of $Thr_{286/287}$ (residue 286 in the α subunit, and 287 in the β subunit) produces Ca^{2+}-independent activity while phosphorylation of $Ser_{305/306}$ eliminates stimulation by Ca^{2+} (Miller *et al.*, 1988; Schworer *et al.*, 1988; Theil *et al.*, 1988; Patton *et al.*, 1990). The importance of $Thr_{286/287}$ to the regulation of kinase activity was further demonstrated by the results of altering this site in the α subunit (Fong *et al.*, 1989; Hanson *et al.*, 1989; Mike Ito, personal communication). Converting the threonine to alanine eliminated the regulation of activity by autophosphorylation, as predicted, while the conversion to aspartate produced a basal level of Ca^{2+}-independent activity which was not increased by

autophosphorylation. This characterization of the autophosphorylation sites critical for regulating enzyme activity provided the direction and motivation for many of the experiments reported in the following chapters of this thesis.

While the characteristics of the CaM kinase have been extensively studied *in vitro* (reviewed in Colbran *et al.*, 1990), relatively little is known about its actual function *in vivo*. Phosphorylation of tyrosine hydroxylase, the rate limiting enzyme in catecholamine biosynthesis, by the CaM kinase alters the ability of an activator protein to stimulate the enzyme (Atkinson *et al.*, 1987). This effect may underlie the activation of tyrosine hydroxylase observed in depolarized neural tissue (El Mestikawy *et al.*, 1983). The CaM kinase can bind to cytoskeletal elements (Saitoh and Schwartz, 1985; Ohta *et al.*, 1986; Erondu, graduate thesis) and affect their assembly *in vitro* via phosphorylation. Such regulation, however, has not been demonstrated *in vivo*.

The association of the CaM kinase with specialized structures known as postsynaptic densities (PSDs) has lead to discussions of the importance of the kinase in the regulation of synaptic activity. PSDs are electron-dense structures found underlying the postsynaptic membrane at both excitatory and inhibitory central nervous system synapses (Landis and Reese, 1974; Shepherd, 1974; Siekevitz, 1981; Carlin *et al.*, 1980). Biochemical methods have been used to isolate these structures from lysed synaptosomes. When analyzed by electron microscopy (Matus, 1981), isolated PSDs appear to be composed of subunits of the same dimensions as kinase holoenzymes (as estimated by their hydrodynamic properties) (Bennett *et al.*, 1983; Kennedy *et al.*, 1983) . The biochemical analysis of PSDs isolated from cerebral

tissue showed that the major protein component of these structures is the α subunit of CaM kinase (Kennedy *et al.*, 1983; Kelly *et al.*, 1984; Goldenring *et al.*, 1984). Quantitative radioimmunoassays indicate that approximately 20% of the protein in PSDs from forebrain is CaM kinase, however, the kinase constitutes only 1-2% of the protein in cerebellar PSDs. Immunocytochemical studies have also demonstrated that the α subunit of the kinase is concentrated in PSDs isolated from rat forebrain (Kennedy *et al.*, 1990).

The localization of the kinase within the PSD brings it in close proximity to several potential substrates, including cytoskeletal elements (Carlin *et al.*, 1983; Bloomberg *et al.*, 1977; Kelly and Cotman, 1978), glutamate receptors (Fagg and Matus, 1984), GABA receptors (Wong and Hornig, 1977), Ca^{2+}-activated K^+ channels (Wu *et al.*, 1985) and several unidentified glycoproteins (Gurd, 1977; Kelly and Cotman, 1977; Gurd, 1980; Gordon-Weeks and Harding, 1983). In addition, elements of the cyclic-nucleotide-dependent phosphorylation system may also be enriched in the PSD (Florendo *et al.*, 1971; Bloom *et al.*, 1979; DeBlas *et al.*, 1979; Wood *et al.*, 1980).

The position of the kinase in the PSD may allow it to respond to local increases in Ca^{2+} concentration induced by activation of ligand or voltage gated channels in the synaptic membrane. The kinase could then modify the response properties and/or ultrastructure of the postsynaptic cell by phosphorylating any number of substrate proteins. The regulation of CaM kinase activity by autophosphorylation has prompted some authors to consider the enzyme as a molecular switch, which, by virtue of its localization within the PSD, may control

long-term changes in synaptic efficacy. This possibility is further explored in Chapters 2 and 3 of this thesis.

II. Role of Second Messenger Systems in Synaptic Plasticity

The previous section described some of the prominent second messenger systems found in all types of tissues and species. It also described several of the best known protein kinases that are the most common effector molecules of these second messenger regulatory mechanisms. It should be noted that only serine/threonine kinases were discussed. A considerable number of tyrosine kinases have been described, however, these enzymes are often associated directly with the activation of growth factor receptors and related transmembrane signalling proteins. Although the pathways of the receptor linked tyrosine kinases and the previously described second messenger systems often overlap, the former enzymes seem to be more important in the control of cell growth than in control of neuronal function. An example of this relationship is the number of cellular oncogene products with tyrosine kinase domains and/or activity. The following sections discuss the role of second messenger systems and serine/threonine kinases in the control of neuronal function, with particular emphasis on the mechanisms underlying various forms of synaptic plasticity.

The role of Ca^{2+} in secretion provides an example of the importance of second messengers in neuronal function, and at the same time demonstrates the difficulties involved in determining their actual mechanisms of action. Despite

extensive studies, the precise mechanism by which Ca^{2+} stimulates secretion is not understood (review in Reichardt and Kelly, 1983, Augustine *et al.*, 1987). There may be several reasons for this intractability. Neurons are complex cells and may have redundant mechanisms, such as the overlap of second messenger systems, for controlling their cellular properties. In addition, the time frame for neuronal events often makes traditional biochemical studies impractical. Finally, the broad range of neuronal cell types, many with unique regulatory properties, makes it difficult to find a "pure" population of cells with which to conduct biochemical studies. Despite these difficulties, considerable progress has been made with some systems.

Cyclic nucleotides, Ca^{2+}, DAG and $InsP_3$, and arachidonic acid metabolites have all been implicated in the control of neuronal function. Such control can either be indirect, via phosphorylation cascades, or direct via binding to ion channels. Unfortunately, most evidence for the involvement of these second messenger systems is correlational. A few regulatory mechanisms, however, are relatively well documented. The following sections will describe the progress made in the delineation of mechanisms underlying the various forms of synaptic plasticity seen in neuronal systems.

Modulation of Ion Channels

A number of ion channels or currents are reportedly controlled by second messengers in neurons (reviewed in Kaczmarek and Levitan, 1987). This control is generally thought to occur via phosphorylation of channels or closely associated proteins. Phosphorylation of these channels affects the electrical properties of the neurons, often producing changes in their spontaneous firing patterns, excitability, or

action potentials.

An example of such control is seen in neuron R15 in the *Aplysia* abdominal ganglion. R15 displays endogenous bursting activity that can be modulated by serotonin (Drummond *et al.*, 1980). Serotonin acts by increasing a potassium current that hyperpolarizes the cell and inhibits bursting (Benson and Levitan, 1983). This response mimics the effect of synaptic activation of R15. A series of experiments (reviewed in Benson and Adams, 1987) have demonstrated that serotonin regulates the potassium current via a cAMP-dependent phosphorylation cascade. First, binding to serotonin activates an adenylate cyclase (Levitan, 1978) linked to the receptor. Also, increasing intracellular cAMP by a variety of mechanisms activates the serotonin-sensitive current ($I_{K(r)}$) (Treistman and Levitan, 1976a and b; Drummond *et al.*, 1980; Lemos and Levitan, 1984). Furthermore, intracellular injection of a specific peptide inhibitor of the cAMP-dependent protein kinase blocks the serotonin stimulated increase in $I_{K(r)}$ (Adams and Levitan, 1982).

These studies of the regulation of bursting activity in R15 provide an example of the types of experiments required to establish the role of a second messenger system in neuronal regulation. Similar work implicates second messenger systems in the regulation of action potentials and repetitive firing in the bag cell neurons of *Aplysia* (reviewed in Strong and Kaczmarek, 1987). Phosphorylation by both the cAMP-dependent protein kinase and protein kinase C appear to regulate bag cell activity via changes in ionic conductances.

In addition to the invertebrate systems described above, a number of modulatory currents and channels have been described in mammalian cells (see

Kaczmarek and Levitan, 1987 for reviews). These include potassium and calcium currents regulated by a variety of neurotransmitters and peptides. The mechanisms by which these currents are regulated are not yet well defined. In part, this is due to the complexity of the mammalian nervous system and the relative difficulty of studying the individual cells.

These studies of activity modulation in R15 and bag cells demonstrate the importance of second messenger systems in determining the overall response properties of neurons. They do not, however, address the issue of synaptic plasticity. The following sections will focus on various forms of synaptic plasticity, in both invertebrate and vertebrate systems, and what is known about their underlying mechanisms.

Synaptic Plasticity in the *Aplysia* Gill Withdrawl Reflex

The gill and siphon withdrawal reflex of *Aplysia* has received considerable attention as a model system for understanding the physiological and biochemical properties of learning and memory. This system displays several forms of simple synaptic plasticity that appear to share a few basic characteristics with behavioral learning phenomena (e.g. acquisition). The best-studied examples of synaptic plasticity in the withdrawal reflex are short-term and long-term facilitation. During repeated stimulation of the sensory neurons in the gill or siphon, the synaptic activation of the motor neurons controlling withdrawal (occurring via activation of an interneuron "follower" cell in the case of the gill) will decrease. This use-dependent decrease in synaptic strength (habituation) lasts minutes and is thought to result from a depletion of synaptic vesicles available for release. In *Aplysia*,

habituation can be reversed, or the basal response enhanced, by sensitizing the animal with a noxious stimulus to the head or tail. This sensitization can last for either minutes or days depending on the number and frequency of stimuli delivered. The effect of head or tail stimulation on the gill/siphon withdrawal reflex is mediated by sensory neurons that are heterosynaptically coupled to the presynaptic terminals of the gill and siphon sensory neurons by faciliatory interneurons.

Effects of Serotonin. Several studies indicate that activation of the cAMP second messenger system in the sensory cells, produced by the release of transmitter from the facilitory interneurons, is responsible for the plasticity observed in this system. A variety of studies suggest that serotonin is one of the transmitters responsible for altering transmission at these synapses. Serotonin decreased K^+ current and enhanced transmitter release in the gill sensory cells (Tomosky-Sykes, 1978). This increase in transmitter release is thought to occur because the decreased K^+ current broadens action potentials in the sensory cell, thus increasing the duration of Ca^{2+} flux through voltage gated channels. The K^+ current modified by serotonin (S current) was found to be distinct from other K^+ currents in the sensory cells, including the early, delayed, and Ca^{2+}-regulated K^+ currents (Klein *et al.*, 1982; Siegelbaum *et al.*, 1986).

The role of cAMP. In addition to modulating the S current, serotonin also increased the level of cAMP in the gill sensory neurons (Bernier *et al.*, 1982). This increase suggested that the second messenger cAMP might be involved in the regulation of the S current in the sensory cell. The intracellular injection of the catalytic subunit of the cAMP-dependent protein kinase into sensory neurons

mimicked the action of serotonin by producing prolonged action potentials and enhancing the postsynaptic response of follower cells (Castellucci *et al.*, 1980). Further support for the involvement of phosphorylation in the regulation of the S current was obtained from the results of injection of a specific peptide inhibitor of the c AMP-dependent protein kinase into sensory cells. Injection of the inhibitor prevented the broadening of action potentials by serotonin application (Castellucci *et al.*, 1980). It also blocked action potential broadening and the enhancement of postsynaptic potentials induced by activation of the facilitory interneuron. Together, these results suggest that serotonin facilitates the synaptic connection between the gill sensory cell and its follower neuron by enhancing cAMP-dependent phosphorylation of a protein that controls a repolarizing K^+ current (S current).

The application of patchclamp technology provided further evidence for the involvement of cAMP-dependent protein phosphorylation in the control of serotonin induced facilitation. Using cell-attached membrane patches, investigators were able to isolate K^+ channels (S channels) with properties characteristic of the S current in sensory cells. These channels were reportedly closed by either application of serotonin to the cell (outside the patch) or injection of cAMP (Siegelbaum *et al.*, 1982). These observations suggest that serotonin regulates the S channel through a diffusible second messenger system that is mimicked by cAMP. Analysis in cell-free patches showed that S channels can also be closed by exposure to the catalytic subunit of the cAMP-dependent protein kinase (Shuster *et al.*, 1985). The fact that this regulation was dependent on Mg-ATP further implicates protein phosphorylation as a critical step in altering gating of the S channel.

This body of evidence is highly suggestive but not yet complete. Proving that the native transmitter is indeed serotonin has been difficult. Also, one does not know if the S channels themselves, or associated proteins, are the targets of the cAMP-dependent phosphorylation. Determining the latter point may require the cloning and expression of the S channel since even the dilution of channels into lipid bilayers may carry along tightly associated proteins.

It seems probable that cAMP-dependent regulation of the S channel underlies the short-term facilitation (hours) of the sensory cell synapses. This is supported by the observation that continued cAMP-dependent kinase activity is required to maintain facilitation (Castellucci *et al.*, 1982) It should be noted, however, that the catalytic subunit did not close S channels in cell-free patches as efficiently as serotonin or cAMP did in whole cell patches. This difference points to a more complex regulation of the channel that might, in part, involve cellular protein phosphatases (Shuster *et al.*, 1985).

Long-term Changes. It has been suggested that serotonin-stimulated, cAMP-dependent phosphorylation of S channels plays a role in long-term (days-weeks) facilitation as well. Behavioral studies show that long-term (7 days) sensitization of the siphon withdrawal reflex is acquired in a trial-dependent manner similar to that seen in mammalian learning paradigms (Frost *et al.*, 1985). Electrophysiological analysis of animals that demonstrated the highest levels of behavioral modification showed an enhancement of the motor cell response to sensory cell depolarization. Part of this enhanced response may result from an increase in the total area of synaptic contact (Baily and Chen, 1983). It is possible, however, that prolonged

changes in the cAMP second messenger system, or S channels themselves, could also be involved.

Greenberg *et al.* (1987) proposed that the proportion of cAMP-dependent protein kinase active at resting levels of cAMP might be altered during long-term facilitation. Their study indicates that such a change may occur via a reduction in the amount of regulatory subunit in sensory cells from trained animals (Greenberg *et al.*, 1987). Unlike short-term facilitation, long-term facilitation requires the synthesis of new protein. Montarolo *et al.* (1985) were able to reconstitute the sensory cell to motor neuron connection *in vitro*, and showed that repeated application of serotonin produced an enhancement of synaptic efficacy similar to that seen in long-term facilitation *in vivo*. The presence of inhibitors of protein or RNA synthesis blocked this serotonin induced long-term facilitation, but did not prevent short-term facilitation. Greenberg *et al.* (1987) suggest that protein synthesis may be required for expression of a protease that would degrade the regulatory subunit. This enhanced proteolysis would decrease the amount of regulatory subunit available to complex with the catalytic subunit, thereby enhancing the level of kinase activity at a given (subsaturating) concentration of cAMP.

Despite the attractiveness of this model, it seems to be an oversimplification. There is evidence that characteristics of other cells in the system change during long-term facilitation (Frost and Kandel, 1984; Frost *et al.*, 85; Eberly and Pinsker, 1984). Also, given the degree of interaction between second messenger systems at all levels (including effects on regulatory molecules, common substrates, and phosphorylation of the kinases themselves) it seems unlikely that no other pathway

is involved.

Although facilitation of the sensory-to-motor cell connections in the *Aplysia* gill/siphon withdrawal reflex has been proposed as a model of simple learning and memory, it does not demonstrate true associative properties. The ability of an animal to form an association between two stimuli, presented within a well defined window of time, is probably the best studied form of learning. Although the delivery of noxious stimuli to the head or tail of *Aplysia* modifies the behavioral response to contact at a different part of the animal, the noxious stimuli need not be paired with activation of the gill or siphon sensory cells. There have been reports that these sensory cell synapses can display a conditioned response (Schwartz *et al.*, 1983; Kandel *et al.*, 1983; Walters and Byrne, 1985), which might involve the some cAMP-dependent protein phosphorylation pathway.

Associative Learning in *Hermissenda*

Attempts have been made to demonstrate associative learning in other invertebrates, including another marine mollusc *Hermisenda* (Lederhendler *et al.*, 1986). Coincident activation of the vestibular and photoreceptor pathways in *Hermisenda* reverses the normal phototactic behavior of these mollusks. This learned response reportedly displays several characteristics of mammalian learning, namely: 1) acquisition - increased aptitude with repeated training, 2) retention - the learned response persists for several days (Crow and Alkon, 1978; Harrigan and Alkon, 1985), 3) extinction - learning becomes more difficult and animals "forget" faster when non-paired stimuli are interposed (Richards *et al.*, 1984), and 4) savings - animals re-learn forgotten behaviors more rapidly than naive animals (Crow and

Alkon, 1978). These characteristics, combined with the absolute requirement for temporal pairing of the stimuli, suggest that the *Hermisenda* system may be a simplified model for some types of mammalian learning.

Detailed analyses of the neuronal circuits involved in the photo/vestibular learning paradigm (reviewed in Alkon, 1987) indicate that some photoreceptors (type B) and hair cells are heterosynaptically coupled, and are capable of modulating the activity of the other receptor cell. The type B photoreceptor also modulates the activity of the type A photoreceptor, the latter of which is coupled to motor neurons controlling the foot. Training alters the activity patterns and excitability of various neurons in this circuit. These changes include an inherent increase in the excitability of the type B photoreceptor, correlated with enhanced membrane resistance (Alkon, 1984; Alkon *et al.*, 1982; Crow and Alkon, 1980; Farley *et al.*, 1983; Goh *et al.*, 1985; West *et al.*, 1982). The affected current appears to be an early K^+ current (I_A) (Alkon *et al.*, 1984). Increased intracellular free Ca^{2+} in the type B photoreceptor may alter the K^+ current via phosphorylation by the type II CaM kinase and/or protein kinase C. Therefore, the ultimate learning-dependent changes may be very similar to that described for *Aplysia*, only with a different second messenger system.

Despite the increased complexity of the circuitry involved in the *Hermisenda* system, the primary locus of "memory" storage appears to reside in the sensory receptors. This is also the case in facilitation of the gill/siphon withdrawal reflex in *Aplysia*. As with *Aplysia*, the conclusions outlined above are probably an oversimplification of the system. There could be significant changes in the inherent

properties of other cells in the circuit, which might not be dramatic enough to be detected in the electrophysiological studies. Aside from these reservations, the *Hermisenda* system offers an example of how a relatively simple neuronal circuit can display a variety of characteristics associated with learning and memory. At least some of these characteristics may result from prolonged changes in the second messenger systems within individual neurons.

Drosophila Learning and Memory

The approach to understanding the cellular and biochemical basis of learning and memory in invertebrates has emphasized the mapping of neuronal circuits, and physiological characterization of the component neurons. A completely different approach was adopted in studies of learning and memory in *Drosophila.* The increased complexity of the *Drosophila* nervous system makes circuit mapping impractical. Molecular genetics, however, provided an alternative approach. By screening flies for their ability to perform an odor discrimination task, investigators were able to isolate several mutant strains of flies that either failed to learn or could not remember (reviewed in Tully, 1984). In addition, mutant strains isolated on the basis of other behavioral anomalies or deficits often displayed irregular performance of the learning/memory tasks (Cowan and Siegel, 1986). Attempts to identify the structural or biochemical basis for the deficits in these flies produced some correlations with the molluscan systems described above.

One mutant strain, rutabaga (rut), has a defect in the Ca^{2+}/calmodulin stimulated adenylate cyclase (Livingstone *et al.*, 1984; Dudai and Zvi, 1985; Livingstone, 1985). A second strain, dunce, has a mutation in the structural gene

(dunce$^+$) for the type II cAMP phosphodiesterase (Chen *et al.*, 1986). Mutations in the dopa-decarboxylase gene (Ddc) also block learning and memory (Temple *et al.*, 1984).

The degree to which these mutations affect both learning and memory is correlated with their relative position in the cAMP regulatory cascade. The disruption of dopamine, norepinephrine and serotonin synthesis in Ddc mutants has the greatest effect on learning and memory, while the loss of phosphodiesterase activity in dunce flies has the least. The way in which these mutants affect learning and memory at the cellular level is not understood. Despite the fact that the rutabaga and dunce mutations have the opposite effect on the resting level of cAMP, they have the same behavioral effect. This suggests that it is not simply the resting concentration of cAMP that controls learning, but rather the ability to manipulate this level in a context-dependent manner. This is supported by the observation that double mutants of dunce and rutabaga have normal levels of cAMP (as measured in fly homogenates) but do not learn (Livingstone *et al.*, 1982). As predicted by the work in *Aplysia*, the dunce and rutabaga mutants are also deficient in the simple learning tasks of habituation and sensitization (Duerr and Quinn, 1982).

Not all of the learning/memory deficient flies have defects in the cAMP cascade. The K^+ channel mutant shaker shows a reduced rate and maximum degree of acquisition of an odor avoidance task (Cowan and Siegel, 1986). Another mutant with altered channel properties (nap^{ts}, faulty sodium channel function) also displayed delayed acquisition of the task. A third mutant, turnip, isolated in the behavioral

screens, was reported to have a deficit in protein kinase C activity, but may actually effect the linkage of membrane receptors or G-proteins. This mutant is also more generally debilitated than the other learning/memory mutants. Amnesiac flies display a specific deficit in their ability to remember certain memory tasks. The underlying reason for this deficit is not known, but it does not appear to be a defect in the cAMP pathway.

Despite the long list above, not all mutant strains of flies are deficient in learning or memory. Also, the learning/memory mutants described above do not appear to be deficient in simple olfactory or visual abilities. Many of these mutants do, however, display other (pleiotropic) effects, such as female sterility. Such pleiotropic effects are not surprising considering the general importance of second messenger systems in cell regulation. The fact that mutants defective in second messenger pathways are not lethal, and have relatively well defined behavioral deficits, may reflect the presence of brain specific isoforms for some of the regulatory enzymes (Dudai and Zvi, 1985).

While much of the work on learning/memory mutants has focused on their biochemical deficiencies, there appear to be some structural changes as well. Two strains of flies with mutations in the structures known as mushroom bodies displayed learning deficits when tested with an olfactory discrimination task (Heisenberg *et al.*, 1985). The defects do not appear to affect the perception of olfactory stimuli, or learning based on visual cues. The intrinsic neurons of the mushroom bodies (Kenyon cells) are either misrouted or degenerate in the mutant strains. Although the number of Kenyon cell fibers is dependent on olfactory

experience, it is not yet known what role the mushroom bodies play in learning/memory. The learning mutants dunce and rutabaga, however, do not display this experience-dependent modulation of Kenyon cell fibers (Balling *et al.*, 1987). This last observation suggests that the biochemical deficits seen in some of the mutants may effect learning and memory on a variety of levels.

The studies conducted with invertebrates suggest that both the cAMP- and Ca^{2+}-dependent phosphorylation of proteins play an important role in the plasticity of synaptic connections. The forms of plasticity studied in these sytems, however, are usually relatively simple. Yet, despite there simplicity, most of these systems are not yet fully understood. The examples of S channel regulation in *Aplysia* and K^+ channel regulation in *Hermisenda* provide convincing evidence for the importance of second messenger systems in the regulation of synaptic connections. These systems also exemplify the difficulties involved in determining second messenger regulatory mechanisms. It is not surprising, therefore, that the forms of synaptic plasticity found in mammalian brain (described in the following section) are not only more complex, they are also less well understood.

Synaptic Plasticity in Mammalian Systems

Changes in synaptic efficacy have been observed in neurons throughout the brain as well as in the peripheral nervous system. Both short-term (seconds to minutes) and long-term (minutes to weeks) forms of plasticity have been described. The various forms of short-term plasticity can be categorized as: 1)facilitation - lasting <1 second, 2)augmentation - lasting 2 to 20 seconds, 3)Posttetanic potentiation - lasting 2 to 5 minutes, and 4)depression - lasting msec to minutes.

Facilitation and augmentation are similar to the short-term sensitization seen in invertebrates, and are thought to arise from high residual Ca^{2+} levels in the synaptic terminals following high frequency firing of the neuron. Posttetanic potentiation probably arises in part from increased residual Ca^{2+}, but may also involve changes in the phosphorylation state of the synaptic vesicle associated protein, synapsin I (Llinas *et al.*, 1985). Short-term depression (described as habituation in *Aplysia*) is a gradual decline in synaptic response during repetitive stimulation. This effect often arises from a depletion of releasable transmitter in the presynaptic cell (Zucker *et al.*, 1989).

Long-term changes in synaptic efficacy are also broken into several catagories based on their functional properties. The principle distinguishing characteristic of the various types of long-term potentiation (LTP) is a dependence on coincident postsynaptic activity. Associative LTP requires depolarization of the postsynaptic cell in combination with activation of the synaptic inputs (Kelso *et al.*, 1986). It should be noted that, unlike most invertebrate neural connections, the inputs from one presynaptic CNS neuron are not sufficient to substantially alter the resting potential of the postsynaptic cell. Instead, postsynaptic cells integrate inputs from large numbers of presynaptic cells. Therefore, associative LTP reinforces synapses that coincidentily activate the postsynaptic cell (Barrionuevo and Brown, 1983). This associative property was originally predicted by Hebb (1949) to explain the results of behavioral learning studies.

The second predominant form of LTP is nonassociative, and requires only the persistent activation of the presynaptic fibers. The postsynaptic cell need not be

depolarized. Both associative and nonassociative LTP can be further broken down on the basis of persistence. Decremental LTP lasts from 15 to 30 minutes, whereas nondecremental LTP persists for hours to weeks. Long-term depression, though rarely seen (Ito, 1989; Levy and Steward, 1983; Bramham and Srebo, 1987; Stanton and Sejnowski, 1989), lasts 10 minutes to hours.

Simple forms of synaptic modulation, such as facilitation and posttetanic potentiation, have been observed throughout the mammalian central and peripheral nervous systems. However, LTP, especially in its associative form, has a much more restricted distribution. Despite this restricted distribution, LTP has been studied extensively. The interest in LTP stems from its possible role in learning and memory. As mentioned above, associative LTP displays cellular characteristics similar to the behavioral phenomenon of associative learning. Also, LTP can be readily demonstrated in an area of the brain, the hippocampus, which is implicated in the formation of memories. Clinical studies of patients with focal lessions in the hippocampus demonstrate that damage to this brain region seriously impares the ability to store new memories (Zola-Morgan *et al.*, 1986).

The hippocampus contains three primary neuronal cell types (dentate granule cells, and CA1 and CA3 pyramidal cells) organized in lamina. These cell types are interconnected by well defined fiber pathways within the hippocampus. The dentate cells receive inputs from neurons in the entorhinal cortex via the perforant path which runs through the adjacent subiculum. The dentate cells synapse on dendrites of the CA3 pyramidal cells via the mossy fiber pathway. The CA3 pyramidal cells send axons through the Schaffer collateral pathway to synapse onto the CA1

neurons. The CA1 cells synapse onto neurons in the subiculum which then project back to entorhinal cortex. This loop is called the trisynaptic circuit. In addition to this primary synaptic pathway, neurons in the hippocampus project to, and receive inputs from other parts of the brain. The trisynaptic circuit receives input from various sensory and associational areas of the brain via the entorhinal cortex. The hippocampus, therefore, is in a position to integrate information of various modalities, and perhaps consolidate it for storage in other areas of the brain (Squire *et al.*, 1989). LTP may be an important mechanism by which such integration and consolidation occurs.

LTP in the Hippocampus

The laminar organization of the cell layers and synaptic pathways has made it possible to study LTP in slices of hippocampal tissue. These studies demonstrated that various forms of LTP can be induced in all the principal neuronal cell types in the hippocampus. Tetanic stimulation produces LTP of the mossy fiber input to CA3 cells (Johnston and Brown, 1984; Harris and Cotman, 1986; Kauer and Nicoll, 1988). Associative LTP can be generated in the perforant path input onto the dentate granule cells (Levy and Steward, 1979), as well as the Schaffer collateral input to CA1 pyramidal cells (Barrionuevo and Brown, 1983). These data demonstrate that LTP is a common mechanism of synaptic modulation within the hippocampus. The pervasiveness of LTP as a means of altering synaptic efficacy within the trisynaptic circuit lead to concerted efforts to determine its cellular mechanism(s) and importance to the function of the hippocampus.

Importance of Ca^{2+}. Several studies have demonstrated that postsynaptic Ca^{2+}

increases are critical to the generation of LTP in hippocampal neurons. Lynch *et al.* (1983) showed that LTP in the Schaffer collateral pathway could be blocked by injection of EGTA into the postsynaptic CA1 pyramidal cells. Studies using the light-sensitive Ca^{2+} chelator nitr-5 produced the same result (Malenka *et al.*, 1988). Using the Ca^{2+}-loaded form of nitr-5, Malenka *et al.* (1988) showed that the release of free Ca^{2+} in CA1 cells produced long-lasting enhancement of the Schaffer collateral inputs. Although this Ca^{2+} induced potentiation may not be identical to LTP, it demonstrates the importance of postsynaptic Ca^{2+} in the regulation of transmission at Schaffer collateral terminals.

In a similar study, the injection of the Ca^{2+} chelators BAPTA and QUIN-2 into CA3 pyramidal cells reportedly blocked LTP in the mossy fiber pathway (Williams and Johnston, 1989). This result is somewhat surprising since LTP at mossy fiber terminals is nonassociative, and therefore, by definition, independent of the resting potential of the postsynaptic cell. This implies that Ca^{2+} flux through voltage and/or NMDA regulated channels (see below) is not necessary for the production of LTP at these synapses. It should be noted that the associational-commisural fiber terminals on the CA3 neurons exhibit associative LTP (Zalutsky and Nicoll, 1990). This finding shows that the CA3 cells are able to support this form of synaptic modulation, even if it is not used at mossy fiber terminals.

The mechanism responsible for the critical increase in postsynaptic Ca^{2+} at terminals that exhibit associative LTP was determined by studies of amino acid transmission in the hippocampus. Glutamate is thought to be the principle excitatory transmitter for hippocampal neurons. There appear to be three

pharmacologically distinct glutamate receptors that regulate ionic conductances (Watkins and Evans, 1981). Two of these receptors, defined by the selective agonists quisqualate and kianate (refered to collectively as Q/K receptors), regulate the conductance of channels selective for Na^+ and K^+ (Mayer and Westbrook, 1985; MacDermott *et al.*, 1986; Jahr and Stevens, 1987). Antagonists of the Q/K receptors can block excitatory transmission in the Schaffer collateral pathway. This suggests that activation of these receptors is normally responsible for the majority of the postsynaptic response in CA1 cells (Collinridge *et al.*, 1983).

Role of NMDA Receptors. A third pharmacological subtype of glutamate receptor, activated by N-methyl-D-aspartic acid (NMDA), has several distinguishing characteristics. The ion channel regulated by the NMDA receptor passes Ca^{2+} in addition to Na^+ and K^+ (Jahr and Stevens, 1987; Mayer and Westbrook, 1987). The channel, however, is blocked at resting membrane potentials by extracellular Mg^{2+} (Nowak *et al.*, 1984). This Mg^{2+} block is relieved by depolarization of the membrane. Therefore, the channel will only pass Ca^{2+} (as well as other ions) when agonist is bound, and the cell is depolarized to a level sufficient to remove the Mg^{2+} block. This dual regulation of conductance seen for the NMDA-sensitive channel is thought to underlie the associative properties observed for LTP in several areas of the hippocampus. The conditions required for Ca^{2+} flux through the NMDA regulated channel could be met either by tetanic stimulation of a single synaptic pathway or by coincident activation of convergent pathways. These conditions are the same as those known to produce LTP at hippocampal synapses.

Collinridge *et al.* (1983) found that antagonists of the NMDA receptor had

no affect on the excitatory postsynaptic potential produced by stimulation of the Schaffer collaterals. The antagonist did, however, block potentiation at these synapses. In contrast, antagonists of the NMDA receptor had no affect on the induction of LTP at mossy fiber terminals on CA3 neurons (Harris and Cotman, 1986; Kauer and Nicoll, 1988). These results are consistent with the proposal that Ca^{2+} flux mediated by NMDA receptor activation is critical for the production of associative LTP.

Studies by Regehr and Tank (1990) support the idea that NMDA receptor activation produces an increase in postsynaptic Ca^{2+} during stimulation of the Schaffer collateral pathway. Using high resolution imaging techniques and the Ca^{2+}-sensitive dye Fura 2, these investigators demonstrated transient increases in Ca^{2+} within the dendrites of CA1 pyramidal cells during high frequency stimulation of Schaffer collateral inputs. A portion of this Ca^{2+} increase, mainly localized in the distal dendrites, was blocked by application of APV (a specific antagonist of the NMDA receptor). Low frequency stimulation (below the threshold for LTP induction) did not elicit an APV-sensitive increase in Ca^{2+}. Both low and high frequency stimulation produced substantial non-NMDA mediated increases in intracellular Ca^{2+}, presumably through voltage gated channels.

The enrichment of NMDA receptors in the dendrites of CA1 and dentate granule cells further supports a role for these receptors in the induction of associative LTP (Monaghan and Cotman, 1985). The dendritic region of CA3 neurons containing the synaptic terminals of the mossy fibers had a much lower concentration of NMDA receptors. This is consistent with the observation that the

mossy fiber pathway exhibits only nonassociative LTP. The fact that CA3 cells do support associative LTP at the associative-commisural fiber terminals could explain the presence of some NMDA receptors in this synaptic region. These results show that the localization of NMDA receptors closely matches the distribution of fiber pathways that display associative LTP.

