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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 describe one character that we have studied for several years, called type II Ca^{++}/calmodulin-dependent protein kinase (CaM kinase II). We then discuss an interesting mechanism by which this particular protein kinase may allow neurons to store information, if only for a short time. Finally, we 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; Bulleit et al. 1988; Miller et al. 1988; Patton et al. 1990; S.S. Molloy and M.B. Kennedy, in prep.).

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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 minutes. Crude synaptosomes and mitochondria were removed from the supernatant solution by centrifugation at 18,000*g* for 10 minutes and then carefully resuspended in a sucrose buffer and layered onto discontinuous sucrose density gradients. After centrifugation at 82,000*g* for 2 hours, an enriched synaptosome fraction was harvested from the 1.0 M:1.2 M sucrose interface. The synaptosomes were diluted fourfold with 0.32 M sucrose in bicarbonate buffer (pH 8.0) and then sedimented by centrifugation at 37,000*g* for 20 minutes. 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 according to 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°C. The lightly fixed synaptosomes were recovered by centrifugation at 17,000*g* for 20 minutes. 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 slowly several times through a 25-gauge needle. Each suspension (100–180 μl) was placed in a tube prewarmed to 58°C. After 15 seconds, 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 according to the method of DeCamilli et al. (1983b). The agarose-embedded synaptosomes were allowed to cool for 1 hour at room temperature. The resulting thin slabs were cut with a razor blade into 2 mm × 2 mm blocks. Six blocks of each sample were placed into each of several small test tubes. The blocks were first incubated for 30 minutes in 2 ml of 0.5 M Tris (pH 7.4) and then incubated for 30 minutes in 0.5 ml of Tris buffer containing 2 mg/ml

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sheep immunoglobulin G (IgG). Finally, they were incubated overnight at 4°C in 0.2 ml of Tris buffer containing 2 mg/ml sheep and either 40–50 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 five changes of 2 ml of solution B (20 mM phosphate [pH 7.4], 0.5 M NaCl) over 40 minutes. They were then incubated for 90 minutes in 0.15 ml of solution B containing 50 mg/ml ovalbumin, 2 mg/ml sheep IgG, and a one-fifth dilution of ferritin-conjugated sheep anti-mouse IgG antisera purchased from Janssen Life Sciences Products. The blocks were washed again with five changes of 2 ml of solution B over 40 minutes. Finally, the blocks were fixed in 2 ml of 1% glutaraldehyde, 0.12 M phosphate (pH 7.4) for 30 minutes at 4°C. The fixed blocks were washed for 20 minutes in 0.12 M phosphate (pH 7.4) at 4°C and then osmicated in ice cold 1% OsO_4 for 1 hour. After two 5-minute 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 micrometer of postsynaptic density (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 the 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^{++} and calmodulin. It is a large heteromultimer composed of 12 homologous subunits. The predominant holoenzyme purified from the forebrain contains, on the average, nine subunits with a molecular weight of 54,000 called α and, on the average, three subunits with molecular weights of 57,000–60,000 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, whereas 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; whereas 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 tissue.

Cellular Distribution of Type II CaM Kinase

An early finding that focused attention on the potential importance of CaM kinase II for central nervous system (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 PSD, a prominent 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 tissue (Kennedy et al. 1983a). This protein had previously been referred to as the major postsynaptic density protein (Kelly and Cotman 1978). Quantitative estimates indicate that the CaM kinase comprises 20–40% of the total protein in the PSD fraction (Miller and Kennedy 1985). Curiously, the content of CaM kinase is much reduced in PSDs isolated from the cerebellum, where the kinase is composed 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 PSDs from brain homogenates requires the isolation of a crude synaptosomal fraction, followed by a 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

