REGIONAL DISTRIBUTION AND SUBCELLULAR ASSOCIATIONS OF TYPE II CALCIUM AND CALMODULIN-DEPENDENT PROTEIN KINASE IN RAT BRAIN

Thesis by

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This thesis is dedicated to my parents

Mr. & Mrs. J. E. Erondu

and to

Ugom on our first anniversary.

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Abstract

Four monoclonal antibodies generated against the Type II CaM kinase have been characterized. Two of these antibodies were used to confirm that both alpha and beta subunits were part of the holoenzyme complex. I also developed liquid phase and solid phase radioimmunoassays for the kinase.

With the solid phase radioimmunoassay, the distribution of the kinase in rat brain was examined. This study revealed that the concentration of the kinase varies markedly in different brain regions. It is most highly concentrated in the telencephalon where it comprises approximately 2% of total hippocampal protein, 1.3% of cortical protein and 0.7% of striatal protein. It is less concentrated in lower brain regions ranging from 0.3% of hypothalamic protein to 0.1% of protein in the pons/medulla. The unusually high concentration of the kinase in telencephalic regions may confer upon their neurons specialized responses to calcium that are different from those of neurons in lower brain regions.

The association of the kinase with elements of the cytoskeleton was also investigated. The results of this study showed that autophosphorylation causes an increase in the association of the enzyme with taxol-polymerized microtubules and F-actin. This increase in association was reversed by dephosphorylating phosphokinase with protein phosphatase. These results suggest that autophosphorylation could constitute a mechanism for the regulation of the subcellular associations of the Type II CaM kinase by neuronal activity.

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Chapter 1

INTRODUCTION

The second messenger role of calcium in the nervous system as well as in other tissues has been well documented (Rasmussen, 1981; Campbell, 1983). Cellular processes believed to be regulated by changes in intracellular Ca^{2+} levels include exocytosis, endocytosis, neurotransmitter synthesis, membrane excitability, long-term potentiation of synaptic transmission, axoplasmic transport, muscle contraction, and glycogen metabolism (Ochs and Iqbal, 1982; Eccles, 1983; Reichardt and Kelly, 1983; Rasmussen, 1986). To understand, at the molecular level, how Ca^{2+} is able to regulate such a diverse number of cellular processes, it will be necessary not only to identify the Ca^{2+} target proteins but in addition to determine their tissue, cellular, and subcellular distribution. I have studied aspects of the cellular and subcellular distribution of a calcium regulated protein kinase called Type II Ca^{2+}/calmodulin-dependent protein kinase. I have characterized monoclonal antibodies that bind to the kinase and have used these antibodies in studies of its regional distribution in brain; and its association with cytoskeletal elements, since the regulation of a number of the above-mentioned biological processes by calcium could be effected by the modulation of cytoskeletal dynamics. In this chapter (i.e., Introduction to the thesis), I will first review the tissue, cellular, and subcellular distribution of calmodulin-dependent protein kinases. In Section II, I will review the components of the cytoskeleton with particular emphasis on brain cytoskeleton.

Section I

There are two major biochemical pathways through which Ca^{2+} regulates cellular processes, namely, the C-kinase branch regulated synergistically by lipid and calcium (Takai et al., 1979) and the calmodulin branch (Cheung, 1980). Studies of several cellular responses such as neurosecretion, skeletal muscle contraction, angiotensin II-mediated aldosterone secretion, glucose-induced insulin

secretion, and the acetylcholine-induced contraction in tracheal smooth muscle have led to the suggestion that the "calmodulin branch is responsible for either brief responses or the initial phase of sustained responses, while the C-kinase branch is responsible for the sustained phase of cellular responses" (Rasmussen, 1986). However, recent findings reveal that autophosphorylation of the Type II Ca^{2+}/calmodulin-dependent protein kinase causes the enzyme to become calcium independent, thereby providing a mechanism by which kinase activity could be prolonged beyond the duration of an initial activating calcium signal (Miller and Kennedy, 1986; Lai et al., 1986). It seems possible, therefore, that in some cases the calmodulin branch could mediate long-lasting cellular responses to transient increases in intracellular calcium ion concentration.

The enzymes regulated by calcium/calmodulin include a cyclic nucleotide phosphodiesterase (Sharma et al., 1980), a protein phosphatase (Stewart et al., 1982), an adenylate cyclase (Brostrom et al., 1978), and several protein kinases (Nairn et al., 1985; Kennedy et al., 1986). The protein kinases have been classified on the basis of their structures and substrate specificities into two general types (Kennedy et al., 1986). One type, termed specialized CaM[1] kinases, includes phosphorylase kinase, myosin light chain kinase, CaM kinase I and CaM kinase III. They have a relatively narrow substrate specificity and appear to perform a small number of specialized functions. The other type, termed broad specificity Ca^{2+}/CaM protein kinases, includes muscle and liver glycogen synthase kinase, brain Type II CaM kinase, and several partially characterized kinases in other tissues. These kinases all have similar substrate specificity and show close structural homologies. Thus, they appear to constitute a family of related protein kinases. This family appears to regulate a variety of different functions.

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[1]CaM stands for calmodulin

The different types of Ca^{2+}/calmodulin-dependent kinases have been identified in many tissues. However, the relative proportion of each type of kinase in the different tissues is highly variable. For example, Type II CaM kinase predominates in brain while phosphorylase kinase and myosin light chain kinase predominate in skeletal muscle. The tissues also differ in the proportion of enzyme present in the soluble and particulate fractions. The tissue, cellular, and subcellular distributions of some of these kinases are discussed below.

A. Phosphorylase Kinase

Phosphorylase kinase is found in many tissues and has been purified to homogeneity from skeletal muscle (Brostrom et al., 1971; Cohen, 1973; Pocinwong et al., 1981), cardiac muscle (Cooper et al., 1980) and liver (Chrisman et al., 1982). It is most abundant in skeletal muscle where it constitutes approximately 0.3% of total protein and 1% of soluble protein. Other tissues contain a lower concentration: approximately 0.03% of total protein in heart and 0.01-0.02% of total protein in mammalian liver, brain, and spleen (Proux et al., 1974; Tiara et al., 1982). It has also been studied in insect flight muscle (Hansford and Sacktor, 1970), insect fat body (Yanagawa and Horie, 1979; Ashida and Wyatt, 1979; Hayakawa, 1985; van Marrewijk et al., 1985) and the fungus *Neurospora crassa* (Gold et al., 1974), but its concentration in these tissues is not known.

Phosphorylase kinase is predominantly cytosolic. In skeletal muscle and liver it is present together with other enzymes of glycogen metabolism in protein-glycogen particles (glycosomes) (Meyer et al., 1970; Scott and Cooper, 1973; Chrisman et al., 1982; Livanova and Poglazov, 1982). Steiner and Marshall (1982) have demonstrated the formation of a complex between glycogen and phosphorylase kinase *in vitro* in the presence of Ca^{2+} and Mg^{2+}. Formation of the complex appears to be mediated by the α-subunit of the kinase (Chan and

Graves, 1982). The existence of such complexes *in vivo* could promote efficient breakdown of glycogen in response to hormonal stimuli. A report on the subcellular localization of the enzyme in rabbit *psoas major* muscle using monoclonal antibodies specific for α-, β- and γ-subunits of rabbit skeletal muscle phosphorylase kinase has appeared, suggesting that phosphorylase kinase, in addition to being cytosolic may be associated with the sarcoplasmic reticulum (Thieleczek et al., 1985).

B. Myosin Light Chain Kinase

Myosin light chain kinase has been purified from mammalian and avian skeletal muscle (Pires and Perry, 1977; Yazawa and Yagi, 1978; Blumenthal and Stull, 1980; Crouch et al., 1981; Edelman and Krebs, 1982; Nunnally et al., 1985), smooth muscle (Adelstein and Klee, 1981; Vallet et al., 1981; Walsh et al., 1982; Higashi et al., 1983), and cardiac muscle (Walsh et al., 1980; Wolf and Hofmann, 1980) as well as from *Limulus* muscle (Sellers and Harvey, 1984). It has also been isolated from bovine brain (Dabrowska and Hartshorne, 1978; Hathaway et al., 1981) and human platelets (Dabrowska and Hartshorne, 1978; Hathaway and Adelstein, 1979). Estimates of its concentration in muscle based on recovery of activity after purification indicate that it is approximately 0.6 to 1.6 μM in the tissue and comprises 0.04 to 0.1% of total muscle protein (Pires and Perry, 1977; Walsh et al., 1980, 1982; Adelstein and Klee, 1981; Nunnally et al., 1985). The enzyme is less concentrated in brain where it is approximately 0.01% of total protein (Hathaway et al., 1981).

In muscle, myosin light chain kinase (MLCK) appears to be associated with the contractile apparatus; however, the strength of this association varies with the different isozymes. Immunocytochemical staining of smooth muscle cells with anti-MLCK antibodies appears diffuse (Cavadore et al., 1982). However, the smooth muscle light chain kinases from avian gizzard (Adelstein and Klee,

1981) and mammalian stomach (Walsh et al., 1982) copurify with myofibrils after homogenization and are solubilized only after extensive extraction in high Mg^{2+}. This tight association is consistent with the obligatory role of the kinase in smooth muscle contraction. The staining pattern of skeletal and cardiac muscle with anti-MLCK antibodies demonstrates an association of the enzyme with the actin I-bands (Guerriero et al., 1981; Cavadore et al., 1982). This staining pattern persists when the antisera are preabsorbed with pure actin but is abolished after preabsorption with MLCK. Curiously, the skeletal and cardiac light chain kinases are more easily extracted after homogenization than the smooth muscle kinase, requiring only low ionic strength buffer (Pires and Perry, 1977; Yazawa and Yagi, 1978; Walsh et al., 1980). While myosin light chain kinase activation appears obligatory for initiation of smooth muscle contraction, the kinase probably plays only a modulatory role in striated muscle. Guerriero et al. (1981) have suggested that the association of the light chain kinase with actin bands rather than with myosin in striated muscle could indicate that light chain phosphorylation cannot occur unless the I and M bands are in register. This localization may underlie an involvement of the kinase in contraction-induced changes in muscle function, for example, post-tetanic potentiation (Manning and Stull, 1979).

In non-muscle cells such as cultured fibroblasts (deLanerolle et al., 1981) and various cell lines at interphase (Guerriero et al., 1981), myosin light chain kinase antibodies stain the actin stress fibers as well as the fibrillar region of the nucleolus (Guerriero et al., 1981). In the fibroblasts, the staining along stress fibers is periodic and is identical to the pattern obtained with anti-myosin antibodies, suggesting a close association between the kinase and its myosin substrate in these cells. In dividing cells, the enzyme is concentrated in the mitotic spindle (Guerriero et al., 1981). The association of the kinase with stress

fibers and with the mitotic spindle suggests a role for it in acto-myosin contraction in the cytosol and in movement of chromosomes along the spindle. The significance of the nucleolar staining is unknown. Myosin light chain kinase has also been found to be associated with the lymphocyte membrane-cytoskeleton complex (Bourguignon et al., 1982). These workers reported a seven to eightfold enrichment of enzyme activity in the cytoskeletal fraction prepared by detergent treatment of plasma membranes. They also provided immunocytochemical evidence that the kinase accumulated directly under Con A-capped structures in these cells. In a control experiment, prefixed cells that had a uniform distribution of Con A receptors on their surface also showed a uniform distribution of enzyme staining in their cytoplasm.

Taken together, the results discussed above corroborate biochemical evidence for an important role for myosin light chain kinase in the calcium dependent regulation of the contractile apparatus in both muscle and non-muscle cells.

C. The Family of Broad Specificity Protein Kinases

Broad specificity CaM kinases have been purified to apparent homogeneity from rat brain (Bennett et al., 1983; Goldenring, et al., 1983; Yamauchi and Fujisawa, 1983; Kuret and Schulman, 1984; McGuinness et al., 1985; Miller and Kennedy, 1985), rabbit skeletal muscle (McGuinness et al., 1983; Woodgett et al., 1983), and rabbit liver (Ahmad et al., 1982; Payne et al., 1983). They have been partially characterized in *Aplysia* nervous system (Novak-Hofer and Levitan, 1983; DeRiemer et al., 1984), torpedo electric organ (Palfrey et al., 1983), turkey erythrocytes (Palfrey et al., 1984), bovine heart (Palfrey, 1984), and rat pancreas (Gorelick et al., 1983; Cohn et al., 1984).

This family of kinases is more highly expressed in brain than in nonneuronal tissues. Measured by radioimmunoassay, mammalian brain Type II CaM kinase makes up about 1% of total brain protein (see Chapter 3). Estimated from specific activities, the concentration of broad specificity CaM kinases in other tissues is considerably lower: 0.09% of total protein in spleen, and less than 0.03% in heart, skeletal muscle, adrenals, liver, and kidney. Within the brain, the Type II CaM kinase is most concentrated in forebrain regions such as the cerebral cortex and hippocampus, where it makes up 1% and 2% of total protein, respectively. Its concentration is less in lower brain regions such as the cerebellum and pons/medulla (0.05-0.3% total protein). The skewed distribution of the kinase within the brain has been confirmed by immunocytochemistry (Ouimet et al., 1984; Chapter 3 this thesis). Thus, it appears that the Type II CaM kinase may carry out specialized functions in forebrain neurons. It has been pointed out that the regions of the brain that contain a high concentration of Type II CaM kinase are those that show forms of calcium-dependent modulation of synaptic strength called long-term potentiation, a class of synaptic changes that may be important for memory formation.

In liver and muscle, the broad specificity CaM kinases have been studied only in the soluble fraction; no evidence has been presented for particulate forms. However, in brain, the Type II CaM kinase is present in both the soluble and the particulate fractions (Kennedy et al., 1983b). A large portion of the particulate kinase can be solubilized in low ionic strength buffer and partially purified. The soluble and particulate forms of the kinase are not distinguishable when their physical and catalytic properties are compared (Kennedy et al., 1983b). It has therefore been proposed that much of the enzyme in brain is cytosolic but that it can associate in a reversible way with particulate subcellular structures. The pattern of Type II CaM kinase immunoreactivity in

neurons supports the notion that at least a portion of the enzyme has a diffuse distribution in the cytoplasm and is present in dendrites, cell bodies, and synaptic terminals (Ouimet et al., 1984).

Biochemical and immunochemical evidence suggests that one of the structures that contains the kinase is the postsynaptic density, a fibrous cytoskeletal structure that adheres to the neuronal membrane at postsynaptic sites (Kennedy et al., 1983a; Goldenring et al., 1984; Kelly et al., 1984, Kennedy and Radice, 1984; Miller and Kennedy, 1985). The kinase makes up approximately 20% of the total protein in isolated postsynaptic density fractions (Miller and Kennedy, 1985). In this location the kinase would be strategically positioned to respond to calcium signals generated by binding of transmitters to their receptors. The concentration of the kinase within the postsynaptic density may be nearly ten times higher than in the surrounding cytosol (Kennedy and Radice, unpublished observations). Because the kinase is tightly bound within the postsynaptic density fraction, this portion of the kinase may not be in equilibrium with the cytosol (Kelly et al., 1984). The Type II CaM kinase is also found associated with synaptic vesicles (Huttner et al., 1983; Palfrey et al., 1983). Since a major brain substrate for the kinase, synapsin I, is a synaptic vesicle-associated protein, the vesicle-associated kinase may be positioned to phosphorylate this protein rapidly. Phosphorylation of synapsin I by the Type II CaM kinase reduces its affinity for synaptic vesicles (Schiebler et al., 1986), and this may unmask sites on the vesicle membrane that promote neurotransmitter release (Llinas et al., 1985).

Several reports have suggested that the Type II CaM kinase may associate with other cytoskeletal elements. Sahyoun et al. (1984a,b; 1985) have reported that the kinase is enriched in both nuclear matrix and crude cytosolic cytoskeletal fractions. Vallano et al. (1985) found a threefold enrichment of the kinase in twice-cycled microtubules. However, in the latter study, the kinase

specific activity was not constant during microtubule purifications as it is for the microtubule-associated cAMP-dependent protein kinase (Sloboda et al., 1975), indicating that the Type II CaM kinase detected in the study may not be an integral component of the microtubules. Saitoh and Schwartz (1985) found a large fraction of *Aplysia* neuronal Type II CaM kinase in the triton-insoluble fraction, which they defined as a membrane-cytoskeletal fraction. A portion of this kinase was released from the triton-insoluble pellet after incubation under conditions that would permit autophosphorylation. However, Suzuki and Tanaka (1986) did not find any release of the kinase from rat cerebral synaptic junction after autophosphorylation.

The tissue, cellular and subcellular distributions as well as the broad substrate specificity of the Type II CaM kinases indicate the multiplicity of Ca^{2+} regulated processes they may be involved in. These findings should provide a useful basis for further studies to determine the *in vivo* functions of this class of protein kinases. Chapter 4 of this thesis concerns studies of the association of the Type II CaM kinase with both crude and purified elements of the cytoskeleton. In the next section of the Introduction (Section II), I will review the components of the cytoskeleton with particular emphasis on the brain cytoskeleton.

Section II

The cytoskeleton can be defined as the complex array of protein filaments that connect different parts of cells to each other and to the cell membrane. A common approach to the study of the cytoskeleton is to treat cells with non-ionic detergents resulting in the removal of membranes, organelles, small molecules, and soluble proteins. The residue left behind retains some of the structural and functional properties of the cytoskeleton in intact cells and is

more accessible to experimental manipulation and analysis. More thorough biochemical studies have revealed three major components of the cytoplasmic cytoskeleton, namely microfilaments, intermediate filaments and microtubules. There is also a distinct submembranous cytoskeleton. A brief discussion of these cytoskeletal components and their accessory proteins follows.

A. Microfilaments

These are 5-7 nm in diameter and consist primarily of actin, a globular protein that can assemble and disassemble rapidly in cells. Together with myosin they constitute the contractile machinery in both muscle and non-muscle cells. It is widely accepted that force generation is effected by the sliding of actin filaments past myosin with concomitant hydrolysis of ATP by the myosin ATPase.

The actin-myosin interaction necessary for force generation is triggered by calcium. In skeletal muscle, an increase in intracellular calcium leads to the activation of Troponin C (a calcium-binding protein), which then relieves the inhibition of actin-myosin interaction imposed by the Troponin I/Troponin T/Tropomyosin complex (Potter and Gergely, 1975). However, in smooth muscle and non-muscle cells, the initiation of contraction involves the activation by calcium of Ca^{2+}/calmodulin-dependent myosin light chain kinase. Phosphorylation of one of the myosin light chains by this kinase leads to an enhancement of the actin-activated ATPase activity of myosin (Adelstein et al., 1981). It is pertinent to note that in the brain, the Type II Ca^{2+}/calmodulin-dependent kinase (Type II CaM kinase) may account for most of the myosin light chain kinase activity (Edelman et al., 1985). It has also been shown that phosphorylation of myosin light chains by the Type II CaM kinase increases the actin-activated myosin ATPase activity (Edelman et al., 1986). The Type II CaM kinase may therefore regulate the actin-myosin contractile apparatus in the

brain. Another regulatory influence is the phosphorylation of the myosin light chain kinase by the cAMP dependent kinase, which results in a decreased affinity for calmodulin and therefore in decreased light chain kinase activity (Adelstein et al., 1981). Switching off the contractile machinery in smooth muscle cells involves both the removal of calcium and the dephosphorylation of myosin light chains. A phosphatase capable of dephosphorylating myosin light chains has, in fact, been isolated (Pato and Adelstein, 1980). While phosphorylation/ dephosphorylation of myosin light chains is widely accepted as the basis of actomyosin regulation in smooth muscle and non-muscle cells, the functional significance of light chain phosphorylation in skeletal muscle is yet to be determined.

There are fundamental differences between the contractile systems in muscle and non-muscle cells. For example, the contractile apparatus in skeletal muscle is a highly ordered paracrystalline array that is very stable. On the other hand, the contractile machinery in non-muscle cells exhibits a considerable degree of temporal and spatial plasticity. Basically, this reflects the ability of actin in non-muscle cells to form networks and the presence of proteins that regulate the state of these networks. Network formation by actin can be effectively regulated by changes in (i) total amount of subunits in polymer forms, (ii) average length of the polymer, and (iii) the density of cross-linkers. Network regulating proteins that effect changes in all three categories have been identified. For example, depolymerizing and polymerization-inhibiting proteins increase the concentration of monomeric actin or make actin unavailable for polymerization, thereby reducing the total amount of subunits in polymeric form. The best studied examples are profilin and DNAse I (Stossel, 1984). End-binding or capping proteins regulate the polymer length. Some of these (villin, gelsolin, fragmin, and severin) require micromolar calcium for the severing or

cutting of actin filaments into shorter fragments (Yin and Stossel, 1982). Gelation factors such as spectrin, actin-binding protein (ABP), and filamin determine the degree of cross-linking between actin polymers (Schliwa, 1986). At high cross-linker to actin ratios, bundling of actin filaments occurs. The interaction of actin networks with other components of the cytoskeleton and with the plasma membrane provides additional means for regulating network structure and rigidity.

A number of laboratories have provided cytological evidence for a structural association between actin filaments and microtubules (Pollard et al., 1984). *In vitro* experiments have also established a specific interaction between actin filaments and microtubules, mediated by the high molecular weight MAPs (Griffith and Pollard, 1982). This cross-linking activity of MAPs was shown to be inhibited by phosphorylation (Selden and Pollard, 1983). It has also been observed that actin filament disrupting agents such as DNAse I and gelsolin can inhibit fast axonal transport (Goldberg et al., 1980). It seems possible that there may be a functional association between the two filament systems, raising the possibility that some microtubule-dependent movements may be effected by an actomyosin based contractile machinery.

Actin filaments have been identified in neuronal growth cones (Yamada et al., 1970, 1971; Letourneau, 1983). Treatment of growing axons with cytochalasin B (which disrupts microfilaments) caused retraction of microspikes, rounding-up of growth cones and cessation of axon elongation (Yamada et al., 1970). This effect was reversible and was shown not to be secondary to drug-induced inhibition of glucose uptake by the neurons (Yamada and Wessells, 1973). The actin network may, therefore, be involved in the locomotory behavior of neuronal growth cones.

B. Intermediate Filaments

These filaments have diameters of 8-12 nm, intermediate between those of microfilaments and microtubules. They were originally thought to be disaggregated forms or breakdown products of some other cytoplasmic component such as microtubules, but biochemical and immunocytochemical studies indicate that they constitute a distinct component of the cytoskeleton. In general, intermediate filaments are insoluble in neutral buffers over a wide range of ionic strength and in non-ionic detergents. Depending on the type of filament, they are soluble, to varying degrees, in low ionic strength solutions, denaturing agents, low pH, or ionic detergents. They have been classified into six major groups (Steinert et al., 1984; McKeon et al., 1986): (i) desmin, found predominantly in striated and smooth muscle cells; (ii) vimentin, characteristic of cells of mesenchymal origin; (iii) keratin, found in epithelial cells; (iv) neurofilaments, found in neurons; (v) glial filaments present in glial cells and astrocytes; and (vi) the lamins, protein components of the nuclear lamina. The cell type-specific expression of intermediate filaments reflected in the above classification has been useful in the correct identification of the histological origin of tumors. However, cells are capable of expressing two different intermediate filament proteins simultaneously. For reviews on intermediate filaments, see Lazarides (1980, 1982) and Traub (1985).

Neurofilaments. Neurofilaments have an average diameter of 10 nm and are found mainly in the axon, being less prominent in dendrites (Lasek et al., 1985). They extend parallel to the long axis uninterrupted for long distances, though a discontinuity may exist at the node of Ranvier (Tsukita and Ishikawa, 1981). As seen in cross-section, they either can be randomly dispersed or may occur in clusters or bundles. In many instances, they are linked to each other and to microtubules by "wispy side arms or cross-bridges" visible in the electron

microscope (Hirokawa et al., 1984). Neurofilaments conform to the description of intermediate filaments by Lazarides (1980) as "mechanical integrators" of cellular space. They have been shown to increase axonal diameter by adding to the volume of the cytoskeleton (Lasek et al., 1983). They also constitute the most abundant component of the cytoskeleton in large diameter axons such as Mauthner axons and the squid giant axons (Lasek et al., 1983). Thus, neurofilaments can affect neuronal function since large diameter axons conduct impulses faster than small diameter axons. However, neurofilaments are not indispensable for the function of neurons. For example, granule cells of the cerebellum contain no neurofilaments in their axons and neither do axons in arthropods (Lasek et al., 1985).

In mammals, neurofilaments are composed of three polypeptides with molecular weights of approximately 200, 150, and 68, kilodaltons (kD). These have been referred to as the "neurofilament triplet" (Hoffman and Lasek, 1975). These three polypeptides are related, sharing some antigenic determinants (Shaw et al., 1984). However, they are distinct proteins and are not derived from a common precursor protein since they do possess unique antigenic determinants and each polypeptide is translated from a different mRNA *in vitro* (Czosnek et al., 1980). Like all intermediate filament proteins, the triplet proteins possess a conserved 40 kD rod-shaped domain, and it has been suggested that they are evolutionarily related to each other (Lazarides, 1982; Lasek et al., 1985). Reptilian and avian neurofilament polypeptides are immunologically related to the mammalian proteins, though they differ in the molecular weights of homologous proteins (Shaw et al., 1984). On the other hand, invertebrate neurofilaments appear to be significantly different from vertebrate neurofilaments (Lasek et al., 1985).

Neurofilament Associated Proteins. As mentioned earlier, the versatility and plasticity of actin-based cellular structures are largely determined by their numerous associated proteins. For this reason, a number of laboratories have attempted to identify and characterize intermediate filament-associated proteins. It has been proposed that neurofilament-associated proteins link neurofilaments to one another as well as to microtubules (Schliwa, 1986). One of these proteins is the high molecular weight component (200 kD) of the triplet protein. Immunoelectron microscopy studies have revealed that this protein is a component of the cross-bridges between neurofilaments, while the 68 kD polypeptide is a component of the "central core" (Hirokawa et al., 1984). This finding is consistent with observations on the *in vitro* assembly of neurofilament from the "triplet" polypeptides. Such experiments have shown that the 68 kD subunit can assemble by itself into intermediate filament-like structures, whereas the other two can co-assemble only with the 68 kD component (Liem and Hutchison, 1982). It has also been shown that the incorporation of the 200 kD proteins in these filaments results in formation of filaments with fuzzy projections, indicating that a portion of the 200 kD protein extends peripherally from the central core (Geisler and Weber, 1981). The 150 kD component also appears to be peripheral, but the results are more equivocal. It should be noted that the above results do not rule out the possibility that portions of the 150 kD and 200 kD proteins could be part of the central core.

Recent morphological studies have provided indirect evidence implicating MAPs as the cross-linkers between microtubules and neurofilaments. Papasozomenos et al. (1985) observed co-localization of a MAP2 epitope with neurofilaments, following a drug-induced segregation of neurofilaments from microtubules and other axoplasmic organelles. Bloom and Vallee (1983) also reported that in cultured brain cells, following depolymerization of microtubules

with vinblastine, MAP2 co-localizes with cables of vimentin filaments. Further support for the suggested role of MAPs has come from *in vitro* studies of the interactions between microtubules and neurofilaments. Runge et al. (1981) reported that MAPs produce an increase in the viscosity of an ATP-induced complex between microtubules and neurofilaments. Leterrier et al. (1982) showed that partial inhibition of microtubule assembly by neurofilaments was due to the association of a complex of high molecular weight MAPs and tubulin with neurofilaments. They further demonstrated saturable binding of MAPs to neurofilaments. Liem et al. (1985) subsequently used ^{32}P-labeled MAPs in protein blotting experiments to show that the MAPs bound to the 68 kD protein, not to either of the other two neurofilament proteins. It should be noted that the absence of binding on blots by no means excludes the possibility that MAPs bind to the 150 and 200 kD proteins *in vivo*. Electron microscopic studies have also shown that MAP-free microtubules adhere in great numbers to neurofilament bearing grids only when such grids had been previously incubated with MAPs (Williams and Aamodt, 1985).

In addition to the high molecular MAPs, tau proteins may also be involved in mediating the interaction between the two filament systems (Liem et al., 1985). They have also proposed a cross-linking role for a 66 kD intermediate filament-associated protein that may be related to the tau proteins. While the data from the above experiments may appear impressive, it remains to be demonstrated unequivocally that the interaction between microtubules and neurofilaments is biologically significant.

Regulation of Neurofilament Organization. Phosphorylation and proteolysis have been proposed as mechanisms for the regulation of neurofilament organization (Lazarides, 1980). A number of laboratories have described phosphorylation of neurofilament proteins by both cAMP-dependent and independent

protein kinases (Leterrier et al., 1981; Pant et al., 1986). Wong et al. (1984) reported that phosphorylation of the 150 kD neurofilament protein increases its association with intact neurofilaments, while dephosphorylation induces dissociation. It has also been reported that the neurofilament triplet proteins in axons are more highly phosphorylated than those in cell bodies and dendrites (Sternberger and Sternberger, 1983). It seems, therefore, that phosphorylation could influence the spatial and consequently the functional organization of neurofilaments.

Tashiro and Ishizaki (1982) have reported the identification of a calcium-dependent protease associated with cytoskeletal preparations which selectively degrades the 150 kD component of neurofilaments. Such subunit specificity led them to suggest that proteolysis could be a mechanism for regulating filament-filament interaction. However, Schlaepfer and Zimmerman (1985) could not find selective degradation of any of the proteins. Adding to the complexity of the system is the co-purification, under certain conditions, of the protease, calcium-dependent protease inhibitor, and the neurofilament specific kinase (Schlaepfer and Zimmerman, 1985). Thus, further experimental studies are needed to elucidate the role, if any, of calcium-activated proteolysis in neurofilament organization and function.

C. Microtubules

These hollow cylindrical structures possess an outer diameter of approximately 25 nm and an inner diameter of 15 nm and are present in all eukaryotic cells. The wall of the microtubule is usually made up of 13 protofilaments arranged in parallel, each consisting of a stack of tubulin subunits. Microtubules with either more or less than 13 protofilaments have been identified but the functional significance of the variability in number of protofilaments is not known (Schliwa, 1986). Microtubules can exist as part of very stable, complex

structures such as flagella and cilia as well as part of dynamic structures that may subserve such diverse physiological functions as maintenance of cell shape, intracellular transport, cell motility and mitosis. Complex assemblies of microtubules such as centrioles, axonemes, basal bodies, cilia and flagella will not be discussed here. The monograph by Dustin (1984) provides a detailed source of information on various aspects of microtubule structure and function.

Certain functions of microtubules depend on the ability of these structures to undergo rapid assembly/disassembly; and the presence in the cell of factors that regulate the rate, extent and site of this process. The functional unit of assembly is a heterodimer, alpha and beta tubulin--two similar but distinct proteins. Like actin, tubulin is highly conserved but substantial micro-heterogeneity of both tubulin subunits occur. Evidence has been presented to show that this microheterogeneity can arise either from multiple tubulin genes or posttranslational modification or both (Dustin, 1984). Posttranslational modifications include C-terminal tyrosination of the alpha subunit and phosphorylation of the beta subunit. It has been suggested that this microheterogeneity could give rise to the formation of microtubules made from different forms of tubulin, thereby establishing functionally different microtubule structures (McKeithan and Rosenbaum, 1984).

The discovery of optimal conditions for the *in vitro* assembly of tubulin (Weisenberg, 1972) opened the way for intensive and fruitful studies on the mechanics and regulation of microtubule assembly. It became possible to correlate dynamics of microtubule behavior with their postulated physiological functions. These studies led to the identification of a number of molecules that specifically interact with tubulin, thereby affecting its polymerization. The interaction of tubulin with some of these molecules is discussed below.

1. Ions:

The tubulin dimer has one or two high affinity and several low affinity binding sites for calcium, and many binding sites for magnesium (Solomon, 1977). Two high affinity binding sites for calcium have been localized to the carboxyl-terminal regions of both subunits (Serrano et al., 1986). *In vitro* millimolar magnesium is required for assembly, while even micromolar calcium is inhibitory. It has been proposed that in addition to direct effects via binding to tubulin, calcium exerts its inhibitory effect through calmodulin. Evidence for this comes from studies which show (1) that anticalmodulin drugs reduce the sensitivity of cellular microtubules to calcium (Schliwa et al., 1981) and (2) that calmodulin potentiates the inhibitory action of calcium on microtubule assembly *in vitro* (Marcum et al., 1978). Two mechanisms have been proposed for the calcium/calmodulin induced microtubule disassembly, namely, the sequestration of MAPs by the formation of a calcium/calmodulin • MAP complex (Lee and Wolf, 1984); and the calcium/calmodulin-dependent phosphorylation of MAP and/or tubulin (Yamamoto et al., 1985) as well as a "cold stability factor" (Margolis et al., 1986). Other ions such as zinc and cobalt cause the formation of abnormal structures and are not believed to be physiologically relevant. They have been used in *in vitro* studies of the tubulin lattice (Dustin, 1984).

2. Nucleotides:

Tubulin has two binding sites per dimer for guanosine nucleotides--the exchangeable (or E) site located on the beta subunit and the nonexchangeable (or N) site (Kirschner, 1978). The GTP at the E site exchanges rapidly with free GTP and is hydrolyzed during polymerization while the GTP at the nonexchangeable site is not hydrolyzed and is released only on denaturation of the tubulin molecule. It was thought that GTP hydrolysis was required for assembly until

the demonstration that nonhydrolyzable analogs of GTP could support microtubule polymerization (Kirschner, 1978). Much attention has since been focussed on elucidating the role of GTP hydrolysis in microtubule dynamics.

It has been postulated that GTP hydrolysis confers polarity on microtubules in the sense that each end of the polymer has a different critical concentration for assembly. At steady state, this would result in net assembly at one end and net disassembly at the opposite end, leading to a "unidirectional flux of tubulin from one end of the microtubule to the other"--a process termed "treadmilling" (Margolis and Wilson, 1981). These authors also suggested that treadmilling could constitute a "molecular machinery" for the movement of chromosomes to the poles during mitosis. However, the prediction of a poleward flow or "treadmilling" of microtubules during mitosis was not borne out by experiments in which measurements were made of fluorescence redistribution after photobleaching labeled microtubules during anaphase (Wadsworth and Salmon, 1986). Rather, most of the spindle microtubules in these tissue culture cells were found to rapidly assemble and then disappear in an asynchronous manner. These results are consistent with an alternative model--the "dynamic instability" model of Mitchinson and Kirschner (1984).

The "dynamic instability" model simply states that individual microtubules can exist in either of two distinct phases--a growing phase or a rapid shrinking phase and that the transition between the two phases is regulated by GTP hydrolysis. The model predicts that microtubules at steady state would comprise two populations: growing microtubules and shrinking ones. This has been confirmed by direct observations of microtubule dynamics, using immunofluorescence and electron microscopy (Cassimeris et al., 1986; Kristofferson et al., 1986). It has also been proposed that tubulin-GTP acts as a cap at the microtubule end, permitting the growth of the microtubule; but when the GTP cap is lost (e.g., by

hydrolysis), the exposed tubulin-GDP undergoes rapid and "catastrophic" disassembly (Mitchinson and Kirschner, 1984). Though the GTP cap remains to be demonstrated, the data obtained from *in vitro* and *in vivo* studies of microtubule dynamics are more consistent with the dynamic instability model.

3. Microtubule-associated Proteins:

These proteins have been identified on the basis of their co-purification with tubulin though a more rigorous definition requires that such proteins remain associated with tubulin through several cycles of assembly/disassembly at relatively constant ratios. Two major classes of microtubule-associated proteins (MAPs) have been identified, namely, a group of high molecular weight proteins with two major polypeptides MAP1 (350 kD) and MAP2 (300 kD); and a group of proteins (55-70 kD) termed tau proteins. It has been suggested that the microheterogeneity of tau proteins results from differences in phosphorylation (Butler and Shelanski, 1986) though its function is unknown. The high molecular weight MAPs have been shown to form regularly spaced projections on the surface of microtubules (Kim et al., 1979). Both high molecular weight and tau MAPs reduce the critical concentration of tubulin for assembly and stimulate polymerization *in vitro* (Kirschner, 1978). They have also been shown to stabilize microtubules against the depolymerizing action of calcium in detergent extracted BSC-1 monkey cells (Schliwa et al., 1981).

Other microtubule-associated proteins of interest include cAMP dependent protein kinase directly associated with MAP2 (Sloboda et al., 1975; Vallee et al., 1981) and the Type II CaM kinase (Vallano et al., 1985; Chapter 4, this thesis). The latter enzyme does not maintain a constant stoichiometry wtih tubulin during purification by cycles of assembly/disassembly--a finding that may reflect a weak interaction. Phosphorylation of MAP2 by the cyclic AMP-dependent protein kinase and phosphorylation of both MAP2 and tubulin by the Type II CaM

kinase cause inhibition of microtubule assembly *in vitro* (Yamamoto et al., 1985). Thus, phosphorylation of MAPs and tubulin could play an important role in the regulation of the structure and, consequently, function of microtubules *in vivo*.

The distribution of MAPs in the nervous system have been studied using immunocytochemical (Caceres et al., 1984; Huber and Matus, 1984; Hirokawa et al., 1984) and biochemical methods (Vallee, 1982). The interpretation of many of these studies is complicated by the fact that each MAP is a family of polypeptides (Vallee and Bloom, 1984). Still, one can conclude that there is a differential cellular and subcellular distribution of MAPs, with MAP2 being predominantly in the soma and dendrites while MAP1 and tau proteins are predominantly axonal. This spatial segregation may have functional implications as has been suggested in the case of spectrin (Lazarides and Nelson, 1983, Lazarides et al., 1984).

A number of studies have been carried out to elucidate the role of MAPs in microtubule function. Evidence implicating MAPs as cross-linkers between microtubules and other components of the cytoskeleton has already been discussed. Hirokawa et al. (1984) used both immunofluorescence and immunoelectron microscopy to demonstrate the presence of MAPs in fine strands that connected MAPs to membrane-bounded organelles--raising the possibility that MAPs may be involved in the fast component of axonal transport.

Based on ultrastructural studies and the effects of drug-induced microtubule depolymerization, it has been postulated that several forms of intracellular organelle and vesicle translocation such as the fast component of axoplasmic transport are microtubule dependent (Dustin, 1984; Schliwa, 1984). It was not clear whether the role of microtubules was passive, i.e., acting as tracks for some other force-generating system or whether they (including MAPs) were

involved directly in force production. Studies designed to resolve these and other issues benefited immensely from two recent developments--the finding that extruded axoplasm from the giant axon of the squid (*Loligo pealei*) remains metabolically active for hours (Lasek, 1974); and the use of video-enhanced differential interference contrast microscopy to directly observe organelle motility in this preparation (Brady et al., 1982).

With the above set up, a number of workers demonstrated the capacity of single microtubules to support bidirectional organelle movement (Allen et al., 1985; Brady et al., 1985; Schnapp et al., 1985). Subsequently Vale et al. (1985) partially purified a 600 kD protein--"kinesin" from squid axoplasm that translocated latex beads in an anterograde direction on single microtubules. This protein was established by a number of criteria to be distinct from either myosin or dynein. A homologous protein was also identified in bovine brain. These workers also demonstrated that retrograde bead movement is supported by factor(s) that are immunologically and pharmacologically distinct from kinesin. It is not yet clear how far these results can be extrapolated to organelle movement in axons. For example, organelle movement unlike bead movement exhibits approximately the same velocity in both directions and organelles rarely reverse their direction of movement (Schnapp et al., 1985). In addition, it has not been shown that kinesin binds to organelles--a requirement for a translocator. In this regard, Gilbert and Sloboda (1986) have reported the identification of an ATP-binding protein that is associated with vesicles that translocate on purified MAP-free microtubules. They suggested, on the basis of immunological cross-reactivity and co-polymerization, that this protein is similar to mammalian brain MAP2. It seems, therefore, that with the available technology, the role of microtubules in this form of transport will be elucidated in the not too distant future.

In the early seventies, colchicine disruption of microtubules was used to show the possible involvement of microtubules in neurite outgrowth (Yamada et al., 1970; Daniels, 1973). Recently, an assembly-promoting drug, taxol, was shown to inhibit neurite outgrowth in cultured sensory neurons from chick embryos (Letourneau and Ressler, 1984). It has also been demonstrated that nerve growth factor regulates neurite outgrowth in PC12 cells via the induction of microtubule-associated proteins (Greene et al., 1983; Drubin et al., 1985). These experimental results are consistent with the notion that controlled assembly of microtubules is crucial in the outgrowth and stabilization of neurites.

D. Membrane-Cytoskeleton Interactions

In order for modulation of cytoskeletal dynamics to result in useful activities such as cell movement, shape changes, or cell surface rearrangements, there ought to be physical connections between the cytoskeleton and the plasmalemma. The erythrocyte has served as a useful model in the attempt to understand these interactions. The basic approach has been to isolate a "membrane skeleton" by treating red cell ghosts with non-ionic detergents, followed by biochemical and immunochemical characterization of the protein components, and to study the interactions between these components *in vitro*. Much data has been accumulated as documented in two recent reviews (Bennett, 1985; Marchesi, 1985).

It has been suggested that the unit structure of the "membrane skeleton" is the ternary complex of spectrin, actin, and band 4.1 (Bennett, 1985; Marchesi, 1985). Spectrin is composed of two subunits with molecular weights of 240 kD (alpha or band 1) and 220 kD (beta or band 2). Both subunits associate side by side to form heterodimers which in turn self-associate at one end to form tetramers. Although spectrin is capable of forming higher oligomers *in vitro*, it

seems that it exists as a tetramer in the ternary complex (Goodman and Weidner, 1980). The binding sites for actin and band 4.1 have been localized to the ends of the spectrin dimer not involved in tetramer formation (Bennett, 1985). Band 4.1, composed of two chemically similar polypeptides promotes spectrin binding to actin and stabilizes their interactions in this complex (Cohen and Foley, 1982). In addition, band 4.1 may anchor the spectrin-actin complex to an integral membrane protein, glycophorin (Cohen and Foley, 1982). Ankyrin, a 200 kD globular protein, links spectrin and presumably the ternary complex to the membrane (Weaver et al., 1984). Its binding site has been localized to the beta subunit of spectrin about 20 nm from the end involved in tetramer formation. Ankyrin links spectrin to band 3, the most abundant integral membrane protein that is also an anion transporter. Other identified components of the "membrane skeleton" include tropomyosin, band 4.9 (an actin bundling protein), myosin, and a calmodulin-binding heterodimer (Fowler, 1986).

The model that has emerged is that of a two-dimensional subplasmalemmal network comprised of complexes of actin, band 4.1, and a number of accessory proteins linked together by long, filamentous spectrin tetramers (Bennett, 1985; Marchesi, 1985). This network is then connected to the erythrocyte membrane by ankyrin and perhaps by band 4.1. It is remarkable that the above model obtained by studying purified components has been essentially confirmed by elegant ultrastructural studies of the erythrocyte membrane skeleton (Byers and Branton, 1985; Shen et al., 1986). These workers used low ionic strength buffers to spread the membrane skeleton without apparently disrupting the linkages holding it together. This protocol enabled them to obtain clear images from negatively stained preparations. They found a highly ordered array of filaments, whose morphology corresponds to purified spectrin tetramers, cross-linked by junctional complexes. Between four and seven spectrin tetramers appear to

insert into each junction, which contains a short, stubby rod, presumably the actin oligomer plus accessory proteins. Distinct globules were seen on the spectrin tetramer at positions that suggested that they were occupied ankyrin binding sites. Confirmation of the molecular organization of this network will now await immunoelectron microscopy.

