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Calmodulin-Dependent Protein Kinase II

Multifunctional Roles in Neuronal Differentiation and Synaptic Plasticity

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Abstract

One of the most important mechanisms for regulating neuronal functions is through second messenger cascades that control protein kinases and the subsequent phosphorylation of substrate proteins. Ca^{2+}/calmodulin-dependent protein kinase II (CaM-kinase II) is the most abundant protein kinase in mammalian brain tissues, and the α-subunit of this kinase is the major protein and enzymatic molecule of synaptic junctions in many brain regions. CaM-kinase II regulates itself through a complex autophosphorylation mechanism whereby it becomes calcium-independent following its initial activation. This property has implicated CaM-kinase II as a potential molecular switch at central nervous system (CNS) synapses. Recent studies have suggested that CaM-kinase II is involved in many diverse phenomena such as epilepsy, sensory deprivation, ischemia, synapse formation, synaptic transmission, long-term potentiation, learning, and memory.

During brain development, the expression of CaM-kinase II at both protein and mRNA levels coincides with the active periods of synapse formation and, therefore, factors regulating the genes encoding kinase subunits may play a role in the cell-to-cell recognition events that underlie neuronal differentiation and the establishment of mature synaptic functions. Recent findings have demonstrated that the mRNA encoding the α-subunit of CaM-kinase II is localized in neuronal dendrites. Current speculation suggests that the localized translation of dendritic mRNAs encoding specific synaptic proteins may be responsible for producing synapse-specific changes associated with the processing, storage, and retrieval of information in neural networks.

Introduction

Synapses are the basic structural and functional subunits of neural tissues and constitute the junctures of cell-to-cell communication. Two of the most important questions in neurobiology are: how is synaptic connectivity formed during brain development and how may it be modified in the mature brain? and, how does the nervous system change in response to experience? Together, changes in these processes are responsible for the brain's ability to learn and remember. Recent discoveries have shown that certain changes in the nervous system that persist for long periods of time are encoded at synapses (Nicoll et al., 1988). To better understand the basis of synaptic modulation, recent efforts have focused on the identification and functional analyses of molecules that are involved in synapse formation and synaptic plasticity.

With the advent of methods to purify synaptic junction (SJ) and postsynaptic density (PSD) fractions in the 1970s, a number of individuals became particularly interested in a protein that was originally called the *major PSD protein* (mPSDp) since it was the major protein constituent of isolated PSDs (Kelly and Cotman, 1977; Kelly and Montgomery, 1982). Developmental studies indicated that the expression and accumulation of the mPSDp in purified SJs paralleled the most active period of synapse formation in the postnatal rat brain (Kelly and Cotman, 1981). Biochemical analyses of the mPSDp indicated that it was extremely insoluble in most chaotropic agents (e.g., bile salts, neutral and ionic detergents; Kelly and Cotman, 1977), displayed a high propensity to crosslink itself and other SJ proteins through disulfide bond formation (Kelly and Cotman, 1976), and was distinct from all known cytoskeletal proteins (e.g., tubulin, actin, α-actinin, and many other filamentous proteins; Kelly and Cotman, 1978). Together, these findings suggested that the mPSDp was a structural element of the postsynaptic region of asymmetric synapses. This simplified view changed dramatically when reports documented that the mPSDp was identical to the α-subunit of CaM-kinase II (Kennedy et al., 1983a; Goldenring et al., 1984; Kelly et al., 1984). The discovery of a catalytic role for the mPSDp was especially surprising since estimates indicated that 30–40% of the total protein in PSDs was this single polypeptide.

Molecular Properties of Cam-Kinase II

Many reports in the past few years have documented the properties of CaM-kinase II in neural tissues, as well as closely related protein kinases in nonneuronal tissues (e.g., liver and skeletal muscle) and cell lines (McGuinness et al., 1983; Schworer et al., 1985; Ohta et al., 1986; Shenolikar et al., 1986). Although CaM-kinase II phosphorylates a broad range of substrates in vitro, the list of known in vivo substrates is short, including synapsin I, tyrosine hydroxylase, and microtubule-associated protein 2 (MAP-2; Kennedy et al., 1983b; Llinas et al., 1985; Schulman et al., 1985). In most cells and tissues CaM-kinase II is a multimeric enzyme complex, or holoenzyme, composed of distinct but related protein subunits (Kuret and Schulman, 1985; McGuinness et al., 1985). The two most common subunits, termed α and β, are approx 85% identical in primary amino acid sequence and are encoded by two separate genes (Hanley et al., 1987; Bulleit et al., 1988). The holoenzyme in rat forebrain contains approx 12 α- and 3 β-subunits (Bennett et al., 1983). The most interesting molecular property of CaM-kinase II is the mechanism by which Ca^{2+}/CaM-dependent autophosphorylation converts the enzyme to a largely Ca^{2+}-independent kinase (Saitoh and Schwartz, 1985; Lai et al., 1986; Miller and Kennedy, 1986; Schworer et al., 1986; Lickteig et al., 1988). This intramolecular autoregulatory process is initiated by the autophosphorylation of as few as one or two subunits per holoenzyme (Lai et al., 1986; Lou, et al., 1986; Miller and Kennedy, 1986; Lickteig, et al., 1988). The continued Ca^{2+}/CaM-independent autophosphorylation of CaM-kinase II in EGTA results

in the generation of distinct phosphopeptides as identified by high-performance liquid chromatography (HPLC), and enzymatic properties that are different than those observed for Ca^{2+}/CaM-dependent activity (Lickteig et al., 1988). These Ca^{2+}/CaM-independent properties include: (1) increased catalytic activity; (2) higher substrate affinity for the phosphorylation of synapsin I; and (3) decreased calmodulin-binding to both CaM-kinase II subunits as analyzed with ^{125}I-calmodulin gel overlays. These results indicate that the autophosphorylation of only one or two subunits/holoenzyme is required to generate the Ca^{2+}/CaM-independent form of CaM-kinase II and they suggest a two-step process by which autophosphorylation regulates CaM-kinase II. *Step 1* requires Ca^{2+}/CaM and underlies initial kinase activation. *Step 2* involves continued autophosphorylation of the Ca^{2+}/CaM-independent kinase and results in increased affinity for its substrate synapsin I and decreased affinity for calmodulin. These results indicate a complex mechanism through which autophosphorylation of CaM-kinase II may regulate its activity in response to transient fluctuations in intracellular calcium.

Many studies have examined the mechanisms underlying autoregulation and have shown that the autophosphorylation of Thr^{286} in the α-subunit or Thr^{287} in the β-subunit are critical steps in the generation of Ca^{2+}/CaM-independent activity (Lai et al., 1987; Miller et al., 1988; Schworer et al., 1988; Thiel et al., 1988). Mutagenesis of Thr^{286} produces a kinase with dramatically altered properties. Changing Thr^{286} to an alanine creates a kinase that can no longer autoconvert into a Ca^{2+}-independent enzyme (Waxham et al., 1990), whereas aspartic acid or glutamic acid substitutions create enzymes that are partially active in the absence of autophosphorylation (Fong et al., 1989; Fong and Soderling 1990; Waldmann et al., 1990). cDNAs encoding α- or β-subunits have been expressed individually in bacteria and eukaryotic cells and each display enzymatic and autoregulatory properties that are very similar to the heterosubunit holoenzyme (Yamauchi, et al., 1989; Waxham et al., 1990). It remains a mystery why two distinct subunits make up the native holoenzyme; however, some have speculated that the α-subunit is involved in targeting the enzyme to the cytoskeleton and/or postsynaptic membrane. Eukaryotic expression studies indicate that the α-subunit may be necessary for heterosubunit holoenzyme formation since CHO cells expressing only the β-subunit contained just the monomeric form of the enzyme, whereas cells expressing only α- or mixtures of α- plus β-subunits contained multimeric holoenzymes (Yamauchi et al., 1990).

The cDNAs encoding the α- and β-subunits of CaM-kinase II have been cloned (Bennett and Kennedy, 1987; Hanley et al., 1987; Lin et al., 1987). Homology comparisons between these cDNAs revealed a sequence of approx 20 amino acids in either subunit that shared 60–70% homology with the CaM-binding domain of myosin light-chain kinase (Fig. 1). To determine if this region encoded the CaM-binding domains of CaM-kinase II, a synthetic peptide encompassing amino acids 290–310 of the α-subunit was prepared (Hanley et al., 1987). This peptide was a potent CaM antagonist and inhibited the calmodulin-dependent activation of brain phosphodiesterase (kinetic experiments indicated an $IC_{50} \approx 2$–5 n*M*). Since the initial identification of the CaM-binding domain, more detailed information about this important regulatory domain has been obtained. For example, a series of overlapping synthetic peptides were made to define the high-affinity CaM-binding domain to just 12 amino acids (residues 298–309 of the α-subunit; Kelly et al., 1989).