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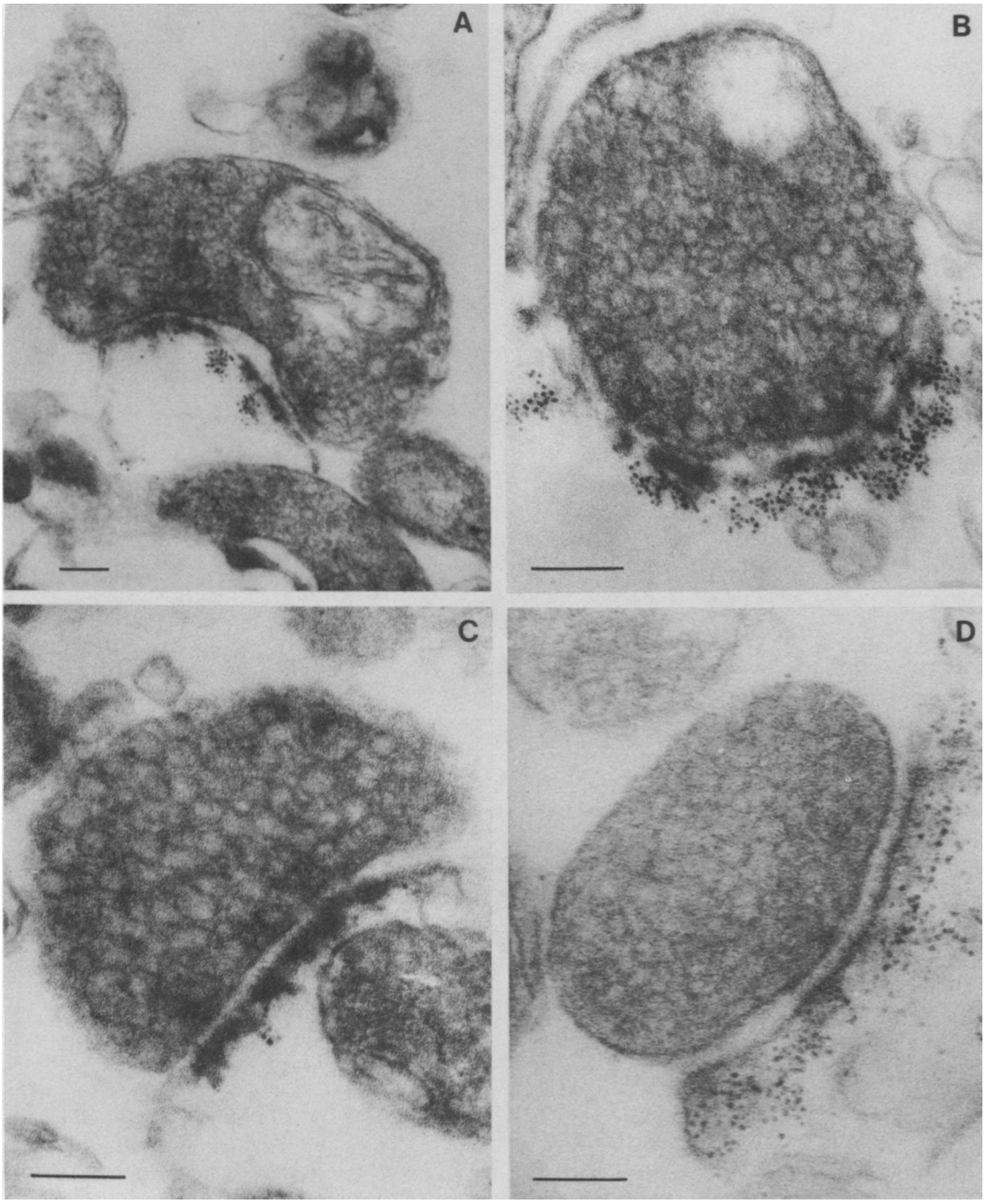

Figure 1. Synaptosomal postsynaptic densities labeled with antibody against the α-subunit of CaM kinase II. Synaptosomes were prepared, embedded in agarose, and labeled with either a specific monoclonal antibody against the α-subunit (6g9) or control mouse IgG and then with ferritin-labeled sheep anti-mouse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

to detect the kinase in PSDs, synaptosomes were kept intact during the incubations with antibodies. Figure 1 shows 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 the cerebellum and from the hippocampus were labeled separately (Fig. 2B), the average density of particles in