The studies described above indicate that NMDA receptor activation is necessary for the induction of LTP. However, NMDA receptor activation alone is apparently not sufficient for LTP induction. Exposure of CA1 neurons in hippocampal slices to depolarizing concentrations of NMDA or glutamate produced a substantial enhancement of synaptic activation (Kauer *et al.*, 1988). Unlike true LTP, however, this pharmacologically-induced enhancement decayed relatively rapidly ($t_{1/2}$ 10-15 minutes; referred to as slowly decaying potential [SDP]). Prior induction of LTP blocked production of SDP for 15 to 20 minutes. This suggests that SDP is an early component of the normal potentiation of these synapses. It also indicates that SDP involves a mechanism separate from and less persistant than that underlying LTP. In addition, these studies suggest that either synaptic activity cannot produce LTP by virtue of glutamate release alone, or that NMDA application does not mimic glutamate release. It is possible that a second modulatory transmitter or peptide, necessary for true LTP, is coreleased from the presynaptic terminal along with excitatory transmitter. The identity and function of this putative second regulatory molecule is not known.

Phases of LTP. Investigators have made numerous attempts to define the biochemical mechanism(s) involved in LTP. The results of these studies have often

been confusing or contradictory. However, several important conclusions can be drawn. Biochemical analyses suggest that LTP can be broken into two related yet distinct phases (Bliss and Lynch, 1988; Nicoll *et al.*, 1988), namely induction and maintenance. The induction phase includes the period during and immediately following synaptic activation. It is during this induction phase that the cellular events that ultimately produce the long-lasting changes in synaptic efficacy are triggered. The maintenance phase refers to the period of sustained potentiation (hours to weeks) following induction. As described below, these two phases of LTP may reflect the action of distinct mechanisms for the enhancement of synaptic transmission.

Role of the C Kinase in LTP. The importance of Ca^{2+} in the induction of LTP prompted several studies designed to help define the role of Ca^{2+}-regulated enzymes. These experiments, and theories of synaptic regulation based on their interpretations, focus on protein kinase C (C kinase) and the type II Ca^{2+}/calmodulin-dependent protein kinase (CaM kinase). There is experimental evidence supporting a role for both of these kinases in LTP.

The C kinase was the first Ca^{2+}-dependent enzyme implicated in LTP. The high concentration of the kinase in synaptic regions of the hippocampus (Saito *et al.*, 1988), combined with its regulation by convergent second messenger systems (DAG and Ca^{2+}) suggested that the enzyme could be important in synaptic modulation. High frequency stimulation sufficient to produce LTP of the perforant path input to dentate granule cells caused a translocation of protein kinase to the cell membrane (Akers *et al.*, 1986). Low frequency stimulation did not produce a

translocation. This translocation is indicative of C kinase activation (Bell, 1986). Application of phorbol ester, a membrane permeant activator of the C kinase, produced a significant enhancement of synaptic transmission at Schaffer collateral terminals (Malenka *et al.*, 1986b). This phorbol ester-induced enhancement is in many ways the same as LTP, however, it decays much more rapidly (approximately 30 min). Therefore, direct activation of endogenous C kinase appears to produce a slowly decaying potential (SDP) much like that induced by application of glutamate or NMDA.

Further evidence for the involvement of C kinase in LTP comes from studies in which either the enzyme itself, or specific peptide inhibitors were introduced into the postsynaptic cells. Hu *et al.* (1987) demonstrated that injection of purified C kinase into CA1 cells potentiated their response to Schaffer collateral stimulation. Unlike similar studies in which the catalytic subunit of the cAMP-dependent kinase was injected into *Aplysia* neurons, the C kinase was not constitutively active. It is not clear, therefore, how the injected C kinase produced this effect. More direct evidence is provided by the results of inhibitory peptide injections. Malinow *et al.* (1989) showed that injection of an inhibitory pseudosubstrate peptide , specific for the C kinase, blocked the induction of LTP in CA1 neurons. However, injection of the peptide <u>after</u> induction of LTP had no effect. Together these results suggest that activation of C kinase in the postsynaptic cell is necessary but not sufficient for the induction of LTP. Also, constitutve kinase activity in the postsynaptic cell is not necessary for the maintenance of LTP.

Role of Type II CaM Kinase in LTP. Its broad substrate specificity, concentration

at the postsynaptic membrane, and regulation by Ca^{2+}-dependent autophosphorylation combine to make the CaM kinase a likely synaptic regulatory molecule. Despite theories concerning its potential role as a Ca^{2+}-activated molecular switch (Miller and Kennedy, 1986; Lisman and Goldring, 1988), relatively little is known about the actual function of the CaM kinase in LTP. Experimental evidence, however, indicates that activation of the enzyme is critical for the production of LTP in the hippocampus.

Malenka *et al.* (1989) reported that the injection of calmodulin binding peptides blocked the induction of associative LTP in hippocampal neurons. These peptides, however, should interfere with the activation of any Ca^{2+}/calmodulin-dependent enzymes normally stimulated during the induction of LTP, not just the CaM kinase. Injection of a peptide inhibitor specific for the CaM kinase also blocked LTP (Malinow *et al.*, 1989). The inhibitor, however, failed to reverse LTP once it was established. These findings indicate that activation of postsynaptic CaM kinase, as well as C kinase, is critical for the induction but not the maintenance of LTP in hippocampal neurons.

It should be noted that bath application of general kinase inhibitors reportedly affects the maintenace of LTP. Unfortunately, interpretation of these results is difficult due to the lack of specificitiy of the inhibitors.

The electrophysiological studies described above provide strong evidence for the involvement of CaM kinase and C kinase in LTP. However, the cellular substrates critical for LTP induction have not been identified. Despite the lack of knowledge regarding its mechanism of action, there is some indication that the C

kinase pathway is affected during the formation of LTP *in vivo*. Nicoletti *et al.* (1988) reported that acquisition of a spatial learning task by rats enhanced the excitatory amino acid-stimulated hydrolysis of phosphoinositide in the hippocampus. Classical conditioning of rabbits apparently causes a translocation of C kinase to the membrane, and a redistribution from the cell bodies to the dendrites of CA1 pyramidal cells (Bank *et al.*, 1988; Olds *et al.*, 1989). Unfortunately, these studies only demonstrate a correlation between C kinase activation and learning, and do not prove that these changes underlie LTP *in vivo*.

A third Ca^{2+}-stimulated enzyme, the protease calpain I, may also contribute to LTP in the hippocampus (Lynch and Baudry, 1984; Siman *et al.*, 1987). Calpain I is found in synaptic membrane fractions (Siman *et al.*, 1983), and can hydrolyze several structural proteins from brain, including MAP-2 (Siman and Noszek, 1988) and fodrin (Siman *et al.*, 1984). The enzyme can also proteolyze the regulatory domains of the CaM kinase (Kwiatkowski and King, 1989) and the C kinase (Inoue *et al.*, 1977), thereby generating constitutively active forms of these enzymes.

The substrate specificity of calpain I suggests two ways by which the enzyme could influence synaptic properties in the hippocampus. Ca^{2+}-stimulated proteolysis of cytoskeletal elements in the postsynaptic cell may alter the physical and electrical properties at the synapse. Alternatively, proteolysis of CaM kinase and C kinase may change the half-life and/or distribution of the active forms of these enzymes. Although calpain I in neurons can be activated by high concentrations of transmitter (Siman and Noszek, 1988), there is no evidence for its requirement in LTP. These potential mechanisms of action, therefore, remain purely

hypothetical.

Cellular Mechanisms of LTP, Pre- Vs Postsynaptic. The studies using specific peptide inhibitors make it clear that kinase activation in the postsynaptic cell is necessary for the induction of associative LTP. However, the relative contributions of pre- and postsynaptic mechanisms to the maintenance of LTP is considerably less well understood. There is evidence for both a presynaptic increase in transmitter release and an increase in responsiveness of the postsynaptic cell. While these two mechanisms are not mutually exclusive, they have yet to be completely reconciled.

In support of a presynaptic mechanism, Bliss *et al.* (1986) reported a strong correlation between LTP in the perforant path in rats and enhanced ^{3}H-glutamate release at the terminals. Whole cell patch recordings of CA1 pyramidal cells revealed a change in synaptic variability and proportion of synaptic failures during LTP (Malinow and Tsien, 1990), which also suggests a presynaptic component of the potentiation. These observations fit with studies of peripheral synapses where LTP clearly arises from enhanced presynaptic release of transmitter (Briggs *et al.*, 1985; Baxter *et al.*, 1985). Initial attempts to measure postsynaptic glutamate sensitivity showed no change with LTP (Lynch *et al.*, 1976; Mohan and Sastry, 1985; Taube and Schwartzkroin, 1988). Two recent studies, however, show a selective enhancement of the Q/K mediated component of the postsynaptic response following induction of LTP (Kauer *et al.*, 1988b; Muller *et al.*, 1988). The response to NMDA was unchanged. This large change in the Q/K-mediated response was detected within 5 minutes of the initiation of LTP. A third study also detected a specific enhancement of the Q/K response, but it developed slowly between 30

minutes and 2 hours after induction of LTP (Davies *et al.*, 1989). While the relative importance of these pre- and post synaptic effects and their temporal relationships remain unclear, both seem to contribute to associative LTP.

One implication of a presynaptic locus for the maintenance of LTP is the requirement for a retrograde signal from the postsynaptic spine. The changes in postsynaptic Ca^{2+} that trigger LTP must somehow be communicated to the presynaptic terminal. The metabolites of arachidonic acid could act as retrograde messengers. These membrane permeant metabolites are known to mediate FMRFamide induced presynaptic inhibition in *Aplysia* (Piomelli *et al.*, 1987). Furthermore, NMDA can stimulate the release of arachidonic acid from neurons by the Ca^{2+}-mediated activation of phospholipase A_2 (Dumis *et al.*, 1988). It is not yet known, however, if this potential retrograde signalling pathway operates at hippocampal synapses.

It should be clear from the preceeding discussion that several questions concerning the induction and maintenance of LTP remain to be answered. Principle among these is the determination of the critical cellular substrates of the CaM and C kinases. It is possible that phosphorylation of the Q/K receptors may activate a previously silent population of channels. Sequence analysis of recently cloned glutamate receptors (Hollmann *et al.*, 1989; Boulter *et al.*, 1990) suggests that they may have phosphorylation sites for the CaM and C kinase, however, actual phosphorylation of these proteins has not yet been demonstrated.

The kinase could also phosphorylate cytoskeletal elements and/or synaptic vesicle associated proteins, thereby producing changes in the presynaptic terminal

that would facilitate transmitter release. Facilitation of transmission at the squid giant synapse could be a model for such regulation (Llinas *et al.*, 1988). While some synaptic receptors and vesicle associated proteins have already been isolated or identified (Lester, 1988; Huttner *et al.*, 1983; Thomas *et al.*, 1988; Trimble and Scheller, 1988), many important synaptic structures (e.g., PSDs) and molecules have not been fully characterized.

Continued progress toward the identification and characterization of synaptic molecules, including the identification of cellular substrates of the CaM and C kinases, will help elucidate the biochemical pathways that produce and sustain LTP in the hippocampus.

How these molecular changes ultimately produce a (semi)permanant enhancement of transmission also remains a question. A cellular oncogene-induced change in the expression of synaptic regulatory molecules and/or the physical rearrangement of synaptic terminals are two likely candidates.

Summary

Second messenger systems are clearly important in the regulation of synaptic transmission in mammalian systems. The complexity of these systems, however, has precluded an understanding of exactly how they operate. We believe that a better understanding of synaptic regulatory molecules, especially how they act in intact neurons, will eventually provide a more complete picture of mammalian synaptic plasticity.

This thesis describes our attempts to clarify the role of the CaM kinase in

neurons. This information should help elucidate the importance of the enzyme to hippocampal neurons, including its potential role in the modulation of synaptic strength. Chapter 2 describes our attempts to determine if the switch-like properties of the CaM kinase are used in neurons. We also tried to determine how the switching of the enzyme to a Ca^{2+}-independent form by autophosphorylation might be regulated in these neurons. Chapter 3 extends the biochemical studies of the kinase by using immunocytochemistry. The work reported in chapter 3 allowed us to visualize the phosphorylated, Ca^{2+}-independent kinase in the hippocampal neurons. Chapter 4 contains an analysis of the tissue and brain region specific expression of the CaM kinase. Chapter 5 provides a brief synopsis of the results of the preceeding chapters along with suggestions about possible future experiments.

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Chapter 2

Autophosphorylation of Type II Ca^{+2}/Calmodulin-Dependent Protein Kinase in Organotypic Cultures of Hippocampal Slices.

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Autophosphorylation of Type II Ca^{2+}/Calmodulin-Dependent Protein Kinase In Organotypic Cultures of Hippocampal Slices

Key words: synaptic regulation, molecular switch

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Abbreviations: CaM kinase, type II Ca^{2+}/calmodulin dependent protein kinase; NMDA, n-methyl-D-aspartic acid.

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ABSTRACT Autophosphorylation of threonine-286 (Thr_{286}) on type II Ca^{2+}/calmodulin-dependent protein kinase (CaM kinase) *in vitro* causes kinase activity to become partially independent of Ca^{2+}. Here we report that Thr_{286} is the major CaM kinase autophosphorylation site occupied *in situ* in organotypic hippocampal cultures. Measurement of Ca^{2+}-independent CaM kinase activity revealed that approximately one third of the kinase is autophosphorylated *in situ* when the basal Ca^{2+} concentration is 15-43 nM. This proportion was substantially reduced 30 min after removal of extracellular Ca^{2+} or treatment of the cultures with protein kinase inhibitors, and was increased by treatment with okadaic acid. Therefore, the high proportion of autophosphorylated kinase at basal Ca^{2+} concentrations appears to be maintained by Ca^{2+}-dependent autophosphorylation. Homogenates of intact hippocampi also contain a high proportion of Ca^{2+} independent type II CaM kinase, 13 to 23% depending on developmental age. Thus, in hippocampal neurons, an important function of the autophosphorylation mechanism may be to produce a relatively high level of CaM kinase activity, even at basal Ca^{2+} concentrations, permitting both upward and downward local regulation by physiological agents.

Brief tetanic stimulation of certain hippocampal synapses produces long-term potentiation (LTP), a long-lasting increase in the strength of synaptic transmission that might play a role in memory formation (1-3). In synapses of the perforant path and Schaffer collateral pathway activation of N-methyl D-aspartate (NMDA) receptors causes an increase in postsynaptic Ca^{2+} that is necessary for induction of LTP (4-6). Potentiation of transmission appears to result from enhanced release of presynaptic transmitter (7-9) and/or enhanced current through quisqualate-type glutamate receptors (10, 11). The molecular mechanisms that link postsynaptic Ca^{2+} influx to the enduring increase in synaptic strength are unknown.

One event thought to be necessary for induction of LTP is activation of type II Ca^{2+}/calmodulin-dependent protein kinase (CaM kinase II) (12, 13). This kinase constitutes as much as 2% of total protein in neurons of the hippocampus (14). It is distributed throughout the neuronal cytosol (15), and is also the major component of the postsynaptic density (PSD) (16,17), where it appears to be positioned to respond to increases in Ca^{2+} concentration produced by activation of NMDA receptors. The kinase is a multimeric holoenzyme composed of approximately twelve catalytic subunits encoded by at least two distinct genes (18-20). When the kinase is activated by Ca^{2+}/calmodulin, each subunit is rapidly autophosphorylated at a threonine residue (Thr_{286} in α and Thr_{287} in β) near the calmodulin-binding domain. Autophosphorylation of this residue in as few as 1 to 3 of the subunits in a holoenzyme causes all the subunits to remain partially active beyond the duration of the initial activating Ca^{2+} signal (21-23). Ca^{2+}-independent activity can be reversed by dephosphorylation of $Thr_{286-287}$ (22).

The lack of appropriate model systems has limited the study of CaM kinase regulation in neurons of the central nervous system. Neuronal cell lines and primary cultures have been useful for physiological studies of synaptic transmission, however they are not ideal for study of the biochemical events associated with synaptic transmission and its regulation. Neuronal cell lines usually display phenotypes more typical of embryonic neurons than of adult neurons. They contain low concentrations of type II CaM kinase (data not shown) and the density of synapses that they form in culture is relatively low. Primary cultures of embryonic hippocampal pyramidal cells begin to express adult levels of CaM kinase II subunits after several weeks in culture (24), but the density of synapses in the cultures is still low (25). Physiologists have successfully used acutely prepared slices of hippocampus to study LTP, but the slices contain a layer of dying tissue on either side that would be included in biochemical samples. Organotypic cultures of slices of rat hippocampus live for eight weeks or more and have been shown to develop the cytoarchitecture, principal neuronal cell types, and major synaptic pathways found in the adult hippocampus (17, 26). In this study, we examined regulation of autophosphorylation of the kinase and its consequent Ca^{2+}-independent activity in these cultures.

Methods

Reagents. Sprague/Dawley albino rats were obtained from Simonsen Laboratories. Rat tail collagen was a gift of Doreen McDowell and Dr. Paul Patterson. Rat type II CaM kinase, bovine synapsin I, and calmodulin were purified as described (27,28). PKI 14-24 amide (Walsh Inhibitor) was purchased from Peninsula

Laboratories. Radiochemicals were purchased from ICN. Pharmacological agents were obtained from Sigma or Tocris Nuramin (London). BAY K8644 was a gift from A. Scriabine of Miles Pharmaceutical Division, W. Haven, CT. H7, HA1004, W7, and W5 were purchased from Seikagaku America.

Preparation of Organotypic Cultures. Organotypic cultures of rat hippocampal slices were prepared by a modification of the roller-tube method of Gähwiler (29). Hippocampi from 4-6 day old Sprague-Dawley albino rat pups were sliced into 400 μm parasagittal sections with a wire grid slicer fabricated at Caltech. Freshly cut slices were incubated at 4°C in Gey's Balanced Salt Solution for 30 min then placed on glass coverslips coated with rat-tail collagen. Slices were immobilized in a clot of polymerized Vitrogen (Collagen Corp., Palo Alto CA) applied in 9:1 dilution with 10X minimum essential medium (MEM), then placed in tubes containing 1ml of medium (29) buffered with 25 mM Hepes, pH 7.4. For most experiments, cultures were treated with a mixture of anti-mitotic agents (0.33-1 μM each of uridine, 5-fluorodeoxyuridine and cytosine β-D-arabinofuranoside) for 20 hr on the 4th or 5th day to limit proliferation of glial cells. Cultures grown in this way are two to four cell layers thick.

Assay for CaM Kinase Activity. Before treatments, medium was replaced with a physiological saline solution (PS) containing 125 mM NaCl, 2 mM KCl, 1.25 mM NaH_2PO_4, 2 mM $MgSO_4$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM D-glucose, buffered either with 25 mM Hepes (pH 7.4) or by equilibration with 5% CO_2/95% O_2. Results were identical with either buffer. After treatment, slices were frozen on 60% propylene glycol (Lacco) precooled on dry-ice, scraped from coverslips,

homogenized on ice in 100 µl H-buffer per culture (20 mM Tris-HCL (pH 8), 1 mM imidazole, 2 mM EDTA, 20 mM $Na_4P_2O_7$, 25 mg/L soybean trypsin inhibitor, 1 mg/L leupeptin, 2 mM DTT and 0.1 mM phenylmethyl-sulfonyl floride) in a glass/teflon homogenizer and immediately assayed for kinase activity. H-buffer inhibited phosphatase and kinase activity, thus preserving the autophosphorylation state of endogenous and exogenously added CaM kinase (data not shown).

For assays, homogenate protein (2-4 µg) was added to a solution at 30°C containing 0.2 mg/ml synapsin I, 50 µg/ml calmodulin, 0.5 µM PKI, 13 mM DTT, 6.6 mM $MgCl_2$, 100 µM [γ-^{32}P]ATP (2-3 x 10^3 cpm/pmole), 0.4 mM EGTA and, for assays in the presence of Ca^{2+}, 0.6 mM $CaCl_2$ in a final volume of 30 µl. Reactions were terminated after 15 s by the addition of 15 µl $NaDodSO_4$-stop solution and incorporation of ^{32}P into synapsin was measured as described previously (27,28).

To determine the rate of Ca^{2+}-independent CaM kinase II activity in homogenates in which 100% of the kinase is in the autophosphorylated state, autophosphorylation of the kinase was initiated as described above, except that synapsin I was excluded and, in some cases, ATP was replaced by ATP-γ thiophosphate (ATPγS). After 5 to 90 s of autophosphorylation, synapsin I or synapsin I plus EGTA (final 2 mM) was added directly to each tube and phosphorylation was terminated after 15 sec.

Labeling of CaM Kinase in Organotypic Cultures. Five to ten slice cultures were rinsed twice with phosphate-free Eagle's Basal Medium with Earle's Salts (BME/E) plus 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, supplemented as described for the culture

medium; 29) then incubated for 12-24 hr at 37°C in phosphate-free BME/E with 2-3 mCi/ml carrier free [^{32}P]-H_3PO_4. At the end of the incubation, cultures were rinsed twice with phosphate-free BME/E, frozen, and homogenized. Homogenates were brought to 1% NP-40, cleared by centrifugation at 10,000 g for 1 min, and incubated for 8-12 hours at 4°C with monoclonal antibodies against type II CaM kinase (4A4, 4A11, and 6E9 [14]; 10-20 μg/ml). Immune complexes were precipitated by incubation for 2 hrs with Rabbit anti-mouse immunoglobulin (R-αMIG) and Protein A-Sepharose CL-4B (20 mg/ml). The beads were collected by centrifugation at 10,000g for 2 min, washed 6 times with NET buffer (18) supplemented with 1% (v/v) NP-40 and twice with NET buffer, resuspended in $NaDodSO_4$-stop solution, and fractionated by $NaDodSO_4$-PAGE. Labeled subunits were detected by autoradiography.

Other Procedures. Tryptic phosphopeptide maps were prepared as described previously (22,30). Protein concentrations were measured by the method of Wallace and Partlow (31), which is insensitive to collagen. $NaDodSO_4$-PAGE was performed by the method of Laemmli (32). Calcium measurements with FURA-2 were performed by Wade Regehr and David Tank as described previously (6).

Results

Autophosphorylation of Thr_{286} *In Situ*. Cultures were incubated overnight in $^{32}PO_4$, then homogenized (Methods). The α and β subunits of the kinase were immunoprecipitated, then separated by $NaDodSO_4$-PAGE (Fig. 1B; insert). The α subunit was cut from the gel and digested with trypsin. Tryptic peptides were fractionated by reverse-phase HPLC to generate a phosphopeptide map (Fig. 1).

The mobilities of the four phosphopeptides that contain Thr_{286} are known from previous studies of purified kinase (Fig. 1A and C; 22, 30). Two peaks are generated when a portion of the amino terminal glutamine cyclizes to pyroglutamate. Two additional peaks arise from these parent peaks by an unknown reversible reaction (22). Most of the labeled phosphate incorporated into the α subunit *in situ* was located on Thr_{286} (Fig. 1B). Some labeled phosphate was also recovered in the peptide containing Ser_{314}, a site that is autophosphorylated *in vitro* only when Ca^{2+} is removed following autophosphorylation of Thr_{286} (30).

Ca^{2+}-Independent Kinase Activity *In Situ* . The extent of phosphorylation of Thr_{286} *in situ* cannot be quantified reliably because of losses of material during the chemical procedures used to generate the peptide map. Therefore, we determined the proportion of the kinase in the Ca^{2+}-independent state by measuring the level of Ca^{2+}-independent CaM kinase activity in culture homogenates. Sets of five cultures were homogenized in a cold buffer containing sodium pyrophosphate which inhibits dephosphorylation of the CaM kinase (Methods). CaM kinase activity was then assayed in the presence and absence of Ca^{2+}.

Control experiments established that the activity in the absence of Ca^{2+} measured by our assay represents Ca^{2+}-independent activity of type II CaM kinase. Monoclonal antibodies against the kinase specifically inhibited Ca^{2+}-independent activity in homogenates to the same extent that they inhibit Ca^{2+}-independent activity of purified kinase with synapsin I as substrate (88 $\pm$ 5% versus 97$\pm$ 1%). In addition, Ca^{2+}-independent activity in homogenates decayed in the absence of pyrophosphate, as expected if the activity depends on autophosphorylation of Thr_{286}.

$_{287}$ (Fig. 2). The decay was blocked by okadaic acid, a potent inhibitor of phosphatases 1 and 2A (33). Finally, more than 90% of the phosphorylation of synapsin I by homogenates in the absence of Ca^{2+} occurred at the sites phosphorylated by purified type II CaM kinase (34; data not shown).

After autophosphorylation, the CaM kinase is only partially activated in the absence of Ca^{2+}. Therefore, in order to convert measured ratios of Ca^{2+}-independent to Ca^{2+}-stimulated activity to actual percentages of CaM kinase molecules in the autophosphorylated state, we determined the maximum proportion of Ca^{2+}-independent activity that would be detected in our assay if all of the CaM kinase molecules in a homogenate were autophosphorylated. Aliquots of homogenates were incubated in the presence of Ca^{2+}/calmodulin and ATP for sufficient time to fully autophosphorylate endogenous CaM kinase at $Thr_{286-287}$. After autophosphorylation, the rate of synapsin phosphorylation in the absence of Ca^{2+} reached a maximum of 45 $\pm$ 1 % of the rate in the presence of Ca^{2+}. This maximum percentage was not changed by substitution of ATP-γS for ATP in the autophosphorylation reaction. (Thiophosphorylated proteins are resistant to dephosphorylation by cellular phosphatases [18, 35]). In subsequent experiments, the measured percentage of Ca^{2+}-independent activity to Ca^{2+}-stimulated activity in each homogenate was divided by 0.45 to convert it to the actual percentage of CaM kinase molecules in the autophosphorylated state.

Substantial levels of Ca^{2+}-independent activity were detected in homogenates of slice cultures, of adult forebrain and hippocampus, and of superfused acute slices of adult hippocampus (Table 1). The amount of CaM kinase in the Ca^{2+}-

independent state was highest in homogenates of slice cultures (34%). The mean level in homogenates of forebrains from animals less than 25 days old was about 23%, while in animals older than 25 days it was about 13%. Thus, there appears to be a reduction in the level of autophosphorylation *in situ* of $Thr_{286-287}$ between 5 and 7 weeks of age.

Basal Ca^{2+} concentrations in neurons in hippocampal cultures, measured in saline (PS) equilibrated with 5% CO_2/95% O_2 as described under Methods, ranged from 15 to 43 nM (mean 31 $\pm$ 2 nM, n=15). Therefore, the high proportion of autophosphorylated CaM kinase appears to be maintained even at the usual low basal cytosolic Ca^{2+} concentrations.

Stability of Ca^{2+}-independent kinase *in situ*. The level of Ca^{2+}-independent activity in the cultures was not altered after incubation in 1mM kynurenic acid for 1hr to 2.5 days to block excitatory amino acid receptors, in 100-200 μM 5-phosphono-aminovaleric acid (AP5) to inhibit NMDA-receptors, in 1 μM tetrodotoxin for 2 hrs to inhibit any spontaneous electrical activity, or in serum free medium for twelve days. Similarly, growth of cultures for 2.5 weeks in 100 μM AP5 and 20 mM Mg^{+}, beginning immediately after their preparation, did not reduce the level of Ca^{2+}-independent activity.

Depolarization of neurons in the cultures by the addition of 60 mM K^{+} to the bathing medium produced an average 38% increase (range 14-78%) in Ca^{2+}-independent activity in approximately half of the experiments (8 of 18), and no detectable change in the other experiments. Application of 100 μM NMDA to the cultures had no effect on the level of Ca^{2+}-independent kinase activity after 10-15

seconds (n=11), although the same treatment consistently produced increases in cytosolic Ca^{2+} concentrations that peaked at about 10 seconds (data not shown). After incubation of the cultures for 30 min in 1-2 μM okadaic acid, a phosphatase inhibitor (33), addition of 100 μM NMDA produced only a slight increase in Ca^{2+}-independent kinase activity (9.6 ± 6.3%, n=9). The addition of glutamate, BAY K8644, nifedipine, picrotoxin, or carbachol, in normal or depolarizing salt solution, did not alter the proportion of Ca^{2+}-independent kinase.

In contrast to treatments that affect membrane channels and receptors, application of the membrane permeant protein kinase inhibitor, H7, and the calmodulin antagonist, W7, reduced the steady state proportion of Ca^{2+}-independent kinase *in situ*. Exposure of cultures to 300 μM H7, which has a K_I for the CaM kinase of 30-50 μM (data not shown), reduced the proportion of Ca^{2+}-independent kinase in homogenates by 35% (range, 10-52%; n=7) after 30 minutes. HA1004 (300 μM), which has a K_I for CaM kinase of 16 μM but is less membrane permeable, reduced the proportion by 26% (range, 22-28%; n=3). W7 (100 μM) produced an average 60% reduction (range, 48-72%; n=2), whereas W5 (100 μM), a much less potent antagonist, produced a 29% reduction (range, 28-29%; n=2). None of these treatments affected total Ca^{2+}-stimulated kinase activity. The proportion of Ca^{2+}-independent kinase was also substantially reduced by removal of Ca^{2+} from the external medium. Two hours after removal of Ca^{2+}, the proportion of Ca^{2+}-independent kinase in homogenates was reduced by 60 to 70% (Fig. 3A). The decline was complete after 30 minutes with a half time of approximately 5-7 minutes (Fig. 3B). Phosphopeptide mapping of CaM kinase labeled *in situ* showed

that this down-regulation of Ca^{2+}-independent activity is correlated with a significant reduction in ^{32}P incorporation into Thr_{286} (see Appendix D). Taken together, these results suggest that continuing Ca^{2+}-stimulated autophosphorylation of the kinase is required to maintain the level of Ca^{2+}-independent activity for longer than 20 to 30 minutes *in situ.*

The steady state proportion of Ca^{2+}-independent kinase was increased by application of okadaic acid, a membrane permeant inhibitor of phosphatases 1 and 2A. Incubation of the cultures with 1-2 μM okadaic acid for 30 minutes increased the proportion of Ca^{2+}-independent kinase by 40 ± 9 % (n=8). Thus, the proportion of Ca^{2+}-independent kinase is dynamic and depends upon the balance between rates of autophosphorylation and phosphatase activity.

Discussion

We have shown that Thr_{286} is the major site autophosphorylated on type II CaM kinase *in situ* in organotypic cultures of hippocampal neurons. Approximately one third of the CaM kinase molecules are in the Ca^{2+}-independent state when the basal Ca^{2+} concentration in the neurons is 15-45 nM. This surprisingly high proportion of Ca^{2+}-independent kinase appears to be maintained by a dynamic steady state between autophosphorylation and dephosphorylation of Thr_{286}. Treatment of the neurons with the protein kinase inhibitor H7 or the calmodulin antagonist W7, reduces the basal proportion of Ca^{2+}-independent kinase, while treatment with the phosphatase inhibitor, okadaic acid, increases it. Removal of external Ca^{2+} also reduces basal Ca^{2+}-independent kinase, implying that continuous Ca^{2+}-dependent autophosphorylation is necessary to maintain the basal Ca^{2+}-independent activity for

longer than 20-30 minutes (Fig. 3B).

The high basal proportion of Ca^{2+}-independent activity is also found in homogenates of freshly dissected rat forebrain and hippocampus. Dissected brain regions were homogenized rapidly under conditions that preserve the endogenous protein phosphorylation state and the homogenates were assayed immediately. Therefore, it is likely that the measured levels of Ca^{2+}-independent activity are a reasonably accurate reflection of the autophosphorylation state of the kinase *in vivo*, although we cannot rule out changes in the levels during dissection. The drop from 23% Ca^{2+}-independent kinase to 13% between postnatal days 23 and 26 suggests a developmental change in expression of a protein phosphatase or perhaps in Ca^{2+}-mobilization or buffering systems. Similarly, the higher level of Ca^{2+}-independent kinase in cultured hippocampal neurons compared to intact tissue may reflect, in part, a difference in the balance of processes that affect the equilibrium autophosphorylation state.

Phosphorylation of CaM kinase II in intact cells or synaptosomes has been demonstrated previously. Addition of serum to a fibroblast cell line stimulated phosphorylation of type II CaM kinase at serine residues (36). Depolarization of isolated synaptosomes with high K^+ enhanced phosphorylation of threonine in the CaM kinase and generated transient Ca^{2+}-independent activity (37). Fukanaga et al. (38) reported that, in cultured cerebellar granule neurons, under basal conditions, 4-7% of the total kinase molecules are in the Ca^{2+}-independent state. In contrast to our results with hippocampal neurons, brief depolarization of the cerebellar neurons with high K^+ reliably produced a transient increase in the proportion of Ca^{2+}-

independent CaM kinase to about 13% of total kinase molecules. Thus, not only do cerebellar neurons in culture have a much lower basal proportion of Ca^{2+}-independent kinase than hippocampal neurons, but this proportion is more readily altered by brief changes in Ca^{2+}-concentration.