While many of the interconnections and associations within the "membrane skeleton" have been determined, not much is known about the factors that regulate them. Several components of the network (spectrin, ankyrin, band 4.1) are phosphoproteins. In addition, a number of kinase activities (cAMP-dependent, Ca^{2+}/calmodulin-dependent, kinase C) have been identified in erythrocyte membranes (Fowler, 1986). However, the only evidence for a role of phosphorylation in regulating this network comes from the finding that phosphorylation of band 4.1 reduced its affinity for spectrin (Eder et al., 1986).

The suitability of the red cell membrane as a model system for study of the submembranous cytoskeleton was enhanced by the identification of analogs of these erythrocyte proteins in non-erythroid cells. In general, these proteins, while retaining major functional features, have substantial differences in antigenic sites and peptide maps from their erythrocyte analogs. It has been suggested that they may have acquired additional specific functions. For example, brain ankyrin binds to microtubules and could link microtubules to the membrane (Bennett, 1985). At least two spectrin subtypes have been identified in both avian and mammalian brains (Levine and Willard, 1981; Lazarides and Nelson, 1983; Goodman and Zagon, 1986)--an erythrocyte form which is present in neuronal cell bodies and dendrites but is excluded from axons, and a brain form that is predominantly axonal in mammals but is found throughout the neuron in chicken cerebellum. Brain spectrin (fodrin) is concentrated at postsynaptic densities (Carlin et al., 1983), synaptic endings and nodes of Ranvier

(Bennett, 1985) and may, therefore, restrict the localization of membrane proteins to specific regions of the neuron (Lazarides and Nelson, 1983). An immunoelectron microscopic study of the subcellular distribution of spectrin in mouse brain revealed an extensive meshwork of spectrin that interconnects organelles such as microtubules, neurofilaments, and mitochondria to one another and also to the plasma membrane (Zagon et al., 1986). However, as the authors pointed out, this finding needs to be confirmed by using techniques that would eliminate the possibility of diffusion of the peroxidase reaction product from the site of antibody binding.

It has been suggested, on the basis of immunological cross-reactivity, peptide mapping and spectrin binding, that synapsin I is the brain analog of band 4.1. It should be noted that the synapsin I sequence deduced from a cDNA clone did not reveal any significant homology with the sequence of a 67 amino acid peptide of erythrocyte band 4.1 involved in spectrin-actin associations (McCaffery and DeGennaro, 1986). It is still possible that the unsequenced regions of band 4.1 might display extensive homology with synapsin I. Equally interesting is the finding that synapsin I contains sequences related to the actin-binding proteins--profilin and villin; pointing to the possibility of an interaction between synapsin I and the neuronal cytoskeleton. Synapsin I is a vesicle-associated protein whose phosphorylation by brain Type II CaM kinase may be responsible for an enhancement in transmitter release in the squid giant synapse (Llinas et al., 1985). A recent immunoelectron microscopic study also indicates that synapsin I may link cytoskeletal elements to the neural membrane as well as to one another (Goldenring et al., 1986).

In summary, much data have been accumulated over the years suggesting the involvement of the cytoskeleton in a variety of cellular processes. With the identification and characterization of a number of the protein components of the cytoskeleton, the stage is now set to determine their specific roles in these processes.

The following chapters of this thesis present the characterization of monoclonal antibodies specific for brain Type II calcium and calmodulin-dependent protein kinase (Type II CaM kinase); the development of liquid-phase and solid-phase radioimmunoassays for the enzyme; and studies of its regional distribution and subcellular associations.

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Chapter 2

CHARACTERIZATION OF MONOCLONAL ANTIBODIES SPECIFIC FOR TYPE II Ca^{2+}/CALMODULIN-DEPENDENT PROTEIN KINASE AND THE DEVELOPMENT OF LIQUID-PHASE RADIOIMMUNOASSAYS

Abstract

Four monoclonal antibodies (4A4, 4A11, 6E9 and 6G9) generated against Type II CaM kinase have been characterized. Three of these hybridomas (4A4, 4A11 and 6E9) bind to the enzyme in solution and inhibit its activity while two (4A4 and 6G9) recognize only the α-subunit of the kinase on immunoblots. Two of the antibodies (4A4 and 6E9) were used to confirm the subunit composition of the enzyme.

Liquid phase radioimmunoassays for the kinase were developed with three of the monoclonal antibodies. For the assays, pure kinase was radioiodinated by the Bolton-Hunter procedure. This resulted in the incorporation of one mole of ^{125}I per three moles of kinase--a specific radioactivity of 700 Ci/mmol. The radioimmunoassays with antibodies 4A11 and 6E9 exhibited a sensitivity of 2.0 ng (3 fmol), $p < 0.05$. The assays were sensitive to the presence of the ionic detergent sodium dodecyl sulfate (SDS) even when a non-ionic detergent (Triton X-100) was included in the assay mix. Furthermore, antibody 6E9 does not recognize the phosphorylated form of kinase while 4A11 recognizes the phosphorylated form poorly. On the other hand, the radioimmunoassay with antibody 6G9 exhibited a sensitivity of 20 ng (30 fmol), $p < 0.05$. This assay was not sensitive to the presence of SDS when a sufficient amount of Triton X-100 was included in the assay mix.

These results suggest the feasibility of using a combination of such radioimmunoassays to determine the proportion of the kinase that exists in either the phosphorylated or non-phosphorylated form under a number of physiological conditions *in vivo*.

Introduction

The Type II CaM kinases are a class of closely related enzymes (Nairn et al., 1985; Kennedy et al., 1986); that are far more highly expressed in brain than in other tissues. Estimates of the concentration of brain Type II CaM kinase have been made using enzyme activity (Bennett et al., 1983; McGuinness et al., 1985). The distribution of Type II CaM kinase in the rat brain has also been studied using activity measurements (Walaas et al., 1983).

However, enzyme activity may not accurately measure the concentration of the kinase in crude homogenates for the following reasons. (1) Kinase in the particulate fraction may not have adequate access to substrate during the assay. (2) Enzyme inhibitors may be present. (3) Activity may not distinguish between kinases. For example, it has been reported that most of the myosin light chain kinase activity in brain may be due to the brain Type II CaM kinase rather than myosin light chain kinase (Edelman et al., 1985).

One way to obviate these difficulties is the development of a radioimmunoassay. Towards this end, a panel of monoclonal antibodies (Köhler and Milstein, 1976) directed against brain Type II CaM kinase was generated. In addition to their use in quantitative radioimmunoassays, these antibodies have been useful experimental tools in the detection of cDNA clones coding for the β-subunit of the kinase (Bennett, 1986), in immunocytochemical localization of the kinase in tissue sections (Chapter 3), in identification of a related kinase in *Drosophila* (Wall, Pugh and Kennedy, unpublished observations) and eventually may be useful in functional studies of the kinase *in vivo*. In this chapter, the characterization of four of these hybridomas and the development of liquid phase radioimmunoassays for brain Type II CaM kinase will be described.

Methods

Materials. [γ^{32}P]ATP and carrier free Na ^{125}I were purchased from ICN and nitrocellulose membranes (BA 85, 0.45 μm pore diameter) from Schleicher and Schuell. ATP, dithiothreitol (DTT), imidazole, EDTA, EGTA, phenylmethyl sulfonyl fluoride (PMSF), Trizma buffer, soybean trypsin inhibitor, chloramine-T, Triton X-100, hemoglobin (bovine Type II), napthol blue black (amido black), polyinosinic-polycytidylic acid (Poly I, Poly C), and mouse IgG were purchased from Sigma. Protein A, Protein A-Sepharose, and Sephadex G-25 were purchased from Pharmacia Fine Chemicals. Leupeptin was purchased from Peninsula Laboratories, Inc. Sprague-Dawley rats (140 to 160 g males) were purchased from Simonsen Laboratories (Gilroy, California), New Zealand female rabbits from Lab Pets (Rosemead, California), and Balb/c ByJ mice from Jackson Laboratories (Bar Harbor, Maine). Rabbit anti-mouse IgG antiserum was obtained from rabbits immunized by repeated subcutaneous injections with mouse IgG in Freund's adjuvant and was affinity purified by chromatography on Sepharose 4B coupled with mouse IgG (March et al., 1974). Protein A was iodinated by the chloramine-T method essentially as described by Fryxell et al. (1983). The specific activity was 2-3 Ci/mmol and 50-60% of it was retained by rabbit anti-mouse IgG coated on plastic microwells. Glycerol was purchased from J. T. Baker Chemical Co.; ^{125}I labeled Bolton-Hunter reagent [^{125}I labeled N-succinimidyl-3-(4-hydroxylphenyl) propionate] was from Amersham. Rabbit anti-mouse Ig sera and immunodiffusion discs (Ouchterlony plates) were purchased from Miles Laboratories. NP-40 was from BDH Laboratories and Protein A-bearing *Staphylococcus aureus* cells (Pansorbin) were purchased from Calbiochem/Behring. RPMI 1640 tissue culture medium, glutamine, penicillin,

and streptomycin were purchased from Gribco Laboratories. Newborn calf serum was purchased from Irvine Scientific and selenous acid (Specpure) was from Johnson Matthey, Inc.

Preparation and Characterization of Hybridomas. The generation and selection of hybridomas secreting anti-kinase monoclonal antibodies was performed as described in Erondu and Kennedy (1985, i.e., Chapter 3). To determine immunoglobulin class, the method of Ouchterlony (Moore et al., 1982) was used. Supernatants from hybridomas grown in serum-free media, and concentrated as described in Bennett et al. (1983, i.e., Appendix I), were pipetted into the central well of an Ouchterlony plate. Peripheral wells contained specific rabbit anti-mouse immunoglobulin subclass antibodies. These were allowed to diffuse and precipitin lines were formed. The plates were rinsed in phosphate buffered saline (PBS) before being stained with Coomassie Blue. Ascites fluids were prepared as described in Kennedy et al. (1983, i.e., Appendix II). These were partially purified by precipitation with 50% ammonium sulfate and dialyzed against 50 mM Tris-HCl, pH 7.4 containing 0.9% NaCl and 0.1% NaN_3 (TSA buffer). For the radioimmunoassay, the dialysate was further purified on a Protein A-Sepharose affinity column as described in Ey et al. (1978). The prominent IgG fraction eluted from the column is subsequently referred to as "Pr. A-purified" antibody. The concentrations of stock solutions of the "Pr. A-purified" antibodies were 0.8 mg/ml (4A4 and 4A11); 0.9 mg/ml (6E9); and 0.6 mg/ml (6G9). Protein was measured by the method of Peterson (1977) with bovine serum albumin as standard.

Inhibition of Kinase Activity by Monoclonal Antibodies. Pure kinase at a concentration of 0.2 mg/ml was diluted 20-fold into culture supernatant from each of the hybridomas. After an 8 hour incubation on ice, the mixtures were

assayed for synapsin I kinase activity by the TCA method as described in Bennett et al. (1983, i.e., Appendix I). In the control experiment, kinase was diluted into RPMI 1640 medium containing 20% calf serum.

Immunoblotting of Kinase with Monoclonal Antibodies. Pure kinase (500 ng per lane) was subjected to SDS/PAGE and transferred onto nitrocellulose by the method of Towbin et al. (1979). After the transfer, the nitrocellulose sheets were incubated with i) TSA buffer containing 5% hemoglobin (2 hr); (ii) partially purified ascites fluid diluted 1000-fold into TSA buffer containing 1% hemoglobin (8 hr); (iii) wash buffer (TSA buffer containing 0.5% hemoglobin) (1 hr with three changes); (iv) affinity purified rabbit anti-mouse IgG 8 μg/ml in wash buffer (2 hr); (v) wash buffer (1 hr with three changes); (vi) ^{125}I-labeled protein A (2-3 × 10^5 cpm/ml) in wash buffer (2 hr); (vii) wash buffer (1 hr with three changes). The nitrocellulose sheets were dried, and bands containing ^{125}I were detected by autoradiography.

Confirmation of the subunit composition of Type II CaM kinase. 15 μg of purified Type II CaM kinase was added to 30 μg of "Pr.A-purified" hybridoma 4A4 or 40 mM Tris-HCl (pH 7.5) in a volume of 0.1 ml and incubated overnight on ice. The resulting immune complexes were pelleted by centrifugation in the microfuge for 5 min. The protein in the supernatant was concentrated by precipitation with 10% trichloroacetic acid, dissolved in 1/3 diluted SDS stop solution (0.062 M Tris-HCl, pH 6.7 containing 3% SDS, 2% v/v β-mercaptoethanol, 5% w/v glycerol and a trace of bromophenol blue) and adjusted to neutral pH with NaOH. The pellets were washed twice with phosphate-buffered saline, dissolved in 1/3 diluted SDS stop solution, and, together with the supernatants subjected to SDS polyacrylamide gel electrophoresis (SDS/PAGE).

Radioiodination of Type II CaM Kinase. Bolton-Hunter procedure: Pure forebrain Type II CaM kinase was radioiodinated by the Bolton-Hunter procedure (Bolton and Hunter, 1973). 0.1 mCi of ^{125}I labeled N-succinimidyl-3-(4-hydroxyphenyl) propionate [^{125}I labeled Bolton-Hunter reagent (2000 Ci/mmol supplied in benzene by Amersham)] was evaporated to dryness under a stream of nitrogen. 10 µg of pure forebrain Type II CaM kinase in 10 µl of 0.1 M borate buffer (pH 8.5) were added to the residue. The reaction mixture was agitated for 15 min at 0°C. Thus, 3-4 mol of labeled ester was reacted per mole of kinase. Excess ^{125}I labeled Bolton-Hunter reagent was reacted with 0.5 ml of 0.2 M glycine in 0.1 M borate buffer (pH 8.5) for 5 min at 0°C. ^{125}I labeled kinase was separated from free ^{125}I labeled Bolton-Hunter reagent and its glycine conjugate by chromatography on a Sephadex G-25 column (0.8 × 20 cm) previously equilibrated with 40 mM Tris, 1 mM imidazole buffer (pH 7.5) containing 0.25% (w/v) gelatin, 10% (v/v) glycerol, 1 mM $MgCl_2$, 2 mM DTT, 0.1 mM PM SF, 1 mg/liter leupeptin, 25 mg/liter soybean trypsin inhibitor, and 0.2% NaN_3. Kinase radioiodinated in this manner routinely incorporated 1 mol of ^{125}I/3 mol protein resulting in a specific radioactivity of approximately 700 Ci/mmol.

Immediately prior to each assay, the ^{125}I-labeled kinase was precleared with SAC (*Staphylococcus aureus* cells, prepared as described below). This was accomplished by adding an equal volume of washed SAC to ^{125}I-labeled kinase. Enough volume of 1X RIA buffer (50 mM Tris, pH 7.4 containing 5 mM EDTA and 1mg/ml Ig free BSA) was added to cause a 10-fold dilution of the stock ^{125}I-labeled kinase. After a 30 min incubation on ice, the mixture was centrifuged for 5 min in a microfuge. The supernatant containing "precleared" ^{125}I-labeled kinase was saved and appropriate amounts used in the radioimmunoassays.

Chloramine-T procedure: 5-10 µg pure forebrain Type II CaM kinase were radioiodinated by the chloramine-T procedure as described by Fryxell et al. (1983) and desalted as described above. Chloramine-T labeled kinase had a specific radioactivity of 400 to 7000 Ci/mmol, depending on the amount of chloramine-T used.

Preparation of SAC. SAC was prepared as described in Kessler (1975). The formalin-fixed SAC was spun at 2000 g for 20 min in a microfuge at 4°C. It was incubated in 0.5% NP-40 in NET buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris, and 0.02% NaN_3 pH 7.4) for 15 min at 23°C. After centrifugation, it was washed once in 0.05% NP-40 in NET and resuspended in the original volume in 0.05% NP-40 NET containing 1 mg/ml BSA.

Effect of Detergent on the Precipitation of ^{125}I-Labeled Kinase by Monoclonal Antibodies. 10 ng of "precleared" ^{125}I-labeled kinase was incubated with a fixed amount (3 to 5 µg) of monoclonal antibody in the presence of varying amounts of ionic (SDS) and non-ionic (Triton X-100) detergents. The assays were conducted in a final volume of 75 µl with the addition of 15µl of 5X RIA buffer. At the end of the 12-hour incubation on ice, affinity purified rabbit anti-mouse IgG antibodies were added and the mix was incubated for another hour. Washed SAC was added and the incubation continued for another 30 min. The reaction mixture was spun for 5 min in a microfuge. Aliquots of the supernatant were saved for counting; the pellet was washed twice with NET buffer and counted in a gamma counter.

Generation of Antibody Dilution Curves. The assays were performed as described above except for the following modifications. The concentration of monoclonal antibody was varied. Triton X-100 at a final concentration of 0.05% (v/v) was the only detergent used in the assays with hybridomas 4A11 and 6E9, while in the 6G9 assays, both Triton X-100 and SDS were present at final

concentrations of 0.5% (v/v) and 0.1% (v/v), respectively. The final volume of the assays was 100 µl with the addition of 20 µl 5X RIA buffer. Additional experiments were also performed in which the ^{125}I-labeled kinase was incubated with monoclonal antibody for 2 hr.

Generation of Standard Competitive Binding Curves. The assays were conducted as already described. The assay tubes contained increasing amounts (0 to 200 ng) of standard forebrain Type II CaM kinase, a constant amount of antibody, and a constant amount of ^{125}I labeled kinase. All the reaction mixtures were made in duplicate. The amount of ^{125}I labeled kinase was 10 ng (10,000 cpm). The amount of antibody was 10 µl of 1:10,000 dilution (4A11 and 6E9) or 1:2,000 dilution (6G9) and was sufficient to precipitate 35 to 45% of the labeled enzyme.

Blank tubes contained only ^{125}I-labeled kinase; i.e., both standard forebrain kinase and monoclonal antibody were omitted. The reaction mixture was incubated for 2 hr (4A11 and 6E9) and 12 hr (6G9) on ice before the addition of affinity purified rabbit anti-mouse IgG antibodies. After 1 hr, washed SAC was added and the incubation continued for another 30 min. Immune complexes were pelleted in a microfuge, washed and counted. The data were plotted on both a semilog scale and a log-logit scale.

Effect of Autophosphorylation of the Kinase on the Standard Competitive Binding Curves. The assays were performed as described above except that autophosphorylated kinase was used as kinase standard. Autophosphorylation of kinase was carried out as described in Erondu and Kennedy (1985, Chapter 3, this thesis) except that the reaction was terminated after varying lengths of time by adding EDTA to a final concentration of 100 mM. To determine the degree of phosphorylation, parallel experiments were performed with radioactive ATP. ^{32}P-labeled subunits were localized in gels by autoradiography, cut out, and their

radioactivity was determined by liquid scintillation spectrometry. Control (non-phosphorylated) kinase was generated by omitting calcium from the phosphorylation mix.

Results

Characterization of Monoclonal Antibodies. The generation and selection of monoclonal antibodies directed against the Type II CaM kinase are described in Chapter 3. In order to maximize the usefulness of these reagents, some of their immunological properties had to be determined.

Hybridomas 4A11, 6E9 and 6G9 were shown to be of the IgG1 subclass by Ouchterlony double-diffusion against subclass specific rabbit anti-mouse antisera. The same technique revealed that hybridoma 4A4 is of the IgG3 subclass. The chromatographic behavior on Pr. A-Sepharose of all four monoclonal antibodies was consistent with this classification: 4A11, 6E9 and 6G9 eluted at pH 6.0, while 4A4 eluted at pH 4.5 (Ey et al., 1978).

The ability of each hybridoma supernatant to inhibit enzyme activity was tested. RPMI 1640 medium plus 20% calf serum served as control. As shown in Table I, hybridomas 4A4, 4A11 and 6E9 are inhibitory, while 6G9 is not. It should be noted that the data presented in Table I are semiquantitative, since the concentration of antibody in each clone supernatant was not determined. Quantitative studies on the inhibition of kinase activity by known concentrations of antibody have since been performed in the laboratory (V. Jennings, unpublished studies). These experiments confirmed the inhibitory activities of 4A4, 4A11 and 6E9.

I used the immunoblot technique (Towbin et al., 1979) as a means of determining which subunit(s) of the kinase is/are recognized by the hybridomas. It should be pointed out that this technique requires that the epitope recognized by

the hybridoma survive denaturation or be exposed after denaturation of kinase. As Figure 1 illustrates, 4A4 and 6G9 react with a single polypeptide band. This band has been shown to be the α-subunit of the kinase (Fig. 2, Appendix II). This property of antibody 6G9 has been used to show the immunochemical relationship between the α-subunit of the kinase and the major postsynaptic density protein (Fig. 1, Appendix II). In addition, 6G9 was used to develop a solid-phase radioimmunoassay for the α-subunit of the kinase (Chapter 3).

Both α and β-subunits of the kinase were co-precipitated from solution by hybridoma 4A4 (Fig. 2). This provides evidence that both subunits are part of the same holoenzyme complex. Antibody 6E9 has also been used previously in such an experiment (Fig. 5, Appendix I). In the 6E9 experiments, control monoclonal antibody did not precipitate either of the kinase subunits.

Table II summarizes the properties of the four hybridomas I have characterized. Three of these hybridomas (4A11, 6E9 and 6G9) have been used in attempts to develop liquid-phase radioimmunoassays for the Type II CaM kinase. These assays would complement the 6G9 solid-phase radioimmunoassay (Chapter 3) in a number of ways: (1) they would measure the holoenzyme, not the α-subunit; (2) they would allow the assay of more samples in a shorter period of time; and (3) they would require smaller quantities of the reagents.

Radioimmunoassay. There are three basic steps involved in the development of a radioimmunoassay: (1) the preparation of a radiolabeled ligand that would be used to "trace" the distribution of the standard between bound and free phases, (2) determination of the optimal concentration of binder (in this case, antibody) that would be used in the assay, and (3) generation of standard curves. The results of my experiments will be presented along these lines.

Radioiodination of Type II CaM Kinase. The goal is to obtain ^{125}I labeled kinase that is of sufficiently high specific radioactivity and still retains its reactivity with the antibodies. A variety of methods are available for the radioiodination of proteins (Hunter, 1974). These methods can be broadly divided into two groups--conjugation methods and direct methods. An example of the conjugation method is the Bolton-Hunter procedure, where a radioiodinated moiety [^{125}I labeled N-succinimidyl-3-(4-hydroxyphenyl) propionate] is conjugated to the ε-amino group of lysine or N terminus of the protein to be labeled (Bolton and Hunter, 1973). Kinase radioiodinated by this procedure routinely incorporated 1 mol of ^{125}I per 3 mol protein resulting in a specific radioactivity of approximately 700 Ci/mmol. More importantly, the ^{125}I labeled kinase could be precipitated by the monoclonal antibodies. Furthermore, the ^{125}I labeled kinase was relatively stable and could be stored up to four weeks at -80°C without any significant damage. Figure 3 illustrates that both α and β-subunits are radioiodinated by this procedure.

An example of the direct method of radioiodination is the chloramine T procedure, where radioactive iodide is oxidized to the more reactive iodine, which is then directly incorporated into tyrosine (and occasionally histidine and phenylalanine) residues of the protein (Hunter and Greenwood, 1962; Greenwood et al., 1963). With this method, kinase could be labeled to very high specific radioactivities (up to 7000 Ci/mmol); but this also led to fragmentation of the ^{125}I labeled kinase. When the amount of chloramine T was reduced, kinase with lower specific radioactivity was obtained. However, despite the reduction in the amount of radioactive iodine incorporated, the ^{125}I labeled kinase did not retain its reactivity with the monoclonal antibodies. The "iodination damage" observed here is not uncommon with the chloramine T method (Fang et al., 1975; Chafouleas et al., 1979). The damage could have been caused by any one or a

combination of the following--the radioiodide solution itself, which comes in 0.1 N sodium hydroxide; chloramine T, which is an oxidizing agent; the incorporation of iodine into a sensitive site on the protein. In conclusion, kinase labeled by the Bolton-Hunter procedure seemed preferable for use in developing the radioimmunoassays.

Effect of Detergent on the Precipitation of ^{125}I Labeled Kinase by Monoclonal Antibodies. A difficulty with radioimmunoassay of Type II CaM kinase is that the enzyme is present in both soluble and particulate fractions of brain homogenates. While some of the particulate kinase can be solubilized by dilution into a low ionic strength buffer (Kennedy et al., 1983b), complete solubilization requires a strong detergent such as SDS. Therefore, for a radioimmunoassay to be useful in measuring total kinase in brain homogenates, it should not be sensitive to ionic detergents such as SDS. Another reason to have detergents in radioimmunoassays is the reduction of non-specific adherence of the labeled protein to surfaces. Unfortunately, even low concentration of SDS could disrupt antigen-antibody complexes (Dimitriadis, 1979). However, in certain cases, a fivefold excess of non-ionic detergents "by virtue of mixed micelle formation" greatly reduces the disruptive effects of SDS (Dimitriadis, 1979; Goelz et al., 1981).

The ability of a large excess of monoclonal antibody to precipitate ^{125}I kinase in the presence of varying concentrations of SDS, an ionic detergent and Triton X-100 a non-ionic detergent was tested as described under "Methods." In the absence of detergents, 4A11 and 6E9 precipitated about 75% of added ^{125}I kinase. As Figure 4A and B illustrate, they were both sensitive to SDS, and the disruptive effect of SDS was reduced only slightly by the addition of

Triton X-100 in A and not reduced at all in B. Triton X-100, alone, did not affect the stability of the ^{125}I kinase-antikinase complex and was, therefore, included in subsequent assays at a final concentration of 0.05% (v/v).

In the absence of detergent, hybridoma 6G9 precipitated only 32% of added ^{125}I kinase and the proportion of precipitated kinase increased in the presence of detergent. A possible explanation for this behavior of 6G9 is that radioiodination may have made the epitope it recognizes somewhat accessible and that the detergents caused a further exposure of the epitope. This explanation is necessary since 6G9 does not precipitate native kinase. As Figure 4C illustrates, the ability of 6G9 to precipitate ^{125}I kinase decreases with increasing concentrations of SDS. However, the disruptive action of SDS is prevented in the presence of a fivefold excess of Triton X-100. Therefore, in subsequent assays with 6G9, both SDS and Triton X-100 were present at concentrations of 0.1% (v/v) and 0.5 (v/v), respectively.

Antibody Dilution Curves. The goal is to determine an optimal antibody concentration to be used in generating a standard curve. This choice is made such that small changes in the concentration of the unlabeled (standard) ligand will produce significant changes in the proportion of labeled ligand bound. As described under "Methods," the generation of the antibody dilution curves involved the incubation of a fixed amount of ^{125}I-kinase with different concentrations of each monoclonal antibody. The assays with hybridoma 4A11 or 6E9 contained 0.05% (v/v) Triton X-100 while the assays with hybridoma 6G9 contained SDS, 0.1% (v/v) and Triton X-100, 0.5% (v/v). The amount of ^{125}I kinase precipitated under each experimental condition is determined as described in "Methods." The data obtained with 4A11 and 6E9 were pooled and plotted on a semilog scale (Fig. 5A), while the data obtained with 6G9 are shown in Figure 5B,

also on a semilog scale. The concentrations of antibodies chosen from these curves were sufficient to precipitate 35-45% of added ^{125}I kinase. This was 10 µl of 1:10,000 dilution (4A11 and 6E9) and 10 µl of 1:2,000 dilution for 6G9.

Generation of Standard Curves. The assays were performed as described under "Methods." The 4A11 and 6E9 assays exhibited a sensitivity of 2 ng (3 fmol) while the 6G9 assay exhibited a sensitivity of 20 ng (30 fmol) $P < 0.05$. The basic principle is drawn from the Law of Mass Action and could be stated thus: in the presence of a fixed amount of antibody, the proportion of kinase bound (relative to free kinase or relative to total kinase) will decrease with increasing amounts of kinase present in the assay. It should be noted that the ^{125}I labeled kinase simply helps to monitor this distribution of bound and free kinase.

There are a number of ways of plotting the data generated from these assays. Most result in nonlinear plots as shown in Figures 6A, 6B, and 8A for hybridomas 4A11, 6E9 and 6G9, respectively. Since unknowns can only be reliably computed from the linear portions of the curves; these nonlinear plots limit the useful range of radioimmunoassays. For this reason, several methods have been developed to linearize radioimmunoassay curves (Rodbard et al., 1969). One such method is the logit transformation shown in Figures 7A, 7B, and 8B for antibodies 4A11, 6E9 and 6G9, respectively. Logit B is defined as $\text{Log}_e\left(\frac{B}{100-B}\right)$, where B is bound tracer expressed as a percent of that bound in the absence of unlabeled standard. It can be shown that in radioimmunoassays of the type developed here (saturation analysis, i.e., antibody saturated with ligand), that a plot of Logit B against log [standard] is of the form $y = mx + c$ where $y \equiv$ logit B; $x \equiv$ log [standard] and m is negative slope.

Effect of Autophosphorylation on 4A11 and 6E9 Standard Curves. Because some antibodies distinguish between "phospho" and "dephospho" forms of a phosphoprotein (Nairn et al., 1982; Sternberger and Sternberger, 1983), I examined

the effect of autophosphorylation of kinase on the standard curves of hybridomas 4A11 and 6E9. It should be noted that antibody 6G9 recognizes both "phospho" and "non-phospho" α-subunit of the kinase on immunoblots (Chapter 3). The effect of autophosphorylation was studied by using kinase containing an average of 0, 8, 20 and 35 mols phosphate/mol holoenzyme as standard kinase in the radioimmunoassays (see "Methods"). The results are shown in Figure 9. They reveal that the "affinity" of antibody 4A11 for kinase diminishes with increasing degree of phosphorylation as reflected by the rightward shift of the standard curves (Fig. 9A). On the other hand, the curves of antibody 6E9 with phospho-kinase (irrespective of degree of autophosphorylation) were flat (Fig. 9B), suggesting that 6E9 does not recognize the "phospho" form of the kinase at all. It may be interesting to repeat these assays with kinase that has even fewer mols phosphate per mol holoenzyme, since both the autophosphorylation induced switch in enzyme activity (Miller and Kennedy, 1986) and autophosphorylation induced increase in association of kinase with microtubules, and F-actin (Chapter 4) require only about 4 mols phosphate/mol holoenzyme.

Discussion

In view of the large number of calcium target proteins identified thus far, it has become necessary to generate specific reagents that would help elucidate the role of each of these proteins in calcium-regulated physiological processes. The generation of a panel of monoclonal antibodies against the Type II Ca^{2+}/calmodulin-dependent protein kinase will meet part of this need. The usefulness of these reagents is reflected in the properties of the four hybridomas characterized in the work reported here.

Hybridomas 4A4 and 6E9 have been used to confirm the subunit composition of the kinase. Another precipitating antibody, 4A11, was used in the immunoprecipitation of *in vitro* translated kinase subunits during the isolation of cDNA clones coding for the β-subunit of the kinase (Bennett, 1986). The ability of 6G9 to recognize the α-subunit on immunoblots has been used in two ways: (1) To demonstrate the immunochemical identity of the α-subunit of the kinase with the major postsynaptic density protein. (2) The development of a solid-phase radioimmunoassay used to study the distribution of the α-subunit in rat brain.

The inhibiting antibodies could be useful in probing the function of the kinase *in vivo* and even in reconstituted systems. For example, it has been suggested that phosphorylation of synapsin I by Type II CaM kinase reduces the affinity of synapsin I for synaptic vesicles, and this somehow leads to enhancement of transmitter release in the squid giant synapse (Llinas et al., 1985). Microinjection of these antibodies could help determine if kinase, in fact, plays a role in this phenomenon.

Though the liquid phase radioimmunoassays developed were not used in any studies because of their sensitivity to SDS, they revealed the feasibility of developing assays that are specific for either phosphorylated, non-phosphorylated, or both forms of the kinase. Such a combination of assays could be used to determine the proportion of kinase that is in the phosphorylated or non-phosphorylated form *in vivo* and also determine if this proportion changes under a number of physiological conditions. This would be especially useful in view of recent findings that autophosphorylation of the kinase induces a switch in enzyme activity (Miller and Kennedy, 1986) and results that suggest that autophosphorylation regulates the subcellular associations of the kinase (Chapter 4).

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Table I

Inhibition of Type II CaM kinase by monoclonal antibodies.

Hybridoma	Synapsin I kinase activity (%)
RPMI plus 20% Calf Serum	100
4A4	30
4A11	22
6E9	53
6G9	108

Table II

Summary of some properties of the hybridomas.

Hybridoma	Type	Subunit Specificity on Immunoblot	Inhibition of Kinase Activity	Precipitation of Kinase
4A4	IgG3	α	+	+
4A11	IgG1	-	+	+
6E9	IgG1	-	+	+
6G9	IgG1	α	-	-[a]

[a]While 6G9 does not recognize native kinase, it does bind radioiodinated kinase, and this binding is enhanced by detergents (see "Results").

Fig. 1. Recognition of kinase by monoclonal antibodies on immunoblots.

Pure Type II CaM kinase (500 ng per lane) was subjected to SDS/PAGE and transferred to nitrocellulose paper (each lane to a separate piece of nitrocellulose paper). Each piece of nitrocellulose paper was reacted with a different hybridoma by the immunoblot method as described under "Methods."

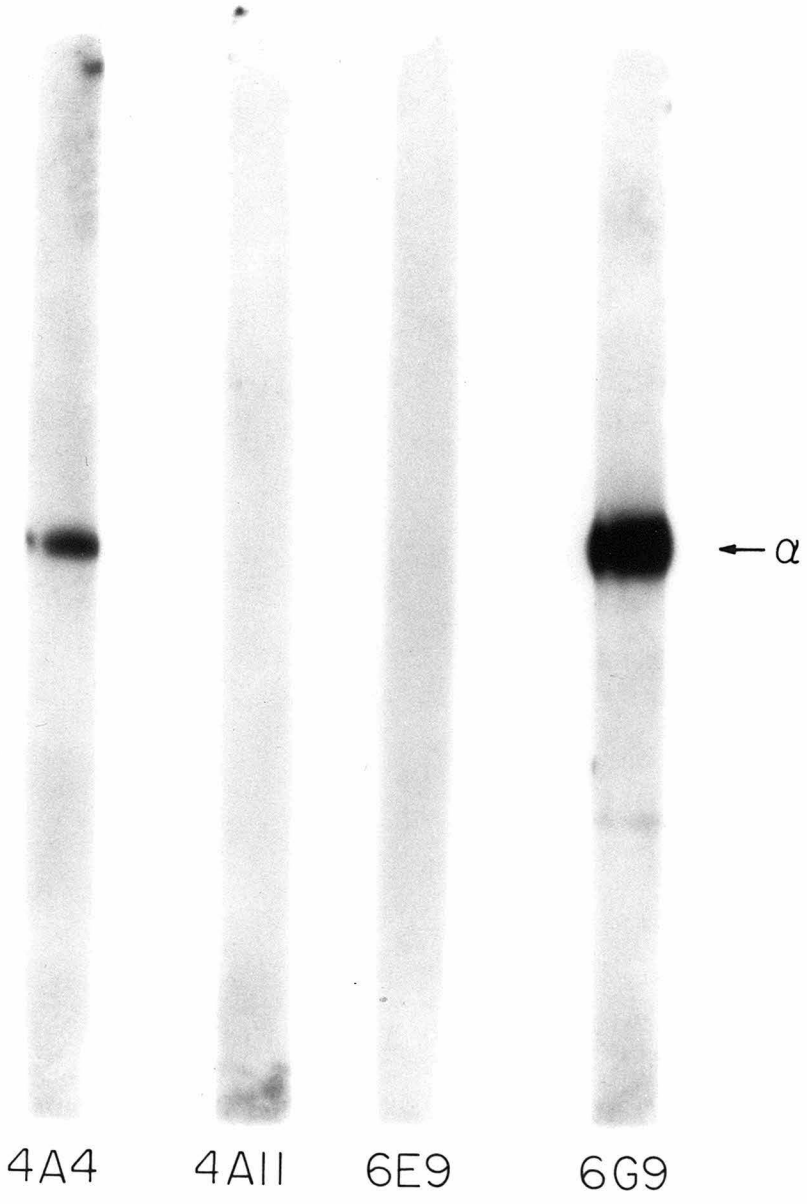

α
4A4
4AII
6E9
6G9

Fig. 2. Immunoprecipitation of kinase by antibody 4A4.

Immunoprecipitation of pure kinase by monoclonal antibody 4A4 was performed as described under "Methods." The protein staining patterns of the supernatant (S) and pellet (P) from incubations containing kinase alone, kinase plus 4A4 and 4A4 alone are shown. IgG-H and IgG-L refer to the heavy and light chains of hybridoma 4A4, respectively.

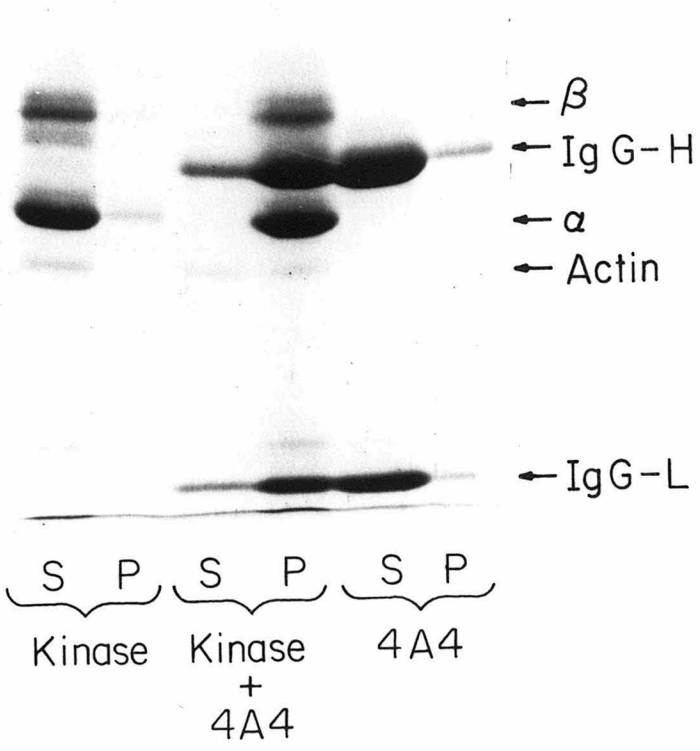

β
Ig G-H
α
Actin
IgG-L
S P
S P
S P
Kinase
Kinase + 4A4
4A4

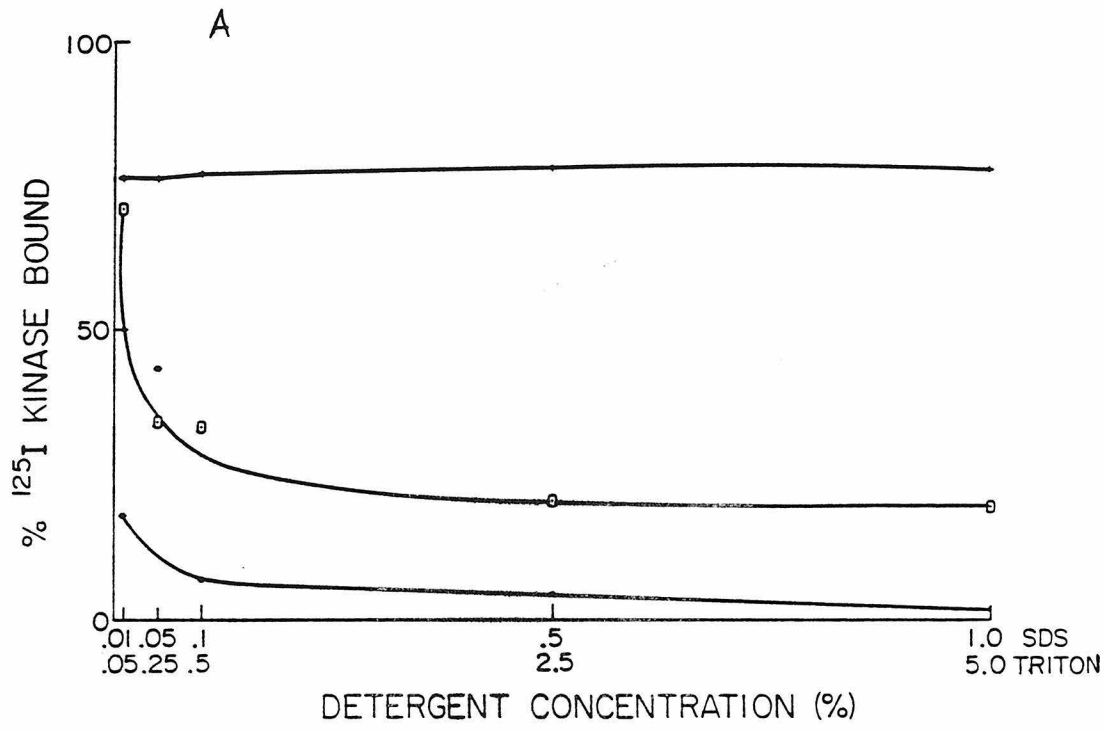
Fig. 3. SDS/PAGE of ^{125}I-labeled kinase.

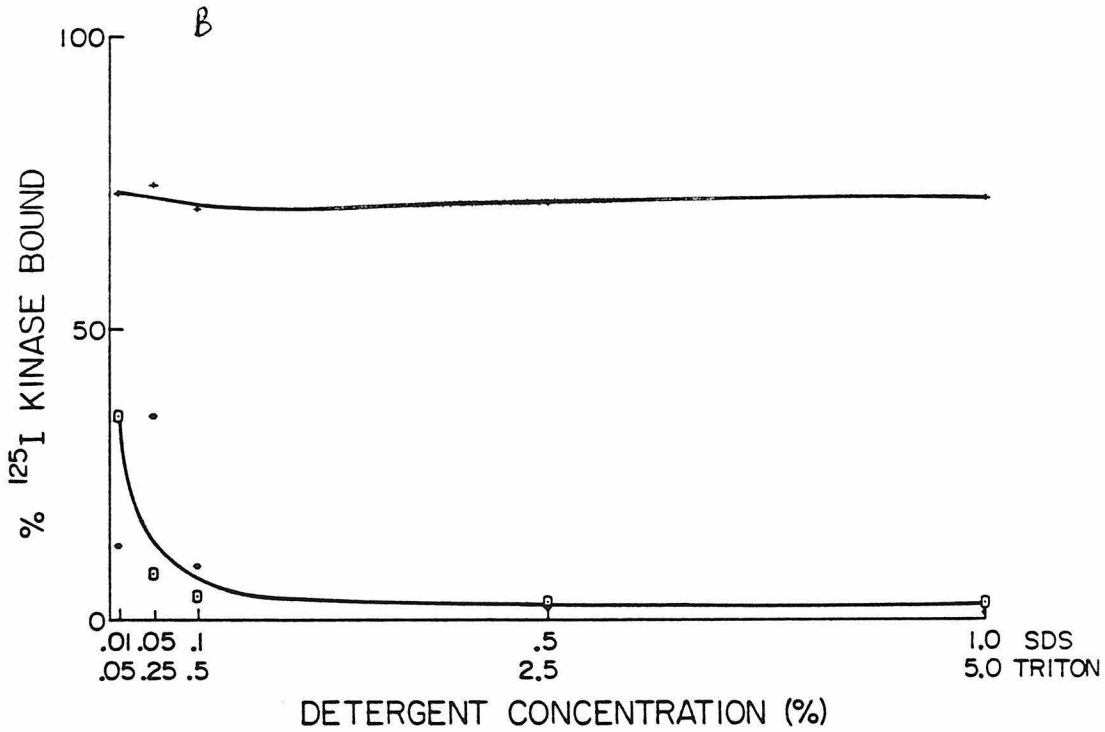
Pure kinase was radioiodinated by the Bolton-Hunter procedure and desalted on a Sephadex G-25 column as described under "Methods." An aliquot of the purified ^{125}I-labeled kinase was subjected to SDS PAGE and ^{125}I-labeled subunits detected by autoradiography.