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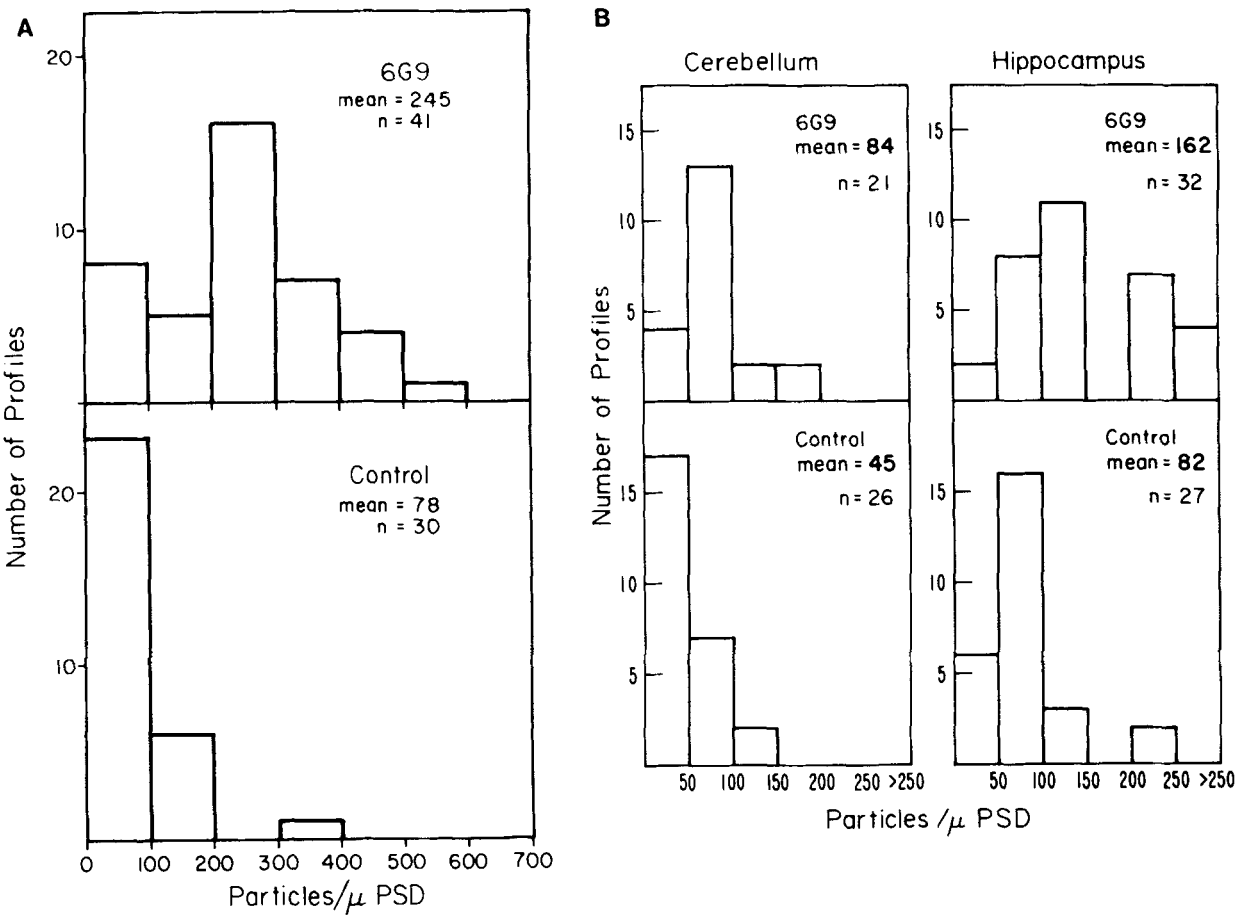