The high, stable proportion of Ca^{2+}-independent CaM kinase in hippocampal neurons indicates that previous hypotheses about the function of the CaM kinase and its activation by autophosphorylation may require revision. For example, the bulk of CaM kinase within hippocampal neurons does not behave as predicted by the model for memory storage proposed by Lisman and Goldring (18, 39, 40). However, it is possible that the neurons contain a small pool of CaM kinase that is sensitive to regulation by NMDA receptors and behaves in a switch-like fashion, as predicted by their model. We may have been unable to detect large changes in autophosphorylation of such a pool with our present methods because of the high background of Ca^{2+}-independent kinase. Nevertheless, our results suggest that a different but important physiological role for the autophosphorylation mechanism in hippocampal neurons may be to keep a substantial portion of the CaM kinase molecules (13-34%) active at low Ca^{2+} concentrations. The level of basal CaM kinase activity in the cytosol of hippocampal neurons predicted by kinetic equations is considerably less than 1% in the absence of activation by autophosphorylation, assuming a basal Ca^{2+} concentration of 50 nM, a calmodulin concentration of 50 μM, four Ca^{2+}-binding sites on calmodulin with K_Ds of 1 μM, and a CaM kinase concentration of 30 μM. The high concentration of CaM kinase in hippocampal neurons (14) may enhance its rate of activation by the few molecules of calmodulin

with four bound Ca^{2+} ions at basal Ca^{2+}. Furthermore, a low concentration of basal phosphatase activity may allow each activation event to produce a long-lived autophosphorylated kinase holoenzyme. This mechanism would permit rapid regulation of kinase activity, both downward by activation of phosphatases, and upward by elevations in Ca^{2+} concentration. However, our inability to induce an increase in the overall proportion of Ca^{2+}-independent kinase by application of pharmacological agents to hippocampal neurons suggests that if such regulation occurs in these neurons it is likely to be highly localized.

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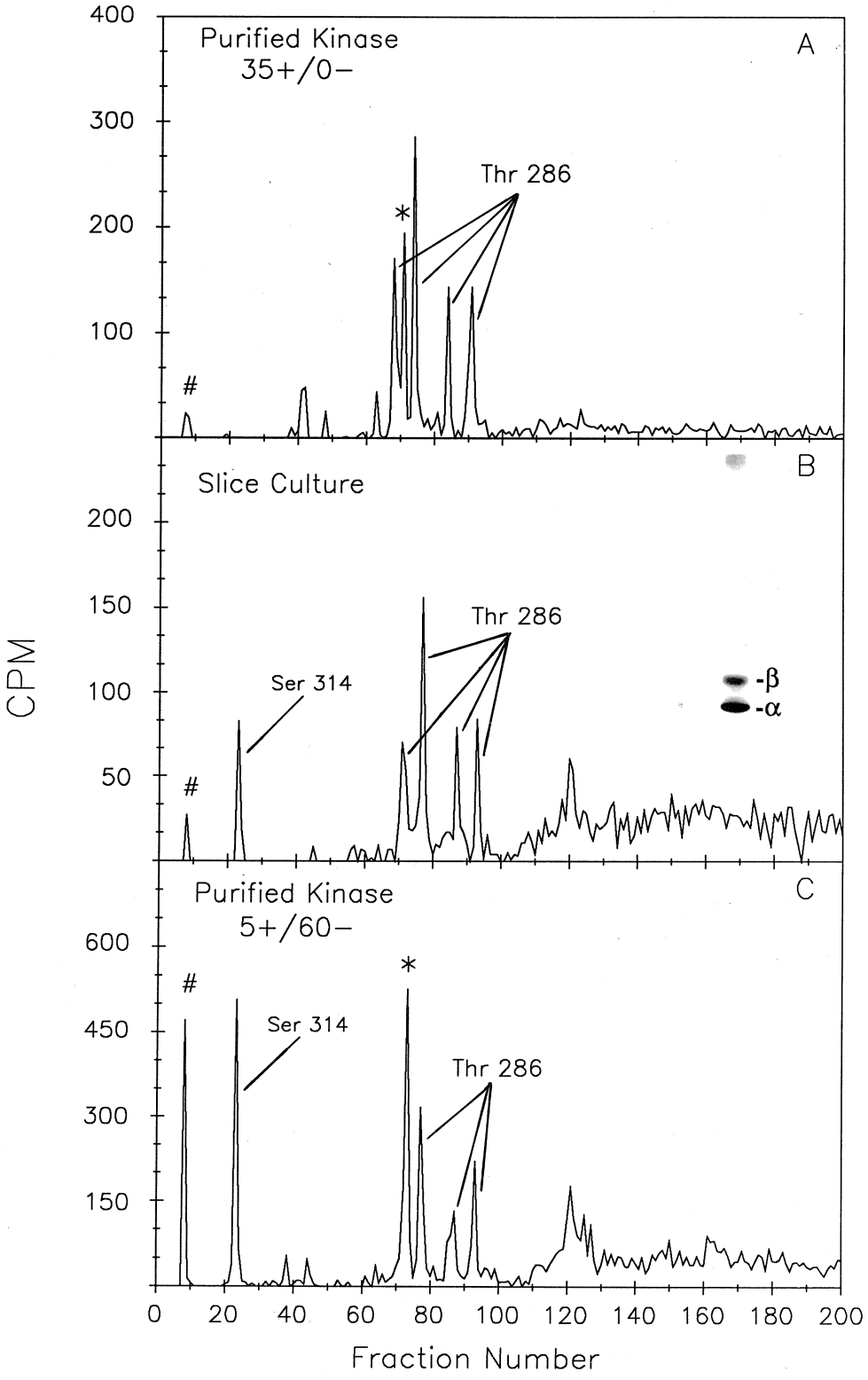
Table 1. Proportion of CaM Kinase Autophosphorylated *In Situ* in Cultures and in Tissues

Tissue Homogenate	N	% Ca^{2+} Independent Activity	% of Total CaM Kinase in Ca^{2+} Independent State
Maximally autophosphorylated homogenates	10	45 ± 1	100
Hippocampal cultures (2-8 weeks in culture)	44	15.5 ± 0.6	34 ± 1
Forebrain (4-24 days old)	12	10.5 ± 0.3	23.3 ± 0.9
Hippocampus (5/6 days old)	2	9.0 ± 0.2	19.8 ± 0.3
Forebrain (27-72 days old)	11	6.0 ± 0.3	13.2 ± 0.7
Hippocampus (25-57 days old)	4	5.8 ± 0.5	13 ± 1
Acutely prepared hippocampal slices (40-70 days old)	8	4.0 ± 0.7	9 ± 2

Cultures and tissues were homogenized and type II CaM kinase activity was measured in the homogenates as described under Methods. The percent Ca^{2+}-independent activity was calculated for each homogenate, then divided by the percent Ca^{2+}-independent activity in maximally autophosphorylated homogenates (45%) to calculate the percent of total CaM kinase in the Ca^{2+}-independent state (see Methods).

Figure 1. Tryptic Phosphopeptide Map of the α Subunit of Type II CaM Kinase Phosphorylated *In Situ* in Organotypic Hippocampal Cultures. Purified kinase holoenzyme was autophosphorylated *in vitro*, as described previously (22, 30) either in the presence of Ca^{2+} for 35 s (A) or in the presence of Ca^{2+} for 5 s, then in the absence of Ca^{2+} for an additional 60 s after Ca^{2+} had been chelated by EGTA (C). Labeled α subunit was isolated from culture homogenates (Methods). An autoradiogram of labeled kinase immunoprecipitated from the homogenate is shown as an inset in (B). Tryptic peptide maps were prepared as described under Methods. In all maps, phosphopeptides containing Thr_{286} appear as a pair of split peaks (22). The N-terminal peptide, which contains a slowly autophosphorylated threonine residue is labeled with an asterisk (*; 30). In (C), this peak has obscurred the leading peak of the first Thr_{286} doublet. Also in (C), several peaks are visible that contain sites autophosphorylated only after the removal of Ca^{2+}. These sites include Ser_{314}, Thr_{305}, and a peptide containing an unidentified site that elutes in the void volume (β; 30). Similar results were obtained in three experiments.


Purified Kinase
35+/0−
A
Thr 286
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Slice Culture
B
Thr 286
Ser 314
-β
-α
#
Purified Kinase
5+/60−
C
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Ser 314
Thr 286
CPM
Fraction Number
400
300
200
100
150
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600
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180
200

Figure 2. The Effect of Phosphatase Inhibition on the Decay of Ca^{2+}-Independent CaM Kinase Activity in Homogenates of Organotypic Hippocampal Cultures.

Four sets of 5 hippocampal cultures were frozen and homogenized as described under Methods. Sodium pyrophosphate was omitted from the homogenization buffer for two sets, one of which contained 1 μM okadaic acid. CaM kinase activity was determined immediately in the absence and presence of calcium. Aliquots of each homogenate were incubated at 20°C for the times indicated, then placed on ice and CaM kinase activity was determined again. ( ), Ca^{2+}-independent activity in homogenates with sodium pyrophosphate; ( ), without sodium pyrophosphate, and ( ) with okadaic acid. Error bars indicate S.D.. Ca^{2+}-independent activity also decayed more rapidly in the absence of sodium pyrophosphate in homogenates of 35 day old rat hippocampi at 0°C. ( ), with sodium pyrophosphate; ( ), without sodium pyrophosphate. Total CaM kinase activity did not change during the incubations. Each point represents quadruplicate assays.

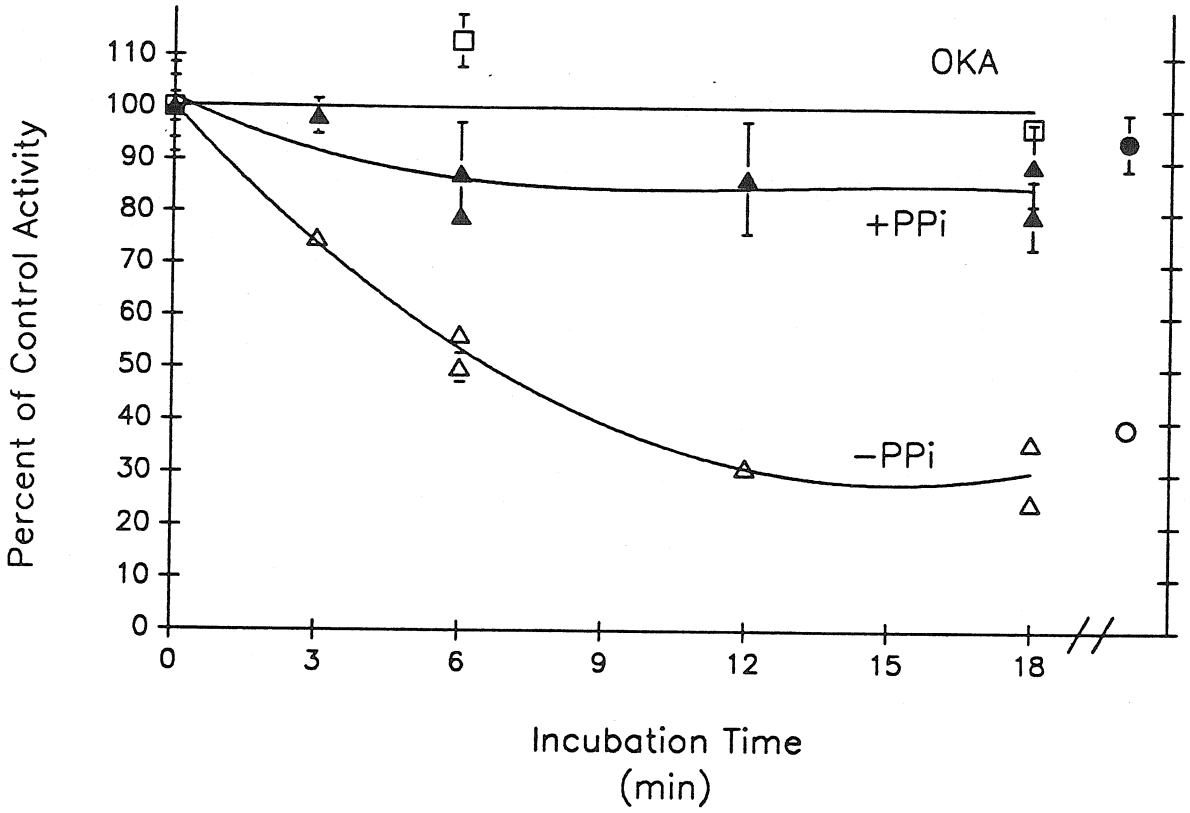
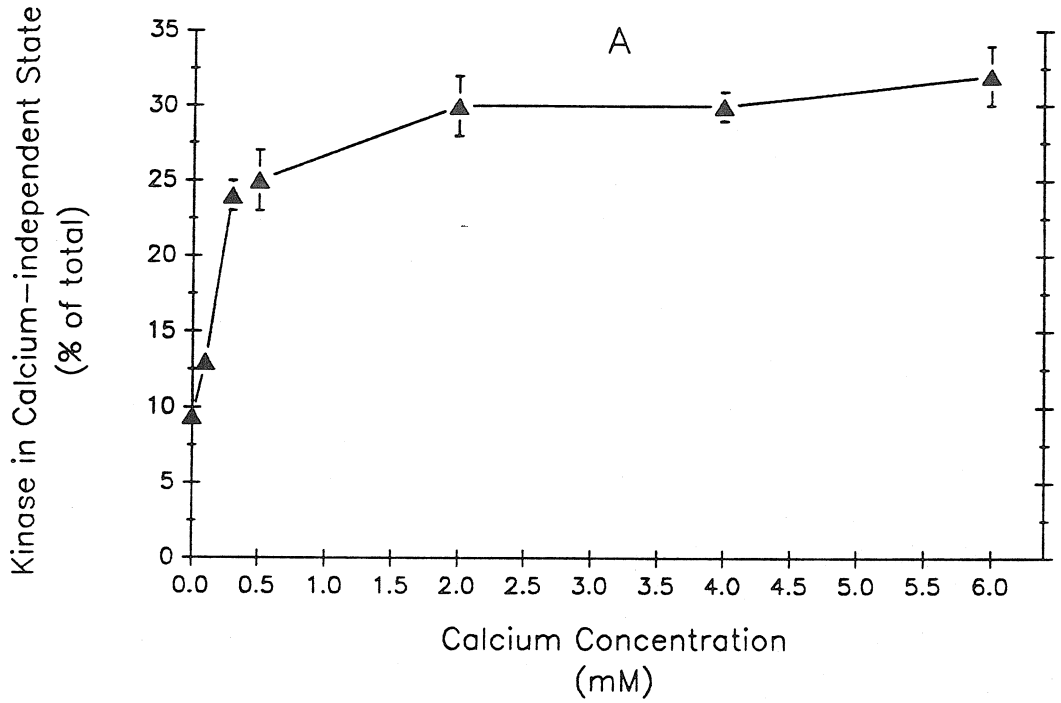
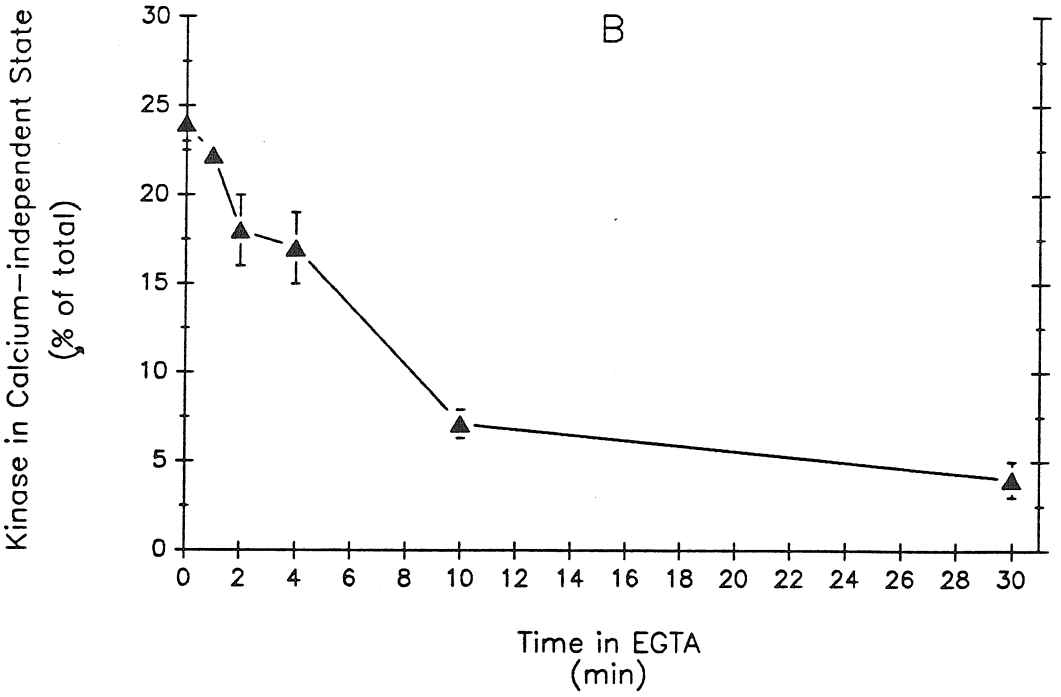

Percent of Control Activity
110
100
90
80
70
60
50
40
30
20
10
0
0
3
6
9
12
15
18
OKA
+PPi
−PPi
Incubation Time
(min)

Figure 3. Effect of External Calcium Concentration on Ca^{2+}-Independent CaM Kinase Activity *In Situ*.

(A) Cultures were equilibrated in a salt solution with the indicated concentration of added calcium for 2-3 hr at 37°C then frozen and homogenized as described under Methods. Ca^{2+}-independent kinase was determined as a percentage of total kinase as described under Methods. (B) Cultures were placed into a salt solution containing 200 μM EGTA at 37°C for the indicated times, after which each culture was frozen and homogenized. The percentage of CaM kinase in the Ca^{2+}-independent state was determined as described under Methods. Changes in external Ca^{2+} concentration had no effect on total CaM kinase activity. Each point represents the mean ± S.E.M. of 2-4 separate experiments.


A
Kinase in Calcium-independent State
(% of total)
35
30
25
20
15
10
5
0
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5 5.0 5.5 6.0
Calcium Concentration
(mM)


B
Kinase in Calcium-independent State
(% of total)
30
25
20
15
10
5
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
Time in EGTA
(min)

Chapter 3

Immunocytochemical Characterization of Hippocampal Slice Cultures: Localization of Autophosphorylated Type II CaM Kinase.

Introduction

Previous studies of the Type II CaM kinase have demonstrated several characteristics of this enzyme which suggest it could play an important role in the regulation of neuronal activity (reviewed in Kennedy *et al.*, 1987; Colbran *et al.*, 1989). Neuronal CaM kinase is found in several brain regions, but is most concentrated in the hippocampus where it constitutes as much as 2% of the total protein (Erondu and Kennedy, 1985). The kinase is localized in the pyramidal and dentate granule cells within the hippocampus where it associates with postsynaptic densities (Kennedy *et al.*, 1983; Kelly *et al.*, 1984; Appendix C). These neuronal cell types display several forms of activity-dependent modifications of synaptic efficacy. One of the most studied forms of such synaptic plasticity is long-term potentiation (LTP; reviewed in Kennedy, 1989). The initiation of LTP in the CA1 pyramidal cells of the hippocampus is dependent on a rise in postsynaptic Ca2+ mediated by activation of the NMDA subtype of glutamate channel . Recent studies using inhibitory peptides showed that activation of the CaM kinase in the postsynaptic cell is also a requirement for the initiation of LTP (Malinow *et al.*, 1989). Activation of protein kinase C also appears to be important. While postsynaptic activation of CaM kinase is clearly necessary for producing LTP, the function of the enzyme is unknown.

Although the role of the kinase in the postsynaptic cell is not understood, progress has been made toward elucidating a presynaptic function of the kinase. CaM kinase appears to be capable of regulating the availability of synaptic vesicles for fusion with the presynaptic membrane via phosphorylation of the vesicle

associated protein synapsin I. Phosphorylation of synapsin I by the type II CaM kinase, but not the cAMP-dependent or type I kinases, reduces the affinity of synapsin I for synaptic vesicles. Studies of synaptic release at nerve terminals suggest that the phosphorylation of endogenous synapsin I by CaM kinase enhances the fusion of vesicles while the introduction of non-phosphorylated synapsin decreases release (Llinas *et al.*, 1985). CaM kinase may be involved in some forms of synaptic plasticity, such as post-tetanic potentiation, by virtue of its effect on synapsin I. However, the effect on synapsin I phosphorylation can only explain presynaptic actions of the CaM kinase.

Determining the postsynaptic role of the CaM kinase should clarify the mechanism of LTP as well as provide an understanding of the importance of this enzyme to the regulatory processes of neurons. We addressed these issues by studying the regulation of CaM kinase in living neurons within cultured hippocampal slices (Chapter 2). Biochemical studies revealed that, under conditions of basal Ca^{2+}, approximately 30% of the kinase is in the Ca^{2+}-independent form within the neurons. This high basal level of Ca^{2+}-independent kinase was maintained by continued autophosphorylation at Thr_{286}. Therefore, the autophosphorylation mechanism produced a much higher level of CaM kinase activity at basal Ca^{2+} than would be predicted based on the affinity of the native (nonphosphorylated) enzyme for Ca2+/calmodulin (see Chapter 1 Discussion).

A high level of CaM kinase activity at basal Ca^{2+} concentrations contradicts hypotheses about the behavior of the enzyme as a "molecular switch" (Miller *et al.*, 1986; Lisman and Goldring, 1988) which would become constitutively active only

after prolonged exposure to increased intracellular Ca^{2+} concentrations. Not only is the proportion of Ca^{2+}-independent kinase high at resting levels of Ca^{2+}, but it is also quite stable to pharmacologically induced increases in intracellular Ca^{2+}. This might, however, reflect limitations of the biochemical assays.

The biochemical studies do not indicate where the autophosphorylated, Ca^{2+}-independent kinase is located within the neurons. It is possible that a small pool of kinase, located in the postsynaptic density (PSD) and/or distal dendrites, is regulated in a switch-like manner by pharmacologically-induced Ca^{2+} fluxes. However, the biochemical assays may not be sensitive enough to detect such changes.

We began an immunocytochemical characterization of the hippocampal slice cultures to determine the location of the phosphorylated form of the kinase, and address the possibility of regional differences in its regulation. This paper describes the examination of synaptic organization in the hippocampal cultures by electron microscopy and immunocytochemistry. It also describes the use of monoclonal antibodies specific to the phosphorylated form of the type II CaM kinase to visualize the location of the Ca^{2+}-independent form of the enzyme.

Methods

Cell Culture. Long-term cultures of rat hippocampal slices were established and maintained as described previously (Gahwiler, 1984).

Monoclonal Antibodies. The monoclonal antibody 6G9 has been described previously (Erondu and Kennedy, 1985). The antibody 22B1 was obtained by Bruce Patton by using phosphorylated synthetic peptide as antigen. Mice were immunized

with phosphorylated peptide linked to keyhole limpet hemocyanin (KLH). The synthetic peptide represented a stretch of primary sequence from the α subunit of the kinase containing the phosphorylation site at Thr_{286}, the site that controls Ca^{2+}-independent activity. After several rounds of immunization, the spleens were removed and their cells fused with myelomas by standard techniques. The resulting hybridomas were screened by solid phase ELISA for reactivity against the phosphorylated and native forms of the kinase. Clones producing antibodies that reacted preferentially with the phosphorylated kinase were tested further for their specificity on western blots. Several clones were isolated that produced antibodies specific for phosphorylated kinase subunits on western blots. These hybridomas were subcloned and one of them, 22B1, was used for the immunocytochemical localization of the phosphorylated kinase in sections of organotypic cultures. The antibody 22B1 was chosen because of its high affinity and specificity for phosphorylated kinase (see Bruce Patton, Caltech graduate thesis).

Immunocytochemistry. Organotypic cultures (2.5-8 wks *in vitro*) were fixed in 0.1M sodium phosphate buffer (pH 7.2) with 4% (w/v) paraformaldehyde and 0.2% (w/v) glutaraldehyde for 1 hr at 4°C. Fixed cultures were then washed twice for 5 min each with phosphate buffer and post-fixed in 0.05% osmium tetroxide for 60 min on ice. After a second rinse in phosphate buffer, the cultures were dehydrated in a graded ethanol series (2 incubations for 5 min each in 70%, 95%, and 100% ethanol). Dehydrated cultures were equilibrated on ice in EMBED 812 resin without activator by one incubation overnight followed by two additional incubations for 60 min each. Finally, the cultures were equilibrated at room temperature (RT)

in complete resin by one incubation for 1 to 4 hr, followed by one incubation overnight and one incubation for 1 hr. The resin was hardened at 60°C for 24 hr and the embedded slice cultures were mounted onto chucks. The cultures were sectioned at 2 μm on a Reichardt ultramicrotome and mounted on subbed slides for staining.

Prior to staining, plastic sections were etched for 10 min at RT with 67% (v/v) absolute methanol, 33% (v/v) propylene oxide and 13.3% (w/v) potassium hydroxide. Reactive aldehyde groups were blocked by incubation in 0.1 M glycine (pH 7.2) for 40 min. The sections were rinsed with distilled water and the remaining aldehyde groups reduced by incubation in 1% (w/v) sodium borohydride for 3-4 min. After brief rinses with distilled water and PBS/Triton (20 mM sodium phosphate [pH 7.2], 0.45 M NaCl, 0.1% [v/v] Triton X-100), the etched sections were incubated overnight at 4°C with primary antibody. Primary antibodies included a monoclonal antibody specific for the type II CaM kinase (6G9, Erondu and Kennedy, 1985), a monoclonal antibody specific for the phosphorylated form of the kinase (22B1), or a polyclonal rabbit sera against synapsin I (a gift from Dr. Louis Reichardt). Antibodies 6G9 and 22B1 were purified form ascites fluid by ammonium sulfate precipitation and dialysis. For primary incubations, 6G9 was diluted 250 to 500-fold in PBS-Triton containing 5% (v/v) horse serum while 22B1 was diluted 2000-fold. The anti-synapsin I serum was diluted 250-fold in PBS/Triton with 5% (v/v) goat serum. After the primary incubation, sections were washed three times for 10 min each with PBS/Triton containing 5% normal serum (horse or goat), then incubated 1 hr at RT with a 1/100 dilution of biotinylated

horse anti-mouse IgG or goat anti-rabbit IgG in PBS/Triton with 1% normal serum. The sections were then washed and incubated in a 1/200 dilution of avidin-peroxidase in PBS/Triton for 1 hr at RT. After a rinse with Tris-buffered saline (TBS; 0.1M Tris-HCL [pH7.5], 0.9% [w/v] NaCl), sections were equilibrated with TBS, 0.025% (w/v) $CoCl_2$, 0.02% (w/v) $NiNH_4SO_4$ for 15 min. The sections were then rinsed with 0.1 M phosphate buffer (pH 7.2) and incubated with 0.5 mg/ml DAB (diaminobenzedine, Sigma) in phosphate buffer for 10 min. Finally, the sections were developed by incubation in DAB, 0.025% (v/v) hydrogen peroxide for 5-15 min, rinsed in distilled water, dried, and coverslipped with Gurr mounting solution. The sections were then photographed on an Olympus Vanox Photomicroscope.

Results

Structure. The overall cytoarchitecture of the cultures closely resembled that of the adult hippocampus, even after several weeks *in vivo* (Figure 1A). The width of the cell body layers was variable, especially in CA1 and dentate. Previous studies of hippocampal slice cultures describe them as monolayers (Gahwiler, 1988), however, our cultures remain 3 to 4 cell layers thick. This increase in the number of viable cells may account for some the spreading of the cell body layers. Electron micrographs (Figure 1B) show the fine synaptic structure that developed in the cultures. Dendritic spines are clearly visible on pyramidal cells within the cultures. Furthermore, these spines contain postsynaptic densities (PSDs), which are cytoskeletal specializations characteristic of many mature CNS neurons.

Localization of Synaptic Molecules. The immunocytochemical localization of synapsin I is nearly identical to that observed for adult tissue (Figure 2A). Heavy staining was observed in and around the cell body layer in CA3. At high magnification, this staining was comprised of large intense puncta that were described in previous studies of adult brain (de Camilli *et al.*, 1983). These large puncta probably represent the mossy fiber terminals of the dentate granule cells that normally synapse on the proximal portion of the apical dendrites of the CA3 pyramidal cells. Considerable staining was also seen in the molecular layers of both CA1 and CA3. The staining in the molecular layer was also granular in appearance, however, the individual puncta were much smaller than that observed near the CA3 pyramidal cell bodies. This finer grained staining is also seen in the molecular layers of adult hippocampus, where it presumably represents the smaller synaptic terminals of the asssociational-commisural fibers. The only deviation from the synapsin I staining pattern of the adult hippocampus seen in the cultures was the presence of large puncta near the proximal apical dendrites of CA3 pyramidal cells.

The staining pattern of staining obtained with 6G9 was also the same as that described for the adult hippocampus. The cell body layers were all heavily stained, including the dentate granule cells and CA1 and CA3 pyramidal cells (Figure 2B, Figure 3 A and C, Figure 4 A and C, and Appendix C). The staining was primarily cytoplasmic and excluded the nuclei. Dendrites were not distinguishable for the dentate granule cells, however, there appeared to be diffuse staining in the hilus (see Appendix C). The CA3 and CA1 pyramidal cells often had clearly distinguishable dendrites which could occasionally be traced for a considerable

distance through the plane of section (Figure 2B inset, Figure 3C, and Figure 4A and C). When fully in the plane of section, apical dendrites of the CA3 pyramidal cells appeared to stain most heavily in close apposition to the membrane. In many instances one could distinguish puncta that seemed to line the dendrites (Figure 2B inset). It was impossible to determine if this punctate dendritic staining was pre- or postsynaptic with the light microscope. In addition to the intense staining of the neuronal cell bodies and dendrites, there was significant staining in all of the molecular layers within the cultures. This staining of molecular layers with 6G9 was also observed in adult tissue and probably represents the presence of synaptic terminals in these regions.

In order to compare the localization of the autophosphorylated, Ca^{2+}-independent form of the kinase to its overall distribution in the cultures (as visualized with 6G9) we stained sections with the phospho-kinase-specific monoclonal antibody 22B1. Figures 3 and 4 show a detailed comparison of the localization of the α subunit (6G9) and the phosphorylated kinase (22B1). As these figures demonstrate, the general localization of the phosphorylated kinase was the same as the overall distribution of the enzyme. The three readily distinguishable neuronal cell types in the hippocampus, granule cells, and CA1 and CA3 pyramidal cells, all reacted with 22B1 (Figure 3 B and C, Figure 4 B and C). The relative intensity of the reactivity between these cell layers matched that observed with 6G9. As with 6G9, 22B1 stained the molecular layers throughout the culture. Overall, the staining with 22B1 completely overlapped that of 6G9 but appeared less intense.

At higher magnification, the 22B1 reactivity appeared to be localized in the cytoplasm. With both 22B1 and 6G9 one could often see a fibrous pattern to the staining in the cell bodies. Staining of the dendrites with 22B1 also displayed the localized concentrations observed with 6G9. Thus, the staining with the phospho-kinase-specific monoclonal 22B1 appears to be the same as that seen with 6G9, both at low and high magnification.

Discussion

The preservation of the cytoarchitecture in the hippocampal slice cultures is consistent with previous studies. This enhances the usefulness of these cultures for studies that require the ability to recognize specific hippocampal cell types and synaptic pathways. Similarly, the presence of dendritic spines and PSDs indicates that the synaptic structures within these cultures develop as they would *in vivo*. This is an important consideration for studies of synaptic regulation, which require the presence of mature synapses.

In addition to having the principle cell types, organization, and synaptic structure normally found in adult tissue, staining of cultures for the presence of synapsin I indicates that they express specialized synaptic molecules as well. The staining pattern observed with the anti-synapsin I sera shows that the cultures express this protein in the same areas where it is normally found in the adult.

The large puncta of staining surrounding the CA3 pyramidal cells probably reflect the presence of mossy fiber terminals from the dentate granule cells. This fiber pathway, as visualized by Timms silver staining, has previously been shown

to develop in hippocampal slice cultures *in vitro* (Gahwiler, 1984). The putative mossy fiber terminals on the proximal basal dendrites of the CA3 pyramidal cells is seen only in the cultures. This probably results from "inappropriate" projections of the dentate granule cells that normally would not occur, or would be displaced during development *in vivo.*

The staining observed with 6G9 shows that synapsin I is not the only synaptic molecule expressed in the hippocampal cultures. The abundance of 6G9 staining is consistent with our previous biochemical studies that indicated that the CaM kinase is expressed at an adult level in the cultures. The immunocytochemical data show that the kinase is expressed in the same cell types and with the same cellular localization normally found *in vivo.* The resolution of the light microscope is insufficient to determine if the kinase is concentrated in PSDs, however, the presence of these structures combined with the high expression of α subunit makes it seem likely.

Our biochemical analysis of the CaM kinase in hippocampal cultures indicated that approximately one third of the enzyme was in its autophosphorylated, Ca^{2+}-independent form. Unfortunately, the biochemical assays were unable to show where the Ca^{2+}-independent kinase was located in the cultures. Therefore, we were unable to determine if all of the kinase in only some of the cells was phosphorylated or a portion in all of the cells was phosphorylated. Furthermore, we could not determine if there was heterogeneity in the level of kinase phosphorylation between cell types within the hippocampus.

The results of the immunocytochemical studies using 22B1 answered several

of the questions concerning the distribution of the phosphorylated kinase. The antibody clearly stained all of the neuronal cell types that stain with 6G9. Therefore, the phosphorylated CaM kinase is not restricted to a subpopulation of the neurons that express the enzyme. Furthermore, since the relative intensity of staining between the neuronal cell types follows the same pattern seen with 6G9, it appears that there is no gross difference between cell types in the proportion of kinase phosphorylated. However, the techniques employed in these studies are not sensitive enough to detecte subtle differences in the proportion of phosphorylated enzyme (see below).

The differences in staining between neurons of the same type also appear to be small. Whenever noticeble differences occurred, they were present in both 6G9 and 22B1 stained sections. This suggests that some heterogeneity in kinase expression may occur between neurons. The high magnification images of 22B1 staining indicate that the phosphorylated kinase is present throughout the cytosol in both the cell bodies and dendrites of pyramidal cells. Therefore, there is no evidence that the phosphorylated enzyme is sequestered within a particular region of the neurons. Taken together, these data indicate that wherever the kinase is located in the cultured hippocampal neurons, a significant proportion of the enzyme is phosphorylated.

