Developmental Changes in Ca^{2+} /Calmodulin-Dependent Protein Kinase II in Cultures of Hippocampal Pyramidal Neurons and Astrocytes

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We have analyzed Ca^{2+} /calmodulin-dependent protein kinase II (CaM-kinase II) localization, activity, and endogenous protein substrates during differentiation and synaptogenesis in cultured hippocampal neurons. Primary cultures from hippocampi from 18 d embryonic rats are composed primarily of pyramidal neurons, with minimal contamination by non-neuronal cells. We have used monoclonal (Mab) and affinity-purified polyclonal antibodies that recognize either or both of the subunits of CaM-kinase II in order to localize the enzyme at progressive stages of neuronal differentiation. Diffuse but specific binding, determined by indirect immunofluorescence analyses, was first detected in cell bodies and growth cones of pyramidal neurons after 4 d in culture. Immunoreactivity increased during the next 3 d of culture, at which time fluorescent labeling was patchy along neuritic processes. By 10 d, intensely fluorescent, discrete spots were observed along processes and on cell bodies. Astrocyte cultures prepared from newborn rat cortex showed no detectable immunofluorescence with anti-CaM-kinase II antibodies. Cytosolic and particulate fractions from cultured pyramidal neurons and astrocytes were analyzed using immunoblot, *in vitro* phosphorylation, 2-dimensional gel electrophoresis, and phosphopeptide mapping techniques. Although pure astrocyte cultures contained low levels of Ca^{2+} /CaM-stimulated protein kinase activity, they did not display detectable levels of immunoreactive 50 kDa subunit nor 50 and 60 kDa phosphoproteins analogous to the autophosphorylated subunits of CaM-kinase II. Immunoblot analysis detected the 60 kDa kinase subunit in particulate and cytosolic fractions from 2 d neurons. By contrast, the 50 kDa subunit of CaM-kinase II was not detected in cytosolic or particulate fractions of pyramidal neurons before 4 d in culture. In 2 d pyramidal neuron cultures, only low levels of Ca^{2+} /CaM-stimulated protein phosphorylation were observed. Ca^{2+} /CaM-dependent phosphorylation of 10 d pyramidal cell proteins was 3–5-fold greater than that of 2 d cultures, and included major phosphoproteins of 48, 50, 56, 58/60, 80–86, 90, 120, 138, 175, and 190 kDa. Phosphopep-

tide maps of 58/60 and 50 kDa phosphoproteins gave patterns very similar to those of the autophosphorylated 60 and 50 kDa subunits, respectively, of purified CaM-kinase II. A phosphoprotein doublet of 83 kDa was identified as synapsin I. Developmental changes in Ca^{2+} /CaM-dependent phosphorylation in pyramidal neuron cultures were very similar to those previously described in subcellular fractions from postnatal rat forebrain.

The nervous system contains a number of protein kinases that are regulated by second messengers, such as cyclic nucleotides, phospholipids, and Ca^{2+} (for review, see Nairn et al., 1985). Protein phosphorylation has been implicated in the transduction of many extracellular messages controlling neuronal differentiation, metabolism, excitability, and neurotransmitter release (for review, see Nestler and Greengard, 1984). A number of protein substrates phosphorylated *in vitro* by Ca^{2+} /CaM-dependent protein kinase II (CaM-kinase II), protein kinase C, or cAMP-dependent protein kinase in synaptosomal, synaptic membrane, synaptic junction, and postsynaptic density fractions have been described (Ueda and Greengard, 1977; Schulman and Greengard, 1978; Kelly et al., 1979; Carlin et al., 1981; Dunkley, 1981; Grab et al., 1981; DeLorenzo, 1982; Rodnight, 1982; Aloyo et al., 1983; Gurd et al., 1983; Walaas et al., 1983a, b; Ackers and Routtenberg, 1985; Kelly et al., 1985). CaM-kinase II is thought to play important roles in synaptic vesicle translocation and transmitter release (Llinas et al., 1985), visual adaptation in *Drosophila* (Willmund et al., 1986), and neuronal modulation in *Aplysia* (DeRiemer et al., 1984; Saitoh and Schwartz, 1985). CaM-kinase II is a member of a large class of enzymes designated Ca^{2+} /calmodulin-dependent multifunctional protein kinases because of their wide range of substrate specificity (McGuinness et al., 1983; Nairn et al., 1985; Shenolikar et al., 1986). In rat forebrain, CaM-kinase II is composed of 2 distinct subunits of 50 (alpha) and 58/60 (beta) kDa in a ratio of approximately 4:1 (Bennett et al., 1983; Kennedy et al., 1983b; Kuret and Schulman, 1985). The holoenzyme displays a native M_r of about 750 kDa and is present in both particulate and cytosolic fractions. Both subunits bind calmodulin in a Ca^{2+} -dependent manner, and subsequent activation of the purified kinase results in the autophosphorylation of both subunits (Bennett et al., 1983; McGuinness et al., 1983; Shields et al., 1984; Kuret and Schulman, 1985; Nairn et al., 1985; Shenolikar et al., 1986). Autophosphorylation *in vitro* converts it to a predominantly Ca^{2+} /CaM-independent kinase, and the return to

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the highly regulated form can be mediated by purified protein phosphatase (Lai et al., 1986; Miller and Kennedy, 1986; Schworer et al., 1986; Kelly et al., 1987a). Thus, CaM-kinase II has been proposed to constitute a molecular switch that transduces Ca^{2+} signals into protein phosphorylation events that modulate cell-cell communication at CNS synapses. Since biochemical analyses have shown that the postsynaptic density of asymmetric synaptic junctions is composed predominantly of the 50 kDa subunit of CaM-kinase II (Kennedy et al., 1983a; Goldenring et al., 1984; Kelly et al., 1984), we have investigated the expression, subcellular distribution, activity, and endogenous substrates of CaM-kinase II in differentiating pyramidal neurons in culture.

Electron-microscopic analysis of the developing rat dentate gyrus showed that the most active period for synapse formation is between 4 and 11 d after birth, during which time the density of synapses increases approximately 20-fold (Crain et al., 1973). From 11 to 25 d, there is a 5-fold increase, whereas after 25 d, there is little, if any, change in synaptic density. Analysis of isolated synaptic junctions from rat forebrain between postnatal days 10 and 25 showed increases in total protein paralleling the increase in synaptic density observed *in situ* during this same developmental period (Kelly and Cotman, 1981). Kelly and Vernon (1985) reported a shift in the distribution of CaM-kinase II, as measured by ^{125}I -CaM binding, from predominantly cytosolic fractions in 5 d rat forebrain (4-fold more 50 kDa subunit in cytosolic than in particulate) to synaptic fractions in adult rat forebrain (4-fold more 50 kDa subunit in particulate than cytosol). Moreover, greater than 60% of the particulate CaM-kinase II was recovered from fractions highly enriched in asymmetric synaptic junctions.