Following these initial peptide studies came the identification of an active site-directed inhibitory domain of CaM-kinase II (Kelly et al., 1988). Analysis of the primary sequences surrounding the CaM-binding domain of α- or β-subunits revealed a consensus phosphorylation motif ($RQET^{286}$) just 12 residues *N*-terminal to the calmodulin-binding domain. The close proximity of a potential autophosphorylation site and the CaM-binding domain contributed to the

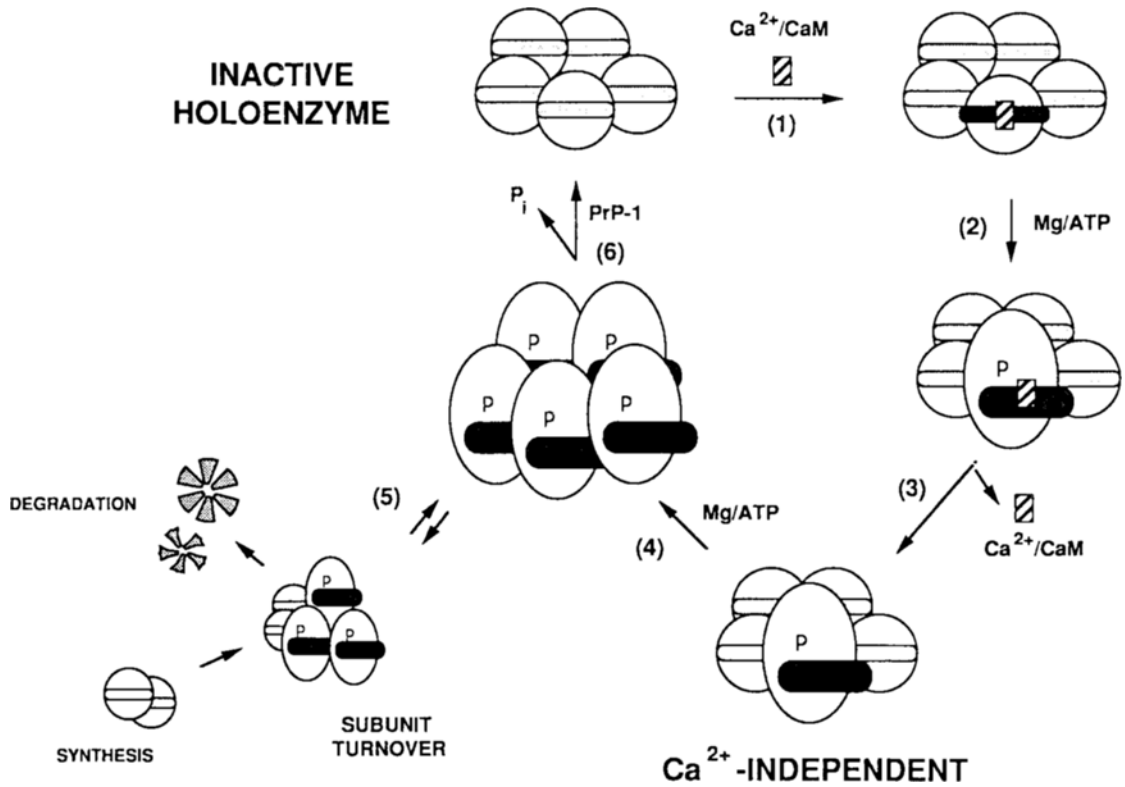

Fig. 1. Schematic representation of the regulation of CaM-kinase II by autophosphorylation, the generation of Ca^{2+}-independent activity, and dephosphorylation by protein phosphatase type-1 (PrP-1). The Ca^{2+}/calmodulin-dependent holoenzyme is depicted in the upper lefthand part of the figure; **(1)** Ca^{2+}/calmodulin binding results in a conformational change that precedes the binding of Mg^{2+}-ATP; **(2)** the holoenzyme binds Mg^{2+}-ATP and undergoes autophosphorylation on one or more of its subunits; **(3)** following autophosphorylation, the Ca^{2+}/calmodulin associated with one or more of the subunits can be removed by chelation of calcium and the Ca^{2+}-independent form of the enzyme remains active; **(4)** the Ca^{2+}-independent holoenzyme is active and subunits continue to autophosphorylate; **(5)** the Ca^{2+}-independent holoenzyme may undergo subunit turnover (*see* Lisman, 1985; Lisman and Goldring, 1988), which allows the Ca^{2+}-independent enzyme to persist for long periods of time; and **(6)** the Ca^{2+}-independent enzyme is reversibly dephosphorylated by PrP-1 to return to its Ca^{2+}-dependent form.

hypothesis that the Ca^{2+}/CaM-dependent autophosphorylation of Thr^{286} was the critical step in relaxing the enzyme's stringent dependence on Ca^{2+}/CaM for activity (Fig. 2). Thus, we initially reasoned that Thr^{286} in the sequence $RQET^{286}$ could be part of an active-site directed inhibitory or pseudosubstrate domain (Hardie, 1988). This resulted in the synthesis of two new peptides, designated CBP (residues 281–309 of α-subunit) and CBP_{-3} (284–309) (Fig. 3; Kelly et al., 1988). As predicted, CBP_{-3} was almost exclusively a CaM antagonist ($IC_{50} \approx 70$ n*M*) and did not inhibit the Ca^{2+}/CaM-independent form of CaM-kinase II. In sharp contrast, CBP, which contains the complete autophosphorylation sequence $RQET^{286}$, displayed bifunctional inhibitory properties. In addition to being a potent CaM antagonist just like CBP_{-3}, CBP was also a potent inhibitor of the Ca^{2+}/CaM-independent form of CaM-KII ($IC_{50} \approx 2$ μ*M*). These studies not only delineated the separation of autoinhibitory and CaM-binding domains but demonstrated that active-site directed inhibitory peptides can in some cases include *bona fide* phosphorylation sites

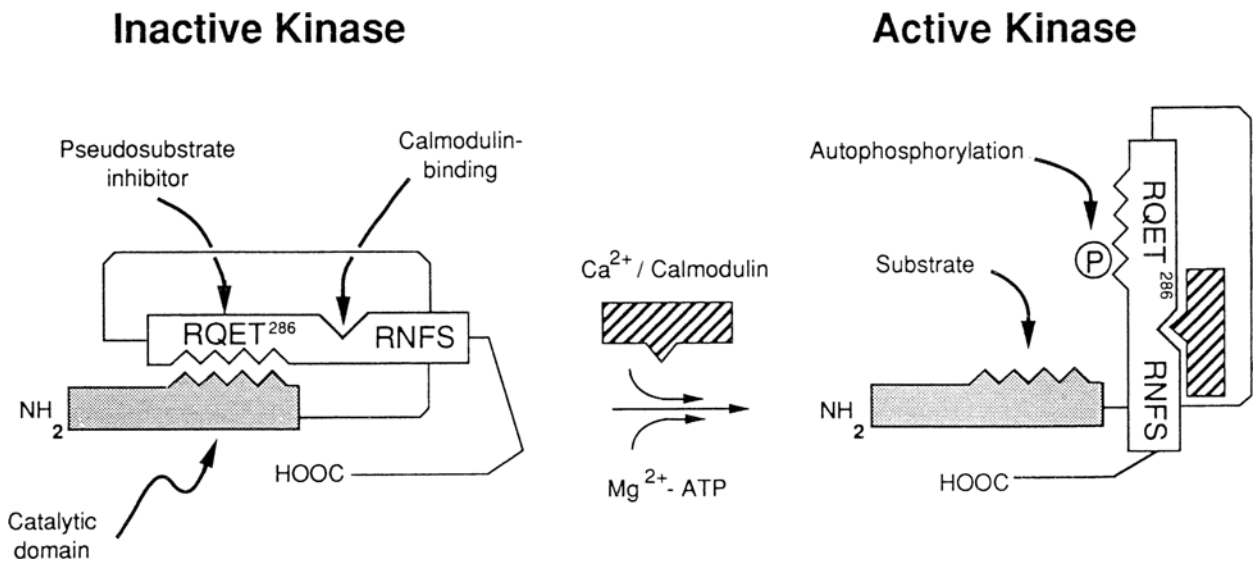

Fig. 2. Schematic figure depicting the alignment of the pseudosubstrate inhibitory domain with the catalytic/substrate-binding domain of CaM-kinase II. Activation of the enzyme by Ca^{2+}/calmodulin results in the autophosphorylation of Thr^{286}/Thr^{287} in the α- or β-subunit, respectively, and results in a kinase that can then phosphorylate substrate proteins. Autophosphorylation of only these threonine residues appears to be requisite for the generation of the Ca^{2+}-independent form of the kinase.