The results obtained with 22B1 help explain our previous biochemical findings. It appears that the biochemical measurements of Ca^{2+}-independent CaM kinase activity reflect the fact that phosphorylated kinase is present in all the neurons that express the enzyme. Therefore, basal free Ca^{2+} concentrations are

sufficient to maintain a substantial portion of the kinase in the autophosphorylated state throughout the hippocampal neurons. This implies that there is significant constituitive CaM kinase activity in the cytosol of these neurons.

The immunocytochemical techniques used in this study are not particularly sensitive to subtle regional differences in the phosphorylation state of the kinase. Such measurements, however, would be valuable in determining if local regulation of autophosphorylation occurs in these cells. We have recently developed a double immunocytochemical staining paradigm that should greatly enhance our ability to detect small local changes in the autophosphorylation of the kinase.

A purified polyclonal rabbit sera specific for nonphosphorylated kinase is used in conjunction with 22B1. The primary antibodies are visualized separately by using species-specific secondary antibodies directly coupled to fluorescent markers (rhodamine [red] and fluorescein [green]). The nonphosphorylated and the phosphorylated kinase can be imaged independently by exciting one or the other fluorescent marker. A double exposure with both markers shows where the two overlap (yellow). Any change in phosphorylation will be amplified in the double image since it represents the difference signal of the two stains. This difference signal should provide considerably more sensitivity than either antibody alone.

Preliminary results using this double staining protocol suggest that there may be subtle differences in the degree of kinase autophosphorylation found in the various regions of the cultured hippocampal slices. An attempt to alter the normal staining pattern by removing extracellular Ca^{2+} from the cultures (a treatment known to dramatically decrease the level of Ca^{2+}-independent CaM kinase activity; see

Chapter 2) resulted in a striking decrease in the phosphorylation of the kinase as visualized by double staining (data not shown). This result demonstrates the potential usefulness of the double staining technique in testing for local regulation of autophosphorylation within hippocampal neurons.

The combination of biochemical and immunocytochemical techniques used to date have revealed that CaM kinase activity is regulated by autophosphorylation throughout the neurons of cultured hippocampal slices. Furthermore, the development of antibodies specific for the phosphorylated kinase has enhanced our ability to assess the importance of this regulation to the physiology of these cells.

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Figure 1. Cytoarchitecture and Ultrastructure of Organotypic Hippocampal Cultures.

(A) Slice cultures (2-4 wks *in vitro*) were fixed in Pipes buffered saline containing 2% formaldehyde, stained with cresyl violet, cleared, and mounted. Areas CA1, CA3, and the dentate gyrus (DG) are labeled. The final magnification is approximately 20 X. (B and C) Cultures were fixed in 2% glutaraldehyde for 45 min, stained with 1% osmium tetroxide, embedded, and sectioned for electron microscopy, in the lab of Dr. Dennis Landis, by standard techniques. Presynaptic terminals (Pre), and postsynaptic densities within dendritic spines (arrowheads) are indicated. Scale bars represent 400 nm (B) and 200 nm (C).

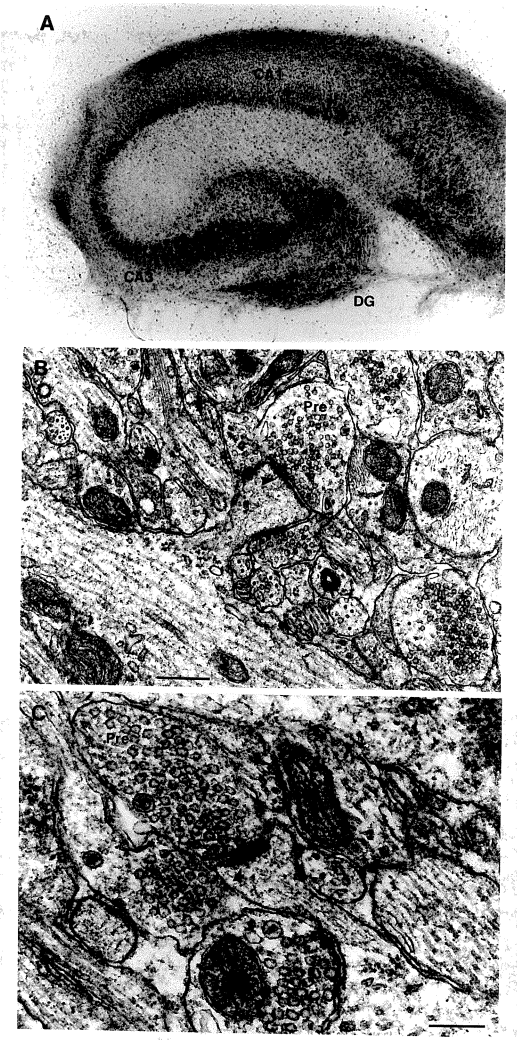

A
CA1
CA3
DG
B
Pre
C
Pre

Figure 2. Immunocytochemical Localization of Synapsin I and Type II CaM Kinase in Organotypic Hippocampal Cultures.

Tissue sections (2 um) were prepared and stained as described under Methods. (A) The section was incubated with a 1/250 dilution of rabbit anti-synapsin I antisera. Antibody binding was visualized by secondary labeling with peroxidase conjugated anti-rabbit antibodies. Punctate staining similar to that previously observed in sections of adult tissue (DeCamilli et al., 1983) is visible around cell bodies and in the molecular layers. Finer grained punctate staining was prevalent in the molecular layers of CA1. Large puncta along proximal dendrites in CA3 (shown) are characteristic of the staining of mossy fiber terminals. Final magnification is 125 X. (B) The section was incubated with a 1/500 dilution of 6G9, a monoclonal antibody against the α subunit of the type II CaM kinase. Antibody binding was visualized as in A. The overall pattern of staining was similar to that previously observed in adult tissue (Ouimet et al., 1984; Erondu and Kennedy, 1985). The antibody stained cell bodies and dendrites of pyramidal cells in both CA3 (shown) and CA1 as well as dentate granule cells. In CA3 neurons with well delineated basal dendrites, punctate staining could often be visualized along the dendrites (insets) suggesting high local concentrations of kinase that might be postsynaptic densities. These local concentrations of kinase have not been visible in thicker sections (Quimet et al., 1984; Erondu and Kennedy, 1985). Final magnification is 250 X. (C and D) Control sections were incubated with either preimmune rabbit sera (C) or ascites fluid from the parent myeloma of hybridoma 6G9 (D) at the appropriate dilution. Final magnifications are 125 X and 200 X.

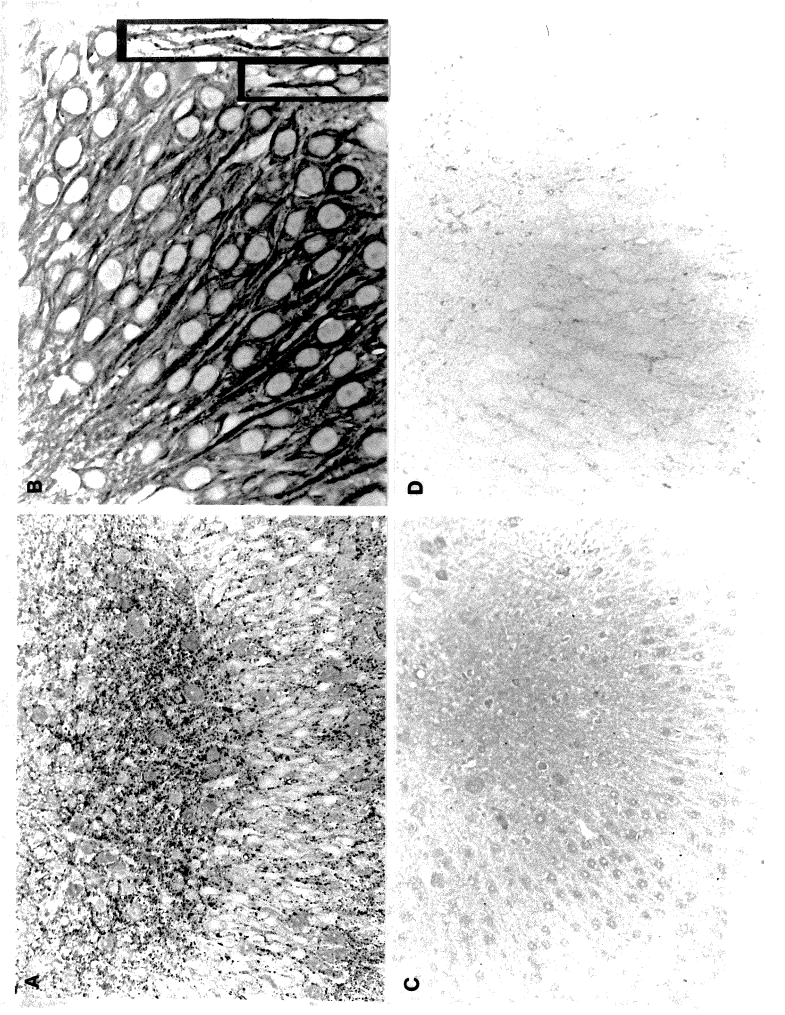

A
B
C
D

Figure 3. Comparison of Total Kinase and Phosphorylated Kinase Localization within Dentate and CA1 Regions of Hippocampal Cultures.

(A and C) The section was processed as described under Methods and Figure 2. The primary incubation was in a 1/250 dilution of 6G9. The cytosol of the dentate granule cell bodies was darkly stained (A), however the dendrites of these cells could not be distinguished. The cytosol of the CA1 pyramidal cells was also heavily stained (C). In the CA1 region, portions of proximal apical dendrites were often visible. (B and D) A section from the same culture used in A and C was incubated with a 1/2000 dilution of the phospho-kinase-specific monoclonal antibody 22B1 (see Methods), and the antibody binding visualized as described. The pattern of dentate granule cell staining obtained with 22B1 (B) was indistinguishable from that seen with 6G9. The overall intensity of staining appeared to be less with 22B1 as the primary antibody. Preliminary studies suggest that this was not due to a limiting amount of primary antibody. The differential in intensity may reflect the fact that only about one third of the kinase appears to be phosphorylated under basal conditions in these cells. (D) The staining pattern of 22B1 in the CA1 region was also indistinguishable from that seen with 6G9 (C), although the difference in intensity was still noticeable. Final magnification is 150 X.

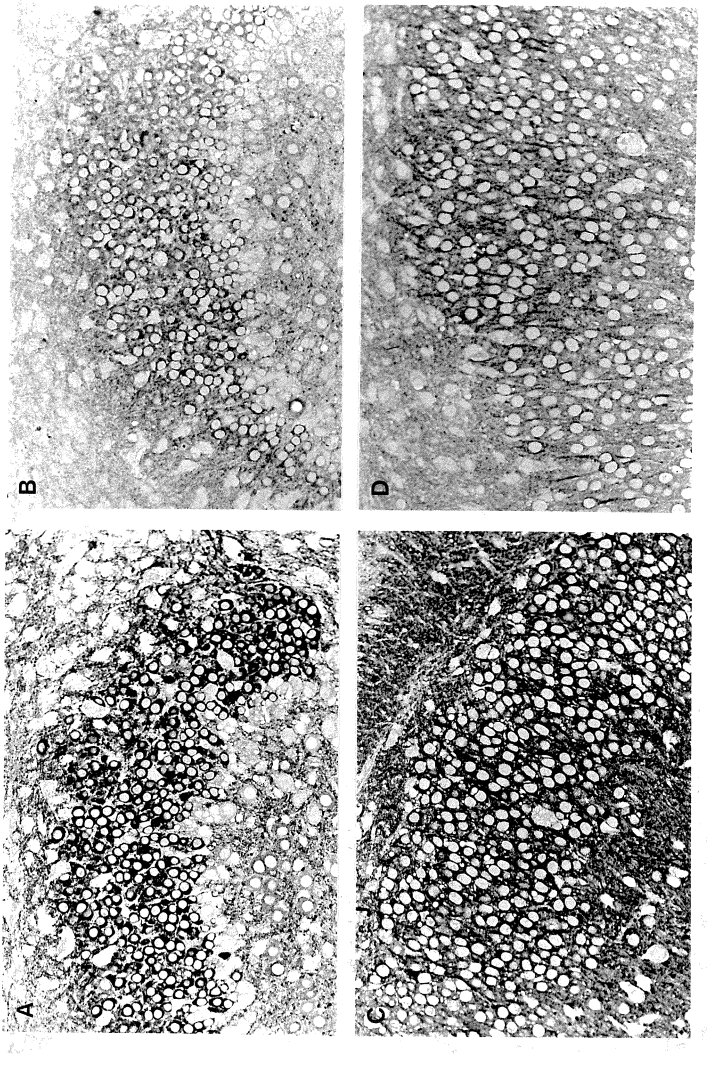
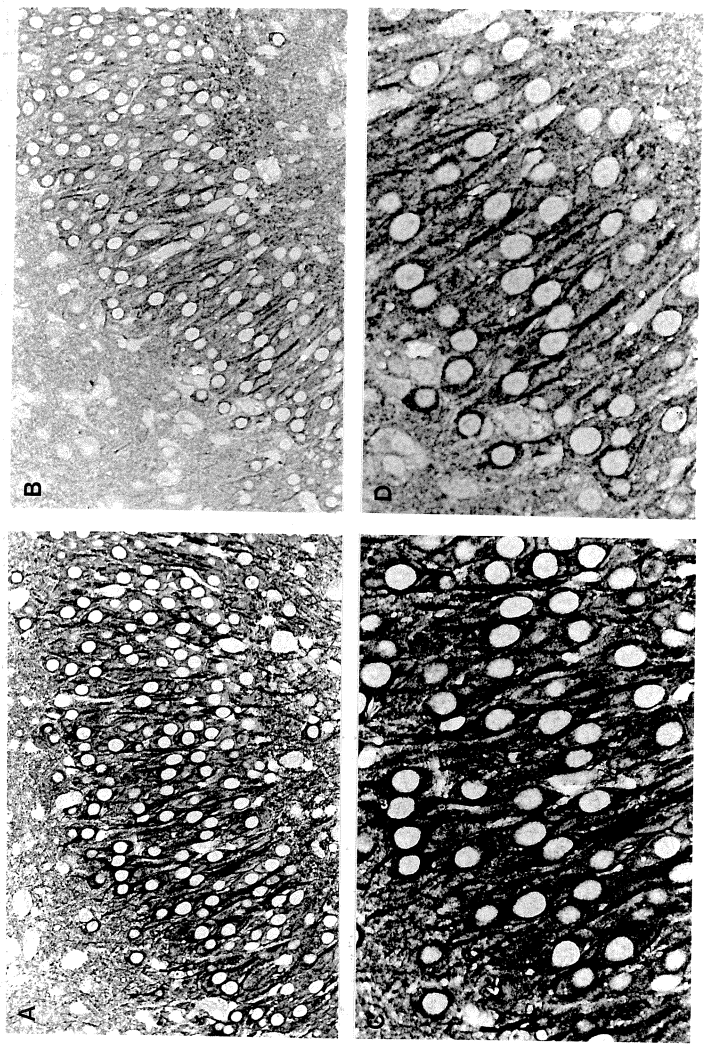

B
D
A
C

Figure 4. Comparison of Total Kinase and Phosphorylated Kinase Localization within the CA3 Region of Hippocampal Cultures.

(A and C) The section was incubated with a 1/250 dilution of 6G9, processed as described in Methods, and photographed at medium and high magnification (A and B 150 X; C and D 300 X). (B and D) A section from the same culture used in A and C was incubated in a 1/2000 dilution of 22B1, processed and photographed as described. Comparison of A and C with B and D shows that the overall staining pattern and the appearance of individually stained neurons was nearly identical with the two monoclonal antibodies. The only noticeable difference was the lower intensity of the 22B1 staining. Local concentrations of staining within neurons were apparent with both antibodies (C and D). The cell bodies and dendrites of the pyramidal cells were stained with roughly the same relative intensity in both cases.


B
D
A
C

Chapter 4

Conserved and Variable Regions in the Subunits of Brain Type II Ca^{+2}/calmodulin-dependent Protein Kinase.

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Conserved and Variable Regions in the Subunits of Brain Type II Ca^{2+}/Calmodulin-Dependent Protein Kinase

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Summary

Brain type II Ca^{2+}/calmodulin-dependent protein kinase is a holoenzyme composed of several copies each of three subunits, α (50 kd), β (60 kd), and β′ (58 kd), in varying proportions. The deduced amino acid sequences of α (reported here) and β are highly similar but not identical. The major difference between them is the deletion from α of two short segments (residues 316-339 and 354-392 in β). cDNAs that appear to encode β′ are identical to β except for the deletion of a segment encoding residues 378-392. Thus, the structural differences among α, β, and β′ arise primarily from deletions (or insertions) in a variable region lying immediately carboxyl to the protein kinase and calmodulin-binding domains. The α and β subunits are encoded by distinct genes expressed primarily, if not exclusively, in brain. Rather than being encoded by a third gene, β′ may arise by alternative splicing of the β gene transcript.

Introduction

Of the six known calcium-regulated protein kinases in brain (Kennedy, 1983; Hunter, 1987), the most abundant is type II Ca^{2+}/calmodulin-dependent protein kinase (type II CaM kinase; Bennett et al., 1983; Goldenring et al., 1983). It constitutes approximately 1% of total brain protein and is particularly concentrated in the forebrain, where it is nearly 2% of total hippocampal protein (Erondu and Kennedy, 1985). It has attracted interest recently because it appears to be concentrated in forebrain postsynaptic densities (Kennedy et al., 1983a; Kelly et al., 1984) and it acquires a calcium-independent activity when it phosphorylates itself. Thus, it may remain active for a period that exceeds the duration of an activating calcium signal (Miller and Kennedy, 1986). The kinase is a large, apparently dodecameric, holoenzyme composed primarily of varying proportions of two related subunits, α (50 kd) and β (60 kd). In different parts of the brain and at different times during development, the two subunits are present in different ratios. The α subunit predominates in the mature forebrain, while the β subunit predominates in the cerebellum (McGuinness et al., 1985; Miller and Kennedy, 1985). The β subunit is expressed early in development; while the α subunit begins to be synthesized in large quantity as the neurons mature (Sayhoun et al., 1985). Although both subunits bind calmodulin (Bennett et al., 1983) and appear to be catalytic (Miller and Kennedy, 1985), the differences in their regional and developmental expression suggest that they have some distinct functions. A third relatively minor subunit, β′ (58 kd), is structurally related to the β subunit as evidenced by the similarity between their peptide maps (Goldenring et al., 1983; Kelly et al., 1984). It is found in small quantities in all brain regions but is more abundant in the cerebellum (Miller and Kennedy, 1985).

The kinase holoenzyme appears to be distributed throughout neurons that contain it (Ouimet et al., 1984). Some of it is soluble and located in the cytosol (Kennedy et al., 1983b); the rest associates with particulate structures such as microtubules (Vallano et al., 1985) and postsynaptic densities (where it is the major protein constituent; Kennedy et al., 1983a; Kelly et al., 1984). There is some evidence that an increase in the proportion of β subunits in the holoenzyme decreases the association of the kinase with the postsynaptic density (Miller and Kennedy, 1985). This may be important for controlling whether or not the kinase is concentrated at postsynaptic sites to enhance its response to local calcium signals.

We recently reported the primary sequence of the β subunit (Bennett and Kennedy, 1987). We have now deduced the primary sequences of the α and β′ subunits from cDNA clones. We show that although the sequences of the three subunits are similar throughout most of their length, major variations in structure occur in a 77 residue segment (in β) that may contain autophosphorylation sites and/or mediate subcellular associations. We also show that the different proportions of the two major subunits in brain regions correlate with a difference in the proportion of their messages and that the brain messages are not expressed in other tissues. After this work was completed, a partial sequence of the α subunit was published by Hanley et al. (1987) and the full sequence was published by Lin et al. (1987). Our study is the first comparison of the sequences of all three subunits.

Results

The α and β Subunits Are Encoded by Distinct Genes

We used two independent probes to select cDNA clones encoding the α subunit. One probe was derived from the sequence of a peptide (Figure 1A) produced by chymotryptic cleavage of the forebrain holoenzyme, which contains mostly α subunits. The sequence was 60% identical to a segment within the predicted amino acid sequence of the β subunit (Bennett and Kennedy, 1987). Because the two subunits are known to be related (Goldenring et al., 1983), this similarity, together with the abundance of the peptide, indicated that it is a frag-

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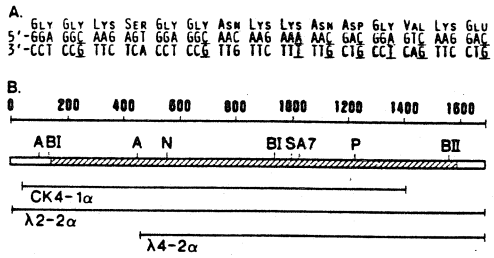

Figure 1. cDNAs Encoding the α Subunit of Brain Type II CaM Kinase

(A) The amino acid sequence of a peptide from the α subunit determined as described in Experimental Procedures. A 45 base oligonucleotide that would encode the peptide sequence is shown on the second line. The ambiguous bases were assigned based on the codon in the similar region of the β sequence or on codon usage frequency. The complementary guessmer probe is shown on the third line. The underlined bases indicate differences from the determined sequence (Figure 2).

(B) Restriction maps of the three longest independently isolated α cDNA clones. The coding region is hatched. The length and position of the clones are marked by solid heavy lines. A, AvaI; A7, Asp700; BI, BglI; BII, BglII; N, NruI; P, PvuII; S, SphI.

ment of the α subunit. A 45 base oligonucleotide "guessmer" was designed based on this sequence (Figure 1). The labeled guessmer hybridized at high stringency to a 5.0 kb band of poly(A)$^+$ RNA that was more abundant in forebrain than in cerebellum (data not shown). At lower stringencies, it also hybridized weakly to a 4.1 kb band that comigrates with the message for the β subunit (Bennett and Kennedy, 1987). The hybridization pattern indicated that the guessmer is highly complementary to a message with the distribution expected for the α subunit and less complementary to the message for the β subunit. The second and independent probe that we used for selection was a restriction fragment from a cDNA encoding the β subunit (Bennett and Kennedy, 1987). The restriction fragment contained the region encoding the amino-terminal half of β and did not contain the region 60% identical to the guessmer. At reduced stringency, this probe hybridized with both the 5.0 kb putative α message and the β message (Bennett and Kennedy, 1987). After screening a size-selected cDNA library prepared from adult rat brain poly(A)$^+$ RNA and cloned into pBR322 (Experimental Procedures), a single clone (CK4-1α) that hybridized with both probes was isolated (Figure 1). The 1.3 kb CK4-1α insert had a restriction map distinct from that of the cDNAs encoding β (Bennett and Kennedy, 1987) and hybridized strongly to the putative 5.0 kb α subunit message. To obtain overlapping clones representing the entire coding region, a second adult rat brain cDNA library cloned into λgt10 was screened with labeled CK4-1α (see Experimental Procedures). Seventeen additional clones were selected. Restriction maps of the two longest inserts, λ2-2α (1.6 kb) and λ4-2α (1.1 kb) overlapped with that of CK4-1α (Figure 1). These two inserts also hybridized to the 5.0 kb putative α message.

The insert from λ2-2α, which contains the sequence of the other two cDNAs (Figure 1B), was sequenced by the dideoxy chain termination method (Figure 2). The sequence reveals a 1434 nucleotide open reading frame that encodes a 478 amino acid protein of molecular weight 54,123, close to that predicted for the α subunit from its mobility on SDS-PAGE (Bennett et al., 1983). The open reading frame begins with an ATG that fulfills Kozak's criteria for an initiation codon (Kozak, 1986). The sequence of the chymotryptic peptide is found at residues 315-329, confirming that the cDNA encodes the α subunit. The sequence is consistent with the partial sequence published by Hanley et al. (1987) and the complete coding sequence published by Lin et al. (1987).

The sequences of the α and β subunits are very similar and likely to have diverged only recently in evolution (Figure 3). The major difference between them is the absence from α of two segments, residues 316-339 and residues 354-392 in the β sequence. The deletions occur near the junction between the amino- and carboxy-terminal halves. The amino-terminal halves of the two subunits are the most similar: 91% identical at the amino acid level, with conservative changes accounting for most of the differences, and 82% identical at the nucleotide level. The carboxy-terminal halves (immediately after the two deletions) are less similar: 76% identical at the amino acid level and 75% identical at the nucleotide level. Again, most of the changes are conservative. The highly conserved regions include known functional domains, for example, the amino-terminal "kinase domain" present in all members of the protein kinase family and containing the putative ATP-binding site (Figures 2 and 3; Zoller et al., 1981; Hanks et al., 1987) and the calmodulin-binding domain (Figure 3; Bennett and Kennedy, 1987; Hanley et al., 1987), which is identical in the two subunits. The functions of the carboxy-terminal halves of the two subunits are not known. They are not similar to domains in other protein kinases. Thus they are probably involved in subunit interactions or in other binding or regulatory functions unique to the type II CaM kinase.

A characteristic of brain type II CaM kinase is that its activity can become independent of calcium following autophosphorylation (Miller and Kennedy, 1986). The relevant autophosphorylation sites have not yet been identified. There are two potential sites that are conserved in both subunits (residues 286 and 314 in α; Figure 3). They are bounded by the sequence R-X-X-S/T, which has been proposed as a consensus sequence for phosphorylation sites in substrates of the type II CaM kinases (Pearson et al., 1985). Two additional consensus sites, at residues 321 and 510 in β, are not conserved in α. There are several other serine and threonine residues in both subunits that could be autophosphorylated; it is not yet known whether the autophosphorylation sites conform to the substrate consensus sequence.

The α and β Subunit Genes Are Expressed in Different Ratios in Different Brain Regions

The most extreme variation in subunit composition of the type II CaM kinase holoenzyme is between the fore-

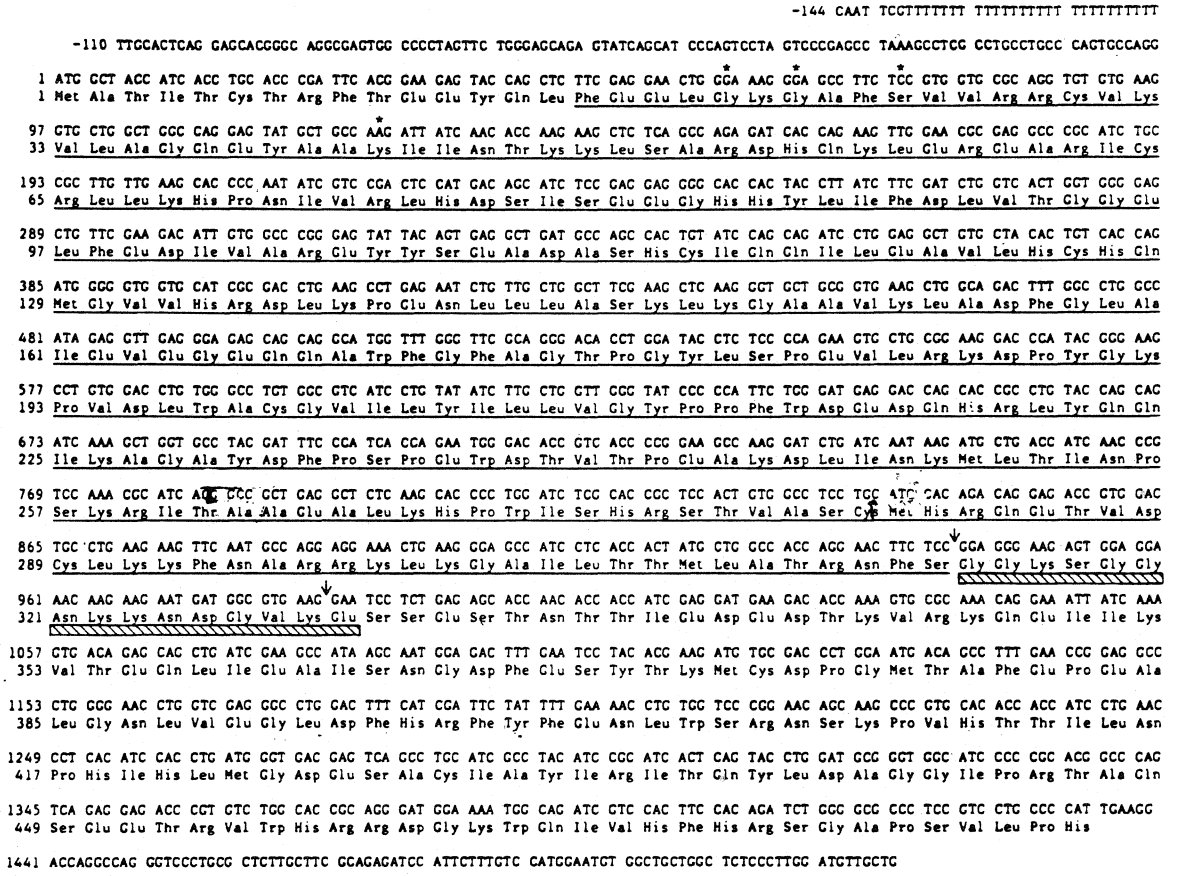

Figure 2. Nucleotide and Predicted Amino Acid Sequences of the α Subunit of Rat Brain Type II CaM Kinase

The sequence of the chymotryptic peptide (Figure 1) is marked by a hatched bar. The kinase domain, which is highly conserved in the sequences of all known kinases, is underlined. Asterisks mark residues of the predicted ATP-binding site, and arrows indicate the points at which the sequence is interrupted by additional segments in β (see Figure 3).

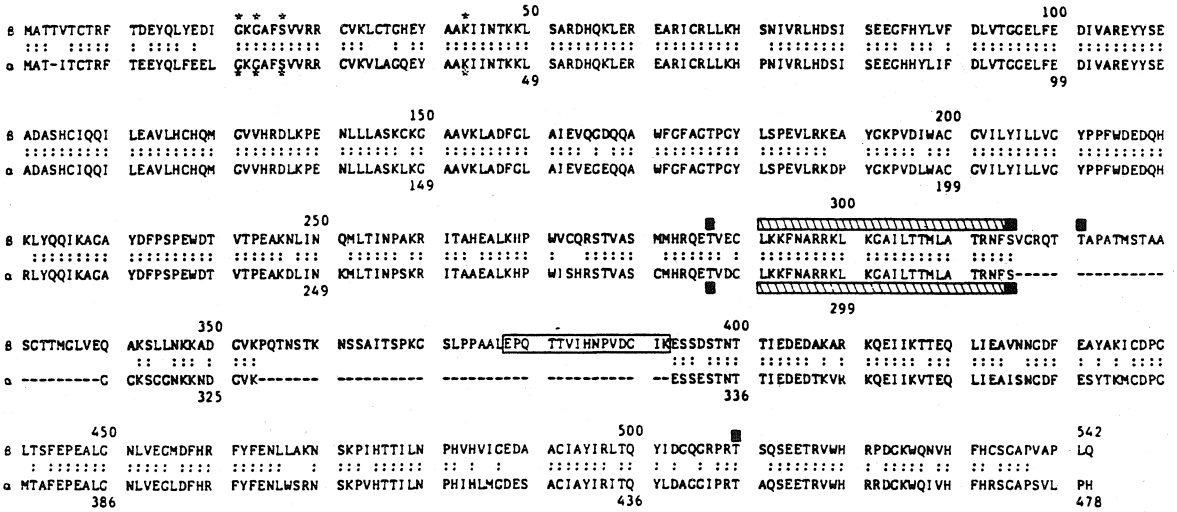

Figure 3. Comparison of the Predicted Amino Acid Sequences of the α and β Subunits of Type II CaM Kinase

The β subunit sequence was reported by Bennett and Kennedy (1987). Gaps are inserted in the α sequence to optimize the alignment and are indicated by dashed lines. Identities between the two sequences are indicated by colons. The segment that appears to be deleted in the β′ subunit is enclosed by a box. Residues in the predicted ATP-binding site are marked by asterisks. Potential autophosphorylation sites contained in a substrate consensus sequence (see Results) are marked by solid bars. The calmodulin-binding domain is marked by a hatched bar.

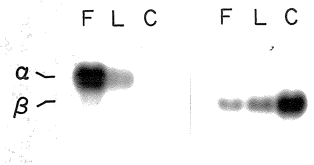

Figure 4. Relative Abundance of Messages for α and β Subunits in Brain Regions

Total RNA (10 μg) from forebrain (F), lower brain (L, including thalamus, hypothalamus, pons, and medulla), and cerebellum (C) was fractionated on formaldehyde agarose gels and transferred to nitrocellulose. The blots were incubated in H buffer at 42°C with full-length inserts from CK4-1α (left) or λ10β5-2 (right; Bennett and Kennedy, 1987). The probes were labeled to a specific activity of 1×19^9 to 3×10^9 cpm/μg as described in Experimental Procedures. The blots were washed in 2× SSC, 1% SDS, at 62°C and exposed to X-ray film for 16 hr.

brain holoenzyme, which contains primarily α subunits, and the cerebellar holoenzyme, which contains primarily β subunits. The level of expression of messages for the two subunits in these brain regions, measured by probing RNA blots with labeled α or β cDNAs, correlates with the difference in subunit composition (Figure 4). Lower brain regions such as the pons and medulla appear to contain approximately equal amounts of the two subunits (Erondu and Kennedy, 1985) and equal amounts of the two messages (Figure 4). This suggests that the subunit composition of the kinase holoenzyme within different brain regions is controlled by the level of expression of the α and β subunit genes.