Immunocytochemical localization of CaM-kinase II in rat brain, using a monoclonal antibody that recognizes both kinase subunits, demonstrated strong reactivity in neuronal cell bodies and dendrites and weak reactivity in nerve terminals (Ouimet et al., 1984). Examination of the regional distribution of CaM-kinase II showed high activity in cortical regions, particularly in the hippocampal formation (Ouimet et al., 1984; Eröndu and Kennedy, 1985). Biochemical analysis showed that Ca^{2+} /CaM-dependent activity was relatively high in the hippocampal formation in both particulate and cytosolic fractions (Walaas et al., 1983a, b). It has also been shown that calmodulin activity, as measured by phosphodiesterase activation, is very high in the hippocampus (Caceres et al., 1983; Zhou et al., 1985). Therefore, cultured hippocampal neurons should represent an ideal cellular system in which to study CaM-kinase II. Previous studies have demonstrated that extensive process outgrowth and synapse formation occur in primary cultures of hippocampal pyramidal neurons (Bartlett and Banker, 1984a, b). We have used these cultures as a model of neuronal differentiation, synaptogenesis, and synaptic function. Other advantages of hippocampal neuronal cultures, described herein, include (1) the near-homogeneity of a defined neuronal population, predominantly of pyramidal cells (Banker and Cowan, 1977, 1979; Bartlett and Banker, 1984a, b), and (2) the neuronal and non-neuronal elements of the hippocampus are physically separated and thus can be analyzed separately at both biochemical and immunohistochemical levels. Using this system, we have identified CaM-kinase II activity and endogenous protein substrates in pyramidal neurons derived from fetal hippocampi and maintained for up to 35 d in culture.

This work was presented in part at the 16th annual meeting of the Society for Neuroscience (Allen et al., 1986).

Materials and Methods

Preparation of pyramidal neuron cultures. Pyramidal neuron cultures were prepared from embryonic day 18 (18E) rats (Holtzman), using a procedure developed by Banker and coworkers (Banker and Cowan, 1977, 1979; Banker, 1980; Bartlett and Banker, 1984a, b), with the following modifications: After trypsinization but prior to tissue trituration, deoxyribonuclease I (type IV; Sigma) was added (250 ng/ml) to digest flocculent material. This treatment increased the yield of neurons. The cells were then plated on 15 mm round glass coverslips (Bellco), prepared by attaching 3 small parafilm feet and coating with 0.5% poly-L-lysine in 0.1 M borate buffer (Banker and Cowan, 1977; G. Banker, personal communication). Cells were plated at $3\text{--}5 \times 10^4$ cells/coverslip in 10% horse serum (HS; Gibco) without antibiotics in Dulbecco's modified Eagle's medium (DMEM; Hazleton Research Products). Following cell attachment (2–4 hr), coverslips were transferred to cultures of confluent astroglia in defined media (N2 supplements, developed by Botstein and Sato, 1979, as modified by Bartlett and Banker, 1984a); biotin was added at a final concentration of 15 nM (supplements were prepared in DMEM). Astrocytes were prepared 10 d prior to neuronal cultures from 2–3 d postnatal cerebral cortices as described by Bartlett and Banker (1984a). To enhance the media-conditioning effects of astrocytes, coverslips containing pyramidal neurons were placed cell-side down, supported just above the glial cells (about 0.8 mm) by small parafilm feet. Cultures were treated on day 4 with 15 μM cytosine- β -D-arabino-furanoside (Sigma) to eliminate non-neuronal cell proliferation. Cultures were maintained up to 5 weeks by exchanging about 12% of the culture media with fresh, defined media on day 10 and once every following week. Astrocyte cultures used for biochemical and immunohistochemical analysis were maintained for 7–12 d in 10% HS in DMEM.

Preparation of monoclonal antibodies. Monoclonal antibodies were prepared by fusion of P3X63Ag8.653 myeloma cells with spleen cells from mice that had been immunized with CaM-kinase II purified from rat brain cytosol, as previously described (Schulman, 1984). Selection of anti-CaM-kinase II antibody-producing hybridomas was based on an enzyme-linked immunosorbent assay using purified, soluble CaM-kinase II bound to polyvinyl chloride microtiter plates. Specificity of the monoclonal antibodies for the 50 and 60 kDa subunits of CaM-kinase II was determined using immunoblots against purified, soluble rat brain kinase and against whole homogenate of rat brain (Fig. 1).

Immunofluorescence analyses. Pyramidal neurons or astrocytes were fixed for 10 min at room temperature in 2% paraformaldehyde and 0.2% glutaraldehyde in Hanks' balanced salt solution. Cells were washed 2 times in Dulbecco's phosphate-buffered saline (D-PBS) and permeabilized in 0.1% Triton X-100 in D-PBS for 10 min at room temperature. Nonspecific antibody binding was reduced by preincubation in 2% fetal calf serum (FCS) and 0.2% BSA in D-PBS (blocking buffer) for 20 min. Monoclonal antibodies (Mabs) in the form of ascites fluid (2–3 mg IgG/ml), or affinity-purified antibodies (1 ng IgG/ml), specific for both 50 and 60 kDa subunits of CaM-kinase II, were diluted 1:2000 and 1:25, respectively, in blocking buffer. Affinity-purified antibodies were prepared as previously described (Shenolikar et al., 1986). Mab to glial fibrillary acidic protein (GFAP; Boehringer Mannheim) was diluted 1:4 in blocking buffer. Cells were incubated with primary antibodies for 2 hr at room temperature. Coverslips were then washed in D-PBS and incubated in the appropriate secondary antibodies (rhodamine-conjugated goat anti-mouse IgG or rhodamine-conjugated goat anti-rabbit IgG; E-Y Laboratories). Coverslips were washed in D-PBS, parafilm feet removed, and mounted in 90% glycerol, D-PBS on glass slides, and sealed with nail polish. Slides were examined by phase-contrast and fluorescence microscopy, and photographed with a Nikon Diaphot microscope with epifluorescence attachment and Nikon FA camera.