β
α

Fig. 4. Effect of various concentrations of SDS (*_*), Triton X-100 (+_+) and a mixture of both detergents (□_□) on ^{125}I kinase -antibody reaction.

10 ng of "precleared" ^{125}I-labeled kinase (10,000 cpm) was incubated with a large excess of antibody 4A11 (A), antibody 6E9 (B) or antibody 6G9 (C) in the presence of increasing amounts of SDS, Triton X-100 or a mixture of both. After the additional incubations with rabbit anti-mouse IgG antibodies and washed SAC, the immune complexes were pelleted and the counts/min in both the supernatant and pellet were determined. The total counts in the pellet as a % of the added counts is plotted as a function of detergent concentration. In the absence of both detergents, 4A11 and 6E9 precipitated approximately 75% of added ^{125}I kinase, while 6G9 precipitated 32% of added ^{125}I kinase.


A
100
50
0
% 125I KINASE BOUND
.01 .05 .1 .5 1.0 SDS
.05 .25 .5 2.5 5.0 TRITON
DETERGENT CONCENTRATION (%)


B
100
50
0
% 125I KINASE BOUND
.01 .05 .1 .5 1.0 SDS
.05 .25 .5 2.5 5.0 TRITON
DETERGENT CONCENTRATION (%)

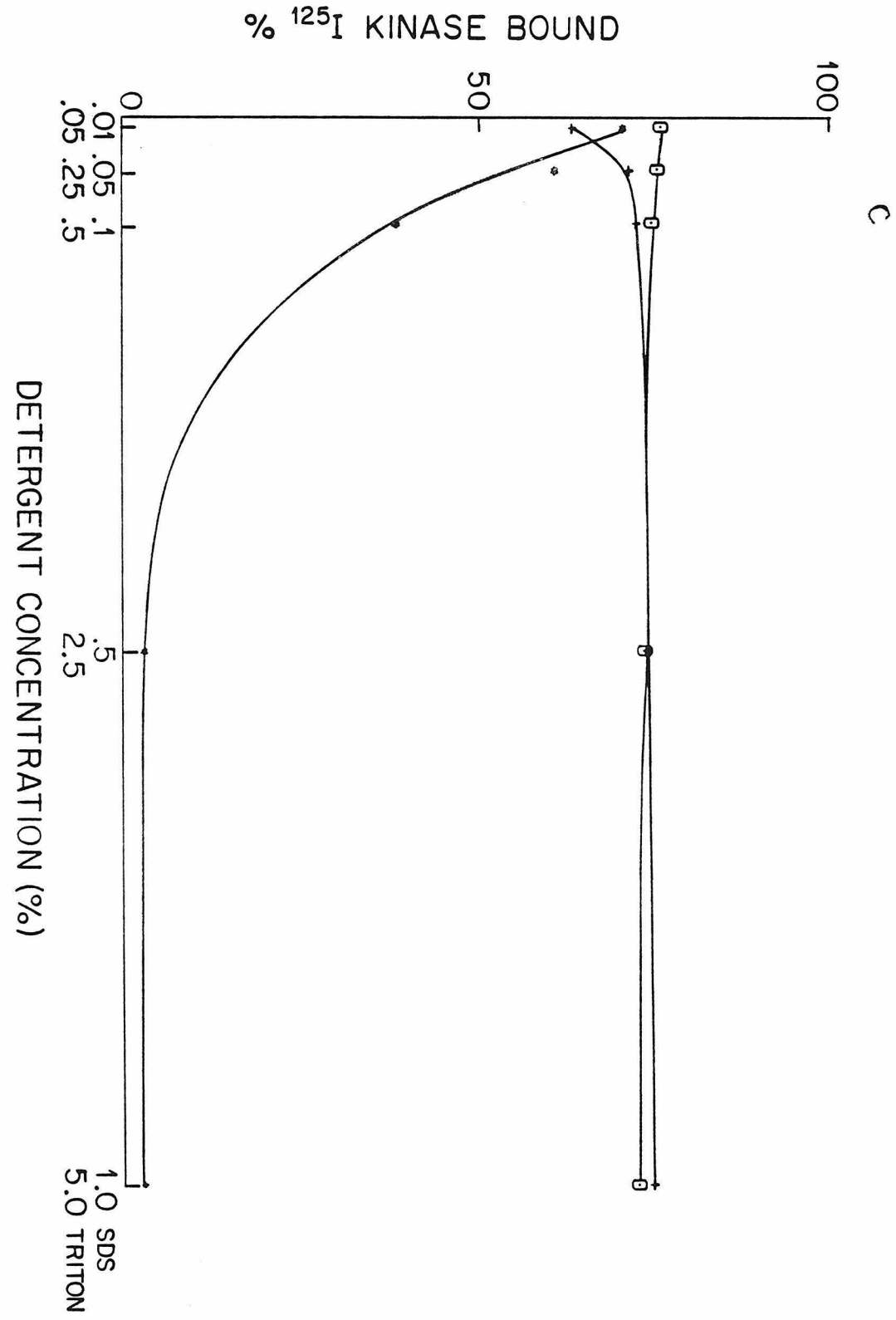
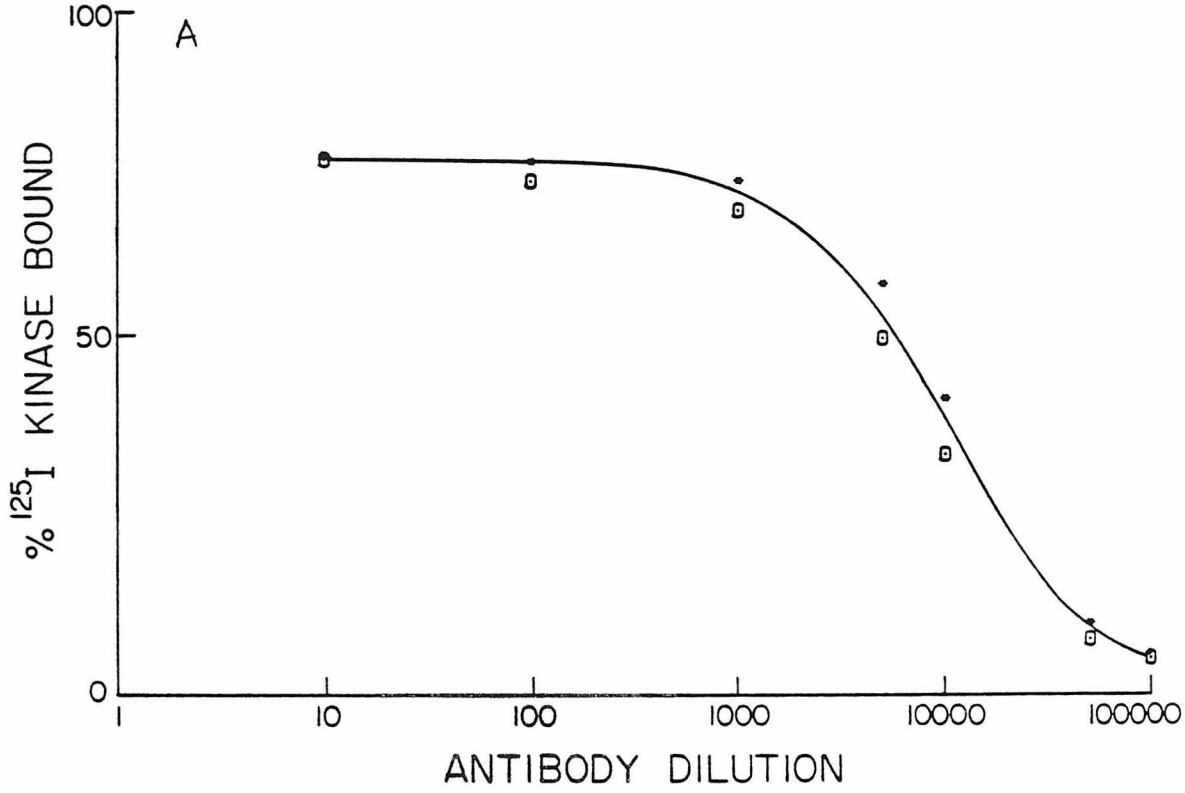

% 125I KINASE BOUND
100
50
0
C
0
.01
.05
.25
.5
1
.5
2.5
1.0
5.0
SDS
TRITON
DETERGENT CONCENTRATION (%)

Fig. 5. Antibody dilution curves. A. 4A11 (*) and 6E9 (⊡); B. 6G9.

The dilution profile was obtained by incubating the appropriate dilutions of each monoclonal antibody with a fixed concentration of ^{125}I kinase (10,000 cpm) in the presence of 0.05% (v/v) Triton X-100 (A); and both Triton X-100, 0.5% (v/v) and SDS, 0.1% (v/v) (B). The samples were then processed as described under "Methods" to determine the % of added kinase that bound to the antibodies.


A
100
50
0
% 125I KINASE BOUND
1
10
100
1000
10000
100000
ANTIBODY DILUTION

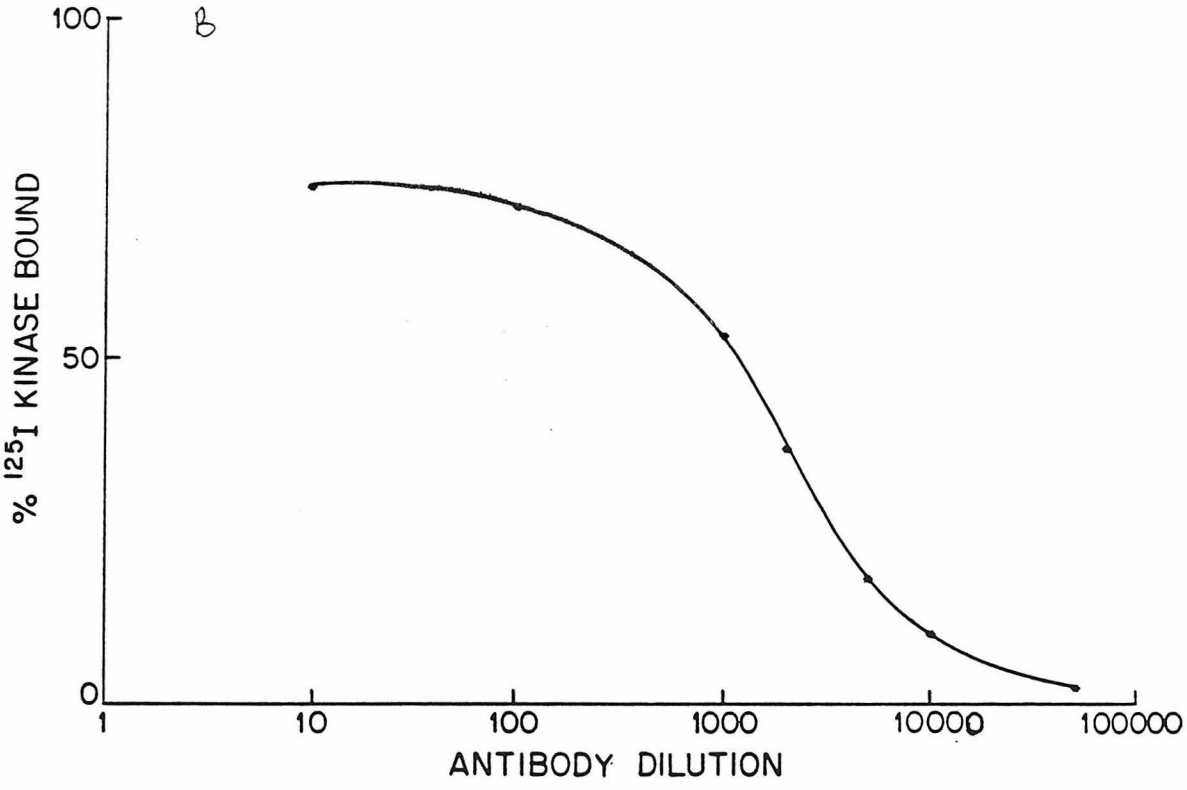
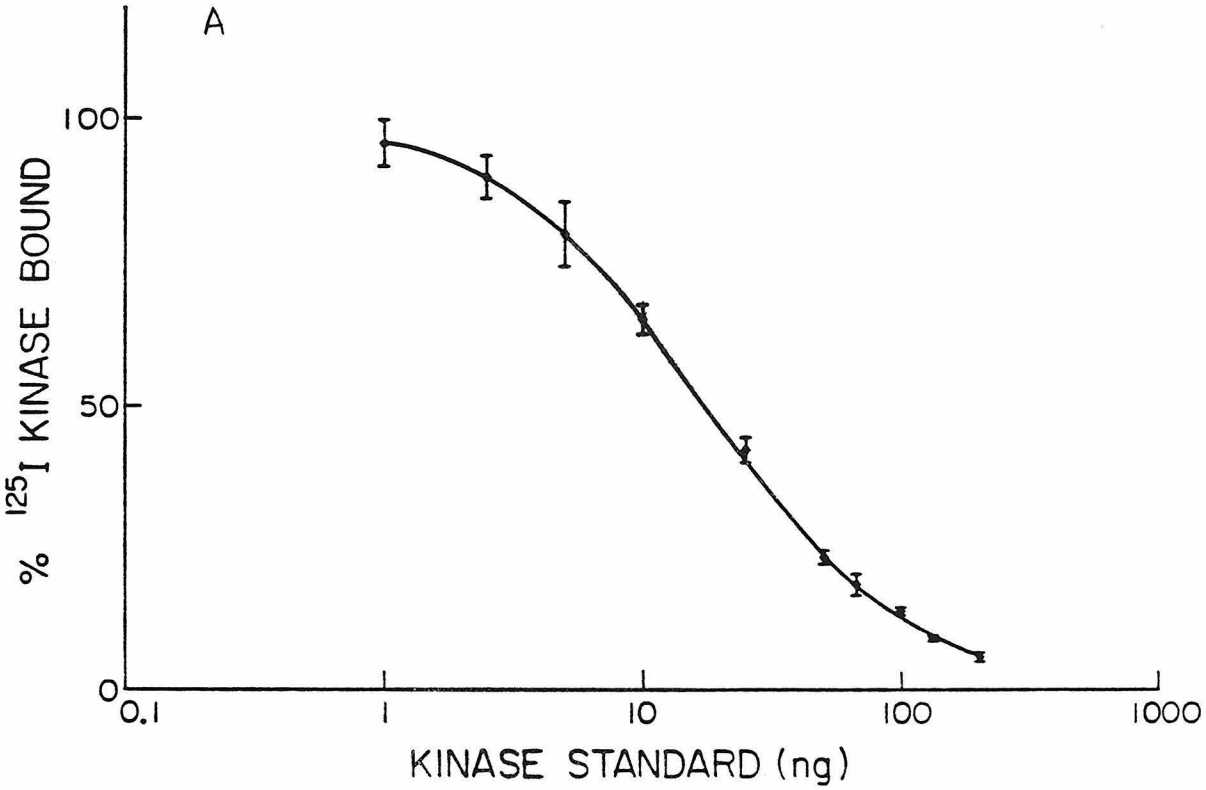

B
100
50
0
% 125I KINASE BOUND
1
10
100
1000
10000
100000
ANTIBODY DILUTION

Fig. 6. Standard curves for 4A11 (A) and 6E9 (B).

The assay tubes contained increasing amounts (0-200 ng) of standard (unlabeled) forebrain Type II CaM kinase, a constant amount of ^{125}I-labeled kinase (10,000 cpm), 0.05% (v/v) Triton X-100 and a constant amount of monoclonal antibody. This amount of antibody was sufficient to precipitate 35 to 45 % of ^{125}I kinase in the absence of standard (unlabeled) kinase and was determined from the antibody dilution curve (Fig. 5A). The assay tubes were processed as described under "Methods" to determine the amount of ^{125}I kinase bound by antibody in the presence of varying amounts of standard kinase. This is expressed as % of ^{125}I kinase bound in the absence of standard kinase and the data plotted on a semilog scale. Nonspecific background (< 200 cpm) was determined by processing assay tubes that contained ^{125}I kinase but no antibody or standard kinase, and this value was subtracted from total counts bound.


A
100
50
0
% ^{125}I KINASE BOUND
0.1
1
10
100
1000
KINASE STANDARD (ng)

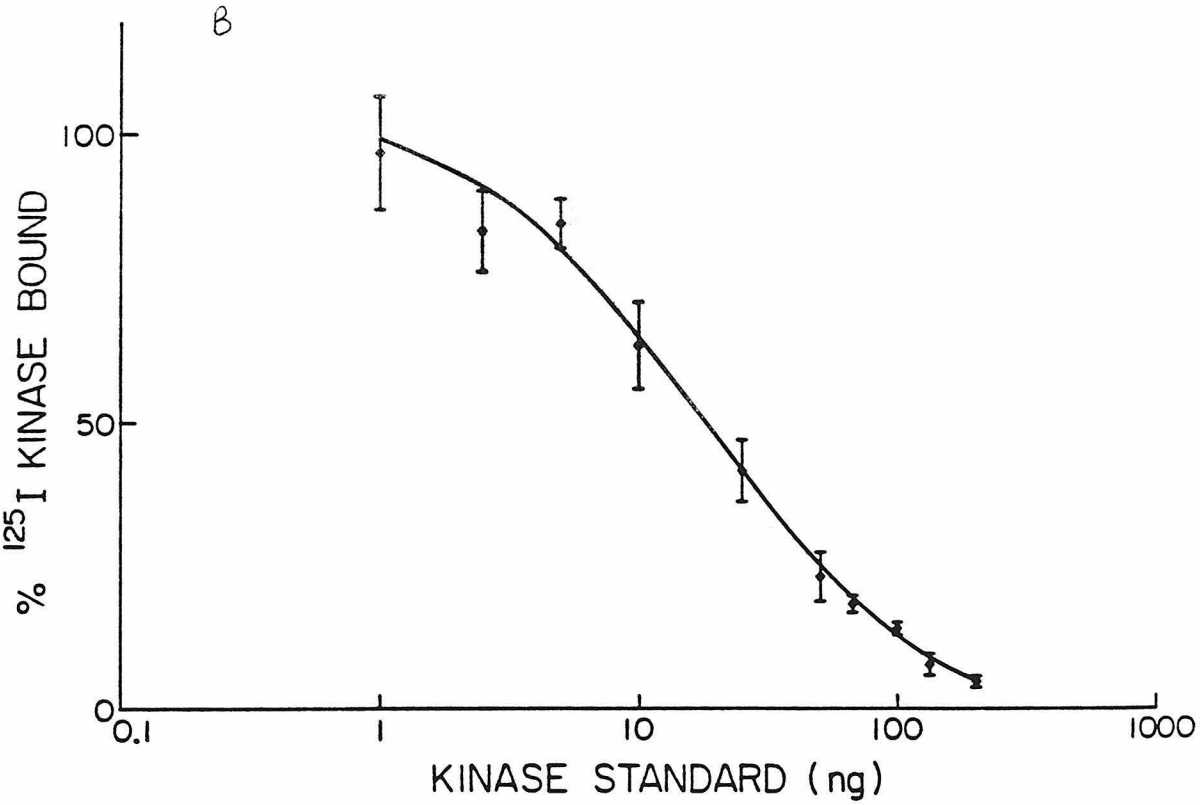
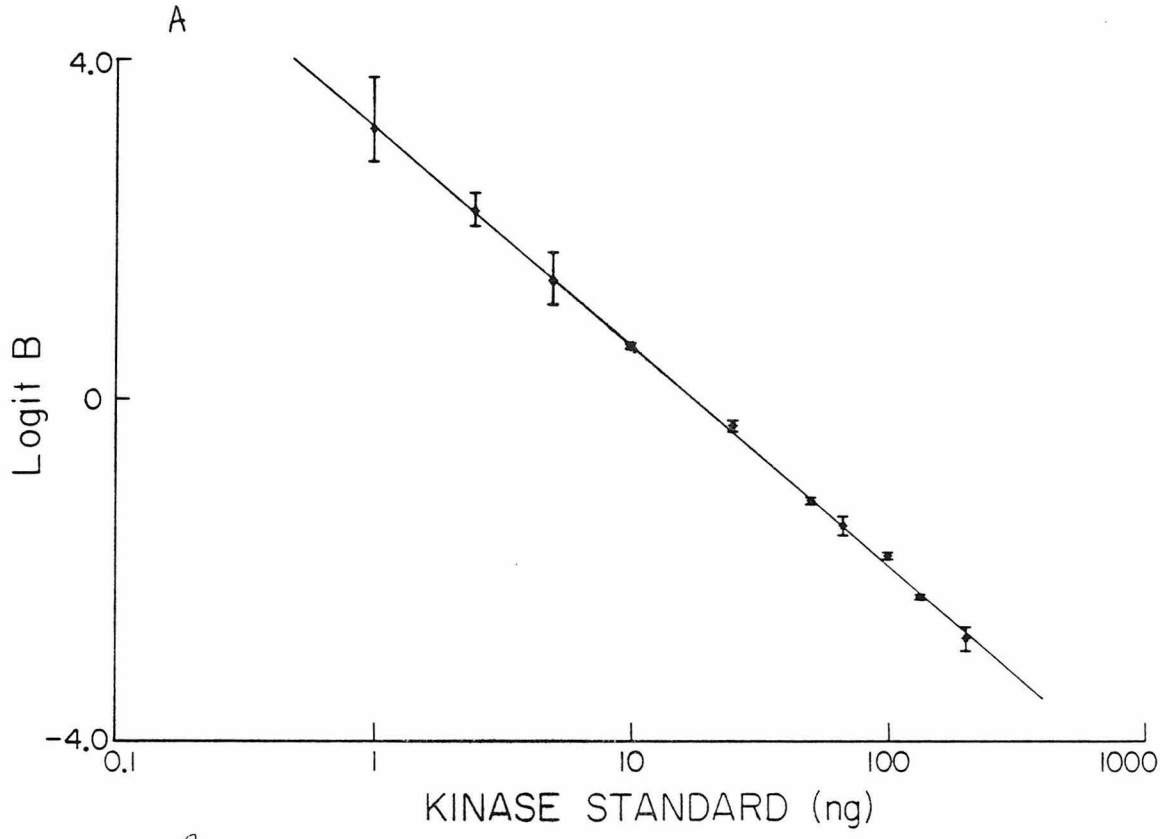

B
100
50
0
% ^{125}I KINASE BOUND
0.1
1
10
100
1000
KINASE STANDARD (ng)

Fig. 7. Standard curves for 4A11 (A) and 6E9 (B) assays.

The data shown in Fig. 6 have been replotted on a log-logit scale. Logit B = $\text{Log}_e \left(\frac{B}{100-B}\right)$, where B is % Bound as defined in Fig. 6.


A
4.0
0
-4.0
Logit B
0.1
1
10
100
1000
KINASE STANDARD (ng)

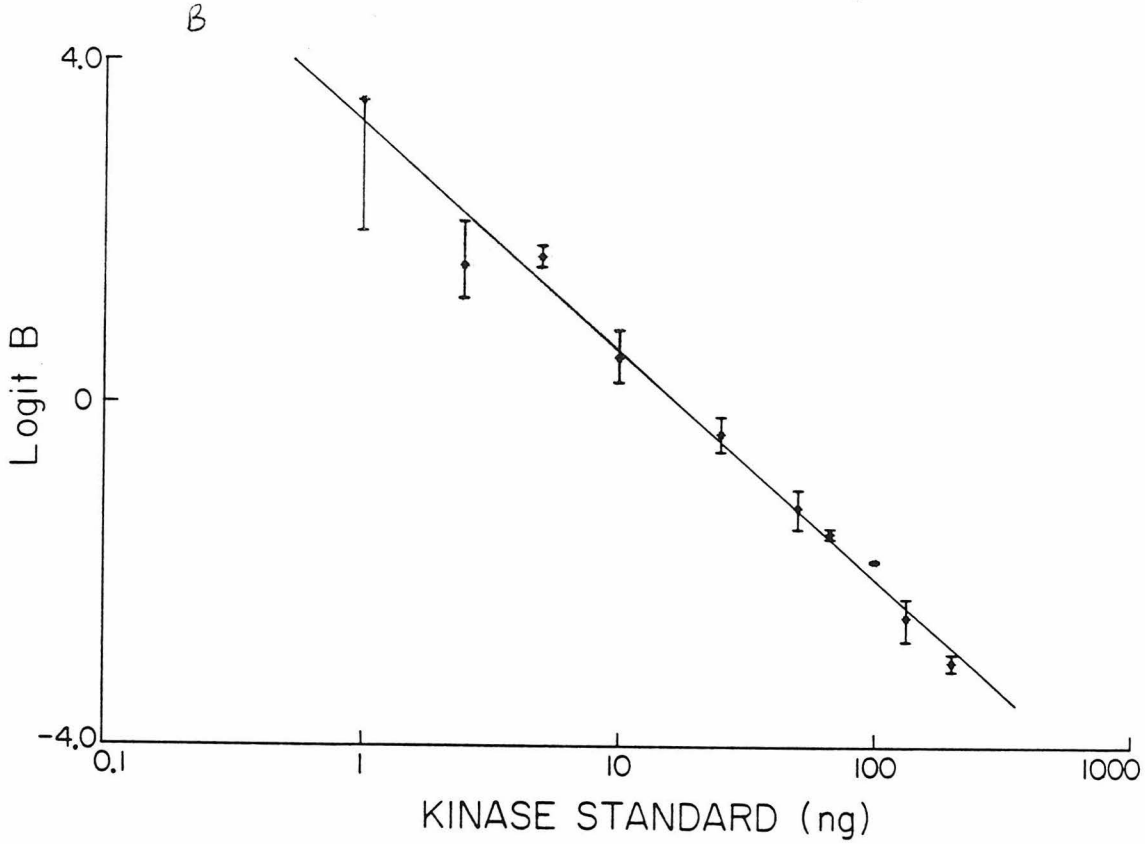
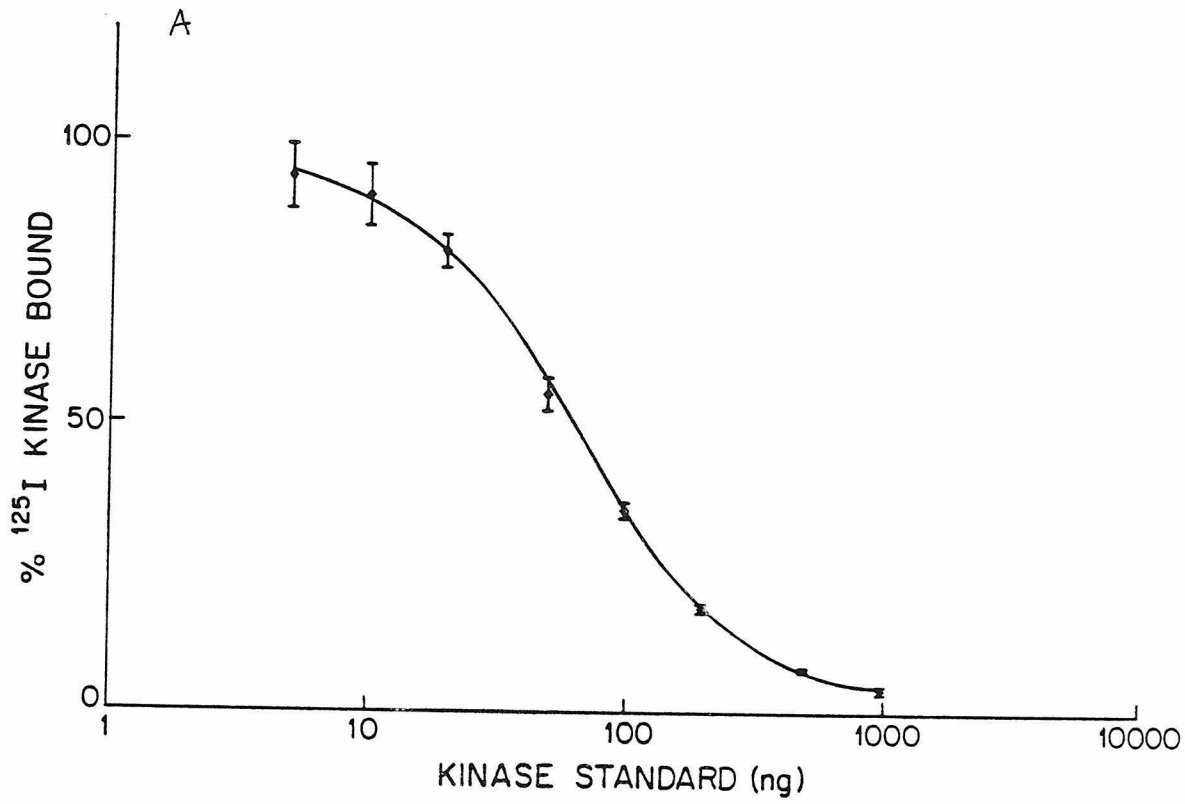

B
4.0
0
-4.0
Logit B
0.1
1
10
100
1000
KINASE STANDARD (ng)

Fig. 8. Standard curve for 6G9.

A. The assays were performed as described in Figure 6 except for the following modifications. A constant amount of 6G9 (sufficient to precipitate about 40% of ^{125}I kinase in the absence of unlabeled kinase see Figure 5B) was used. Both Triton X-100, 0.5% (v/v) and SDS, 0.1% (v/v) were present in the assay mix. The amount of ^{125}I kinase bound to 6G9 in the presence of varying amounts of standard kinase was determined as described in Figure 6, and plotted on a semilog scale.

B. The data obtained above were plotted on a log-logit scale where Logit B = $\text{Log}_e \left(\frac{B}{100-B}\right)$, and B is % Bound as defined in Figure 6.


A
100
50
0
% ^{125}I KINASE BOUND
1
10
100
1000
10000
KINASE STANDARD (ng)

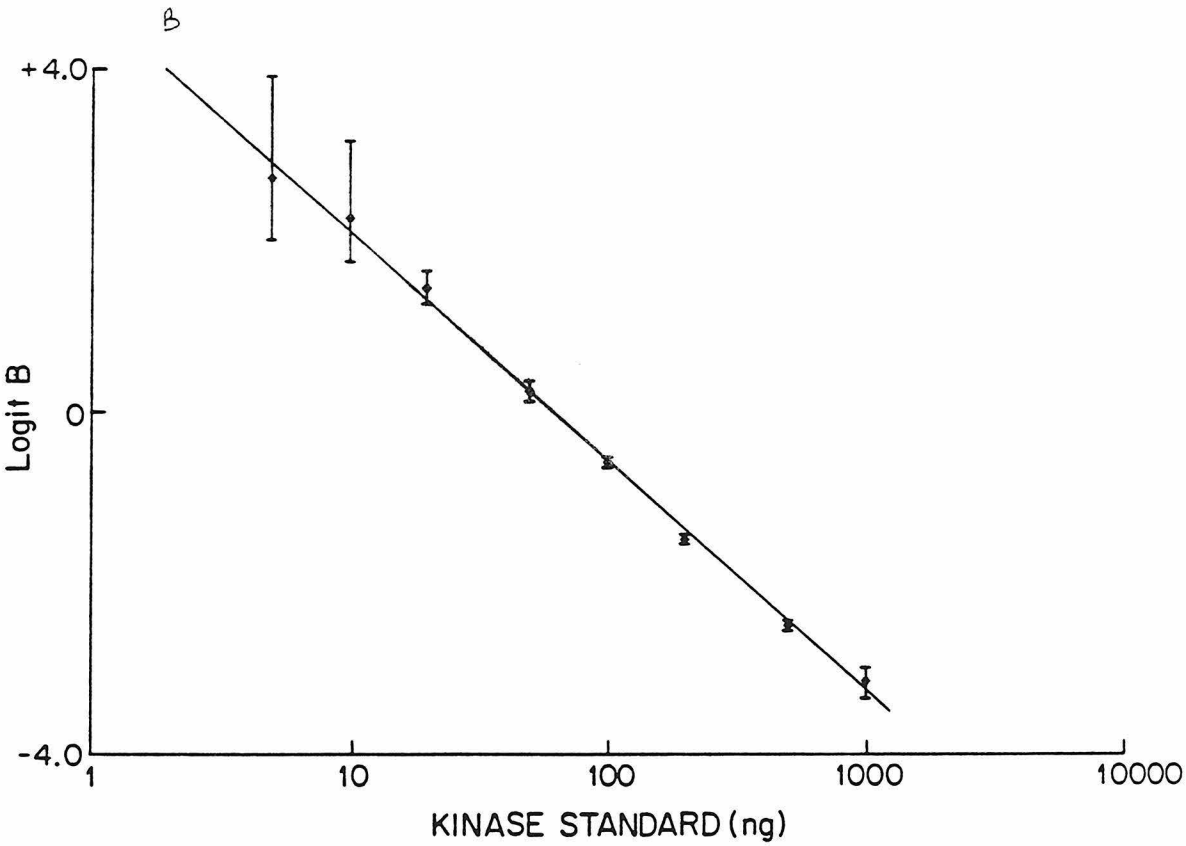
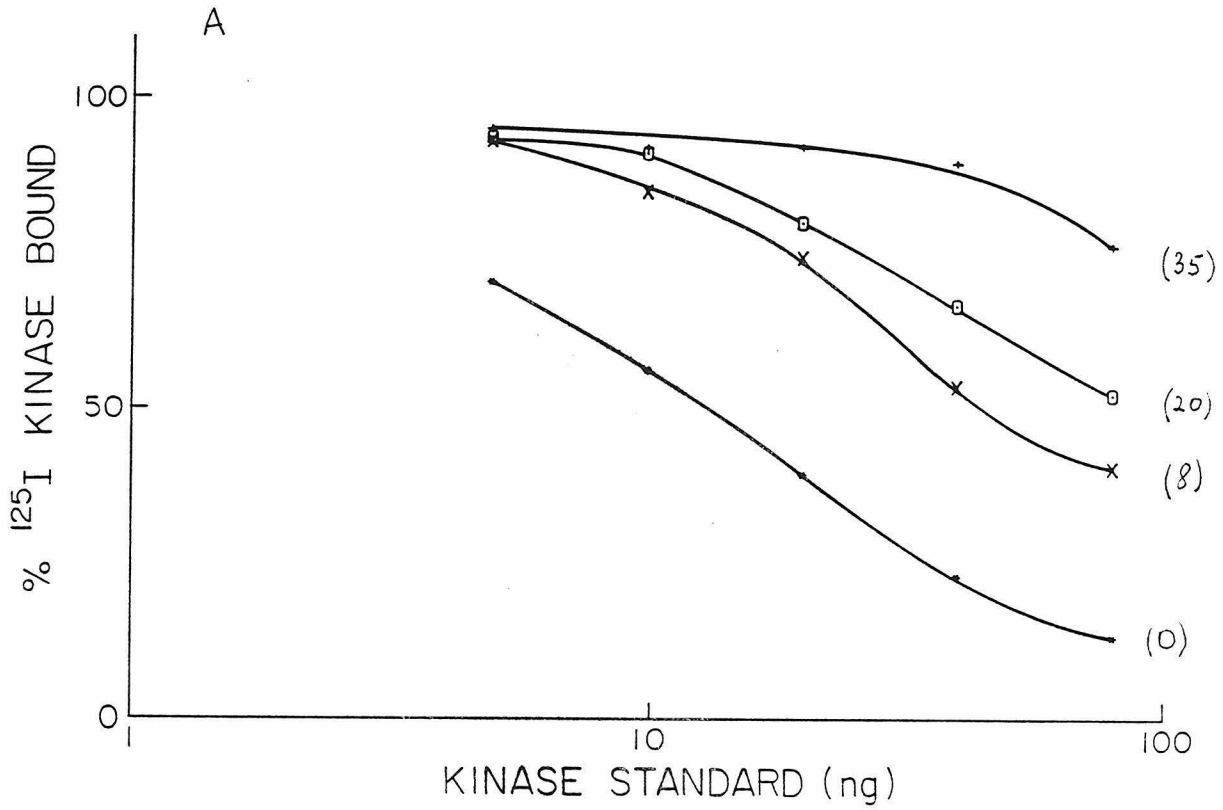
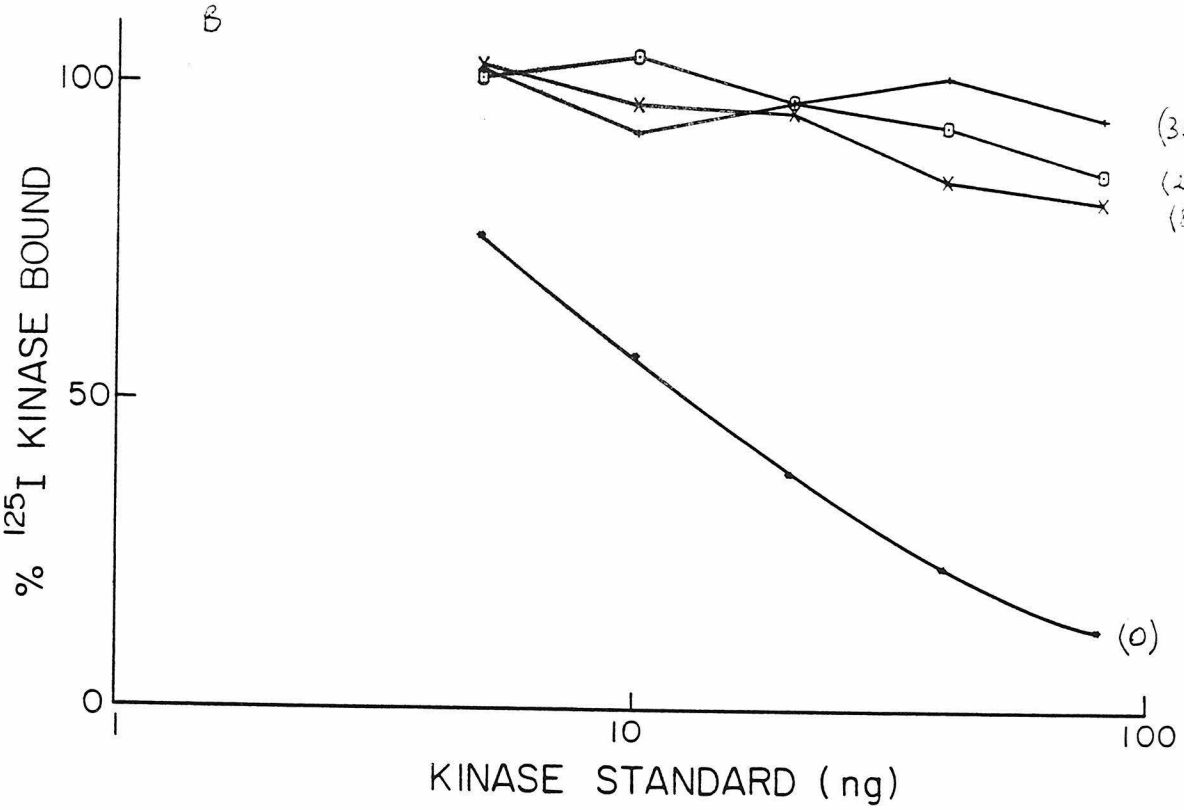

B
+4.0
0
-4.0
Logit B
1
10
100
1000
10000
KINASE STANDARD (ng)

Fig. 9. Effect of autophosphorylation of kinase on the standard curves of 4A11 (A) and 6E9 (B).

The assays were performed as described in Fig. 6, except that kinase autophosphorylated with non-radioactive ATP was used as kinase standard. The amount of phosphate incorporated per mole holoenzyme was determined as described under "Methods" and is shown in parentheses. ^{125}I-labeled kinase bound to monoclonal antibody was determined as described in Fig. 6 and expressed as % of that bound in the absence of kinase standard. Nonspecific background has been subtracted, and the data have been plotted on a semilog scale.


A
100
50
0
% ^{125}I KINASE BOUND
(35)
(20)
(8)
(0)
10
100
KINASE STANDARD (ng)


B
100
50
0
% ^{125}I KINASE BOUND
(35)
(20)
(8)
(0)
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100
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Chapter 3

REGIONAL DISTRIBUTION OF TYPE II Ca^{2+}/CALMODULIN-DEPENDENT PROTEIN KINASE IN RAT BRAIN

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Regional Distribution of Type II Ca^{2+}/Calmodulin-dependent Protein Kinase in Rat Brain[1]

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Abstract

The distribution of type II Ca^{2+}/calmodulin-dependent protein kinase has been mapped in rat brain by immunochemical and immunohistochemical methods using an antibody against its α-subunit. The concentration of the kinase, measured by radioimmunoassay, varies markedly in different brain regions. It is most highly concentrated in the telencephalon where it comprises approximately 2% of the total hippocampal protein, 1.3% of cortical protein, and 0.7% of striatal protein. It is less concentrated in lower brain structures, ranging from about 0.3% of hypothalamic protein to 0.1% of protein in the pons/medulla. The gradient of staining intensity observed in brain sections by immunohistochemistry corroborates this distribution. Neurons and neuropil of the hippocampus are densely stained, whereas little staining is observed in lower brain regions such as the superior colliculus. Within the diencephalon and midbrain, dense staining is observed only in thalamic nuclei and the substantia nigra. The skewed distribution of α-subunit appears to be due in part to the occurrence in the cerebellum and pons/medulla of forms of the kinase with a high ratio of β- to α-subunits. However, most of the variation is due to the extremely high concentration of the kinase in particular neurons, especially those of the hippocampus, cortex and striatum. The unusually high expression of the kinase in these neurons is likely to confer upon them specialized responses to calcium ion that are different from those of neurons in lower brain regions.

Changes in the intracellular concentration of calcium regulate a number of important neuronal processes (Greengard, 1981; Reichardt and Kelly, 1983). In order to understand at the molecular level how calcium ion carries out its regulatory roles, it will be necessary to identify the calcium target proteins in neurons and to understand how these proteins compete for calcium to generate a coordinated physiological response. In order to explain the fact that different classes of neurons respond to calcium ion in different ways, it will also be important to understand how distinct sets of calcium target proteins in different neurons generate specialized responses to calcium ion.