The Brain Subunit Messages Are Not Present in Other Tissues

The brain kinase is one of a family of calmodulin-dependent protein kinases found in several tissues (Ahmad et al., 1982; McGuinness et al., 1983; Payne et al., 1983; Woodgett et al., 1983; Schulman et al., 1985). Members of this family have similar subunit compositions and the same relatively broad substrate specificity. To determine whether nonneural tissues contain messages similar to those encoding the brain subunits, we examined total RNA from several rat tissues for transcripts that hybridized with cDNAs encoding the brain subunits (Figure 5). Skeletal muscle contains a band of 4.3 kb that hybridized strongly to probes coding for the N- and C-terminal halves of both α and β. Hybridization at higher stringency revealed that this message is more similar to β than to α (data not shown). Its molecular size is larger than the brain β message. An additional 2.7 kb band in muscle hybridized only with the C-terminal portion of the α probe. Intestine contains a 2.9 kb band that hybridized with both halves of the β probe. Several other tissues contain bands that hybridized weakly with one or more of the probes and may encode homologous kinases. Thus, messages for both of the brain subunits are found primarily, if not exclusively, in brain. The related kinases found in other tissues appear to be synthesized from distinct messages.

The β′ Subunit Message May Be Generated by Alternative Splicing

The β′ subunit is a minor 58 kd subunit found in most brain regions (Bennett et al., 1983; Miller and Kennedy, 1985). It appears more abundant in preparations of the cerebellar holoenzyme, which has a greater proportion of β subunits than the forebrain holoenzyme (McGuinness et al., 1985; Miller and Kennedy, 1985). While studying brain cDNAs encoding β, we selected and sequenced two independent cDNA clones (Figure 6) containing a deletion of 45 bases from the middle of the coding region (bases 1132–1176 in β; Bennett and Kennedy, 1987). Full-length β messages with this deletion would

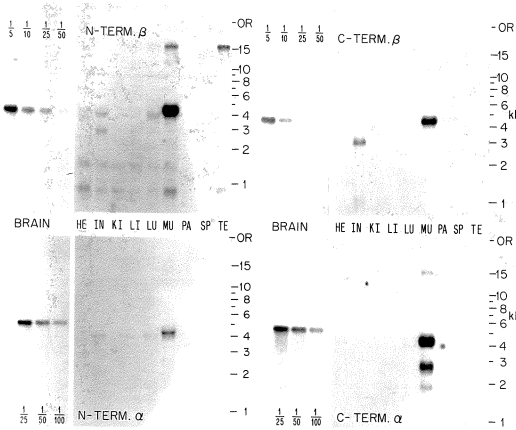

Figure 5. Expression of Messages Similar to Brain α and β Subunits in Different Tissues

Total RNA was isolated from rat tissues, fractionated on formaldehyde agarose gels (10 μg/lane), and transferred to nitrocellulose. For comparison, each gel included forebrain total RNA diluted into Drosophila poly(A)$^-$ RNA (10 μg/lane). Nitrocellulose blots were incubated with fragments of λ10β5-2 encoding the amino or carboxyl domain of β or with fragments of λ2-2α encoding the amino or carboxyl domain of α. The probes were prepared and incubations were performed as described in Experimental Procedures. The dried blot labeled with the N-terminal α probe was exposed to X-ray film for 6 days; the others were exposed for 4 days. HE, heart; IN, intestine; KI, kidney; LI, liver; LU, lung; MU, skeletal muscle; PA, pancreas; SP, spleen; TE, testis.

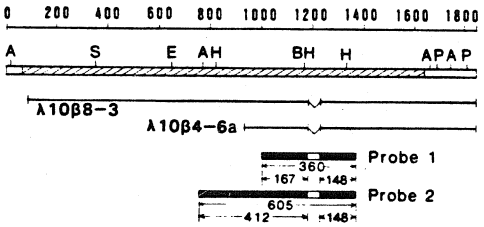

Figure 6. cDNAs Encoding a β′ Subunit of the Type II CaM Kinase

The restriction map of the β subunit is from Bennett and Kennedy (1987). The hatched bar represents the coding region. The length and position of two independently isolated clones, λ10β8-3 and λ10β4-6a, are shown below. They were selected as previously described (Bennett and Kennedy, 1987). The sequence of λ10β4-6a was determined from the 5′ end to the HincII site at base 1337. The full sequence of λ10β8-3 was determined except for bases 820–1050. The region deleted in these clones, but present in cDNAs encoding β, is indicated by breaks in the lines. The sizes of the two probes used in the S1 nuclease protection experiments and of the predicted fragments are indicated by the solid bars. A, AvaI; BH, BamHI; E, EcoRV; H, HincII; P, PstI.

encode a protein of molecular weight 58,705 containing trypsin-sensitive sites nearly identical to those in β. These are the properties expected of β′. The segment deleted from the putative β′ is the last 15 residues (378–392) of the second segment deleted from α (see Figure 3).

In order to ensure that the β′ clones were not the result of a cloning artifact, we employed S1 nuclease protection, a technique for detecting rare mRNAs, to test whether messages with this deletion are present in the brain (Figures 6 and 7). Because S1 nuclease hydrolyzes only single-stranded nucleotide chains, pieces of single-stranded DNA mixed with mRNA are protected from hydrolysis only if they are bound to a complementary message. Two labeled DNA probes spanning and containing the 45 base region were prepared from cDNAs encoding β (Figure 6). We predicted that the probes would be protected from hydrolysis if bound to messages encoding β. However, if bound to messages containing the 45 base deletion, they would be partially hydrolyzed. Probe 1 would be digested into pieces of 167 and 148 bases; probe 2, into pieces of 412 and 148 bases. When the labeled probes were hybridized with an excess of brain poly(A)$^+$ RNA, then subjected to digestion with S1 nuclease, most of the probe was protected. However, a small portion of each was hydrolyzed to pieces of the predicted sizes, indicating that messages with the 45 base deletion exist in the brain (Figure 7A). A greater portion of the probe was hydrolyzed when bound to cerebellar mRNA than when bound to forebrain mRNA, presumably reflecting the greater concentration of β′ subunit in the cerebellum.

The β′ message could arise by transcription of a separate β′ gene or by alternative splicing of the β gene transcript. Genomic restriction analyses suggest that there is only one gene encoding the sequence of the β subunit (Figure 7B). Furthermore, the sequences flanking and including the deletion are consistent with the possibility that the deleted sequence is an alternatively spliced exon (see Discussion). Therefore, the present data favor the possibility that β′ arises by alternative splicing. Proof

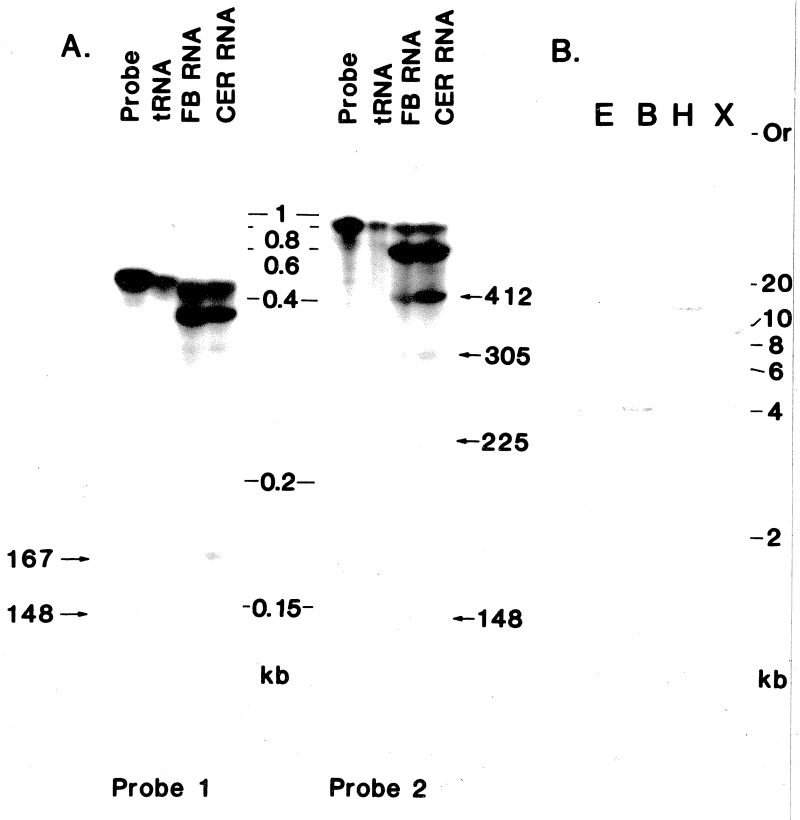

Figure 7. Existence of the Predicted β′ Messages in Brain

(A) S1 nuclease protection experiment. Hybridization and S1 nuclease digestion were carried out as described in Experimental Procedures. One half of each digestion reaction was fractionated on an 8% polyacrylamide sequencing gel, which was then exposed to X-ray film. The left and right panels show protection of probes 1 and 2 (Figure 6), respectively, with yeast tRNA (tRNA), forebrain total RNA (FB RNA), and cerebellar total RNA (CER RNA). The left-most lanes show undigested probe (one-thirtieth of the amount used in the protection experiment). Molecular size markers were ^{32}P-labeled fragments of λ DNA digested with EcoRI and HindIII and a sequence ladder from a β subunit cDNA clone.

(B) Hybridization of rat genomic DNA with β subunit cDNA. Rat genomic DNA was digested with EcoRI (E), BamHI (B), HindIII (H), or XbaI (X), fractionated on an 0.8% agarose gel, and transferred to a nylon membrane as described in Experimental Procedures. The DNA blot was incubated with a fragment of λ10β5-2 encoding the carboxyl domain of the β subunit and washed as described in Experimental Procedures. The blots were exposed to X-ray film for 6 days.

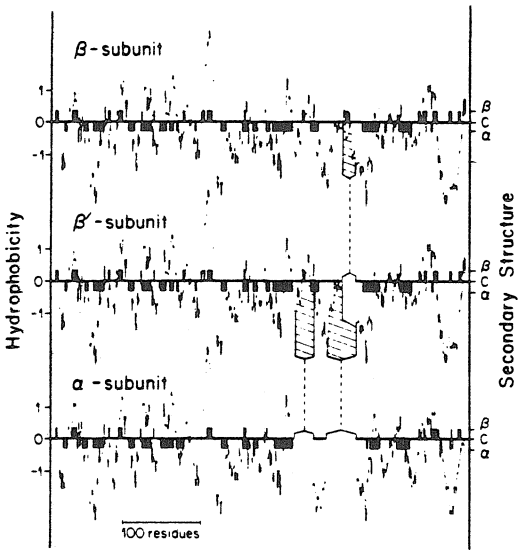

Figure 8. Relative Hydrophobicity and Predicted Secondary Structure of the Subunits of Brain Type II CaM Kinase

Hydrophobicity was calculated by the procedure of Kyte and Doolittle (1982) with a window of 11 amino acids. The result is plotted as a continuous thin solid line. Secondary structure was predicted by the algorithm of N. Qian and T. J. Sejnowski (submitted). Coiled (relatively unstructured) regions of the sequence are indicated as a solid line on the horizontal axis (C). Regions predicted to have more definite structure are indicated by solid bars, either above the line (β-pleated sheet, β) or below the line (α-helix, α). The predictions are similar to those made by the method of Chou and Fasman (1978; data not shown), except that the borders of predicted regions are more definite. Regions missing in the β′ and α subunits are indicated by gaps in the horizontal axis; the corresponding regions in β or β′ are highlighted by hatching.

of this hypothesis will require analysis of the structure of the β gene.

Additional fragments of 305 and 225 bases were produced by the S1 nuclease digestion. Fragments of approximately this size would be produced if messages with deletions of nucleotides 946–1017 in β were present in brain. These nucleotides encode the first 24 residue segment that is missing in α (residues 316–339 in β; Figure 3). Thus, this segment may also be encoded by an alternatively spliced exon, and there may be two distinct β′ transcripts (see Discussion).

Variations in Structure among the β, β′, and α Subunits Occur Primarily in a 77 Residue Segment

The relative hydrophobicity, predicted secondary structure, and distribution of charge are conserved throughout most of the length of the three subunits (Figures 8 and 9). The major structural differences are confined to the regions where segments are absent from the α and β′ subunits. The two segments absent in α are mostly neutral and are predicted to have a "coiled" or relatively random secondary structure (Figure 8). Their removal forms one strongly hydrophilic domain in α at a position where there are two hydrophilic domains in β separated by a neutral coil. It brings together in α two regions of high charge density, forming a single long highly charged

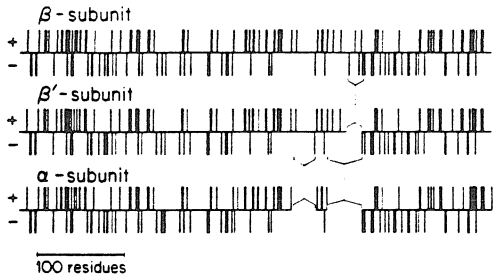

Figure 9. Distribution of Charged Amino Acids in the Subunits of Brain Type II CaM Kinase

The horizontal axis indicates the residue position. Vertical lines above the horizontal axis indicate positively charged residues (lysine, arginine, or histidine). Those below the horizontal axis indicate negatively charged residues (aspartate or glutamate). Regions absent in the β′ and α subunits are indicated by gaps in the horizontal axis; the corresponding regions in β or β′ are indicated by brackets.

domain (Figure 9). The shorter deletion in β′ decreases the length of the neutral region between the hydrophilic domains. The deletions from α and β′ have little effect on the overall charge of the two proteins. This is consistent with an earlier observation that β, β′, and α have nearly identical isoelectric points (Kennedy et al., 1983a).

There are several notable structural features that are highly conserved among the three subunits. The kinase domain (residues 17–290) is primarily a series of predicted α-helixes alternating with coiled regions. However, one prominent feature is a very hydrophobic predicted β sheet from residues 196–215. A similar hydrophobic region is also present in myosin light chain kinase and cAMP-dependent protein kinase (data not shown). The calmodulin-binding amphiphilic α-helix (residues 291–315; Bennett and Kennedy, 1987; Hanley et al., 1987), which just follows the kinase domain, is entirely conserved in all three subunits. Finally, although the carboxyl halves of the subunits are less conserved in sequence, they contain some conserved structural features. Particularly apparent is a strongly hydrophilic predicted coil at the carboxyl terminal beginning with residue 500 in β. This region is part of a domain that is relatively rich in positively charged residues (458–542 in β). It is preceded by a region relatively rich in negatively charged residues (392–457 in β). These domains of alternating overall charge could be involved in symmetrical subunit associations within the holoenzyme or in other subcellular associations shared by all three subunits.

Discussion

The three subunits of brain type II CaM kinase can be divided into several discrete regions based on the extent of their conservation and their proposed functions (Figures 3 and 10). Three regions present and highly conserved in all of the subunits are termed C1, C2, and C3. C1 extends over the entire amino-terminal half of each subunit and contains the protein kinase domain (Hanks, 1987) as well as the calmodulin-binding domain (Ben-

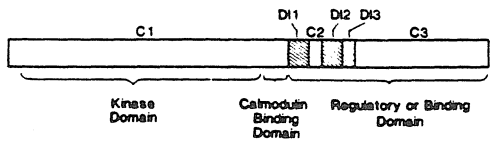

Figure 10. Summary of Structures of the Subunits of Brain Type II CaM Kinase

Regions that are highly conserved in all the subunits are represented as open bars (C1, C2, and C3). Segments that are absent from α or β′ subunits, and thus were probably deleted or inserted as the subunits evolved, are represented as hatched and cross-hatched bars (DI1, DI2, and DI3). Functional domains such as the kinase domain, the calmodulin-binding domain, and the proposed regulatory and/or binding domains are also labeled.

nett and Kennedy, 1987; Hanley et al., 1987). C2 is embedded within a highly variable region. C3 comprises approximately the carboxy-terminal one-third of each subunit. Three segments deleted (or inserted) in different subunits are termed DI (for deletion/insertion) 1, DI2, and DI3. DI3 is deleted in the β′ subunit; all three DI segments are deleted in the α subunit. A single continuous 77 residue region composed of DI1, C2, DI2, and DI3 contains most of the variation in structure among the subunits. The deletion of DI3 in β′ shortens a neutral segment with a predicted coil structure that connects two hydrophilic domains in β. The further deletion of DI1 and DI2 from α joins these two hydrophilic domains to form a single new, highly charged domain (Figures 8 and 9).

The carboxy-terminal halves of the subunits, composed of C2 and C3 plus one or more of the DI segments, are not similar in sequence to any other known protein. Thus they are likely to be important for functions unique to the type II CaM kinase, such as subunit associations or interactions with organelles. The variations in structure among the subunits may lead to corresponding functional differences among the subunits. For example, the forebrain holoenzyme, which is richer in α subunits, appears to associate more strongly with postsynaptic densities than the cerebellar holoenzyme, which is richer in β subunits (Miller and Kennedy, 1985). Thus, the deletion of the DI segments in α may create a postsynaptic density binding site. Another difference between the α and β subunits is in their number of autophosphorylation sites. Each dodecameric holoenzyme (containing an average of nine α's and three β's in the forebrain) can incorporate about 30 mol of phosphate in the presence of calcium and calmodulin. We have previously shown that autophosphorylation of the first three to five sites (less than one per subunit) is sufficient to trigger a new calcium-independent kinase activity. Thus, it appears that autophosphorylation of only a few of the subunits at the triggering site can activate all of the subunits in a holoenzyme by an allosteric mechanism (Miller and Kennedy, 1986). Several other sites on both subunits are autophosphorylated more slowly (unpublished observations). None of the autophosphorylation sites has yet been identified. However, β incorporates a total of approximately 3 mol of phosphate per mol in the presence of calcium, while α incorporates only 2 mol per mol (Miller and Kennedy, 1986). Therefore, at least one of the β autophosphorylation sites may be located in a segment that is absent in α. There are several serines and threonines in each of the DI segments, including one consensus substrate site in DI1. Each of these is a potential autophosphorylation site.

The β subunit (containing all of the regions described in Figure 10) and the α subunit (missing DI1, DI2, and DI3) are transcribed from distinct genes (Figure 3). Their strong similarity suggests that the two genes diverged recently in evolution. They may have evolved by gene duplication followed by either insertion or deletion of the DI regions and gradual mutational drift of the rest of the sequence. Heterogeneity of the type II CaM kinase holoenzyme is generated by differential expression of these two genes in different brain regions. In the forebrain, the α subunit message predominates; in the pons and medulla, the two messages are present in roughly equal amounts; while in the cerebellum, the β subunit predominates (Figure 4). The composition of the holoenzyme in each of these regions reflects the level of messages for each subunit.

Rather than being encoded by a separate gene, the β′ subunit (missing region DI3) may be generated by alternative splicing of the transcript of the β gene. Restriction analysis of genomic DNA suggests that there is only one gene encoding β sequences (Figure 7B). The sequences of the two cDNAs with DI3 deleted are identical to that of the β cDNA outside the region of the deletion (Figure 6). It is unlikely that a distinct message so similar to that of the β message is transcribed from a different gene. Rather, it seems likely that a single exon encoding region DI3 is removed to generate β′. The putative exon (nucleotides 1132–1176 in the β coding region; Bennett and Kennedy, 1987) begins with a G, as specified by the consensus sequence for the 5′ end of an exon at an intron-exon splice junction (Mount, 1982). It ends with AAG, as specified for the 3′ end of an exon at an exon–intron splice junction. The sequence CTG (nucleotides 1129–1131) immediately preceding the putative exon has been found at the 3′ end of exons and matches 2 of the 3 nucleotides of the consensus sequence for the 3′ end of an exon (Mount, 1982). The nucleotide immediately following the putative exon (1177) is a G, as specified for the beginning of a new exon. Interestingly, in the S1 protection experiment, fragments were produced that suggest the presence of a small quantity of a second alternatively spliced β′ message with segment DI1 rather than DI3 deleted (Figure 7A). This message would produce a protein with a molecular weight of about 57,000. A doublet at the position of β′ is in fact often observed in purified type II CaM kinase, most prominently in the cerebellar holoenzyme (Miller and Kennedy, 1985; unpublished observations). Proof that β′ is generated by alternative splicing will require characterization of the structure of the β gene.

Tissues other than the brain contain calmodulin-dependent protein kinases that appear to be related to the brain type II CaM kinase because they share several

properties (Bennett et al., 1983; McGuinness et al., 1983; Schulman et al., 1985). They are large holoenzymes composed of several subunits in the 50 to 60 kd range. All of the subunits bind calmodulin, appear to be catalytic, and have the same relatively broad substrate specificity. They differ in the exact molecular weights of their subunits and in the number of subunits per holoenzyme. Hybridization analysis of RNA from several tissues suggests that the related kinases are encoded by genes which are distinct from those encoding the brain α and β subunits (Figure 5). Skeletal muscle contains a 4.3 kb message that is similar to both the α and β messages but is distinct from either of them. Several other tissues contain messages that appear only distantly related to the brain messages. The spleen, which is known to contain a protein kinase activity similar to that of the brain kinase (Erondu and Kennedy, 1985), nevertheless contains no detectable cross-hybridizing message. The results are consistent with those of Lin et al. (1987), who detected only traces of messages similar to a brain α subunit cDNA in nonneural tissues. We have shown that the genes for both the α and the β subunits appear to be expressed only in the brain. Thus there may be several other related but distinct genes that are expressed in their own narrow range of tissues.

The large amounts of type II CaM kinase expressed in the brain and the difference in expression of its two major subunits in different brain regions suggest important specialized functions for each of its subunits in neuronal regulation. The structural analysis of the subunits provides a basis for study of their distinct functions in vivo as well as in vitro. Present evidence suggests that they differ in their regulatory autophosphorylation sites (Miller and Kennedy, 1986) and their affinity for postsynaptic densities (Miller and Kennedy, 1985). When functional differences among the subunits are understood, it may be illuminating to look for consistent physiological differences among neurons that express them in different proportions.

Experimental Procedures

Materials

Restriction endonucleases, S1 nuclease, and the Klenow fragment of DNA polymerase were obtained from Boehringer Mannheim (Indianapolis, IN). T4 DNA ligase, Maloney murine leukemia virus reverse transcriptase, and ribonuclease H (E. coli) were obtained from Bethesda Research Laboratories (Gaithersburg, MD). The Cyclone System subcloning kit, T4 DNA polymerase, and Sequel-SS/KF Sequencing Biosystem sequencing kit were obtained from International Biotechnologies, Inc. (New Haven, CT). Chymotrypsin was obtained from Sigma (St. Louis, MO). E. coli DNA ligase and EcoRI linkers were obtained from New England Biolabs (Beverly, MA). Polynucleotide kinase and the Oligolabeling kit were obtained from Pharmacia (Piscataway, NJ). [α-^{32}P]dCTP (~3000 Ci/mmol) and [α^{32}P]dATP (~800 Ci/mmol) were obtained from Amersham (Arlington Heights, IL). [γ-^{32}P]ATP was obtained from ICN Biomedicals, Inc. (Costa Mesa, CA).

Peptide Sequence Analysis

Brain type II CaM kinase was purified from rat forebrain as described previously (Bennett et al., 1983). Purified kinase (0.4 mg) was digested with chymotrypsin (800 ng) at 30°C for 1.5 hr. The proteolyzed kinase was brought to 2% SDS and 3% β-mercaptoethanol, placed in a boiling water bath for 2 min, and loaded onto an HPLC gel filtration column. The absorbance of the column effluent was monitored at 214 nm. Peak fractions were pooled and analyzed on a 15% polyacrylamide gel. Fractions containing a single abundant 20 kd peptide were pooled and dialyzed against 10 mM NH_4HCO_3, 0.02% SDS, for 40 hr. The dialyzed peptide was lyophilized and redissolved in 50 μl of double-distilled H_2O. Approximately 1 nmol of the peptide was subjected to automated Edman degradation on a gas phase sequenator (Hunkapiller et al., 1983). Phenylthiohydantoin-amino acids were identified in two separate runs by high pressure chromatography on a reverse-phase column (Hunkapiller and Hood, 1983). Repetitive yields were 90% or higher. Fifteen amino acids were identified reliably (Figure 1A).

Preparation of Probes

A 45 nucleotide guessmer was designed based on the sequence of the peptide (Figure 1) and synthesized in the microchemical facility at Caltech. The oligonucleotide was purified on a preparative 20% polyacrylamide gel containing 7 M urea. It was eluted into 3 ml of 0.5 M ammonium acetate, 0.1 M magnesium acetate, 1 mM EDTA, 0.1% SDS, at 37°C overnight. The solution was concentrated to 200 μl by butanol extraction. It was desalted on a Sepharose G-25 column, lyophilized, and redissolved in H_2O to a concentration of 1 mg/ml. The oligonucleotide was labeled at the 5′ end to a specific activity of 1×10^9 to 2×10^9 cpm/μg by reaction with [γ-^{32}P]ATP in the presence of polynucleotide kinase (Maniatis et al., 1982).

The second probe, a restriction fragment of the β subunit cDNA (Bennett and Kennedy, 1987) encoding the amino terminal, was prepared by digestion of the β cDNA with BamHI and HincII. It was labeled to a specific activity of 1×10^9 to 3×10^9 cpm/μg by incubation with [α-^{32}P]dCTP in the presence of the Klenow fragment of DNA polymerase, after priming with a set of random oligonucleotides (Pharmacia).

Screening of cDNA Libraries

The first library screened was an adult rat brain cDNA library inserted into PBR322 (obtained from Gary Schull, University of Cincinnati; Shull et al., 1985). Two successive screenings of 3×10^4 recombinants were performed with the two independent probes. One set of duplicate filters, prepared as described previously (Shull et al., 1985), was incubated with labeled guessmer in 2× SSC (0.3 M NaCl, 0.03 M sodium citrate) 10× Denhardt's (0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% BSA), 0.1% SDS, 100 μg/ml hydrolyzed yeast RNA, at 50°C. It was washed in 2× SSC, 1% SDS, at 50°C. The second set of filters was incubated with the labeled β restriction fragment in H buffer (0.9 M NaCl, 0.06 M NaH_2PO_4, 0.006 M EDTA, 50% formamide, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% BSA, 1% SDS, 200 μg/ml salmon sperm DNA) at 42°C, followed by washing in 2× SSC, 1% SDS, at 68°C. One recombinant (ck4-1α) was selected.

The second library, which was screened to obtain a full-length α cDNA, was a λgt10 library synthesized from forebrain poly(A)$^+$ RNA as described by Gubler and Hoffman, 1983. EcoRI linkers were added by ligation with T4 DNA ligase to blunt-ended cDNA. The cDNA was then fractionated on a Sepharose 2B 300 column prepared as described by Huynh et al. (1985). Large cDNA was inserted into the EcoRI site of λgt10, packaged, and cloned in E. coli c600 (Huynh et al., 1985). The library contains 9×10^5 recombinants, with an average insert size of 1.5 kb. It was amplified and screened essentially as described by Maniatis et al. (1982). Filters were incubated with the full-length insert of ck4-1α (labeled as described above for the β restriction fragment) in H buffer at 42°C and washed in 0.4× SSC, 1% SDS, at 68°C.

DNA Sequencing

The 1.6 kb EcoRI fragment containing the full insert of clone λ2-2α (Figure 1) was inserted into M13mp19 in both orientations (Norrander et al., 1983). A sequential series of overlapping subclones was generated from the M13 clones by exonuclease digestion with T4 DNA polymerase as described by Dale et al. (1985). Subclones were sequenced by the dideoxy chain termination method with [α-^{32}P]dATP (800 Ci/mM) as label (Sanger et al., 1977).

Blot Hybridization

Total RNA was isolated from rat tissues as described (Goldman et

al., 1985), separated on formaldehyde agarose gels (10 μg/lane), and blotted to nitrocellulose (Maniatis et al., 1982). In blots of RNA from tissues other than brain, a set of standards of forebrain total RNA diluted into Drosophila poly(A)⁻ RNA (10 μg/lane; provided by D. S. Leonard) was included. An RNA ladder (Bethesda Research Laboratories) containing 9.5, 7.5, 4.4, 2.4, 1.4, and 0.3 kb RNAs and stained with methylene blue provided molecular size standards. One set of blots was incubated in H buffer at 42°C with either of two fragments produced by digestion of the β cDNA λ10β5-2 (Bennett and Kennedy, 1987) with BamHI and EcoRI, one fragment encoding the amino domain and the other the carboxyl domain of β. Another set was incubated under the same conditions with either of two fragments produced by digestion of the α cDNA λ2-2α (Figure 1) with BglI and BglII, one fragment encoding the amino domain and the other the carboxyl domain of α. The probes were labeled as described for the β cDNA fragment. Following hybridization, the blots were washed in 2× SSC, 1% SDS, at 62°C and exposed to X-ray film for 4–6 days. The integrity of the RNA was verified by staining the blots with methylene blue following exposure.

For DNA blots, rat genomic DNA was isolated by the method of Blin and Stafford (1976). DNA (6 μg) was digested with EcoRI, BamHI, HindIII, or XbaI and separated on an 0.8% agarose gel. DNA was transferred to charged nylon membranes (New England Nuclear, Boston, MA) in the presence of 0.4 M NaOH (Reed and Mann, 1985). The membranes were hybridized with a fragment of the β cDNA (Bennett and Kennedy, 1987) produced by digestion with BamHI and EcoRI and encoding the carboxy-terminal domain of β. The probe was labeled to a specific activity of 1×10^9 to 3×10^9 cpm/μg by priming with a random set of oligonucleotides as described above. Blots were incubated in H buffer at 42°C and washed in 0.1× SSC, 1% SDS, at 70°C. The membranes were exposed to X-ray film for 6 days.

S1 Nuclease Protection Experiment

The probes for the S1 nuclease protection experiment (Figure 6) were prepared from the coding strand of two incomplete β cDNAs containing only a part of the coding region (Bennett and Kennedy, 1987). Probe 1 was generated from cDNA beginning at nucleotide 964; probe 2, from a cDNA beginning at nucleotide 719. The cDNAs were inserted into M13, and the single-stranded vectors were primed by hybridization with a synthetic β subunit–specific primer (complementary to nucleotides 1307–1324 of the β coding region). In each case the primer was extended from the 3′ end with DNA polymerase Klenow fragment in the presence of [α-^{32}P]dATP. The products of the extension reaction were digested with HindIII or AvaI, which recognized sites in the vectors just beyond the ends of the β subunit inserts. The labeled probes were separated from the template DNA by polyacrylamide gel electrophoresis (Maniatis et al., 1982). Probe 1, generated from M13 clone 5-3a2 by digestion with HindIII, was 420 nucleotides in length and was complementary to 360 nucleotides of the β subunit sequence. Probe 2, generated from M13 clone 5-18 by digestion with AvaI, was 800 nucleotides in length and was complementary to 605 nucleotides of the β subunit sequence. Both probes had a specific activity of 2×10^8 cpm/μg and had identical 5′ ends. Each probe (1.5 ng, 3×10^5 cpm) was hybridized with either 15 μg of yeast RNA, 15 μg of forebrain total RNA, or 15 μg of cerebellar total RNA. Hybridization was carried out at 50°C for 10 hr in 70% formamide, 40 mM PIPES (pH 6.4), 0.4 M NaCl, 1 mM EDTA. The reaction was diluted 10-fold with 50 mM sodium acetate (pH 4.5), 0.2 M NaCl, 1 mM $ZnSO_4$, 0.5% glycerol, 2 μg of denatured salmon sperm DNA, 200 U of S1 nuclease, and incubated at 37°C for 30 min. The samples were deproteinized by extraction with an equal volume of phenol, then phenol, chloroform (1:1), and finally chloroform. They were then precipitated in ethanol, redissolved in sequencing gel buffer, and fractionated on an 8% polyacrylamide DNA sequencing gel (Maniatis et al., 1982). Radioactive bands were detected by autoradiography.

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Chapter 5

Summary

The physical and biochemical characteristics of the type II Ca^{2+}/calmodulin-dependent protein kinase have been studied at great length. The results of these studies indicate that both the expression and activity of the enzyme are regulated in a complex manner. The overall expression of the kinase is highly tissue-specific, however, the pattern of expression is differentially controlled for each subunit. The subunits also have distinct developmental time courses of expression. These observations suggest that the regulatory elements of the genes coding for these subunits are relatively sophisticated, and would be well worth analyzing. The biochemical properties of the kinase, however, are equally complex. The unique physical and enzymatic properties of the enzyme, combined with its specific expression in nervous tissue, provided the motivation and direction for the studies reported in this thesis.

The distribution of messenger RNA coding for the α and β subunits of the kinase was investigated to determine if the tissue and brain region-specific expression of the enzyme was controlled at the level of transcription. The results of these studies, as described in Chapter 4, indicate that the α and β subunit mRNAs are selectively transcribed in the brain. Alternatively, the mRNAs could be posttranscriptionally modified in the brain, thereby increasing their half life compared to the messages in other tissues. Interestingly, smaller cross-reacting RNA species were in other tissues; these may represent messages encoding additional CaM kinase subunits (γ and δ) recently cloned on the basis of sequence similarity. Furthermore, regions of the brain that have kinase holoenzymes with distinct subunit compositions also contain different levels of α and β mRNA. These

results suggest that both the overall level of kinase expression, and the subunit stoichiometries of the various forms of the holoenzyme could be determined by the rate of transcription (or possibly degradation) of the mRNA. This supports the impression that the regulatory system controlling expression of the subunit genes is quite sophisticated. Unfortunately, confirmation of this idea requires the isolation and characterization of genomic clones for the kinase.