Preparation of cellular fractions. Pyramidal neurons were harvested after 1–2, 9–10, and 29–31 d in culture. Cultures were washed twice with DMEM before carefully removing the parafilm feet and scraping cells from each coverslip with a razor blade into a small volume (800–1200 μl) of homogenization buffer (5 mM Tris, 0.5 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 5 $\mu\text{g/ml}$ leupeptin, and 5 $\mu\text{g/ml}$ pepstatin). Astrocytes were washed twice and harvested from 60 mm dishes with a rubber policeman. All harvesting and processing of subcellular fractions was done at 4°C. Cells were disrupted

in a Teflon-glass homogenizer with a motor-drive pestle using 20 passes at 1200 rpm. The homogenate was centrifuged at $20,000 \times g$ for 20 min. Supernatants (cytosol) were collected and pellets (total particulate fraction) were resuspended in 30 μ l of homogenization buffer. To standardize protein concentrations, an estimated 2 and 4 μ g of each fraction were examined by SDS-PAGE, followed by the silver-staining method of Oakley et al. (1980). Staining intensities were compared with standard amounts of synaptic junction (SJ) or synaptic plasma membrane (SPM) fractions prepared as previously described (Kelly and Vernon, 1985). The amount of particulate protein ranged from 1 μ g/coverlip in 1–2 d cultures to 2.5 μ g/coverlip in cultures maintained longer than 2 weeks. The amount of cytosolic protein per coverlip varied little among cultures, averaging 0.83 μ g/coverlip. Each coverslip contained about 30,000 neurons. Cytosolic fractions were concentrated about 5-fold to 100 μ g/ml, using Centricon microconcentrators (Amicon; 10,000 Da cutoff). The concentration of particulate fractions was adjusted to 100 μ g/ml with homogenization buffer. Protein concentration determined by the method of Lowry et al. (1951) showed less than 16% variance among particulate fractions. Cytosolic fractions showed more variation, due probably to residual proteins, especially ovalbumin, from defined media. Subcellular fractions were stored at -80°C (4–8 weeks) until a complete set of neuronal and astrocyte fractions was obtained. Six sets (i.e., neuronal cultures at 1–2, 9–10, and 29–30 d of culture and astrocytes from 7 to 12 d cultures) were prepared and analyzed as described below.

Ca²⁺- and calmodulin-stimulated phosphorylation. Phosphorylation was performed as described previously (Kelly et al., 1984). Subcellular fractions (2 μ g) were added to buffer in a volume of 25 μ l at the following final concentrations: 5 mM MgCl₂, 0.5 mM dithiothreitol, 10 mM HEPES, 1 mM CaCl₂, and 20 μ g/ml calmodulin (Calbiochem) or 2 mM EGTA replacing CaCl₂ and calmodulin (basal phosphorylation). When indicated, 2 μ g of purified synapsin I (a generous gift from Drs. Haycock and Greengard, Rockefeller University) was added per reaction. Ca²⁺/phospholipid-dependent phosphorylation was performed as described for Ca²⁺/CaM-stimulated phosphorylation except that the reaction buffer contained 50 μ g/ml phosphatidylserine and 5 μ g/ml diacylglycerol instead of calmodulin. Reactions were initiated by adding 5 μ Ci (γ -³²P)-ATP (Amersham) to a final concentration of 15 μ M (final volume, 30 μ l) and shifting to 35°C for 35 sec. Reactions were stopped by adding 10 μ l of 4 $\times$ sample buffer and heating at 70°C for 4 min. Half of each sample was analyzed by SDS-PAGE, as described by Laemmli (1970), using a 7–16% exponential polyacrylamide gradient. The other half was analyzed by the 2-dimensional method of O'Farrell (1975), as modified by Kelly et al. (1985). In the isoelectric focusing (IEF) dimension, 2.7% (vol/vol) of each of the following ampholyte mixtures was used: pH 3.5–10, pH 5–7 (LKB), and pH 3–10 (Pharmacia). Molecular-weight standards included on 1- and 2-dimensional gels were lysozyme, soybean trypsin inhibitor, carbonic anhydrase, ovalbumin, BSA, and phosphorase B. Radioactive phosphoproteins were detected by autoradiography at -80°C using intensifying screens (DuPont) and RPI x-ray film (AGFA-Gevaert). Phosphorylation of synapsin I was quantified by cutting the protein bands from dried gels and liquid-scintillation counting. After counting, bands were washed with ether, dried, and analyzed by peptide mapping.

Peptide mapping. Peptide mapping of phosphorylated proteins cut from dried gels was performed by the technique of Cleveland et al. (1977), using limited proteolysis with 2.0 μ g *S. aureus* V.8 protease (Miles Scientific) per band. Phosphopeptides were resolved on 15–20% gradient gels by SDS-PAGE and processed for autoradiography.

Immunoblot analyses. Protein samples (40 μ g/lane) were separated by SDS-PAGE and transferred to nitrocellulose according to the method of Towbin et al. (1979). Nitrocellulose sheets were stained with Ponceau red (0.4% in 8% trichloroacetic acid, 2% acetic acid) to visualize protein patterns, and each lane was cut into 3 vertical strips. Strips were preincubated 4–16 hr in 2% FCS, 0.2% BSA in Elisa buffer (10 mM Na-phosphate, pH 8.0, 0.5 M NaCl, and 0.05% Tween-20), and incubated overnight at 4°C with anti-50 kDa Mab, anti-60 kDa Mab (ascites fluids diluted 1:2000) or affinity-purified antibodies specific for both 50 and 60 kDa subunits of CaM-kinase II. Immunoreactive bands were visualized by 2 methods: (1) incubation for 2 hr in 0.25 μ Ci/ml ¹²⁵I-protein A (30 μ Ci/ μ g; Amersham), followed by exhaustive washing and autoradiography, or (2) incubation for 1 hr in alkaline phosphatase-conjugated secondary antibodies (0.13 μ g/ml), followed by visualization of antigen-antibody complexes with a mixture of nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Promega Biotec). The im-

Figure 1. Immunoblot analysis of Mab specificity. Aliquots of purified kinase (0.5 μ g, lanes *k*) or rat brain homogenate (100 μ g, lanes *h*) were subjected to SDS-PAGE and transferred to nitrocellulose. The nitrocellulose was then reacted with anti-50 or anti-60 kDa Mabs (ascites fluids, 1:2000 dilution), as described in Materials and Methods. *A*, Amido black stain. *B*, Anti-50 kDa Mab. *C*, Anti-60 kDa Mab.

munoblots shown in Figure 1 were prepared similarly, except that preincubation was in Tris-saline buffer (20 mM Tris-HCl, pH 7.3, 0.15 M NaCl) containing 5% BSA, 0.1% Triton X-100, and antibodies were diluted in Tris-saline containing 5% FCS, 0.5% BSA, and 0.05% Tween-20. Immunoreactivity was visualized by method (2), above. Proteins transferred to nitrocellulose were stained with 0.1% Amido black in 50% methanol, 10% acetic acid, followed by destaining in a solution containing 70% methanol, 5% acetic acid.