Figure 2. Quantitation of ferritin-labeling of synaptosomal postsynaptic densities. The number of ferritin grains per micrometer of PSD was determined for several synaptosomal profiles after labeling with antibodies as described in the legend to Fig. 1. (*A*) Labeling of forebrain PSDs with monoclonal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

hippocampal PSDs was approximately twice that of 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 100–400 μM. This is five to ten times higher than the concentration of α-subunits in the cytosol (19–37 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^{++} current that is generated by activation of *N*-methyl-D-aspartate 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 long-term potentiation (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^{++}/calmodulin (Bennett et al. 1983). This autophosphorylation is the basis of an interesting mechanism for controlling CaM kinase activity. Nonphosphorylated CaM kinase is catalytically active only in the presence of Ca^{++}/calmodulin. However, if the kinase is briefly autophosphorylated by incubating it for 5 seconds in the presence of Ca^{++}/calmodulin and ATP before it is added to assay tubes containing exogenous substrate, a new Ca^{++}-independent activity becomes apparent (Fig. 3) (Miller and Kennedy 1986). The magnitude of this activity depends on the substrate (Patton et al. 1990). With a synthetic peptide substrate,

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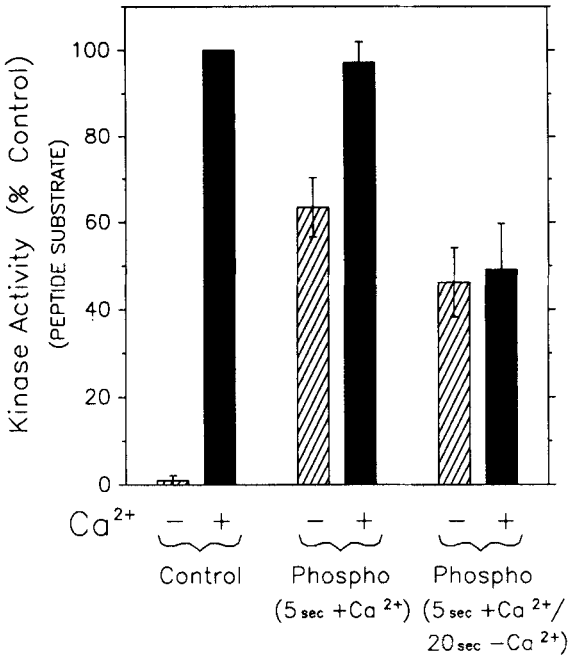

Figure 3. Effects of autophosphorylation on the kinase activity of CaM kinase II. Purified kinase was autophosphorylated and then assayed with a synthetic peptide substrate (calmodulin-dependent protein kinase substrate analog, purchased from Peninsula Laboratories) as described previously (Patton et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

such as that used in the experiment shown in Figure 3, phosphorylation proceeds in the absence of Ca^{++} at about 60% of the rate in the presence of Ca^{++}.

This switch to a partially Ca^{++}-independent state has four important features. First, the Ca^{++}-independent activity is fully activated after addition of as little as 2–3 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^{++}-independent. Third, the effects of autophosphorylation are reversible. Ca^{++}-independent activity is lost when the kinase is dephosphorylated by protein phosphatases (Lai et al. 1986; Miller and Kennedy 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^{++} and calmodulin for activity. When sufficient autophosphorylation has occurred, the kinase is switched to state 2 in which it has a substantial Ca^{++}-independent kinase activity with exogenous substrates. Furthermore, in state 2, Ca^{++}-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^{++} 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^{++}-independent autophosphorylation and the local catalytic rate of cellular phosphatases.