The biochemical properties of the CaM kinase suggest that it could play an important role in the regulation of neurons. In contrast to the limited tissue distribution of the kinase, it can phosphorylate a broad range of proteins *in vitro*. Some of these proteins (e.g., MAP-2, tyrosine hydroxylase) are known to be important in the expression of neuronal phenotypes. The high concentration of the kinase, and its distribution throughout the cytosol of neurons indicate that the enzyme may have many more cellular substrates. The fact that the kinase is found in both presynaptic terminals and postsynaptic densities within dendritic spines, suggests that the enzyme may function, at least in part, to control the synaptic properties of neurons.

A considerable body of evidence (reviewed in Chapter 1) points to the involvement of Ca^{2+}-stimulated protein kinases in the regulation of synaptic strength in invertebrate and mammalian neurons. While the synaptic regulatory mechanisms of some invertebrate neurons have been unravelled, the biochemical events that underlie long-term changes in synaptic strength in mammalian brain are still unclear. The hippocampus is unique among brain regions in the degree to which its neurons display long-term changes in synaptic efficacy. This region of the brain also has

the highest concentration of CaM kinase. The combination of these two characteristics prompted us to establish cultures of hippocampal slices in which to study the CaM kinase in intact neurons.

The discovery of the effect of autophosphorylation on CaM kinase activity suggested a way in which the enzyme could produce long-term changes in synaptic properties. Biochemical studies *in vitro* demonstrated that autophosphorylation at $Thr_{286/287}$ made the kinase independent of Ca^{2+}. It was proposed that prolonged increases in Ca^{2+}, as would occur during repeated synaptic activation, could support a level of autophosphorylation sufficient to make the kinase completely independent of Ca^{2+}. This would result in synaptic terminals filled with constituitively active CaM kinase. Continuous phosphorylation of CaM kinase substrates could alter the physical and chemical properties of the synapse. There was, however, no evidence that regulation of CaM kinase activity by autophosphorylation occurs *in vivo*.

This paucity of information regarding the regulation of the CaM kinase in intact neurons provided the motivation for our biochemical analyses of the enzyme in cultured hippocampal slices. These studies clearly demonstrate that the kinase does autophosphorylate in intact neurons. Furthermore, this phosphorylation occurs primarily at Thr_{286} on the α subunit and produces a concomitant Ca^{2+}-independent activity. An additional unexpected result was that a substantial proportion of the kinase was in the autophosphorylated, Ca^{2+}-independent form in the neurons at rest. This observation contradicts the proposal that prolonged, synaptically driven increases in Ca^{2+} were required to stimulate autophosphorylation of the enzyme and induce constituitve activity.

The sensitivity of this Ca^{2+}-independent activity in the neurons to reductions in external Ca^{2+}, and application of kinase or phosphatase inhibitors, indicates that the level of Ca^{2+}-independent enzyme reflects the balance between continuous phosphorylation and dephosphosphorylation of the kinase. Although we were unable to detect any pharmacologically induced changes in the basal level of Ca^{2+}-independent enzyme activity, the dynamic nature of this pool of kinase suggests that localized, synaptically driven modulation could occur. Since basal Ca^{2+} concentrations are sufficient to sustain a significant level of phosphorylated kinase, this modulation could either be upward or downward.

The production of monoclonal antibodies specific for the phosphorylated form of the kinase allowed us to localize the Ca^{2+}-independent enzyme in the hippocampal slices. These immunocytochemical studies showed that the kinase is phosphorylated throughout the cell bodies and dendrites of the pyramidal and dentate granule cells. These antibodies might also provide a means by which to test for local changes in kinase phosphorylation at hippocampal synapses.

A combination of biochemical, physiological, and immunocytochemical techniques may ultimately reveal whether or not changes in CaM kinase phosphorylation participate in the long-term enhancement of synaptic transmission in the hippocampus. Ideally, one could potentiate the synapses in a single pathway, or even on individual neurons, and assess the degree of CaM kinase phosphorylated at these sites by immunocytochemistry. We are currently developing the techniques that would make such experiments possible.

Unfortunately, these experiments would not help in determining the important

cellular substrates of the kinase. The lab, however, is currently pursuing other approaches that could reveal the identity of these proteins. The *in situ* labeling techniques used to demonstrate autophosphorylation of the kinase in hippocampal cultures are also useful for identifying substrates. By immunoprecipitating particular synaptic proteins (e.g., glutamate receptors) or stuctures (e.g., postsynaptic densities [PSDs]) from homogenates of labeled cultures, one could determine if their phosphorylation state was dependent on the level of Ca^{2+}-independent CaM kinase. In addition, efforts to identify, clone, and sequence PSD proteins phosphorylated by the CaM kinase may reveal important, but rare substrates localized at the synapse (e.g., receptor and channel proteins).

The studies reported in this thesis have contributed to our understanding of the CaM kinase and its regulation in intact neurons. The results of the ongoing projects described above will provide additional valuable information regarding the actual function(s) of the enzyme *in vivo*. It is enticing, however, to speculate that the highly specific control of kinase expression and subunit composition, combined with regulation of activity by autophosphorylation, endows neurons with unique mechanisms by which to modulate their synaptic properties.

Appendix A

Type II CaM Kinase in Cell Lines.

INTRODUCTION

A tractable model system was needed in which to study the regulation of the CaM kinase in intact cells. Desired characteristics of such a model system include: a) a high level of kinase expression, b) neuronal phenotype, including the expression of synaptic molecules and second messenger regulatory systems, and c) the capacity to support long-term experiments (days).

Some of the hybrid cell lines generated by the Nirenberg laboratory are known to display neuronal characteristics in culture, including the ability to form synapses with co-cultured muscle cells (Nirenberg *et al.*, 1983; Malouf *et al.*, 1984). The expression of CaM kinase by these cells, however, had not been studied. We examined three of the best characterized of these cell lines, NCB-20, NG108-15, and N18-RE-105, to determine if they expressed the brain type II CaM kinase, and if so, to determine the subunit composition and biochemical properties of the enzyme. This information would allow us to determine if these cell lines could prove useful for future biochemical and cell biological studies of CaM kinase regulation in intact cells.

METHODS

Cell lines were obtained from Dr. Nirenberg and maintained as previously described (Nirenberg *et al.*, 1983). The basic growth conditions included cell culture medium supplemented with 10% fetal calf serum and buffered with

bicarbonate and 5% $C0_2$. In some cases dibuteryl-CAMP was added to increase intracellular CAMP levels and promote neuronal differentiation.

For measurements of CaM kinase activity, cells were grown to near confluency then detached from their flasks with Ca^{+2}- and Mg^{+2}-free phosphate buffered saline (PBS) supplemented with trypsin (0.25% [w/v]). Medium containing serum was added to the suspended cells to inactivate the trypsin, and the cells spun down, washed, and resuspended in ice cold buffer. The cells were then lysed by sonication and the cell extract (2000 x g supernatant) immediately assayed for CaM kinase activity with synapsin I as an exogenous substrate (see Chapter 2 Methods). In some cases, monoclonal antibodies against the CaM kinase were added to the reaction to assess their ability to inhibit the cell line kinase.

For analysis of endogenous CaM kinase substrates, cell extracts were prepared and assayed as described (Chapter 2) except higher specific activity ^{32}P-ATP was used and exogenous synapsin I was left out of the reaction.

Immunoblotting of cell line extracts was performed as described in Erondu and Kennedy (1985). Aliquots of cell extracts, along with brain homogenates and purified forebrain kinase, were run on SDS-PAGE gels then electrophoretically transferred to nitrocellulose. The nitrocellulose blots were blocked to prevent nonspecific binding then incubated with either 6G9, a monoclonal antibody directed against the α subunit of the brain type II CaM kinase, or the polyclonal rabbit serum Darcy, which recognizes the β subunit of the enzyme.

RESULTS

All three cell lines had detectable levels of Ca^{2+}-stimulated kinase activity against synapsin I. The level of the activity, however, was low when compared to homogenates of adult rat brain. The specific activity in cell line homogenates was 10 to 20% of that measured in forebrain homogenate controls. Two criteria indicated that Ca^{2+}-stimulated phosphorylation of synapsin I by cell extracts resulted from CaM kinase activity. First, digestion of phosphorylated synapsin I revealed that greater than 90% of the ^{32}P was incorporated at the site recognized by the CaM kinase *in vitro*. Second, a monoclonal antibody to the kinase, 4A11, inhibited the activity in cell line extracts when added to the reaction. Interestingly, a second monoclonal antibody, 6E9, which inhibited purified brain type II CaM kinase was ineffective at inhibiting the cell line kinase. The specific activity of this CaM kinase-like activity in the cell line extracts was not reliably enhanced by treatments that promote differentiation in these cells, namely, treatment with high K^+ or dibuteryl cAMP.

Analysis of endogenously phosphorylated proteins in cell extracts by SDS-PAGE revealed bands of approximately 50 kD and 60 kD which showed enhanced labeling in the presence of Ca^{+2}/calmodulin (Figure 1). The 60 kD band comigrates with the β subunit of CaM kinase; however, the lower molecular weight band migrates slightly slower than the α subunit. The endogenous phosphoproteins were further analyzed by SAP-mapping. Following autoradiography, the bands were excised from the dried gel, digested with *Staph aureus* protease, and run on SDS-

PAGE to separate the fragments (Figure 2). The pattern of proteolytic fragments obtained from the endogenous 60 kD band was identical to that seen for the β subunit. The pattern of proteolytic fragments obtained from the endogenous band in the 50 kD region was strikingly similar to genuine α subunit. The digest of the 50 kD band shared three proteolytic fragments with α, but also contained another predominant band. These results suggest that the cell line extracts contain endogenous phosphoproteins that are closely related to the α and β subunits of the CaM kinase.

To further investigate the similarities of these endogenous phosphoproteins to the subunits of the CaM kinase, we subjected cell line extracts to western blot analysis. When protein blots were incubated with the monoclonal antibody 6G9, α subunit could be readily detected in homogenates of rat or mouse brain, but not in the cell line extracts. Even though several-fold more total protein was used, the cell line extracts had no cross-reacting bands. Incubation with the polyclonal rabbit serum Darcy, which recognizes the β subunit of the kinase, showed a cross-reacting band in the cell line homogenates at approximately 60 kD. This band appeared to comigrate β subunit. Quantitative blotting of the cell line extracts using Darcy indicated that the amount of cross-reacting 60 kD protein was consistent with the observed level of CaM kinase-like activity found in the extracts.

DISCUSSION

All three of the cell lines examined expressed some CaM kinase-like activity, and had phosphoproteins in the 50-60 kD region. The observation that proteolytic

digestion of the 60 kD phosphoprotein and β subunit produced the same set of peptides, combined with cross-reactivity with β subunit antiserum, suggests that the cell lines express this subunit of the kinase. The evidence for expression of α subunit in the cell lines is less clear. The phosphopeptide maps of 50 kD phosphoprotein and α subunit were not identical, and no cross-reactivity with 6G9 was detected. These data suggest that a protein similar to, but not the same as α is present in the cell lines.

The low expression of α subunit is similar to young brain tissue, where peak expression lags behind that of the β subunit. This implies that the cell lines most closely resemble immature neurons with respect to CaM kinase expression. Therefore, despite possessing some neuronal characteristics, these cell lines are not appropriate model systems for studying the regulation of the kinase in adult neuronal tissue. The cell lines could, however, be useful for developmental studies of α subunit expression. It might be possible to use the cell lines to screen potential developmental regulatory factors for their ability to enhance expression of the α subunit.

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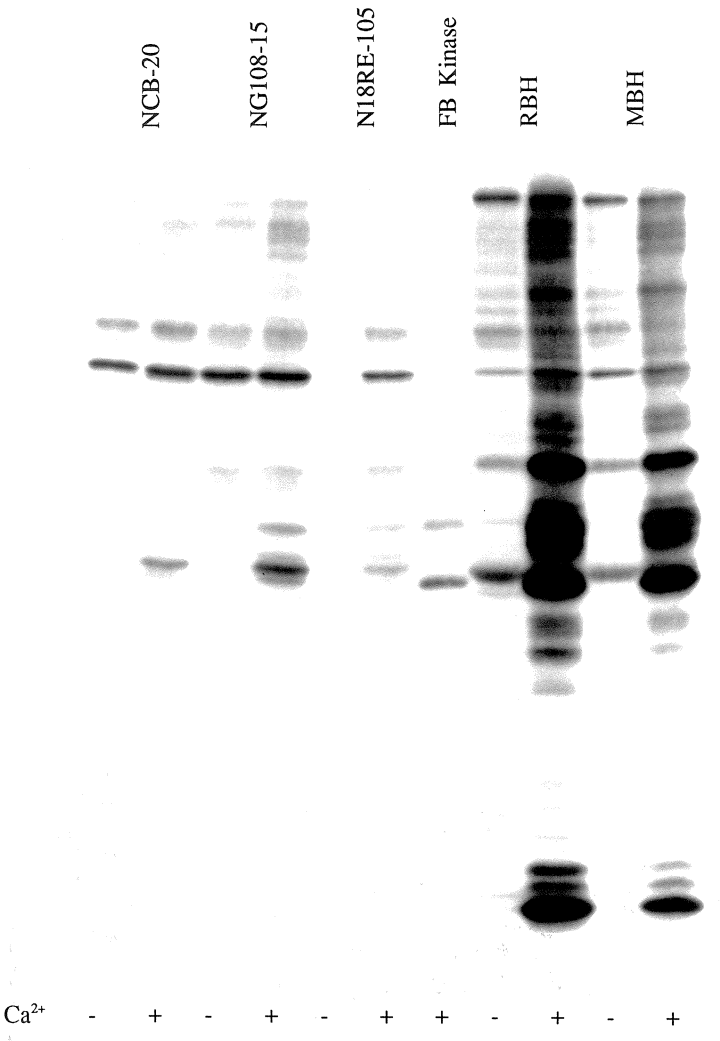
Figure 1. Endogenous Phosphorylation of Proteins in Cell Line Extracts.

Extracts of cultures were made as described in Methods. The extracts were diluted into an assay mix containing buffer, Mg-ATP (^{32}P-labeled), and calmodulin (with or without Ca^{2+} [+ or -]), and incubated at 30°C for 30 sec. The proteins were separated by SDS-PAGE, and the labeled bands visualized by autoradiography. Autophosphorylated CaM kinase and brain homogenates (mouse and rat) were used as controls.


NCB-20
NG108-15
N18RE-105
FB Kinase
RBH
MBH
Ca2+ - + - + - + + - + - +

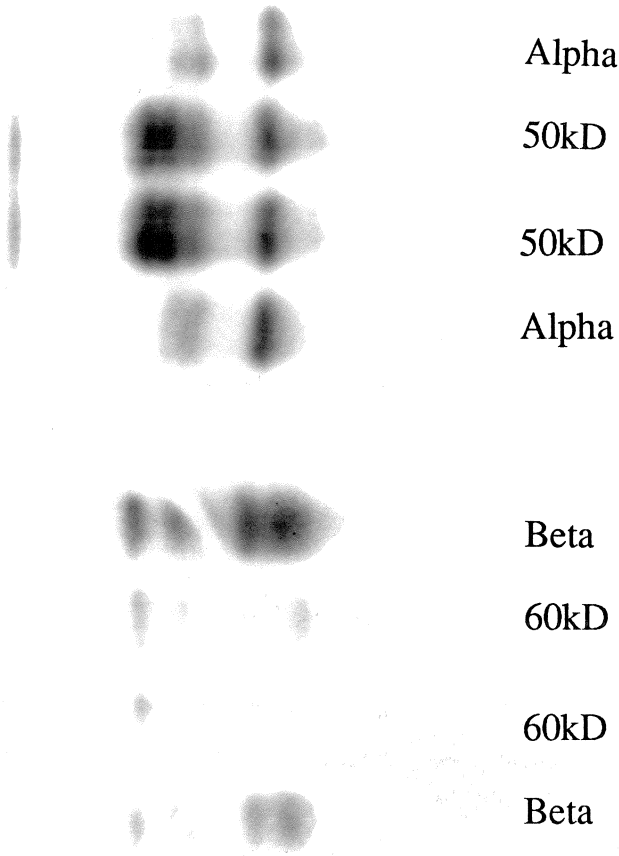

Alpha
50kD
50kD
Alpha
Beta
60kD
60kD
Beta

Figure 2. Phosphopeptide Mapping of Endogenously Labeled Proteins from Cell Line Extracts.

Phosphorylated protein bands were excised from the 50 kD and 60 kD regions of an SDS-PAGE gel (see Figure 1). The proteins in the gel pieces were digested with *Staph aureus* protease and run on a second, high percentage acrylamide gel to separate the proteolytic fragments. The ^{32}P-labeled fragments were visualized by autoradiography. Autophosphorylated α and β subunits of purifed CaM kinase were digested as controls.

Appendix B

Monoclonal Antibodies Against Phosphorylated Type II CaM Kinase.

INTRODUCTION

One limitation of biochemical studies of CaM kinase regulation in neurons is the inability to directly measure or visualize the phosphorylated form of the enzyme. In order to eliminate this deficiency, we attempted to generate antibodies specific for the phosphorylated, Ca^{2+}-independent form of the kinase. Such antibodies could recognize either the phosphorylation sites that control the production of Ca^{2+}-independent activity (i.e., $Thr_{286/287}$) or a conformational state specific to the Ca^{2+}-independent enzyme. Such antibodies would facilitate the immunocytochemical localization of the phosphorylated enzyme, and provide a means of detecting small local changes in the regulation of the kinase. Also, the antibodies could be incorporated into a more sensitive quantitative assay for phosphorylated kinase, perhaps allowing for the testing of SDS-denatured samples. Antibodies specific for the non-phosphorylated kinase could be just as useful for some of these experiments.

The generation of antibodies to the autophosphorylated form of the kinase is feasible since the phosphorylation sites must be accessible to the active site of the enzyme, and therefore are likely to be near the surface of the molecule. Also, the phosphorylation sites should be hydrophilic, and therefore less likely to be buried in the hydrophobic core of the molecule.

METHODS

A potential problem in the generation of phospho-specific antibodies to the kinase is the successful presentation of the antigen. Serum proteases could rapidly

dephosphorylate the kinase at the critical threonine autophosphorylation sites, thereby preventing the desired antigenic response. To reduce this possibility we used gamma thio-ATP in preparation of phosphorylated CaM kinase for immunizations. The kinase is able to use thio-ATP as a substrate, but the resultant thio-phosphorylated enzyme is considerably more resistant to phosphatase activity (Miller and Kennedy, 1986). Therefore, the thio-phosphorylated form of the kinase may be more likely to produce an antigenic response *in vivo*.

Thio-phosphorylated kinase was injected into New Zealand white rabbits according to standard protocols, and their sera screened by western blotting and solid phase RIA to test the antigenicity of the enzyme. Injection of the thio-phosphorylated kinase produced a significant amount of anti-kinase antibodies in the rabbit sera. Unfortunately, most of the reactivity in the sera was not specific to the phosphorylated form of the kinase. When tested on solid phase RIA, the sera were equally reactive with either phosphorylated or non-phosphorylated kinase. Western blots, however, revealed a clear selectivity for the phosphorylated kinase. This indicated that a small population of phospho-specific antibodies were present in the sera. The results with the polyclonal sera suggested that it was possible to produce phospho-specific antibodies even though these antibodies did not dominate the antigenic response. Since phosphorylation-related epitopes of the kinase were not dominant, we attempted to generate monoclonal antibodies that would allow us to select those clones that were producing the phospho-kinase-specific antibodies.

In our initial attempt to produce phospho-kinase-specific monoclonals, we chose a protocol previously used in the laboratory to generate antibodies against the

native (non-phosphorylated) form of the enzyme. In addition, a second attempt was made, using immunosuppression techniques introduced by Matthew and Patterson (1983) to selectively enhance the antigenic response to the phosphorylated kinase. The immunosupression method was originally used to increase the response to a subpopulation of proteins within a larger mixture. The technique should also work to enhance the response to specific epitopes within different forms of the same protein.

The following sections will summarize the protocols and results for each approach. These sections will be followed by an overall conclusion.

METHODS/FUSION 1

The summary of the first fusion protocol (Figure 1) shows the type of mice, the amounts of antigen, the injection schedule, and the screening method used in this procedure. Antigen was prepared for injection by phosphorylating purified rat forebrain kinase for 2 min at 30°C in a reaction mixture containing 0.4 mg/ml kinase, 0.2mg/ml calmodulin, 100uM gamma thio-ATP, 10 mM MgCl, 10 mM DTT, 20 mM Tris (pH 8.0), and approximately 300 uM free Ca^{+2}. The reaction was terminated by adding an excess of EDTA and placing the reaction tube on ice. The phosphorylated kinase was precipitated by adding ammonium persulfate to 30% saturation. This step eliminated unreacted ATP and much of the calmodulin. The precipitate was collected by centrifugation at 10,000 x g for 10 min and the pellet resuspended in PBS (0.9% NaCl [w/v] and 40 mM sodium phosphate [pH 7.4]). An equal weight of poly I-poly C was added as adjuvant and the concentration of

kinase adjusted to 100 ug/ml by adding PBS. One half of the antigen was injected into each mouse subcutaneously (500ul) while the remainder was injected interperitoneal.

Sera was collected from the mice at various times to check the titer of anti-kinase antibodies. A drop of blood was collected from the tail of each animal and the red blood cells removed by clotting and centrifugation. The sera were then screened by solid phase RIA and western blotting against phosphorylated CaM kinase. The results from these screens were used to choose the mouse used in the fusion.

For the fusion, the spleen was removed from the mouse and disrupted by maceration. The liberated spleenocytes (1.35×10^8 cells) were mixed with 6.8×10^7 6531 myeloma cells in the presence of polyethylene glycol and then plated at clonal density (3×10^5 spleenocytes/well). The developing clones were maintained in HAT selection medium.

To screen the clones for the production of anti-phospho-kinase antibodies, we developed a dot blot protocol using SDS denatured antigen. This technique successfully replicated the phospho-kinase-specific staining of western blots seen with the polyclonal rabbit sera. Each dot contained 30 ng of SDS denatured native or phosphorylated kinase which had been applied to nitrocellulose after dilution in a 10 mM solution of sodium borate. The nitrocellulose dots were blocked with Tris buffered saline containing 10% fetal calf serum and 0.5% Tween 20 for 2 hours then incubated with culture supernatants from the growing clones for an additional 2 hours at room temperature. A nitrocellulose dot containing native kinase and

another with phosphokinase was incubated with each culture supernatant in order to provide a test of differential staining. Clones with supernatants that reacted selectively with either phosphorylated or native kinase were saved and rescreened. Clones having highly reactive supernatants that reacted with both forms of the kinase were also saved.

RESULTS/FUSION 1

The results form the primary screening of the first fusion are shown in Table 1. Only 1.3% of the screened clones were specific for the phosphorylated form of the kinase using the dot blot assay. Of these 5 phospho-specific clones, only 1 was specific when tested with the solid phase RIA assay. This single clone, 3F10, was further characterized by examining its sensitivity to antigen fixation (paraformaldehyde/glutaraldehyde) and phosphate incorporation. Treatment of the kinase with fixative prior to antibody exposure slightly reduced the reactivity of 3F10 in the solid phase RIA but had no effect on the specificity for the phosphorylated form (approximately 100-fold). The level of phosphate incorporation required to produce maximal reactivity with 3F10 was the same as that required to fully stimulate the Ca^{2+}-independent activity of the kinase (Figure 2). These results indicate that the 3F10 epitope was stable and that its appearance correlated with the shift in Ca^{2+} dependence of the enzyme. The rapid plateau in 3F10 reactivity vs. kinase phosphorylation suggests that the epitope may be a conformational state of the enzyme rather than an individual phosphorylation site. Even the most rapidly filled sites do not have such a fast time course.

Together these data indicate that despite the relatively low antigenicity of this molecule, it is possible to isolate hybridomas that produce antibodies specific for the phosphorylated form of the type II CaM kinase. The stability of the 3F10 epitope to fixation of the antigen suggests that such antibodies would be useful in immunocytochemical studies of kinase regulation. Unfortunately, the 3F10 clone was lost during expansion for subcloning and was never recovered. Our ability to isolate such a clone, however, provided the impetus to continue these studies.

METHODS/FUSION 2

A summary of the protocol for the second fusion is illustrated in Figure 1. In order to increase the possibility of isolating phospho-specific clones, we adapted the immunosuppression technique of Matthew and Patterson (1983). We hoped to increase the percentage of spleen cells secreting antibodies specific to the phosphorylated kinase by suppressing the antigenic response to the native enzyme. If successful, this would increase the percentage of hybridomas secreting the desired antibodies. We used RBF mice for the second set of fusion to reduce the risk of selected clones losing the ability to secrete antibody. These mice have a chromosomal translocation that results in the colocalization of the immunoglobulin genes with the gene responsible for aminopterin resistance. Therefore, if hybridomas from this mouse line lose their Ig genes, they can no longer survive in the selection medium.

The preparation of antigen was as described for the first fusion. The injection protocol was also similar, except that injections of native kinase were

followed by injections of cyclophosphamide (100 mg/kg) to kill the responding spleenocytes. Only one round of this suppression was used, followed by 3 injections with phospho-kinase at 2 week intervals. One mouse was used as a control and was not given the cyclophosphamide injections.

Mice were bled and their sera screened as described above. The reactivity of the sera from the suppressed mice was substantially lower than that of the serum from the control mouse. This indicated that the suppression was having an effect on the production of antibodies against the kinase. This suppression, however, appeared to affect the reactivity to both native and phosphorylated kinase. Only the final bleed from the suppressed mice, taken at the time of the fusion, had a higher reactivity to the phosphorylated form of the kinase. The results obtained from screening the sera prompted us to use all three mice in the fusions.

The spleen cells from the suppressed mice were pooled for one fusion and the spleen from the control mouse was used separately. Of the 3 x 10^8 cells obtained from the experimental mice, 1.2 x 10^8 spleenocytes were fused with 6 x 10^7 HL1-653 myeloma cells. For the control mouse, 1.2 x 10^8 spleenocytes were fused with 6 x 10^7 myeloma cells. The hybridomas were plated at clonal density and maintained in selection medium (HAT). The supernatants from growing clones were assayed by solid phase RIA for reactivity to native and phosphorylated kinase.

RESULTS/FUSION 2

A summary of the screening results is shown in Table 1. Of the nearly 700 clones screened (268 from the control mouse, 429 from the suppressed mice) 35 had

3-fold or greater selectivity for one form of the kinase. Two of these clones were approximately 10-fold more reactive with the native (non-phosphorylated) kinase. The clones that were most selective for the phosphorylated kinase showed an approximately 7-fold difference in reactivity. Therefore, none of the clones tested from the second set of fusions had the degree of specificity observed for 3F10. Both the control and suppressed mice yielded several strong but non-selective clones (approximately 9% of the total screened for each group). The suppressed mice produced a higher percentage of selective clones than the control mouse. However, the control mouse yielded the clones with the highest degree of selectivity, including the two clones selective for the native kinase.

Our attempts to expand and subclone some of the most selective clones indicated that antibody production in these cells was unstable. Even when subclones were obtained, the reactivity of the culture supernatants was significantly lower than that observed during the initial screening. Only the two clones that were selective for the native enzyme retained their reactivity throughout the subcloning procedure. Yet, when these native kinase-selective subclones were thawed from frozen stocks, they also began to loose their reactivity. A second round of subcloning provided more stable cells for these two clones. We are currently testing the potential of these native kinase-selective clones for use in immunocytochemical studies of CaM kinase regulation in neurons (see Chapter 2 and Bruce L. Patton, graduate thesis).

CONCLUSIONS

Our attempts to generate monoclonal antibodies specific for the

phosphorylated form of the type II CaM kinase indicate that such antibodies can be obtained. However, such phosphorylation-state-specific antibodies appear to be rare. The application of immunosuppression techniques seems to have enhanced the likelihood of obtaining these antibodies when using the phosphorylated holoenzyme as an antigen.

The success of Steve Miller and Bruce Patton in determining the primary sequence of the critical autophosphorylation sites on the kinase offered a more direct approach to obtaining phospho-kinase-specific antibodies. Bruce Patton has developed techniques to phosphorylate synthetic peptides containing the phosphorylation site Thr_{286} from the α subunit. Using this peptide as an antigen, in both its phosphorylated and non-phosphorylated forms, Bruce Patton has generated a polyclonal rabbit serum selective for the non-phosphorylated kinase as well as monoclonal antibodies specific for the phosphorylated kinase. These monoclonal antibodies are highly specific, as was the 3F10 antibody generated in the study reported above. One of these antibodies has already proven useful in determining the localization of the phosphorylated kinase in hippocampal neurons (see Chapter 3).

The combination of monoclonal antibody technology described in this study with the use of phosphorylation site peptides as antigens has provided us with the tools to conduct experiments that were impossible or impractical with standard biochemical techniques.

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Table 1

Fusion	I	II	
		Control	Suppressed
Total Screened	389	268	429
Saved	31 (8%)	47 (18%)	107 (25%)
Non-sel.	1 (>1%)	23 (9%)	38 (9%)
Selective	10* (3%)	7 (3%)	28 (7%)

Table 1 shows the total number of clones screened and saved from each fusion. The data from the second fusion is subdivided into the hybridomas obtained from the control mouse and those obtained from the suppressed mice. Non-selective clones in the second set of fusions were saved only if they were highly reactive. Therefore, this number underestimates the number of positive clones from these fusions. Selective clones were those that showed a 3-fold or greater difference in reactivity between the phosphorylated and non-phosphorylated kinase on RIA (second set of fusions). * Of the 10 clones from the first fusion that reacted preferentially with the phosphorylated kinase on dot blots, only 1 was highly reactive and specific on RIA.

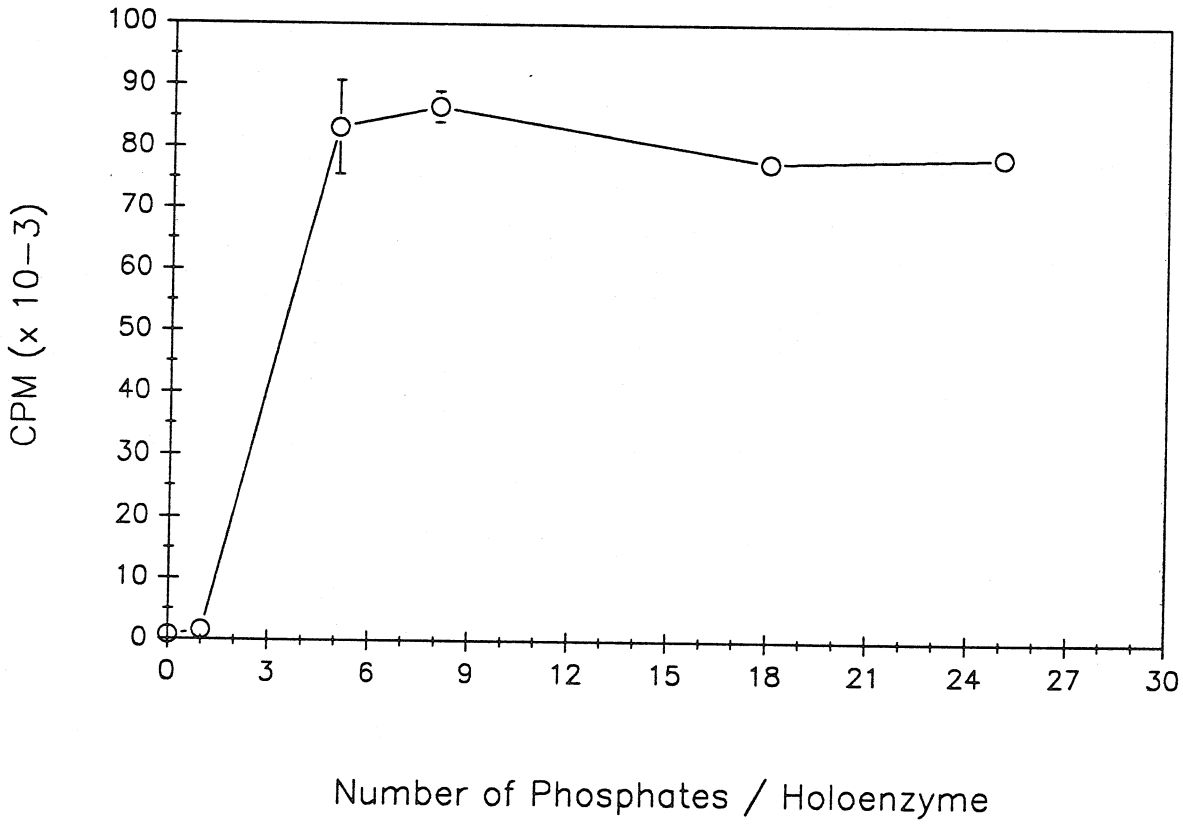
Figure 1. Summary of Fusion Protocols Used to Isolate Mononclonal Antibodies to the Phosphorylated Form of the Type II CaM Kinase.

Fusion Protocols

	I	II
Mice	Balb C	RBF
Primary Injection	50 ug phospho-kinase	50 ug native native kinase
Suppression	none	100 mg/kg cyclophos. 3 consecutive days
Test Bleed	none	day 10 post inject.
Interval	1 month	2 weeks
Secondary Injection	50 ug phospho-kinase	100 ug phospho-kinase
Test Bleed	day 4 post inject.	day 10 post inject.
Interval	1 month	2 weeks
Tertiary	50 ug phospho-kinase 4 consecutive days **FUSION** on day 5	100 ug phospho-kinase
Test Bleed		day 10
Interval		2 weeks
Quaternary		100 ug phospho-kinase **FUSION** on day 2
Screen	dot blot	solid phase RIA

Figure 2. Reactivity of Antibody 3F10 with CaM Kinase at Various Stages of Autophosphorylation.