Results

Immunofluorescent localization of CaM-kinase II in differentiating pyramidal neurons

In order to localize CaM-kinase II within cells and investigate the distribution of the kinase during neuronal differentiation, monoclonal and affinity-purified antibodies specific for either or both of the CaM-kinase II subunits were used to stain neurons at increasing periods in culture (1–30 d). In general, each of the CaM-kinase II antibodies recognized all pyramidal neurons and showed very similar staining patterns. One exception was observed, however, in neurons cultured for 4 d. At this point, the anti-50 kDa Mab recognized only a small percentage of the total pyramidal neurons that were recognized by affinity-purified anti-60/50 kDa antibodies or anti-60 kDa Mab. Immunoreactivity detected during the first 4 d in culture was very low and diffuse (Fig. 2, *a*, *b*). Appearance of CaM-kinase II immunoreactivity in discrete spots was observed after 1 week in culture (Fig. 2, *c–e*, arrows). The appearance of these apparent antigen clusters was gradual, in that fluorescence was first observed at growth cones (arrowheads, Fig. 3*a*) and in 2–6 μ m patches along

neuritic processes (arrows, Figs. 2*c*; 3, *c*, *e*) before coalescing into smaller (less than 1 μ m), more discrete spots of immunoreactivity between 10 and 15 d (Figs. 2*e*, 3*i*). Immunoreactive spots were observed on the bottom side of cell bodies of approximately 25% of pyramidal cells cultured for 10 or more days (Fig. 2*c*). Figure 2*d* shows CaM-kinase II immunoreactivity in a low-density culture. The processes of this neuron, which have not contacted processes of other neurons, expressed CaM-kinase II in their growth cones. Using anti-GFAP antibodies, it was shown that less than 10% of cells in pyramidal neuron cultures were astrocytes. Although a very small contribution of non-neuronal cells was observed in neuronal cultures, virtually homogeneous astrocyte cultures analyzed by immunofluorescence displayed no detectable reactivity with anti-CaM-kinase II antibodies (results not shown).

In order to examine very-low-density cultures of pyramidal neurons (25 cells/cm²), it was necessary to maintain neurons in mixed cultures, i.e., neurons and astrocytes grown on the same coverslip. Secondary astrocyte cultures on which the pyramidal neurons were plated contained no CaM-kinase II immunoreactivity. These extremely low-density mixed cultures demonstrated that initial expression of CaM-kinase II at 2–4 d did not require interneuronal contacts with other pyramidal neurons (Fig. 3*a*). Neurons grown at high densities (15,000 cells/cm²) in mixed cultures displayed immunoreactive spots along neurites at 10 d (Fig. 3, *c*, *e*); however, the number of spots per neurite was less than that described above for 10 d pure pyramidal cultures (Fig. 2*e*). In addition, small, intensely fluorescent spots observed on the bottom surface of cell bodies of neurons cultured directly on substrate were not observed in pyramidal neurons cultured directly on a confluent bed of astrocytes for 10–20 d (Fig. 3, *c*, *e*, *i*).

Ca²⁺/calmodulin-stimulated phosphorylation of endogenous proteins during neuronal differentiation. Ca²⁺/CaM-dependent protein kinase activities in pyramidal neuron particulate and cytosolic fractions were analyzed at 2, 10, and 29 d after plating (Fig. 4). Ca²⁺/CaM-stimulated ³²P-incorporation into endogenous proteins was 3- to 5-fold greater in particulate (Fig. 4, lanes 1–3) than in cytosolic (lanes 5–7) fractions at all culture times. Levels of endogenous protein phosphorylation in particulate and cytosolic fractions increased approximately 5- and 3-fold, respectively, from day 2 to day 10, and decreased slightly from day 10 to day 29. Endogenous proteins that displayed Ca²⁺/CaM-dependent phosphorylation in subcellular fractions from 2 d neuronal cultures were 56 kDa in both particulate and cytosolic fractions, 48 kDa in only particulate fractions, and 100 and 190 kDa polypeptides in only cytosolic fractions (Fig. 4, lanes 1 and 5). Major phosphoproteins in 10 d neuronal cultures included 190, 120, 58/60, and 56 kDa proteins in both particulate and cytosolic fractions, and 175, 138, 90, 80–87, and 48–50 kDa phosphoproteins enriched in particulate fractions (Fig. 4, lanes 2 and 6). The 50 kDa subunit of CaM-kinase II in neuronal particulate fractions comigrated on 1-dimensional gels with a 48 kDa phosphoprotein; however, this phosphoprotein was clearly distinguished by 2-dimensional gel electrophoresis and peptide mapping (see below). After 29 d in culture, neuronal proteins phosphorylated in a Ca²⁺/CaM-stimulated manner were similar to those at 10 d, with the exception of a more prominent 50 kDa subunit of CaM-kinase II in cytosolic fractions (Fig. 4, lane 7).

Ca²⁺/CaM-dependent phosphorylation of exogenous synapsin I. During neuronal differentiation, CaM-kinase II activity per

microgram of cellular protein was estimated by its ability to phosphorylate saturating amounts of exogenous synapsin I in a Ca²⁺/CaM-dependent manner. Neuronal particulate or cytosolic fractions displayed increases in synapsin I phosphorylation of about 2- and 4.5-fold, respectively, between 2 and 29 d in culture (Fig. 5*A*). Figure 5 shows a representative experiment using the same fractions and protein concentrations depicted in Figure 4 (3 independent experiments were performed). Phosphorylation of exogenous synapsin I was subsequently analyzed by phosphopeptide mapping. The phosphorylation of synapsin I by CaM-kinase II occurs preferentially on the 30 kDa peptide generated by V.8 proteolysis (Huttner et al., 1981). Greater than 90% of the Ca²⁺/CaM-stimulated phosphorylation of synapsin I by pyramidal neuron fractions was associated with the 30 kDa phosphopeptide; phosphorylation of this peptide was equally predominant at all culture ages. When relative kinase activities were calculated per coverslip (i.e., per 30,000 neurons), the activity in particulate fractions increased more dramatically than that in cytosolic fractions during culture, such that by 29 d, activity in particulate was approximately 4.6-fold greater than cytosolic fractions (Fig. 5*B*).