Several calcium target proteins have been identified in the nervous system (Kennedy, 1983; Reichardt and Kelly, 1983). Among them are three classes of calcium-activated protein kinases: the C-kinase (Takai et al., 1979) and at least two different classes of calmodulin-dependent protein kinases (Yamauchi and Fujisawa, 1980, 1983; Kennedy and Greengard, 1981; Bennett et al., 1983; Goldenring et al., 1983). The type II Ca^{2+}/calmodulin-dependent protein kinases (type II CaM kinases) are a class of closely related enzymes all of which have a similar broad substrate specificity. They are large oligomeric proteins composed of subunits with M_r = 50,000 to 60,000. Their exact subunit composition varies from tissue to tissue (Ahmad et al., 1982; Bennett et al., 1983; Goldenring et al., 1983; McGuinness et al., 1983; Woodgett et al., 1983) and in different brain regions (McGuinness et al., 1985; Miller and Kennedy, 1985).

This class of kinases is far more highly expressed in brain than in other tissues. Estimates of the concentration of brain type II CaM kinase, based on its fold-purification, suggest that it makes up at least 0.3% of total brain protein (Bennett et al., 1983). In contrast, the type II CaM kinases expressed in liver and skeletal muscle are less than 0.02% of the total protein in those tissues (Payne et al., 1983; Woodgett et al., 1983). The most prominent form of the enzyme in brain is purified from the forebrain. It contains an average of nine α-subunits (50 kilodaltons (kd)) and three β/β'-subunits (60/58 kd) (Bennett et al., 1983). The cerebellum contains a variant of the brain enzyme, composed of an average of eight β/β'-subunits and two α-subunits (McGuinness et al., 1985; Miller and Kennedy, 1985).

A report by Walaas et al. (1983) suggested that type II CaM kinase activity in rat brain showed more regional variation than either cAMP-dependent kinase or C-kinase activities. In this study we have examined the distribution of the type II CaM kinase by immunochemical methods. We developed a radioimmunoassay based on a monoclonal antibody that binds on immunoblots with high affinity and high specificity to the α-subunit of the brain kinase. Using this assay, we have confirmed the high concentration of the kinase in brain and have demonstrated a marked variation in its concentration in different brain regions. For example, we estimate that the kinase holoenzyme is about 2% of the total protein in the hippocampus and only about 0.1% of the protein in the pons/medulla. We have also used immunohistochemical methods to examine more precisely the distribution of the kinase in neurons within the brain. These results generally confirm those obtained by immunoassay, revealing, for example, dense staining of all neurons in the hippocampus, and very light or no staining of neurons in the medulla. In addition, the histochemical results reveal that, within the diencephalon and midbrain, the thalamic nuclei and the substantia nigra stain more darkly than do other areas. A preliminary report of this work has appeared (Erondu et al., 1983).

Materials and Methods

Materials. [γ-^{32}P]ATP and carrier-free Na^{125}I were purchased from ICN (Irvine, CA), and nitrocellulose membranes (BA 85, 0.45-μm pore diameter)

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were purchased from Schleicher and Schuell Inc. (Keene, NH). ATP, dithiothreitol (DTT), imidazole, EDTA, EGTA, phenylmethylsulfonyl fluoride (PMSF), Trizma buffer, soybean trypsin inhibitor, diaminobenzidine, Triton X-100, hemoglobin (bovine type II), naphthol blue black (amido black), polyinosinic-polycytidylic acid (Poly I, Poly C), and mouse IgG were purchased from Sigma Chemical Co. (St. Louis, MO). Protein A, protein A-Sepharose, and agarose C were purchased from Pharmacia Fine Chemicals (Piscataway, NJ). Leupeptin was purchased from Peninsula Laboratories, Inc. (Belmont, CA), and ultrapure sucrose was purchased from Schwartz/Mann (Spring Valley, N. Y.). Horseradish peroxidase-conjugated rabbit anti-mouse IgG was purchased from Accurate Chemical and Scientific Corp. (Westbury, NY). Glutaraldehyde was purchased from Polysciences, Inc. (Warrington, PA). Sprague-Dawley rats (140- to 160-gm males) were purchased from Simonsen Laboratories (Gilroy, CA), New Zealand female rabbits were obtained from Lab Pets (Rosemead, CA), and BALB/c ByJ mice were from Jackson Laboratories (Bar Harbor, ME). Rabbit anti-mouse IgG antiserum was obtained from rabbits immunized by repeated subcutaneous injections with mouse IgG in Freund's adjuvant and was affinity-purified by chromatography on Sepharose 4B coupled with mouse IgG (March et al., 1974). Protein A was iodinated by the chloramine T method essentially as described by Fryxell et al. (1983). The specific activity was 2 to 3 Ci/mmol, and 50 to 60% of it was retained by rabbit anti-mouse IgG coated on plastic microwells.

Preparation of hybridoma 6G9. A 6-week-old BALB/c ByJ female mouse was immunized acording to a schedule adapted from Stähli et al. (1980). For each set of injections, 50% pure type II CaM kinase, purified through the calmodulin-Sepharose step as described by Kennedy et al. (1983a), was mixed with an equal weight of Poly I, Poly C in a volume of 300 μl. About half of the mixture was administered intraperitoneally, the other half was given subcutaneously. The mouse was primed with 150 μg of kinase and boosted with 220 μg 3 weeks later. After an additional week, the mouse was bled from the tail and the titer of anti-kinase antibodies was measured by solid phase radioimmunoassay as previously described (Kennedy et al., 1983a). Six weeks after the boost, 195 μg of antigen were administered each day for 4 days in a row. On the fifth day, spleen cells were fused with NS1/SP2 myeloma cells by standard techniques, and fused hybrids were selected in hypoxanthine/aminopterin/thymidine medium (Köhler and Milstein, 1976; Moore et al., 1982). Of 160 antibody-secreting clones, 80 produced antibody against the crude antigen as measured in a solid phase well-binding assay similar to that described by Moore et al. (1982). One of these, 6G9, bound with high specificity and high affinity to the α-subunit on immunoblots. This hybridoma was subcloned and ascites fluids were prepared as previously described (Kennedy et al., 1983a). Antibody from ascites fluids was partially purified by precipitation with 50% saturated ammonium sulfate and dialyzed against buffer A (50 mM Tris-HCl, pH 7.4/0.9% NaCl/0.1% NaN_3). This preparation (20 mg of protein/ml) was used for immunocytochemistry. For the radioimmunoassay, the dialysate was further purified on a protein A-Sepharose affinity column (Ey et al., 1978). The IgG_1 fraction eluted from the column is subsequently referred to as "Pr. A-purified" 6G9.

Immunoassay of brain regions. Male Sprague-Dawley rats (140 to 160 gm body weight) were stunned and decapitated, and their brains were placed in ice-cold buffer B (40 mM Tris (pH 7.5), 1 mM imidazole, containing 150 mM sodium perchlorate, 250 mM sucrose, 5 mM EDTA, 1 mM EGTA, 2 mM DDT, 0.1 mM PMSF, 2 mg/liter of leupeptin, and 25 mg/liter of soybean trypsin inhibitor). Dissections were performed in an ice-cooled Petri dish according to a modification of the method described by Glowinski and Iversen (1966), as shown in Figure 1. The olfactory bulbs, cerebellum, and pons/medulla were separated from the rest of the brain. Then, a sagittal section was made to divide the brain into two halves. The hypothalamus was dissected along the lines shown in Figure 1, using the anterior commissure as a reference point. The midbrain/thalamus was then gently separated from the rest of the brain, exposing the striatum. The striatum and the hippocampus were removed by blunt dissection, and the rest of the brain was taken as the cortex. With this dissection procedure, the "striatum" consists of the basal ganglia of the telencephalon without the amygdala, whereas the "cortex" corresponds to the telencephalon without the "striatum." Each brain region was weighed. The wet weight varied in different dissections by less than 10%. The brain regions were homogenized in 10 vol of buffer B with 12 up-and-down strokes in a glass/Teflon homogenizer rotating at 1500 rpm.

The different brain regions were assayed for α-subunit using a quantitative immunoblot method. Aliquots of the homogenates were subjeted to polyacrylamide gel electrophoresis (PAGE) in the presence of SDS according to the method of Laemmli (1970). Stacking gels were 3.5% acrylamide/0.09% bisacrylamide. Running gels were 10% acrylamide/0.27% bisacrylamide. Standard amounts of purified kinase, containing 35, 70, 140, 210, and 350 ng of α-subunit were loaded onto each gel. Immediately after electrophoresis, the region of the gel containing proteins of M_r = 30,000 to 70,000 was cut out, and the separated proteins were transferred onto nitrocellulose paper by a modification of the method of Towbin et al. (1979). The transfer was effected at a constant current of 350 mA for 10 hr in 10 mM sodium borate, pH 9.5. After the transfer, the nitrocellulose sheets were incubated with: (*1*) buffer A containing 5% hemoglobin (2 hr); (*2*) Pr. A-purified 6G9 (3 to 5 μg/ml) diluted into buffer A containing 1% hemoglobin (8 hr); (*3*) wash buffer (buffer A containing 0.5% hemoglobin) (1 hr with three changes); (*4*) rabbit anti-mouse IgG (3 to 5 μg/ml) in wash buffer (2 hr); (*5*) wash buffer (1 hr with three changes); (*6*) ^{125}I-labeled protein A (2 to 3 × 10^5 cpm/ml) in wash buffer (2 hr); and (*7*) wash buffer (1 hr with three changes). The nitrocellulose sheet was dried, and the ^{125}I-labeled bands were located by autoradiography, cut out, and counted in a gamma counter. Background counts were obtained by cutting out and counting portions of the nitrocellulose sheet that contained no α-subunit. After subtracting background counts, standard curves were prepared by plotting on a double log scale the counts in labeled bands from lanes containing pure kinase against the amount of α-subunit in each lane. These standard curves were used to compute the amount of α-subunit present in unknown samples of brain region homogenates.

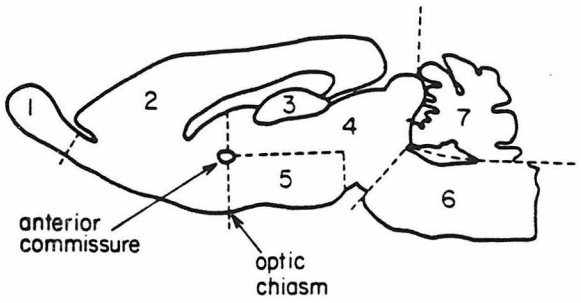

Figure 1. Diagrammatic representation of the procedure used in the dissection of rat brain. *Dashed lines* indicate positions of the sections: *1*, olfactory bulb; *2*, cerebral cortex; *3*, hippocampus; *4*, midbrain/thalamus; *5*, hypothalamus; *6*, pons/medulla; and *7*, cerebellum. Adapted from Glowinski and Iversen (1966).

Immunohistochemical staining of α-subunit in brain regions. Male Sprague-Dawley rats (140 to 160 gm body weight) were perfused through the heart for 6 to 8 min with 0.1 M sodium phosphate buffer, pH 7.2, 4% paraformaldehyde, 0.1% glutaraldehyde. After an additional 10 min, brains were removed and placed in the same fixative at 4° C overnight. Fifty-micrometer sections were cut on an Oxford Vibratome under cold 0.1 M phosphate buffer, pH 7.2. The sections were stained by a modification of the method described by Pickel (1981). They were washed briefly in 0.1 M Tris buffer, pH 7.5, 0.9% NaCl (Tns-saline) to inactivate remaining glutaraldehyde (DeCamilli et al., 1983), then transferred for 15 min to Tns-saline containing 0.2% Triton. Some sets of sections (noted in figure legend) were not treated with Triton. The sections were washed twice for 8 to 10 min each in Tris-saline, then incubated for 30 min in 3% non-immune rabbit serum in Tris-saline. They were again washed twice for 8 to 10 min each in Tris-saline and transferred to experimental or control antibody solutions prepared in Tris-saline plus 3% non-immune rabbit serum. Experimental antibody solutions contained ammonium sulfate-purified 6G9 ascites fluid diluted 1/200. Control solutions contained either ammonium sulfate-purified ascites fluid from the NS1/SP2 parent tumor diluted 1/200 or no antibody. Sections were incubated in these solutions at 4°C for 8 to 12 hr, then washed twice for 8 to 10 min each in Tris-saline plus 1% non-immune rabbit serum. They were then transferred to a solution of horseradish peroxidase-conjugated rabbit anti-mouse IgG diluted 1/50 in Tris-saline plus 1% non-immune rabbit serum, incubated for 1 hr at room temperature, and washed twice for 8 to 10 min each in Tris-saline. Reaction product was developed by incubation for 6 to 8 min in 0.0125% diaminobenzidine and 0.003% peroxide in 0.1 M Tns buffer, pH 7.5. The reaction was stopped by two quick rinses in 0.1 M Tris buffer. The mounted sections were examined with a Zeiss Universal microscope and then photographed with a Nikon Multiphot apparatus using the diascopic illuminator.

Agarose slabs containing kinase were prepared by mixing various amounts of pure kinase with a prewarmed solution of 3% agarose in 5 mM phosphate buffer, pH 7.2, to produce solutions containing 1.5% agarose, and 0.5, 0.25, 0.1, 0.05, or 0.02 mg/ml of kinase in a volume of 80 μl. The solutions were quickly formed into 0.17-mm-thick slabs by pipetting them into prewarmed molds as described by DeCamilli et al. (1983). The cooled slabs were fixed

for 30 min at room temperature in 4% paraformaldehyde, 0.1% glutaraldehyde, 0.1 M phosphate buffer, pH 7.2, and then overnight at 4°C in the same solution. The fixed slabs were stained with 6G9 as described above.

Assay for calmodulin-dependent synapsin I kinase activity. Brain regions or other tissues were homogenized in 20 mM Tris (pH 7.5), 1 mM imidazole containing 2 mM DTT, 0.1 mM PMSF, 1.0 mM $MgCl_2$, 25 mg/liter of soybean trypsin inhibitor, and 1 mg/liter of leupeptin. Calmodulin-dependent synapsin I kinase activity was assayed, with minor modifications, as previously described (Bennett et al., 1983; Kennedy et al., 1983b). The reaction mix contained, in a final volume of 100 μl, 50 mM Tris (pH 8.0), 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 1 μg of calmodulin, 20 μg of synapsin I, 50 μM[γ-^{32}P]ATP (1 to 2 × 10^3 cpm/pmol), either 0.4 mM EGTA (minus calcium) or 0.4 mM EGTA/0.7 mM $CaCl_2$ (plus calcium), and 2 to 25 μg of crude homogenate protein. Tubes were warmed for 60 sec to 30°C; reactions were initiated by addition of ATP and terminated after 20 sec (to ensure initial rates) by addition of 50 μl of SDS-stop solution. Synapsin I phosphorylation was analyzed by SDS-PAGE as described (Kennedy et al., 1983b). For each brain region or tissue, assays were performed in duplicate with varying amounts of homogenate protein to determine the range in which the rate of phosphorylation was linear with protein. The type II CaM kinase specifically phosphorylates a site on synapsin I that is recovered in a 30-kd fragment after digestion with *Staphylococcus aureus* V8 protease. Therefore, after assays of brain region homogenates, labeled synapsin I was digested and phosphorylation of this site was determined as described previously (Kennedy and Greengard, 1981). Under the present assay conditions, 75 to 90% of calcium-stimulated phosphorylation of synapsin I occurred on the site recovered in the 30-kd fragment. Specific activities were calculated by dividing the rate of phosphorylation of the site contained in the 30-kd fragment by the amount of homogenate protein in the assay. Protein was measured by the method of Peterson (1977) with bovine serum albumin as standard.

Phosphorylation of the kinase subunits. Incorporation of ^{32}P into the kinase subunits was measured as above with the following modifications. Two hundred nanograms of pure kinase were used per assay, and synapsin I and homogenate protein were omitted. [γ-^{32}P]ATP was used at a higher specific activity (1 to 2 × 10^4 cpm/pmol) and the reaction was terminated after varying lengths of time by the addition of SDS-stop solution. ^{32}P-labeled subunits were localized in gels by autoradiography, cut out, and counted by liquid scintillation spectrometry. For measurement of phosphorylated α-subunit by the immunoblot assay, cold (nonradioactive) ATP was used instead of [γ-^{32}P]ATP.

Results

Radioimmunoassay for the α-subunit of brain Type II CaM kinase. A difficulty with radioimmunoassay of brain type II CaM kinase is that the kinase is found in both soluble and particulate fractions of brain homogenates. Some of the particulate kinase can be solubilized by dilution into a low ionic strength buffer (Kennedy et al., 1983b), but complete solubilization requires a strong detergent. To circumvent this problem, we devised a radioimmunoassay based on quantitative immunoblots. Such an adaptation of the immunoblot technique has been used previously for the quantitation of gap junction proteins (Traub et al., 1983) and P_0 glycoprotein of peripheral nerve myelin (Nunn and Mezei, 1984). In this method, the protein sample is completely solubilized in SDS and fractionated by SDS-PAGE before the immunoassay is performed. To develop the assay, we used a monoclonal antibody (6G9) that binds on immunoblots with high specificity and high affinity to the α-subunit of the type II CaM kinase (Kennedy et al., 1983a).

Antibody 6G9 binds specifically to the α-subunit of the kinase in all of the brain regions examined in this study (Fig. 2). In most of the homogenates the antibody reacts with a single band of 50 kd. This band can be removed from homogenates by immunoprecipitation with a different anti-kinase monoclonal antibody, 4A11 (data not shown). In homogenates of the hypothalamus, midbrain/thalamus, and pons/medulla, 6G9 binds to a closely spaced doublet, both bands of which can be immunoprecipitated by 4A11. The second band appears to be an isomeric variant of the α-subunit which is present in these regions, rather than a post-translationally modified form, since *in vitro* translation of whole brain messenger RNA also produces two closely spaced bands, both of which are precipitated by anti-kinase antibodies (M. K. Bennett, unpublished observations).

Because some antibodies distinguish between "dephospho" and "phospho" forms of a phosphoprotein (Nairn et al., 1982; Sternberger and Sternberger, 1983), we compared the binding of 6G9 to unphosphorylated and phosphorylated α-subunit (Fig. 3). After 15 sec of autophosphorylation, approximately 1 mol of phosphate was incorporated/mol of subunit and the amount of 6G9 bound was not significantly changed. After 10 min, approximately 3 mol of phosphate were incorporated/mol of subunit, its mobility had shifted considerably (see Bennett et al., 1983), and the amount of 6G9 bound was approximately twice that bound to the same amount of unphosphorylated kinase. Thus, in theory, the amount of α-subunit measured in the various brain regions could depend, within a factor of two, on the proportion of phosphorylated subunit in each region. However, in all of the assays reported in this study, the α-subunit band appeared sharp, rather than diffuse or shifted in mobility. We concluded that the level of phosphorylation in the crude homogenates was not sufficient to affect the results.

The linear range of the immunoassay with purified kinase is 20 to 500 ng of α-subunit (28 to 700 ng of kinase) (Fig. 4). The limit of detection ($p < 0.05$) of α-subunit is 3.5 ng (5 ng of kinase). A standard curve similar to that shown in Figure 4 was used to calibrate each radioimmunoassay as described under "Materials and Methods." The range in which the amount of α-subunit measured in brain homogenates is linear with homogenate protein is shown in Figure 5. The presence of up to 100 μg of homogenate protein does not

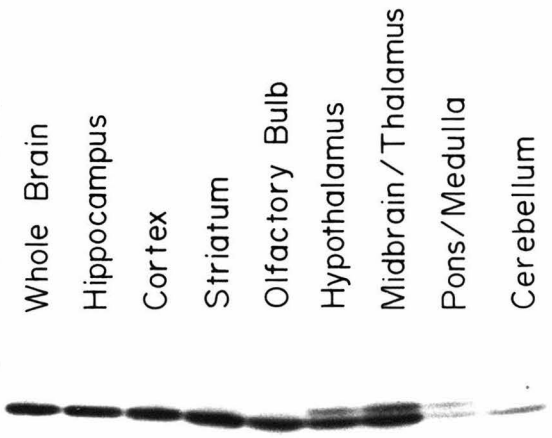

Figure 2. Autoradiogram illustrating the reaction of antibody 6G9 with crude homogenates from different regions of the rat brain. Aliquots of crude homogenates were subjected to SDS-PAGE and the proteins on the gel were transferred to nitrocellulose paper. The nitrocellulose paper sheet was then reacted with 6G9 by the immunoblot technique as described under "Materials and Methods." Whole brain, 24 μg; hippocampus, 11 μg; cortex, 18 μg; striatum, 29 μg; olfactory bulb, 70 μg; hypothalamus, 57 μg; midbrain/thalamus, 92 μg; pons/medulla, 164 μg; cerebellum, 171 μg.

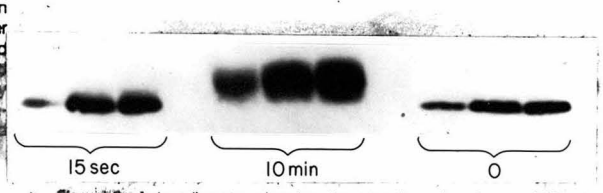

Figure 3. Autoradiogram showing the reaction of antibody 6G9 with "phospho" and "dephospho" forms of the α-subunit. Pure kinase was autophosphorylated for the lengths of time indicated. Aliquots of the reaction mixture containing 35, 70, and 85 ng of the α-subunit were subjected to SDS-PAGE, transferred to nitrocellulose paper and reacted with 6G9 by the immunoblot technique as described under "Materials and Methods."

affect detection of added standard α-subunit (data not shown); although it does increase the error of detection and decrease the linear range of the assay to approximately 20 to 250 ng of α-subunit.

Distribution of α-subunit in brain regions measured by radioimmunoassay. We measured the concentration of the α-subunit in several brain regions, as described under "Materials and Methods." Two important findings emerge from the results summarized in Table I. First, when measured by radioimmunoassay, the α-subunit is 3 times as abundant in brain homogenates as was originally estimated from purification results (Bennett et al., 1983). It constitutes approximately 0.7% of the total protein in whole brain homogenates. Therefore, the entire type II CaM kinase holoenzyme, which is composed of both α- and β-subunits in approximately a 3 to 1 ratio, makes up nearly 1% of the total brain protein. Second, there is a marked regional variation in the concentration of the α-subunit. It is highly concentrated in the hippocampus where it is 1.4% of total protein. Therefore, the kinase holoenzyme is 2% of the hippocampal protein. It is also highly concentrated in other parts of the telencephalon, whereas its concentration is considerably lower (0.05 to 0.2% of total protein) in the pons/medulla, cerebellum, and midbrain/thalamus. We determined that this difference was not due to enhanced degradation of the α-subunit by measuring purified α-subunit after mixing it with cerebellar homogenates (data not shown).

TABLE I

Distribution of the α-subunit of type II CaM kinase in rat brain

Brain Region	Concentration of α-Subunit: Nanograms/100 μg of Total Protein[a]	Concentration of α-Subunit: Percentage of Total Protein	No. of Determinations	No. of Animals
Whole brain	740 ± 42	0.74	15	2
Hippocampus	1420 ± 87	1.4	16	4
Cerebral cortex	860 ± 82	0.86	10	4
Striatum	460 ± 39	0.46	12	4
Olfactory bulb	260 ± 20	0.26	11	4
Hypothalamus	220 ± 13	0.22	8	4
Midbrain/thalamus	160 ± 10	0.16	12	4
Cerebellum	57 ± 6	0.06	16	6
Pons/medulla	50 ± 3	0.05	8	2

[a] Mean ± SEM.

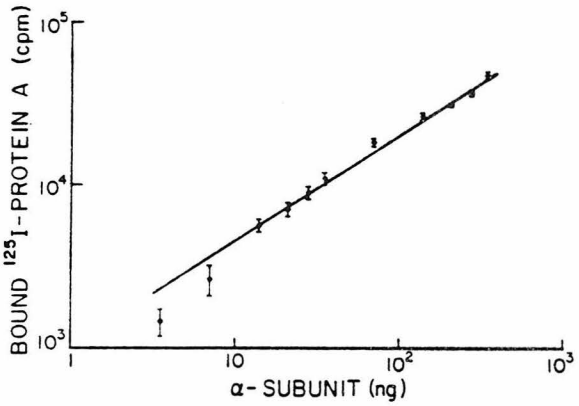

Figure 4. Standard curve. Varying amounts of pure kinase were subjected to SDS-PAGE and transferred to nitrocellulose paper. The paper sheet was then reacted with antibody 6G9 by the immunoblot technique, and labeled bands were cut out and counted as described under "Materials and Methods." The *line* was calculated by weighted linear regression of the data. Each *point* represents the mean ± SD of four determinations.

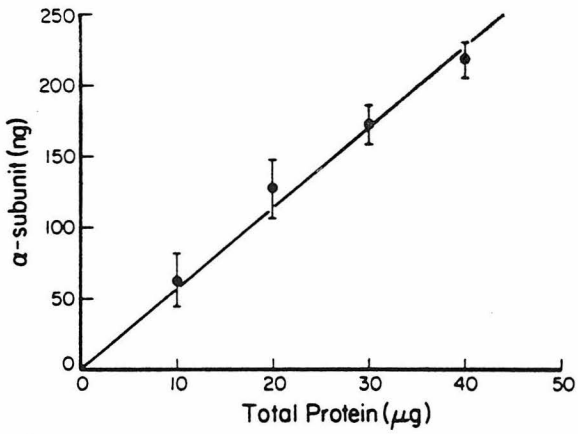

Figure 5. Proportionality between the α-subunit content of whole brain homogenate and total brain protein assayed. Increasing amounts of crude whole brain homogenate were assayed for α-subunit by the immunoblot method as described under "Materials and Methods." The values of α-subunit computed from the standard curve were then plotted against the total protein in the homogenate. The *line* was calculated by linear regression of the data. Each *point* represents the mean ± SD of four determinations.

Immunohistochemical staining for the α-subunit in brain sections. In order to determine which cell types and brain nuclei contain the α-subunit, we used antibody 6G9 and a horseradish peroxidase sandwich method to stain fixed sections of rat brain (Fig. 6). The results generally confirmed the distribution measured by radioimmunoassay. Dark, specific staining was observed in all cortical areas, the septal nuclei, and the caudatoputamen. The most intense staining, however, was in the hippocampus and amygdaloid nuclei. In the diencephalon and midbrain, the most consistently stained structures were the thalamic nuclei and the substantia nigra. Within the same sections, staining of structures such as the superior colliculus and the central gray nucleus was barely perceptible. In the cerebellum, Purkinje cells were stained nonspecifically, and we could not reliably detect specific staining.

In agreement with the results of Ouimet et al. (1984), specific staining in all areas was confined to neuronal cell bodies and/or neuropil (data not shown). We saw no evidence for staining of glia. Staining of neuropil regions was enhanced by treatment of fixed sections with dilute Triton prior to incubation with antibodies (cf. hippocampal staining in Fig. 6, *B* and *C*). Presumably, the brief detergent treatment allowed better access of the antibodies to the interior of small neuropil structures. The Triton-treated sections revealed that neuropil in the hippocampus and amygdala stains more intensely than in the cortex.

Although 6G9 is specific for the α-subunit on immunoblots, it was possible, as with any monoclonal antibody, that tissue fixation had destroyed the 6G9 epitope on the α-subunit and/or exposed a similar epitope in another protein. We used several controls to rule out this possibility. Incubations were performed in which 6G9 ascites fluid was replaced by buffer or by ascites fluid prepared from the parent NS1/SP2 tumor line. All control incubations produced very little staining within brain sections and a band of nonspecific reaction product around the outside (Fig. 6*F*). These controls indicated that 6G9 was necessary for specific staining. In addition, we tested whether the concentration of kinase measured in brain regions by the radioimmunoassay could account for the range of staining intensity shown in Figure 6. Thin agarose slabs containing kinase concentrations from 0.5 mg/ml to 0.02 mg/ml were prepared as described under "Materials and Methods." They were fixed and stained with 6G9 together with control slabs containing no kinase. Specific staining was observed in the blocks that contained kinase, and the intensity increased with kinase concentration (data not

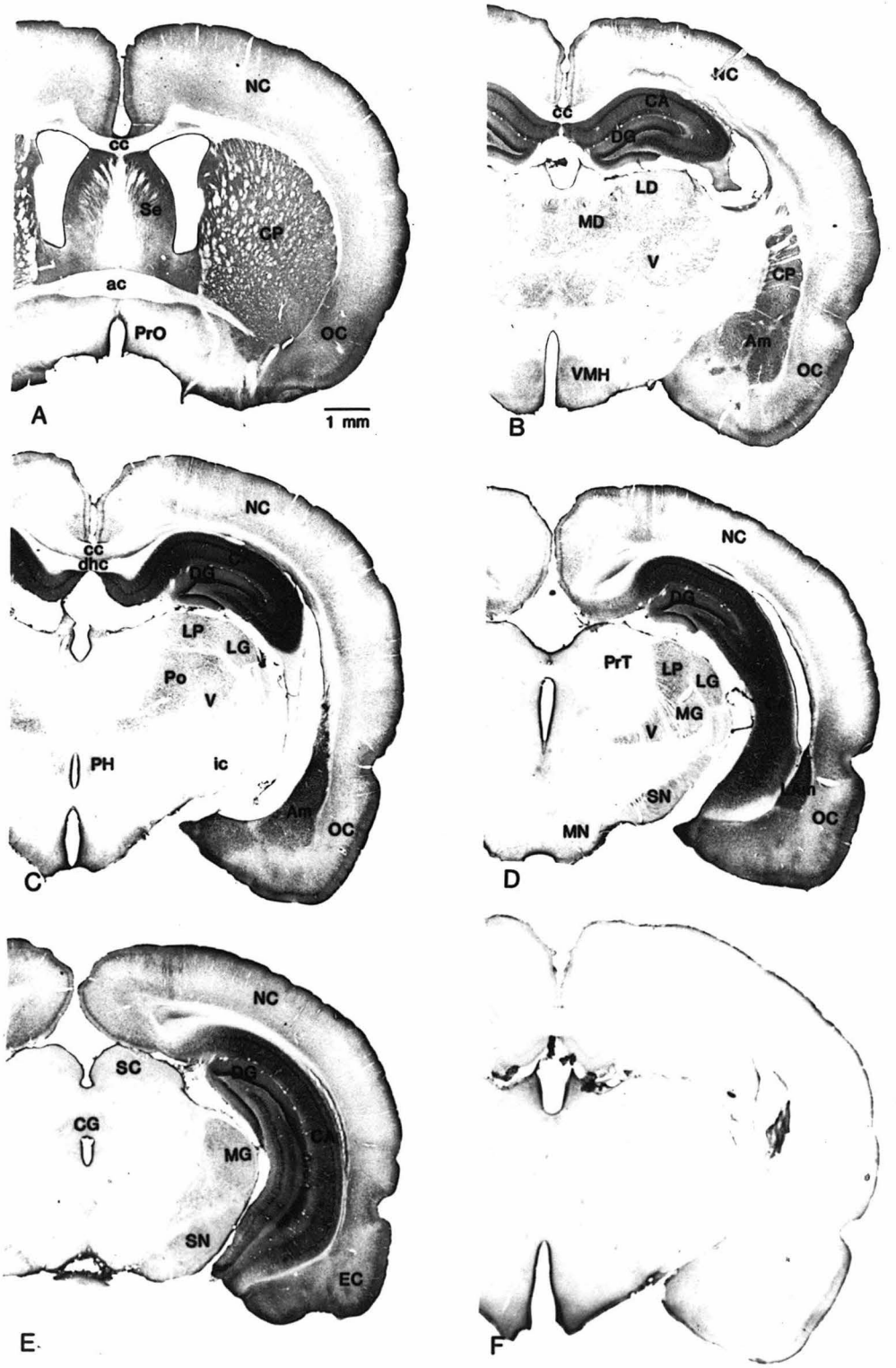
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Figure 6

shown). Thus, the presence of the fixed kinase molecule was sufficient to produce the staining intensities observed in brain sections. Finally, we note that the intensity of staining in brain regions varied in parallel with the concentration of α-subunit measured by radioimmunoassay. Taken together, the evidence is quite strong that the staining patterns shown in Figure 6 reflect the variable concentration of α-subunit in different brain regions.

Type II CaM kinase activity in brain regions. The intepretation of these results is complicated by the recent finding that the subunit composition of the type II CaM kinase varies in certain brain regions (McGuiness et al., 1985; Miller and Kennedy, 1985). In particular, the kinase from the cerebellum contains, on average, more β-subunit and less α-subunit than does the forebrain kinase. We measured the specific activity of type II CaM kinase in the different brain regions in order to determine how closely the concentration of the α-subunit parallels the concentration of kinase activity. The results (Table II) indicated a rather close parallel in all brain regions except cerebellum and the pons/medulla. In these two regions, the kinase activity per α-subunit was significantly higher than that of other brain regions. Thus, the average subunit composition of the type II CaM kinase in the pons/medulla may be between that of the forebrain and that of the cerebellum. The results also suggest that the kinase in the olfactory bulb, the hypothalamus, and the midbrain/thalamus may have, on average, a slightly lower proportion of α-subunit than in the hippocampus and cortex.

Measurement of α-subunit in non-neuronal tissues. Because several non-neuronal tissues contain kinases that appear homologous to the brain type II CaM kinase, we examined these tissues to see whether the α-subunit is expressed at low levels. Even in the liver, where the molecular weights of the homologous kinase subunits are near 50,000 (Ahmad et al., 1982; Payne et al., 1983), we could not detect any α-subunit (Table III). Thus, the α-subunit may be specific to neuronal tissue.

TABLE III

Distribution of type II CaM kinase activity and the α-subunit in non-neuronal tissues

Tissue	Kinase Specific Activity[a] (%)	α-Subunit (ng/100 μg of total protein)
Brain	100.0	740 ± 42
Spleen	9.0	<3.5
Testis	3.2	<3.5
Heart	3.0	<3.5
Skeletal muscle	2.4	<3.5
Adrenals	1.4	<3.5
Liver	0.8	<3.5
Kidney	0.5	<3.5

[a] Activity in brain was 16.1 nmol/min/mg. Specific activities were determined as described under "Materials and Methods."

Discussion

We have used a quantitative immunochemical technique, together with immunocytochemistry, to map the distribution within the brain of the α-subunit of type II CaM kinase. The most important finding emerging from the study is the extent of differential expression of the type II CaM kinase in different brain regions. The kinase is highly expressed in most telencephalic neurons (hippocampus, cortex, striatum, septum, and amygdala) and in thalamic neurons. Its concentration is highest in the hippocampus where it is about 2% of the total protein. In contrast, the kinase comprises only about 0.1% of the total protein in the pons/medulla. Intermediate concentrations are found in other brain areas. Immunocytochemical staining of brain sections confirms the variation in concentration of the α-subunit measured by radioimmunoassay and reveals its distribution more precisely. In the hippocampus and amygdala, neurons and neuropil appear densely stained. In cortical areas, staining of the neuropil is lighter and more variable. In the diencephalon and midbrain only the thalamic nuclei and the substantia nigra are stained, and in lower brain areas very little specific staining is apparent.

TABLE II

Ratio of type II synapsin I kinase activity and α-subunit in brain regions

Brain Region	Specific Activity[a] (nmol/min/mg of protein)	Activity/α-Subunit[b] (nmol/min/μg of α-subunit)
Whole brain	14.4	1.9
Hippocampus	16.6	1.2
Cortex	12.9	1.5
Striatum	7.4	1.6
Olfactory bulb	4.8	1.8
Cerebellum	4.0	7.0
Hypothalamus	4.3	2.0
Midbrain/thalamus	3.0	1.9
Pons/medulla	1.9	3.8

[a] Measured as described under "Materials and Methods."

[b] Calculated from specific activities and concentrations of α-subunit listed in Table I.

Some of the regional variation in concentration of the α-subunit is due to variation in the subunit composition of the type II CaM kinase. Purified cerebellar kinase has a considerably higher proportion of β-subunit (α-to-β ratio of 1:4; Miller and Kennedy, 1985) than does the forebrain kinase (α-to-β ratio of 3:1; Bennett et al., 1983). This is reflected in the correspondingly high level of kinase activity per α-subunit measured in cerebellar homogenates (Table II). In the pons/medulla, kinase activity per α-subunit is intermediate between that in forebrain and cerebellum, suggesting that the α- and β-subunits may be present there in nearly equal amounts. We recently found that the forebrain kinase isozyme is 10 to 20 times more concentrated in the postsynaptic density fraction from forebrain than is the cerebellar isozyme in cerebellar postsynaptic densities. Thus, the different proportions of α- and β-subunits may affect the subcellular distribution of type II CaM kinase within different cell types (Miller and Kennedy, 1985).

Although the α-subunit has been shown to be identical to the "major postsynaptic density protein" (Kennedy et al., 1983; Goldenring et al., 1984; Kelly et al., 1984) and is probably concentrated in postsynaptic densities *in situ* (Kennedy and Radice, 1984), biochemical studies indicate that a substantial portion of it is free in the cytosol. Immunocytochemical localization of the α-subunit is consistent with a diffuse distribution of the kinase within neurons. Neuronal cell bodies, dendrites, and neuropil regions are stained, as well as

Figure 6. Coronal sections through rat brain stained immunohistochemically for the α-subunit. Sections were fixed, cut, and stained as described under "Materials and Methods." Approximate positions of each section anterior to the interaural line were estimated from those of Paxinos and Watson (1982): *A*, 8.5 mm; *B*, 6.0 mm; *C*, 5.0 mm; *D*, 4.0 mm; *E*, 3.0 mm; *F*, 5.5 mm. All sections except *B* were treated briefly with Triton before incubation with antibody, as described under "Materials and Methods." *ac*, anterior commissure; *Am*, amygdaloid nuclei; *CA*, Ca fields of the hippocampus; *cc*, corpus collosum; *CG*, central gray; *CP*, caudatoputamen; *DG*, dentate gyrus of the hippocampus; *dhc*, dorsal hippocampal commissure; *EC*, entorhinal cortex; *ic*, internal capsule; *LAm*, lateral amygdaloid nucleus; *LD*, laterodorsal thalamic nucleus; *LG*, lateral geniculate thalamic nucleus; *LP*, lateroposterior thalamic nucleus; *MG*, medial geniculate thalamic nucleus; *MN*, mammilary nuclei; *NC*, neocortex; *OC*, olfactory cortex; *PH*, posterior hypothalamic nucleus; *Po*, posterior thalamic nuclei; *PrO*, preoptic area; *PrT*, pretectal area; *SC*, superior colliculus; *Se*, septal nuclei; *SN*, substantia nigra; *V*, ventral thalamic nuclei; *VMH*, ventromedial hypothalamus. Magnification × 8.

some axons in the corpus collosum (Fig. 6C). This agrees with the localization of the type II CaM kinase at the electron microscope level reported by Ouimet et al. (1984). It is consistent with the notion that the kinase regulates several neuronal functions.

At present, the available structural and enzymatic data can only give clues about what these functions may be. In response to calcium, the kinase phosphorylates the synaptic vesicle-associated protein synapsin I at a high rate (Bennett et al., 1983). This phosphorylation reduces the affinity of synapsin I for synaptic vesicles (Huttner et al., 1983). Thus, the kinase may regulate vesicle function in presynaptic terminals (Nestler et al., 1984). The type II CaM kinase also phosphorylates at least two components of the neuronal cytoskeleton *in vitro*; MAP_2 at a high rate (Yamauchi and Fujisawa, 1982; Bennett et al., 1983; Schulman, 1984) and tubulin at a lower rate (Goldenring et al., 1983; R. DeLorenzo and M. B. Kennedy, unpublished observations). These findings, the association of the kinase with the postsynaptic density fraction, and its association with the cytoskeleton in *Aplysia* (Saitoh and Schwartz, 1985) suggest that it is involved in regulating the neuronal cytoskeleton. Such regulation could affect neurite outgrowth, receptor clustering, the shape and size of dendrites and spines, and/or transport of materials through neuronal processes. There is as yet no evidence that the kinase phosphorylates ion channels. Better understanding of the various functions of the type II CaM kinase will require more precise structural data, information about additional kinase substrates, and the development of simplified systems in which its role can be studied in intact cells.

The high concentration of type II CaM kinase in telencephalic neurons could specifically affect their functioning in several ways. First, it will allow the kinase to compete more effectively with other calmodulin-binding proteins for calcium-bound calmodulin. As a result, the cascade of functional changes produced by activation of the kinase could begin to dominate the response to calcium in these neurons. Second, the overall rate of phosphorylation by activated kinase will be higher in these neurons, since the velocity of an enzymatic reaction depends on the concentration of enzyme as well as the concentration of substrate. Because the turnover numbers of protein kinases are rather low, 2 to 20/sec, an extremely rapid response by the kinase to a calcium signal may require that its concentration be unusually high. Finally, the kinase may play a structural role in these neurons. Expression of an enzyme at the level of 1 to 2% of total protein is unusual in any cell. Most highly abundant brain proteins, such as tubulin and actin, which are approximately 10% and 7% of total brain protein, respectively (Gordon et al., 1977; Fulton and Simpson, 1979), perform structural roles. The myosin ATPase, which is present at a high concentration in muscle cells, has a structural role in addition to its enzymatic one. By analogy, it is possible that the type II CaM kinase may have both structural and enzymatic roles, especially in neurons in which it is highly expressed. The finding that the kinase is a major component of the postsynaptic density fraction supports this possibility. However, its diffuse distribution within neurons suggests that any structural role may not be confined to postsynaptic densities.

Whatever its precise role, the high concentration of type II CaM kinase in certain sets of neurons undoubtedly confers on them a set of specialized responses to changes in calcium concentration. It is interesting to note that many of the regions that are especially enriched in the α-subunit are also those that have been implicated in memory formation (Thompson et al., 1983) and that display robust long-term potentiation (Swanson et al., 1982; Racine et al., 1983). It is, therefore, possible that the type II CaM kinase and, in particular, its α-subunit, may be important in the cascade of events that lead to long-term, activity-dependent changes in synaptic efficacy.

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Chapter 4

ASSOCIATION OF TYPE II CA^{2+}/CALMODULIN-DEPENDENT PROTEIN KINASE WITH BRAIN MICROTUBULES AND F-ACTIN

Abstract

Microtubules isolated from rat brain cytosol after taxol-induced polymerization at 30°C were enriched 2-fold for Type II CaM kinase (approximately 1 mol kinase/80 mols MAP2/720 mols tubulin dimer), but there was no enrichment when the polymerization was carried out at 0°C. However, there was a 4-fold enrichment at both temperatures when the polymerization was done in the presence of calcium and calmodulin, conditions under which autophosphorylation of the kinase would occur.

To understand and characterize this phenomenon, the association between pure microtubules and pure kinase was studied. The proportion of the kinase recovered in the microtubule pellet was 2-3x higher for phosphokinase than for non-phosphokinase with stoichiometries of approximately 1 mol kinase/26 mols MAP2/240 mols tubulin dimer and 1 mol kinase/80 mols MAP2/720 mols tubulin dimer, respectively. We also determined that the incorporation of as few as 4 moles phosphate per mole holoenzyme (out of a possible total of 30 phosphate groups per mole holoenzyme) was sufficient to cause maximal association of kinase with microtubules. Dephosphorylation of phosphokinase by protein phosphatase reduced its association to the level observed for non-phosphokinase. The association of thio-phosphokinase with microtubules was not reduced by protein phosphatase. Examination in the electron microscope of microtubules with associated kinase, after negative staining, revealed neither the presence of abnormal microtubules nor any obvious structural differences that could be attributed to the presence of the kinase. Phosphorylation also caused a reversible increase in the association of the Type II CaM kinase with F-actin. These results suggest that autophosphorylation of the kinase could constitute a mechanism for the regulation of its subcellular associations by neuronal activity.