(*see* Hardie, 1988). Recent studies have more precisely defined the active-site directed inhibitory (or pseudosubstrate) domain of CaM-KII to residues 281–302 of the α-subunit (Malinow et al., 1989; Nichols et al., 1990). Synthetic peptides spanning this region are relatively potent inhibitors of the holoenzyme (IC_{50} = 2–10 μ*M*) and do not bind calmodulin. More importantly, they are specific to CaM-KII and display very little if any inhibitory activity toward other serine and/or threonine protein kinases (e.g., PKC or PKA).

Cellular Properties of CaM-Kinase II

CaM-kinase II or closely related enzymes have been implicated in a variety of cellular processes. In nonneuronal systems, recent in vitro results have indicated that CaM-kinase II's phosphorylation of the cyclic-AMP response element binding protein (CREB) increases its in vitro transcription enhancing activity (Dash et al., 1991; Sheng et al., 1991). In sea urchin eggs, the intracellular injection of specific peptide inhibitors of CaM-kinase II prevents nuclear membrane breakdown during mitosis (Baitinger et al., 1990). Certain neuropathological conditions are associated with changes in CaM-kinase II. For example, cerebral ischemia (Taft et al., 1988), or the kindling model of epilepsy (Goldenring et al., 1986) produce decreases in CaM-kinase II activity in hippocampal homogenates and isolated PSDs. In the visual system, CaM-kinase II immunoreactivity and mRNA content increase in visual cortex following monocular deprivation (Hendry and Kennedy, 1986; Bensen et al., 1991), and visual adaptation in Drosophilia produces long-term changes in the autophosphorylation and subcellular distribution of CaM-kinase II (Willmund et al., 1986). In invertebrate nervous systems, changes in the subcellular localization and autophosphorylation of CaM-kinase II have been observed following neuronal stimulation (Saitoh and Schwartz, 1985), and conditioning-induced changes in photoreceptor K^+-currents appears to

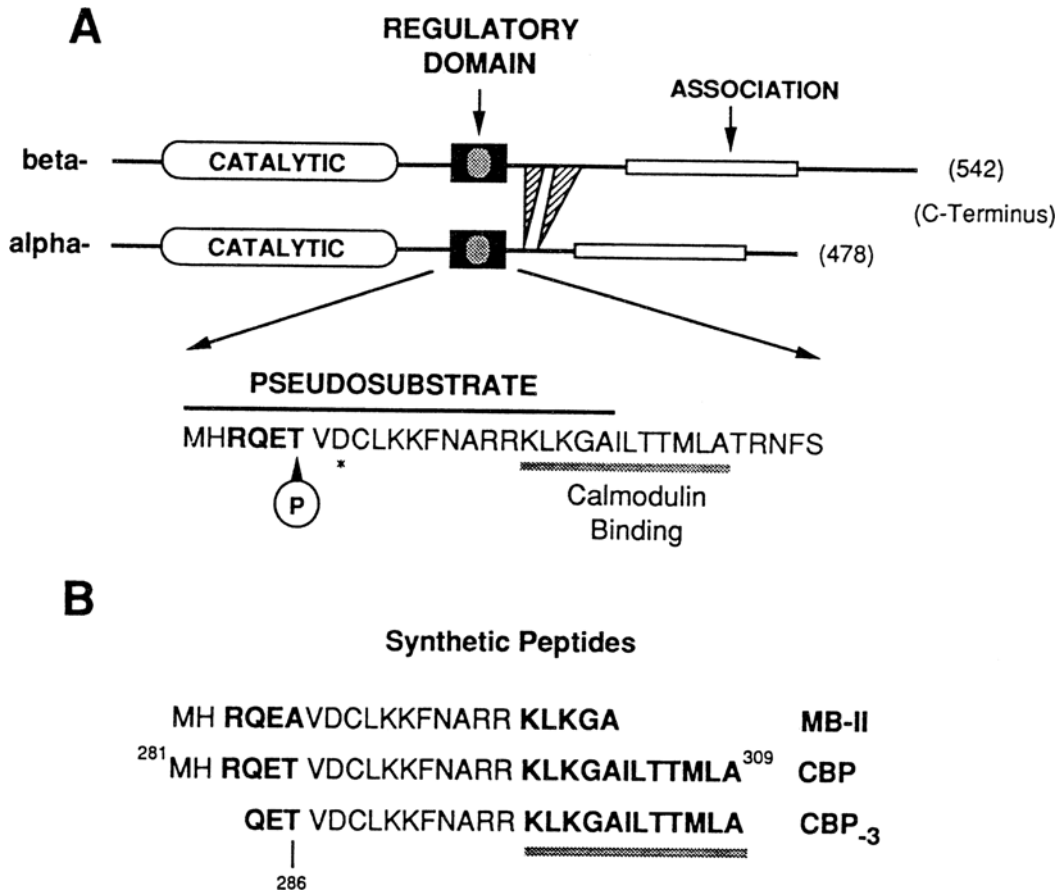

Fig. 3A. Linear map of catalytic, regulatory, and association domains of the α- and β-subunits (50 and 60 kDa proteins, respectively) of CaM-kinase II. The crosshatched triangles with their bases on the β-subunit (60 kDa) indicate two short inserted amino acid sequences that are distinct to the β-subunit (Bennett and Kennedy, 1987; Bulleit et al., 1988). The expanded amino acid sequence of the regulatory domains of either subunit is indicated with the minimum calmodulin-binding (Kelly et al., 1989) and pseudosubstrate inhibitory (Kelly et al., 1988; Malinow et al., 1989; Nichols et al., 1990) domains designated with horizontal bars (the asterisk denotes the only conservative amino acid difference in this region between the α- and β-subunits). B. Amino acid sequences of the synthetic peptides used to inhibit CaM-kinase II activity in vitro and in vivo (numbers indicate the position of amino acids in the α-subunit).

involve Ca^{2+}/CaM-dependent protein kinase(s) (Neary and Alkon, 1986).

One of the better understood roles of CaM-kinase II is its involvement in presynaptic processes underlying vesicle mobilization and synaptic transmission. Greengard and coworkers have studied the role of synapsin I in transmitter release. Depolarization-dependent Ca^{2+} influx activates CaM-kinase II, which then phosphorylates synapsin I on specific serine residues. This causes synapsin I to dissociate from synaptic vesicles and facilitates vesicle mobilization and evoked transmitter release (Llinas et al., 1985; Lin et al., 1990; Nichols et al., 1990). An important part of the synapsin I story was uncovered when it was shown to interact with cytoskeletal elements and thus tether vesicles to these structures until being phosphorylated by CaM-KII (Goldenring et al., 1986; Bahler and Greengard, 1987). The involvement of synapsin I and its phosphorylation by CaM-kinase II in presynaptic terminals has been studied in a number of preparations, includ-

ing the squid giant synapse (Lin et al., 1990), goldfish Mauthner axons (Hackett et al., 1990), and rat brain synaptosomes (Nichols et al., 1990). In general, the results from these different studies are consistent in that the phosphorylation of synapsin I in presynaptic terminals is intimately involved with the mobilization of synaptic vesicles for the evoked release of neurotransmitters, without modulating the release mechanism itself.

In support of the proposed role of synapsin I phosphorylation in transmitter release, recent studies have shown that the extracellular application of specific peptide inhibitors of CaM-kinase II reduces the *in situ* phosphorylation of synapsin I and attenuates synaptic transmission in hippocampal slices (Waxham et al., 1991). We observed that the extracellular application of peptide inhibitors of CaM-kinase II (CBP or MB-II; *see* Fig. 3B) attenuated (decreased by 60%) synaptic transmission in the CA1 region of hippocampal slices (Waxham et al., 1991). A number of control peptides, or a peptide inhibitor specific for protein kinase C (PKC) (House and Kemp, 1987), had little or no effect on synaptic transmission. Parallel studies examining the *in situ* phosphorylation of synapsin I revealed that the extracellular application of peptide inhibitors of CaM-kinase II that attenuated synaptic transmission also inhibited synapsin I phosphorylation. This inhibition was specific to the sites on synapsin I that are known to be phosphorylated by CaM-kinase II. Additional studies using peptide inhibitors together with the calcium ionophore A23187 indicated that the inhibition of synapsin I phosphorylation was not owing to nonspecific decreases in Ca^{2+} influx across neuronal membranes. Additional control experiments further showed that peptide inhibitors of CaM-kinase II did not alter; (a) postsynaptic responses to iontophoretically applied glutamate, or (b) the amplitude or latency to peak of presynaptic fiber volleys. Whole-cell patch clamp recordings of postsynaptic neurons demonstrated that inhibitory peptides produced no changes in input resistance or holding currents, but attenuated EPSCs. Together, these results support a presynaptic locus as the site of action of these inhibitory peptides and indicate that CaM-kinase II activity in presynaptic terminals, and its phosphoylation of synapsin I, plays an important role in normal synaptic transmission in the hippocampus (Waxham et al., 1991).