Endogenous protein substrates of CaM-kinase II in homogenates of neuronal cultures. Endogenous CaM-kinase II substrates were further analyzed by peptide mapping (Fig. 6). In cytosolic fractions, the 50 kDa phosphoprotein (Fig. 6, lanes 1 and 2) was resolved as a distinct band on 1-dimensional gels and produced a peptide map that was indistinguishable from the 50 kDa subunit of CaM-kinase II (lane 3). In contrast, peptide mapping of the same *M_r* region from 9 and 31 d particulate fractions (lanes 4 and 5) resulted in a more complex pattern of peptides that were generated from a distinct 48 kDa phosphoprotein (*M_r* 10, 13, and 14 kDa), which were superimposed on the 50 kDa kinase subunit phosphopeptides (*M_r* 12, 18, 23, and 29 kDa). Comparisons of these 50 kDa-specific phosphopeptides between 9- and 31-d-old particulate fractions demonstrated that this subunit of CaM-kinase II was more highly phosphorylated in the older cultures. Phosphopeptide maps of the 60 kDa phosphoprotein from neuronal cultures (Fig. 6, lanes 8 and 9) were very similar to those of the autophosphorylated 60 kDa subunit of purified CaM-kinase II (lane 7). Ca²⁺/phosphatidylserine diacylglycerol stimulated the phosphorylation of an endogenous protein in 29 d neuronal particulate fractions, which demonstrated the same phosphopeptides as the 48 kDa protein (lane 6); these conditions resulted in no detectable labeling of the 50 kDa kinase subunit. The peptide map of a phosphoprotein doublet in neuronal fractions of apparent molecular weight 83 kDa (*M_r* 30 kDa, lanes 10 and 11) was very similar to that of purified synapsin I phosphorylated by purified CaM-kinase II (lane 12). An 87 kDa phosphoprotein that comigrated with synapsin I in 1-dimensional gels was also phosphorylated in the presence of EGTA. Phosphorylation of particulate fractions from neurons cultured for 29 d by endogenous Ca²⁺/phosphatidylserine/diacylglycerol-stimulated phosphorylation substantially increased the labeling of phosphopeptides associated with the 87 kDa protein (*M_r* 9 and 13 kDa, lane 13).

Analysis of endogenous phosphoproteins from 29 d neuronal particulate fractions on 2-dimensional gels clearly distinguished phosphoproteins with similar molecular weights but different isoelectric points (pI; Fig. 7). Synapsin I and the 87 kDa phosphoprotein, which comigrate in 1-dimensional PAGE, were easily separated by 2-dimensional gel electrophoresis, since synapsin I has a very basic pI, while the 87 kDa phosphoprotein

Figure 2. CaM-kinase II localization in cultured pyramidal neurons. Pyramidal neurons from 18E rat hippocampi were examined at 2, 10, 23, and 29 d in culture using an anti-50 kDa Mab or affinity-purified antibodies recognizing both 50 and 60 kDa subunits of CaM-kinase II. *a, b*, Anti-50 kDa Mab binding and phase-contrast micrographs of pyramidal neurons at 2 d in culture. *c*, Anti-50 kDa Mab binding to pyramidal neurons cultured for 10 d. *d*, Affinity-purified antibody reactivity to an isolated neuron in a very-low-density culture of 23 d pyramidal neurons. *e, f*, Affinity-purified antibody reactivity and phase-contrast micrograph of a pyramidal neuron cultured for 29 d. In *c–f*, *arrowheads* denote growth cones and *arrows* denote antigen clusters in neurites. *g, h*, Immunofluorescence and phase-contrast micrograph, respectively, of 29 d neurons in which no primary antibody was used. *Open arrows* in *a, b, g*, and *h* denote cell bodies. Calibration bars: 40 μ m (*a, d*), 20 μ m (*c, e, g*).

Figure 4. Ca^{2+} /CaM-stimulated phosphorylation of endogenous proteins during neuronal differentiation. Two micrograms of endogenous particulate (paired lanes 1–4) or cytosolic (paired lanes 5–8) proteins from cultured pyramidal neurons at increasing numbers of days in culture (lanes 1–3, 5–7) or astrocytes (lanes 4 and 8) were phosphorylated by endogenous Ca^{2+} /CaM-dependent protein kinase (+) or under basal conditions (2 mM EGTA; –), as described in Materials and Methods. Lane 9 shows synaptic plasma membrane from adult rat forebrain phosphorylated in the presence of Ca^{2+} plus CaM. Autoradiograph exposure was 12 hr for lanes 1–4 and 9, and 24 hr for lanes 5–8.

has a very acidic pI (Fig. 7a). The 50 kDa subunit of CaM-kinase II has a broad isoelectric point and was clearly separated from the 48 kDa phosphoprotein of acidic pI. The phosphorylation of the 50 and 60 kDa subunits of CaM-kinase II, 48 kDa and synapsin I, was stimulated in the presence of Ca^{2+} /CaM, while that of the 87 kDa phosphoprotein was not (Fig. 7, compare *a* and *b*). The 48 and 87 kDa proteins were phosphorylated in a Ca^{2+} /phospholipid-stimulated manner (results not shown). A 56 kDa phosphoprotein with an acidic pI was also phosphorylated in a Ca^{2+} /CaM-stimulated manner (Fig. 7a).

Immunoblot analysis of CaM-kinase II from neuronal cultures. Immunoreactive 50 and 60 kDa subunits of CaM-kinase II in particulate and cytosolic fractions from pyramidal cultures were investigated on immunoblots of electrophoretically separated proteins (Fig. 8). In the youngest fractions examined (1 d cultures), the 60 kDa subunit of CaM-kinase II, but not the 50 kDa subunit, was detected with affinity-purified antibodies (Fig. 8C, lanes 1 and 4) and anti-60 kDa Mab (Fig. 8B, lane 5). Although 60 kDa immunoreactivity in neuronal cytosolic fractions (Fig. 8B, lanes 5–7; Fig. 8C, lanes 4–6) was detected earlier than in

particulate fractions (Fig. 8B, lanes 1–3; Fig. 8C, lanes 1–3), levels of 60 kDa in the former never reached those observed in particulate fractions. The 50 kDa subunit of CaM-kinase II was readily detected in particulate and cytosolic fractions of neurons by 1 week in culture (Fig. 8A, lanes 2 and 6; Fig. 8C, lanes 2 and 5). At 29 d, the amount of 50 kDa immunoreactivity had increased in particulate and cytosolic fractions with the largest increase observed in the latter.