Introduction

A number of important neuronal processes are regulated by changes in the intracellular concentration of calcium (Greengard, 1981; Reichardt and Kelly, 1983). Many of these physiological processes are mediated through the regulation, by calcium, of various protein phosphorylating systems (Kennedy, 1983; Browning et al., 1985). To understand at the molecular level how calcium is able to regulate such a diverse number of cellular processes, it will be necessary to characterize the calcium target proteins in terms of their tissue, cellular, and subcellular associations.

Several calcium-activated protein kinases have been identified in the nervous system: the C-kinase, regulated by calcium and phospholipid (Takai et al., 1979) and two classes of calmodulin-dependent kinases, namely the "specialized" calmodulin-dependent kinases and the "broad specificity" calmodulin-dependent kinases (Nairn et al., 1985; Kennedy et al., 1986; see Introduction to this thesis). The family of "broad specificity" protein kinases is more highly expressed in brain than in non-neuronal tissues. Brain Type II Ca^{2+}/calmodulin-dependent protein kinase (Type II CaM kinase) belongs to this class and makes up about 1% of total brain protein (Erondu and Kennedy, 1985, i.e., Chapter 3 this thesis). In this chapter, kinase will be used interchangeably with Type II CaM kinase to refer to this enzyme.

In brain, the Type II CaM kinase is present in both soluble and particulate fractions (Kennedy et al., 1983b). A large portion of the particulate enzyme can be solubilized in low ionic strength buffer. The particulate and soluble forms are indistinguishable when their physical and catalytic properties are compared (Kennedy et al., 1983b). One of the structures that contain the enzyme is the postsynaptic density, a fibrous cytoskeletal structure that adheres to neuronal membranes at postsynaptic sites (Kennedy et al., 1983a, Goldenring et al., 1984;

Kelly et al., 1984; Kennedy and Radice, 1984; Miller and Kennedy, 1985). In this location, the kinase is in a position to respond to calcium signals generated by the binding of neurotransmitters to their receptors. The Type II CaM kinase is also found associated with synaptic vesicles (Huttner et al., 1983). Synapsin I, a synaptic vesicle-associated protein, is a major substrate for the kinase. Phosphorylation of synapsin I by the kinase reduces its affinity for synaptic vesicles (Schiebler et al., 1986), and this may unmask sites on the vesicle membrane that promote transmitter release (Llinas et al., 1985).

Several reports have provided evidence suggesting that the Type II CaM kinase may associate with or phosphorylate elements of the cytoskeleton. Sahyoun et al. (1984a,b; 1985) have reported that the kinase is enriched in crude cytoskeletal fractions as well as in the nuclear matrix. Saitoh and Schwartz (1985) found a large proportion of *Aplysia* neuronal Type II CaM kinase in the triton-insoluble fraction of *Aplysia* ganglia. A portion of this kinase was released from the triton insoluble pellet after incubation under conditions that would permit autophosphorylation. It has also been reported that the Type II CaM kinase can phosphorylate myosin light chains leading to an enhancement of actin activated myosin ATPase activity (Edelman et al., 1986; Tanaka et al., 1986). Both tubulin and microtubule associated proteins--MAP2 and tau--are substrates for the kinase; and phosphorylation of any of these proteins inhibits assembly of microtubules (Yamamato et al., 1983, 1985; Yamauchi and Fujisawa, 1983; Wandosell et al., 1986).

While it has been clearly demonstrated that about one third of cAMP-dependent protein kinase in brain cytosol binds tightly to MAP2 (Sloboda et al., 1975; Vallee et al., 1984), no precise data are available on the interaction between Type II CaM kinase and microtubules. In this chapter, studies on the association of Type II CaM kinase with both crude and purified elements of the

cytoskeleton will be described. Microtubules were isolated from rat brain cytosol in one step by the use of an assembly promoting drug, taxol (Schiff et al., 1979; Vallee, 1982). These microtubules contained endogenous Type II CaM kinase and the proportion of cytosolic kinase recovered in the microtubule pellet increased when the polymerization was allowed to take place in the presence of calcium. Further investigation of this phenomenon with pure components revealed that autophosphorylation of pure Type II CaM kinase causes a reversible increase in its association with both taxol-polymerized microtubules and F-actin.

Methods

Materials. [γ-^{32}P] ATP and carrier free Na ^{125}I were purchased from ICN and nitrocellulose membranes (BA 85, 0.45 μm pore diameter) from Schleicher and Schuell. ATP, ATP-γS, GTP, dithiothreitol (DTT), imidazole, EDTA, EGTA, phenylmethyl sulfonyl fluoride (PMSF), Trizma buffer, Pipes buffer, soybean trypsin inhibitor, hemoglobin (bovine type II), naphthol blue black (amido black), polyinosinic-polycytidylic acid (Poly I, Poly C), and mouse IgG were purchased from Sigma. Protein A and Protein A-Sepharose were purchased from Pharmacia Fine Chemicals. Leupeptin was purchased from Peninsula Laboratories, Inc., and ultrapure sucrose from Schwartz/Mann. Bovine serum albumin (BSA) was purchased from Boehringer Mannheim, and glycerol was from J. T. Baker Chemical Co. Taxol was obtained from the National Cancer Institute. A stock solution (10 mM) was prepared in dimethyl sulfoxide, and stored at -80°C in small aliquots. Synapsin I was prepared by a modification of the method of Ueda and Greengard (1977) as described previously (Bennett et al., 1983, i.e., Appendix I). Calmodulin was purified from bovine brain by the method of Watterson et al. (1976). Protein phosphatase was purified from rabbit skeletal muscle and was a gift from B. Patton of this laboratory. Rat forebrain Type II CaM kinase was

purified by the method of Bennett et al. (1983, i.e., Appendix I) with minor modifications as described in Miller and Kennedy (1985). Skeletal muscle powder was kindly provided by Dr. Horst Hinssen of the Division of Biology, Caltech. Sprague-Dawley rats (140 to 160 g males) were purchased from Simonsen laboratories (Gilroy, California), New Zealand female rabbits from Lab Pets (Rosemead, California), and Balb/c ByJ mice from Jackson Laboratories (Bar Harbor, Maine). Rabbit anti-mouse IgG antiserum was obtained from rabbits immunized by repeated subcutaneous injections with mouse IgG in Freund's adjuvant and was affinity purified by chromatography on Sepharose 4B coupled with mouse IgG (March et al., 1974). Protein A was iodinated by the chloramine T method essentially as described by Fryxell et al. (1983). The specific activity was 2-3 Ci/mmol, and 50-60% of it was retained by rabbit anti-mouse IgG coated on plastic microwells.

Preparation of microtubule protein. Rat brain microtubules were prepared using the assembly promoting drug, taxol, essentially as described by Vallee (1982) and shown in Figure 1. Aliquots of cytosol, taxol supernatant and microtubule pellet were assayed for Type II CaM dependent synapsin I kinase activity as well as for α-subunit concentration.

For studies of the association between pure kinase and pure microtubules, the latter were prepared from rat brain by two cycles of assembly-disassembly (Shelanski et al., 1973) and stored either at -80°C or in liquid nitrogen in Buffer A (0.1 M Pipes buffer pH 6.9 containing 1 mM $MgSO_4$, 2 mM EGTA) containing 1 mM GTP. Before each experiment, the microtubules were thawed and spun in the microfuge for 5 min to remove aggregated material. These microtubules are referred to as 2X MTs.

Measurement of the Effect of Autophosphorylation on the Association of Pure Type II CaM Kinase with Taxol-Polymerized Microtubules. The measurement of the effect of autophosphorylation involved three steps as shown in Figure 2. In the first step, we polymerized 2X MTs at a concentration of 1.5 mg/ml using taxol for 30 min at 30°C. In the second step, pure forebrain Type II CaM kinase was autophosphorylated by a slight modification of the method described in Miller and Kennedy (1986). The reaction mixture contained 0.2 mg/ml kinase, 50 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 0.1 mg/ml calmodulin, 0.4 mM EGTA, 0.7 mM $CaCl_2$, 10 mM DTT, 0.2 mg/ml BSA and 50 μM unlabeled (non-radioactive) ATP. After preincubation for 30 sec at 30°C, the reaction was initiated by the addition of kinase. The entire reaction was quenched after 30 sec by the addition of EDTA to a final concentration of 100 mM. For control kinase (non-phosphokinase[1]), ATP was added after the reaction had been stopped with EDTA. In the third step, an aliquot of the reaction mix was added to the taxol-polymerized 2X MTs bringing the final concentration of kinase and tubulin dimer to 44 nM and 8.9 μM, respectively. The mixture was spun through a 5% sucrose cushion at 25° or 4°C to a total of 1.1×10^{10} rad^2/sec in a 50 Ti rotor (approximately 50,000 RPM for 10 min). For experiments performed at 4°C, the taxol-polymerized 2X MTs and the kinase solution were separately precooled on ice for 2 min before being mixed. For control experiments at 25°C, 2 mg/ml BSA + 20 μM taxol was substituted for taxol-polymerized 2X MTs, while at 4°C, ice-cold 2X MTs with no taxol (depolymerized 2X MTs) were substituted. Aliquots of the supernatant and

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[1]Non-phosphokinase refers to non-phosphorylated kinase, i.e., kinase that has not undergone autophosphorylation after its isolation and purification from rat brain.

resuspended pellet were assayed for calmodulin-dependent synapsin I kinase activity and α subunit. Protein was measured by the method of Peterson (1977) with bovine serum albumin as standard.

Determination of the Threshold of Autophosphorylation Required to Produce the Increased Association of Kinase with Taxol-Polymerized Microtubules. Kinase was prephosphorylated for 15 sec as already described, except that the concentrations of $MgCl_2$ used in the assay were varied to produce different levels of phosphate incorporation (Miller and Kennedy, 1986). The association of phosphokinase[1] with taxol-polymerized microtubules was then measured as described above. To determine the degree of autophosphorylation, parallel experiments were carried out with radioactive ATP. ^{32}P-labeled subunits were localized in gels by autoradiography, cut out, and counted by liquid scintillation spectrometry.

Dephosphorylation of Phosphokinase by Protein Phosphatase. Kinase at a concentration of 0.4 mg/ml was prephosphorylated with radioactive ATP for 15 sec in the presence of either 0.5 mM or 1 mM $MgCl_2$, as described above. The reaction was stopped with EDTA, and the assay mix was then incubated on ice with an equal volume of protein phosphatase, bringing the final concentrations of kinase and phosphatase to 0.16 mg/ml and 0.07 mg/ml, respectively. Aliquots of this mixture were taken at different times during the incubation and the amount of phosphate remaining on kinase was determined by the "gel method" as described in Kennedy et al. (1983b).

Effect of Dephosphorylation on the Association of Phosphokinase with Taxol-Polymerized Microtubules. Kinase was prephosphorylated with unlabeled ATP or ATP-γS for 15 sec in the presence of 0.5 mM $MgCl_2$ as described above. The reaction was quenched with EDTA. To generate a non-phosphokinase

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[1]Phosphokinase refers to kinase autophosphorylated in the presence of ATP.

control, ATP was added after the reaction had been stopped with EDTA. One-half of each assay mix was incubated on ice with protein phosphatase for 30 min and the other half was incubated on ice with buffer for 30 min. At the end of the incubation, the association of control (non-phospho), phospho and dephosphorylated kinase with taxol-polymerized microtubules was examined as previously described. In some experiments, protein phosphatase was added to phospho and thio-phosphokinase[1] after kinase had been preincubated for 3 min with taxol-polymerized microtubules. At the end of the incubation with phosphatase, the proportion of kinase recovered in the microtubule pellet was determined as previously described.

Assay for Calmodulin-Dependent Synapsin I Kinase Activity. Calmodulin-dependent synapsin I kinase activity was assayed by measuring the initial rate of phosphorylation of synapsin I. We performed these reactions for 30 sec at 30°C, using 10-20 μg synapsin and 0.1-10 μg sample protein. The amount of the sample used in these assays was such that the rate of synapsin I phosphorylation was linear. The incorporation of phosphate into synapsin I was measured either by a method in which phosphorylated protein was precipitated with trichloroacetic acid (the TCA method), described in Bennett et al. (1983, Appendix I), or, after separation of proteins by SDS-PAGE (the gel method), as described in Kennedy et al. (1983b).

Measurement of α Subunit. The quantity of the α subunit of the Type II CaM kinase in the various samples was determined by radioimmunoassay as described in Erondu and Kennedy (1985, Chapter 3).

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[1]Thio-phosphokinase refers to kinase autophosphorylated in the presence of ATP-γS (thiophosphate).

Electron Microscopy. A 10 µl sample from the resuspended microtubule pellet was placed on a carbon coated grid that had been prewetted with bacitracin. After 15 sec, the sample was displaced with 8 drops of either 2% uranyl acetate or 2% phosphotungstic acid. The excess stain was removed from the grid with filter paper; the grid was air dried and viewed under a Phillips 420 electron microscope. Some samples were fixed with 0.2% glutaraldehyde before being stained.

Association of Pure Type II CaM Kinase with F-Actin. G-actin, purified from skeletal muscle powder according to the method of Spudich and Watt (1971) was polymerized to F-actin by incubating at 30°C for 30 min in the presence of 0.2 mM ATP and 7.5 mM KCl.

The association of phospho and non-phosphokinase with F-actin was studied as described for 2X MTs, except that F-actin at a concentration of 1.5 mg/ml was substituted for taxol-polymerized microtubules. Similarly, the effect of protein phosphatase on this association was determined as described for 2X MTs.

Results

Endogenous Type II CaM Kinase in Microtubule Preparations. Microtubules isolated from rat brain cytosol by the reversible assembly/disassembly method (Shelanski et al., 1973) were shown to contain endogenous Type II CaM kinase (Vallano et al., 1985). However, the kinase specific activity did not remain constant with multiple cycles of assembly/disassembly as is the case with microtubule associated cAMP dependent protein kinase (Sloboda et al., 1975). In view of the possibility that a weak interaction might be disrupted during the cycles of polymerization and depolymerization, I isolated microtubules from rat brain cytosol, using the assembly promoting drug, taxol (Schiff et al., 1979; Vallee, 1982; see Fig. 1). The advantages of this procedure include the ability to obtain

pure microtubules in one step, the stability of taxol-polymerized microtubules to cold, presence of Ca^{2+} and absence of Mg^{2+}, as well as the ability of taxol to induce some polymerization of microtubules in the cold and in the presence of Ca^{2+}.

Figure 3 shows the concentration of the α-subunit of the Type II CaM kinase in the various fractions obtained after taxol-induced polymerization of microtubules in rat brain cytosol. This was determined by radioimmunoassay as described in Chapter 3. The microtubule pellet isolated at 30°C (Fig. 3A) in the absence of calcium was enriched twofold for the Type II CaM kinase (approximately 1 mol kinase/80 mols MAP2/720 mols tubulin dimer[1]) but there was no enrichment when the polymerization was carried out at 0°C (Fig. 3B). However, there was a fourfold enrichment at both temperatures (Fig. 3A & B), when the polymerization was done in the presence of calcium and calmodulin (approximately 1 mol kinase/40 mols MAP2/360 mols tubulin dimer[1]). Table I reveals that at 30°C, a significant proportion of the cytosolic kinase associates with microtubules (34%[2]), and this proportion increases to 69% when the polymerization is carried out in the presence of calcium. The reduced proportion of kinase recovered in the microtubule pellet at 0°C is due to the fact that the microtubule pellet isolated under these conditions is about 15% of that isolated at 30°C. Table II reveals the fact that the specific kinase activity (activity/α-subunit) in the microtubule pellet is less than that in the cytosol and

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[1]In calculating these stoichiometries, we assumed that the microtubules consist of MAP2 and tubulin dimer in a molar ratio of 1:9 (Kim et al., 1979).

[2]% in pellet calculated as:

$$\frac{\text{Amount in pellet}}{\text{Amount in pellet} + \text{Amount in Supernatant}} \times 100$$

This approach assumes that any loss encountered during the fractionation is distributed evenly between the two fractions.

the supernatant, suggesting that denatured enzyme may be preferentially associating with microtubules. However, the activity/α-subunit in the supernatant is not greater than that of the cytosol, as would be expected if the supernatant had been selectively cleared of denatured enzyme. It is equally reassuring that in the experiments done on ice, there was not much difference in the specific activity of the kinase in the various fractions (Table III). Therefore, the association of endogenous kinase with taxol-polymerized microtubules is not likely to be a consequence of enzyme denaturation.

Effect of Autophosphorylation on the Association of Pure Type II CaM Kinase with Taxol-Polymerized Microtubules. One possible effect of calcium and calmodulin that might have promoted the association of endogenous kinase with microtubules is autophosphorylation of the enzyme. To investigate this possibility, I studied the association between pure microtubules and pure Type II CaM kinase (non-phosphorylated and phosphorylated forms).

The concentrations of pure kinase and pure 2X MTs used in this study were chosen to reflect concentrations determined in the cytosol experiments described above. In preliminary experiments, I had obtained results showing that the association between pure kinase and pure taxol-polymerized 2X MTs at 25°C was quite rapid; it appeared to be complete within a minute (data not shown). Thus, in subsequent experiments, the two components were incubated for 1-3 min before spinning through a sucrose cushion. As shown in Figure 4A, the proportion of phosphokinase recovered in the microtubule pellet is 3X that of non-phosphokinase. This difference is not due to reduction in the pellet size, since in these experiments there was less than 10% variability in the protein content of the microtubule pellet. The stoichiometries obtained in these experiments (approximately 1 mol non-phospho kinase/80 mols MAP2/720 mols tubulin dimer and 1 mol phosphokinase/26 mols MAP2/240 mols tubulin dimer) are similar to

those obtained in the cytosol experiments. In the control experiment, 2 mg/ml BSA + 20 μM taxol was substituted for taxol-polymerized microtubules, and approximately 2% and 8% of non-phospho and phosphokinase respectively, was recovered in the pellet. Therefore, non-specific sedimenting of kinase cannot account for the kinase recovered in the microtubule pellet.

As in the experiments with crude cytosol, activity/α-subunit in the pellet is less than that in the starting material (unspun mixture) and supernatant (Table IV). Again, this raised the possibility that denatured enzyme may be preferentially associating with microtubules. However, the specific kinase activity in the taxol supernatant is not as high as one would expect if it was being selectively depleted of denatured enzyme. This issue was also addressed by repeating the above experiments at 4°C, a temperature where denaturation of the kinase is reduced. This is reflected in Table V, which shows that the total activity (and hence activity/α-subunit) of kinase in each of the three fractions is greater than that observed at 25°C (Table IV). At 4°C as Figure 4B shows, more phosphokinase than non-phosphokinase is recovered in the microtubule pellet. This result, which is similar to that obtained at 25°C (Fig. 4A), also suggests that the increased association of phosphokinase with taxol-polymerized microtubules is not simply a consequence of enzyme denaturation. It should be noted that at both temperatures, recovery of total activity of phosphokinase after centrifugation[1] is low (Tables IV and V). It is, therefore, likely that the loss of activity of kinase in the pellet occurs during centrifugation as well as during resuspension of the microtubule pellet rather than before the association of kinase with microtubules.

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[1] $\text{Recovery} = \dfrac{\text{total activity in supernatant + total activity in pellet}}{\text{total activity in unspun mixture}}$

The reduction of total activity of phosphokinase relative to non-phosphokinase (Tables IV and V) is consistent with reports in the literature (Miller and Kennedy, 1986; Lai et al., 1986). This appears to result partly from the increased thermal lability of phosphokinase (Lai et al., 1986) and partly from a phosphorylation-induced conformational change that appears to be reversible by dephosphorylation (Miller and Kennedy, 1986; see results of reversal experiments below).

Electron Microscopy. Taxol-polymerized microtubules with and without associated kinase were examined under the electron microscope for the following reasons: (1) to determine if kinase was associating with abnormal microtubules; (2) to determine if kinase itself had been induced to form abnormal structures such as filaments; and (3) to determine if there was any evidence of kinase on microtubules such as periodicity. Examination of these microtubule pellets, after negative staining, showed the presence of normal microtubules (Fig. 5). There were no significant differences between fixed and unfixed samples; neither were there any obvious differences between samples stained with uranyl acetate and those stained with phosphotungstic acid. Furthermore, there were no obvious structural differences that could be attributed to the presence of kinase (data not shown).

Minimum Level of Phosphorylation Required to Produce the Increased Association of Type II CaM Kinase with Taxol-Polymerized Microtubules. In view of recent findings (Miller and Kennedy, 1986) that submaximal incorporation of phosphate into the enzyme (3-12 out of a possible total 30 phosphate groups per holoenzyme) is sufficient to produce a complete change in regulation of activity; I performed experiments to determine the threshold level of phosphorylation required to cause the increased association of kinase with microtubules. The concentration of Mg^{2+} in the autophosphorylation mix was

varied to produce different levels of phosphorylation (Miller and Kennedy, 1986). The results of these experiments, shown in Figure 6, indicate that the incorporation of an average of four moles of phosphate per mole holoenzyme is sufficient to cause maximal binding of kinase to microtubules. Maximal binding was taken as that exhibited by kinase that was prephosphorylated for 30 sec in the presence of 10 mM $MgCl_2$ (Fig. 4). Prephosphorylation of kinase for as long as 2 min, which would lead to the incorporation of up to 27 moles phosphate per mole holoenzyme, did not cause an additional increase in its association with taxol-polymerized microtubules. It therefore seems possible that both the production of calcium-independent kinase activity and the increased association of kinase with microtubules could result from the same autophosphorylation-induced conformational change.

Effect of Dephosphorylation on the Association of Phosphokinase with Taxol-Polymerized Microtubules. To test whether the effect of autophosphorylation on association with microtubules was reversible, kinase was first prephosphorylated to 5.1 moles phosphate per mole holoenzyme and then incubated with protein phosphatase on ice. After 30 min, protein phosphatase removed 80% of the ^{32}P incorporated into both subunits of the enzyme (Fig. 7). Measurement of the association of phospho and dephosphorylated kinase with microtubules showed that dephosphorylation by protein phosphatase completely reversed the increase in association (Fig. 8). The figure also shows that the reversal is due to dephosphorylation and not due to presence of phosphatase by itself since phosphatase had no effect on the association of control (non-phospho) kinase with microtubules. Additional evidence is provided by the fact that reversal could be obtained by dephosphorylation after phosphokinase has associated with taxol-polymerized microtubules (Fig. 9). Thio-phosphokinase was generated by autophosphorylating kinase in the presence of ATP-γS. In general,

thio-phosphorylated proteins are poor substrates for protein phosphatases (Gratecos and Fischer, 1974). As Figure 9 shows, protein phosphatase did not affect the association of thio-phosphokinase with taxol-polymerized microtubules. Thus, it seems that the increased association of kinase with taxol-polymerized microtubules is caused by autophosphorylation rather than by irreversible denaturation.

Following dephosphorylation there is an increase in the total synapsin I kinase activity (Table VI). This suggests that the reduced activity of phosphokinase relative to non-phosphokinase seen in Tables IV & V and also reported by Miller and Kennedy (1986) is partly due to autophosphorylation and not wholly accounted for by the thermal instability of phosphokinase (Lai et al., 1986).

The Association of Pure Kinase with F-Actin. Two questions were raised by the experimental results presented above. (1) Does the kinase associate with other elements of the cytoskeleton? (2) Would autophosphorylation of the kinase affect its association with such a cytoskeletal component? To address these questions, I carried out preliminary studies on the association of pure kinase with purified F-actin, using the same protocol that was worked out for microtubules. F-actin was chosen for three reasons. (1) Actin is a persistent contaminant during purification of the kinase from rat brain (Bennett et al., 1983, i.e. Appendix I). (2) Actin is not a substrate for the kinase. (3) Smooth muscle myosin light chain kinase binds to F-actin (Dabrowska et al., 1982); and the Type II CaM kinase may account for most of the myosin light chain kinase activity in brain (Edelman et al., 1985).

The results of these preliminary studies are shown in Figures 10-12 and Tables VII and VIII. Figure 10 shows that kinase associates with F-actin as measured by recovery of kinase in the actin pellet. In addition, there is a two- to threefold difference in the proportion of phosphokinase relative to

non-phosphokinase recovered in the pellet. This increase in association can be reversed by dephosphorylating phosphokinase with protein phosphatase either before the association (Fig. 11) or after the association with F-actin (Fig. 12). However, as illustrated by Figure 12, when the dephosphorylation is performed after the association, a greater proportion of both phosphorylated and dephosphorylated kinase is recovered in the pellet (cf. Fig. 11). It should be noted that under these conditions, kinase is incubated with F-actin for 30 min longer than the time of incubation in the experiment shown in Figure 11. Figure 12 also shows that when control (non-phospho) kinase is incubated for that length of time with F-actin, a greater proportion of kinase is recovered in the actin pellet (cf. Fig. 10). This suggests that the association of kinase with F-actin at 4°C is not as rapid as the association of kinase with microtubules.

A number of similarities between these studies with F-actin and the studies with microtubules are evident in Tables VII and VIII. The kinase specific activity in the actin pellet is less than that in either the unspun mixture or the supernatant. The activity of phosphokinase is less than that of non-phosphokinase (Table VII), and this reduction in activity can be partially reversed by dephosphorylating phosphokinase with protein phosphatase (Table VIII). Despite these similarities, the experiments with F-actin were plagued by low recovery of kinase. Part of this problem could be attributed to a difficulty with pipetting the F-actin gel. This caveat notwithstanding, these preliminary results suggest that autophosphorylation of the Type II CaM kinase causes a reversible increase in its association with F-actin.

Discussion

I have used radioimmunoassay and activity measurements to study the association of endogenous Type II CaM kinase with microtubules isolated from rat brain cytosol after a taxol-induced polymerization. Two important findings emerged from this study. First, a significant proportion (34%) of cytosolic kinase is recovered in the microtubule pellet. Secondly, this proportion increases to 69% when the polymerization is performed under conditions that would permit autophosphorylation of the kinase.

To understand and characterize this phenomenon, I examined the association of pure kinase with purified, taxol-polymerized microtubules. I used co-sedimentation of these pure components as an index of association. While this technique is not ideal, it has served as a useful tool for answering questions about the interaction between macromolecules. It has been used in studies of the interaction of phosphofructokinase with F-actin (Luther and Lee, 1986); binding of myosin light chain kinase to actin (Dabrowska et al., 1982); the binding of synapsin I to microtubules (Baines and Bennett, 1986); the association between neurofilaments and microtubules (Runge et al., 1981); the binding of MAPs to 70 kD neurofilament protein (Heimann et al., 1985) and the binding of tubulin to the postsynaptic density fraction (Sahyoun et al., 1986). Thus, while co-sedimentation by itself is not sufficient proof for a specific interaction between macromolecules, results obtained from such experiments can provide a useful framework for further studies *in situ* and *in vivo*.

The results I obtained by studying the association of pure Type II CaM kinase with pure microtubules essentially confirmed the studies in crude cytosol. Autophosphorylation of the kinase increased its association with purified, taxol- polymerized microtubules. The study also revealed that this change can be produced after submaximal incorporation of phosphate

(approximately 4 mol/mol holoenzyme out of a possible total of 30). This phenomenon was also reversed by dephosphorylating phosphokinase with protein phosphatase. The threshold level of autophosphorylation and the reversal by phosphatase reported here are reminiscent of the findings on the autophosphorylation-induced production of calcium-independent activity (Miller and Kennedy, 1986). Thus, the same autophosphorylation induced conformational change may be responsible for the two phenomena.

There are alternative explanations for my experimental results. One of them is that the observed association of kinase with microtubules is an artifact resulting from enzyme denaturation. This is suggested by the finding that kinase specific activity in the microtubule pellet is less than that in either the starting material (unspun mixture) or supernatant. However, the reversal of the increased association of phosphokinase by dephosphorylation argues against this alternative. In addition, the kinase specific activity in the taxol supernatant is not as high as would be expected if the supernatant was being selectively depleted of denatured enzyme. Furthermore, the low recovery of kinase activity after centrifugation (see "Results") indicate that the loss in the activity of kinase in the microtubule pellet occurs during centrifugation, and resuspension of the pellet, i.e., after the association rather than before the association of kinase with the microtubules. A second alternative explanation is that kinase is simply binding to its substrates--tubulin and MAP2. However, the experiments that showed that autophosphorylation of the kinase caused a reversible increase in its association with F-actin, though actin is not a substrate argue against this alternative. Thus, the evidence suggests that autophosphorylation may regulate the subcellular association of the kinase.

It is not uncommon for phosphorylation to regulate the subcellular associations of proteins. Examples include the distribution of vimentin and desmin during myogenesis *in vitro* (D. Gard, 1982); the association of phosphofructokinase with F-actin (Luther and Lee, 1986); the distribution of some cytoplasmic microtubule associated proteins (Pallas and Solomon, 1982) and the association of synapsin I with synaptic vesicles (Schiebler et al., 1986). It should be pointed out that the increased association, following autophosphorylation of kinase observed in my study is not completely consistent with the observations of Saitoh and Schwartz (1985). They reported an autophosphorylation-dependent release of *Aplysia* neuronal Type II CaM kinase from detergent-extracted ganglia. In this study it was not clear how much of the kinase remaining in these "ganglia" was still phosphorylated. It is possible that the association of kinase with the different components of their "membrane-cytoskeleton" preparation may be regulated differently. It is also possible that the regulation of the subcellular association of kinase in *Aplysia* is different from that in the mammalian nervous system. In this regard, Suzuki and Tanaka (1986) did not find any release of kinase from a rat brain synaptic junction preparation, following autophosphorylation of the enzyme.

The results of this study offer some clues as to how the Type II CaM kinase could modulate neuronal function in response to changes in intracellular calcium ion concentration. Autophosphorylation of kinase could constitute a mechanism for the regulation of the subcellular associations of the enzyme by neuronal activity. Thus, the kinase could directly affect the structure and function of microtubules and the actomyosin-based contractile apparatus, by regulating the state of phosphorylation of tubulin, microtubule associated proteins and myosin light chains. Further studies are needed to show that the Type II CaM kinase associates with microtubules and microfilaments *in vivo*, and that

autophosphorylation of the kinase regulates such an association. Such studies will be challenging, but the results of my experiments suggest that they may be worth pursuing.

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Yamamoto, H., Fukunaga, K., Groto, S., Tanaka, E. and Miyamato, E. (1985) J. Neurochem. **44:** 759-768.

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Table I

Proportion of endogenous α-subunit recovered in the microtubule pellet after taxol induced polymerization of microtubules in brain cytosol.

Temperature (°C)	Ca^{2+}	% α-Subunit in MT Pellet[a]
30	-	34 ± 2
30	+	69 ± 4
0	-	3 ± 0.4
0	+	10 ± 1

[a]Calculated by the formula:

$$\% \text{ in pellet} = \frac{\text{amount in pellet}}{\text{amount in pellet} + \text{amount in supernatant}} \times 100$$

The error bars represent the standard error of the mean from three separate experiments.

Table II

Endogenous Type II CaM kinase activity in fractions from brain cytosol after taxol induced polymerization of microtubules at 30°C in the absence or presence of calcium.

Fraction	Total Synapsin I[a] Kinase Activity (nmol/min)		Activity/α-Subunit[b] (nmol/min/μg)	
	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$
Cytosol	70 ± 9	20 ± 5	4.7 ± 0.7	1.3 ± 0.3
MT pellet	4.3 ± 0.4	1.4 ± 0.2	0.8 ± 0.1	0.2 ± 0.03
Taxol supernatant	36 ± 4	4.7 ± 0.9	3.6 ± 0.5	1.1 ± 0.3

[a]Mean ± SEM

[b]Calculated from total synapsin I kinase activity and total α-subunit.

Table III

Endogenous Type II CaM kinase activity in fractions from brain cytosol after taxol induced polymerization of microtubules at 0°C in the absence or presence of calcium.

Fraction	Total Synapsin I[a,c] Kinase Activity (nmol/min)		Activity/α-Subunit[b] nmol/min/μg	
	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$
Cytosol	29 ± 2	27 ± 1	1.7 ± 0.1	1.4 ± 0.1
MT pellet	0.6 ± 0.2	2.2 ± 0.4	1.0 ± 0.4	1.0 ± 0.2
Taxol Supernatant	24 ± 2	21 ± 3	1.1 ± 0.1	1.0 ± 0.2

[a]Mean ± SEM.

[b]Calculated from total synapsin I kinase activity and total α-subunit.

[c]The values are lower than those in Table II for two reasons: 1) 10 μg of synapsin I not 20 μg was used in these assays. (2) The incorporation of phosphate into synapsin I was measured by the "gel method" and not by the "TCA method."

Table IV

Kinase activity recovered after association of pure Type II CaM kinase with taxol-polymerized microtubules at 25°C.

Fraction	Total Synapsin I[a] Kinase Activity (nmol/min)		Activity/α-Subunit[b] (nmol/min/μg)	
	non-phosphokinase	phosphokinase	non-phosphokinase	phosphokinase
Unspun mixture	33 ± 4.0	9.6 ± 1.3	3.0 ± 0.6	0.6 ± 0.1
MT pellet	2.3 ± 0.5	1.6 ± 0.3	1.4 ± 0.3	0.3 ± 0.07
Taxol Supernatant	24 ± 1.4	2.9 ± 0.5	3.5 ± 0.6	0.7 ± 0.1

[a]Mean ± SEM.

[b]Calculated from total synapsin I kinase activity and total α-subunit.

Table V

Kinase activity recovered after association of pure Type II CaM kinase with taxol polymerized microtubules at 4°C.

Fraction	Total Synapsin I[a] Kinase Activity (nmoles/min)		Activity/α-subunit[b] (nmols/min/μg)	
	non-phosphokinase	phosphokinase	non-phosphokinase	phosphokinase
Unspun mixture	38 ± 0.5	20 ± 1.0	3.8 ± 0.4	1.3 ± 0.1
MT pellet	1.9 ± 0.1	1.8 ± 0.2	1.2 ± 0.2	0.4 ± 0.05
Taxol Supernatant	30 ± 2.9	8.5 ± 0.5	5.1 ± 0.6	1.7 ± 0.05

[a]Mean ± SEM

[b]Calculated from total synapsin I kinase activity and total α-subunit.

Table VI

Kinase activity recovered after association of phosphokinase and dephosphorylated kinase[a] with taxol-polymerized microtubules at 4°C.

Fraction	Total Synapsin[b] Kinase Activity (nmol/min)		Activity/α-subunit[c] nmol/min/μg	
	phosphokinase	dephosphorylated kinase	phosphokinase	dephosphorylated kinase
Unspun mixture	15 ± 0.6	22 ± 0.01	2.7 ± 0.1	3.6 ± 0.4
MT pellet	2.3 ± 0.3	1.3 ± 0.4	1.0 ± 0.2	1.2 ± 0.4
Taxol Supernatant	7.0 ± 0.1	18 ± 1.0	2.4 ± 0.2	4.6 ± 0.3

[a]Phosphokinase was dephosphorylated by incubation with protein phosphatase for 30 min on ice (see Methods).

[b]Mean ± S.D.

[c]Calculated from total synapsin I kinase activity and total α-subunit.

Table VII

Kinase activity recovered after association of

pure kinase with F-actin at 4°C.

Fraction	Total Synapsin I Kinase Activity[a] (nmols/min)		Activity/α-Subunit[b] (nmols/min/μg)	
	non-phosphokinase	phosphokinase	non-phosphokinase	phosphokinase
Unspun mixture	43 ± 5.0	24 ± 1.4	7.5 ± 0.9	4.5 ± 0.4
Actin pellet	0.7 ± 0.2	0.8 ± 0.3	1.4 ± 0.4	0.8 ± 0.3
Actin supernatant	23 ± 1.5	12 ± 1.2	6.8 ± 0.5	5.3 ± 0.9

[a]Mean ± S.D.

[b]Calculated from total synapsin I kinase activity and total α-subunit.

Table VIII

Kinase activity recovered after association of phosphokinase and dephosphorylated kinase[a] with F-actin at 4°C.

Fraction	Total Synapsin I Kinase Activity[a] (nmols/min)		Activity/α-Subunit[c] (nomols/min/μg)	
	phosphokinase	dephosphorylated kinase	phosphokinase	dephosphorylated kinase
Unspun mixture	20 ± 3.5	30 ± 4.6	4.2 ± 0.7	6.5 ± 1.2
Actin pellet	0.5 ± 0.07	0.7 ± 0.03	0.6 ± 0.2	2.1 ± 0.2
Actin supernatant	6.4 ± 1.8	14 ± 1.0	5.4 ± 1.6	6.7 ± 1.1

[a]Phosphokinase was dephosphorylated by incubation with protein phosphatase for 30 min on ice (see Methods).

[b]Mean ± S.D.

[c]Calculated from total synapsin I kinase activity and total α-subunit.

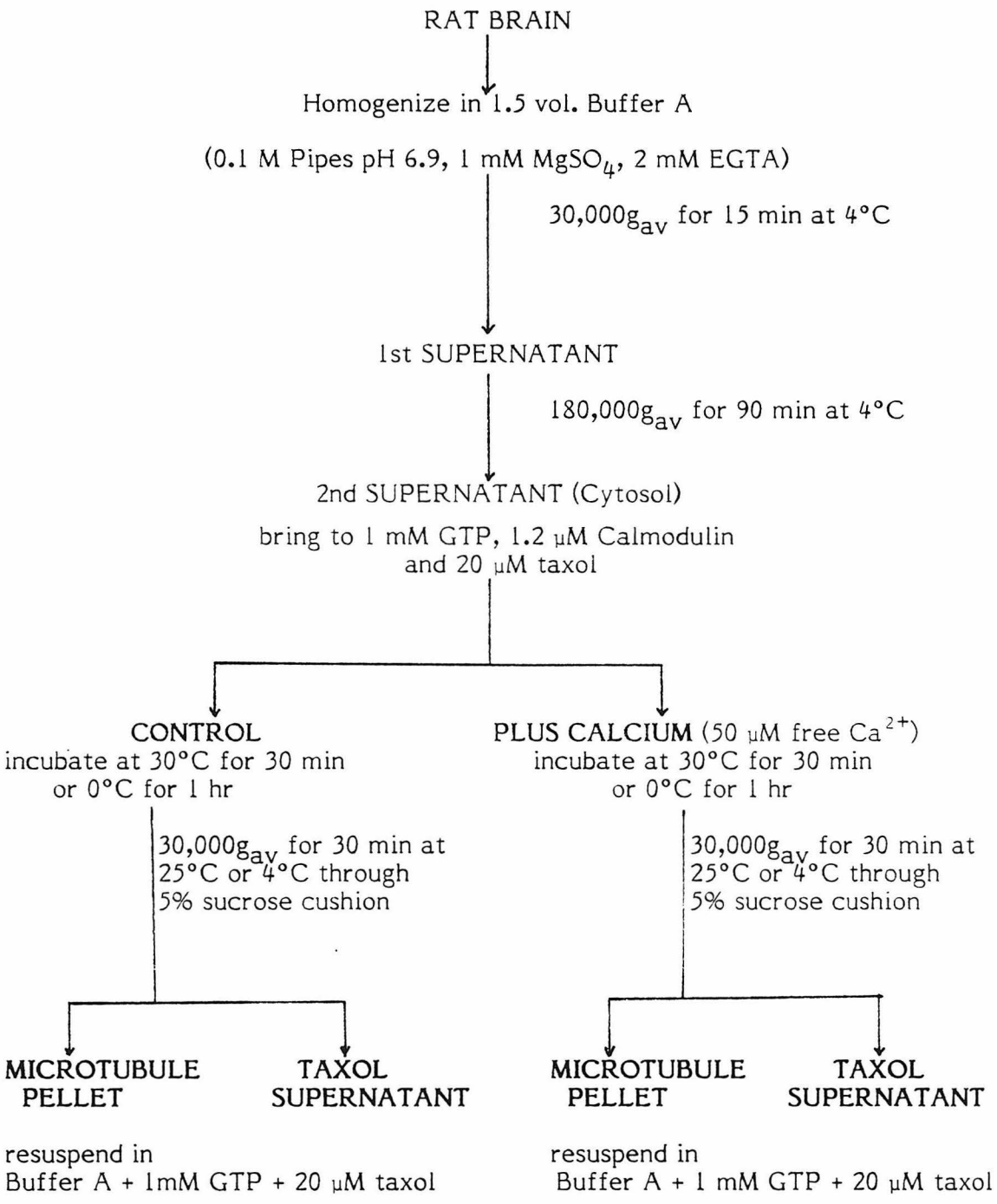


Fig. 1 Scheme for the isolation of microtubules from rat brain, using taxol in the absence or presence of calcium. Free calcium was calculated by using the curves of Portzehl et al. (1964).