Changes in CaM-Kinase II During Development

The first examination of CaM-KII in developing brain tissue was done prior to its identification as a protein kinase (Kelly and Cotman, 1981). In postnatal rat forebrain, the greatest accumulation of the mPSDp (or α-subunit of CaM-KII) occured during the third postnatal week, which temporally corresponds to the active stage of synapse formation. This early correlation fostered the notion that the mPSDp represented one of the major structural building blocks of the postsynaptic apparatus. Although it is now clear that the mPSDp functions as an enzymatic molecule, the possibility that it also serves another function such as structural integrity and/or interprotein crosslinking remains an attractive but unproven hypothesis. In this context, CaM-KII could be analogous to myosin, the latter of which is a mechanocontractile protein with both structural and enzymatic functions.

The subcellular distribution of CaM-KII changes dramatically during brain development in both rodent (Kelly and Cotman, 1981; Kelly and Vernon, 1985; Sahyoun et al., 1985; Kelly et al., 1987) and chicken (Weinberger and Rostas, 1988) forebrains. In either species, there is a progressive shift or translocation of CaM-KII (α- and β-subunits) from cytosolic to particulate subcellular fractions during postnatal/posthatching development. In rat, over four times as much CaM-KII is cytosolic relative to particulate in newborn brain, whereas this proportion is reversed in adult brain. Large increases in particulate CaM-KII are observed during postnatal development in rat synaptic membrane (fivefold) and synaptic junction (14-fold) fractions. Although the

molecular mechanism(s) underlying the translocation of CaM-KII from cytosolic to particulate/synaptic compartments is unknown, this developmental change is accompanied by a major reversal in the subunit composition of the forebrain holoenzyme (Kelly and Cotman, 1981; Kelly and Vernon, 1985; Kelly et al., 1987; Weinberger and Rostas, 1988). In newborn rat forebrain, the β-subunit constitutes the major catalytic and immunoreactive subunit of the cytosolic holoenzyme, which has a β- to α-subunit ratio of approx 4:1, whereas this ratio in adult is 1:3. This developmental shift in subunit ratios is even more exaggerated in synaptic junctions purified from newborn and adult rat forebrains where the β-to α-ratios are approx 6:1 and 1:7, respectively (Kelly et al., 1987). The temporal correlation between increased levels of α-subunit expression and holoenzyme composition has led to the hypothesis that this subunit mediates in part the translocation of CaM-KII from the cytoplasm to particulate fractions and postsynaptic membranes. Thus, the high α:β ratio in many adult brain regions is consistent with observations that a majority of CaM-KII is particulate. Exceptions to this notion are found in the adult rat cerebellum and the developing chick brain. In cerebellum, the β-subunit is the major constituent of cytosolic, particulate, and PSD-associated enzymes (Groswald et al., 1983; McGuinness et al., 1985; Miller and Kennedy, 1985). Moreover, during chick brain development, the ratio of α:β subunits changes much before the translocation of the holoenzyme occurs (Weinberger and Rostas, 1988), and a greater percentage of total CaM-KII in cerebellum is particulate (about 85%), compared to approx 60% in forebrain (Miller and Kennedy, 1985). Additional evidence indicating that the translocation of CaM-KII may not be mediated by the α-subunit comes from eukaryotic expression studies. Either α- or β-subunits have been expressed at high levels in CHO or insect cells and the subcellular distribution of each has been determined (Kelly, Waxham, and Aronowski, unpublished results). These studies indicate that the partitioning or translocation of α- and β-subunits between cytosolic and particulate fractions is approximately equivalent and suggests that the association of subunits with particulate structures is a complex process that is not mediated simply by one subunit or the other. In this context, accessory proteins may be required for the crosslinking and/or association of CaM-KII with cytoskeletal structures or synaptic membranes (Sahyoun et al., 1986). Finally, previous studies in invertebrates (Saitoh and Schwartz, 1985) and insects (Willmund et al., 1986) suggested that the autophosphorylation of CaM-kinase II may be involved in translocation, although similar results have yet to be demonstrated in vertebrate neural tissues.

Early studies examined the developmental properies of CaM-KII in brain tissues containing heterogeneous neuronal and nonneuronal cell types. To address the issue of cellular heterogeneity, Scholz et al. (1988) examined the expression and subcellular localization of CaM-KII in a defined cellular setting using primary cultures of pyramidal neurons from embryonic rat hippocampus. Although pyramidal neurons are cocultured with nonneuronal cells that condition the serum-free media in which they grow, the two cell types are physically separated, allowing direct examinaion and manipulations of a virtually pure neuronal population (Banker and Cowan, 1977; Bartlett and Banker, 1984). These studies also used antibodies specific for either α- or β-subunits and observed that their expression in differentiating pyramidal neurons was very similar to that observed in whole forebrain in vivo. The expression of the β-subunit preceded that of the α-subunit, and the progressive translocation of the enzyme from the cytosolic to particulate/synaptic compartments increased with neuronal maturation. These studies showed that CaM-kinase II was very abundant in an identified neuronal population, and there was no indication that the kinase was present in nonneuronal cells (e.g., astrocytes). One important finding from these studies was the analysis of neuronal differentiation in very low density cultures where single neurons grow too far apart to make interneuronal contacts. Studies

with low density cultures tested the hypothesis that interneuronal contact was critical for the developmental expression of CaM-KII. Under these conditions, the expression of CaM-KII (α- and β-subunits) appeared to proceed along a developmental course that was similar to that observed in high density cultures where numerous synaptic connections are formed. Interestingly, isolated pyramidal neurons in low density cultures appeared to make synapses on themselves and localize immunoreactive CaM-kinase II at synapse-like "hot spots." These results indicated that the developmental expression of CaM-kinase II in hippocampal pyramidal neurons may be regulated in part by intrinsic factors that do not require interactions between neurons.

As discussed elsewhere, the transcription of the α-subunit mRNA is developmentally regulated and increases dramatically at the time of active synapse formation (Hanley et al., 1987; Burgin et al., 1990). In addition, the very high levels of CaM-kinase II expression in brain (where it may comprise 1% of total tissue protein; Erondu and Kennedy, 1985) relative to other tissues suggests that important factors are regulating the transcription of α-subunit mRNA at the genomic level. The expression of CaM-kinase II among the myriad of cell types in brain is highly concentrated in neurons vs nonneuronal cells (Ouimet et al., 1984; Scholz et al., 1988). Even among neurons, there are substantial differences in the levels of α- and β-subunit mRNA expression between different neuronal populations. For example, in adult brain, the α-subunit mRNA is expressed in high levels in hippocampus and cerebral cortex; whereas α-mRNA is very low in cerebellum, especially in granule neurons (Hanley et al., 1987; Burgin et al., 1990). In contrast, β-mRNA is expressed at high levels in cerebellum, particularly in granule cells (Bennett and Kennedy, 1987; Burgin et al., 1990). Together, these findings show that a neuron's genetic program and/or extrinsic factors such as cellular environment, trophic factors, and synaptic connectivity may play important roles in regulating the differential expression of the genes encoding the α- and β-subunits of CaM-KII during development and in the adult brain.

Comparisons of α-subunit (Hanley et al., 1987; Lin et al., 1987; Bulleit et al., 1988) and β-subunit cDNA sequences (Bennett and Kennedy, 1987) indicate that although the two subunits are structurally and functionally related, they are clearly products of distinct genes. Previous studies have shown that CaM-KII displays distinct patterns of expression in different brain regions (Ouimet et al., 1984; Erondu and Kennedy, 1985) and during development (Kelly and Vernon, 1985; Kelly et al., 1987). We used *in situ* hybridization histochemistry to determine if the level of α- and β-subunits in different neuronal populations was a direct reflection of the expression of their respective mRNAs. Synthetic oligonucleotides specific for the α- or β-mRNA were prepared and used to perform *in situ* hybridization on frozen tissue sections from different aged brains (Burgin et al., 1990). Unlike previous immunohistochemical studies on intact brain tissue which evaluated the distribution of CaM-kinase II with either an α-subunit specific antibody (Erondu and Kennedy, 1985) or an α/β-subunit crossreacting antibody (Ouimet et al., 1984), we were able to examine the distribution of each subunit's mRNA in adjacent sections. We observed that CaM-kinase II mRNA hybridization intensities generally paralleled the distribution of CaM-kinase II immunoreactivity in adult tissue (Ouiet et al., 1984; Erondu and Kennedy, 1985), and the temporal pattern of protein expression during development (Kelly and Montgomery, 1982; Kelly and Vernon, 1985; Sahyoun et al., 1985; Kelly et al., 1987). Quantitative northern slot-blot analyses of CaM-kinase II mRNA from microdissected brain regions during development further supported the correlation between levels of mRNA and protein expression. Together, these results indicate that the expression of CaM-kinase II subunits is regulated primarily at the level of gene transcription.