Ca^{2+} /CaM-stimulated kinase activity in astrocytes. Astrocytes displayed low Ca^{2+} /CaM-stimulated kinase activity, as measured by the phosphorylation of endogenous substrates (Fig. 4, lanes 4 and 8). These substrates included a 56 kDa phosphoprotein in particulate fractions and a 100 kDa phosphoprotein detected only in cytosolic fractions. A similar 100 kDa phosphoprotein was also detected at low levels in cytosolic fractions of 2 d pyramidal cultures. Ca^{2+} /CaM-stimulated kinase activity in astrocyte subcellular fractions, as measured by the phosphorylation of exogenous synapsin I (Fig. 5a), was 58 and 102% of the activity per μg protein compared to 2 d pyramidal neuron particulate and cytosolic fractions, respectively. Similar to CaM-

Figure 3. Immunofluorescent localization of CaM-kinase II in pyramidal neurons cultured directly on astrocytes. *a, b*, Anti-60 kDa Mab reactivity and phase-contrast micrograph of a pyramidal neuron cultured for 4 d. *c, d*, Anti-50 kDa Mab reactivity and phase-contrast micrograph of a pyramidal neuron at 10 d in culture. *e, f*, Two adjacent cell bodies of 10 d neurons reacted with anti-60 kDa Mab and viewed by fluorescent and phase-contrast microscopy, respectively. *g, h*, Fluorescent and phase-contrast micrographs of 10 d neurons in which no primary antibody was used. *i, j*, Anti-60 kDa Mab reactivity and phase-contrast micrograph, respectively, of pyramidal neurons cultured for 17 d. Arrowheads in *a–d* denote growth cones. Arrows in *c–f, i*, and *j* denote antigen clusters along neurites. Open arrows in *a, b, e–h* denote cell bodies. Calibration bars, 20 μm .

Figure 5. Ca^{2+} /CaM-dependent phosphorylation of exogenous synapsin I. *A*, Excess synapsin I (2 μg) was added to 1 μg of each cell fraction (■, particulate; ●, cytosolic) and phosphorylated in a Ca^{2+} /CaM-dependent manner. Phosphate incorporation into synapsin I under basal conditions was subtracted for each sample. Phosphate incorporation by astrocyte cultures is shown at right (AST). *B*, The same data recalculated to represent synapsin I phosphorylation per coverslip (i.e., 30,000 cells).

kinase II activity in neuronal fractions, Ca^{2+} /CaM-stimulated phosphorylation of exogenous synapsin I by astrocyte fractions was associated with the 30 kDa phosphopeptide generated by V.8 proteolysis (results not shown). The Ca^{2+} /CaM-stimulated phosphorylation of a minor 50 kDa polypeptide in astrocyte fractions was detected by 2-dimensional gel electrophoresis (Fig. 7, *c*, *d*); however, the concentration or phosphate incorporation associated with this protein was insufficient to determine whether it was related to the 50 kDa kinase subunit. An immunoreactive band analogous to the 50 kDa subunit of CaM-kinase II was not detected in astrocyte fractions (Fig. 8*A*, lanes 4 and 8). In contrast, a tentative 58 kDa subunit of CaM-kinase II was detected by immunoblot analysis of astrocyte cytosolic proteins using an anti-60 kDa Mab (Fig. 8*B*, lane 8); however, no Ca^{2+} /CaM-stimulated autophosphorylation of the 58/60 kDa subunit of CaM-kinase II was detected. While the 60 kDa subunit was often resolved as a 58/60 kDa doublet in pyramidal neuron cultures, astrocytes appeared to express only a 58 kDa polypeptide. At all astrocyte culture ages, the 50 and 60 kDa immunoreactivity in astrocyte fractions was significantly lower than that observed in pyramidal neurons (Fig. 8). This is also evident in immunofluorescent analyses using each anti-CaM-kinase II antibody in mixed cultures, which showed no astrocyte immunoreactivity (Fig. 3, *a*, *e*, *i*).

We do not believe that the presence of Ca^{2+} /CaM-stimulated phosphorylation and anti-CaM-kinase II-immunoreactive proteins from astrocyte cultures is due to neuronal contamination. Freshly prepared astrocyte cultures (subconfluent) contained less than 0.5% neurons; neurons were easily detected by anti-50 or

anti-60 kDa Mabs. The astrocytes used for biochemical analyses were confluent primary cultures that contained less than 0.005% neuronal contamination. When these astrocytes were replated, no immunoreactive cells could be detected by immunofluorescence analyses using any of the 3 classes of anti-CaM-kinase II antibodies. In addition, greater than 95% of the cells in confluent astrocyte cultures showed anti-GFAP immunoreactivity.

Discussion

Studies on rat brain have shown that CaM-kinase II is developmentally regulated; developmental changes in its subcellular distribution (Kelly and Vernon, 1985), endogenous substrates (Katz et al., 1985; Kelly et al., 1987b), and holoenzyme composition (Sahyoun et al., 1985; Kelly et al., 1987b) have been reported. These studies suggest that CaM-kinase II plays a role in neuronal maturation as well as in adult function, especially synaptic transmission. In order to refine such studies on the role of CaM-kinase II in neuronal differentiation and synapse formation at cellular and molecular levels, a culture system of homogeneous pyramidal neurons was used. Pyramidal cultures are ideal because (1) they represent a nearly homogeneous population of one neuronal type with few non-neuronal cells, and (2) differentiation within cultures is relatively synchronous.

Changes in CaM-kinase II expression in pyramidal neurons with increasing days in culture were similar to those reported in developing rat brain. CaM-kinase II activity per microgram of neuronal protein, measured as the Ca^{2+} /CaM-dependent phosphorylation of exogenous synapsin I, increased approximately 4.5- and 2-fold from 2 to 29 d in culture in cytosolic and particulate fractions, respectively. In addition, immunoblot analyses using anti-CaM-kinase II antibodies showed increasing immunoreactivity with days in culture. During neuronal differentiation in culture, particulate fractions accounted for an increasing percentage of the total cellular protein, i.e., 38% at 2 d, 54% at 10 d, and 85% at 29 d in culture. Taking this into account, it can be shown that the amount of CaM-kinase II at 2 d in culture is equally distributed between particulate and cytosolic fractions. By contrast, by 29 d in culture, the amount of particulate CaM-kinase II is approximately 5-fold higher than that of the cytosolic.

The Ca^{2+} /CaM-stimulated phosphorylation of endogenous proteins in cultured pyramidal neurons increased between 1 and 10 d of culture. Levels of endogenous phosphorylation per microgram of protein at 10 and 29 d were approximately 4-fold higher in particulate than in cytosolic fractions (Fig. 4), whereas CaM-kinase II activity per microgram of neuronal protein, as measured by synapsin I phosphorylation, was approximately equal in particulate and cytosolic fractions at these ages (Fig. 5). This suggests that the greater amount of phosphorylation of endogenous particulate proteins relative to cytosolic proteins is due to the ability of proteins in the former to act as substrates for Ca^{2+} /CaM-stimulated protein phosphorylation. Alternatively, there may be a difference in substrate specificity between particulate and cytosolic CaM-kinase II.