Autophosphorylated kinase was prepared as described in Methods. The reaction time and concentration of Mg^{+2} were varied to achieve the desired level of phosphorylation. The phosphorylated kinase was used to coat the wells of RIA plates which were then incubated with culture supernatant from the clone 3F10. Following the primary incubation, the plates were washed and incubated with purified rabbit anti-mouse IgG antibodies. After the secondary incubation, the plates were washed and exposed to ^{125}I-labeled protein A. After a final wash, acetic acid was used to strip the wells and the eluted protein A quantified with a gamma counter. Contols showed that the binding of protein A was not saturated at 80,000 CPM.


100
90
80
70
60
50
40
30
20
10
0
CPM (x 10−3)
0
3
6
9
12
15
18
21
24
27
30
Number of Phosphates / Holoenzyme

Appendix C

Structure and Regulation of Type II Calcium/Calmodulin-Dependent Protein Kinase in Central Nervous System Neurons.

CSH Symp. Quant. Biol. LV (in press)

Structure and Regulation of Type II Calcium/Calmodulin-Dependent Protein Kinase in Central Nervous System Neurons

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In a recent talk at Caltech, David Baltimore suggested that molecular biology (the study of gene expression) and neurobiology are at similar stages. Both fields have identified many of the cast of important characters, but we still have much to learn about mechanisms and algorithms. In molecular neurobiology, the "mechanisms" are the ways that individual proteins work together to regulate release of transmitter or to modulate receptors and ion channels. The "algorithms" are the ways that these mechanisms are coordinated to allow neurons to maintain homeostasis, while at the same time adapting to changes in the external environment and storing information through molecular changes that alter the behavior of neural networks.

In the last several years, the field of molecular neurobiology has appropriately placed great emphasis on identification of the relevant "characters." By characters, we mean the proteins that make up synaptic vesicles and other synaptic organelles, transmitter receptors, ion channels, and neuronal regulatory molecules. In this paper, we will first describe one "character" that we have studied for several years, called type II Ca^{2+}/calmodulin-dependent protein kinase (CaM kinase II). Then, we will discuss an interesting mechanism by which this particular protein kinase may allow neurons to store information, if only for a short time. Finally, we will describe an experimental system that we hope to use to learn how the CaM kinase, together with other neuronal proteins, participates in regulatory algorithms that are important for brain function.

EXPERIMENTAL PROCEDURES

Detailed procedures for most of the experiments were presented previously (Erondu and Kennedy, 1985; Miller and Kennedy, 1985, 1986; Miller et al., 1988; Bulleit et al., 1988; Patton et al., 1990; Molloy and Kennedy, in preparation).

Immmunocytochemical staining of synaptosomes. Synaptosomes were prepared from forebrains, hippocampi and cerebelli of 8 to 12 young adult rats, by a modification of the method of Cohen et al. (1977) which is briefly summarized here. Brain regions were dissected and homogenized in a sucrose buffer. Large particles were removed by centrifugation at 1500 g for 10 min. Crude synaptosomes and mitochondria were removed from the supernatant solution by centrifugation at 18,000 g for 10 min, then carefully resuspended in a sucrose buffer and layered onto discontinuous sucrose density gradients. After centrifugation at 82,000 g for 2 hrs, an enriched synaptosome fraction was harvested from the 1.0M : 1.2 M sucrose interface. The synaptosomes were diluted four-fold with 0.32 M sucrose in bicarbonate buffer (pH 8.0), then sedimented by centrifugation at 37,000 g for 20 min. The pellet was gently resuspended in 1 ml or less of 0.32 M sucrose in bicarbonate buffer.

The synaptosomes were fixed and stained essentially by the method of DeCamilli et al. (1983b). Each resuspended synaptosome pellet was fixed by slow 20 fold dilution into 4% paraformaldehyde, 0.1% glutaraldehyde, 20 mM cacodylate buffer (pH 7.4), 0.05 mM $CaCl_2$, 0.32 M sucrose at 4°. The lightly fixed synaptosomes were recovered by centrifugation at 17,000 g for 20 min. The pellets were gently scraped with a teflon rod into a small volume of 0.12 M phosphate (pH

7.4) and homogenized by hand in a small teflon/glass homogenizer. The resuspended pellet was passed several times, slowly, through a 25 guage needle. Each suspension (100-180 µl) was placed in a tube prewarmed to 58°. After 15 sec, prewarmed 3% low melting point agarose (100-180 µl) dissolved in 5 mM phosphate (pH 7.4) was added. The mixture was stirred with a prewarmed pipette and quickly placed into warm frames constructed as described in DeCamilli et al. (1983b). The agarose embedded synaptosomes were allowed to cool for 1 hr at room temperature. The resulting thin slabs were cut with a razor blade into 2mm x 2mm blocks. Six blocks of each sample were placed into each of several small test tubes. The blocks were first incubated for 30 min in 2 mls 0.5 M Tris (pH 7.4), then for 30 min in 0.5 mls Tris buffer containing 2 mg/ml sheep IgG. Finally, they were incubated overnight at 4° in 0.2 mls Tris buffer containing 2 mg/ml sheep IgG and either 0.04 µg/ml 6g9 monoclonal antibody purified from Ascites fluid by chromatography on a Protein A affinity column or 40 µg/ml mouse IgG. The blocks were washed at room temperature with 5 changes of 2 mls solution B (20 mM phosphate [pH 7.4], 0.5 M NaCl) over 40 minutes. They were then incubated for 90 min in 0.15 ml solution B containing 50 mg/ml ovalbumin, 2 mg/ml sheep IgG, and a 1/5 dilution of ferritin-conjugated sheep anti-mouse IgG antisera purchased from Janssen Life Sciences Products. The blocks were washed again with 5 changes of 2 mls solution B over 40 minutes. Finally, the blocks were fixed in 2 mls 1% glutaraldehyde, 0.12 M phosphate (pH 7.4) for 30 min at 4°. The fixed blocks were washed for 20 min in 0.12 M phosphate (pH 7.4) at 4°, then osmicated in ice cold 1% OsO_4 for 1 hr. After two 5 min washes in 0.1 M

phosphate (pH 7.4), the blocks were dehydrated in a graded alcohol series and embedded in epon by standard methods for sectioning. Ultrathin sections were examined and photographed with a Phillips 301 electron microscope. The number of bound ferritin grains per μ of PSD was determined from photographs with the aid of a Tektronix digitizing tablet.

RESULTS AND DISCUSSION

Molecular Structure of Brain Type II CaM kinase. Type II CaM kinase is a calmodulin-dependent protein kinase that was first purified from brain with the use of an assay that measured its ability to phosphorylate synapsin I (Bennett et al., 1983; McGuinness et al., 1985; Miller and Kennedy, 1985) or tubulin (Goldenring et al., 1983) in the presence of Ca^{2+} and calmodulin. It is a large hetero-multimer composed of twelve homologous subunits. The predominant holoenzyme purified from the forebrain contains, on the average, nine subunits of molecular weight 54,000 called α and, on the average, three subunits of molecular weights 57,000 to 60,000 that are alternative products of the same gene and are called β and β'. The subunits appear to associate randomly into dodecameric holoenzymes; the ratio of subunits in holoenzymes from a particular brain region is approximately the same as the ratio of the subunit messages from that region (McGuinness et al., 1985; Miller and Kennedy, 1985; Bulleit et al., 1988). This ratio varies considerably. For example, the forebrain holoenzyme contains three times as many α subunits as β subunits, while the cerebellar holoenzyme contains approximately four times as many β subunits as α subunits.

The α and β subunits are neuron specific. The gene encoding the α subunit

is expressed at highest levels in mature forebrain neurons; while the gene encoding the β subunit is expressed more uniformly in all neurons (Bulleit et al., 1988). Recently, two additional CaM kinase genes encoding γ and δ subunits have been isolated and sequenced (Tobimatsu et al., 1988; Tobimatsu and Fujisawa, 1989). The γ and δ subunits are highly homologous to the α and β subunits but are expressed uniformly in many tissues including brain.

Cellular Distribution of type II CaM kinase. An early finding that focused attention on the potential importance of CaM kinase II for CNS function was its high concentration in the brain. The kinase is particularly highly concentrated in forebrain neurons where it comprises approximately 2% of total hippocampal protein and 1% of cortical protein (Erondu and Kennedy, 1985). Within the forebrain, about half of the kinase is soluble and distributed throughout the cytosol (Kennedy, et al., 1983b; Ouimet et al., 1984). The rest is associated with particulate structures (Kennedy, et al., 1983b).

Association of the CaM kinase with postsynaptic densities. At least one of the particulate structures that the kinase associates with is the postsynaptic density (PSD), a prominant specialization of the submembranous cytoskeleton that is attached to the postsynaptic membrane at CNS synapses (Cotman, et al., 1974). The α subunit was found to be identical to a protein that is a major constituent of highly enriched PSD fractions prepared from brain (Kennedy et al., 1983a; Kelly et al., 1984). This protein had previously been refered to as the "major postsynaptic density protein" (Kelly and Cotman, 1978). Quantitative estimates indicate that the CaM kinase comprises 20% to 40% of the total protein in the PSD fraction (Kelly

et al., 1984; Miller and Kennedy, 1985). Curiously, the content of CaM kinase is much reduced in PSDs isolated from the cerebellum where the kinase is comprised mainly of β subunits. We have postulated that the α subunit may contain a binding site for a PSD receptor protein (Miller and Kennedy, 1985).

Preparation of purified postsynaptic densities from brain homogenates requires the isolation of a crude synaptosomal fraction, followed by treatment of that fraction with detergent, either triton X-100 (Cohen et al., 1977) or sodium lauroyl sarcosinate (Cotman et al., 1974). Finally, PSDs relatively free of membrane lipids are isolated by differential or density gradient centrifugation. Because of the necessity for detergent treatment to remove synaptosomal membranes, there has been controversy about the relationship between the composition of the PSD fraction and the true composition of PSDs *in vivo*. Some proteins can become denatured by detergent and may associate artifactually with the PSD fraction (Matus, et al., 1980). To confirm the presence of the CaM kinase in PSDs prior to detergent treatment, we stained synaptosomes immunocytochemically with a monoclonal antibody against the CaM kinase (Fig. 1). Because these experiments were specifically designed to detect the kinase in PSDs, synaptosomes were kept intact during the incubations with antibodies. Figure 1 contains examples of forebrain synaptosomes labeled with either anti-α subunit or control mouse antibodies and then with ferritin-labeled sheep anti-mouse IgG secondary antibodies. Figure 2A summarizes the extent of labeling with control and anti-kinase antibodies. On the average, the concentration of ferritin particles was three times higher in PSDs stained with the specific anti-α subunit antibody. When synaptosomes from cerebellum and from hippocampus were labeled

separately (Fig. 2B), the average density of particles in hippocampal PSDs was approximately twice that in cerebellar PSDs. This is consistent with the earlier finding that less CaM kinase is associated with cerebellar PSDs than with forebrain PSDs; although the difference between PSDs from the two regions is greater when measured biochemically (Miller and Kennedy, 1985). The results support the hypothesis that the CaM kinase is highly concentrated in PSDs *in vivo*. It is important to remember, however, that the kinase is not uniquely located in PSDs. About half of it is soluble and appears to be distributed throughout the neuronal cytosol (Ouimet et al., 1984; Erondu and Kennedy, 1985). From the ferritin-labeling data, we estimate that the effective concentration of α subunits in forebrain PSDs is approximately 100 to 400 μM. This is 5 to 10 times higher than the concentration of α subunits in the cytosol (19 to 37 μM), estimated from its abundance in forebrain homogenates (1% of total protein).

The high concentration of CaM kinase in PSDs in the hippocampus suggests that it may be an important target for the Ca^{2+} current that is generated by activation of NMDA receptors. This current is necessary for induction of long-term potentiation (Malenka et al., 1988). Recent physiological studies from the laboratories of R. Nicoll and R. Tsien indicate that inhibition of postsynaptic CaM kinase blocks induction of LTP, strengthening the hypothesis that type II CaM kinase plays a role in the generation of LTP (Malenka, et al. 1989, Malinow et al., 1989).

Regulation of CaM kinase II by autophosphorylation. Each individual CaM kinase subunit can be autophosphorylated when the holoenzyme is activated in the presence

of Ca^{2+}/calmodulin (Bennett et al., 1983). This autophosphorylation is the basis of an interesting mechanism for controlling CaM kinase activity. Non-phosphorylated CaM kinase is catalytically active only in the presence of Ca^{2+}/calmodulin. However, if the kinase is briefly autophosphorylated by incubating it for five seconds in the presence of Ca^{2+}/calmodulin and ATP before it is added to assay tubes containing exogenous substrate, a new Ca^{2+}-independent activity becomes apparent (Fig 3; Miller and Kennedy, 1986). The magnitude of this activity depends upon the substrate (Patton et al., 1990). With a synthetic peptide substrate, such as that used in the experiment shown in Fig. 3, phosphorylation proceeds in the absence of Ca^{2+} at about 60% of the rate in the presence of Ca^{2+}.

This switch to a partially Ca^{2+}-independent state has four important features. First, the Ca^{2+}-independent activity is fully activated after addition of as little as 2 to 3 moles of phosphate to the CaM kinase per mole of dodecameric holoenzyme (Miller and Kennedy, 1986). Therefore, the activation appears to be cooperative; autophosphorylation of one or two of the subunits in a holoenzyme produces activation of the other subunits. Second, the autophosphorylated kinase continues to phosphorylate itself as well as exogenous substrates; thus, autophosphorylation becomes Ca^{2+}-independent. Third, the effects of autophosphorylation are reversible. Ca^{2+}-independent activity is lost when the kinase is dephosphorylated by protein phosphatases (Miller and Kennedy, 1986; Lai et al., 1986; Miller et al., 1988). Finally, autophosphorylation is restricted to individual holoenzymes. Autophosphorylated subunits within a holoenzyme can cause autophosphorylation of neighboring subunits, but one activated holoenzyme does not autophosphorylate

another (Miller and Kennedy, 1986).

Taken together, these properties suggest that the CaM kinase can act as a kind of "switch" (Fig. 4). In state 1, the kinase is completely dependent on Ca^{2+} and calmodulin for activity. When sufficient autophosphorylation has occured, the kinase is switched to state 2 in which it has a substantial Ca^{2+}-independent kinase activity with exogenous substrates. Furthermore, in state 2, Ca^{2+}-independent autophosphorylation can oppose dephosphorylation by cellular phosphatases, which would return the kinase to state 1. This switch mechanism may allow the CaM kinase to retain information *in vivo* about prior activating Ca^{2+} signals. This information would be "read out" as continuing phosphorylation of functionally significant substrate proteins. The length of time that the information would be retained would depend on the balance between the rate of Ca^{2+}-independent autophosphorylation and the local catalytic rate of cellular phosphatases.

Identification of regulatory autophosphorylation sites. In order to learn more precisely how the switch mechanism operates, the specific autophosphorylation site within the CaM kinase that controls Ca^{2+}-independent activity has been identified Threonine-286, located on the amino-terminal side of the calmodulin binding domain (Fig. 5), is autophosphorylated rapidly when the kinase is activated by Ca^{2+}/calmodulin (Thiel et al., 1988; Miller et al., 1988; Schworer et al., 1988). The rate of autophosphorylation and dephosphorylation of this site correlates closely with the onset and decay, respectively, of Ca^{2+}-independent activity (Miller et al., 1988). The importance of threonine-286 for control of Ca^{2+}-independent activity has been confirmed by experiments in which threonine-286 was changed to leucine by *in*

vitro mutagenesis (Hanson et al., 1989). The mutated kinase does not show Ca^{2+}-independent activity upon autophosphorylation. The location of threonine-286 suggests a model in which its autophosphorylation partially mimics binding of calmodulin and prevents refolding of the kinase into an inactive conformation (Fig. 6).

When Ca^{2+} is removed from the autophosphorylation reaction after the kinase is in state 2, a new site becomes autophosphorylated and the kinase is now insensitive to further stimulation by Ca^{2+}/calmodulin (Fig. 3; Hashimoto et al., 1987). We have identified two additional sites that are rapidly autophosphorylated only after Ca^{2+} is removed from an ongoing autophosphorylation reaction. One of these, threonine-305 (Fig. 5; Patton et al., 1990), is located in the middle of the calmodulin binding domain in a sequence of five amino acids that is required for high affinity binding of calmodulin (Payne et al., 1988). The other site, serine-314, is located at the carboxyl-terminal end of the calmodulin binding domain. Inhibition of sensitivity to calmodulin and reversal of this inhibition correlate well with autophosphorylation and dephosphorylation of threonine-305, respectively. Autophosphorylation of serine-314 causes only a two-fold reduction in affinity for calmodulin (Patton et al., 1990). Curiously, phosphoserine-314 is resistant to dephosphorylation by protein phosphatases.

The sequence of regulatory events governing activity of one subunit of the CaM kinase is summarized in Figure 6. Inactive kinase is represented in 6A. In the presence of Ca^{2+}, calmodulin binds to a specific sequence resulting in a conformational change that opens the active site and allows phosphorylation of

exogenous substrates (6B). At the same time, threonine-286, next to the calmodulin binding domain, is rapidly autophosphorylated. When Ca^{2+} is removed from the reaction, calmodulin is released from the kinase (6C). However, the phosphate group on threonine-286 prevents complete refolding of the kinase. In this state, the kinase is still active although at a somewhat reduced rate. An additional site, threonine-305, located in the middle of the calmodulin binding domain, is now autophosphorylated. In the state depicted in C, the kinase has a substantial Ca^{2+}-independent activity, but cannot be further stimulated by Ca^{2+}/calmodulin. It is returned to the inactive state A by dephosphorylation by cellular phosphatases. This model depicts the cycle for one subunit and does not illustrate the cooperative activation of subunits within a holoenzyme. Cooperative activation may occur by either of two mechanisms. Autophosphorylated subunits may activate adjacent subunits through allosteric conformational changes transmitted through subunit-subunit interactions. Alternatively, autophosphorylated subunits may be able to directly phosphorylate neighboring subunits within the holoenzyme.

Organotypic cultures of hippocampal neurons. We are interested in studying how the CaM kinase functions *in situ* within hippocampal neurons, where it is expressed at high concentration and has been implicated in the initiation of long-term potentiation. Physiologists have successfully studied synaptic transmission between hippocampal neurons in slices prepared acutely from adult brain (Nicoll, 1988). However, this system may not be ideal for biochemical studies because damaged tissue at the surface of the slice cannot be separated from intact neurons at the center of the slice. Dissociated cultures of hippocampal neurons are healthy, but

synapses are made randomly within the cultures and at relatively low density (Bartlett and Banker, 1984). We have explored the use of a third preparation, organotypic cultures of hippocampal slices. This preparation was first developed by Gahwiler in Switzerland (Gahwiler, 1988) and we have modified it slightly for our purposes (Molloy and Kennedy, in preparation). To prepare the cultures hippocampi are dissected from 4 to 6 day old rats and cut into 400 μ slices. The slices are fastened onto collagen coated cover slips with a drop of liquid collagen. When the collagen has polymerized, the cover slips are placed into test tubes in 1 ml of liquid medium (Gahwiler, 1984; Molloy and Kennedy, in preparation) and incubated on a roller so that the cultures are exposed periodically to air. On the fourth day, the cultures are treated with mitotic inhibitors to reduce the division of glial cells. After about two weeks, the cultures have shed dead tissue and flattened to a thickness of 2 to 3 cells (50 to 80 μ). They retain many anatomical characteristics of the hippocampus *in vivo*, including a mossy fiber projection from dentate granule cells to area CA3 and a Schaffer collaterol pathway from CA3 to CA1 neurons (Gahwiler, 1988). The neurons contain CaM kinase at a concentration similar to that *in vivo*. Immunocytochemical staining of the cultures for the α subunit of CaM kinase (7; upper) or for the synaptic vesicle associated protein synapsin I (7; lower) produces patterns of staining similar to those in fixed tissue from adult brain (Ouimet et al., 1984; DeCamilli et al., 1983a). CaM kinase staining is dense in dendrites and cytosol with dark patches along dendrites that may represent concentrations of kinase at postsynaptic densities (Molloy and Kennedy, in preparation). Synapsin I staining is concentrated in small punctate structures that

may represent presynaptic terminals. Our goal is to use these cultures to answer several specific questions. How is the CaM kinase regulated *in situ*? Is the switch mechanism described above used *in situ*? What are the substrate proteins for the CaM kinase in specific parts of the neurons. Finally, what functions does the CaM kinase regulate.

Autophosphorylation of CaM kinase II in situ. We attempted to determine whether threonine-286, the site that controls Ca^{2+}-independent activity *in vitro* could be labeled with ^{32}P phosphate *in situ* (Fig. 8, Molloy and Kennedy, in preparation). Several cultures were incubated overnight in a medium containing $^{32}PO_4$ to label ATP pools and phosphorylated proteins. The cultures were homogenized in a buffer that suppresses the activity of protein phosphatases. The CaM kinase was immunoprecipitated with specific anti-kinase monoclonal antibodies. After separation of the subunits by SDS page, the α subunit was digested with trypsin, and the labeled tryptic phosphopeptides were fractionated by HPLC to generate a peptide map. Comparison of this map to similar maps of purified CaM kinase autophosphorylated *in vitro* (Miller et al., 1988; Patton et al., 1990) permitted identification of the most prominent site labeled with $^{32}PO_4$ *in situ* as threonine-286 (Fig. 8; Molloy and Kennedy, in preparation). Thus, kinase molecules can be autophosphorylated at this site *in situ*, even in the absence of exogenous stimulation.

In order to determine the extent of autophosphorylation of threonine-286 *in situ*, we measured the proportion of CaM kinase in the Ca^{2+}-independent state in culture homogenates. We first maximally autophosphorylated the CaM kinase in a

set of homogenates by incubating them with ATP in the presence of Ca^{2+}. We then determined that, in these homogenates with synapsin I as sustrate, CaM kinase activity in the absence of Ca^{2+} was 27% of the full activity in the presence of Ca^{2+}. Synapsin I was used as substrate in these experiments because, unlike synthetic peptides, its phosphorylation in the absence of Ca^{2+} in culture homogenates is catalyzed entirely by CaM kinase II (Molloy and Kennedy, in preparation). We next determined kinase activity in the absence and presence of Ca^{2+} in homogenates prepared under conditions designed to preserve the state of autophosphorylation *in vivo*. The value was 8.4±0.4%, indicating that approximately 31% of the kinase in culture homogenates is in the Ca^{2+}-independent state. This percentage was not reduced by extended treatment of the cultures with a variety of glutamate receptor antagonists before homogenization. Thus, the high proportion of autophosphorylated kinase in the cultures does not depend on spontaneous electrical activity. The proportion was reduced, however, by treatment of the cultures with H7, a general protein kinase inhibitor that crosses cellular membranes, or by reduction of the concentration of external Ca^{2+} in the culture medium. When Ca^{2+} was removed from the medium, the proportion of kinase in the Ca^{2+}-independent state decreased to approximately 5% in 25 to 30 minutes (Molloy and Kennedy, in preparation). Therefore, both Ca^{2+} and continuing kinase activity are required to maintain the autophosphorylated state *in vivo*.

A high percentage of type II CaM kinase is in the Ca^{2+}-independent state in hippocampi from intact rats. Forebrains and hippocampi were dissected from rats of increasing age, and homogenized as described for hippocampal cultures. The

percentage of kinase in the Ca^{2+}-independent state in these homogenates was 23 ± 0.9 for rats of 6 to approximately 24 days of age, and 13 ± 0.7 for rats older than 25 days, suggesting a developmental change around day 25 postnatal (Molloy and Kennedy, in preparation).

The functional significance of this high proportion of Ca^{2+}-independent CaM kinase activity is at present unknown. It will be important to determine the subcellular location of the autophosphorylated kinase, as well as the identity of substrate proteins of the kinase *in situ*. However, the experiments with hippocampal cultures have demonstrated unequivocally that the autophosphorylation switch mechanism is used *in situ*. It seems likely that organotypic cultures of defined brain regions will be used extensively in the future to study the biochemistry of neuronal plasticity. They provide a system in which new methods can be developed to follow modulatory reactions through time and at particular sites within neurons.

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Figure 1. Synaptosomal postsynaptic densities labeled with antibody against the α subunit of type II CaM kinase. Synaptosomes were prepared, embedded in agarose and labeled with either a specific monoclonal antibody against the α subunit (6g9) or control mouse IgG, then with ferritin-labeled sheep anti-mouse IgG, as described under Experimental Procedures. Panels A and C are representative synaptosomes labeled with control mouse IgG. Panels B and D are representative synaptosomes labeled with monoclonal antibody 6g9. The calibration bars are 100 nm.

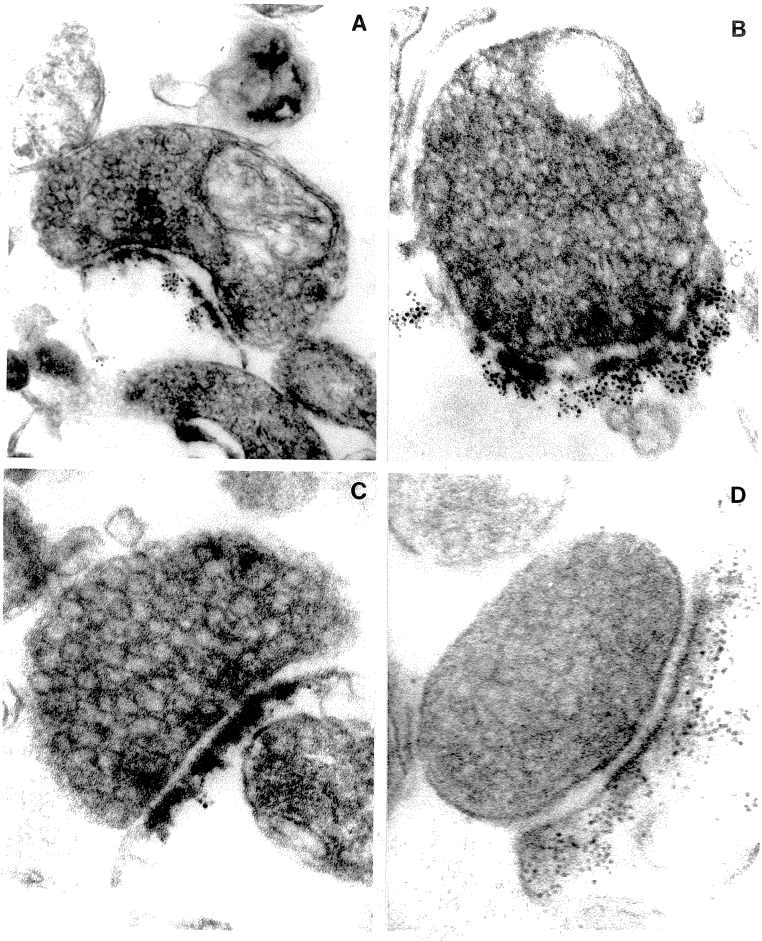

A
B
C
D

Figure 2. Quantitation of ferritin-labeling of synaptosomal postsynaptic densities. The number of ferritin grains per micron of PSD was determined for several synaptosomal profiles after labeling with antibodies as described in the legend to Figure 1. A. Labeling of forebrain PSDs with monoclonal antibody 6g9 or control mouse IgG. B. Labeling of cerebellar and hippocampal PSDs with monoclonal antibody 6g9 or control mouse IgG.

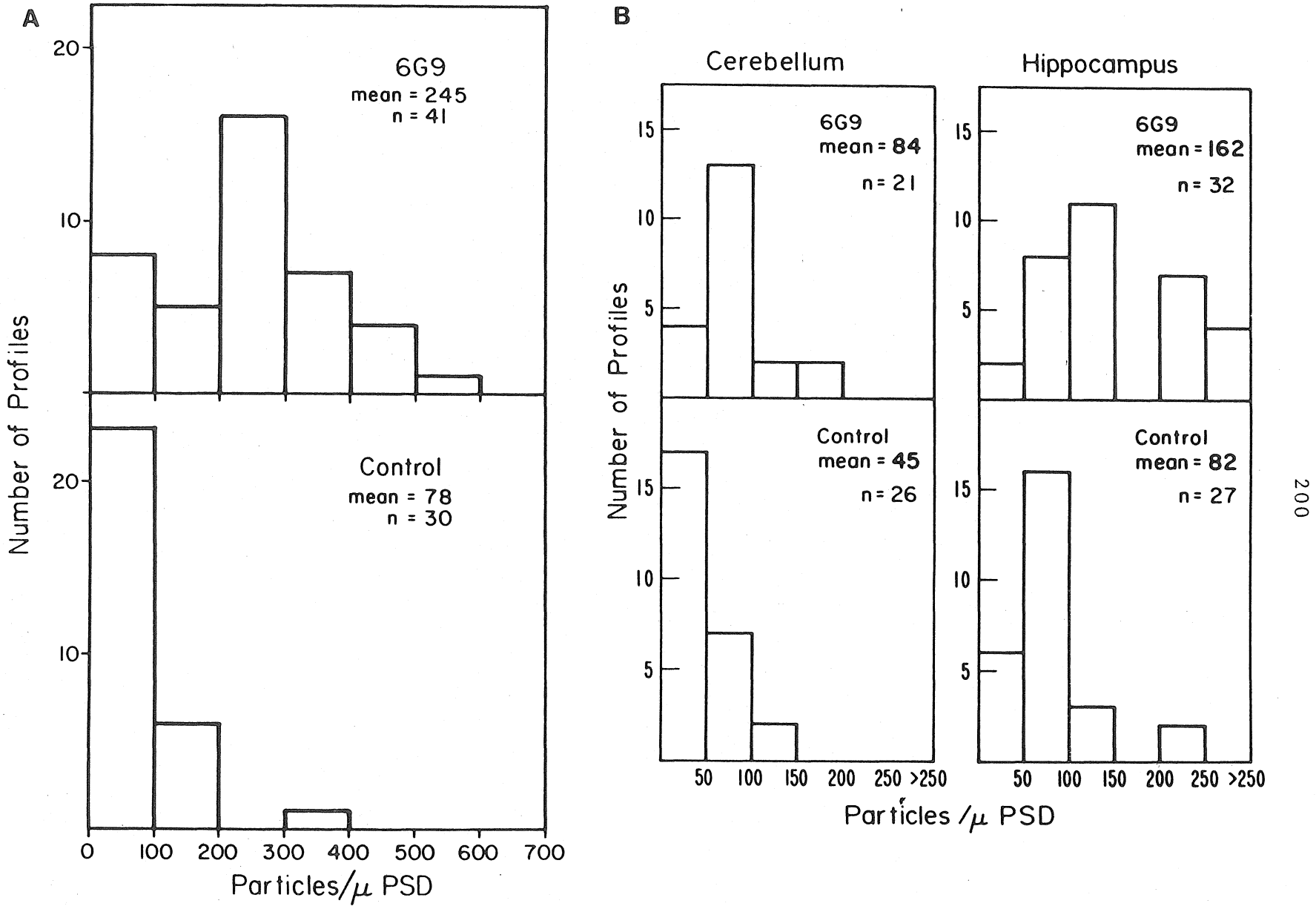

A
6G9
mean = 245
n = 41
Control
mean = 78
n = 30
Number of Profiles
Particles/μ PSD
B
Cerebellum
Hippocampus
6G9
mean = 84
n = 21
Control
mean = 45
n = 26
6G9
mean = 162
n = 32
Control
mean = 82
n = 27
Number of Profiles
Particles /μ PSD

Figure 3. Effects of autophosphorylation on the kinase activity of type II CaM kinase. Purified kinase was autophosphorylated and then assayed with a synthetic peptide substrate ("Calmodulin-dependent protein kinase substrate analog", purchased from Peninsula Laboratories) as previously described (Patton et al., 1990). Kinase was autophosphorylated for the indicated times in the presence or presence followed by absence of Ca^{2+}. Control and autophosphorylated kinase was incubated in the autophosphorylation mix without Ca^{2+}. Autophosphorylated kinase was then diluted into a second assay mix containing γ^{32}P-ATP, peptide substrate, and either EGTA or Ca^{2+}/calmodulin (Patton et al., 1990). Bars represent initial rates of kinase activity expressed as a percent of control activity in the presence of Ca^{2+}.