A potentially important finding from *in situ* hybridization studies was the observation that the α-, but not β-subunit mRNA was localized to den-

dritic compartments of hippcampal pyramidal and granule neurons, as well as neurons of the cerebral cortex (Burgin et al., 1990). The distribution of hybridization signal was diffuse in these neuropil-enriched regions and did not correlate with the location or distribution of interneurons and glial cell bodies. These results indicated that α-subunit mRNA can be localized to dendrites of certain CNS neurons (Burgin et al., 1990). Thus far, only mRNAs encoding the α-subunit of CaM-kinase II, MAP-2 (Tucker et al., 1989), certain small neuropeptides (Dirks et al., 1989; Bloch et al., 1990) and the nontranslated RNA called BC1 (Tiedge et al., 1991) have been localized to dendrites. The localization of a discrete subset of neuronal RNAs in dendritic compartments suggests an important linkage between postsynaptic functions and the translation of polyribosomes that encode dendritic polypeptides that underlie and/or modulate these functions.

Roles of CaM-Kinase II in Synaptic Functions

Interests in synthetic peptide inhibitors of protein kinases stemmed from the need to develop kinase-specific inhibitors that could ultimately determine the role(s) of CaM-kinase II and other protein kinases in synaptic functions. To accomplish this goal, experiments were carried out to study the effects of microinjecting peptide inhibitors into postsynaptic neurons using the in vitro hippocampal slice preparation. The model of neurotransmission and synaptic plasticity that was studied is the phenomenon of long-term potentiation (LTP). Prior to these experiments, a variety of studies had implicated a pivotal role for increases in postsynaptic $[Ca^{2+}]_i$ in the mechanisms underlying the induction and/or expression of LTP (Lynch et al., 1983; Malenka et al., 1988). The central rational for using peptide inhibitors of CaM-kinase II in living neurons was that they are very specific and would not inhibit PKC or cAMP-dependent protein kinase (PKA), and once injected postsynaptically, they would not able to diffuse from the injected neuron and enter neighboring presynaptic terminals. Malenka et al. (1989) found that the intracellular injection of the nonspecific kinase inhibitor H-7, or the general calmodulin antagonist calmidazolium (both of which are membrane permeable), blocked LTP induction in hippocampal CA1 pyramidal neurons. More importantly, LTP was blocked by the postsynaptic injection of synthetic peptides that are potent CaM-antagonists (e.g., CBP_{-3}), or are active site-directed inhibitors of CaM-kinase II (e.g., CBP). These studies demonstrated an essential role for postsynaptic calmodulin and protein kinase activity, in the induction of LTP (Malenka et al., 1989). Malinow et al. (1989) reported equivalent results using similar peptide inhibitors, but made one additional and important observation. They were able to use two independent stimulation pathways and a difficult postinjection paradigm to show that inhibitory peptides did not block LTP expression when injected into postsynaptic neurons after tetanus-induced potentiation. These studies, both using similar peptide inhibitors, indicate that the mechanism(s) responsible for LTP induction require(s) CaM-kinase II, whereas processes responsible for the maintenance or expression of LTP appear not to require persistent CaM-kinase II activity. Malinow et al. also reported that a PKC inhibitory peptide produced essentially the same results previously described for the peptide inhibitor of CaM-kinase II (Malinow et al., 1989). We tried postsynaptic injections of the PKC inhibitory peptide (residues 19–31; *see* House and Kemp, 1987) and saw no effects on LTP (Malenka et al., unpublished results). Finally, O'Dell et al. (1991) recently reported that inhibitors of tyrosine kinases blocked LTP induction in the hippocampus. Thus, the involvement of multiple protein kinases in LTP induction suggests that at least three parallel pathways may underlie this important triggering event, or that a complex cascade of protein kinases and substrate phosphoproteins may be involved.

Studies with protein kinase inhibitors have underscored the role of CaM-kinase II in events that trigger LTP induction. There are additional

reasons why protein kinases are thought to be important in LTP and basal synaptic transmission. First, Ca^{2+} influx through *N*-methyl-D-aspartate (NMDA) receptor/channels appears to be critical for LTP induction (Lynch et al., 1983; Collingridge et al., 1988; Collingridge and Singer, 1990), and Ca^{2+} is an important regulator of CaM-kinase II (and PKC and some adenylate cyclases). Second, experiments have shown that LTP induction in neurons analyzed by whole-cell recordings exhibited considerable "wash-out" behavior. Thus, nystatin perforated patch techniques were used to demonstrate LTP in pairs of cultured neurons (Bekkers and Stevens, 1990), whereas LTP induction was unattainable 30 min after whole-cell access of the same type of neurons in hippocampal slices (Malinow and Tsien, 1990). This suggests the involvement of important diffusible factors in postsynaptic mechanisms underlying LTP induction (Malinow and Tsien, 1990). Third, studies using whole-cell patch clamping of cultured hippocampal neurons observed a rapid washout of NMDA channel activity (50% reduction in 150 s), whereas the loss of kainate responsiveness was minimal (MacDonald et al., 1989). The washout of NMDA responsiveness could be prevented or reversed by including Mg-ATP or an ATP regenerating system in patch pipets. These results suggest that normal NMDA-receptor/channel activity may require phosphorylation. Recent studies on non-NMDA glutamate receptors in hippocampal neurons have demonstrated that cAMP-dependent protein phosphorylation enhances whole-cell currents, as well as single channel opening frequency and mean open-times (Greengard et al., 1991; Wang et al., 1991). In addition, preliminary results indicate that PKC may regulate the function of NMDA-type receptor/ion channels expressed in frog oocytes (Nomura et al., 1991). Together, these results strongly indicate that the phosphorylation of postsynaptic glutamate receptors plays an important role in receptor function and synaptic plasticity.

The ability of CaM-KII to become Ca^{2+}-independent following autophosphorylation has made it an attractive candidate as a memory molecule that may underlie long-term changes in synaptic plasticity. CaM-kinase II could encode the history of synaptic activity by its autophosphorylation and resulting increases in Ca^{2+}/CaM-independent activity (Miller and Kennedy, 1986; Kennedy, 1987). Lisman and coworkers viewed the autophosphorylation of CaM-kinase II as a potential storage mechanism for long-term memory (i.e., synapse strengthening), whereas the dephosphorylation of autophosphorylated CaM-kinase II could be responsible for synapse weakening (Lisman, 1985; 1989; Lisman and Goldring, 1988). Support for this hypothesis comes from in vivo studies demonstrating the presence of Ca^{2+}-independent CaM-KII activity in neural tissues and cultured neurons. Early experiments by Fukanaga et al. (1989) showed that cultured cerebellar granule cells expressed Ca^{2+}-independent CaM-KII activity in response to depolarization with high K^+. The generation of Ca^{2+}-independent activity required extracellular calcium, was transient lasting only a few minutes, and constituted approx 10% of the total cerebellar CaM-KII activity. The generation of Ca^{2+}-independent activity could be mimicked by calcium ionophores and could be potentiated by okadaic acid, a membrane-permeable inhibitor of protein phosphatases type-1 and type-2A. Interestingly, under nonstimulated basal conditions, about 5% of the total CaM-KII activity in granule cultures was Ca^{2+}-independent. Recent studies using acutely prepared hippocampal slices demonstrated similar results on the generation of Ca^{2+}-independent activity by high K^+ depolarization (Ocorr and Schulman, 1991). Other studies using organotypic hippocampal slice cultures showed that under basal conditions, as much as 34% of the total CaM-KII activity was Ca^{2+}-independent (Molloy and Kennedy, 1991). The latter studies showed that Ca^{2+}-independent activity was increased by high K^+ depolarization, however, this effect was variable and occurred in less than half of the experiments. These experiments also showed that a variety of conditions expected to stimulate or inhibit excitatory neurotransmission in the hippocampus (e.g., treatments with kynurenic acid,