The earliest detection of CaM-kinase II by immunoblot analysis was at 1 d after plating. At this time, only the 60 kDa subunit of CaM-kinase II could be detected in cytosolic, but not particulate, fractions. The 50 kDa subunit was detected by immunoblot at 4 d in culture in both neuronal subcellular fractions. In cytosolic fractions, the 60 kDa subunit displayed much smaller developmental increases during pyramidal neuron differentiation than did the 50 kDa subunit, such that by 29 d in culture,

Figure 6. Peptide mapping of neuronal phosphoproteins. Lanes 1, 2, 4, 5, and 8–11 show *S. aureus* V.8 protease digests of neuronal proteins phosphorylated by endogenous kinase(s) in the presence of Ca^{2+} plus CaM. Lanes 1 and 2 show the 50 kDa protein from cytosolic fractions of pyramidal neurons cultured for 10 and 29 d, respectively. Lanes 4 and 5 show the 50 kDa protein from particulate fractions of pyramidal neurons cultured for 10 and 29 d, respectively. Lanes 8 and 9 show the 60 kDa protein from particulate fractions of pyramidal neurons cultured for 10 and 29 d, respectively. Finally, lanes 10 and 11 show the 83–87 kDa phosphoproteins from particulate fractions of pyramidal neurons cultured for 10 and 29 d, respectively. Lanes 3 and 7 show the peptide maps of the autophosphorylated 50 and 60 kDa subunits of purified CaM-kinase II, respectively. Lane 12 shows the peptide map of purified synapsin I phosphorylated by purified CaM-kinase II. Lanes 6 and 13 show the peptide maps of the 48 and 87 kDa endogenous proteins, respectively, from particulate fractions of 29 d neurons phosphorylated in the presence of phosphatidylserine/diacylglycerol/ Ca^{2+} .

50 kDa immunoreactivity was significantly higher than 60 kDa. In particulate fractions of pyramidal neurons, similar developmental increases in the ratio of 50:60 kDa subunits during neuronal maturation were observed using affinity-purified antibodies recognizing both subunits of CaM-kinase II. Similarly, Kelly et al. (1987b) reported a change in the relative amounts of 50 and 60 kDa subunits of CaM-kinase II in synaptic junctions and purified cytosolic CaM-kinase II prepared from rat forebrains at increasing ages. Immunoblot analyses showed a 60:50 kDa ratio of 6:1 in synaptic junctions from 5 d forebrain, approximately 1:1 at 12–18 d, and 1:7 at 24 d after birth. In addition, Sahyoun et al. (1985) and Kelly et al. (1987b) reported a decrease in the 60:50 kDa subunit ratio of cytosolic CaM-kinase II during forebrain development. Such analyses of cytosolic fractions from brain tissues are complicated by an increasing cytosolic contribution of non-neuronal cells, as well as by an increasing diversity of neuronal cells accompanying brain maturation. We have shown a similar shift in relative amounts of 60 and 50 kDa subunits of CaM-kinase II in subcellular fractions of cultured pyramidal neurons, with 60 kDa appearing first and the 50 kDa kinase subunit predominating after 10 d in culture.

Using mixed neuronal cultures derived from 16 d rat embryonic cerebral cortex and midbrain, Sahyoun et al. (1985) observed Ca^{2+} /CaM-stimulated phosphorylation of endogenous

proteins in cytoskeletal and cytosolic preparations. In contrast to the results presented herein, Sahyoun et al. (1985) did not detect autophosphorylated 50 and 60 kDa subunits in their mixed neuronal cultures. Using ^{125}I -CaM overlays, they showed increases in a 60 kDa CaM-binding protein between 1 and 14 d in culture; however, developmental increases in 50 kDa proteins were not observed. Moreover, elevated levels of the 50 kDa subunit of CaM-kinase II were not detected even after 8 weeks in culture and under a variety of culture conditions. In sharp contrast to these observations, the results contained herein demonstrated large increases in both subunits of CaM-kinase II in differentiating pyramidal neurons and are consistent with previous results in developing rat forebrain (Katz et al., 1985; Kelly and Vernon, 1985; Sahyoun et al., 1985; Kelly et al., 1987b).

Endogenous Ca^{2+} /CaM-stimulated phosphoproteins detected in pyramidal neuron cultures were similar to those previously described. An 83 kDa doublet was very similar, if not identical, to synapsin I by 1-dimensional PAGE, 2-dimensional gel electrophoresis, and peptide mapping. The 50 and 60 kDa subunits of CaM-kinase II were identified in cultured pyramidal neurons by immunoreactivity and biochemical analyses and were shown to autophosphorylate in the presence of Ca^{2+} and CaM. A protein that was not phosphorylated in a Ca^{2+} /CaM-dependent manner was very similar to a previously described protein ki-

Figure 7. Two-dimensional gel analysis of Ca^{2+} /CaM-dependent phosphorylation. *a*, Endogenous proteins from particulate fractions of pyramidal neurons at 29 d in culture. The 50 kDa and 60 kDa subunits of CaM-kinase II are indicated. *b*, Phosphorylation of the same fraction as in *a*, except under basal conditions (i.e., 2 mM EGTA). *c*, *d*, Two-dimensional gel analysis of astrocyte particulate fractions in the presence of Ca^{2+} and CaM and under basal conditions, respectively.

nase C substrate protein in rat brain synaptosomes, namely an “87 kDa” phosphoprotein (Wu et al., 1982). A 48 kDa protein, which was enriched in neuronal particulate fractions, was phosphorylated by Ca^{2+} /CaM- and phospholipid/ Ca^{2+} -stimulated kinases. This protein was similar to a number of proteins shown to be substrates for protein kinase C in different neuronal systems, such as B-50, protein F1, pp46, or GAP-43 (Aloyo et al., 1983; DeGraan et al., 1985; Nelson and Routtenberg, 1985; Jacobson et al., 1986; Meiri et al., 1986). A 56 kDa phosphoprotein present in particulate and cytosolic fractions of pyramidal neurons at all culture days displayed a 2-dimensional gel migration pattern similar to that of tubulin, which has been shown to be a substrate of Ca^{2+} /CaM-dependent protein kinase (Goldenring et al., 1983). In addition, the latter was one of only 2 major proteins in astrocytes whose phosphorylation was stimulated by Ca^{2+} /CaM.

Astrocyte particulate and cytosolic fractions displayed much lower levels of Ca^{2+} /CaM-stimulated protein phosphorylation than did neuronal fractions. The amount of phosphate incorporated into exogenous synapsin I by astrocyte Ca^{2+} /CaM-dependent protein kinase activity was similar to the activity of 2 d pyramidal neuron fractions. Major endogenous substrates of

Ca^{2+} /CaM-stimulated phosphorylation included proteins of 56 and 100 kDa. Little, if any, CaM-kinase II was detected in astrocytes by either immunofluorescence or immunoblot analyses. A 58 kDa astrocyte protein was detected by anti-60 kDa Mab and affinity-purified antibodies; however, its Ca^{2+} /CaM-dependent autophosphorylation was not observed. Immunoblot analysis using anti-50 kDa Mab showed no immunoreactivity in astrocyte particulate or cytosolic fractions. Although Ca^{2+} /CaM-dependent phosphorylation of a 50 kDa protein, with a 2-dimensional gel migration similar to that of the 50 kDa subunit of CaM-kinase II, was detected in astrocyte particulate fractions, its phosphorylation, however, was too low to determine a precise correspondence to the 50 kDa subunit of CaM-kinase II. These results suggest that Ca^{2+} /CaM-dependent protein phosphorylation of astrocyte polypeptides was mediated by a kinase that is distinct from neuronal CaM-kinase II.