Identification of Regulatory Autophosphorylation Sites

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

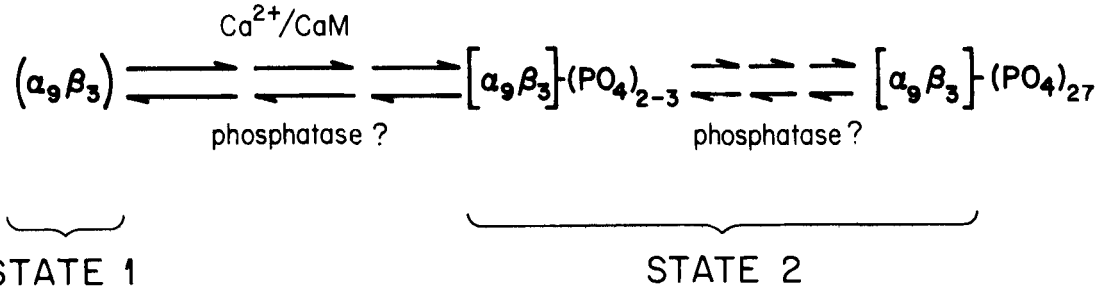

Figure 4. Hypothetical switch model of regulation of type II Ca^{++}/calmodulin-dependent protein kinase by autophosphorylation and dephosphorylation. (Modified from Miller and Kennedy 1986.)

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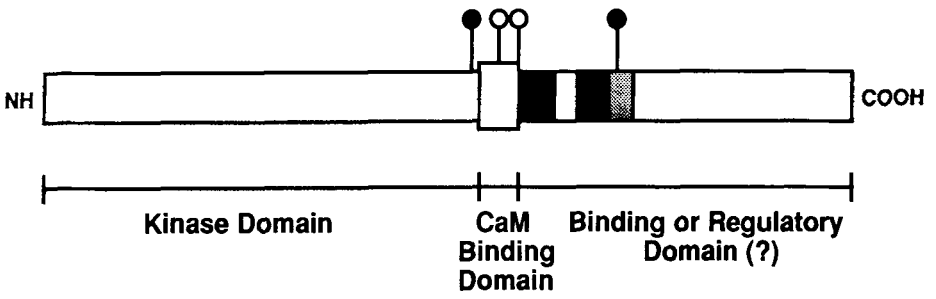

Figure 5. Location of autophosphorylation sites in the sequence of type II Ca^{++}/calmodulin-dependent protein kinase. (●) Sites that are autophosphorylated rapidly when the kinase is activated by Ca^{++}/calmodulin. The site to the left of the calmodulin-binding domain is Thr-286 in the α-subunit (287 in the β-subunit). (○) Sites that are autophosphorylated only when Ca^{++} is removed from the reaction after autophosphorylation of Thr-286. The site in the middle of the calmodulin-binding domain is Thr-305 in the α-subunit (Thr-306 in the β-subunit). The site to the right of the calmodulin-binding domain is Ser-314 in the α-subunit (Ser-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 Patton et al. 1990.)

in which Thr-286 was changed to leucine by in vitro mutagenesis (Hanson et al. 1989). The mutated kinase does not show Ca^{++}-independent activity upon autophosphorylation. The location of Thr-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^{++} 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^{++}/calmodulin (Fig. 3) (Hashimoto et al. 1987). We have identified two additional sites that are rapidly autophosphorylated only after Ca^{++} is removed from an ongoing autophosphorylation reaction. One of these, Thr-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, Ser-314, is located at the carboxy-terminal end of the calmodulin-binding domain. Inhibition of sensitivity to calmodulin and reversal of this inhibition correlate well with autophosphorylation and dephosphorylation of Thr-305, respectively. Autophosphorylation of Ser-314 causes only a twofold reduction in affinity for calmodulin (Patton et al. 1990). Curiously, phosphoserine 314 is resistant to dephosphorylation by protein phosphatases.