2-amino-5-phosponopentanoic acid (AP5), tetrodotoxin, high Mg^{2+}, NMDA, glutamate, picrotoxin, or calcium channel blockers), did not significantly alter the proportion of Ca^{2+}-independent CaM-KII activity in slice cultures. These results are both surprising and paradoxical in light of the proposed role for postsynaptic CaM-KII in synaptic plasticity. First, why is such a large amount of CaM-KII in the Ca^{2+}-independent state under nonstimulated or basal conditions? Moreover, why is this activity unaffected by the application of glutamate and/or NMDA, treatments thought to be involved in the induction of LTP? These results indicate that a large proportion of the in vivo CaM-KII activity is Ca^{2+}-independent and largely unregulated, with the exception of the generation of additional Ca^{2+}-independent activity by the rather nonphysiological conditions of high K^+ depolarization. Third, and possibly more confounding, are the reports that the postsynaptic injection of specific (synthetic peptides) or nonspecific (H-7) inhibitors of CaM-KII have no detectable effects on basal synaptic transmission (Malenka et al., 1989; Malinow et al., 1989). One interpretation of these observations is that if large amounts of Ca^{2+}-independent CaM-KII are present in postsynaptic compartments such as dendritic spines, it is not accessible to the action of kinase inhibitors (despite the fact that postsynaptic inhibitors block the induction of LTP). On the other hand, postsynaptic compartments may contain negligible amounts of Ca^{2+}-independent CaM-KII activity under basal conditions. Alternatively, postsynaptic CaM-KII may not play a role in the processes underlying basal synaptic transmission. Future studies will be necessary to fully understand which neuronal compartments these large amounts of Ca^{2+}-independent CaM-KII reside (e.g., pre- and/or postsynaptic, dendritic vs cell body), or what role(s) it plays in synaptic plasticity and/or neurotransmission.

Finally, the involvement of persistent Ca^{2+}-independent protein kinase activity in the expression of LTP remains somewhat controversial. Whereas early experiments with the extracellular application of H-7 (a general kinase inhibitor) indicated that persistent kinase activity was involved in the maintenance/expression of LTP (Malinow et al., 1988), recent observations have shown that H-7 treatments failed to attenuate synaptic transmission in potentiated pathways more than the control nonpotentiated pathways (Muller et al., 1990; Leahy and Vallano, 1991). Recent evidence suggests that mechanisms underlying the expression of LTP may have a presynaptic locus (Bekkers and Stevens, 1990; Malinow and Tsien, 1990). In this context, it is difficult to reconcile how these presynaptic mechanisms would not also participate in events required for basal synaptic transmission, or why they would be sensitive to attenuation by kinase inhibitors (e.g., H-7) only during the expression of LTP. Consistent with the notion of presynaptic expression, it remains possible that a constitutively active protein kinase in presynaptic terminals may be responsible for LTP expression. Future experiments, possibly using whole-cell recordings of synaptically coupled pairs of neurons, may resolve these issues. Clearly, there is much to learn about the complex mechanisms that underpin synaptic plasticity, as well as the pre- vs postsynaptic locus of LTP expression.

Protein Phosphorylation

CaM-Kinase II Functions

As previously discussed, postsynaptic CaM-kinase II activity is necessary for LTP induction (Malenka et al., 1989; Malinow et al., 1989). Although little is known about the identity of postsynaptic proteins phosphorylated by CaM-kinase II in vivo they are logical candidates to be involved in the modulation of synaptic transmission and synaptic plasticity. We and others have reported the preliminary characterization of proteins phosphorylated by CaM-kinase II in subcellular fractions enriched in synaptic junctions (SJs) (Robinson and Dunkley, 1983; Gurd 1985; Kelly et al., 1985; Dunkley et al., 1986) and PSDs (Grab et al., 1981; Gurd 1985; Gurd and Bissoon, 1985; Kelly et al., 1985; Rostas et al., 1986). Recent

correlative results suggest that certain synaptic proteins phosphorylated by CaM-kinase II in vitro are also phosphorylated in vivo (Yip and Kelly, 1989), however, the kinase(s) that actually phosphorylates these substrates in vivo is unknown. Historically, many of the proteins initially identified as in vitro substrates of CaM-kinase II (i.e., tyrosine hydroxylase, MAP-2, and synapsin 1) have since been shown to be phosphorylated by CaM-kinase II in vivo. One expects that a detailed understanding of synaptic junction proteins that are in vitro substrates for CaM-kinase II will ultimately be required to understand their in vivo role(s) in synaptic function.

Previous studies examined the subcellular distribution of phosphoproteins from hippocampal slices and factors that regulated their phosphorylation and dephosphorylation *in situ* (Yip and Kelly, 1989). High K^+ depolarization in the presence of Ca^{2+} markedly increased $^{32}P_i$-incorporation into endogenous proteins. The effects of Ca^{2+} stimulation were significantly reduced by Ca^{2+}-channel blockers and the calmodulin antagonist W-13. Certain proteins were dephosphorylated *in situ* and their dephosphorylation was dependent on both Ca^{2+} and depolarization. A number of proteins phosphorylated *in situ* were similar to those previously characterized in synaptic fractions phosphorylated in vitro under conditions that activate PKA, PKC, or CaM-KII. Hippocampal phosphoproteins were identified on the basis of mol wt, isoelectric point, immunoreactivity, and phosphopeptide mapping; these included the 87-kDa substrate of protein kinase C (MARCKS; Wang et al., 1989), synapsin I, the α- and β-subunits of CaM-kinase II, tubulin, B-50 (GAP-43), the α-subunit of pyruvate dehydrogenase, and myelin basic proteins. The *in situ* phosphorylation of CaM-kinase II was stimulated about twofold by high K^+ depolarization and was found in cytosolic, detergent-soluble and detergent-insoluble fractions. Interestingly, the apparent specific activity of $^{32}P_i$ labeling in Triton-insoluble fractions (which is enriched in synaptic junction complexes and CaM-KII) was the lowest of all three subcellular fractions. This result indicates that the PSD-like compartment of CaM-kinase II may undergo the least amount of autophosphorylation *in situ*. Peptide mapping indicated that CaM-kinase II phosphorylated *in situ* appeared similar but not identical to its in vitro autophosphorylated counterpart, but indicated that the Ca^{2+}-independent form of the kinase was generated *in situ*. Phosphopeptide mapping analysis of *in situ* labeled synapsin I and MARCKS indicated that cAMP-, Ca^{2+}/calmodulin-, and Ca^{2+}/phospholipid-dependent protein kinases were active in slice preparations under basal conditions. Membrane depolarization produced increased $^{32}P_i$-labeling of many hippocampal proteins and indicated that these three protein kinases were stimulated either directly or indirectly by depolarization.

One can speculate about the identity of potentially important substrates that could be phosphorylated in vivo when postsynaptic CaM-kinase II is activated. Logical substrates include ion channels (e.g., voltage-dependent Ca^{2+}-channels), neurotransmitter receptors (e.g., NMDA- and AMPA-type [amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid] glutamate receptors), cytoskeletal proteins that regulate synapse and/or spine shape (e.g., MAP-2 and fodrin), enzymes involved in the synthesis of hypothetical retrograde messengers (e.g., phospholipase A_2, nitric oxide synthetase, or lipoxygenases), or transcription factors (e.g., CREB; Dash et al., 1991; Sheng et al., 1991). One potentially important class of postsynaptic molecules is a family of glycoproteins that were initially identified, localized, and purified on the basis of their affinity for the plant lectin concanavalin A (Con A) (Kelly et al., 1976; Kelly and Cotman, 1977; Gurd et al., 1983a,b). Previous studies from our group and others indicated that these glycoproteins are anchored in the PSD, span the postsynaptic membrane, and extend their carbohydrate moieties into the synaptic cleft. One member of this group, a 170–180K glycoprotein (GP-170), has been shown to be phosphorylated in vitro by a PSD-associated tyrosine kinase(s) (Gurd and Bissoon, 1990), as well as CaM-kinase II and cyclic-AMP dependent protein kinase (Gurd et al., 1983a; Gurd and

Bissoon, 1985; Kelly et al., 1985), and is known to incorporate $^{32}P_i$ in vivo (Gurd and Bissoon, 1985). GP-170 could transduce changes in intracellular messengers that regulate these two protein kinases to functional alterations at the surface of the postsynaptic membrane. Unfortunately, the function of any member of this family of postsynaptic membrane glycoproteins is unknown.

In addition to GP-170, SJ substrates of mol wt 240–250K, 205–210K, and 140K display substantial phosphorylation in vitro (Gurd, 1985; Kelly et al., 1985). They are phosphorylated almost exclusively on serine residues and their phosphorylation in purified SJs is stimulated 10–20-fold by Ca^{2+}/calmodulin. Recent studies on *in situ* phosphorylation in hippocampal tissue slices revealed proteins of comparable 2-D electrophoretic properties to these in vitro SJ substrates of CaM-kinase II. Although it is unknown if these proteins are identical to the in vitro substrates described above, or whether CaM-kinase II actually phosphorylates them in vivo, their phosphorylation in hippocampal tissue slices is stimulated approx twofold when slices are incubated for 1 min in media containing high K^+ and Ca^{2+} (Yip and Kelly, 1989).