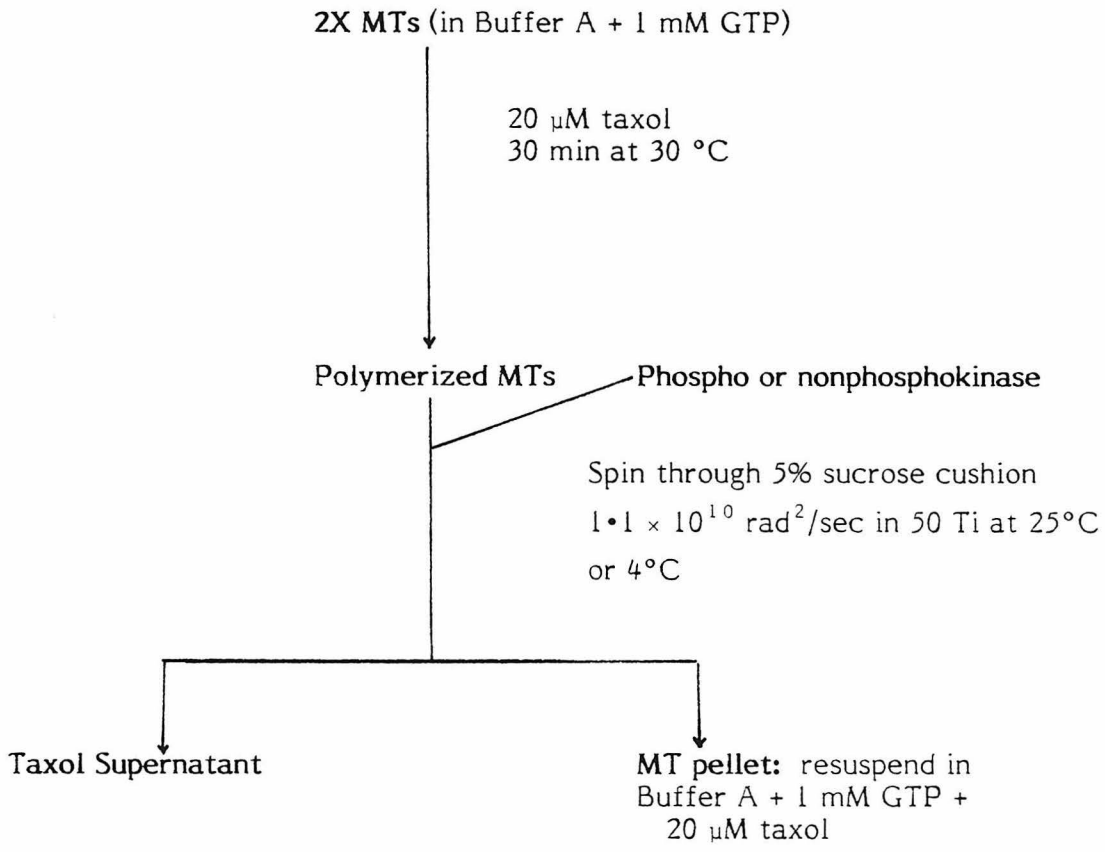
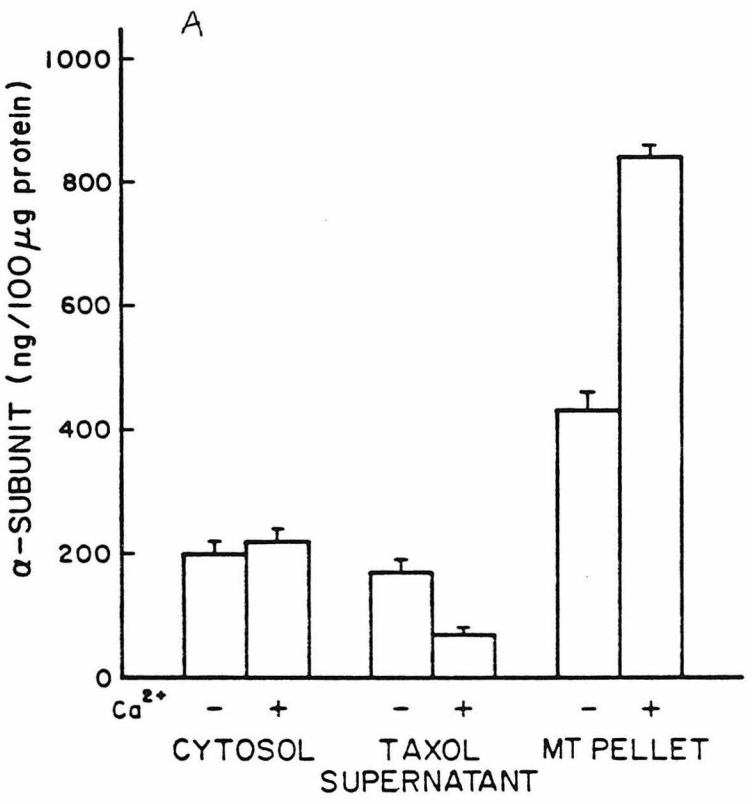


Fig. 2. Scheme for studying the association of Pure Type II CaM kinase with purified, taxol-polymerized microtubules. 2X MTs refers to microtubules purified from brain cytosol by two cycles of assembly/disassembly (Shelanski et al., 1973). Buffer A is 0.1 M pipes pH 6.9, 1 mM $MgSO_4$, 2 mM EGTA.

Fig. 3. Concentration of endogenous α-subunit in taxol-polymerized microtubules from brain cytosol.

Microtubules were isolated from rat brain by taxol polymerization at 30°C (A) or 0°C (B) in the absence (-) or presence (+) of 50 μM free calcium as described under Methods. Aliquots of the various fractions were assayed for α-subunit by radioimmunoassay. Free calcium was calculated by using the curves of Portzehl et al. (1964). Protein was determined in each fraction by the method of Peterson (1977). The error bars represent the standard error of the mean from three separate experiments.


A
α-SUBUNIT (ng/100μg protein)
1000
800
600
400
200
0
Ca2+ - + - + - +
CYTOSOL
TAXOL
MT PELLET
SUPERNATANT

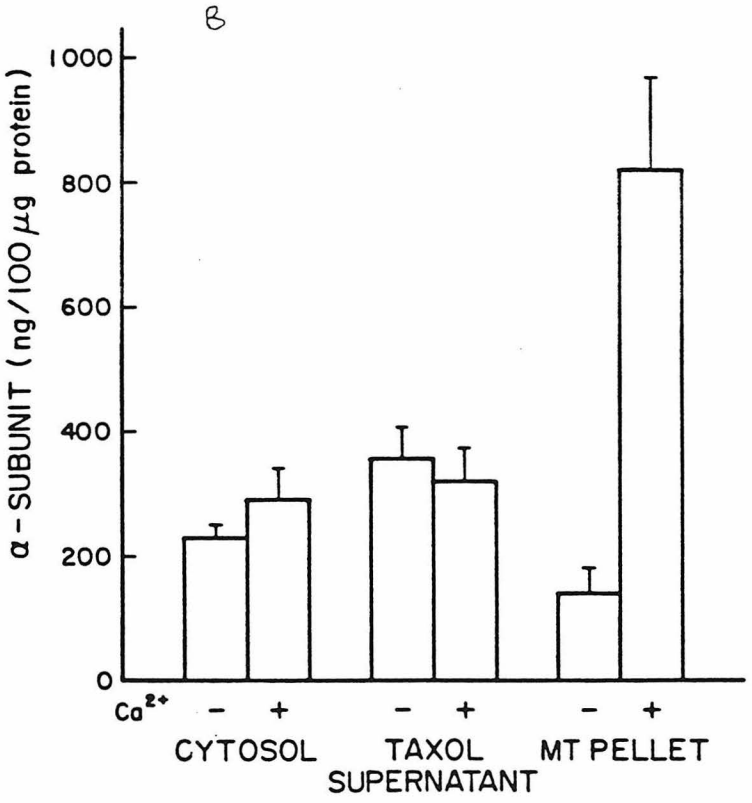

B
α-SUBUNIT (ng/100μg protein)
1000
800
600
400
200
0
Ca2+ - + - + - +
CYTOSOL
TAXOL
MT PELLET
SUPERNATANT

Fig. 4. The effect of autophosphorylation on the association of pure Type II CaM kinase with purified, taxol-polymerized microtubules.

Kinase was prephosphorylated for 30 sec with non-radioactive ATP in the presence of calcium and calmodulin as described under "Methods." Non-phosphokinase was incubated in the absence of ATP. The reaction was stopped with EDTA. An aliquot of the reaction mix was added to taxol-polymerized microtubules bringing the final concentrations of kinase and tubulin dimer to 44 nM and 8.9 μM, respectively. The mixture was immediately spun through a 5% sucrose cushion at 25°C (A) or 4°C (B). In control experiments, BSA was substituted for microtubules (A), and depolymerized MTs were substituted for taxol-polymerized MTs (B). Aliquots of the supernatant and the resuspended pellet were assayed for α-subunit by radioimmunoassay. The % α-subunit recovered in the pellet was calculated by the formula:

$$\% \text{ in pellet} = \frac{\text{amount in pellet}}{\text{amount in pellet} + \text{amount in supernatant}} \times 100$$

The error bars represent the standard error of the mean from four separate experiments (A) and three experiments (B).

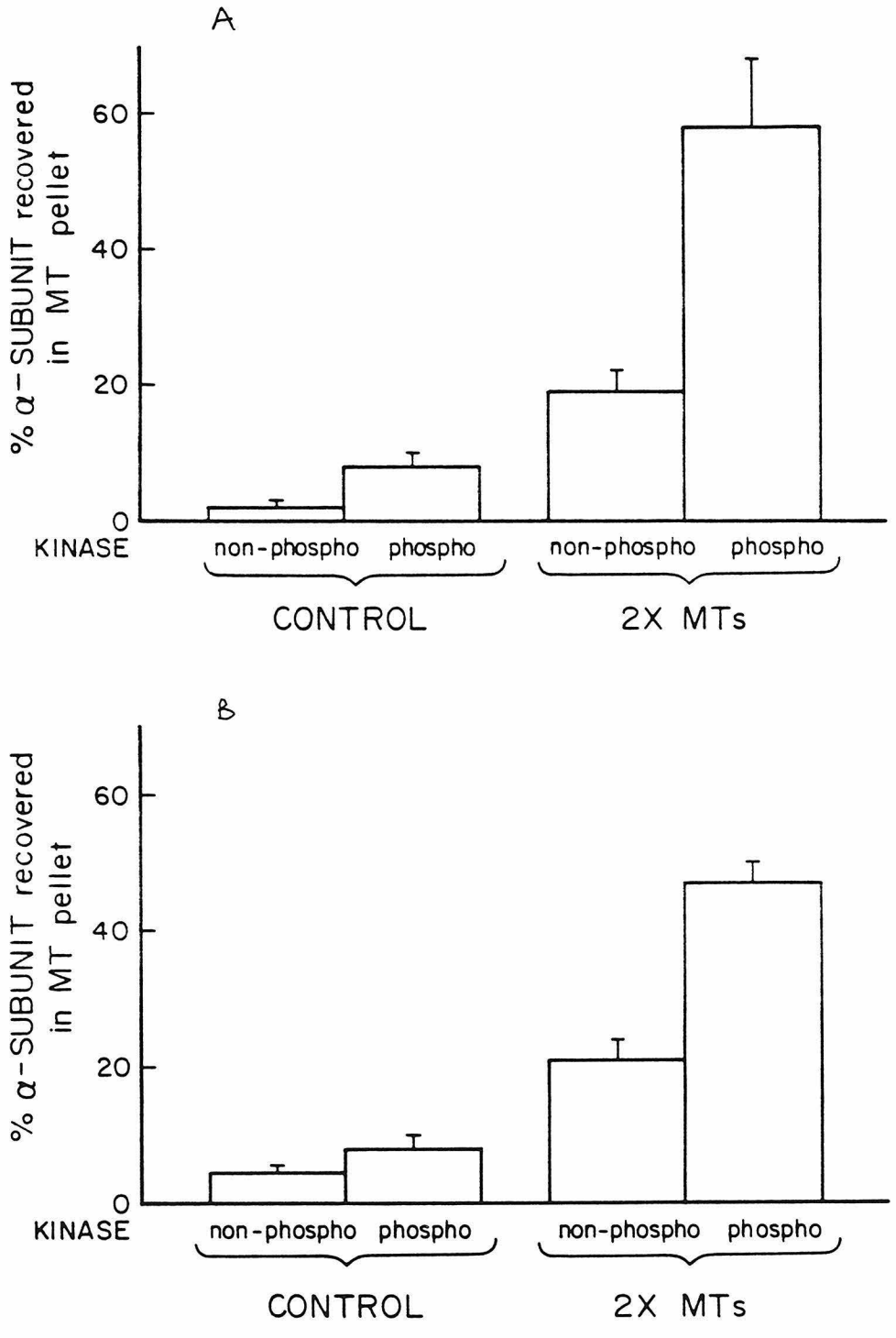

A
% α-SUBUNIT recovered in MT pellet
60
40
20
0
KINASE
non-phospho
phospho
non-phospho
phospho
CONTROL
2X MTs
B
% α-SUBUNIT recovered in MT pellet
60
40
20
0
KINASE
non-phospho
phospho
non-phospho
phospho
CONTROL
2X MTs

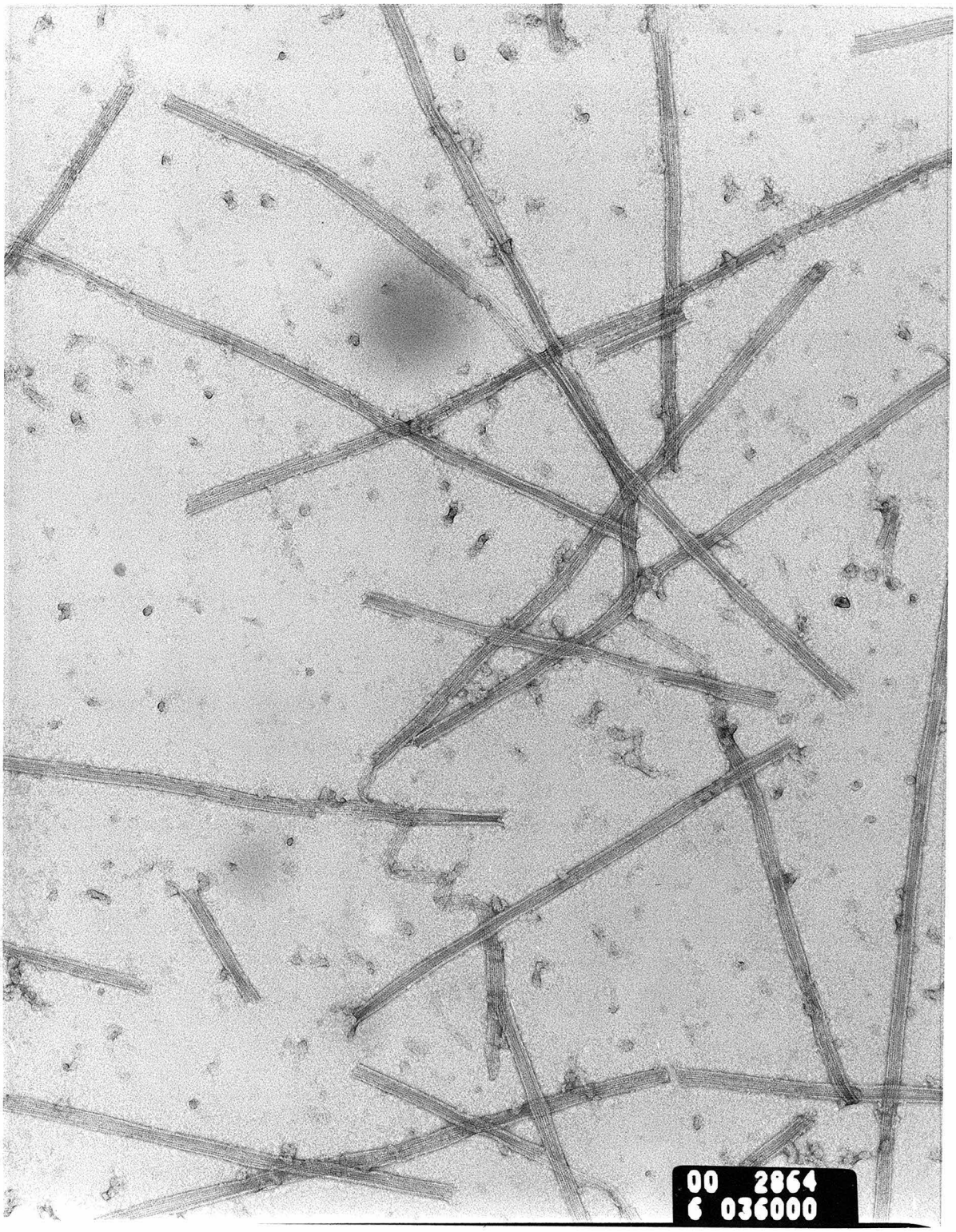
Fig. 5. Electron microscopy of taxol-polymerized microtubules.

Twice cycled microtubules (2X MTs) at a concentration of 1.5 mg/ml were polymerized at 30°C for 30 min in the presence of 20 μM taxol. The microtubules were spun through a 5% sucrose cushion as described in the legend of Figure 4. 10 μl of the resuspended microtubule pellet were placed on a carbon coated grid that had been prewetted with bacitracin. The sample was stained with 2% uranyl acetate and examined in the electron microscope.

A: 76,000 X

B: 174,000 X

A



B

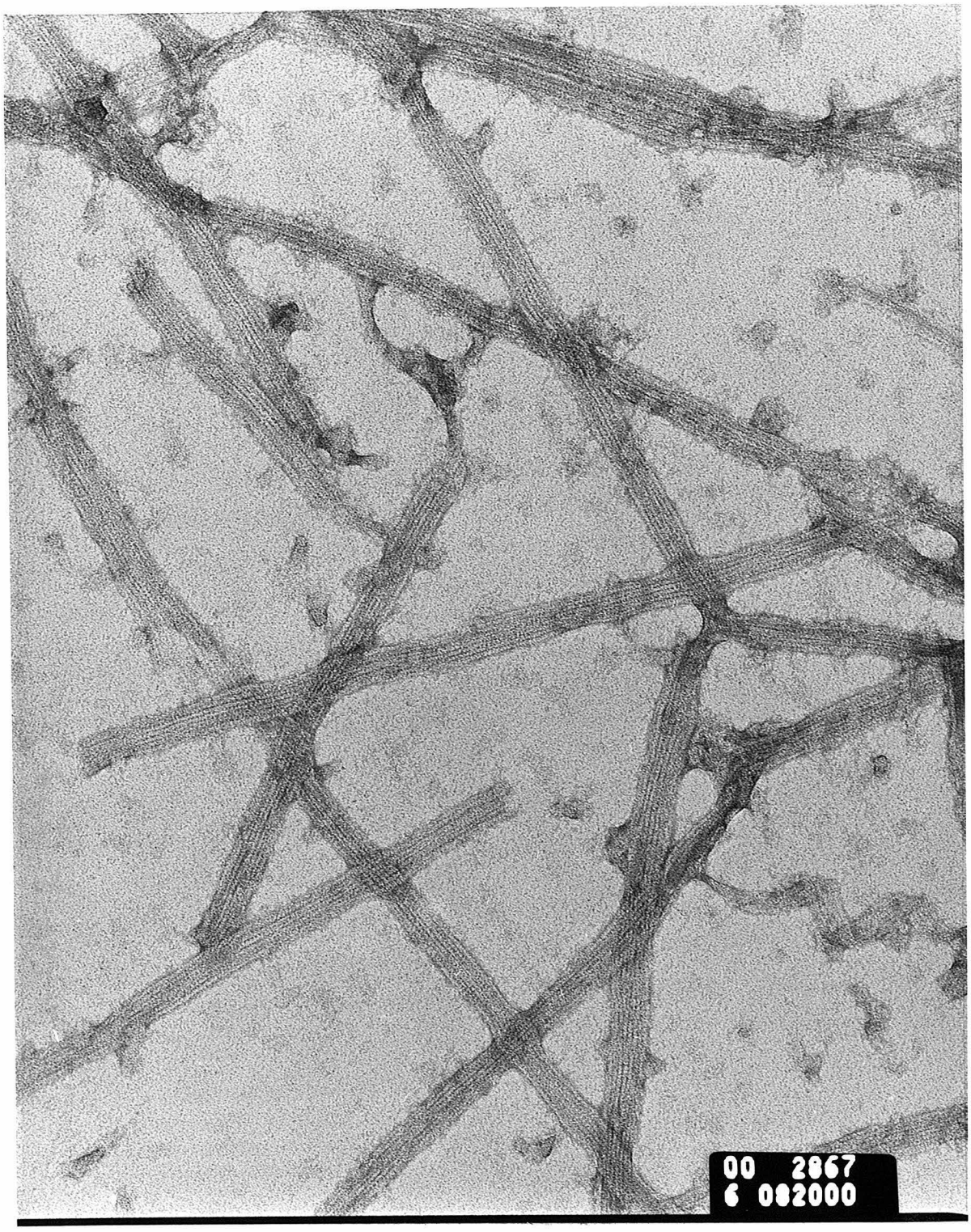


Fig. 6. The minimum level of autophosphorylation required to produce the increased association of Type II CaM kinase with microtubules.

Kinase was prephosphorylated for 15 sec as described in Figure 4, except that the concentrations of $MgCl_2$ used in the assay were varied to produce different levels of phosphate incorporation. To determine the degree of autophosphorylation, parallel experiments were performed with radioactive ATP. The mixture of phosphokinase and taxol-polymerized microtubules was immediately centrifuged through a 5% sucrose cushion as described in Figure 4. We determined the amount of kinase in the pellet and supernatant fractions, using both synapsin I kinase activities and α-subunit measurements. Maximum binding was taken as that exhibited by kinase prephosphorylated for 30 sec in the presence of 10 mM $MgCl_2$. Prephosphorylation of kinase for as long as 2 min did not cause an additional increase in its association with taxol-polymerized microtubules. The error bars represent one standard deviation, and data were pooled from two separate experiments.

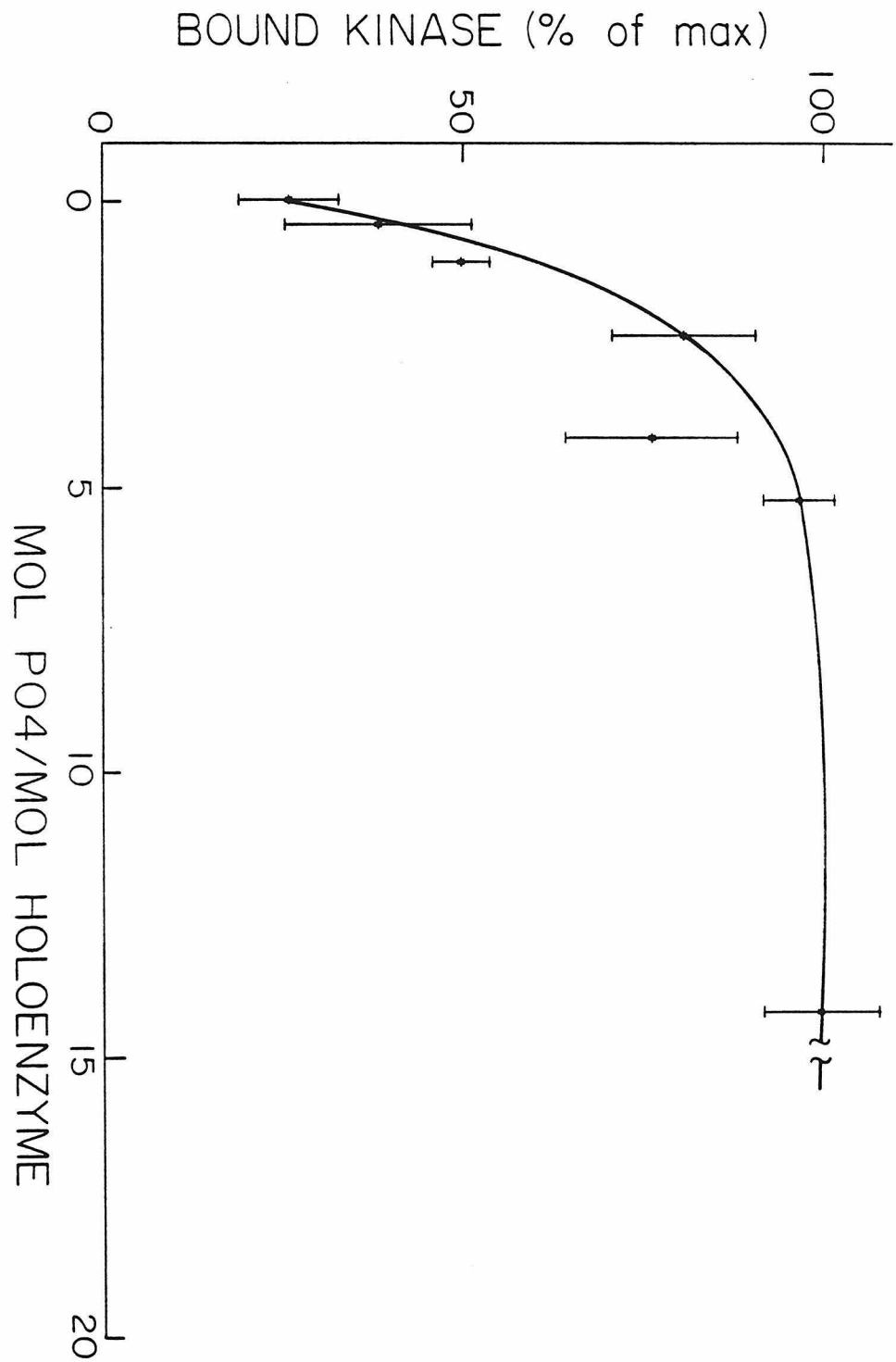

BOUND KINASE (% of max)
0
50
100
0
5
10
15
20
MOL PO4/MOL HOLOENZYME

Fig. 7. Dephosphorylation of phosphokinase by protein phosphatase.

Kinase was prephosphorylated with radioactive ATP for 15 sec in the presence of either 0.5 mM or 1 mM $MgCl_2$, as described in Figure 6. The reaction was stopped with EDTA and the assay mix was then incubated on ice with protein phosphatase. At the indicated times, aliquots were taken, and phosphate remaining on the kinase was determined by the gel method as described under Methods. These values were then plotted as percent of the phosphate in phosphokinase at time zero. One hundred percent is 5.1 moles PO_4/mol holoenzyme (X) and 8.6 moles PO_4/mol holoenzyme (O).

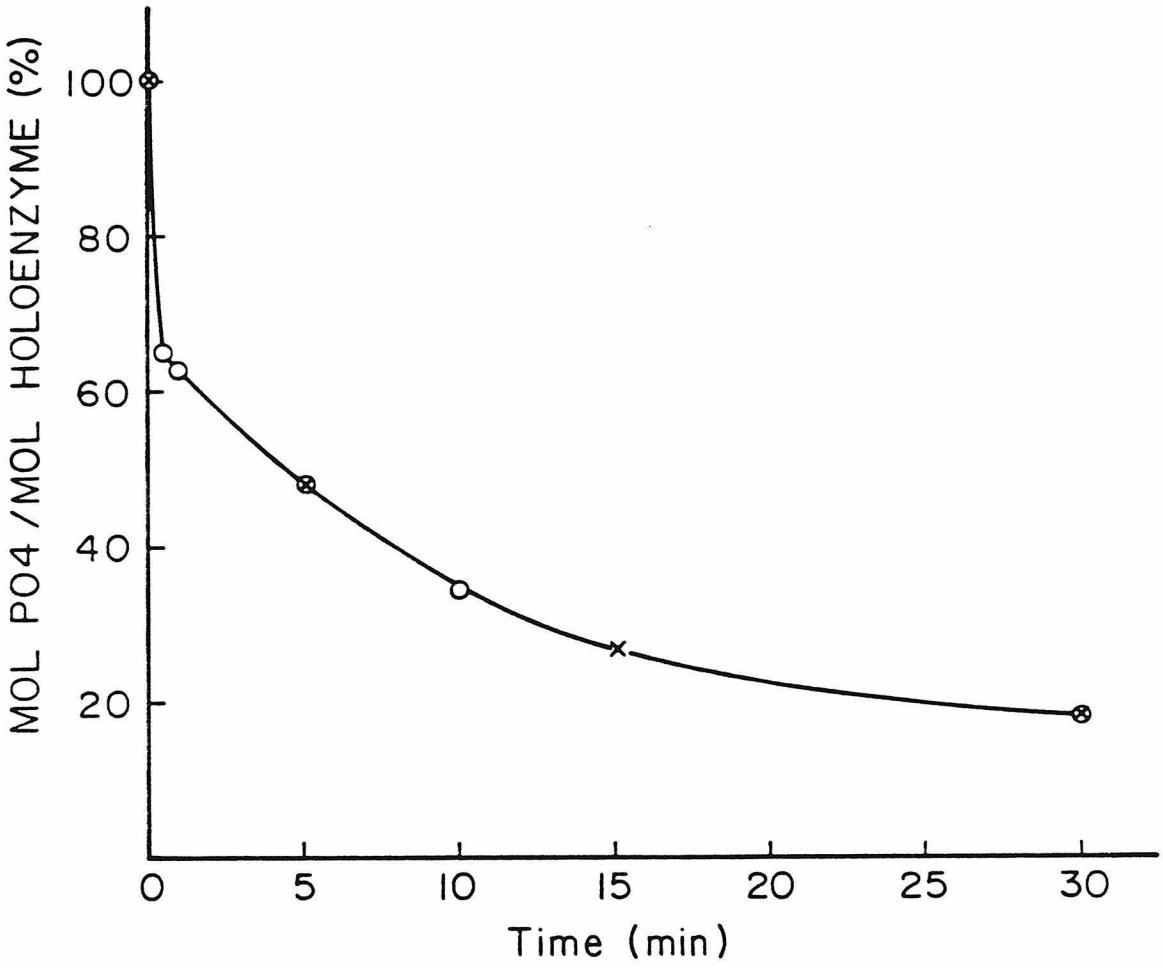

MOL PO4 /MOL HOLOENZYME (%)
100
80
60
40
20
0
5
10
15
20
25
30
Time (min)

Fig. 8. Effect of dephosphorylation on the association of phosphokinase with microtubules; dephosphorylation performed before association.

Kinase was prephosphorylated under conditions where 5.1 moles PO_4 are incorporated per mol holoenzyme (see Fig. 7). The reaction was stopped with EDTA. Control kinase was incubated identically except that ATP was added after the reaction had been quenched with EDTA. One-half of each assay mix was incubated on ice with protein phosphatase for 30 min, and the other half was incubated on ice with buffer for 30 min. The association of both phospho and control kinase with taxol-polymerized microtubules was examined as described in Figure 4B. The proportion of α-subunit recovered in the pellet is shown. The error bars represent the standard deviation from two separate experiments.

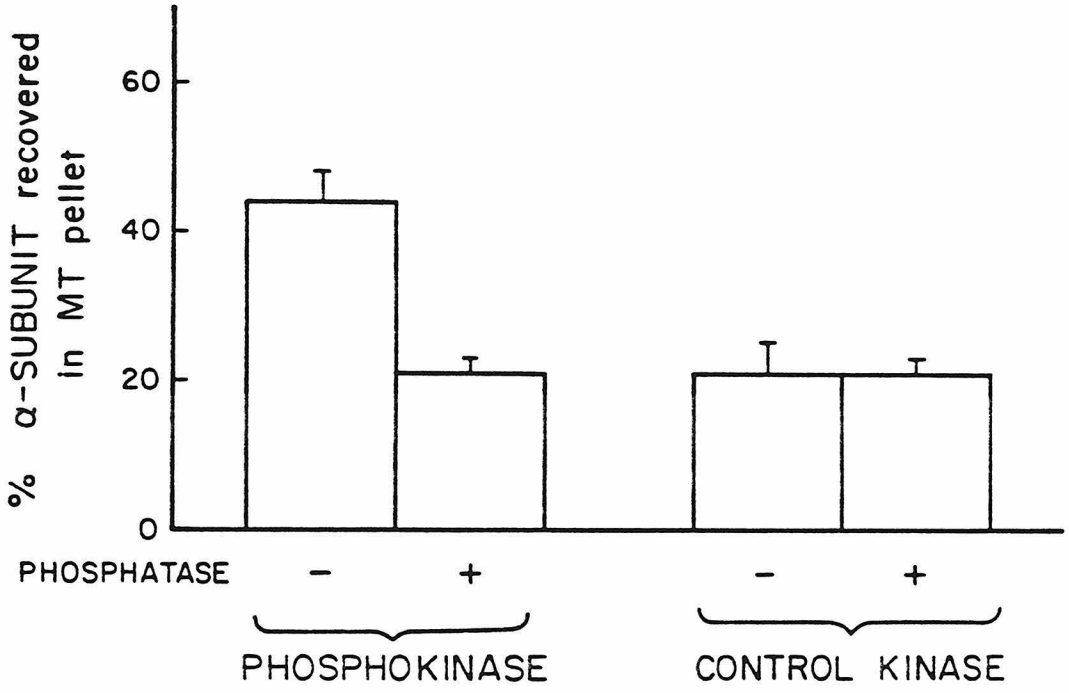

% α-SUBUNIT recovered in MT pellet
60
40
20
0
PHOSPHATASE
−
+
−
+
PHOSPHOKINASE
CONTROL KINASE

Fig. 9. Effect of dephosphorylation on the association of phosphokinase with microtubules; dephosphorylation performed after association.

This experiment was performed as described in Figure 8, except that protein phosphatase or buffer minus phosphatase was added to phosphokinase after kinase had been preincubated with taxol-polymerized microtubules for 3 min on ice. A parallel experiment was performed with kinase autophosphorylated with ATP-γS. The proportion of α-subunit recovered in the pellet is shown. The error bars represent the standard deviation from two separate experiments.

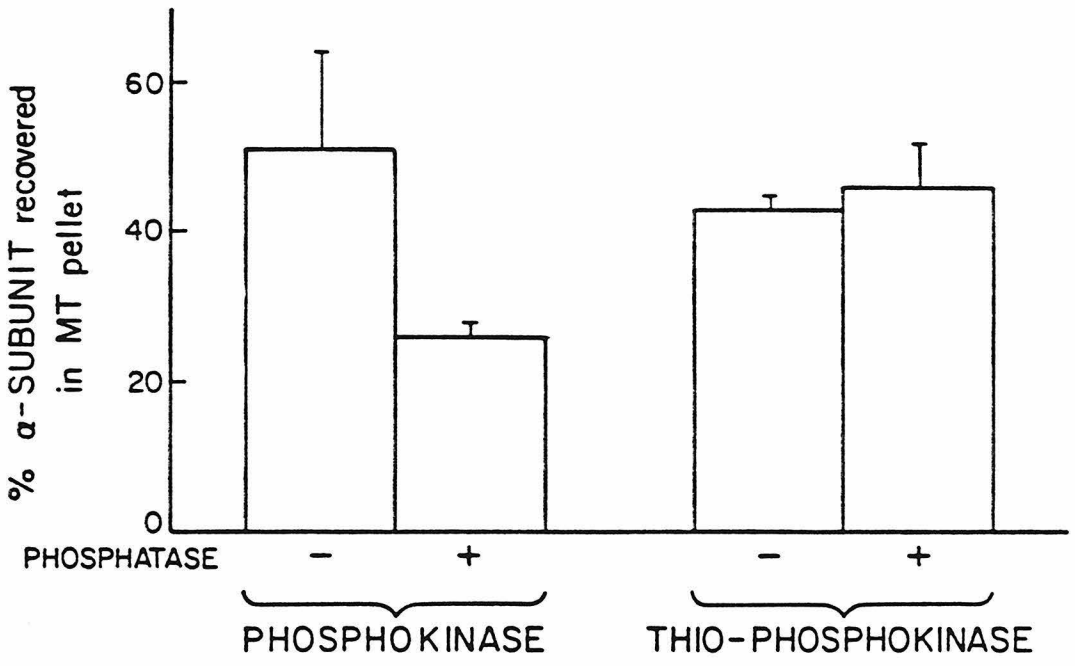

% α-SUBUNIT recovered in MT pellet
60
40
20
0
PHOSPHATASE
−
+
−
+
PHOSPHOKINASE
THIO-PHOSPHOKINASE

Fig. 10. The effect of autophosphorylation on the association of pure Type II CaM kinase with F-actin at 4°C.

The experiment was performed as described in Figure 4, except that F-actin was substituted for taxol-polymerized microtubules. The proportion of α-subunit recovered in the actin pellet is shown. The error bars represent the standard deviation from two separate experiments.

Fig. 11. Effect of dephosphorylation on the association of phosphokinase with F-actin; dephosphorylation performed before association.

The experiment was performed as described in Figure 8, except that F-actin was substituted for taxol-polymerized microtubules. The proportion of α-subunit recovered in the actin pellet is shown. The error bars represent the standard deviation from two separate experiments.

Fig. 10

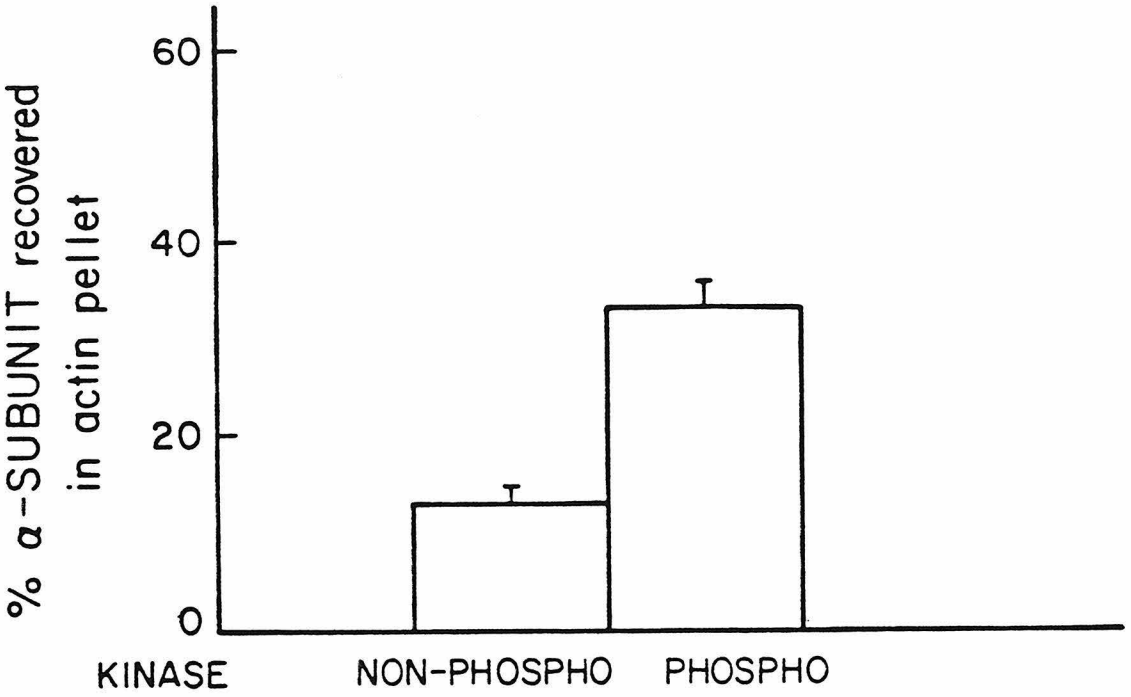

% α-SUBUNIT recovered in actin pellet
60
40
20
0
KINASE
NON-PHOSPHO
PHOSPHO

Fig. 11

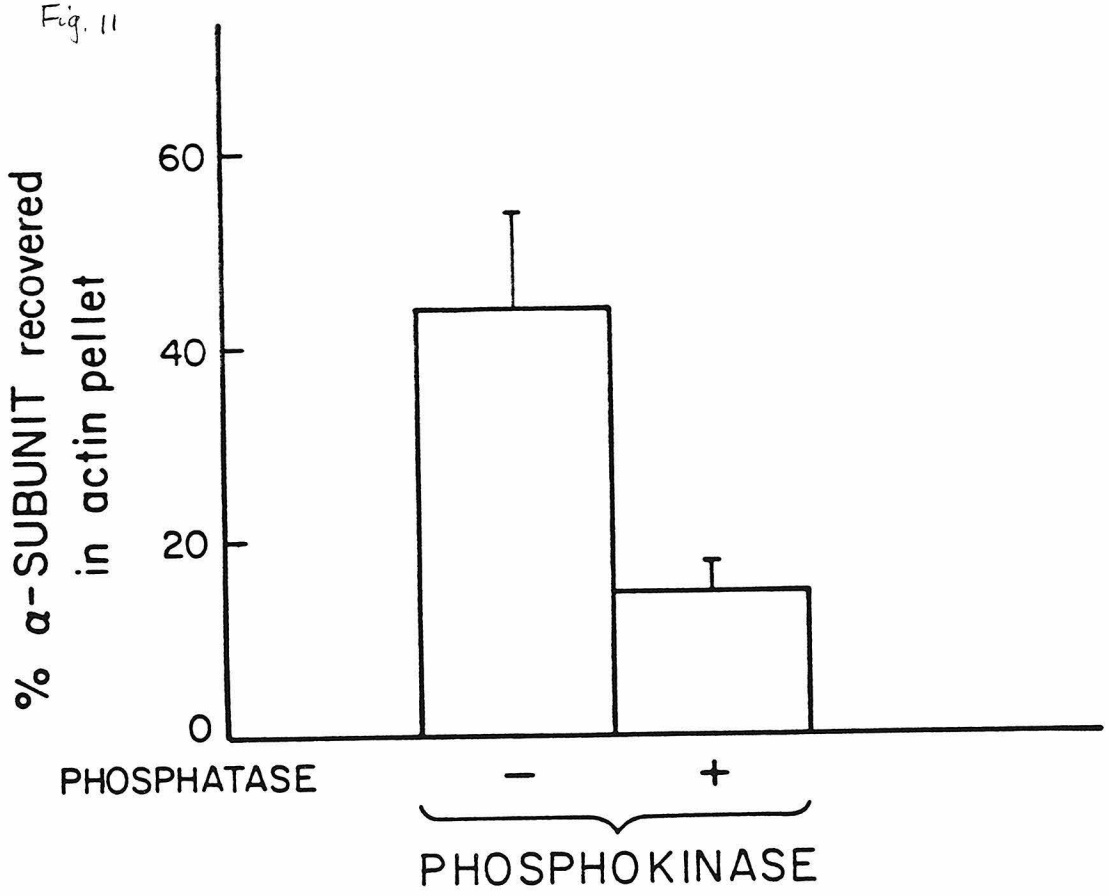
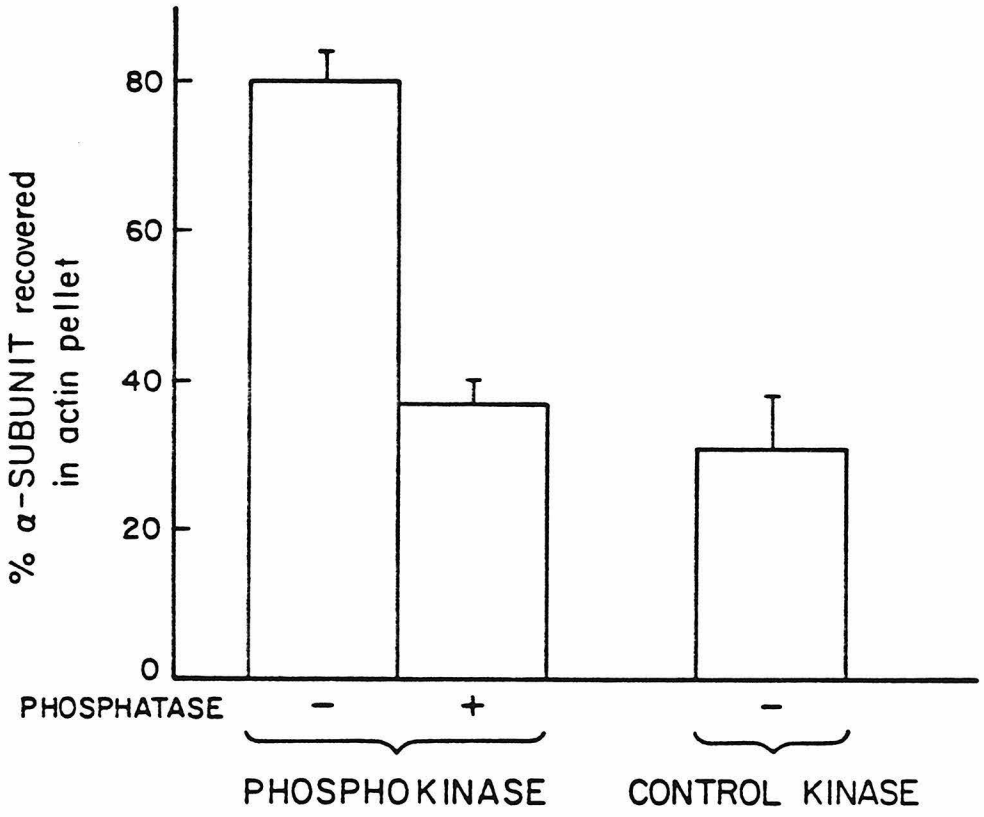

% α-SUBUNIT recovered in actin pellet
60
40
20
0
PHOSPHATASE
−
+
PHOSPHOKINASE

Fig. 12. Effect of dephosphorylation on the association of phosphokinase with F-actin; dephosphorylation performed after association.