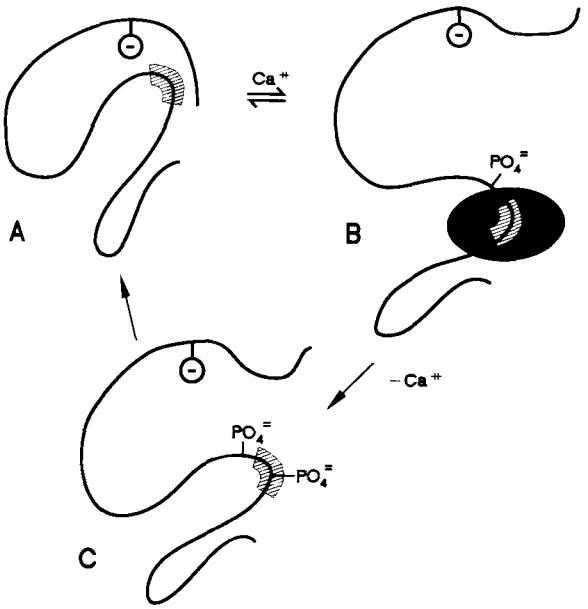

Figure 6. Schematic model of regulation of a subunit of type II Ca^{++}/calmodulin-dependent protein kinase by autophosphorylation. See text for explanation and discussion.

The sequence of regulatory events governing activity of one subunit of the CaM kinase is summarized in Figure 6. Inactive kinase is shown in Figure 6A. In the presence of Ca^{++}, calmodulin binds to a specific sequence, resulting in a conformational change that opens the active site and allows phosphorylation of exogenous substrates (Fig. 6B). At the same time, Thr-286, next to the calmodulin-binding domain, is rapidly autophosphorylated. When Ca^{++} is removed from the reaction, calmodulin is released from the kinase (Fig. 6C). However, the phosphate group on Thr-286 prevents complete refolding of the kinase. In this state, the kinase is still active although at a somewhat reduced rate. An additional site, Thr-305, located in the middle of the calmodulin-binding domain, is now autophosphorylated. In the state depicted in Figure 6C, the kinase has a substantial Ca^{++}-independent activity but cannot be further stimulated by Ca^{++}/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 phosphorylate neighboring subunits directly within the holoenzyme.

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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 a high concentration and has been implicated in the initiation of LTP. Physiologists have successfully studied synaptic transmission between hippocampal neurons in slices prepared acutely from adult brain tissue (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