CaM-Kinase II Messenger RNA: A Role in Dendritic Plasticity

Localization of the Protein Synthetic Apparatus in Dendrites

The identification of components of the neuron's translational machinery in axons and dendrites, and the mechanisms that regulate this machinery, is important to understand neuronal polarity and synaptic plasticity. Early biochemcal studies indicated that RNA species were present in the axons of Mauthner neurons in fish (Koenig, 1979). Recent *in situ* hybridization studies have demonstrated that mRNA encoding oxytocin is present in the axons of the rat hypothalamo-neurohypophysial tract (Jirikowski et al., 1990). mRNAs encoding neuropeptide hormones have been detected in axons and axon terminals of snail neurons (Dirks et al., 1989), as well as in proximal dendrites of neurons in the rat hypothalamus (Bloch et al., 1990). Likewise, the mRNA for tyrosine hydroxylase has been localized in neurites in the human ventral mesencephalon (Dumas et al., 1990). The mRNA encoding the α-, but not β-subunit of CaM-kinase II is localized in dendrites of hippocampal neurons (Burgin et al., 1990). The only other mRNA known to be localized to these dendrites encodes MAP-2 (Garner et al., 1988; Tucker et al., 1989), which is an in vivo substrate for CaM-kinase II (Schulman, 1984; Schulman et al., 1985). mRNA encoding MAP-2 has also been localized in dendrites but not axons of cultured sympathetic neurons (Bruckenstein et al., 1990). Indeed, *in situ* hybridization studies indicate that most mRNAs are restricted to neuronal cell bodies (Bruckenstein et al., 1990; Burgin et al., 1990; Kleiman et al., 1990). For many years, dendrites have been known to contain polyribosomes, particularly in close proximity to dendritic spines (Peters et al., 1991). Studies on the rat dentate gyrus have shown that up to 60% of all neuronal spines contain ribosomes (Steward et al., 1988). Dendritic ribosomes have been proposed to trans-late specific mRNAs encoding important postsynaptic proteins (Steward, 1983b; 1987; Steward and Falk, 1985). Steward and coworkers have shown that the probability of finding polyribosomes beneath dendritic spines is significantly increased during development (Steward and Falk, 1985; 1986) and during lesion-induced reactive synaptogenesis in hippocampus (Steward, 1983a). In rat forebrain, the expression of α-subunit mRNA and protein sharply increases during the most active period of synaptogenesis (Kelly and Vernon, 1985; Hanley et al., 1987; Kelly et al., 1987), a time coincident with increases in the number of polyribosomes beneath dendritic spines. These observations suggest that the translation of dendritic mRNAs may be important in the formation of postsynaptic structures during development, or their modification during synaptic plasticity.

Functional Implications of Dendritic mRNA

In terms of the functional significance of protein synthesis in dendrites, or possible posttranscriptional events in axons and/or presynaptic terminals, it is interesting to speculate that LTP or other types of synaptic plasticity could require or be modulated by local protein synthesis in dendrites and axons. Frey et al. (1989) successfully induced and maintained LTP in hippocampal CA1 regions in which dendrites (i.e., a majority of stratum radiatum) had been surgically amputated from their respective neuronal cell bodies. They were able to maintain LTP (40–50% enhancement of the field EPSP) for 3 h. Although this enhancement returned to baseline in 5–6 h, their ability to produce nondecremental synaptic enhancement for 3 h indicates that the cellular machinery supporting LTP is localized in part to postsynaptic dendrites that display functional autonomy from the cell body. Likewise, let us not forget that one of the common preparations for LTP studies is the isolated CA1 field in which a cut is made through the Schaffer collaterals and commissual axons to reduce seizure activity by transecting CA3 neuronal somas from their terminals (Malenka, 1991). The many studies demonstrating LTP in this preparation clearly show that presynaptic processes mediating LTP do not require critical functions/factors supplied from cell bodies or initial axon segments. Although a totally cell-free preparation (i.e., surgically isolated presynaptic terminals plus postsynaptic dendrites) has yet to be tested, the ability to induce and maintain LTP in such a preparation may be possible. If true, then substantial autonomy exists in axon terminals and dendrites to initiate and maintain LTP for long periods of time (>3 h). These observations further support a role for localized dendritic processes, possibly the translation of mRNAs, in the causal events contributing to synaptic plasticity.

As previously discussed, CaM-kinase II activity has been shown to be essential for LTP induction, presumably through the phosphorylation of critical postsynaptic substrate protein(s). Although somewhat controversial, previous studies have shown that LTP induction requires protein synthesis (Stanton and Sarvey, 1984; Deadwyler et al., 1987; Frey et al., 1988; Otani et al., 1989). A variety of translational inhibitors have been shown to prevent LTP induction or lessen the degree of potentiation if added to hippocampal slices before (15–30 min) or during high-frequency stimulation (Stanton and Sarvey, 1984; Deadwyler et al., 1987). In general, there is little or no effect on the maintenance of LTP if inhibitors are added 15 min after high-frequency stimulation (Otani et al., 1989). These results suggest that LTP induction may require a protein with a very short half-life, or a protein whose translation is regulated by synaptic activity. Because LTP is believed to be a synapse-specific event (Nicoll et al., 1988), it is difficult to reconcile how such a protein could be synthesized in the cell body, specifically transported to the appropriate synapse (i.e., the potentiated synapse), and serve an important function in the early events of LTP induction. It is possible that proteins important in the expression of LTP, or other examples of synaptic plasticity may be synthesized at the base of synaptic spines via the translation of dendritically localized mRNA (*see* Fig. 4). It is also interesting to speculate that a translational apparatus at the spine may produce proteins or peptides that would be exported as retrograde messengers to presynaptic terminals (Williams et al., 1989).

Is the presence of mRNA in dendrites simply a consequence of these processes being an extension of the neuronal cell body? This seems highly unlikey. First, the transport of newly synthesized RNAs into neuronal dendrites is energy-dependent (Davis et al., 1987;1990). Moreover, the vast majority of mRNAs localized by *in situ* hybridization studies are absent from dendritic compartments. Together, these findings suggest that important neuronal functions have evolved that require the targeting of specific mRNAs to dendrites. It is not known why some mRNAs are in dendrites and others are not. Several possibilities exist. First, certain proteins could be trans-