The experiment was performed as described in Figure 9, except that F-actin was substituted for taxol-polymerized microtubules. In a parallel experiment, control kinase (non-phosphokinase) was incubated with F-actin for the same length of time as phospho and dephosphorylated kinase (33 min). The proportion of α-subunit recovered in the actin pellet is shown. The error bars represent the standard deviation from the two separate experiments.


% α-SUBUNIT recovered in actin pellet
80
60
40
20
0
PHOSPHATASE
−
+
−
PHOSPHOKINASE
CONTROL KINASE

Conclusion

Changes in the concentration of intracellular calcium regulates a number of important neuronal processes (Greengard, 1981; Reichardt and Kelly, 1983). The identification and characterization of calcium target proteins will be a useful approach in understanding how calcium is able to regulate such a diverse number of physiological processes.

Brain Type II CaM kinase is one of several calcium target proteins that have been identified in the nervous system (Bennett et al., 1983; Kennedy, 1983; Reichardt and Kelly, 1983). The immunochemical characterization of the Type II CaM kinase reported in this thesis provides some clues as to how it could modulate neuronal function in response to changes in intracellular calcium ion concentration. As determined by radioimmunoassay, the kinase is highly concentrated in mammalian brain, making up to 1% of total brain protein. There is also a marked regional variation in its concentration: it is most highly concentrated in the forebrain, where it comprises approximately 2% of total hippocampal protein. Such a high concentration of the kinase in forebrain neurons would confer on them a set of specialized responses to changes in calcium ion concentration. In addition, these neurons may be able to respond very rapidly to a calcium signal. It is interesting that the regions of the brain that are especially enriched in the kinase are also those that display robust long-term potentiation (Swanson et al., 1982; Eccles, 1983; Racine et al., 1983), raising the possibility that the kinase may be involved in long-term activity dependent changes in synaptic efficacy. The results of recent experiments are consistent with this possibility: Miller and Kennedy (1986) showed that autophosphorylation of the kinase causes the enzyme to become calcium independent, thereby providing a mechanism by which kinase activity could be prolonged beyond the duration of an initial activating calcium signal.

It has also been suggested that the Type II CaM kinase could regulate cytoskeletal structure and function (Yamamoto et al., 1983, 1985; Yamauchi and Fujisawa, 1983; Edelman et al., 1986; Tanaka et al., 1986; Wandosell et al., 1986). The observation (reported in this thesis) that kinase can associate with F-actin and microtubules is consistent with this suggestion. My experimental results also suggest that autophosphorylation of the enzyme could constitute a mechanism for the regulation of its subcellular associations by neuronal activity.

Further studies will be necessary in order to extend the results presented in this thesis to *in vivo* and reconstituted systems. The reagents characterized and the methods developed in my experiments should be useful in these studies. The results of such studies would contribute immensely in establishing the role of the Type II CaM kinase in the regulation of neuronal function by calcium.

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Yamamoto, H., Fukunaga, K., Groto, S., Tanaka, E. and Miyamoto, E. (1985) J. Neurochem. **44:** 759-768.

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Appendix I

PURIFICATION AND CHARACTERIZATION OF A CALMODULIN-DEPENDENT PROTEIN KINASE THAT IS HIGHLY CONCENTRATED IN BRAIN

This material has appeared previously in The Journal of Biological Chemistry **258:** 12735-12744.

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Purification and Characterization of a Calmodulin-dependent Protein Kinase That Is Highly Concentrated in Brain*

(Received for publication, May 10, 1983)

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A calcium and calmodulin-dependent protein kinase has been purified from rat brain. It was monitored during the purification by its ability to phosphorylate the synaptic vesicle-associated protein, synapsin I. A 300-fold purification was sufficient to produce kinase that is 90–95% pure as determined by scans of stained sodium dodecyl sulfate-polyacrylamide gels and has a specific activity of 2.9 μmol of ^{32}P transferred per min/mg of protein. Thus, the kinase is a relatively abundant brain enzyme, perhaps comprising as much as 0.3% of the total brain protein.

The Stokes radius (95 Å) and sedimentation coefficient (16.4 S) of the kinase indicate a holoenzyme molecular weight of approximately 650,000. The holoenzyme is composed of three subunits as judged by their co-migration with kinase activity during the purification steps and co-precipitation with kinase activity by a specific anti-kinase monoclonal antibody. The three subunits have molecular weights of 50,000, 58,000, and 60,000, and have been termed α, β′, and β, respectively. The α- and β-subunits are distinct peptides, however, β′ may have been generated from β by proteolysis. All three of these subunits bind calmodulin in the presence of calcium and are autophosphorylated under conditions in which the kinase is active. The subunits are present in a ratio of about 3 α-subunits to 1 β/β′-subunit. We therefore postulate that the 650,000-Da holoenzyme consists of approximately 9 α-subunits and 3 β/β′-subunits.

The abundance of this calmodulin-dependent protein kinase indicates that its activation is likely to be an important biochemical response to increases in calcium ion concentration in neuronal tissue.

The functions of specialized cell types within different tissues are coordinated by a variety of extracellular agents. Each of these triggers a series of intracellular regulatory events that ultimately alter the functional state of the cell. The first step in the action of many regulatory agents is to increase the intracellular concentration of a second messenger, such as a cyclic nucleotide or calcium ion (1–3). A complete understanding of the general principles of cellular regulation will require a description of the molecular events triggered by each of these second messengers.

We are interested in the mechanisms of action of calcium ion in nervous tissue. A number of important neuronal processes are regulated by changes in the intracellular concentration of calcium (4–7). These changes are brought about both by the actions of specific extracellular agents such as neurotransmitters and neurohormones, and by electrical activity which activates voltage-sensitive calcium channels (8). Changes in calcium concentration are important in synaptic processes such as post-tetanic potentiation and long-term potentiation that involve modulation of the quantity of transmitter released per impulse (9–11). In certain neurons, the conductances of specific potassium (12) or calcium (13) membrane channels are regulated by intracellular calcium ion. Such transient changes in the strength of specific synapses, or in the electrical properties of individual neurons can modulate the flow of information through complex neuronal circuits (14). Information about the properties and distributions in different types of neurons of proteins that are regulated by physiological changes in calcium concentration will be necessary to understand, at the molecular level, the responses of various neurons, and of individual synaptic terminals, to changes in calcium flux.

It is generally accepted that the cyclic nucleotides regulate cell physiology primarily, if not exclusively, by activating protein kinases (15, 16). Distinct kinases that are activated by either cyclic AMP or cyclic GMP have been purified and characterized (17, 18). In contrast, the mechanisms by which calcium alters cellular functions appear to be quite diverse (19). Nevertheless, the importance of calcium-regulated protein kinases has recently been recognized (5, 20). Initial characterization of this distinct class of kinases has revealed that, unlike the cyclic AMP-dependent protein kinases, there are a number of calcium-regulated protein kinases that differ in several characteristics, including mechanism of regulation by calcium, substrate specificity, and tissue distribution (21–29).

Calcium and calmodulin-dependent protein kinase activities have been observed in crude brain homogenates and in various neuronal subcellular fractions (26, 27, 29–34). Although two well characterized calmodulin-dependent protein kinases from non-neuronal tissues, myosin light chain kinase, and phosphorylase kinase, are present in brain, they do not account for a large portion of brain calmodulin-dependent protein kinase activity (26, 27). This paper reports the purification and subunit structure of a distinct calmodulin-dependent protein kinase that is highly concentrated in brain. The enzyme was first observed in brain homogenates as a calmodulin-dependent kinase that phosphorylated synapsin I, a protein associated with synaptic vesicles (26, 35). For clarity, we continue to refer to the enzyme as "calmodulin-dependent synapsin I kinase," although we believe that it is likely to be involved in the phosphorylation and regulation of a number of other brain proteins.

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The structure of the synapsin I kinase is different from either myosin light chain kinase or phosphorylase kinase (22–25). However, it is similar but not identical to a glycogen synthase kinase recently purified from liver (36, 37), and another calmodulin-dependent protein kinase recently purified from rat brain (38). Thus, it may be one of a class of related calmodulin-dependent protein kinases.

EXPERIMENTAL PROCEDURES

Materials—ATP, 3′,5′-cAMP, dithiothreitol, imidazole, EDTA, PMSF,[1] fast green FCF, Coomassie brilliant blue R, fibrinogen, bovine serum albumin, ovalbumin, phosphorylase *b*, carbonic anhydrase, soybean trypsin inhibitor, actin, alcohol dehydrogenase, glycogen synthase, trypsin, casein, phosvitin, arginine-rich histone, Type II hemoglobin, insulin, and transferrin were purchased from Sigma. Thyroglobulin, ferritin, catalase, Protein A and Sepharose 4B were purchased from Pharmacia Fine Chemicals. Leupeptin was purchased from Peninsula Laboratories, Inc. Myosin was purchased from Bethesda Research Laboratories. Lactoperoxidase was purchased from Calbiochem. Chymotrypsinogen was purchased from Worthington. [γ-^{32}P]ATP was purchased from ICN Nutritional Biochemicals. Na^{125}I and [^{3}H]3′,5′-cAMP were purchased from New England Nuclear. Trifluoperazine was obtained from Smith, Kline, and French. Ultrapure sucrose was purchased from Schwarz/Mann. DEAE-cellulose (DE-52) was purchased from Whatman. Nitrocellulose membranes (BA85, 0.45 μm) were purchased from Schleicher and Schuell. RPMI 1640 tissue culture medium, glutamine, penicillin, and streptomycin were purchased from Gibco Laboratories. Newborn calf serum was purchased from Irvine Scientific. Selenous acid (Specpure) was purchased from Johnson Matthey, Inc., Synapsin I was purified from bovine brain by a modification of the procedure of Ueda and Greengard (39), as described in Huttner *et al.* (40). The modifications were as follows. A crude brain particulate fraction was used at step 2, rather than the M-1 fraction. The pH 6 supernatant was adjusted to pH 8 and subjected to chromatography on CM-cellulose, hydroxylapatite and Sephadex G-100. The final purified fractions were concentrated by ammonium sulfate precipitation and dialyzed against 25 mM Tris-HCl, pH 7.0. Calmodulin was purified from bovine brain by the method of Watterson *et al.* (41). Microtubule protein prepared from rat brain by the method of Shelanski *et al.* (42), was a gift of James Soha of our laboratory. Gizzard myosin light chain was a gift of Dr. Robert Adelstein of the National Institutes of Health. Affinity-purified rabbit anti-calcineurin was a gift of Dr. Claude Klee of the National Institutes of Health. Calmodulin-Sepharose was prepared by the method of March *et al.* (43). Simonsen albino rats (140–160 g males) were purchased from Simonsen Laboratories.

Assay for Calmodulin-dependent Synapsin I Kinase Activity—Calmodulin-dependent synapsin I kinase was assayed, with minor modifications, as previously described (26, 35) at 30 °C in a reaction mixture (final volume, 100 μl) containing 50 mM Tris (pH 8.0), 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 1 μg of calmodulin, 50 μM [γ-^{32}P]ATP (0.5–2 × 10^3 cpm/pmol), varying amounts of enzyme, either 0.4 mM EGTA (minus calcium) or 0.4 mM EGTA/0.7 mM $CaCl_2$ (plus calcium), and either no synapsin I, or 10 μg of synapsin I. After preincubation for 1 min, the reaction was initiated by the addition of [γ-^{32}P]ATP, and terminated after 30 s by the addition of 50 μl of a "stop solution" containing 0.3 M EDTA and 2 mg/ml of bovine serum albumin (as a carrier) followed immediately by 1 ml of ice-cold 10% trichloroacetic acid. After 10 min at 4 °C, the reaction mixture was centrifuged in a Beckman microfuge B for 2 min, and the supernatant was removed by aspiration. The pellet was redissolved in 100 μl of 0.1 N NaOH and immediately reprecipitated with 1 ml of 10% trichloroacetic acid. After dissolving the final pellet in 100 μl of 0.1 N NaOH, 1 ml of Aquasol (New England Nuclear) was added and the radioactivity was quantitated by liquid scintillation spectrometry. Phosphorylation of synapsin I by the calmodulin-dependent kinase was measured as the difference between calcium-stimulated incorporation of ^{32}P into trichloroacetic acid-insoluble material in the absence and presence of synapsin I. In the later steps of the purification (after DEAE-cellulose chromatography), phosphorylation of synapsin I in the absence of calcium was minimal, and incorporation of ^{32}P into trichloroacetic acid-insoluble material was measured only in the presence of calcium. A previous report presented evidence that brain homogenates contained two calmodulin-dependent synapsin I kinase activities that recognized different sites in the synapsin I molecule. The kinase activities were named "30-kDa region" kinase and "10-kDa region" kinase, after the *Staphylococcus aureus* V8 protease peptides that contained the respective sites (26). In the present report, phosphorylation of synapsin I was measured at pH 8, rather than at pH 7. At this pH, 95% or more of the radioactive phosphate incorporated into synapsin I in the presence of calcium was present in the site recovered in the 30-kDa fragment, even in crude brain homogenates. Thus, this assay condition eliminated the need to subtract phosphorylation by the 10-kDa region kinase.

Phosphorylation of the Kinase Subunits—Incorporation of ^{32}P into the kinase subunits was measured under the conditions used to measure synapsin I kinase activity except that synapsin I was omitted from the assay, [γ-^{32}P]ATP was used at a lower concentration (10 μM) and higher specific activity (2–4 × 10^4 cpm/pmol), and the reaction was terminated after varying lengths of time by the addition of 50 μl of a stop solution containing 9% SDS, 6% (v/v) 2-mercaptoethanol, 15% (w/v) glycerol, 0.186 M Tris-HCl (pH 6.7) and a trace of bromphenol blue. The solution was then boiled for 2 min and 120 μl was subjected to SDS/PAGE. The gel was dried and the ^{32}P-labeled proteins were localized by autoradiography (26). When quantitation was necessary, the labeled bands were cut out of the dried gel, placed in liquid scintillation fluid, and subjected to liquid scintillation spectrometry.

Substrate Specificity—Incorporation of phosphate into various substrate proteins was measured under standard assay conditions using 60 ng of purified kinase and varying amounts of possible substrate proteins. The reaction was terminated after 30 s by addition of 50 μl of SDS stop solution. The samples were boiled for 2 min and subjected to SDS/PAGE. After the gels were stained and dried, ^{32}P-labeled proteins were located by autoradiography. For quantitation, labeled bands were cut out of the dried gel, placed in liquid scintillation fluid and subjected to liquid scintillation spectrometry.

Immunoprecipitation of Kinase Activity—Hybridomas that secrete monoclonal antibodies specific for the synapsin I kinase were selected from cells formed by fusion of NS1/SP2 myeloma cells with spleen cells from mice that had been immunized with kinase purified through the calmodulin-Sepharose step. Details of the preparation and selection of these hybridomas, including the one used in this study (VIE9, anti-kinase) will be published separately.[2] For immunoprecipitation experiments, VIE9 and a control hybridoma (VF3, anti-sodium channel, kindly supplied by Dr. Larry Fritz of Caltech) were grown to 2 × 10^5 cells/ml in RPMI 1640 medium supplemented with 20% calf serum, 2 mM glutamine, 1 mM pyruvate, 0.5 mM oxaloacetic acid, and 1 mg/ml of penicillin-streptomycin. The cells were harvested by centrifugation and washed once in an equal volume of the same medium without calf serum and with 5 μg/ml of insulin, 0.1 mg/ml of transferrin, 20 μM ethanolamine, and 0.4 μg/ml of H_2SeO_3 (selenium) (44). They were then resuspended in the supplemented serum-free medium and kept for 24 h at 37 °C in a CO_2-controlled incubator. Cells were removed by centrifugation from the medium which contained secreted antibodies. The antibodies were concentrated 100-fold by precipitation with 50% ammonium sulfate, and dialyzed against 40 mM Tris-HCl (pH 7.5).

For precipitation experiments, purified synapsin I kinase (15 μg) was added to 40 μg of either VIE9 anti-kinase antibody, control monoclonal antibody, or 40 mM Tris-HCl (pH 7.5) in a volume of 0.1 ml and incubated overnight on ice. The resulting immune complexes were pelleted by centrifugation in a Beckman microfuge B for 2.5 min and 10-μl aliquots of the supernatants were assayed for synapsin I kinase activity. The protein in the remaining supernatants was concentrated by precipitation with 10% trichloroacetic acid, dissolved in 1/3 diluted SDS stop solution, and adjusted to neutral pH with NaOH. Pellets containing the immune complexes were washed twice with phosphate-buffered saline, dissolved in 1/3 diluted SDS stop solution, and, together with the supernatants, subjected to SDS/PAGE.

Densitometric Scans of SDS-Polyacrylamide Gels—The purified kinase was subjected to SDS/PAGE and protein bands were stained with either Coomassie blue (0.075% in 25% isopropanol, 10% acetic acid) or fast green (0.5% in 50% methanol, 10% acetic acid) (45).

[1] The abbreviations used are: PMSF, phenylmethylsulfonyl fluoride; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

[2] N. E. Erondu, M. B. Kennedy, V. Krieger, and C. B. Shapiro, manuscript in preparation.

Lanes were cut from the gels and scanned with a Beckman ACTA III recording spectrophotometer at 560 nm for Coomassie blue and 635 nm for fast green. Peak areas were quantitated using a Tektronix 4052 minicomputer interfaced to a Tektronix 4956 digitizing tablet. The areas of the major peaks were found to be linearly proportional to the amount of protein loaded on the gel over a range of 7.5–30 μg with the fast green stain. The linear range was more variable with the Coomassie blue stain, but was usually from 2–10 μg.

Immunoblots—Proteins from SDS gels were transferred onto nitrocellulose paper as described by Towbin *et al.* (46) in a Bio-Rad Trans-Blot Cell. After transfer, the nitrocellulose sheets were incubated with: 1) Buffer D (50 mM Tris (pH 7.4), 0.9% NaCl, 0.1% NaN_3) containing 5% hemoglobin (2 h); 2) the appropriate antibody diluted into Buffer D containing 1% hemoglobin (8–12 h); 3) wash buffer (Buffer D containing 0.5% hemoglobin) (1 h with 3 changes); 4) affinity-purified rabbit anti-mouse IgG diluted 1/500 into wash buffer (2 h); 5) wash buffer (1 h with 3 changes); 6) ^{125}I protein A ($2–3 \times 10^6$ cpm/ml) in wash buffer (2 h); 7) wash buffer (1 h with 3 changes). The nitrocellulose sheet was then dried, and bands containing ^{125}I were detected by autoradiography.

Other Procedures—Polyacrylamide gel electrophoresis in the presence of SDS was performed by the method of Laemmli (47). The stacking gel (2.5 × 16 cm × 1.5 mm) contained 3.5% acrylamide/0.09% bisacrylamide and the running gel (14 × 16 cm × 1.5 mm) contained 10% acrylamide/0.27% bisacrylamide. Molecular mass standards used in SDS/PAGE were: microtubule-associated protein$_2$, 300 kDa; phosphorylase *b*, 94 kDa; bovine serum albumin, 68 kDa; α-tubulin, 56 kDa; β-tubulin, 54 kDa; ovalbumin, 43 kDa; alcohol dehydrogenase, 41 kDa; carbonic anhydrase, 29 kDa; chymotrypsinogen, 25 kDa; soybean trypsin inhibitor, 21.5 kDa. The sedimentation coefficient ($s_{20,w}$) of the kinase was determined by sucrose density gradient centrifugation as described by Martin and Ames (48) with ovalbumin ($s_{20,w}$ = 3.5 S), fibrinogen ($s_{20,w}$ = 7.9 S), catalase ($s_{20,w}$ = 11.3 S) and thyroglobulin ($s_{20,w}$ = 19.2 S) as standards. The Stokes radius (Å) of the kinase was determined by gel filtration on Sepharose 4B using the analysis described by Porath (49). Protein standards used for calibration of the gel filtration column were: catalase, Stokes radius = 52 Å; ferritin, Stokes radius = 61 Å; thyroglobulin, Stokes radius = 85 Å; fibrinogen, Stokes radius = 107 Å; myosin, Stokes radius = 200 Å. Protein was determined by a modification of the method of Lowry *et al.* (50) with bovine serum albumin as a standard. Iodinated tryptic peptide maps were prepared by the method of Elder *et al.* (51). Calmodulin-stimulated phosphodiesterase was assayed as described by Rangel-Aldao *et al.* (52).

RESULTS

Purification of Calmodulin-dependent Synapsin I Kinase

The initial steps of the purification (through calmodulin affinity chromatography) were as previously described by Kennedy *et al.* (35) with modifications. In that study it was shown that synapsin I kinase is present in both soluble and particulate fractions of brain homogenates and that kinases partially-purified from each of the two fractions are indistinguishable by several criteria. For practical reasons, we have used as our enzyme source, the soluble fraction alone. Under the conditions described, it contains about 60% of the total synapsin I kinase activity. All purification steps were carried out at 0–4 °C.

Preparation of Crude Extract—Brains (1.4 g each) were removed from 90 rats and homogenized immediately by 12 up-and-down strokes with a Teflon/glass homogenizer at 900 rpm in 10 volumes of Buffer A (20 mM Tris (pH 7.5), 1 mM imidazole, 0.1 mM $CaCl_2$, 1 mM dithiothreitol, 0.1 mM PMSF) containing 1 mM $MgCl_2$, 25 mg/liter of soybean trypsin inhibitor and 1 mg/liter of leupeptin. The homogenate was centrifuged at 10,000 × *g* for 20 min and the resulting supernatant was centrifuged at 170,000 × *g* for 1 h.

DEAE-cellulose Chromatography—The 170,000 × *g* supernatant was brought to 1 mM iodoacetate, adjusted to pH 7.5, and loaded onto a DEAE-cellulose column (5 × 17 cm) previously equilibrated with Buffer A. The column was washed with one column volume of 0.05 M NaCl in Buffer A and was then developed with a 2-liter linear gradient of 0.05–0.30 M NaCl in Buffer A.

Ammonium Sulfate Fractionation—The DEAE column fractions containing synapsin I kinase activity were pooled, adjusted to 0.1 M Tris (pH 7.5), 1 mM dithiothreitol, 0.1 mM PMSF, and brought to 40% saturation by the addition of solid ammonium sulfate. After 3–12 h, precipitated protein was collected by centrifugation and redissolved in a small volume of Buffer B (40 mM Tris (pH 7.5), 0.2 mM $CaCl_2$, 1 mM dithiothreitol, 0.1 mM PMSF) containing 0.2 M NaCl.

Calmodulin Affinity Chromatography—The redissolved ammonium sulfate precipitate was cleared by centrifugation for 10 min at 10,000 × *g*, then applied to a calmodulin-Sepharose affinity column (1.5 × 7 cm) equilibrated with Buffer B containing 0.2 M NaCl. The column was then washed overnight with Buffer B containing 2 M NaCl (~120 ml), followed by one column volume of Buffer B. Synapsin I kinase was eluted with 40 mM Tris (pH 7.5), 2 mM EGTA, 1 mM dithiothreitol, 0.1 mM PMSF. Recovery of enzyme activity from the column was 50–60% with a 13-fold purification.

Sepharose 4B Gel Filtration—The calmodulin-Sepharose column fractions containing synapsin I kinase activity were pooled and adjusted to 40% ammonium sulfate as described above. The precipitate was redissolved in a small volume of Buffer C (40 mM Tris (pH 7.5), 2 mM EGTA, 0.2 M NaCl, 1 mM dithiothreitol) containing 20% (v/v) glycerol and loaded on a Sepharose 4B column (0.75 × 120 cm) equilibrated with the same buffer. The column was developed at a flow rate of 1.8 ml/h. The elution profile obtained is shown in Fig. 1. It contains a single peak of kinase activity that coincides with the major protein peak. Analysis of the peak fractions by SDS/PAGE indicated that a major 50 kDa protein and two bands in the 60-kDa region (58 and 60 kDa) co-migrated with the peak of kinase activity (data not shown). This step resulted in a 2-fold purification with 40–50% recovery of activity.

Sucrose Density Gradient Centrifugation—The peak Sepharose 4B fractions were pooled and protein was concentrated by precipitation with 40% ammonium sulfate. The precipitate was redissolved in a small volume of Buffer C and loaded onto two 12-ml linear gradients of 5–20% sucrose in Buffer C. The gradients were subjected to centrifugation at 40,000 rpm for 12 h in a Beckman SW 40 Ti rotor. They were then fractionated with an Isco Model 184 density gradient fractionator. As shown in Fig. 2, the gradients contained a single major peak of both synapsin I kinase activity and protein. The minor peak closer to the bottom of the tube may represent an enzyme dimer. The peak fractions were pooled and concentrated by calmodulin affinity chromatography (to remove sucrose), followed by ammonium sulfate precipitation. The ammonium sulfate precipitate was dissolved in a small volume of Buffer C so that the protein concentration was 1–2 mg/ml. After this step, the kinase showed variable stability when stored at 0 °C, but could be stored at −80 °C for long periods with virtually no loss of activity.

The purification procedure is summarized in Table I. The concentrated sucrose pool represented a 290-fold purification over the homogenate with 1.2% recovery of activity from the homogenate and 2.9% recovery from the 170,000 × *g* supernatant. After the final step, 90–95% of the protein present was associated with peptides that co-migrated with kinase activity throughout the purification and were co-precipitated with kinase activity by an anti-kinase monoclonal antibody (see below).

One reason for the rather low recovery was a variable and unusually poor recovery after DEAE-cellulose chromatogra-

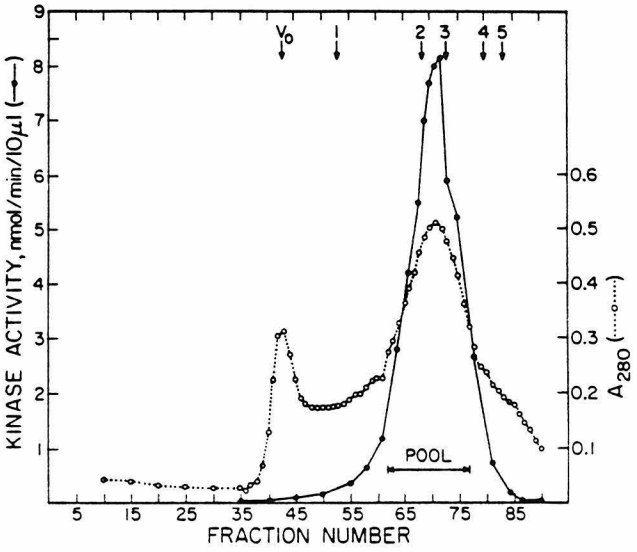

FIG. 1. **Sepharose 4B elution profile (purification step 7).** Gel filtration on Sepharose 4B was carried out as described in the text. Fractions of 0.6 ml were collected and assayed for synapsin I kinase activity. Aliquots of each fraction were diluted as required to obtain a linear rate of synapsin I phosphorylation in a 30-s assay. The elution positions of standard proteins were determined in separate runs by absorbance at 280 nm and are indicated by *arrows*. Standard proteins were: 1) myosin (Stokes radius = 200 Å); 2) fibrinogen (107 Å); 3) thyroglobulin (85 Å); 4) ferritin (61 Å); and 5) catalase (52 Å).

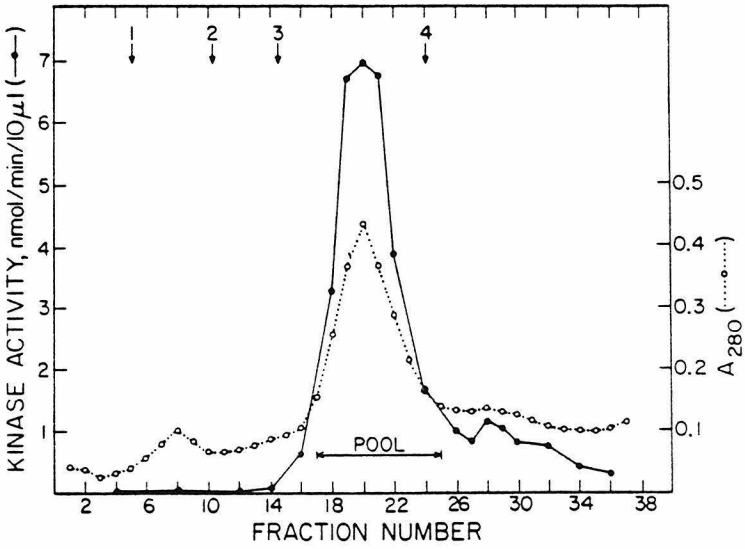

FIG. 2. **Sucrose density gradient sedimentation profile (purification step 8).** Sucrose density gradient centrifugation was carried out as described in the text. Fractions of 0.33 ml were collected (starting from the *top* of the gradient) and assayed for synapsin I kinase activity. Aliquots of fractions were diluted for assay as described in the legend of Fig. 1. The positions of standard proteins were determined on parallel gradients by absorbance at 280 nm and are indicated by *arrows*. Standard proteins were: 1) ovalbumin ($s_{20,w}$ = 3.5 S); 2) fibrinogen (7.9 S); 3) catalase (11.3 S); and 4) thyroglobulin (19.2 S).

TABLE I

Summary of purification of rat brain calmodulin-dependent synapsin I kinase

Step	Total activity*	Total protein*	*n*	Specific activity	Purification	Recovery
	μmol/min	*mg*		*μmol/min/mg*	*-fold*	%
1. Homogenate	132 ± 27	13,100 ± 1,900	9	0.010	1	100
2. 10,000 × *g* Supernatant	82.7 ± 23	3,660 ± 755	9	0.023	2.3	63
3. 170,000 × *g* Supernatant	55.8 ± 16	3,010 ± 747	9	0.019	1.9	42
4. DEAE-cellulose eluate	17.4 ± 6.7	725 ± 84	7	0.024	2.4	13
5. 40% Ammonium sulfate	17.4 ± 5.4	225 ± 45	7	0.077	7.7	13
6. Calmodulin-Sepharose eluate	9.5 ± 3.1	9.6 ± 3.1	7	0.99	99	7.2
7. Sepharose 4B eluate	3.8 ± 0.8	2.1 ± 0.5	5	1.81	181	2.9
8. Sucrose gradient pool	1.6 ± 0.7	0.55 ± 0.2	2	2.90	290	1.2

* Values shown are mean ± S.D. of *n* experiments.

phy. In large scale preparations, recovery at this step has varied from 18 to 40%. These recoveries are lower than those first reported for smaller scale preparations (35). This may be due to denaturation of the enzyme with time on the column. We have tried recombining the enzyme fraction with other pooled column fractions and with proteins stripped from the column by 2 M salt and have seen no stimulation of activity. Thus, we have no evidence that we have lost a factor required for maximal activity. Substitution of other DEAE-resins has not improved the recovery. Recoveries for subsequent purifi-

cation steps, as reported in Table I, include small losses during concentration of pooled fractions. Recoveries during the chromatography and centrifugation steps themselves were generally 50% or greater. In recent preparations, we have eliminated the calmodulin-Sepharose concentration step following sucrose density gradient centrifugation, and this has improved recovery.

Structure and Properties of the Calmodulin-dependent Synapsin I Kinase

Identification of the Kinase Subunits—The peptide composition of the enzyme fractions at each stage of the purification is shown in Fig. 3. The purified kinase fraction contained a major peptide band at 50 kDa, a less prominent band at 60 kDa, and minor bands at 58 and 45 kDa. Two lines of evidence indicate that the 50-, 58-, and 60-kDa peptides (termed α, β', and β respectively) are associated with enzyme activity and are present in a holoenzyme complex.

The amount of these three proteins peaked in the same fractions as kinase activity during gel filtration and sucrose density gradient centrifugation. An example is shown in Fig. 4. In this experiment, the purified kinase was subjected to analytical sucrose density gradient centrifugation. Fractions were assayed for synapsin I kinase activity and subjected to SDS/PAGE. The gels were stained with Coomassie blue and the amount of protein in each band was determined from the area of the optical density peaks on densitometric scans. Although the β and β'-bands were separated on the gel, the optical density peaks on the scans were not well resolved (see Fig. 6). The ratio of the β' "shoulder" to the β peak was constant, so that β and β'-bands were grouped together and treated as one "band" for this analysis. They will be referred to as the β/β'-band. As shown in Fig. 4, both the α and β/β'-bands peak in the same fraction as enzyme activity, and both of the peaks are asymmetric, tailing toward the bottom of the gradient. The exact ratio of the α to β/β'-bands across the gradient was slightly variable, but this probably reflects variability in the staining with Coomassie blue (see below), rather than true differences in the ratio of the bands.

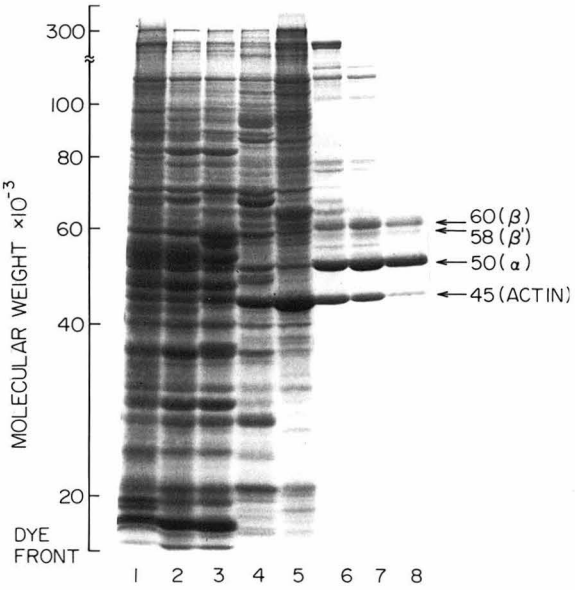

FIG. 3. Purification of synapsin I kinase as monitored by SDS/PAGE. Samples from each step of the purification were subjected to SDS/PAGE and stained with Coomassie blue. The purification step and amount of protein corresponding to each *lane* are as follows: *lane 1*, homogenate, 100 μg; *lane 2*, 10,000 × *g* supernatant, 100 μg; *lane 3*, 170,000 × *g* supernatant, 100 μg; *lane 4*, DEAE-cellulose eluate, 75 μg; *lane 5*, 40% ammonium sulfate precipitate, 75 μg; *lane 6*, calmodulin-Sepharose eluate, 15 μg; *lane 7*, Sepharose 4B eluate, 10 μg; *lane 8*, sucrose gradient pool, 7.5 μg.

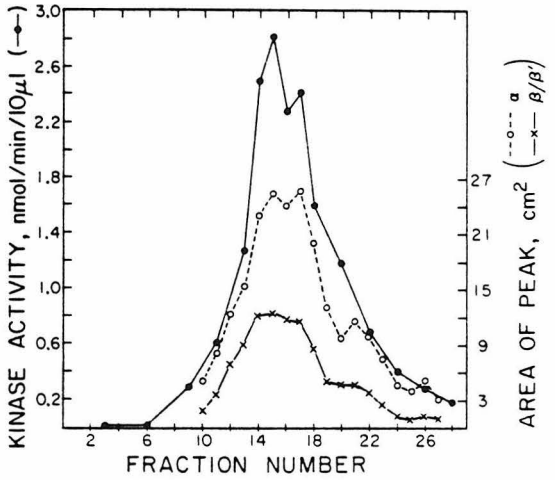

FIG. 4. Co-migration of α and β/β' peptides with kinase activity during sucrose density gradient centrifugation. A portion of the concentrated sucrose pool from purification step 8 (600 μg) was loaded on a 4.8-ml linear gradient of 5–20% sucrose in Buffer C. The gradient was subjected to centrifugation at 65,000 rpm for 4 h in a Beckman SW 65 Ti rotor. Fractions of 0.18 ml were collected and an aliquot of each was diluted and assayed for kinase activity as described in the legend of Fig. 1. A 75-μl aliquot of each fraction was subjected to SDS/PAGE. The gel was stained with Coomassie blue and the amount of α and β/β' peptide in each fraction was quantitated by densitometric scans.

Further evidence that these bands are indeed subunits of the kinase comes from the use of a monoclonal antibody raised against the partially purified kinase. Incubation of the kinase with an appropriate amount of this antibody, as described under "Experimental Procedures," resulted in the precipitation of 92–94% of the enzyme activity along with the α, β, and β' protein bands (Fig. 5). These same three proteins were also specifically precipitated from less pure enzyme fractions that contained several other proteins (data not shown). Incubation without antibody or with a control monoclonal antibody did not precipitate the enzyme activity or the protein bands. The results of the sucrose gradient experiment (Fig. 4) and the immunoprecipitation experiment (Fig. 5) indicate that the α, β, and β' proteins are contained in a complex that has calmodulin-dependent protein kinase activity.

The α and β enzyme subunits appear to be distinct peptides by two criteria. First, phosphopeptide maps of the phosphorylated forms of the two subunits are different and do not support the notion that, for example, the α-subunit was generated from the β-subunit by proteolysis (35). Second, two monoclonal antibodies that react with the α-subunit on immunoblots, do not react with the β or β'-subunits.[2] On the other hand, β' may have been generated from β by proteolysis, since phosphopeptide maps of these two subunits are consistent with this possibility (35). Further structural studies will be necessary to clarify the relationship of the two β-subunits.

The 45-kDa protein band contained in the purified kinase has been identified as actin by the following criteria (data not shown): 1) It co-migrated with skeletal muscle actin during

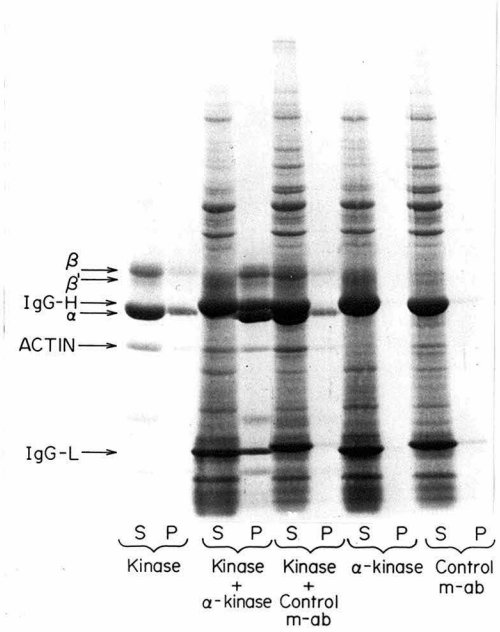

FIG. 5. **Immunoprecipitation of kinase activity.** Immunoprecipitation of kinase activity was carried out as described under "Experimental Procedures." The protein staining patterns of the supernatant (*S*) and pellet (*P*) from incubations containing kinase alone, kinase plus either anti-kinase antibody (*α-kinase*) or control monoclonal antibody (*m-ab*), and the two antibodies alone are shown. Kinase activity remaining in the supernatant after incubation with α-kinase antibody was 7.8% of the activity in the kinase control and 5.6% of the activity remaining in the supernatant after incubation with the control antibody. The lightly staining, low molecular weight bands seen in the kinase supernatant and in the immunoprecipitate are breakdown products which gradually appear after prolonged storage. These breakdown products are recognized by the α-kinase antibody on immunoblots and are precipitated with the enzyme activity (*kinase + α-kinase* pellet). *IgG-H* and *IgG-L* refer to the heavy and light chains of mouse IgG, respectively.

SDS/PAGE. 2) Radioiodination of the 45-kDa and skeletal muscle actin bands followed by digestion with trypsin produced essentially identical iodinated tryptic peptides. 3) A monoclonal antibody that recognizes only the 45-kDa protein on immunoblots, also recognized skeletal muscle actin. The amount of actin in the purified enzyme fractions has varied from 2 to 5% as measured by densitometry of stained gels (see Fig. 6). It appeared to be in the form of heterogeneous F-actin since it was distributed uniformly throughout both the gel filtration and sucrose gradient fractions. Because the amount of actin present was variable, and decreased in proportion to protein kinase with each purification step (see Fig. 1), we have concluded that it is a persistent contaminant and is not a stoichiometric part of the holoenzyme complex. However, we cannot rule out that the kinase has a specific but low affinity actin-binding site that results in the co-purification of actin. It is of interest that a portion of the actin was precipitated with enzyme activity by the anti-kinase monoclonal antibody (Fig. 5). This may have been the result of a specific interaction between actin and the enzyme, or could simply have been caused by trapping of F-actin in the antibody-enzyme matrix.

Two other low molecular weight protein bands appear slowly in the purified kinase when it is stored at 0 °C. These can be seen in Fig. 5, one slightly above and one below the light chain of IgG. These are not present in freshly purified kinase (Fig. 6), thus we have concluded that they are breakdown products of the kinase.

Determination of the Subunit Composition of the Holoenzyme—Densitometric scans of gels stained with both Coomassie blue and fast green were used to determine the molar ratio of the synapsin I kinase subunits (Fig. 6). As in the experiment of Fig. 4, the β and β'-subunits were treated as a single subunit for this analysis. The areas of each of the optical density peaks were normalized by dividing the area of the peak by the apparent molecular weight of the protein band. The molar ratio of the subunits was then calculated as the ratio of the normalized peak areas. Results from the fast green-stained gel (Fig. 6*A*) indicated a molar α to β/β' ratio of 3:1, whereas those from the Coomassie blue-stained gel (Fig. 6*B*) showed a ratio of 5:1. Coomassie blue-stained gels have shown variable subunit ratios (ranging from 2.5:1 to 5:1), while fast green-stained gels have consistently shown a 3:1 ratio. For this reason, we believe the 3:1 ratio is more reliable. Determination of the exact subunit ratio will await the results of protein sequencing experiments.

The approximate molecular weight of the kinase holoenzyme was determined from its hydrodynamic properties. The Stokes radius of the enzyme, as determined by gel filtration on Sepharose 4B (Fig. 1), was 95 Å, and the sedimentation coefficient ($s_{20,w}$), as determined by sucrose density gradient centrifugation (Fig. 2), was 16.4 S. From these values, a molecular weight of 650,000 and a frictional ratio of 1.67 was calculated for the kinase holoenzyme as described in Table II.

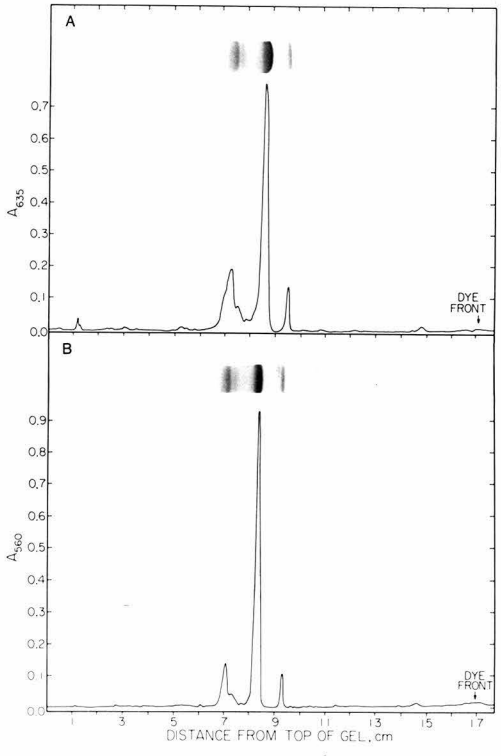

FIG. 6. **Densitometric scans of fast green and Coomassie blue-stained gels of the purified kinase.** *A*, fast green-stained gel (22.5 μg of purified kinase) and densitometric scan at 635 nm. *B*, Coomassie blue-stained gel (7.5 μg of purified kinase) and densitometric scan at 560 nm.