Developmental increases in CaM-kinase II expression in pyramidal neurons, as determined by biochemical methods, correlated with (1) the appearance of diffuse CaM-kinase II immunoreactivity only in the cell body and ends of processes at 4 d in culture, (2) immunoreactivity in growth cones and relatively large spots along processes at 7 d, and (3) spots of CaM-

Figure 8. Immunoblot analysis of CaM-kinase II. Nitrocellulose transfers of subcellular fractions separated by SDS-PAGE were reacted with anti-50 kDa Mab (A) or anti-60 kDa Mab (B), followed by alkaline phosphatase-conjugated secondary antibodies or affinity-purified antibodies recognizing both 50 and 60 kDa subunits of CaM-kinase II, followed by 125 I-protein A (C). Lane assignments for A and B are as follows: 1, 2, and 3 contain particulate fractions of pyramidal neurons at 2, 10, and 29 d in culture, respectively; 4 contains cultured astrocyte particulate fraction; 5–7 contain cytosolic fractions of pyramidal neurons at 2, 10, and 29 d in culture, respectively; 8 shows cultured astrocyte cytosolic fraction; and 9 shows adult rat synaptic plasma membrane. C, Lanes 1–3 show particulate fractions of neurons at 2, 10, and 29 d in culture, respectively. Lanes 4–6 show cytosolic fractions of neurons at 2, 10, and 29 d in culture, respectively. Lane 7 shows synaptic plasma membranes from adult rat forebrain.

kinase II immunoreactivity that became smaller, more discrete, and concentrated in cell processes and on the bottom surface of the neuronal cell body at 10 d in culture. We believe the spots of CaM-kinase II along neurites are associated with asymmetric synaptic junctions for the following reasons: First, increases in CaM-kinase II and the appearance of immunoreactive spots on cell bodies and processes with increasing days in culture (culture days 7–12 are equivalent to postnatal days 4–9 *in vivo*) take place at a similar developmental period to that at which synapse formation is most active in the rat hippocampus (Crain et al., 1973). Second, an EM comparison of synapse development between cultured superior cervical ganglia and spinal cord explants demonstrated that the first definitive sign of synapse formation was the appearance of a postsynaptic density (Rees et al., 1976). Third, asymmetric synapses with prominent dendritic postsynaptic densities are the major synaptic type of cultured pyramidal neuron (Bartlett and Banker, 1984b). Finally, the postsynaptic density of synaptic junctions is composed predominantly of the 50 kDa subunit of CaM-kinase II (Kennedy et al., 1983a; Goldenring et al., 1984; Kelly et al., 1984).

As an extension of this reasoning, localization of CaM-kinase II immunoreactivity on the bottom of neuronal cell bodies in 7–10 d cultures may represent synapses formed with axons passing under the cell body. Alternatively, extensive postsynaptic specializations may form through the interaction of the cell surface plasma membrane with the poly-L-lysine-coated glass substrate. Indeed, Peng and Cheng (1982) have shown that poly-L-lysine coated latex beads can induce postsynaptic specializations in cultured muscle cells in the absence of neurons. When

pyramidal neurons were plated directly onto astrocytes rather than poly-L-lysine-coated glass, we observed no immunoreactive spots on the bottom surface of cell bodies and fewer spots on neurites. While these observations suggest that poly-L-lysine-coated glass induced the accumulation of CaM-kinase II at atypical postsynaptic specializations, a second factor, namely, direct contact with astrocytes, may have an opposing effect of decreasing postsynaptic specializations on the bottom surface of the cell body and synapses along processes. Others have shown that reintroduction of astrocytes to cerebellar explants previously depleted of mature astrocytes by cytosine arabinoside treatment resulted in a significant decrease in the number of synapses on Purkinje cell bodies (Meshul et al., 1987). We are currently using EM immunohistochemistry to precisely define the subcellular localization of CaM-kinase II in cultured pyramidal neurons.

Katz et al. (1985) have examined Ca^{2+} /CaM-stimulated protein phosphorylation in growth cone particles from 17 d embryonic rat brain. The 80, 52, 46, and 43 kDa phosphoprotein substrates described by Katz et al. (1985) were also observed in cultured pyramidal neurons. The autophosphorylated subunits of CaM-kinase II were not observed in growth cone particles. In contrast, the results contained herein demonstrate that both CaM-kinase II subunits were clearly observed by immunofluorescence microscopy in growth cones of pyramidal neurons after 4 d in culture. Although we observed Ca^{2+} /CaM-dependent activity in pyramidal neurons after 1 d in culture, immunoreactivity and autophosphorylation of the 50 kDa subunit of CaM-kinase II were not observed until after 4 d in culture. Therefore, the differences between these two studies may be due

to the earlier age at which the growth cones were examined or to the heterogeneity of the growth cones studied by Katz et al. (1985).

In the absence of interneuronal contact, we might expect a neuron to remain synapse-free. Therefore, plating neurons at very low density on poly-L-lysine-coated glass could provide a useful synapse-free neuronal culture in which to study the dependence of CaM-kinase II expression on interneuronal contact and synapse formation. In very-low-density cultures and in the apparent absence of cell-cell contact, we observed CaM-kinase II in growth cones extending from cell bodies (Fig. 2*d*). However, when neurites failed to make contact with neighboring neurons, these processes returned to form a net of neurites around the cell body. These entangled processes of a single neuron display intense spots of CaM-kinase II immunoreactivity, and thus a neuron may form synapses on itself in very-low-density cultures.

We have shown that CaM-kinase II is abundant in cultured pyramidal neurons and that its expression is developmentally regulated. Many of the developmental changes in CaM-kinase II expression, distribution, and activity reported in rat brain subcellular fractions also occur in culture. This culture system offers the advantage of analyzing discrete cellular and physiological events because of the relative homogeneity of cultured hippocampal neurons as compared to developing brain tissue. We are currently investigating other protein kinases present in pyramidal neurons. This neuronal culture is also attractive as an *in situ* system for studying the role of phosphorylation during neuronal differentiation and synaptic transmission.

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