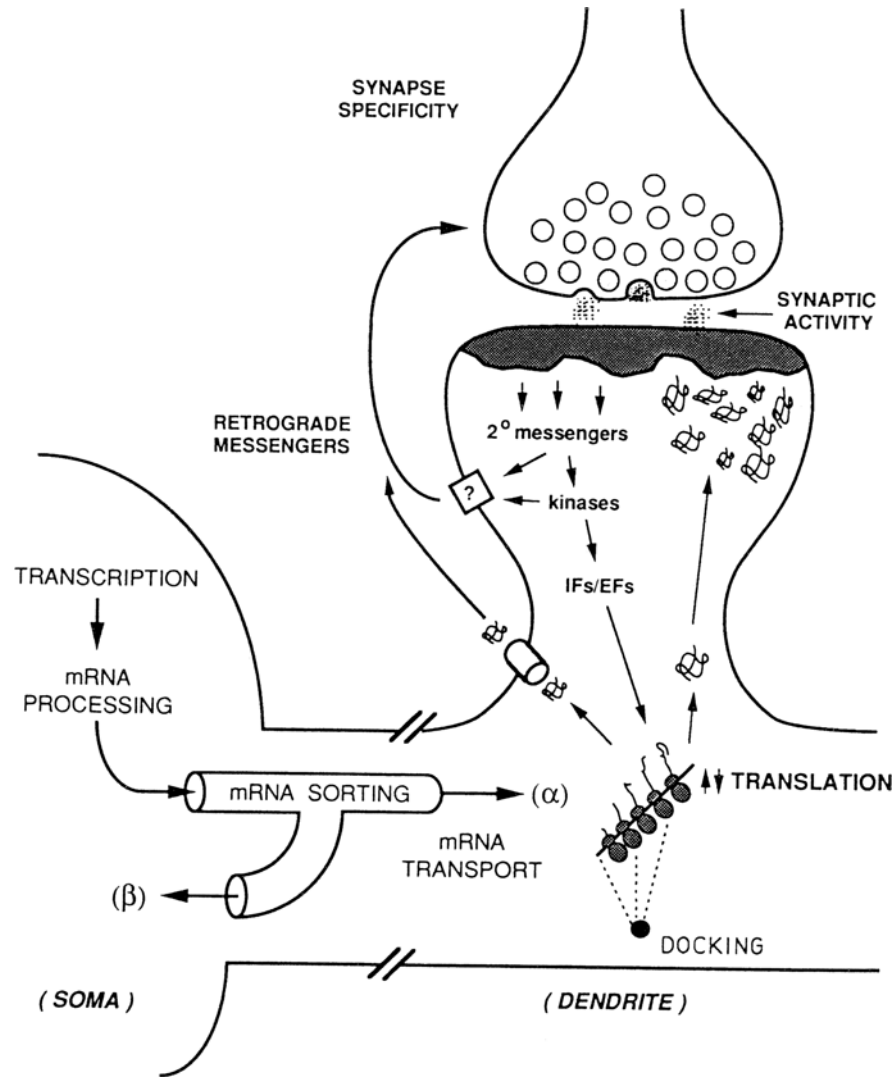

Fig. 4. This displays a number of hypothetical events and putative regulatory mechanisms that may be involved in the localization of specific mRNAs in the dendrites of neurons (*see* text for details). It depicts that a sorting process takes place at the dendrite/soma junction, and that transport mechanisms actively move mRNAs to their final "docking" sites along dendritic shafts and at the base of spines. This model also poses the possibility that one of the products of dendritic protein synthetic machinery could be a retrograde messenger that communicates plastic changes in synaptic efficacy from post- to presynaptic terminals. IFs and EFs correspond to initiation and elongation factors, respectively.

lated from dendritic mRNAs at specific synapses in response to synaptic activation. For example, an "activator" of translation may be required to induce postsynaptic changes (i.e., LTP or LTD) following specific stimuli. On the other hand, a retrograde "signal" protein may need to be synthesized and secreted to induce changes in presynaptic activity following LTP. Second, the half-life of certain proteins may be too short to survive the time required for transport from the soma to distal dendrites. Third, dendritic localization could protect mRNAs from fast turnover rates in cell bodies. Finally, specific proteins may be required to arrive at postsynaptic structures

in unmodified forms (e.g., unglycosylated or unphosphorylated). *De novo* protein synthesis at or near synaptic contacts could prevent unwanted posttranslational modifications that may occur during transport from the cell body. For example, MAP-2 has its maximal affinity for microtubules when about 1/3 of its phosphorylation sites are modified and its affinity for microtubules decreases following dephosphorylation (Brugg and Matus, 1990). The synthesis of MAP-2 from polysomes at the base of dendritic spines could target it to postsynaptic structures before it becomes phosphorylated and tightly bound to dendritic shaft microtubules. Interestingly, dendritic spines contain appreciable levels of MAP-2, but virtually no microtubules (Morales and Fifkova, 1989). In an analogous fashion, monomeric α-subunits could be required as crosslinking molecules to integrate themselves or CaM-kinase II holoenzymes into PSDs (Miller and Kennedy, 1985). Indeed, it has been shown that the α:β subunit ratio in synaptic junctions is two times higher than in the cytosolic holoenzyme (Kelly et al., 1987). By synthesizing α-subunits from dendritic mRNA, this subunit may avoid spontaneous association into heterosubunit holoenzymes, as occurs in cell bodies.

So as to not overstate the functional importance of dendritic mRNA, what is the evidence that these RNAs are actually translated into proteins? Synthetic labeling studies by Steward and coworkers lend supportive evidence that the synthesis and glycosylation of proteins takes place in the local environment of dendrites and nerve-ending particals (Steward, et al., 1988; Rao and Steward, 1991). These experiments used short labeling periods (10 min) to minimize the contribution of dendritic transport of newly synthesized or glycosylated proteins, and employed dissociated cell cultures where individual dendrites of pyramidal neurons can be easily identified by autoradiography. Additional studies examining the kinetics of protein labeling in dendrites using ^{3}H-leucine or ^{3}H-fucose, combined with the use of inhibitors of *N*-linked glycosylation (i.e., tunicamycin) or the export of newly synthesized glycoconjugates from the Golgi (i.e., monensin), provide evidence that the labeling of proteins and/or glycosylation reflects localized dendritic synthesis.

Using a different system, Davis and Kater (1990) examined the local protein synthesis in isolated growth cones of cultured snail neurons. Although growth cones are not truly analagous to the dendrites of mature neurons, they may share certain features by which the neuron's protein synthetic apparatus is localized in neuritic processes. The autonomous properties of growth cones is exemplified by the behavior of isolated growth cones in culture, which is very similar to intact growth cones for at least 24 h following transection. The autonomy of isolated growth cones appears to extend to the level of protein synthesis (Davis and Kater, 1990). Two–6 h after transection, isolated growth cones were labeled with ^{3}H-leucine for 10 min. Autoradiographic analysis showed that silver grain densities over isolated growth cones were equivalent to the labeling of growth cones whose cytoplasm was continuous with the neuronal soma. ^{3}H-Leucine incorporation was observed in isolated growth cones located over 600 μm from cell bodies before transection and incorporation over growth cones was blocked by the appropriate protein synthesis inhibitors. Studies with dendrites and growth cones suggest that local protein synthesis in these structures may not only subserve different cellular functions than those carried out in the neuron's soma, but that the regulation of such dendritic functions may be mediated by local environmental and/or synaptic stimuli. The schematic in Fig. 4 indicates a few possibilities in which synaptic activity may regulate local protein synthesis in dendrites. One obvious mechanism involves the production of second messengers in the spine that would activate protein kinases. As recently reviewed by Hershey (1989), there are many steps in the protein synthetic pathway that have been shown to be regulated through the phosphorylation of initiation and elongation factors, ribosomal proteins, and aminoacyl-tRNA synthetases. The regulation of protein synthesis by kinases is complex, since the phosphorylation

of certain factors stimulates synthesis, whereas the phosphorylation of others is inhibitory. Since most translational controls work at the level of initiation (Hershey, 1989), these events may be more sensitive to control by synaptic activity. In the context of Ca^{2+}-regulated kinases, elongation factor 2 (eEF-2) has been shown to be inactivated via the phosphorylation by CaM-kinase III (Nairn and Palfrey, 1987).

Mechanisms of Dendritic mRNA Targeting

The mRNA encoding the α-subunit of CaM-kinase II (α-mRNA) is localized throughout the proximal-distal extent of hippocampal pyramidal neuron dendrites (Burgin, 1989), whereas the mRNA encoding MAP-2 appears to be restricted to more proximal regions of the same dendrites (Garner and Matus, 1988; Tucker et al., 1989). This difference in distribution suggests that the two mRNAs may be targeted to dendrites by different mechanisms, or that the α-mRNA may be more efficiently transported. We propose that elements in the α-mRNA may be necessary and sufficient for the dendritic targeting of this and possibly other mRNAs. For example, we have identified a large region of potential secondary structure in the 3' UTR of the α-mRNA (Burgin, 1989). Of course, the potential for secondary structure does not necessarily indicate functional relevancy. We favor the notion that targeting sequences in the α-mRNA reside in either 3' or 5' UTRs, since the coding regions of the α-and β-mRNAs are so similar (Bulleit et al., 1988; Burgin et al., 1990), and the latter is not detected in dendrites (Burgin, 1989). It is also possible that the region of the α-mRNA responsible for dendritic targeting does so by binding to a cytoplasmic protein or RNA molecule that mediates dendritic transport. Alternatively, regions of secondary and tertiary structure in transported mRNAs could direct their own transport via a ribozyme-like activity (Celander and Cech, 1991). On the other hand, targeting specificity could reside in the nascent polypeptide instead of the mRNA, analogous to signal peptides of integral-membrane or secreted proteins. Thus, what may be targeted to dendrites could be ribonucleoprotein complexes that have just initiated translation. This scenario may provide a more efficient mechanism to transport multiple components of the translational ensemble at the same time.

Since prior studies indicated that the translocation of RNA to dendrites was dependent on cellular energy metabolism, cytoskeletal motors may be involved not only in the movement of mRNA, but different motors could display differential selectivity towards the types of RNA that they transport (e.g., kinesin vs dynein-like cytoskeletal motors; Black and Baas, 1989; McIntosh and Porter, 1989). The movement of macromolecules and organelles in dendritic compartments is a dynamic process and there is evidence that dendritic transport systems are bidirectional (Black and Baas, 1989). Therefore, one could speculate that a docking mechanism might exist to anchor specific mRNAs to the dendritic cytoskeletal scaffolding, possibly near the base of spines where localized protein synthesis may be critical. The docking of specific mRNAs seems necessary if the regulation of protein synthesis at the base of spines is tightly coupled at both temporal and spatial levels and provides a vehicle through which synapse-specific changes can be made in response to synaptic activity.

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