TABLE II

Physical properties of rat brain calmodulin-dependent protein kinase

Property	Method of determination	Value
Stokes radius (a)[a]	Gel filtration	94.7 ± 1.2 Å
Sedimentation coefficient ($s_{20,w}$)[a]	Sucrose density gradient	16.4 ± 0.7 S
Molecular weight (M_r)[b]	Stokes radius and sedimentation coefficient	650,000
Frictional ratio (f/f_0)[c]	Stokes radius and sedimentation coefficient	1.67
Subunit structure ($\alpha:\beta'/\beta$)	Molecular weight and subunit ratio	9:3 (M_r = 654,000) 10:2 (M_r = 644,000)

[a] Values shown are mean ± S.D. of five separate experiments.

[b] Calculated according to the equation

$$M_r = 6\pi\eta_{20,w} \cdot s_{20,w} \cdot a \cdot N/(1 - \bar{v}\rho_{20,w})$$

where N = Avogadro's number, $\eta_{20,w}$ = viscosity of water at 20 °C, $\rho_{20,w}$ = density of water at 20 °C, and $\bar{v}$ = partial specific volume, for which a value of 0.725 ml/g was assumed (53).

[c] Calculated according to the equation (53)

$$f/f_0 = a(4\pi N/3\bar{v}M_r)^{1/3}$$

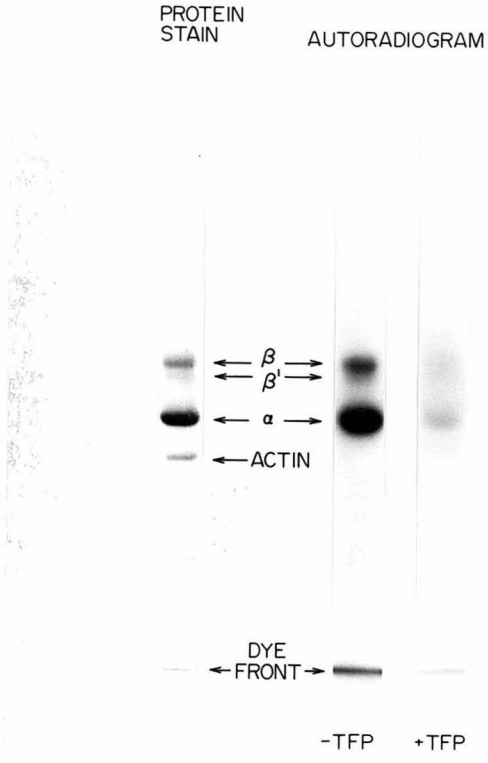

FIG. 7. **^{125}I Calmodulin binding to kinase subunits.** Purified calmodulin was iodinated by the lactoperoxidase method to a specific activity of 3.6 × 10^6 cpm/μg as described by Carlin *et al.* (54). ^{125}I calmodulin co-migrated with unlabeled calmodulin during SDS/PAGE and was able to activate the purified synapsin I kinase. Purified kinase (6 μg/lane) was subjected to SDS/PAGE. The binding of ^{125}I calmodulin to peptides in the gel was carried out as described by Carlin *et al.* (54) in the absence (−TFP) or presence (+*TFP*) of 1.0 mM trifluoperazine. Following ^{125}I calmodulin binding, the gels were stained with Coomassie blue, and labeled bands were detected by autoradiography.

These numbers indicate that the kinase is a large, somewhat elongated molecule made up of multiple subunits.

From the molecular weight of the enzyme and the ratio of its subunits, we can deduce the subunit structure of the holoenzyme. A subunit ratio ($\alpha:\beta/\beta'$) of 3:1 would indicate a holoenzyme consisting of nine α-subunits and three β/β'-subunits, whereas a molar ratio of 5:1 would indicate a holoenzyme consisting of 10 α-subunits and two β/β'-subunits. The physical properties of the kinase are summarized in Table II.

Identification of the Calmodulin-binding Subunits—Calmodulin-binding proteins in the purified kinase were identified by a calmodulin SDS/PAGE overlay technique (54). ^{125}I calmodulin bound specifically to the α, β, and β'-subunits (Fig. 7). The binding was nearly completely inhibited by trifluoperazine. Calmodulin did not bind to actin or to bovine serum albumin included in gels as a control (data not shown). The binding of ^{125}I calmodulin at the dye front may have been due to interaction with small breakdown products of the kinase. The amount of ^{125}I calmodulin bound to the α and β/β'-subunits was determined by γ-emission spectrometry of excised gel pieces. The ratio of ^{125}I bound to the α-subunit to that bound to the β/β'-subunit was 2.9:1. This result is consistent with a 3:1 subunit ratio, if each subunit has one calmodulin binding site/molecule.

Autophosphorylation of the Subunits—Incubation of the purified kinase under standard assay conditions resulted in phosphorylation of both the α- and β-subunits. The time course of this autophosphorylation is shown in Fig. 8. The incorporation of phosphate was both rapid and stoichiometrically significant. After 1 min, each subunit incorporated at least 1 mol of [^{32}P]phosphate/mol of protein. After 10 min, the α-subunit contained 2 mol of phosphate and the β-subunits 3 mol/mol of protein. As phosphorylation proceeded, labeled protein bands with apparent molecular weights of 64,000 and 54,000 gradually appeared. The appearance of

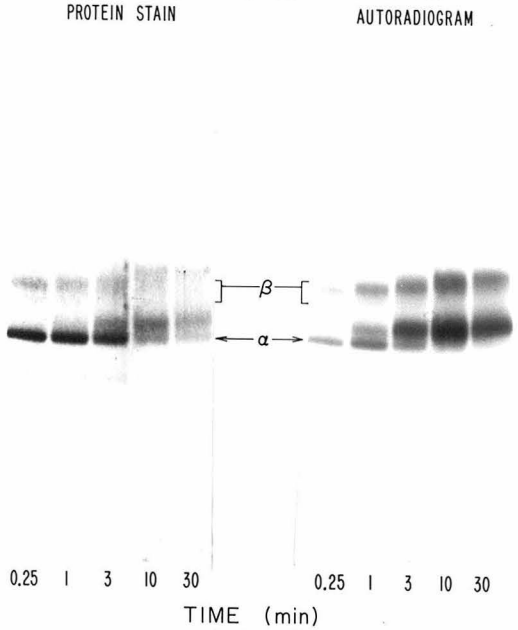

FIG. 8. **Autophosphorylation of kinase subunits.** Purified kinase (3.0 μg) was endogenously phosphorylated as described under "Experimental Procedures" for varying lengths of time. The enzyme subunits were separated by SDS/PAGE and stained with Coomassie blue. ^{32}P incorporation was detected by autoradiography.

TABLE III
Substrate specificity of rat brain calmodulin-dependent kinase

Substrate	Concentration in assay	Rate of calcium-stimulated phosphorylation of substrate
	mg/ml	%
Synapsin I	0.1	100[a]
Microtubule protein[b]	0.4	35
Smooth muscle myosin light chain	0.4	34
Arginine-rich histone[c]	0.1	18
Phosvitin	0.4	15
Casein	0.4	4
Phosphorylase *b*	0.4	0
Glycogen synthase	0.1	0

[a] The rate of calcium-stimulated phosphorylation of synapsin I was 57 pmol/min.

[b] The microtubule protein sample consisted of approximately 95% tubulin and 5% microtubule-associated protein (MAP). Only MAP_2 was phosphorylated.

[c] The arginine-rich histone sample consisted of approximately 30% histone H3, which was the only protein phosphorylated.

these bands was correlated with a decrease in both protein staining and ^{32}P content in the original α- and β-subunit bands. Our interpretation of these findings is that both subunits can be multiply phosphorylated. Phosphorylation of the first sites has no effect on mobility of the subunits on SDS gels, whereas phosphorylation of subsequent sites results in a considerable shift in mobility.

Substrate Specificity—In order to facilitate comparison of the synapsin I kinase to other protein kinases, the ability of the purified kinase to phosphorylate a number of substrates frequently used for assaying protein kinases was examined. As shown in Table III, microtubule protein, smooth muscle myosin light chain, histone H3, and phosvitin were all phosphorylated at a significant rate, whereas casein, phosphorylase *b*, and glycogen synthase were phosphorylated poorly or not at all. The microtubule protein consisted of both tubulin (95%) and microtubule-associated proteins (MAPs, 5%). Only MAP_2 was phosphorylated. The small amount of MAP_2 present in the assay and its relatively high incorporation of phosphate suggests that it is a particularly good substrate for the purified kinase. As a control, each of the substrates was included in an assay with synapsin I to determine if any component of the substrate was inhibiting kinase activity. The glycogen synthase (as purchased) had to be dialyzed against 40 mM Tris (pH 7.5) to remove an inhibitory component before being tested as a substrate. Ten times the usual concentration of calmodulin had to be used in the histone assays.

DISCUSSION

We have reported the purification to near homogeneity of a calcium and calmodulin-dependent protein kinase from rat brain. The kinase is a multisubunit complex with a molecular weight of about 650,000. It contains three subunits; α (50 kDa), β (60 kDa), and β' (58 kDa). The α- and β-subunits are distinct peptides, however β' may have been generated from β by proteolysis. All three of these peptides co-migrate with kinase activity during the purification steps and are co-precipitated with kinase activity by a specific anti-kinase monoclonal antibody. They all can bind calmodulin in the presence of calcium and are autophosphorylated under conditions in which the kinase is active. From the ratio of the subunits, and the holoenzyme molecular weight, we postulate a subunit composition for the kinase of approximately nine α-subunits and three β/β'-subunits.

The recovery of purified synapsin I kinase suggests that it is a relatively abundant brain enzyme constituting approximately 0.3% of the total brain protein. This estimate assumes that the losses of catalytic activity during each purification step were due to losses of enzyme protein rather than to a decrease in the activity of individual enzyme molecules. However, two additional lines of argument suggest that the estimate is essentially correct. First, the specific activity of the purified kinase is as high or higher than that of many other purified protein kinases (36–38, 55, 56). Thus, it seems unlikely that there has been a dramatic decrease in the activity of individual enzyme molecules during the purification. Second, a similarly high concentration of the α-subunit in brain homogenates has been measured by a radioimmunoassay utilizing the anti-kinase monoclonal antibodies.[2]

In an earlier paper (35), we suggested, on the basis of studies carried out with partially purified synapsin I kinase, that three, prominent "substrate" proteins for a calmodulin-dependent protein kinase in brain homogenates might actually be autophosphorylated subunits of the calmodulin-dependent synapsin I kinase itself. The characterization of the subunits of the purified kinase presented in this study confirms that all three of these substrate proteins are in fact part of the synapsin I kinase holoenzyme. Grab *et al.* (34) and Cohen *et al.* (57) have shown that a similar set of three substrate proteins are present in purified cerebral postsynaptic densities, fibrous structures that are located on the cytoplasmic surface of postsynaptic cells, in the region underlying the presynaptic terminal. One of these substrates is a prominent postsynaptic density protein termed the "major 52K PSD protein" by Kelly and Cotman (58). In a separate study, we have shown by biochemical and immunochemical criteria that the α-subunit of the synapsin I kinase is identical to this major 52K PSD protein (70). This suggests that the kinase may be concentrated in postsynaptic densities *in vivo* and thus may be involved in the regulation of postsynaptic as well as presynaptic processes. The presence of the kinase in postsynaptic densities could account for a portion of the synapsin I kinase that is associated with the particulate fraction of brain homogenates (35).

The α- and β-subunits of the kinase each incorporate at least two mol of phosphate/mol of protein in the presence of calcium, calmodulin, magnesium, and ATP. Incorporation of phosphate into the first site does not affect the mobility of the kinase subunits on SDS gels, whereas phosphorylation of additional sites causes a shift in their mobility (Fig. 8). Preliminary experiments have not yet revealed any large effect of this autophosphorylation on the kinetic or structural properties of the purified kinase. Another possible function of the autophosphorylation is the regulation of the association of the kinase with subcellular structures *in vivo*. We have previously noted that the synapsin I kinase is distributed between the soluble and particulate fractions in brain homogenates and that the kinases purified from each of the two fractions appear identical by several criteria (35). Recent experiments in which we examined the autophosphorylation of the kinase subunits in crude soluble and in crude particulate fractions suggested that the soluble form of the kinase, but not the particulate form, may already contain endogenous phosphate at one or more of the autophosphorylatable sites (data not shown). This supports the notion that autophosphorylation may regulate the intracellular location of the kinase.

The calmodulin-dependent synapsin I kinase is different in subunit composition and holoenzyme molecular weight from muscle myosin light chain kinase (23–25) and phosphorylase kinase (22), however, it is similar to two other recently char-

acterized calmodulin-dependent protein kinases (36–38). One of these is a calmodulin-dependent protein kinase from rat brain that phosphorylates smooth muscle myosin light chain and also other endogenous substrates. It was purified by Fukunaga *et al.* (38) and reported to be a 640-kDa holoenzyme composed of 11 to 14 identical 49 kDa subunits. It did not contain the 58- and 60-kDa proteins that we have termed β-subunits. The 49-kDa subunit was autophosphorylated in the presence of calcium, calmodulin, magnesium, and ATP. The recovery of this enzyme suggested that it could constitute as much as 0.1% of the total brain protein. The substrate specificity of this kinase resembles that of the synapsin I kinase. Both enzymes phosphorylate smooth muscle myosin light chain, microtubule-associated protein and arginine-rich histone. However, the kinase of Fukunaga *et al.* (38) phosphorylates casein well and phosvitin poorly, whereas the synapsin I kinase phosphorylates phosvitin at a moderate rate, and does not phosphorylate casein (Table III). Because of the close similarity of this protein kinase to the synapsin I kinase described in this report, we have made a comparison between the results of Fukunaga *et al.* (38) and our own, to determine whether the two enzymes might be related.

The first purification step used by Fukunaga *et al.* (38) was precipitation of protein at pH 6.1. This step left 99% of the calmodulin-dependent myosin light chain kinase activity in the supernatant. In contrast, we have found that precipitation at pH 6.1 leaves only about 10% of the calmodulin-dependent synapsin I kinase activity in the supernatant. The rest is irreversibly inactivated. This difference in recovery could mean either that the two protein kinase activities are properties of distinct molecules or that there is some difference in the methods of assay used by each group. Fukunaga *et al.* (38) used an incubation time of 10 min in their standard assay. We have found that the rate of phosphorylation of synapsin I falls off rapidly after about 30 s. Thus, the total incorporation of phosphate into synapsin I after 10 min is not a reflection of the initial enzymatic rate, and does not accurately measure the amount of enzyme present. In order to see whether the same situation might hold for phosphorylation of myosin light chain in brain homogenates, we determined the recovery of calmodulin-dependent myosin light chain kinase activity after pH 6.1 precipitation using both 30-s and 10-min assays. The assays were performed as described by Fukunaga *et al.* (38) with gizzard myosin light chains as substrate. We found an 80% recovery using a 10-min assay, and a 20–25% recovery using a 30-s assay (data not shown). Thus, it is possible that Fukunaga *et al.* (38) overestimated the recovery of kinase activity during their purification.

The major difference between the purification procedure used by Fukunaga *et al.* (38) and our own was this pH 6.1 precipitation step. Their other steps included chromatography methods similar to our own. We think that it is possible that their protein kinase contains α-subunits identical to those we have described, but has lost the β-subunits, perhaps at the acid precipitation step. Although a molecular weight of 640,000 was estimated for their enzyme from gel filtration data, its hydrodynamic properties indicate that it is actually smaller than the synapsin I kinase described in this report. Its Stokes radius, determined by gel filtration, is 81 Å and its sedimentation coefficient, determined by sucrose density gradient centrifugation, is 15.3 S (38), whereas the comparable values for the synapsin I kinase are 95 Å and 16.4 S. The molecular weight of the Fukunaga kinase, calculated from both its Stokes radius and its sedimentation coefficient as described in Table II, would be ~512,000, 140,000 less than the value of 650,000 calculated for the synapsin I kinase. This apparent difference in size is consistent with the notion that the difference between the two kinases could be the presence or absence of the β-subunits.

The evidence that the β-subunits are a part of the complex that contains synapsin I kinase activity comes from two distinct experiments and is quite strong (see "Results" and previous "Discussion"). However, there have been reports that under some circumstances, protein kinases can form multienzyme complexes with other functionally related molecules (59, 60). We therefore sought to determine whether the β-subunits might be one of two previously described calmodulin-binding proteins with molecular weights of about 60,000, calcineurin A, or calmodulin-dependent cyclic nucleotide phosphodiesterase. Calcineurin is an abundant brain protein thought to have protein phosphatase activity (61). Calcineurin A, its larger, calmodulin-binding subunit, has a molecular weight of 61,000 (62). We obtained polyclonal affinity-purified rabbit anti-calcineurin from Dr. Claude Klee and tested by the immunoblot procedure (see "Methods") for the presence of calcineurin at various stages in the purification of the kinase. Calcineurin was easily detected in the 170,000 × *g* brain supernatant but was not detectable in the final purified kinase preparation (data not shown). Calmodulin-dependent cyclic nucleotide phosphodiesterase is a dimer of two identical calmodulin-binding 59-kDa subunits (63, 64). When purified from brain, it has a specific activity of 160–300 μmol/min/mg and is quite stable. We tested for the presence of this protein at various stages in the purification of the kinase by measuring phosphodiesterase activity according to the method of Rangel-Aldao *et al.* (52). We found a level of calcium-activated phosphodiesterase activity in the 170,000 × *g* supernatant that was consistent with that reported by other laboratories (63, 64). However, the specific activity in the purified kinase was less than 0.008 μmol/min/mg, the limit of detection in this assay (data not shown). Thus, the β-subunits do not appear to be either calcineurin A or calmodulin-dependent phosphodiesterase. It should be emphasized, however, that we do not know the specific functions within the kinase holoenzyme of the α- or the β-subunits. Thus, the possibility remains that one of the subunits may have a function that is not directly involved in catalysis of protein phosphorylation.

The second, recently characterized, calmodulin-dependent protein kinase that resembles synapsin I kinase is glycogen synthase kinase. It is a holoenzyme of about 300,000 Da, composed of roughly equal amounts of autophosphorylatable subunits of 50 and 53 kDa (36, 37). The association of autophosphorylatable, 50–53 kDa subunits into a multimeric holoenzyme is reminiscent of the structure of synapsin I kinase. However, there appear to be significant differences in the substrate specificities of the two enzymes. For example, although both of them phosphorylate smooth muscle myosin light chain (36, 37), synapsin I kinase does not phosphorylate glycogen synthase (see Table III). In addition, casein is a poor substrate for synapsin I kinase (Table III), but a good substrate for glycogen synthase kinase (Ref. 36, but see Ref. 37). Nevertheless, the structural similarities of these two kinases suggest that they may be members of a family of related calmodulin-dependent protein kinases that have evolved from a common precursor.

Although the physiological role of the calmodulin-dependent synapsin I kinase is not yet known, several of its properties suggest that it is involved in the regulation of both pre- and postsynaptic functions. For example, one of its brain substrate proteins, synapsin I, is located primarily in synaptic terminals (65, 66) where it is specifically associated with synaptic vesicles (67). Phosphorylation of synapsin I by the calmodulin-

dependent synapsin I kinase can be triggered both *in vivo* and *in vitro* by depolarization of terminals in the presence of calcium (68, 69). In addition, we have recently shown that the synapsin I kinase itself may be a prominent constituent of certain brain postsynaptic densities based on the identity of one of its subunits with the "major 52K postsynaptic density protein" first described by Kelly and Cotman (58, 70). The molecular characterization of synapsin I kinase presented in this report, and the generation of antibodies that specifically recognize it,[2] will aid in the elucidation of its full range of substrate proteins, and of its distribution in different parts of the nervous system and within individual nerve cells. Such information should be useful in the development of a molecular model for synaptic function and its regulation.

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Appendix II

BIOCHEMICAL AND IMMUNOCHEMICAL EVIDENCE THAT THE "MAJOR POSTSYNAPTIC DENSITY PROTEIN" IS A SUBUNIT OF A CALMODULIN-DEPENDENT PROTEIN KINASE

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Biochemical and immunochemical evidence that the "major postsynaptic density protein" is a subunit of a calmodulin-dependent protein kinase

(synaptic structure/brain phosphoproteins/synaptic regulation)

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ABSTRACT **By three criteria, two biochemical and one immunochemical, the major postsynaptic density protein (mPSDp) is indistinguishable from the 50-kilodalton (kDa) α subunit of a brain calmodulin-dependent protein kinase. First, the two proteins comigrate on NaDodSO$_4$/polyacrylamide gels. Second, iodinated tryptic peptide maps of the two are identical. Finally, a monoclonal antibody (6G9) that was raised against the protein kinase binds on immunoblots to a single 50 kDa band in crude brain homogenates and to both the α subunit of the purified kinase and the mPSDp from postsynaptic density fractions. The purified kinase holoenzyme also contains a 60-kDa subunit termed β. A comparison of the peptide map of β with the maps of 60-kDa proteins from the postsynaptic density fraction suggests that β is present there but is not the only protein present in this molecular weight range. These results indicate that the calmodulin-dependent protein kinase is a major constituent of the postsynaptic density fraction and thus may be a component of type I postsynaptic densities.**

Many synaptic junctions in the central nervous system contain a prominent specialized structure called the "postsynaptic density" (PSD) (1–5). When viewed by electron microscopy, it is a fibrous, electron-opaque thickening lying opposite the presynaptic terminal, on the cytoplasmic side of the postsynaptic membrane. The morphology of postsynaptic densities is variable. Some are thick [20–60 nm (5)] and appear to cover the entire postsynaptic surface area, whereas others are thin, often discontinuous, patches (1, 6). The former have been termed type I PSDs, while the latter are called type II (1). Some investigators have suggested that different types of densities are associated with synapses of particular types. For example, type I PSDs most often occur in synapses that are thought to be excitatory (1, 7, 8), whereas type II PSDs are seen in synapses thought to be inhibitory (9, 10). This hypothesis suggests that morphologically distinct PSDs may also contain distinct proteins that serve specialized functions associated with their particular transmitter type.

In order to understand the structure and function of PSDs, various research groups have developed subcellular fractionation methods to isolate highly enriched preparations of PSD-like material (11–13). These procedures involve osmotic lysis of a subcellular fraction enriched in synaptosomes, followed by purification of junctional membrane complexes and extraction of membrane components by treatment with detergent. The detergent-insoluble residue, purified by density gradient fractionation, consists of electron-opaque, fibrous, disk-shaped structures, 20–60 nm thick and 200–500 nm in diameter (11–15). These structures are similar in appearance and staining characteristics to type I PSDs in intact fixed tissue, and they are not produced by detergent treatment of other subcellular organelles such as mitochondria or myelin (11). Thus, this subcellular fraction is considered to be highly enriched in type I PSDs.

The PSD fraction contains 20–30 proteins (13, 16). Among them are the cytoskeletal proteins tubulin (17, 18), actin (18, 19), and fodrin (20). It has been proposed that they are primarily structural, serving to anchor membrane or cytosolic proteins in the region of the postsynaptic membrane (21). The fraction is enriched in cyclic nucleotide phosphodiesterase activity (22, 23), calmodulin (24), and both cAMP- (25–27) and calmodulin-dependent protein kinase activities (27–29). Immunocytochemical evidence suggests that a calmodulin-dependent protein phosphatase, calcineurin, may also be located in PSDs *in situ* (30, 31). The presence of these regulatory enzymes suggests a role for the PSD in regulation of postsynaptic properties, such as receptor clustering, receptor sensitivity, or gating of ion fluxes. The major component of PSD fractions, making up 10–30% of the total protein, is a 50-kilodalton (kDa) protein of unknown function termed the "major PSD protein" (mPSDp) (18). This protein binds calmodulin (32, 33) and is also a substrate for a calmodulin-dependent protein kinase (28, 32).

We have recently purified and characterized a brain calmodulin-dependent protein kinase (34) that phosphorylates synapsin I (35, 36) as well as other brain proteins (34, 37). We will refer to it as synapsin I kinase to distinguish it from other calmodulin-dependent protein kinases. It is found in both soluble and particulate fractions of brain homogenates (37) and is a relatively abundant enzyme, making up as much as 0.3% of the total brain protein. When purified from the soluble fraction, it is a 650-kDa holoenzyme composed of three subunits with molecular masses of 50, 58, and 60 kDa (termed α, β', and β, respectively) (34). The α and β subunits are distinct peptides; however, β' may have been generated from β by proteolysis. The holoenzyme contains approximately nine α subunits and three β/β' subunits. All of the subunits bind calmodulin and are autophosphorylated under conditions in which the kinase is active. They are labeled with ^{32}P in endogenously phosphorylated brain homogenates (34, 37–39), where they account for a major portion of the endogenous "substrates" for calmodulin-dependent protein kinase in the 50- to 60-kDa region of NaDodSO$_4$/polyacrylamide gels (37).

Proteins of the same molecular weights are phosphorylated by a calmodulin-dependent protein kinase in purified PSD preparations (28, 29). The 52-kDa phosphoprotein is the major PSD protein referred to earlier. The identities of the 58- and 60-kDa phosphoproteins have not been established. These pro-

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Abbreviations: PSD, postsynaptic density; mPSDp, major postsynaptic density protein; kDa, kilodalton(s); INT, *p*-iodonitrotetrazolium violet; Bicine, *N,N*-bis(2-hydroxyethyl)glycine; SM, synaptic membrane.

teins are similar to the phosphorylated proteins observed in crude brain particulate fractions (38). Consequently, Grab *et al.* (28) have suggested that the phosphorylated proteins in this molecular mass range in brain particulate fractions are probably PSD in origin.

The similarities between the 50-, 58-, and 60-kDa proteins phosphorylated in PSD fractions and the subunits of synapsin I kinase suggested that the two sets of proteins might be identical. To test this possibility, we have compared the proteins in PSD preparations with the subunits of synapsin I kinase by immunochemical and biochemical methods. We report here that the mPSDp is indistinguishable by three criteria from the α subunit of calmodulin-dependent synapsin I kinase. In addition, the β subunit of the kinase is present in the PSD fraction, although it is not the only protein present in the 60-kDa molecular mass range.

METHODS

Materials. $Na^{125}I$ (carrier free) was purchased from New England Nuclear. Dithiothreitol, *N,N*-bis(2-hydroxyethyl)glycine (Bicine), *p*-iodonitrotetrazolium violet (INT), sodium phosphate (mono- and dibasic), succinic acid, hemoglobin (bovine type II), mouse IgG, and napthol blue black (amido black) were purchased from Sigma. Trypsin treated with 1-tosylamido-2-phenylethyl chloromethyl ketone was purchased from Worthington, ultrapure sucrose from Schwarz-Mann, sodium *N*-lauroylsarcosinate from ICN, and staphylococcal protein A from Pharmacia. Cellulose-coated thin-layer chromatography plates were purchased from Eastman, and nitrocellulose membranes (BA85, 0.45 μm pore diameter) from Schleicher & Schuell. NS1/SP2 myeloma cells were a gift of Jeremy Brockes. BALB/c ByJ mice were purchased from The Jackson Laboratory, Simonsen albino rats (140- to 160-g males) from Simonsen Laboratories (Gilroy, CA), and New Zealand female rabbits from Lab Pets (Rosemead, CA).

Rabbit anti-mouse IgG antiserum was obtained from rabbits immunized by repeated subcutaneous injections with mouse IgG in Freund's adjuvant and was affinity purified by chromatography on Sepahrose 4B coupled with mouse IgG (40). ^{125}I-Labeled protein A (2–3 Ci/mmol; 1 Ci = 3.7×10^{10} Bq) was prepared by the chloramine-T method (41). Calmodulin-dependent synapsin I kinase was prepared as described by Bennett *et al.* (34).

Preparation of Synaptic Membrane (SM) and PSD Fractions. SM and PSD fractions were prepared from 15 rats by the method of Cotman *et al.* (11). The PSD pellet adheres to glass and plastic; therefore, to maximize recovery of protein, it was resuspended in a small volume of 1% $NaDodSO_4$/2 mM Bicine. The reduction of INT by mitochondria that is used to increase their density produces an insoluble precipitate called formazan that adheres to the SM and PSD fractions. This precipitate interfered with determination of protein by the Lowry method (42). However, we estimate from Coomassie blue-stained gels that we obtained 40–60 mg of SM protein and 200–300 μg of PSD protein.

Selection of Hybridoma 6G9. Hybridomas that secrete monoclonal antibodies specific for synapsin I kinase were selected from a fusion of NS1/SP2 myeloma cells with spleen cells of BALB/c ByJ mice that had been immunized with kinase purified through the calmodulin-Sepharose step (34, 37). Details of the preparation and selection of these hybridomas will be published separately. Hybridoma 6G9 was initially selected by a solid-phase radioimmunoassay similar to that described by Moore *et al.* (43) in which culture supernatants were tested for the presence of antibodies that bound to the crude antigen. 6G9 culture supernatant was then tested for binding to individual peptides by the immunoblot procedure described below. The 6G9 hybridoma was subcloned, then ascites fluid was prepared by injection of $1–3 \times 10^6$ cells intraperitoneally into pristane-primed BALB/c ByJ mice. The antibody was partially purified by precipitation with 50% saturated ammonium sulfate, redissolved to a protein concentration of 20 mg/ml, and dialyzed against 20 mM Tris·HCl, pH 7.0.

Immunoblots. Proteins from $NaDodSO_4$/polyacrylamide gels were transferred onto nitrocellulose paper as described by Towbin *et al.* (44). After transfer, the nitrocellulose sheets were incubated with (*i*) buffer D (50 mM Tris·HCl, pH 7.4/0.9% NaCl/0.1% NaN_3) containing 5% hemoglobin (2 hr); (*ii*) 6G9 partially purified ascites fluid diluted into buffer D containing 1% hemoglobin (8–12 hr); (*iii*) wash buffer (buffer D containing 0.5% hemoglobin) (1 hr with three changes); (*iv*) rabbit anti-mouse IgG (2–7 μg/ml) in wash buffer (2 hr); (*v*) wash buffer (1 hr with three changes); (*vi*) ^{125}I-labeled protein A ($2–3 \times 10^5$ cpm/ml) in wash buffer (2 hr); (*vii*) wash buffer (1 hr with three changes). The nitrocellulose sheet was dried, and bands containing ^{125}I were detected by autoradiography.

Other Procedures. Peptide mapping was performed by the method of Elder *et al.* (45) as modified by Kelly and Cotman (18). Polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ was performed by the method of Laemmli (46). Stacking gels were 3.5% acrylamide/0.09% bisacrylamide. Running gels were 10% acrylamide/0.27% bisacrylamide. Molecular mass standards were phosphorylase *b*, 94 kDa; bovine serum albumin, 68 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 29 kDa. Crude rat brain homogenate was prepared as described by Bennett *et al.* (34) and subjected to centrifugation at $10,000 \times g$ for 10 min to remove unbroken cells and nuclei.

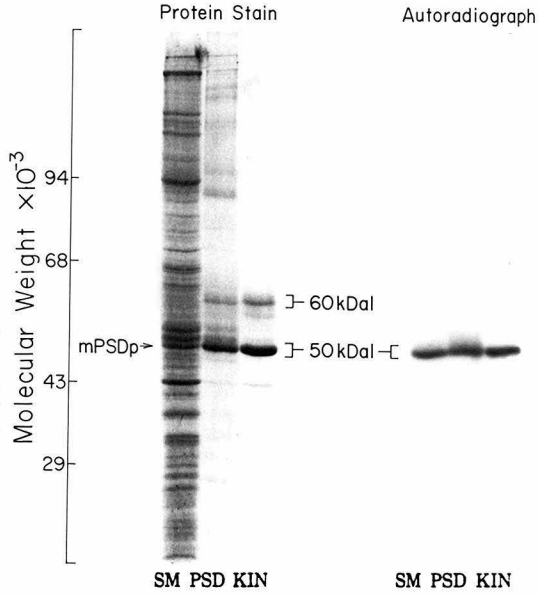

FIG. 1. Immunochemical relationship between the α subunit of synapsin I kinase and the mPSDp. (*Left*) The SM fraction (5 μl, ≈70 μg), the PSD fraction (30 μl, ≈25 μg), and synapsin I kinase (KIN, 6 μg) purified to 95% homogeneity (34) were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis and stained with Coomassie blue. The position of mPSDp is noted. (*Right*) The SM fraction (5 μl), the PSD fraction (7 μl, ≈6 μg), and purified synapsin I kinase (1 μg) were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis, transferred to nitrocellulose, and tested for reaction with antibody 6G9 (diluted 1:500).

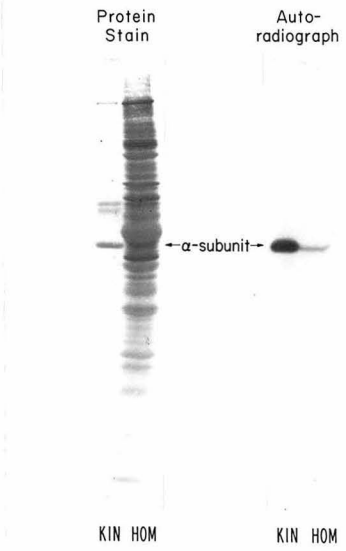

FIG. 2. Binding specificity of antibody 6G9 tested by the immunoblot method. Synapsin I kinase (KIN, 6 μg), partially purified through the calmodulin-Sepharose step (34, 37), and crude rat brain homogenate (HOM, 150 μg) were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis in duplicate lanes and transferred to nitrocellulose paper. One pair of lanes (*Left*) was stained with amido black (44). The other pair (*Right*) was tested for reaction with antibody 6G9 (diluted 1:1000) by the immunoblot method. The calmodulin-Sepharose purified kinase is approximately 50% pure. The major band in the 50-kDa region is the α subunit (37). This is the preparation that was originally used as antigen for the generation of antibody 6G9.

RESULTS

The SM and PSD fractions prepared as described in *Methods* had protein compositions (Fig. 1 *Left*) similar to those published by Kelly and Cotman (16). The protein previously defined as mPSDp migrated with the α subunit of purified synapsin I kinase (marked 50 kDa).

For immunochemical comparison of the α subunit and the mPSDp, we used a monoclonal antibody (6G9) that binds with high affinity to the α subunit of the kinase on immunoblots (Figs. 1 and 2) and recognizes a single protein band of the same molecular weight in crude brain homogenates (Fig. 2). Proteins from the SM and PSD fractions were tested for binding to 6G9 as described for Fig. 1 *Right*. The results indicated that the epitope recognized by 6G9 is contained in the mPSDp as well as the α subunit of the kinase. Since 6G9 appears to react with only one protein band even in crude brain homogenates, its binding to both the α subunit and the mPSDp is strong evidence that the two are chemically related.

Additional and independent evidence that the two proteins are closely related was obtained by comparison of their iodinated tryptic peptide maps. Gel pieces containing the two proteins were cut from gels similar to those pictured in Fig. 1. The proteins were iodinated within the gel and digested with trypsin as described (18). Maps of the resulting iodinated peptides were indistinguishable (Fig. 3). In two separate experiments, both major and minor peptides were identical. The maps resemble those published by Kelly and Cotman of the mPSDp cut from $NaDodSO_4$ gels of the PSD fraction (18). This experiment provides further evidence that the mPSDp and the α subunit of the kinase are closely related and suggests that they may be identical.

The PSD fraction contained protein bands in the 60-kDa region that migrated near the position of the β subunit of the purified synapsin I kinase (Fig. 1, marked 60-kDa). We do not yet have a monoclonal antibody that reacts with the β subunits of the kinase on immunoblots, so we were unable to make an immunochemical comparison of the β subunits and the 60-kDa proteins of the PSD fraction. However, we have compared the two by peptide mapping. The regions marked by the 60-kDa bracket in Fig. 1 were cut from gels of the PSD fraction and the purified kinase. Iodinated tryptic peptide maps of the proteins are shown in Fig. 4. The eight major peptides in maps of the β subunit (marked with arrows) appeared to be prominent in maps of the 60-kDa proteins from the PSD fraction. Thus, a protein related to the β subunit is apparently present in the PSD fraction, but it is not the only protein present in this molecular mass range.

DISCUSSION

We have shown that the protein termed the major PSD protein (18) has several similarities to the α subunit of a recently purified brain calmodulin-dependent protein kinase, synapsin I

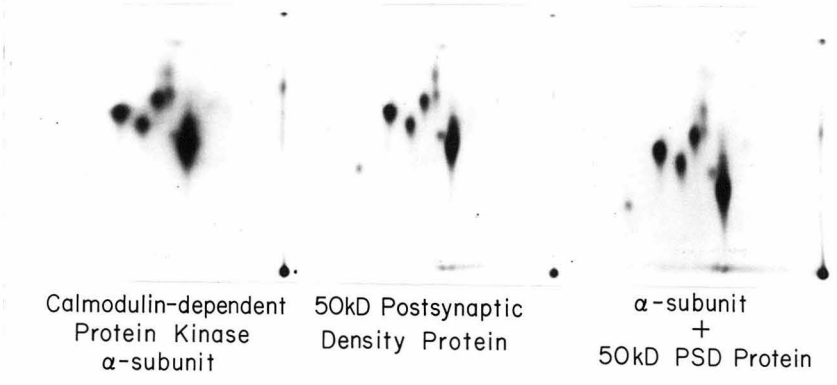

FIG. 3. Iodinated tryptic peptides of the α subunit of synapsin I kinase and the mPSDp. The PSD fraction (30 μl, ≈25 μg) and purified synapsin I kinase (6 μg) were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis. The bands marked 50 kDa in Fig. 1 were cut from the gel. Iodinated tryptic peptide maps of each were prepared as described (18). Two microliters of peptide solution was applied to the first two plates; 1.5 μl of each was applied to the third. The origin was at the lower right. The anode was to the right and the cathode to the left; chromatography was from bottom to top.

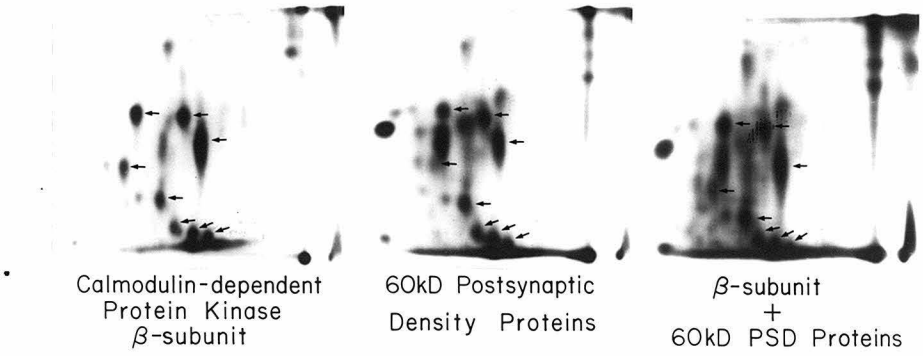

FIG. 4. Iodinated tryptic peptides of the β subunit of synapsin I kinase and 60-kDa proteins from the PSD fraction. The bands marked 60-kDa in Fig. 1 were cut from the same gel used in the experiment of Fig. 3. Iodinated tryptic peptide maps were prepared from each; 2 μl of peptide solution was applied to the plates. Electrophoresis and chromatography were as in Fig. 3.

kinase (34). They have the same mobility on $NaDodSO_4$/polyacrylamide gels. They both bind calmodulin (28, 34) and are phosphorylated in the presence of calcium, calmodulin, Mg^{2+}, and ATP (30, 34). They are both recognized by monoclonal antibody 6G9. Finally, iodinated tryptic peptide maps of the two are identical. These similarities do not rule out small differences between them in amino acid sequence or covalent modification. Nevertheless, they indicate that the two proteins are closely related and may be identical. One of the other two subunits of synapsin I kinase, β, also appears to be present in the PSD fraction. A third subunit, β', occurs in lower amounts than the other two in the kinase holoenzyme and may be a proteolytic product of β (34, 37). We have preliminary evidence, based on peptide maps, that β' is also present in the PSD fraction (data not shown).

The α subunit and the mPSDp are similar in two additional respects not described in this report. Immunochemical measurements indicate that, like the mPSDp (27, 47), the α subunit is much less concentrated in cerebellum than in the forebrain (48). Also, like the mPSDp (18), the α subunit streaks in the isoelectric focusing dimension of two-dimensional gels, and its recovery is low (data not shown). This probably occurs because the kinase holoenzyme is not completely dissociated by nonionic detergents.

Although several laboratories have shown that the PSD fraction contains structures that correspond in morphology and staining characteristics to postsynaptic densities, the possibility of artifactual association of proteins with this fraction during homogenization or treatment with detergent has not been completely ruled out (although see ref. 13). Thus, we cannot yet firmly conclude that the mPSDp/α subunit is associated with PSDs *in vivo*. Immunocytochemical experiments would be helpful in confirming this conclusion, but antibodies to the mPSDp are not produced when the PSD fraction is used as immunogen (49). We have, however, been able to produce antibodies to the α subunit by using soluble synapsin I kinase holoenzyme as immunogen. These should be useful in determining the location of this protein in intact tissue.

When it was originally described, it was postulated that the mPSDp might be a specific marker for the PSD (18, 50). However, Flanagan *et al.* (51) recently presented evidence that it may exist in soluble as well as particulate form. Our results support this idea, since we have previously shown that the α subunit is present in both soluble and particulate fractions of brain homogenates and appears to exist in a dynamic equilibrium between them (37). Kelly and Cotman (52) and Ratner and Mahler (21) have demonstrated that the mPSDp is crosslinked to PSD proteins by disulfide bonds. Thus, disulfide bonds are probably involved in the association of the kinase with the PSD fraction. They have proposed that control of formation of these bonds may be involved in regulation of the assembly and function of PSDs *in vivo*. It is possible that the extent of disulfide bonding may be influenced by autophosphorylation or dephosphorylation of the kinase subunits.

Evidence from other laboratories suggests that the synapsin I kinase is also associated with synaptic vesicles. For example, synapsin I, a principle substrate of the kinase, is primarily associated with vesicles (25, 53). Moreover, highly enriched vesicle fractions contain an endogenous protein kinase activity that phosphorylates synapsin I at the sites that are phosphorylated by purified synapsin I kinase (54, 55). In addition, partially purified synaptic vesicles from rat brain (56) and *Torpedo* electric organ (55) contain endogenous substrates for calmodulin-dependent protein kinase that have the same molecular weights as the autophosphorylated subunits of synapsin I kinase. The presence of the synapsin I kinase in several subcellular compartments would be consistent with the notion that it is involved in the regulation of many functions (34).

The Stokes radius of the synapsin I kinase holoenzyme is 95 Å, as determined by gel filtration (34), thus its diameter in solution is approximately 200 Å. Electron micrographs of isolated PSDs show that they contain particles with a median diameter of 180 Å that appear to be held together by a lattice of fibers (13–15). The similarity of the two diameters suggests that the kinase holoenzyme could be equivalent to the PSD "particles." This idea is supported by the observation that PSDs isolated from the cerebellum lack both the mPSDp and the particles (27).

The identification of the mPSDp as a subunit of calmodulin-dependent synapsin I kinase will allow us to use antibodies to the kinase to test for its presence in PSDs in intact tissue. If its presence there is confirmed, it will strengthen the idea that PSDs play an important role in postsynaptic regulation by calcium ion.

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