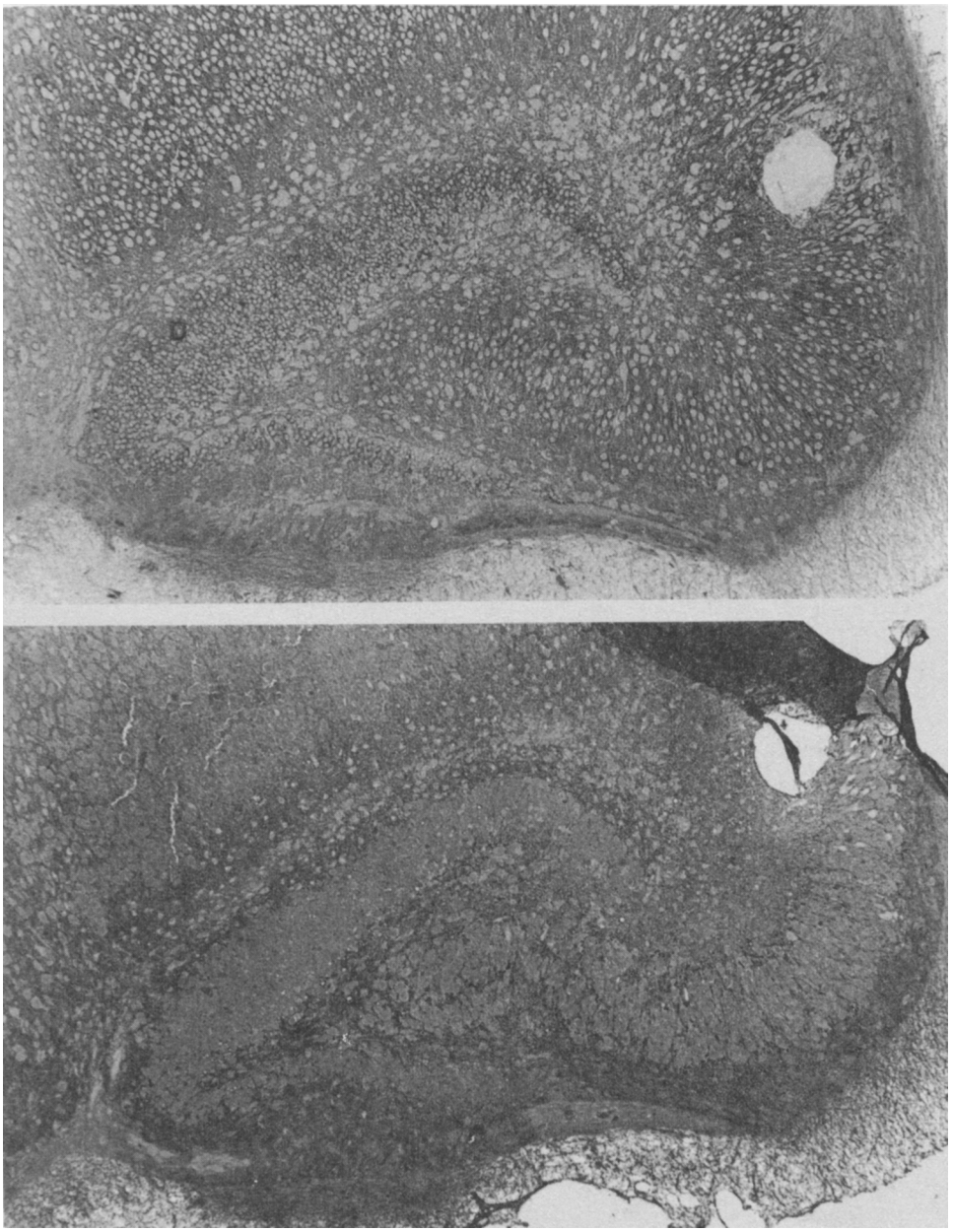

Figure 7. Immunocytochemical staining of organotypic cultures of hippocampus for the α-subunit of CaM kinase II and synapsin I. Organotypic cultures were fixed, embedded in plastic, and cut into 2-μm sections as described previously (DeCamilli et al. 1983a; S.S. Molloy and M.B. Kennedy, in prep.). The sections were etched with peroxide and then incubated with anti-α-subunit monoclonal antibody 6g9 (*top*) or rabbit antisera against synapsin I (*bottom*). The sections were then incubated with appropriate secondary antibodies coupled to horseradish peroxidase. Antibodies were visualized by reaction with diaminobenzidine, and then the sections were photographed with an Olympus Vanox microscope (S.S. Molloy and M.B. Kennedy, in prep.). 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 (*top*). Also note the large punctate mossy fiber terminals in CA3 stained for synapsin I (*bottom*). At higher magnification, smaller punctate structures stained for synapsin I can be visualized throughout the molecular layers. Magnification, 55 $\times$.

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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 (1988) in Switzerland, and we have modified it slightly for our purposes (S.S. Molloy and M.B. Kennedy, in prep.). To prepare the cultures, hippocampi are dissected from 4- to 6-day-old rats and cut into 400-μm slices. The slices are fastened onto collagen-coated coverslips with a drop of liquid collagen. When the collagen has polymerized, the coverslips are placed into test tubes in 1 ml of liquid medium (Gahwiler 1984; S.S. Molloy and M.B. Kennedy, in prep.) 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 2 weeks, the cultures have shed dead tissue and flattened to a thickness of 2–3 cells (50–80 μm). 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 collateral 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 (Fig. 7; top) or for the synaptic-vesicle-associated protein synapsin I (Fig. 7; bottom) produces patterns of staining similar to those in fixed tissue from adult brain (DeCamilli et al. 1983a; Ouimet et al. 1984). CaM kinase staining is dense in dendrites and cytosol with dark patches along dendrites that may represent concentrations of kinase at postsynaptic densities (S.S. Molloy and M.B. Kennedy, in prep.). 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 Thr-286, the site that controls Ca^{++}-independent activity in vitro, could be labeled with [^{32}P]phosphate in situ (Fig. 8) (S.S. Molloy and M.B. Kennedy, in prep.). 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-polyacrylamide gel electrophoresis, the α-subunit was digested with trypsin, and the labeled tryptic phosphopeptides were fractionated by high-performance liquid chromatography (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 Thr-286 (Fig. 8) (S.S. Molloy and M.B. Kennedy, in prep.). Thus, kinase molecules can be autophosphorylated at this site in situ, even in the absence of exogenous stimulation.