Effects of Autophosphorylation on Activity

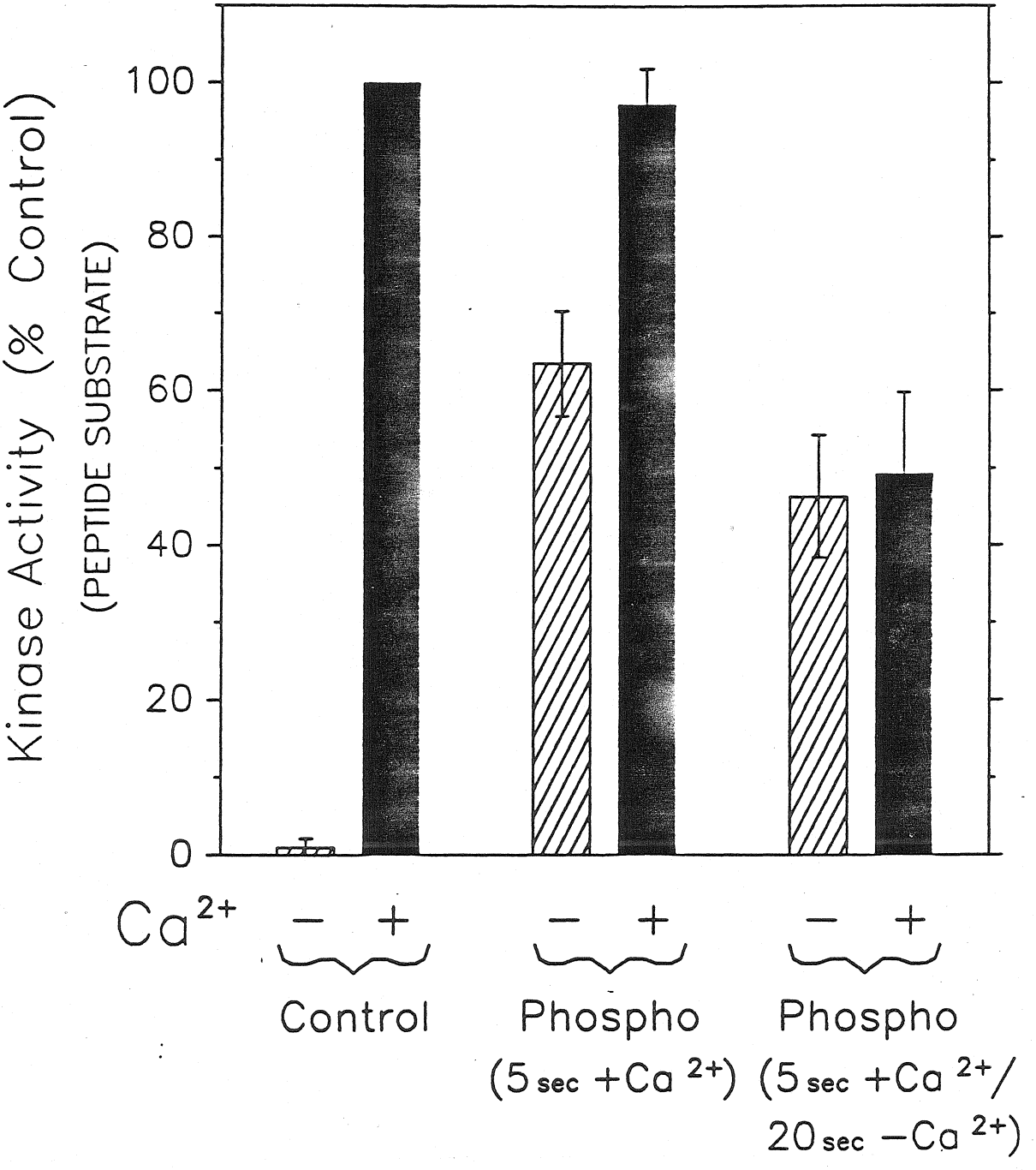


Figure 4. Hypothetical "switch" model of regulation of type II Ca^{2+}/calmodulin-dependent protein kinase by autophosphorylation and dephosphorylation. Modified from Figure 7 of Miller and Kennedy, Cell 44, 861-870, 1986, Cell Press Cambridge.

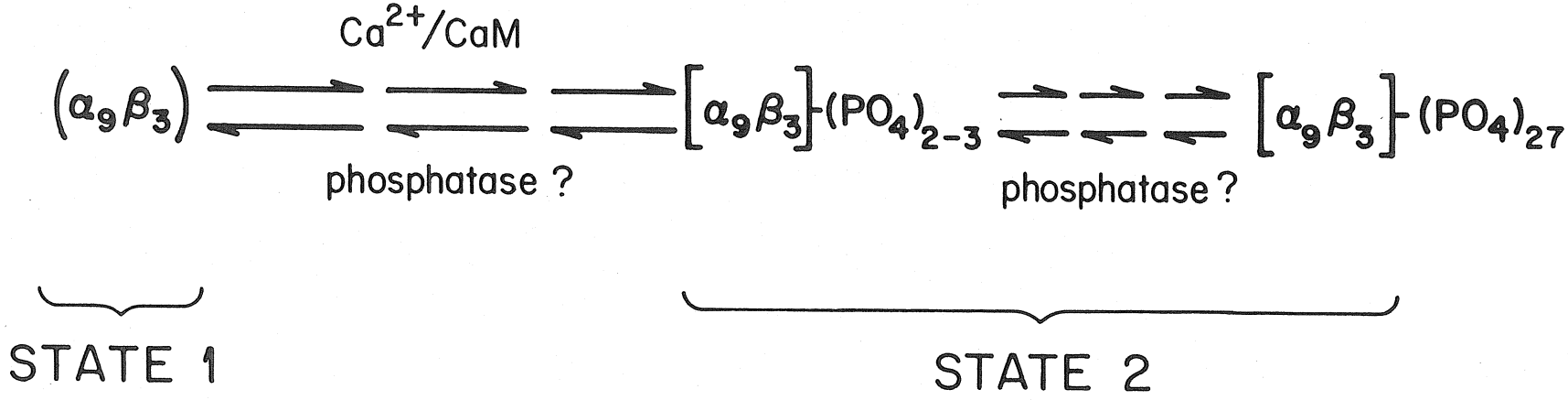

Ca2+/CaM
(α9β3)
[α9β3]-(PO4)2-3
[α9β3]-(PO4)27
phosphatase ?
phosphatase ?
STATE 1
STATE 2

Figure 5. Location of autophosphorylation sites in the sequence of type II Ca^{2+}/calmodulin-dependent protein kinase. Closed circles represent sites that are autophosphorylated rapidly when the kinase is activated by Ca^{2+}/calmodulin. The site to the left of the calmodulin binding domain is threonine-286 in the α subunit (287 in the β subunit). Open circles represent sites that are autophosphorylated only when Ca^{2+} is removed from the reaction after autophosphorylation of threonine- 286. The site in the middle of the calmodulin binding domain is threonine-305 in the α subunit (threonine-306 in the β subunit). The site to the right of the calmodulin binding domain is serine-314 in the α subunit (serine-315 in the β subunit). The regions marked by dark bars or stippled bars are present only in the sequence of the β subunit. The region marked by the stippled bar is spliced out of the β' subunit. Modified from Figure 9, Patton et al., J. Biol. Chem. **265**, in press, 1990, American Society for Biochemistry and Molecular Biology, Betheda.

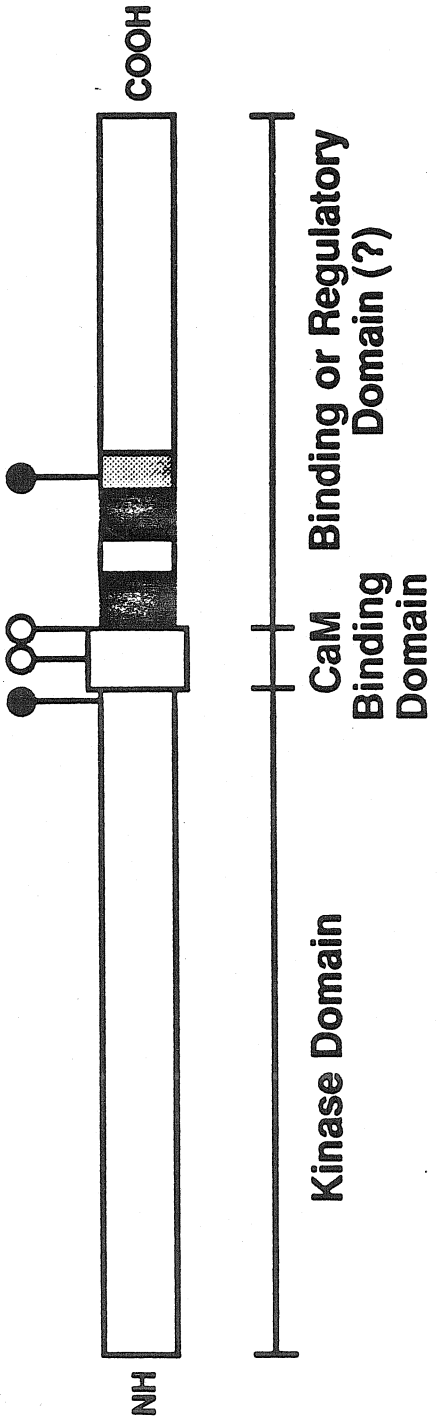

COOH
NH
Kinase Domain
CaM Binding Domain
Binding or Regulatory Domain (?)

Figure 6. Schematic model of regulation of a subunit of type II Ca^{2+}/calmodulin-dependent protein kinase by autophosphorylation. See text for explanation and discussion.

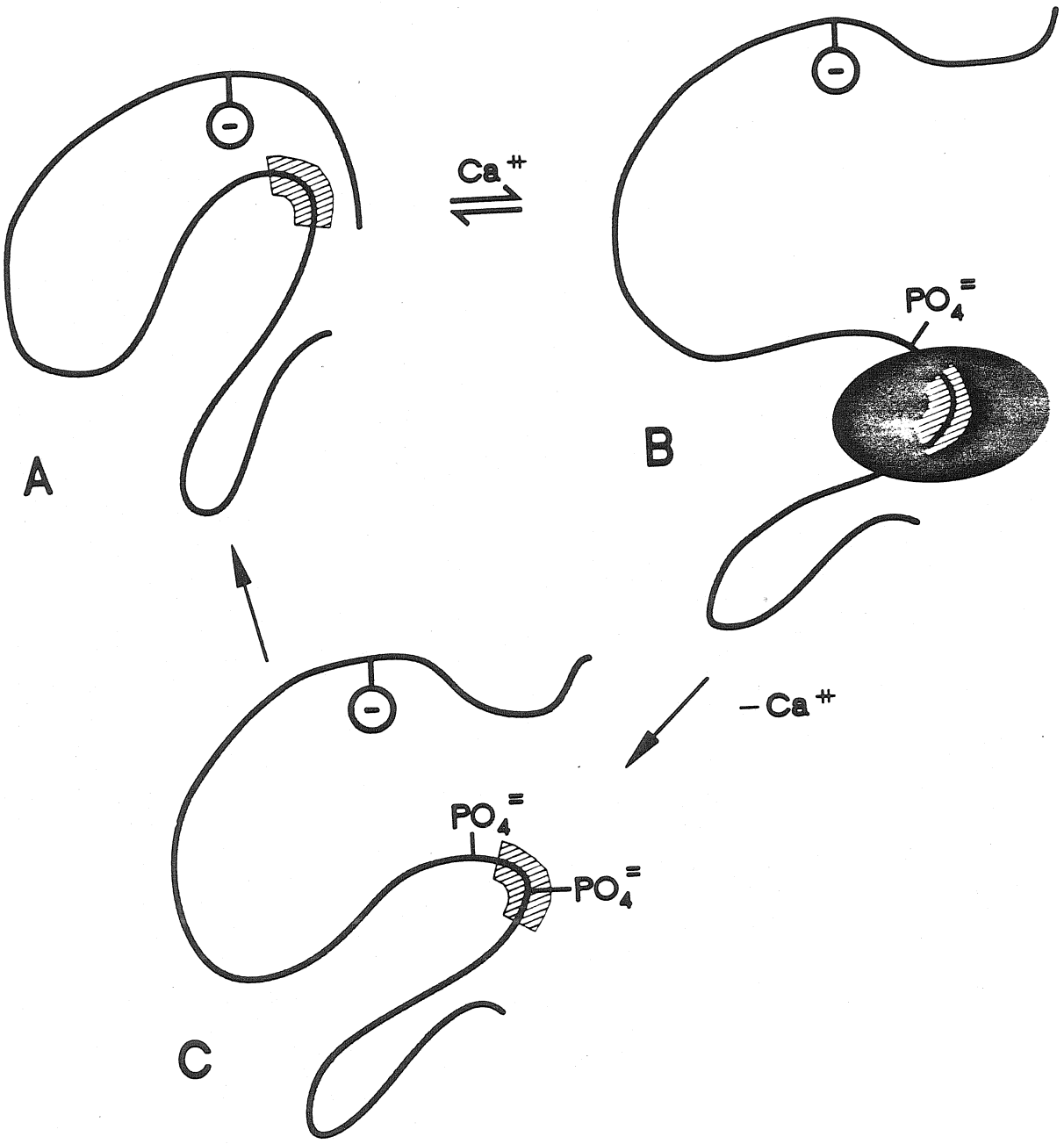

Ca++
A
B
PO4=
C
PO4=
PO4=
−Ca++

Figure 7. Immunocytochemical staining of organotypic cultures of hippocampus for the α subunit of type II CaM kinase and synapsin I. Organotypic cultures were fixed, embedded in plastic and cut into 2 μ sections as previously described (DeCamilli et al., 1983a; Molloy and Kennedy, in preparation). The sections were etched with peroxide, then incubated with (TOP) anti-α subunit monoclonal antibody 6g9, or (BOTTOM) rabbit antisera against synapsin I. The sections were then incubated with appropriate secondary antibodies coupled to horseradish peroxidase. Antibodies were visualized by reaction with diaminobenzidine, then the sections were photographed with an Olympus Vanox microscope (Molloy and Kennedy, in preparation). The preservation of the dentate gyrus (D) and the CA pyramidal fields (C) in the cultures is evident. Cell bodies in both of these areas have spread out as the cultures flattened. Note the dark staining of cytosol and dendrites for CaM kinase in the Top photograph. Also note the large punctate mossy fiber terminals in CA3 stained for synapsin I in the Bottom photograph. At higher magnification, smaller punctate structures stained for synapsin I can be visualized throughout the molecular layers. Magnification is 71 X. [Editor note: 71X should be changed to reflect proportional reduction of the photographs for printing.]

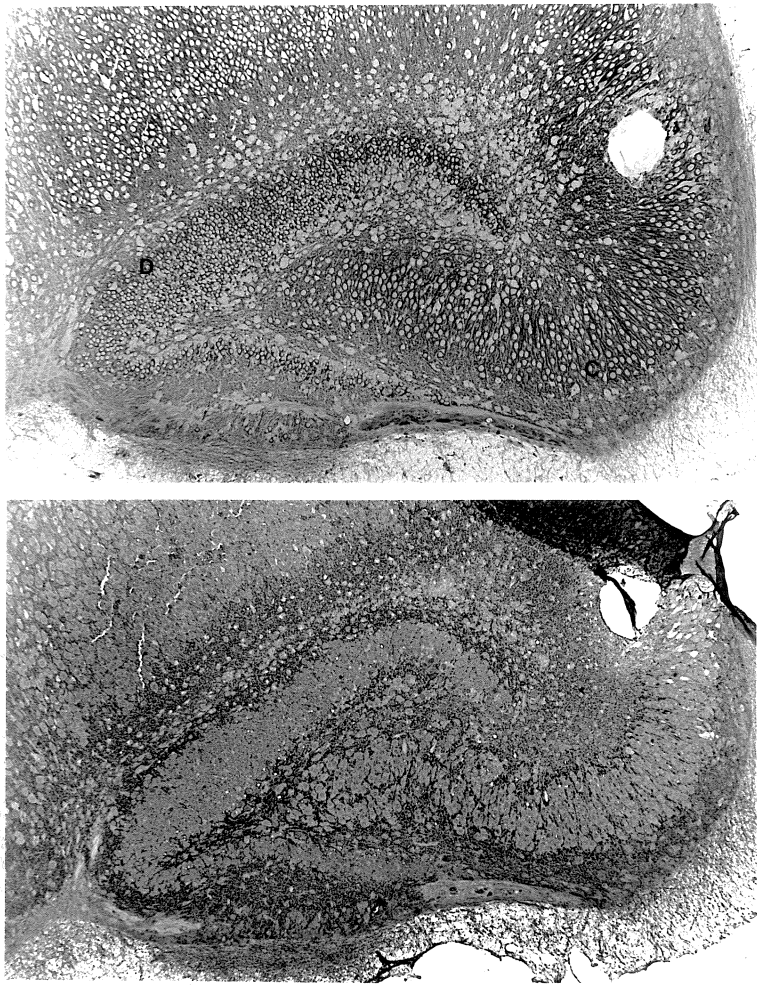
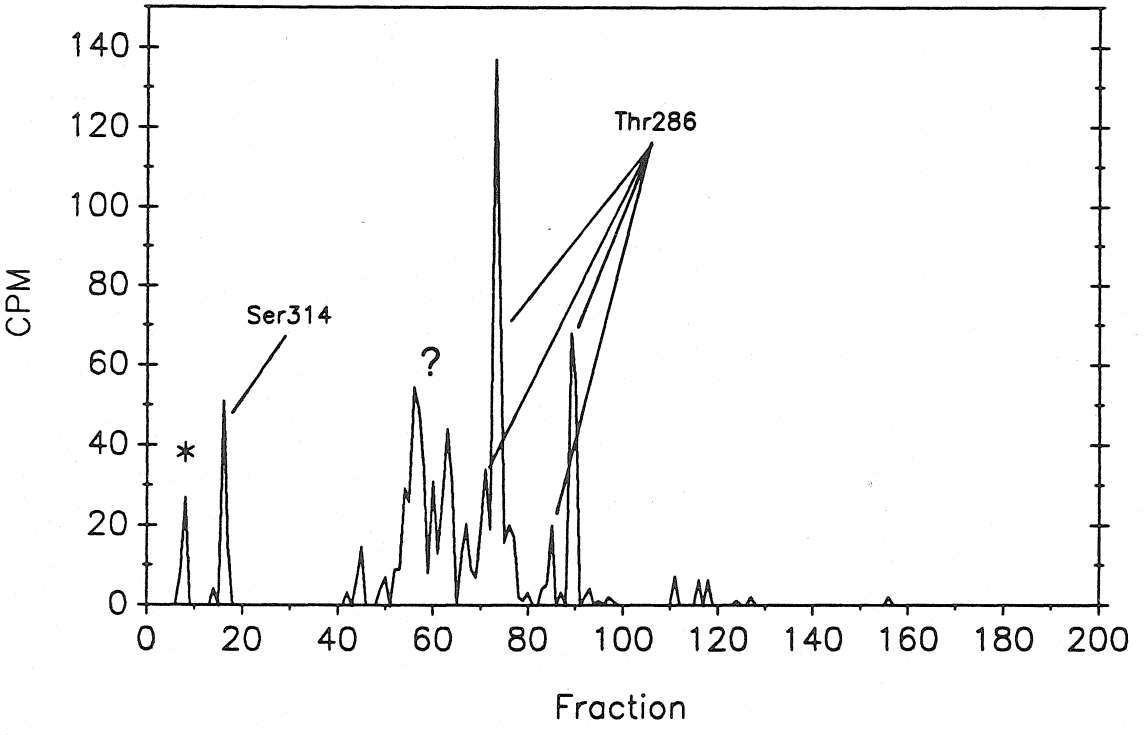

D
C

Figure 8. Phosphotryptic peptide map of the α subunit of type II Ca^{2+}/calmodulin-dependent protein kinase from cultures labeled with $^{32}PO_4$ *in situ*. Organotypic hippocampal cultures were incubated overnight in medium containing $^{32}PO_4$, then homogenized. CaM kinase was immunoprecipitated from the homogenate, its subunits were separated and phosphotryptic peptides were prepared as previously described (Miller et al., 1988; Patton et al., 1990; Molloy and Kennedy, in preparation). The peptides were fractionated by reverse-phase HPLC and radioactivity in the fractions was counted. The four peptides marked Thr286 were identified as phosphotryptic peptides containing phosphothreonine-286 by comparison to similar maps of the α subunit of purified CaM kinase (Miller et al., 1988). The peptide identified as containing serine-314 is also marked. (*) marks the void volume, and (?) indicates unidentified peptides that are occasionally present in maps prepared from both purified kinase and kinase labeled *in situ*. They may be chemically altered forms of peptides containing threonine-305 (Patton et al., 1990; Molloy and Kennedy, in preparation).


CPM
140
120
100
80
60
40
20
0
0
20
40
60
80
100
120
140
160
180
200
Fraction
Thr286
Ser314
?
*

Appendix D

The Effect of Removal of External Calcium on the Labeling of CaM Kinase Autophosphorylation Sites *in situ*.

Figure 1. Tryptic Peptide Map of CaM Kinase Autophosphorylation Sites Labeled in Hippocampal Cultures *in situ.*

The type II CaM kinase in hippocampal cultures was labeled as described in Chapter 2 (Methods) with the following exceptions. The labeling medium contained 100 μM phosphate rather than no added phosphate. The presence of a low concentration of carrier phosphate enhanced the incorperation of label into phosphorylated proteins. This allowed for a reduction in labeling time from 18-24 hr to 8 hr. Following labeling, the cultures were equilibrated in salt solution (Chapter 2, Methods) containing 2 mM Ca^{+2} for 30 min then frozen and homogenized. The CaM kinase was immunoprecipitated from the homogenate as described in Chapter 2 (Methods) except that the rabbit anti-kinase antisera Annette was used instead of the mixture of monoclonals. The antibody complexes were then precipitated directly with protein A sepharose beads. Subunits of the immunoprecipitated kinase were separated by SDS-PAGE and the α subunit processed for tryptic peptide mapping as described in Chapter 2. The ^{32}P-labeled tryptic fragments containing the Ca^{+2}-dependent autophosphorylation site at threonine 286 are indicated (Thr_{286}). The peptide containing the site at serine 314 (Ser_{314}) is also labeled.

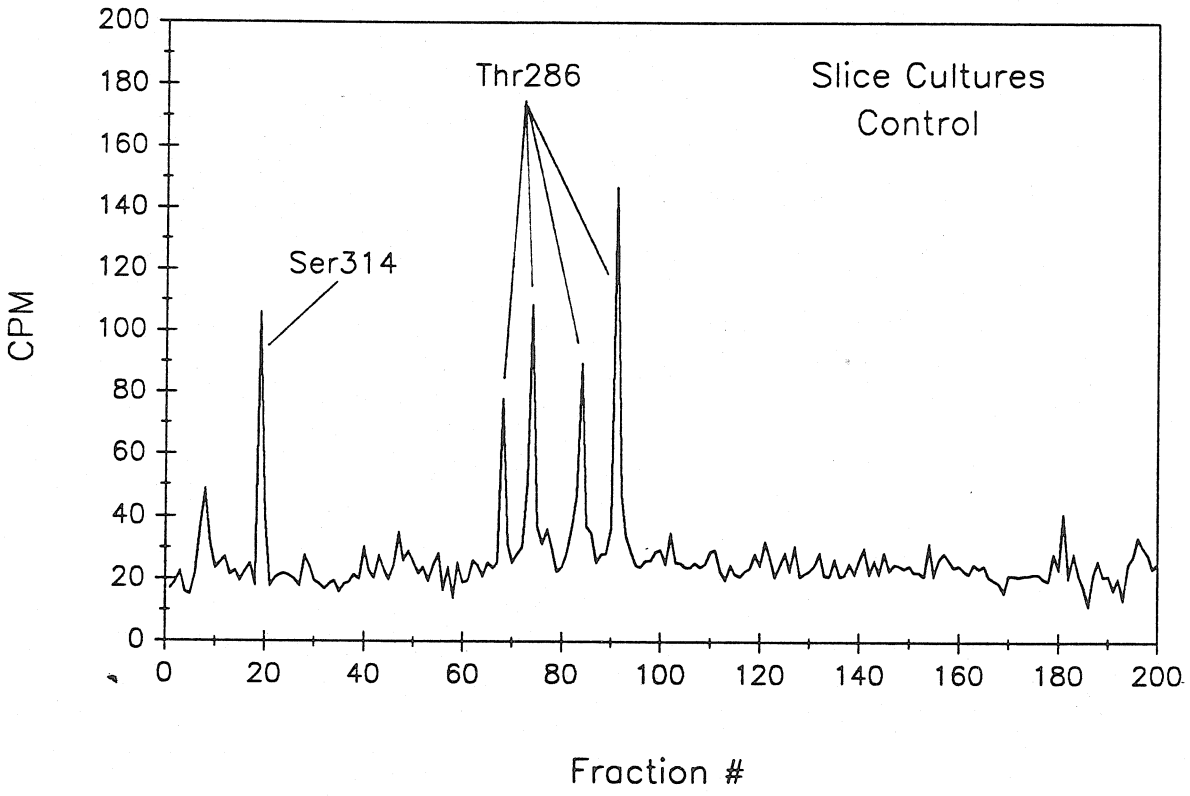

Thr286
Slice Cultures
Control
Ser314
CPM
Fraction #
0
20
40
60
80
100
120
140
160
180
200

Figure 2. Effect of External Calcium Removal on the Labeling of CaM Kinase Autophosphorylation Sites in Hippocampal Cultures *in situ*.

Cultured hippocampal slices were labeled as described in Figure 1. Following the labeling period, the cultures were equilibrated in salt solution containing 200 μM EGTA in place of 2 mM Ca^{+2}. Labeled CaM kinase was isolated as described in Figure 1 and tryptic peptide maps generated as in Chapter 2. The relative number of counts recovered in tryptic fragments containing Thr_{286} were greatly reduced in the EGTA treated cultures compared to controls (Figure 1). The large decrease in the labeling of Thr_{286} compared to Ser_{314} suggests that this site is dephosphorylated *in situ* during the EGTA equilibration. This finding agrees with the decrease in Ca^{+2}-independent CaM kinase activity seen following removal of external Ca^{+2} (Chapter 2, Figure 3). The strong peak obtained for the Ser_{314} peptide suggests that unlike Thr_{286}, this site may be resistant to rapid dephosphorylation in the cultures. Ser_{314} is resistent to dephosphorylation by phosphatases 1 and 2A *in vitro* (Patton et al., 1990).

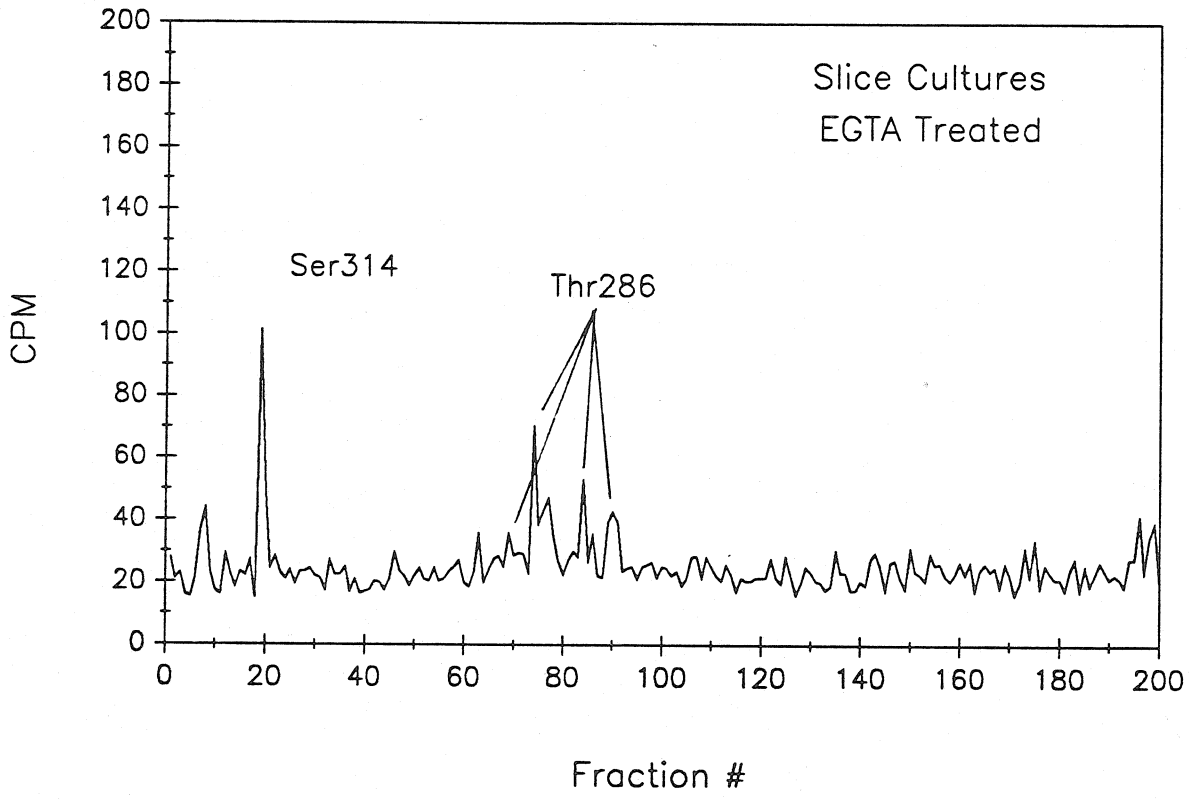
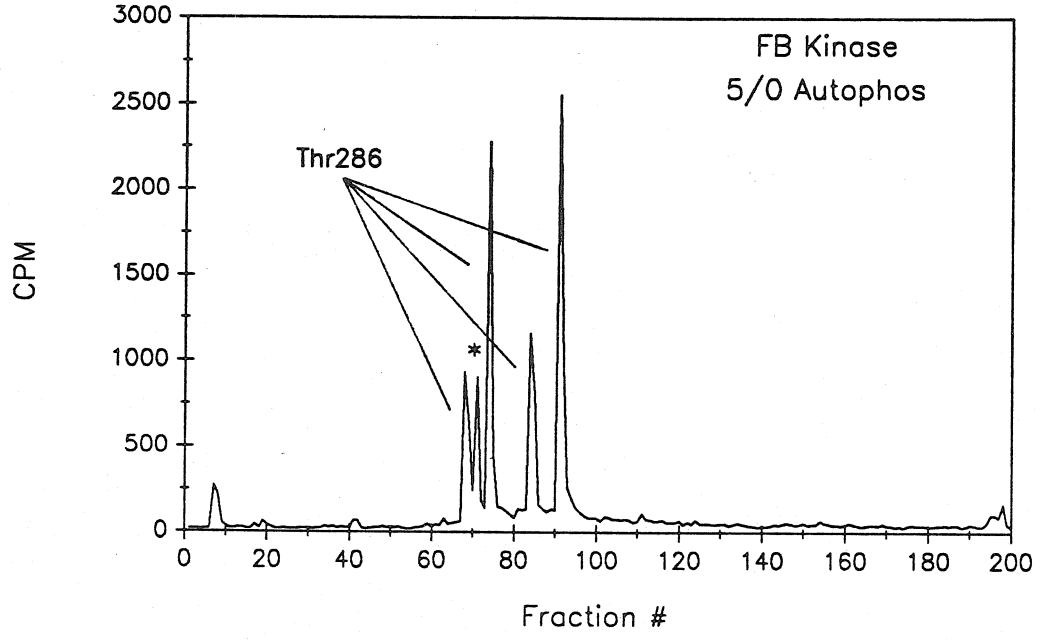

Slice Cultures
EGTA Treated
Ser314
Thr286
CPM
Fraction #
0
20
40
60
80
100
120
140
160
180
200

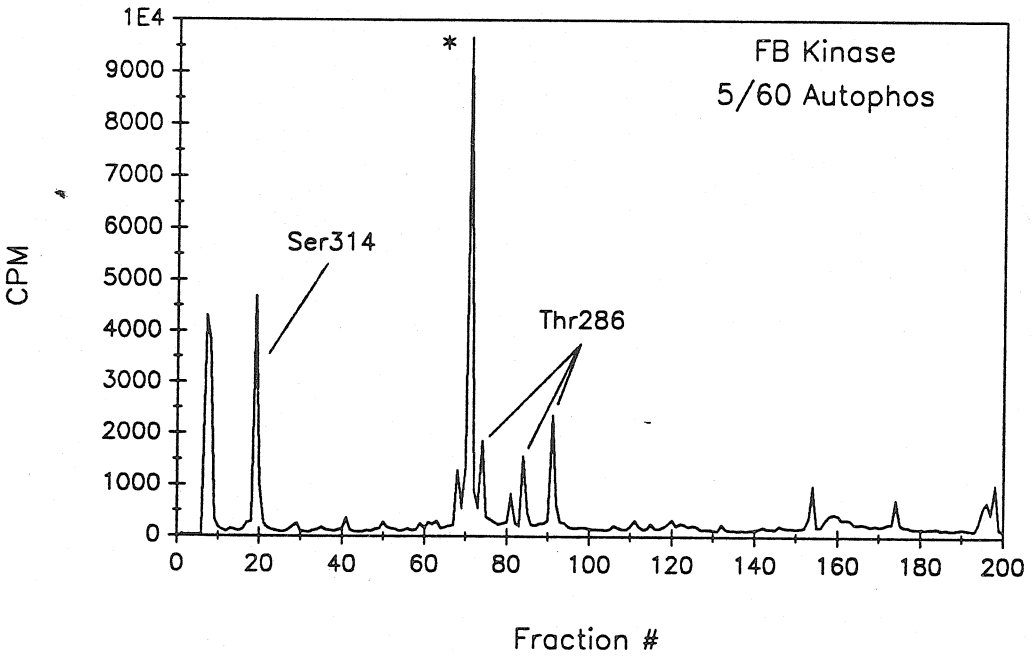
Figure 3. Tryptic Peptide Map of CaM Kinase Autophosphorylated *in vitro*.

(A) Purified CaM kinase was autophosphorylated for 5 sec in the presence of Ca^{+2} as described in Chapter 2 (Methods). The subunits of the kinase were separated by SDS-PAGE and a tryptic map of the α subunit prepared as previously described. The asterix marks the peptide containing a slowly phosphorylated threonine site. (B) The kinase was autophosphorylated for 5 sec in the presence of Ca^{+2} and an additional 30 sec after the removal of Ca^{+2}. These maps of CaM kinase autophosphorylated *in vitro* were parallel controls for the experiments shown in Figures 1 and 2. The peaks representing peptides which contain autophosphorylation sites are labeled as described in Figure 1.

A


FB Kinase
5/0 Autophos
Thr286
*
CPM
Fraction #
3000
2500
2000
1500
1000
500
0
0
20
40
60
80
100
120
140
160
180
200

B


FB Kinase
5/60 Autophos
*
Ser314
Thr286
CPM
Fraction #
1E4
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
0
20
40
60
80
100
120
140
160
180
200