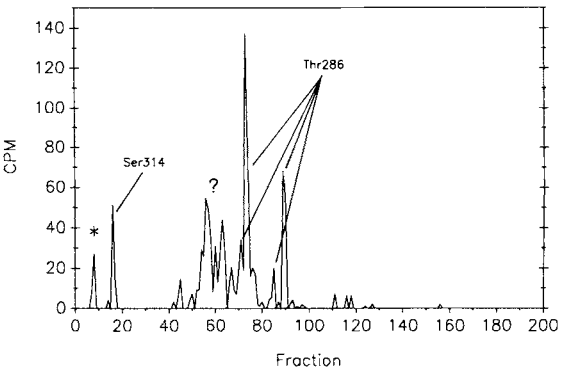

Figure 8. Phosphotryptic peptide map of the α-subunit of type II Ca^{++}/calmodulin-dependent protein kinase from cultures labeled with $^{32}PO_4$ in situ. Organotypic hippocampal cultures were incubated overnight in medium containing $^{32}PO_4$ and then homogenized. CaM kinase was immunoprecipitated from the homogenate, its subunits were separated, and phosphotryptic peptides were prepared as described previously (Miller et al. 1988; Patton et al. 1990; S.S. Molloy and M.B. Kennedy, in prep.). The peptides were fractionated by reverse-phase HPLC, and radioactivity in the fractions was counted. The four peptides marked threonine-286 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 Ser-314 is also marked. The asterisk marks the void volume, and the question mark 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 Thr-305 (Patton et al. 1990; S.S. Molloy and M.B. Kennedy, in prep.).

To determine the extent of autophosphorylation of Thr-286 in situ, we measured the proportion of CaM kinase in the Ca^{++}-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^{++}. We then determined that, in these homogenates with synapsin I as substrate, CaM kinase activity in the absence of Ca^{++} was 27% of the full activity in the presence of Ca^{++}. Synapsin I was used as substrate in these experiments because, unlike synthetic peptides, its phosphorylation in the absence of Ca^{++} in culture homogenates is catalyzed entirely by CaM kinase II (S.S. Molloy and M.B. Kennedy, in prep.). We next determined kinase activity in the absence and presence of Ca^{++} in homogenates prepared under conditions designed to preserve the state of autophosphorylation in vivo. The value was $8.4 \pm 0.4\%$, indicating that approximately 31% of the kinase in culture homogenates is in the Ca^{++}-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 auto-

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phosphorylated 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^{++} in the culture medium. When Ca^{++} was removed from the medium, the proportion of kinase in the Ca^{++}-independent state decreased to approximately 5% in 25–30 minutes (S.S. Molloy and M.B. Kennedy, in prep.). Therefore, both Ca^{++} and continuing kinase activity are required to maintain the autophosphorylated state in vivo.

A high percentage of CaM kinase II is in the Ca^{++}-independent state in hippocampi from intact rats. Forebrains and hippocampi were dissected from rats of increasing age and homogenized according to the method described for hippocampal cultures. The percentage of kinase in the Ca^{++}-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 (S.S. Molloy and M.B. Kennedy, in prep.).

The functional significance of this high proportion of Ca^{++}-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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